Non-Lipopolysaccharide Protective Antigens of Vibrio Cholerae

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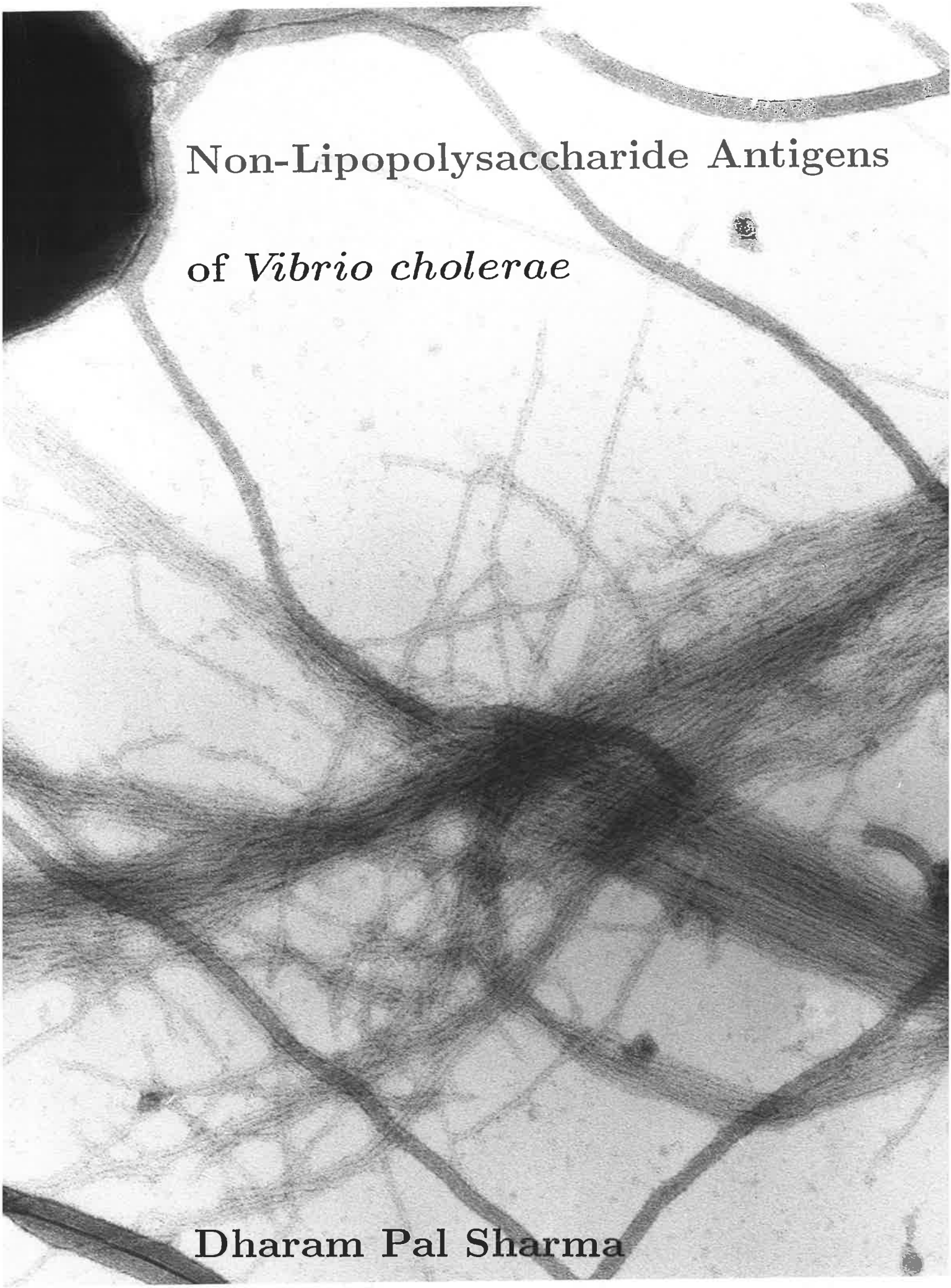

Non-Lipopolysaccharide Antigens of *Vibrio cholerae*

Dharam Pal Sharma

I dedicate this thesis to my wife and to my children for their love and patience. Thank you for your loyal support

Abstract

This thesis covers the investigation of non-LPS antigens expressed by both biotypes (Classical and El Tor) and serotypes (Ogawa and Inaba) of *Vibrio cholerae* and the determination of the extent to which these are shared or strain-specific, since this will be a critical determinant of their vaccine potential. The established infant mouse model was used to study both virulence and protection. The non-LPS antigens are expressed both by Classical and El Tor strains tested except for four old isolates of the El Tor biotype. The protective activities of antibodies to the shared antigens correlated with their capacities to inhibit the *in vitro* attachment of vibrio organisms to isolated murine enterocytes.

Subsequent studies were directed towards identifying the non-LPS antigen(s). Studies from overseas suggested *t*oxin-coregulated *p*ili (TCP) is an important pilus colonization factor. Considerable time was spent in trying to find expression of this factor *in vitro* and then with the aid of SDS-PAGE, immunoblotting and electron microscopy techniques including immunogold-labelling it was found to be expressed only by Classical strains.

The infant mouse cholera model was used to evaluate the relative importance of TCP as protective antigens of *V. cholerae* O1. Antibodies to TCP were sufficient to confer protection against two Classical strains, and were more efficient if the challenge vibrios were cultured for TCP expression. In contrast, such antibodies did not protect mice against challenge with any of four strains of El Tor biotype. Since two of the latter have previously been shown to possess

non-LPS protective antigens, these results suggest that TCP are not the only such antigen in this model.

A serum containing antibodies to non-LPS protective antigens of *V. cholerae* has been used, after extensive absorption, to facilitate the cloning of genes involved in the synthesis of TCP. A gene bank was constructed from *V. cholerae* Z17561 DNA using a mobilizable cosmid vector in *E. coli*, and subsequently transferred by conjugation into *V. cholerae* O17. This strain does not produce TCP *in vitro* and lacks non-LPS protective antigens. Eight positive clones were isolated, and of these, four produced TCP as determined by electron microscopic and immunoblotting analyses. TCP positive O17 clones were 70-fold more virulent than TCP-negative clones or O17 in the infant mouse cholera model. Only the former could remove protective antibodies by absorption and upon immunization elicit protective antibodies specific for non-LPS components. As a corollary, serum containing antibodies to TCP protected mice from challenge with TCP-positive clones, but not with TCP-negative clones or O17, demonstrating that antibodies to TCP are sufficient to mediate protection in this system. The data indicate that TCP is both a virulence determinant and a protective antigen in the infant mouse cholera model. This work to the best of my knowledge, has demonstrated for the first time the protective effect of anti-TCP antibodies albeit in the infant mouse cholera model

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.

Dharam Pal Sharma

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Publications

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Abbreviations

Ap	ampicillin
CFA	colonization factor agar
CFB	colonization factor broth
CP	clone probing
CT	cholera toxin
DIC	duodenal inoculation with cecal ligation
dNTP	deoxy-nucleoside triphosphate
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EM	elctron microscopy
ETEC	enterotoxigenic
G_{M1}	monosialosyl ganglioside
HAs	hemagglutinins
ID	infectious dose
kDa	kilodalton
km	kanamycin
LD	lethal dose
LPS	lipopolysaccharide
MFRHA	D-mannose-fucose-resistant hemagglutinin
MoAb	monoclonal antibody
MSHA	D-mannose-sensitive hemagglutinin
NA	nutrient agar
NB	nutrient broth

OD	optical density
OMP	outer membrane protein
ORS	oral rehydration solution
ORT	oral rehydration therapy
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PP	Peyer's patches
R	resistant
RBC	red blood cells
Sark-OMP	Sarkosyl-insoluble OMP
S	sensitive
sc	subcutaneous
SDS	sodium dodecyl sulphate
SE	standard error
SHA	soluble hemagglutinin
Sm	streptomycin
SRBC	sheep red blood cells
TBS	Tris-buffered saline
Tc	tetracycline
TCP	*t*oxin *c*oregulated *p*ilus
TSA	Tris-buffered saline containing sodium azide
TTBS	Tris-buffered saline containing Tween 20
μg	microgram
μl	microliter(s)
V. cholerae	*Vibrio cholerae*

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Chapter 1

Introduction

1.1 Historical Background

...blue lips, a shrunken face and hollow eyes, a knotted belly, with limbs cramped and curled as if by fire, breath clinging to the warrior's face; fingers that are probed, snaky and clawed; and in writhings the patient expires, the victim of Siva.

Inscription at Viziannugger Temple (cited in Vought 1893)

Cholera (or *murree* as described above) is a disease of antiquity recognised in ancient Indian medical literature. It probably originated in the deltaic regions of the Ganges and the Brahmaputra in the state of Bengal (now known as West Bengal [India] and Bangladesh) where for centuries it has been, and still is, endemic. During the late fifteenth century, Portuguese explorers gave vivid descriptions of cholera outbreaks on the Indian subcontinent; however, the disease did not spread westwards until the nineteenth century (see Pollitzer 1959).

In 1817, cholera broke out with a high mortality in its endemic home; and in the next five years, it spread over much of Asia and the Middle East. By 1832, the disease had spread to Europe and America (Rosenberg 1962; Morris & Black 1985). From 1817 to 1923, six pandemic waves of cholera spread across the world. Religious pilgrimages, the movement of

troops and the sea trade route centered around the endemic home all contributed to the dissemination of the disease (reviewed by Pollitzer 1959).

During this period, two important observations were made that greatly advanced the understanding of the disease: one regarding transmission and the other etiology. In 1855, John Snow published an important and influential treatise on cholera epidemiology (Snow 1855). He clearly established that the disease involved fecal-oral transmission and discussed factors promoting this mode of dissemination, highlighting the significance of water (to be discussed further in Section 1.7.3). Subsequently, Koch confirmed that cholera was indeed "an affectation of the alimentary canal" (Snow 1855). He identified the causative agent as a bacillus, *Vibrio cholerae*, which he cultured from the intestinal contents of cholera victims (Koch 1883, 1884 - cited by Pollitzer 1959). This confirmed earlier observations made in 1854 by Pacini of the association of the disease with vibrios (Hugh 1964).

In 1905, Gotschlich (cited by Pollitzer 1959) isolated a new strain of *V. cholerae* from the cadavers of pilgrims returning from Mecca to the quarantine camps at El Tor on the Sinai Peninsula, Egypt. This strain proved to be different from the previous "Classical" strains and was assigned a new biotype, El Tor. From 1923 to 1961, cholera was once again reduced to its endemic form being confined mainly to the Bengal region, with small foci of endemicity reported in countries to the east of India. In 1937 "paracholera" caused by *V. cholerae* (El Tor biotype) was observed by de Moor (1938 - cited by Kamal 1974) in the Celebes from where it gave rise to sporadic cases in adjacent countries over the next 20 years.

In 1961, the seventh (and current) pandemic began. Unlike previous pandemics, this was caused by vibrios of El Tor biotype (Barua & Cvjetanovic 1970; Kamal 1974). It began in Sulawesi [Celebes] (Kamal 1974), the endemic focus described by de Moor (de Moor 1938 - cited by Kamal 1974) and quickly spread to the Pacific islands, across Asia into the Middle East, the U.S.S.R. and Africa, reaching the North American continent by 1973 (Weissman *et al.*

1974). Other than the United States, no country in North, South or Central America reported cases until 1983, when cholera was detected in the Caribbean Coast of Mexico (Black 1986). The spread of the disease during this current pandemic was facilitated by the greater mobility of people today and by mass migration of refugees.

1.2 Etiology

1.2.1 Characteristics of the bacterium

V. cholerae is a gram-negative comma-shaped bacillus which possesses a sheathed polar flagellum of variable length. In addition, under certain cultural conditions, the presence of pili (or fimbriae) on the organism has recently been described (Tweedy *et al.* 1968; Taylor *et al.* 1987; Hall *et al.* 1988). The bacillus is a non-spore-forming aerobe or facultative anaerobe. Biochemically, it is recognised by being oxidase positive; it ferments glucose (without gas) and sucrose, but not lactose; it utilizes citrate, lysine and ornithine but not arginine; it is Voges-Proskauer (VP) positive and is sensitive to 0/129 (Pteridine) discs (150 μg).

1.2.2 Classification

In 1773, the genus name *Vibrio* was given by Müller. Subsequently, the species name, *cholerae,* was given in honor of Pacini, the first to give a valid description of the organism (cited in Gallut 1974). The genus *Vibrio* now contains, in addition to *V. cholerae*, other vibrios recently upgraded to species level (see below).

V. cholerae strains are classified on the basis of the production of a cell wall polysaccharide antigen designated O1. Two different classification systems for O groups are used (Sakazaki & Shimada 1977; Smith 1979). In both systems O-group 1 (O1) strains are responsible for true cholera infection. The O1 vibrios can be divided into two biotypes, Classical and

El Tor (Bauman *et al.* 1984), which can be differentiated in several ways: the latter can agglutinate chicken red blood cells (Finkelstein & Mukerjee 1963), and is resistant to polymyxin B (Gangarosa *et al.* 1967) and to Mukerjees's type IV cholera phage (Mukerjee 1963). Each biotype is further differentiated into antigenically related serotypes, Inaba, Ogawa and, rarely, Hikojima, based on the presence of 3 antigenic factors (A, B, & C). A, B and C are found in both Ogawa and Hikojima whereas Inaba possesses only A and C antigens (Redmond 1979).

Vibrios that produce polysaccharide antigens other than O1 are commonly referred to as NAG (non-agglutinable) vibrios or NCV (non-cholera vibrios; reviewed by Finkelstein 1973). NAG is a misnomer since, although these organisms are not agglutinated by 01 sera, they do agglutinate in homologous antisera. Such strains produce diarrhea indistinguishable from cholera (Morris & Black 1985). Some of the NAG vibrios causing enteritis were upgraded to species level on the basis of DNA hybridization studies; in 1985, identified species included *V. parahaemolyticus*, *V. mimicus*, *V. fluvialis* and *V. hollisae* (Blake 1980; Morris & Black 1985).

1.3 Pathogenesis and Pathophysiology

Cholera is an enterotoxic enteropathy. The causative bacteria are strictly non-invasive, remaining confined to the lumen and epithelial surface of the gut. The essential pathogenic events involve: (1) ingestion and entrance of viable *V. cholerae* into the small intestine; (2) colonization of the epithelial surface; (3) multiplication and release of enterotoxin and (4) hypersecretion of isotonic fluid by the intestinal epithelium in response to the enterotoxin.

1.3.1 Ingestion and infectious dose

Following ingestion in either water or food, *V. cholerae* must survive the acid environment of the stomach, the first line of defence in the host. It is claimed that infectious dose (ID) is high in healthy adults. Studies with Western volunteers have suggested that 10^8-10^{11} organisms are required to cause diarrhea in 50% adults (ID_{50}; Hornick *et al.* 1971; Cash *et al.* 1974c). The significance of gastric acidity as a barrier to choleraic infection is revealed by studies which have shown that prior neutralization of gastric acid by administration of sodium bicarbonate dramatically reduces the ID_{50} to 10^3-10^6 organisms (Hornick *et al.* 1971; Cash *et al.* 1974c; Levine *et al.* 1981b).

The ID_{50} among the inhabitants of endemic areas remains unknown, but malnutrition would be expected to reduce the efficacy of the gastric acid barrier. In such areas individuals with achlorhydria are predisposed to this disease (Gitelson 1971; Sack *et al.* 1972). The ID is also lowered if vibrios are ingested in contaminated food, which presumably protects the organisms during their passage through the stomach (Levine *et al.* 1981b).

1.3.2 Penetration of the mucus layer

The viable organisms which reach the small intestine encounter two additional non-specific host defence mechanisms; namely, intestinal peristalsis (Dixon 1960) and a mucus layer (Florey 1933; Freter & Jones 1976; Schrank & Verwey 1976) which the organisms must penetrate in order to reach the epithelial surface. *V. cholerae* uses an array of virulence properties in overcoming these barriers. The flagellum is responsible for motility and allows the bacteria to respond to chemotaxins such as L-aminoacids and simple sugars which diffuse away from the mucus surface (Freter & O'Brien 1981a, 1981b; Freter *et al.* 1981).

Several workers using animal or *in vitro* models have demonstrated that motile *V. cholerae* can be rapidly detected in the intervillous spaces and crypts of the small intestine (Guentzel & Berry 1975; Nelson *et al.* 1976; Schrank & Verwey 1976; Freter *et al.* 1981). Mutant strains that are non-motile but enterotoxigenic colonize these regions much less efficiently and therefore exhibit reduced virulence (Guentzel & Berry 1975; Schrank & Verwey 1976; Pierce *et al.* 1988). Electron microscopy (EM) studies have shown vibrios adherent to enterocytes as well as associated with mucin (Nelson *et al.* 1976; Guentzel *et al.* 1981).

To gain entrance into the mucus gel covering the epithelial surface, *V. cholerae* releases a potent mucinase (Burnet & Stone 1947; Burnet 1949; Freter 1955b). *In vitro* studies have indicated that the forward movement of vibrios is impeded by mucus, although the organisms can migrate through channels in the mucus gel (Jones *et al.* 1976). It is suggested that, *in vivo*, such channels result from the action of the mucinase. Various studies have suggested that a soluble hemagglutinin possessing protease activity (see Section 1.3.3.3) might contribute to virulence of *V. cholerae* (reviewed by Booth & Finkelstein 1986), possibly by fulfilling a mucinase function *in vivo* (Schneider & Parker 1978; Finkelstein *et al.* 1983).

1.3.3 Adherence to the epithelium of the small intestine

Adhesion secures the bacteria against the effects of intestinal peristalsis and facilitates delivery of the enterotoxin. The precise molecular mechanism by which the organism attaches to enterocytes of the crypts and villi is not entirely clear. A number of cell surface components (adhesins) could be involved. These include flagellar sheath proteins and other flagellar antigens (Eubanks *et al.* 1977; Attridge & Rowley 1983a, 1983b), lipopolysaccharide (Chitnis *et al.* 1982a, 1982b), hemagglutinins and *cholera lectin* (Finkelstein *et al.* 1983), and pili (Taylor *et al.* 1987; Herrington *et al.* 1988).

1.3.3.1 Flagellar proteins

There is little doubt that the flagellar structure plays an important role in the colonization of *V. cholerae*. Studies in the infant mouse model showed that motile strains were more virulent than non-motile derivatives obtained by mutagenesis (Guentzel & Berry 1975), and microscopic observations of intestinal sections showed that this difference correlated with a more effective colonization by the former (Guentzel *et al.* 1977). In a later study, mixed suspensions of related motility variants were fed to infant mice, and the motile bacteria shown to enjoy a significant advantage in terms of intestinal colonization (Attridge & Rowley 1983a). These reports are supported by numerous *in vitro* studies which show that motile vibrios attach more efficiently to a variety of substrates (Guentzel & Berry 1975; Freter & Jones 1976; Jones & Freter 1976; Attridge & Rowley 1983a, 1983b).

Whether the involvement of the flagellum extends beyond its role as an agent of motility remains uncertain, although reports suggest that this structure also plays a role in the process of attachment. Jones & Freter (1976) showed that flagellated vibrios could bind to brush border membranes *in vitro* but that non-motile organisms could not, even if they were compacted onto the substrate by centrifugation. Attridge & Rowley (1983a) reported that flagellated vibrios which had been immobilized by preincubation with antibodies to LPS, retained the capacity to adhere to segments of intestinal tissue, whereas non-flagellated organisms were non-adherent. The only apparent difference between the two populations was the presence of an immobilized flagellum, suggesting the existence of a flagellar adhesin. In this respect, some investigators have described a flagellar sheath protein (Follet & Gordon 1963; Hranitzky *et al.* 1980). Antibody directed against this protein appears protective against cholera in two animal models; and it has been suggested that this protein may serve as an adhesin (Eubanks *et al.* 1977; Yancey *et al.* 1979). Nonetheless, EM studies indicate that vibrios do not attach by their

flagellum - the flagellum sticks into the lumen of the gut (Nelson *et al.* 1976). It could be that the initial contact is made by the flagellum and the organism then adheres horizontally to allow greater surface contact.

1.3.3.2 Lipopolysaccharide (LPS)

Some evidence has accumulated to suggest that LPS may play a role in adhesion of *V. cholerae* to intestinal mucosa. Using the rabbit ileal-loop model, Chitnis and co-workers (1982b) have demonstrated that purified LPS prepared from an Inaba strain of *V. cholerae* (569B), but not from *Escherichia coli*, can significantly reduce the attachment of other Inaba strains to the mucosal epithelium. Furthermore, several workers have shown the role of antibodies to LPS (Ogawa and Inaba) in the inhibition of attachment of *V. cholerae* strains to intestinal mucosa both *in vitro* and *in vivo* (Freter & Jones 1976; Chitnis *et al.* 1982a, 1982b; Attridge & Rowley 1983c). Lastly, Finkelstein and co-workers, using monoclonal antibodies (MoAbs) directed against *V. cholerae* LPS (presumably directed against specific O-antigen determinants) were able to inhibit the hemagglutination reaction caused by vibrios or LPS (Booth *et al.* 1986).

1.3.3.3 Hemagglutinins (HAs)

Although hemagglutination systems have been widely used in the study of *V. cholerae* adherence, the mechanisms of attachment to erythrocytes (RBCs) and to intact mucosal surfaces may differ (Attridge & Rowley 1983b). Before using RBCs as a convenient substitute for a more relevant substrate, it is therefore important to initially confirm that the characteristics of attachment are similar in the two systems (Jones *et al.* 1976; Jones 1980).

V. cholerae agglutinate certain species of RBCs and this property correlates with adherence to brush border membranes (Jones & Freter 1976). Adhesion to the intestinal

epithelium and hemagglutination may be inhibited by different sugars (Jones & Freter 1976). It is now known that *V. cholerae* strains produce four distinct HAs which can be divided into two classes: cell-associated and soluble (Hanne & Finkelstein 1982). Three cell-associated HAs have been described so far: a D-mannose-sensitive HA (MSHA) produced by El Tor strains; an L-fucose-sensitive HA (FSHA) expressed by Classical strains; and a mannose-fucose-resistant HA (MFRHA) expressed by both El Tor and Classical strains. The fourth HA is soluble, possesses protease activity (SHA/protease) and is found in all strains regardless of biotype or serotype.

There is some evidence that cell-associated HAs of *V. cholerae* are involved in adherence and may represent adhesins (Guinée *et al.* 1985; Finn *et al.* 1987) although a direct correlation between intestinal attachment and HA expression has not always been seen (Freter & Jones 1976; Teppema *et al.* 1987). More recently, *in vitro* adherence studies of *V. cholerae* to formalin-fixed human villus cells (Yamamoto *et al.* 1988) or human mucus coat (Yamamoto & Yokota 1988) have shown that adherence did correlate with cellular HA levels, irrespective of biotype or serotype. Similar inhibition patterns were obtained between adherence and hemagglutination reaction, indicating that cellular HAs might play a role, at least in part, in adherence (Yamamoto *et al.* 1988). The relevance of these studies to the pathogenesis of the disease is not known. Finn *et al.* (1987) isolated an MSHA$^-$ mutant from a *V. cholerae* strain JBK70 (a mutant El Tor Inaba strain lacking the cholera *tox* gene), and the mutant was much less able to colonize the ileal mucosa following oral administration to rabbits. This suggests that the El Tor MSHA might be considered as a putative attachment factor.

The SHA/protease, previously referred to as *cholera lectin* (Finkelstein *et al.* 1978), has now been purified and characterized (Finkelstein & Hanne 1982). It was originally thought to be an adhesive factor as it inhibited the attachment of vibrios of either biotype to epithelial cells both *in vitro* and *in vivo* (Finkelstein *et al.* 1978). However, there is, at present, no con-

vincing evidence that it could be an adhesin. Although the above studies suggest that expression of HAs could promote bacterial adhesion *in vitro*, it remains to be seen whether any of these HAs are operating as adhesins during infection. As discussed below, pili have recently been described on *V. cholerae* (Taylor *et al.* 1987) and it is unclear whether these are associated with HA properties as has been described for other bacteria possessing adhesive pili on their surface (Duguid & Old 1980; Gaastra & de Graaf 1982).

1.3.3.4 Pili/fimbriae

Pili are non-flagellar, filamentous protein appendages projecting outward from the surface of many gram-negative bacteria and are associated with the colonization of the natural environment (Brinton J. 1978; Smith & Linggood 1971; Nagy *et al.* 1977). These appendages were independently described and introduced as fimbriae by Duguid *et al.* (1955) and as pili by Brinton (1959). Evidence is accumulating to suggest that the actual adhesins of these bacteria consist of minor protein molecules at the tip of surface pili. This has been demonstrated in both enterotoxigenic (ETEC) and uropathogenic strains of *E. coli* (Gaastra & de Graaf 1982). By analogy, it has therefore been assumed that pili would be involved in the pathogenesis of cholera but pili expression by *V. cholerae*, although the subject of much discussion, led to conflicting reports in the sixties and seventies (Finkelstein & Mukerjee 1963; Barua & Chatterjee 1964; Lankford & Legsomburana 1965; Tweedy *et al.* 1968; Nelson *et al.* 1976). But in the eighties, reports have appeared confirming pili expression by *V. cholerae* (Faris *et al.* 1982; Al-Kaissi & Mostratos 1985; Ehara *et al.* 1986; Taylor *et al.* 1987).

To date, four types of pili of *V. cholerae* have been described. Ehara *et al.* (1986) described flexible pili on *V. cholerae* during the colonization of the upper small intestine in rabbits. This pilus has now been purified and the structural subunit is a protein of 16 kDa (Ehara *et al.* 1987, 1988) shown to be shared by O1 (both El Tor and Classical strains) and non-O1

V. cholerae (Nakasone & Iwanaga 1988). Antisera from convalescent patients contained antibodies against the pili subunit whilst antisera raised against pili subunits neutralized the hemagglutinating activity of pili preparations (Ehara *et al.* 1988).

Recent reports have described the discovery of a pilus structure on the surface of Classical Ogawa O395 (Taylor *et al.* 1987) confirmed by Hall *et al.* (1988). This pilus is now referred to as a *t*oxin *c*o-regulated *p*ilus (TCP). In addition, Hall *et al.* (1988) have described that *V. cholerae* can express two other morphologically distinct pili besides the TCP; namely, Type B with a wavy morphology and Type C being more rigid. Whether or not any of these two types are related to or identical to that described by Ehara *et al.* (1986, 1988) remains to be established. The significance of these latter types has yet to be elucidated. There is compelling evidence that TCP may be an important colonization factor of *V. cholerae*. This significance is discussed further in subsequent chapters.

1.3.4 Production of enterotoxin and fluid accumulation

After successfully attaching to the enterocytes, the *V. cholerae* organisms enter a phase of active growth, during which they elaborate an enterotoxin. This molecule comprises two types of subunits, A and B (see Section 1.8.3), the latter mediating binding to the enterocyte membrane (Cuatrecasas 1973) via a receptor now identified as a sialidase-resistant monosialosyl ganglioside (G_{M1}) (van Heyningen *et al.* 1971; King & van Heyningen 1973). It is the oligosaccharide portion of the ganglioside to which the toxin binds and there is a correlation between G_{M1} content and binding ability of cells (Holmgren *et al.* 1975a; Hansson *et al.* 1977). Once attachment has occurred, the A subunit, an enzyme, then passes across the cell membrane into the cytoplasm and activates the enzyme adenylate cyclase. This in turn leads to elevated intracellular levels of cyclic 3'5'-adenosine monophosphate (cyclic AMP), resulting in an irreversible hypersecretory response (Petersen *et al.* 1972; Holmgren *et al.*

1975a) by cells in the crypt and on the sides of the villi. The secreted fluid is low in protein and rich in electrolyte including Na^+, K^+, Cl^- & HCO_3^-. Although the toxin is rapidly bound, there is a lag period ranging from 10 minutes to 1 hour before changes in ion transport are seen. This delay probably relates to the time taken for the A subunit to penetrate the cell membrane (Fishman 1980).

In both clinical (Greenough 1965; Banwell *et al.* 1970) and experimental (Carpenter *et al.* 1968) cholera, the fluid loss occurs from all segments of the small bowel with a decreasing gradient from duodenum to ileum. The resultant loss of large volumes of isotonic fluid is responsible for the characteristic clinical profile of the disease. As mentioned below, there is no evidence that any structural lesions are produced by the toxin.

It has been proposed that the action of cholera toxin (CT) promotes the intestinal growth of *V. cholerae* by making available nutrients secreted by epithelial cells (Mekalanos 1985). Construction of site-specific *ctx* mutants has provided the most convincing evidence that toxin production is beneficial for growth in the intestinal environment (Mekalanos 1985). These non-toxinogenic mutant strains colonize rabbit intestines less efficiently than their parental strains (Pierce *et al.* 1988).

Although CT is largely responsible for the voluminous diarrhea characteristic of cholera, two observations suggest *V. cholerae* O1 produces another toxin(s) capable of causing diarrhea. First, there is evidence that naturally occurring CT-negative El Tor strains - whether environmental in origin or associated with cases of diarrhea - cause intestinal secretion when inoculated into certain animal models (Nishibuchi *et al.* 1983; Sanyal *et al.* 1983). Secondly, mild or moderate diarrhea was seen in about 30% of healthy adults fed with viable O1 organisms, of either biotype, that were CT-negative; these were strains whose CT genes had been inactivated by mutagenesis or deleted by recombinant DNA techniques (Levine *et al.* 1988a). The exact nature of non-CT toxin is not known although it could involve the shiga-like

toxin said to be produced by many *V. cholerae* O1 organisms (O'Brien *et al.* 1984). In addition, El Tor strains have been shown to produce a soluble hemolysin which has cytotoxic and enterotoxic activity (Yamamoto *et al.* 1986).

1.3.5 Pathology

The host cellular response to infection is minimal, involving primarily a non-specific increase in mucus-producing goblet cells. There is no major histological damage to the gastrointestinal tract in either man (Gangarosa *et al.* 1960; Fresh *et al.* 1964) or in experimental animals (LaBrec *et al.* 1965; Norris & Majno 1968; Ghosh 1970). Biopsy specimens collected from different regions of the small intestine reveal an intact epithelium. There is edema of the submucosa, dilated crypts and minimal numbers of inflammatory cells in the lamina propria. The intercellular tight-junctions and capillary endothelial cells are also normal. The brush border remains intact and the organisms do not invade the mucosa.

1.4 Clinical Manifestations

Cholera is a disease ranging from asymptomatic infection with mild diarrhea, to a fulminant fatal syndrome, in which form it is one of the most rapidly fatal illnesses known. The incubation period may vary from a few hours to a few days with or without prodromal symptoms. Acute, potentially fatal infection is comparatively rare; the large majority of cases are mild and usually indistinguishable from other forms of gastroenteritis.

In its severest form, the disease progresses from the first liquid stool to shock in 4-12 hours with death following within 18 hours. It is characterized by the sudden onset of effortless vomiting and profuse diarrhea (Greenough 1979) which initially lacks the "rice-water" appearance. As the diarrhea progresses the fecal fluid takes on the typical "rice-water" appearance

due to the presence in the stool of flecks of mucus discharged by the goblet cells as a result of the action of the cholera toxin. Dehydration accounts for the majority of the signs and symptoms seen in cholera. These include thirst, muscular cramps, sunken eyes, cold extremities, thready pulse and sunken fontanelles in babies. In untreated severe cases a patient can lose up to twice his body weight in liquid stools (Hirschhorn *et al.* 1968).

Complications of cholera can occur in both children and adults. These include altered consciousness which in adults is manifested by a detached mental state, while in children unconsciousness or convulsions can occur as a warning of impending hypoglycemia. Electrolyte imbalance leads to hypokalemia and hypernatremia as well as acidosis, particularly in children. Aspiration with fatal sequelae is a serious complication in patients with a combination of altered consciousness and vomiting (Greenough 1979).

1.5 Diagnosis

The most rapid and effective way of diagnosing cholera in the laboratory is by examining a fresh fecal specimen under dark-field illumination. The vibrios can be recognized by their characteristic *darting* motility when present in large numbers. Addition of homologous antisera will immobilize the vibrios and immediate diagnosis can be made (Benenson *et al.* 1964). When stools cannot be examined at once they are best placed in a transport medium such as Cary-Blair medium (Cary & Blair 1964) or Monsur's medium (Monsur 1963).

In the laboratory, the specimens are inoculated into both (a) an enrichment medium (alkaline-peptone water pH 8.4-9.2) and (b) a selective medium (TCBS agar; Kobayashi *et al.* 1963). Growth from enrichment medium is sub-cultured onto the selective medium. All putative *V. cholerae* colonies that are oxidase positive on the selective medium are then subjected to agglutination with a polyvalent O1 serum before being characterized biochemi-

cally. The confirmed isolates of *V. cholerae* can then be phage typed for epidemiological purposes (Glass *et al.* 1983).

1.6 Treatment

The treatment of cholera is simple, both in concept and execution, involving the replacement of the water and salts lost in the cholera stools. Replacement therapy in the form of intravenous infusion of fluids was shown to prevent deaths from severe cholera as early as 1831 (Latta 1831-1832; O'Shaughnessy 1831-1832). Improvement in rehydration therapy based on accurate measurements of salts lost in the cholera stool was subsequently propagated as a correct and totally effective treatment (Watten *et al.* 1959). When promptly applied this replacement therapy reduced the adult mortality rate to less than one percent of those cases severe enough to warrant hospitalization (Hirschhorn *et al.* 1974). However, this form of therapy is technically difficult and expensive.

Successful rehydration can be more conveniently achieved by the oral administration of fluids containing glucose and ions of sodium, potassium, chloride and bicarbonate (Oral Rehydration Therapy-ORT; Nalin & Cash 1974). The glucose facilitates the uptake of water and electrolytes by the small intestine and helps to resolve hypoglycemia whilst potassium prevents the establishment of hypokalemia (Nalin & Cash 1974). The oral rehydration solution (ORS) recommended by the World Health Organization has undergone several clinical trials and has been found to be safe and useful in the treatment of diarrhea from all causes in all patient groups (Mahalanabis 1981; Mahalanabis & Merson 1986). In rural communities of Bangladesh, the ORS has significantly reduced diarrheal morbidity and mortality (Rahaman *et al.* 1979; Chen *et al.* 1980). In the last 10 years, evidence has accumulated showing the importance of nutrition during diarrhea. ORS accompanied with feeding has been shown to rapidly stabilize and then reverse the weight loss and nitrogen imbalance (International Study

Group 1977; Brown *et al.* 1979a; Rabbani 1986). In the worst cases, however, such as severely dehydrated children who are comatosed with high rates of purging and/or uncontrollable vomiting, intravenous replacement therapy is required (Greenough 1980). As soon as the patient is stabilized, ORS can be instituted. With prompt and adequate fluid replacement the patient usually recovers within a few days.

Antibiotics are often administered to reduce the duration and volume of diarrhea, the most commonly employed being tetracycline, which kills *V. cholerae* leading to bacteriologically negative stools after 24 hours (Greenough *et al.* 1964; Carpenter *et al.* 1966). However, widespread use of antibiotics has led to the emergence of plasmid-mediated drug resistance in *V. cholerae* (Mhalu *et al.* 1979; Glass *et al.* 1980; Tabtieng *et al.* 1990), making it imperative to look for alternative means of treatment. One such approach would be pharmacological inhibition of toxin-induced intestinal secretion, the most promising inhibitor being chlorpromazine. However, its attendant sedative effect would limit its clinical usefulness (Greenough & Rabbani 1986).

1.7 Epidemiology

1.7.1 Incidence

Annual worldwide incidence rates of cholera has been estimated to be nearly 8 million cases resulting in approximatley 124,000 deaths (Black 1986). In endemic areas, cholera has the highest attack rate in children, with a peak incidence in the 2 to 9-year old age group (Glass *et al.* 1982). Attack rates decline rapidly with age and the disease is rare after the age of 40 (McCormack *et al.* 1969). This distribution pattern is thought to reflect the induction and maintenance of active immunity as a result of repeated environmental exposure to *V. cholerae* (Mosley *et al.* 1968). This is supported by observations that, when cholera outbreaks occur in

non-endemic regions, individuals of all age groups are equally susceptible (Baine *et al.* 1974), a finding consistent with the absence of (age-dependent) natural immunity.

Since the onset of the seventh pandemic, the epidemiology of cholera has been changing. Although either biotype of *V. cholerae* is capable of causing cholera in its severest form, epidemiological studies revealed that the infection to severe case ratio was approximately ten times higher for the El Tor biotype (Bart *et al.* 1970; Shahid *et al.* 1984). The more recent predominance of the El Tor biotype could be due to the fact that these strains can survive longer in water and in food (Felsenfeld 1963, 1965) as well as in night-soil samples (Bart *et al.* 1970). In Bangladesh, the Classical biotype was replaced by the El Tor biotype in 1973; but in September 1982, Classical cholera reappeared as the cause of a major epidemic and has since remained as the predominant biotype (Samadi *et al.* 1983; Khan *et al.* 1984). In India, however, the El Tor biotype continues to be the principal cause of disease.

In endemic areas the disease has a seasonal occurrence which differs not only in different countries but also in areas which are very close geographically (Martin *et al.* 1969). In Bangladesh before 1972 the peak for cholera (Classical) was post-monsoonal (December) but in 1973 it shifted to October as the Classical biotype was replaced by the El Tor. With the return of the Classical biotype (Samadi *et al.* 1983), cholera peaks twice annually, in December/January and in April/May (Colwell *et al.* 1987). Yet not far away in Calcutta, the peak is pre-monsoonal (Khan & Greenough 1985). The underlying basis for the seasonal incidence of cholera remains unknown, but one possible contributing factor is discussed below.

1.7.2 Aquatic reservoirs

Traditionally, cholera epidemiologists considered that the only natural reservoir of *V. cholerae* was the human intestine, with only a brief period of survival possible outside the human body (Felsenfeld 1974). However, epidemiological studies undertaken in Australia and

Southern U.S.A. suggest the existence of aquatic reservoirs for *V. cholerae* El Tor and thereby offer an explanation for the periodic occurrence of cholera cases in these areas (Blake *et al.* 1980; Bourke *et al.* 1986). It is thought that, like other vibrios, *V. cholerae* may be an autochthonous organism of estuarine and brackish waters (Colwell *et al.* 1977, 1987; Hood & Ness 1982), or at least that the organism is able to persist and multiply in such an environment for long periods of time (Nalin 1976; Blake *et al.* 1980).

In 1973, the first indigenous case in the North American continent in over sixty years occurred in Texas (Weissman *et al.* 1974). Since then cases have been detected on the Gulf Coast of Texas and Louisiana (Blake *et al.* 1980; Shandera *et al.* 1983), and indeed this is now recognized as an endemic area. The most recent cases occurred in Baltimore in 1984 (Lin *et al.* 1986) and in Louisiana and Florida in 1986 (C.D.C. 1986). Epidemiological studies have attributed outbreaks in U.S.A. (Blake *et al.* 1980) and Sardinia (Salmaso *et al.* 1980) to the ingestion of locally caught contaminated seafood. In both locations there had been no cholera in the preceding five years, arguing against the notion that the disease is maintained by regular transmission from infected individuals to water. Instead, they support the hypothesis of the existence of an aquatic reservoir of *V. cholerae*.

Laboratory studies have shown that *V. cholerae* in pure culture can survive for prolonged periods in warm waters without nutrients wherein survival is influenced primarily by salinity, pH and temperature (Singleton *et al.* 1982a, 1982b; Colwell *et al.* 1985b). In such an environment *V. cholerae* can reach a viable but non-culturable stage (Colwell *et al.* 1985a) yet regain its culturable status on human passage (Brayton *et al.* 1987). This suggests that *V. cholerae* can maintain its pathogenic potential during a period in an aquatic environment.

The seasonal incidence of cholera may be at least partly attributable to variations in plankton populations. Oppenheimer and colleagues (1978) have shown that *V. cholerae* can associate with planktonic copepods. Observations in Bangladesh revealed that the zooplankton

population decreases during the monsoon season (May-July) in response to a reduction of nutrients and alteration of salinity. Subsequently, the population increases during August and September, shortly before the appearance of the first cholera cases which initiate the annual epidemic. *V. cholerae* have been shown to produce chitinase (Dastidar & Narayanaswami 1968), to adsorb to a chitin substrate and multiply on such a surface (Nalin *et al.* 1979). Since copepods and crustacea have chitin in their exoskeletons (Huq *et al.* 1986), association with such organisms may therefore explain the abundance of *V. cholerae* in surface waters at certain times of the year (Colwell *et al.* 1987).

1.7.3 Transmission

Historically, most epidemiologists have been proponents of the concept of cholera as an exclusively or primarily waterborne disease. This has been based on the 19th century outbreaks, and in particular, as argued by Feachem (1982), on misinterpretation of the pioneering work of Snow. Snow's investigation of the outbreaks in London revealed that there was a clustering of cases linked with the route of one of the city's two main water supplies (Snow 1855). As pointed out by Feachem (1982), throughout his writings, Snow (1855) maintained a concept of fecal-oral cholera transmission, of which waterborne transmission is but one special case. But person-to-person spread, as a mode of fecaloral transmission, was not overlooked even in Snow's time (reviewed by Feachem 1982).

Epidemiological evidence suggests that person-to-person transmission can occur, particularly in situations where there is overcrowding and poor hygiene, such as hospitals (Mhalu *et al.* 1984; Cliff *et al.* 1986), prisons and refugee camps (Morris *et al.* 1982). In Tanzania, this mode of transmission has been implicated in an outbreak of cholera in a pediatric infectious diseases ward (Mhalu *et al.* 1984). Further opportunity for person-to-person transmission occurs at burial ceremonies, where local tradition dictates the cleansing of the intestines of the

deceased (even of cholera victims) prior to burial. After the ceremony, the participants share a meal served on a communal plate and eating is done with fingers (Mandara & Mhalu 1980).

Recent case control studies have confirmed that, in addition to water, food can serve as a vehicle for the transmission of cholera (Tauxe *et al.* 1988). Foodstuffs incriminated to date include raw or partially cooked shellfish in Malaysia (Dutt *et al.* 1971), Italy (Baine *et al.* 1974) and Portugal (Blake *et al.* 1977), raw fish in the Gilbert Islands (McIntyre *et al.* 1979), and insufficiently cooked crabs in the United States (Blake *et al.* 1980). As already discussed, food may have the effect of lowering the ID of *V. cholerae* by protecting the organisms from gastric acidity during their passage through the stomach. Thus, an ID of 10^6 El Tor vibrios produced severe diarrhea in volunteers when ingested in food, whereas the same number given in water did not (Levine *et al.* 1981b).

In the light of growing evidence for the existence of an aquatic reservoir for *V. cholerae* (see Section 1.7.2), Miller *et al.* (1985) have recently postulated primary and secondary modes of disease transmission. They suggest that in primary transmission vibrios pass from the aquatic reservoir to humans via drinking water or edible aquatic flora or fauna. Subsequently secondary fecal-oral transmission involves person-to-person spread, either directly or via contaminated food or water. This concept is in agreement with the studies of Deb *et al.* (1986). In areas such as Australia (Rogers *et al.* 1980) and the United States (Blake *et al.* 1980), where there are high standards of hygiene and sanitation, little or no secondary transmission would be expected to occur, and as a result an outbreak may comprise primary cases only. The transmission of cholera is therefore complex and it is not always easy to determine the exact mode of dissemination of the disease.

1.8 Potential protective antigens of Vibrio cholerae

The identification and isolation of protective antigens offers one approach to the problem of developing vaccines against bacterial pathogens. Virulence determinants identified by studies on the pathogenesis of infection are obvious candidates for protective antigens. For example, an appreciation of the role of K88 pili in the colonization of the piglet gut by enterotoxigenic *E. coli* led to the development of an effective vaccine comprising purified K88 (Rutter & Jones 1973; Rutter *et al.* 1976). Although various components are thought to assist the pathogenesis of *V. cholerae* (see Section 1.3.3) it remains unclear as to whether any can function as protective antigens for human infection. The evidence implicating these various factors as protective antigens is briefly reviewed.

1.8.1 LPS

The LPS of all serotypes consists of three regions: the lipid A, core oligosaccharides and the repeating O-antigen polysaccharide subunits which represent the outermost surface of the vibrio. The lipid A is hydrophobic and is responsible for the endotoxic biological activity of LPS. The O-antigen region is responsible for antigenic variability and its chemical composition has been determined (Hisatsune *et al.* 1979; Kenne *et al.* 1979, 1982; Redmond 1979). Unlike the LPS of most gram-negative bacteria, the 2-keto-3-deoxyoctonate which links the core sugars to the lipid A is absent in *V. cholerae* LPS; instead, fructose is present (Jann *et al.* 1973; Kabir 1982) and this presumably serves the same linkage function.

Observations made in a variety of experimental models have shown that antibodies directed against the LPS determinants of *V. cholerae* have the potential to mediate protection. This is the case with "open" systems such as the infant mouse model (Neoh & Rowley 1972) or the DIC (duodenal inoculation with cecal ligation) model in adult rabbits (Jansen *et al.* 1988).

Antibodies to LPS are also sufficient to mediate protection in the most commonly employed "closed" system, the rabbit ileal loop model (Svennerholm 1975; Chitnis *et al.* 1982a).

As discussed below (Section 1.8.3), it is generally accepted that resistance to clinical disease is mediated by antibodies directed against antigens of the bacterium rather than the toxin. The relative significance of antibodies to LPS determinants remains uncertain, although circumstantial evidence suggests that LPS may be a protective antigen for human infection. First, field and clinical testing of cholera vaccines has occasionally resulted in serotype-specific protection (Mosley *et al.* 1970; Cash *et al.* 1974a); since antigens associated with LPS are the only ones known to differ between the serotypes, this protection was presumably mediated by antibodies directed against LPS. Second, a purified LPS vaccine conferred transient protection upon adult recipients in a Bangladesh field trial (Joo 1974). Finally, epidemiologic studies have identified inverse correlations between increased levels of anti-LPS antibodies and decreased incidence of disease (Mosley *et al.* 1969; Glass *et al.* 1985).

1.8.2 Non-LPS antigens

Until recently, little interest had been shown in the possible vaccine significance of the non-LPS components of *V. cholerae*. Studies in the infant mouse model had indicated that antibodies to such components were sufficient to mediate protection and that in this system at least these antibodies appeared to be more efficient in this respect than antibodies specific for LPS (Neoh & Rowley 1970; Attridge & Rowley 1983c). The nature of the putative non-LPS protective antigens remains undefined, but candidates would include outer membrane proteins (OMPs), flagellar antigens, HAs and pili.

1.8.2.1 OMPs

Characterization of the outer membranes of *V. cholerae* began comparatively recently (Kabir 1980; Kelly & Parker 1981; Manning *et al.* 1982; Jonson *et al.* 1989). When OMPs of *in vitro* grown organisms are analyzed by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE), approximately 8-10 major proteins ranging in apparent molecular weight from 27-94 kilodaltons (kDa) can be resolved. Major proteins include 3-4 proteins in the range of 45-48 kDa, which probably represent the porins (Kabir 1980; Kelly & Parker 1981; Manning *et al.* 1982; Kabir 1983; Lang & Palva 1987; Jonson *et al.* 1989), a 40 kDa protein, OmpT and a 38 kDa protein, OmpU (Peterson & Mekalanos 1988; Jonson *et al.* 1989). Other OMPs include a 35 kDa OmpA-like protein (Alm *et al.* 1986; Jonson *et al.* 1989) and a 25 kDa protein, OmpV (Stevenson *et al.* 1985; Pohlner *et al.* 1986a, 1986b).

Recent work indicates that the OMP profile of *V. cholerae* is significantly influenced by the growth conditions employed. The most striking example of culture-dependent protein synthesis is the production of pili such as TCP (Section 1.3.3.4). Sciortino & Finkelstein (1983) reported that *V. cholerae* recovered from the intestines of infant rabbits expressed novel OMPs when compared with *in vitro* grown organisms. Culture in iron-depleted medium resulted in a similar OMP profile, suggesting that this was a convenient means of studying proteins synthesized in response to *in vivo* conditions. Earlier studies had shown that *V. cholerae* OMPs are immunogenic in man (Kabir 1983; Sears *et al.* 1984). With the exception of TCP and the flagellar structure, the contribution of any of these OMPs to pathogenesis (and thus the potential protective significance) is unknown.

1.8.2.2 Flagellar (H) antigens

Studies in two different cholera models raise the possibility that *V. cholerae* might bear a protective antigen associated with the flagellar structure. Eubanks *et al.* (1977) reported that an antigen present in a crude flagella fraction was able to elicit protective antibodies in a modification of the infant mouse model. This antigen was not present on a non-flagellated mutant strain, nor did it seem to be associated with the flagellar core protein. Subsequent experiments revealed that the same component was a protective antigen in the adult rabbit ligated ileal loop system (Yancey *et al.* 1979). Although this antigen remains undefined, these studies are of considerable interest in view of the possibility that the flagellum carries an attachment factor which plays a role in colonization (see Section 1.3.3.1).

1.8.2.3 HAs

There is paucity of evidence to suggest that anti-HA antibodies are protective. Chaicumpa & Atthasishtha (1979) reported that an antibody to a cell-associated HA from *V. cholerae* El Tor O17 was marginally protective in the infant mouse cholera model. Other studies in the same system, however, led to the conclusion that the O17 strain lacks non-LPS protective antigens (Attridge & Rowley 1983c). Finkelstein & Hanne (1982) found that Fab fragments of antibodies against purified SHA/protease could inhibit the *in vitro* attachment of *V. cholerae* to infant rabbit small bowel. Subsequent experiments failed to demonstrate protective activity associated with such antibodies (Booth & Finkelstein 1986). A clearer appreciation of the likely vaccine significance of the various HAs produced by *V. cholerae* must await further studies. With the cloning of the gene encoding a *V. cholerae* HA (Franzon & Manning 1986) it may be possible to determine whether or not HAs possess protective activity in cholera.

1.8.2.4 Pili

At the commencement of these studies, nothing was known of the protective significance of antibodies directed against *V. cholerae* pili mainly because the question of pilus expression remained controversial. Recently, however, Taylor *et al.* (1987) described cultural conditions which allowed TCP synthesis by *V. cholerae* and showed that these pili played a critical role in colonization, at least in the infant mouse model. By analogy with other systems, one might speculate that anti-pilus antibodies could be protective against *V. cholerae* by neutralizing the colonization capacity of the bacteria. Thus, pilus vaccines prepared from diarrheagenic strains of *E. coli* and given parenterally to pregnant sows and cows have significantly reduced neonatal deaths from diarrheal disease (Rutter & Jones 1973; Morgan *et al.* 1978; Nagy *et al.* 1978). Protection was shown to be mediated by anti-pilus antibody passively transmitted in maternal colostrum (Rutter & Jones 1973; Morgan *et al.* 1978; Nagy *et al.* 1978). Pilus vaccines have also been effective in preventing experimental *Moraxella bovis* infection in cows (Tramont & Boslego 1985). There is, therefore, a strong possibility that TCP will achieve the status of a protective antigen.

1.8.3 CT

The other major antigen produced by cholera vibrios is the CT (reviewed by Finkelstein 1973; Finkelstein & Dorner 1985). It has been purified to homogeneity (Finkelstein & LoSpalluto 1969, 1970; Richardson & Noftle 1970), and its subunit structure and mechanism of action elucidated (Lonnroth & Holmgren 1973; Holmgren & Lonnroth 1976). CT is an 84 kDa polymeric protein composed of two protomers: an A (27 kDa) or adenylate cyclase stimulating component (choleragen) and a B (58 kDa) protomer (choleragenoid) associated solely with binding (LoSpalluto & Finkelstein 1972; Pierce 1973; van Heyningen 1977). The A

subunit comprises two polypeptide chains, A_1 (22 kDa) and A_2 (5 kDa) (Sattler *et al.* 1975; Lai *et al.* 1976) while five identical subunits (11.6 kDa each) make up the B protomer (Gill 1976; Nakashima *et al.* 1976). The various physiological manifestations of the toxin on different tissues, including the hypersecretory response elicited in the intestinal epithelium, have been found to be based on a single molecular mechanism. Following the binding of the B subunit to the G_{M1} ganglioside of the cell membrane (see Section 1.3.4), the A subunit is cleaved and enters the cell, activating adenylate cyclase and leading to elevated cytoplasmic levels of cyclic AMP (Pierce *et al.* 1971; Gill 1977).

CT is highly immunogenic with nearly all the antibodies directed against the B subunit (Svennerholm 1980; Holmgren 1981). Ninety percent of North American volunteers developed rises in serum IgG antitoxin after an experimental cholera infection and levels were still detectable two years later (Levine *et al.* 1981c). Approximately 60% of volunteers manifested rises in sIgA antitoxin in intestinal fluid, but the response tended to be relatively short-lived with antibody levels dropping one month after challenge (cited by Levine *et al.* 1983).

Studies in various animal models have indicated that, whether passively acquired or induced by active immunization, antibodies to the toxin can protect against experimental cholera (reviewed by Finkelstein 1973, 1975; Finkelstein & Dorner 1985; Holmgren *et al.* 1975b; Yoshiyama & Brown 1987). In contrast, volunteer studies have consistently failed to demonstrate the involvement of such antibodies in resistance to clinical infection (Cash *et al.* 1974a; Levine *et al.* 1979, 1981a). Indeed, a recent report makes it clear that immune responses to the toxin are not necessary for protection in man (Levine *et al.* 1988b). Only two of the three cholera vaccines currently being developed have the potential to generate anti-toxic immunity (see Section 1.10.2).

1.9 Immunity to cholera

It is now clear that cholera infection gives rise to substantial protective immunity to subsequent exposures to the disease. This has been convincingly demonstrated by the fact that the incidence of the disease rapidly declines with increasing age (see Section 1.7.1), and that recurrent infections are rare in the communities where the disease is endemic (Svennerholm 1980; Glass *et al.* 1982). In addition, Levine *et al.* (1981a) reported that, three years after an induced choleraic episode, volunteers were able to resist challenge with a strain of homologous or heterologous serotype.

The nature of the protective immune response remains unclear. Available evidence suggests that antibodies directed against determinants of the bacterium rather than the toxin are primarily responsible for immunity to clinical disease (Cash *et al.* 1974a; Levine *et al.* 1979). In addition, despite the rises in serum antibodies elicited by parenteral vaccines, such formulations have proved ineffective in conferring protection. This indicates that the location, and possibly the isotype, of the antibodies is also critical. Evidently the IgG and IgM antibodies induced by parenteral immunization do not reach the gut in sufficient concentration to make an effective contribution to enteric defence. It is now generally accepted that an effective vaccine will need to be orally administered (Section 1.10.2) to generate antibodies in the intestine to prevent colonization of *V. cholerae*.

1.9.1 Systemic antibody responses to V. cholerae

Infection with *V. cholerae* is associated with a rise in titer of a variety of circulating antibodies, including vibrocidal antibodies (Finkelstein 1962; Mosley 1969) and antibodies to CT (Pierce *et al.* 1970), LPS (Clements *et al.* 1982; Svennerholm *et al.* 1984b) and OMP (Sears *et al.* 1984). Early studies demonstrated that vibriocidal antibody was acquired early in life in

endemic areas; levels increase with age and remain elevated, presumably as a consequence of repeated environmental exposure to *V. cholerae* (Mosley 1969; Glass *et al.* 1985). Sharp increases in titers of both vibriocidal and antitoxic antibodies have also been demonstrated in volunteer studies and in infected individuals from non-endemic areas (Cash *et al.* 1974c; Levine *et al.* 1981c; Snyder *et al.* 1981; Clements *et al.* 1982). However, in contrast to the situation in endemic areas, levels of such antibodies in these individuals are not sustained (Levine *et al.* 1981c; Clements *et al.* 1982).

As mentioned above (Section 1.9), the circulating antibodies generated by inactivated parenteral vaccines do not confer significant protection against the non-invasive *V. cholerae*. Nevertheless, early epidemiological studies revealed an inverse correlation between vibriocidal antibody titers and the incidence of cholera in the field (Mosley 1969). Although such a relationship was not apparent in a volunteer study in which vibriocidal antibodies were elicited by parenteral immunization (Cash *et al.* 1974a), such antibody responses were associated with resistance to challenge in a recent evaluation of an oral cholera vaccine (Tacket *et al.*, In press). Evidently, if vibriocidal antibodies arise as a consequence of oral presentation of *V. cholerae* antigens, they reflect the occurrence of a protective local immune response. Vibriocidal antibody responses may therefore offer a convenient and helpful index of immunogencity to assist the development of improved cholera vaccines (Tacket *et al.*, In press). Since *V. cholerae* is a non-invasive pathogen producing disease at the gut mucosa only, it would be likely that the secretory immune system is the major protective mechanism in limiting infection and the severity of the disease.

1.9.2 Development of intestinal sIgA antibody reponses

Induction of the intestinal response occurs primarily in the Peyer's patches (PP; Woodruff & Clarke 1987; Mestecky & McGhee 1987). These are dome-like swellings on the epithelial surface of the gut which serve as a staging area for migrating lymphoid cells to come in contact with luminal antigens (Husband & Gowans 1978). This interaction begins with uptake of antigen by specialized epithelial cells residing over the PP (Bhalla & Owen 1983). These cells were initially characterized by the presence of luminal surface microfolds rather than microvilli lending them the name M cells for microfold cells (Owen & Jones 1974). When microvilli were observed on these cells, the name persisted and now refers to "membranous" epithelial cells because of the very thin rim of cytoplasm separating the intestinal lumen from the lymphoid cells below (Wolf & Rye 1984). In addition these cells are devoid of proteolytic enzymes and covered with scant mucus thus designed to sample and transport antigen without degrading it (reviewed by Sneller & Strober 1986). Absorbed substances from the lumen are pinocytosed by these M cells and are transmitted to underlying PP in an undenatured form, to be subsequently presented by accessory cells to T and B lymphocytes (Sminia *et al.* 1982; Wilders *et al.* 1983).

The factors which determine whether bacteria present in the gut will be "sampled" by M cells remain unclear. Uptake by absorption may be facilitated by specific membrane receptors or glycoproteins shown by lectin-binding experiments to be present on the surface of M cells (Owen 1983). Further research is necessary to determine whether specific receptors would enhance selective antigenic endocytosis and transport. Owen *et al.* (1986) has recently demonstrated that viable, but not inactivated, *V. cholerae* are indeed ingested by M cells and transferred, apparently intact, to mononuclear cells in underlying lymphoid follicles. They suggested that critical surface antigens or structures, apparently missing from inactivated

bacteria, are required for uptake (Owen *et al.* 1986). Inman *et al.* (1986) have shown that the nature of the surface polysaccharide can influence the rate of *E. coli* sampling by M cells.

Following antigen sensitization, the primed lymphocytes leave the PP via efferent lymphatics without further differentiation (Craig & Cebra 1971). They then pass through mesenteric nodes and enter the thoracic duct from which they migrate entering the systemic circulation to home back to the gut lymph nodes. The concept of a common mucosal immune system linked by emigrating IgA precursor cells has been supported by experimental studies. These have shown that the circulating intestinally-primed lymphoblasts not only return to the sub-epithelial tissues of the gut, but migrate also to the lungs and genitourinary tract as well as to exocrine glands (salivary, lacrimal, mammary) (McDermott & Bienenstock 1979; Bienenstock & Befus 1980; Bienenstock *et al.* 1983). This ensures that the activation of lymphoblasts in a particular mucosal site can result in the deployment of appropriate effector cells throughout that mucosa as well as other mucosal surfaces.

Evidence has now accumulated to indicate that humans also have a common mucosal immune system. Primed lymphoblasts have been detected in the peripheral blood after early intestinal exposure to antigen; in addition, oral immunization results in the secretion of specific sIgA antibodies in saliva, tears, milk and nasal secretions (reviewed by Mestecky 1987). In this context, Lycke *et al.* (1985) found that Swedish volunteers immunized orally with a combined bacterial-toxoid vaccine generated peripheral blood lymphocytes which differentiated into specific cholera IgA antitoxin and anti-LPS antibody-producing cells during a period of *in vitro* culture. Other studies have demonstrated that oral immunization of volunteers or natural disease in cholera induces anti-*V. cholerae* antibody responses in saliva and breast milk (Jertborn *et al.* 1986) also reflecting the gut mucosal responses is part of the common mucosal-associated immune system.

Recent evidence shows that cell surface molecules selectively expressed in mucosal organs are required for lymphocyte homing to these sites (Streeter *et al.* 1988). To enter intestinal tissues, lymphocytes must reach the postcapillary venules in PP, other lymphoid aggregates or the mucosa, and must adhere to the endothelium to begin their migration. It is now clear that this is due to selective and specific interactions between surface determinants of lymphocytes and the endothelial cells (referred to as high endothelial venules, HEV) of the postcapillary venules of the lymphoid tissues. Surface determinants that permit these interactions are found on both the lymphocytes and the endothelial cells. The determinants involved in mucosal tissues differ from those involved in peripheral lymph nodes (reviewed in Möller 1989).

It is becoming increasingly evident that the IgA response is T cell dependent (reviewed in Mestecky & McGhee 1987), particularly in the induction and differentiation of IgA^+ B cells. They have been shown to induce B cells to switch from expression of surface IgM to surface IgA as well as influencing the proliferation and terminal differentiation of B cells through the production of interleukins IL-4 and IL-5, respectively (reviewed by Mestecky & McGhee 1987). The dimeric IgA produced by these cells in the lamina propia is transported into the gut lumen through the columnar epithelial cells via a specific receptor, secretory component, which is located on the basal surface of these cells (Mostov *et al.* 1984) and produced by epithelial cells of a variety of other secretory organs (Brandtzaeg 1985). The secretory component on the sIgA molecule confers some resistance to proteolysis, an important characteristic because of the environment in which sIgA exerts its activity (Lindh 1975). Recently, it has been demonstrated that intestinal antitoxin formation and protection against toxin challenge after oral immunization with CT is thymus-dependent and likely to be under T-cell control (Lycke *et al.* 1987a).

1.9.3 sIgA anti-V. cholerae responses display immunological memory

The intestinal sIgA response to cholera is of relatively short duration, lasting for a few weeks to a few months (Svennerholm *et al.* 1984b). This contrasts with protective immunity in cholera which appears to last for several years (Levine 1980; Glass *et al.* 1982). This paradox has been recently resolved by experimental and clinical studies which document long-term sIgA memory to cholera antigens (Svennerholm *et al.* 1984a; Holmgren & Lycke 1986; Lycke *et al.* 1987b). For example, women in Bangladesh who had been given 2 oral immunizations with cholera B subunit 15 months earlier responded with intestinal IgA antitoxin production more rapidly, and to a lower dose of antigen, than a concurrently tested, previously unimmunized group of volunteers (Lycke & Holmgren 1987). Similarly, 2 years after initial oral immunization of mice, a second dose of antigen evoked a rapid intestinal antibody response which correlated with heightened resistance of gut loops to CT challenge (Lycke & Holmgren 1987). In addition, immunological memory has been shown to be carried by B cells and that it is possible to adoptively transfer long-term memory by these cells isolated from donor animals 1 year after priming (Lycke & Holmgren 1989). These studies all show that the intestinal secretory IgA system has a very efficient immunologic memory.

1.9.4 Mechanism of sIgA antibody-mediated protection against V. cholerae

Various studies have demonstrated that the biological function of sIgA is to prevent initiation of infection by organisms using the mucosal surface as their primary location of attachment during development of disease. While sIgA is neither opsonic nor possesses bacteriocidal activities (reviewed by Underdown & Schiff 1986), it can efficiently cross-link antigens, prevent adhesion and inactivate toxins. sIgA can render bacteria mucophilic (Mag-

nusson & Stjernstrom 1982). It is well known that vibrios are associated with mucus *in vivo* (Jones *et al.* 1976; Nelson *et al.* 1976).

The specific mechanism by which the mucosal immune system functions is poorly understood. Purified sIgA antibodies have been shown to prevent attachment of bacteria to mucosal surfaces. Intestinal loops prepared in orally immunized mice were partially protected from the effects of live cholera organisms in experimental *V. cholerae* infection (Fubara & Freter 1973). The protection appeared to be due to the action of specific antibodies excluding the organisms from attaching to the gut wall. Steele *et al.* (1974) reported that purified sIgA directed against *V. cholerae* LPS was protective in the infant mouse cholera model; subsequent studies in this system suggested that the antibody function necessary for protection is the capacity to inhibit bacterial attachment (Attridge & Rowley 1983c).

Several studies have shown that IgA antitoxin antibodies induced by experimental *V. cholerae* infection, or by oral administration of CT, are associated with protection of intestinal loops against CT challenge (Pierce *et al.* 1978; Tamaru & Brown 1985; Vaerman *et al.* 1985). sIgA can neutalize toxin (Svennerholm 1980). Lycke *et al.* (1987a) have recently demonstrated that IgA antitoxin antibodies synthesized *in vitro* by lamina propia cells have protective or neutralizing activity. Evidence suggests that the essential mechanism is by preventing the B subunits from binding to the G_{M1} ganglioside receptors on the epithelial cells; indeed, most antitoxin antibodies are directed against the B subunit rather than the toxic A subunit after both infection and oral immunization (Holmgren 1981; Lindholm *et al.* 1983).

1.10 Vaccination against cholera

1.10.1 Past approaches to vaccination

A century has elapsed since the first cholera vaccine was administered to human beings, yet there is still no acceptable, effective vaccine for cholera (reviewed by Finkelstein 1984). The history of vaccination against cholera began in 1885 when a vaccine composed of broth cultures of living cholera vibrios was injected subcutaneously by a Spanish physician named Jaime Ferrán y Clua (Bornside 1981). In 1892, Waldeman Mordecai Haffkine, a Russian, developed a regimen of two subcutaneous inoculations of a vaccine which had been "attenuated" by culture at 39 C. Although the vaccine caused malaise, fever, pain and swelling, these side effects were considered acceptable and the vaccine suitable for administration (Bornside 1982). However, due to ethical considerations, live vaccines were then replaced by the killed vaccines of Kolle and other workers (Pollitzer 1959).

1.10.1.1 Conventional killed vaccines

These vaccines were prepared by inactivation, either by phenol, formalin or heat treatment, of suspensions of *V. cholerae* grown on agar or in liquid media. Only in the 1960s, with the advent of controlled field trials as a means of evaluating vaccine efficacy, did it become apparent that such vaccines were not sufficiently effective to have an impact on the incidence and prevalence of cholera (reviewed by Finkelstein 1984). Although some formulations conferred high levels of protection, the immunity quickly waned (Joo 1974; Feeley & Gangarosa 1980). Furthermore, killed parenteral vaccines were generally more effective in older people, for whom the vaccines were thought to boost environmentally acquired natural immunity (Finkelstein 1962). More frequent administration of vaccine was required to observe

similar rates of protection in young children (Joo 1974; Feeley & Gangarosa 1980), the group at highest risk of acquiring infection.

The oral administration of killed vaccines had been considered several times prior to 1960 (Pollitzer 1959; Freter & Gangarosa 1963), but the impetus for such an approach was provided by a report that college students fed heat-killed vibrios mounted both serum and intestinal antibody responses (Freter & Gangarosa 1963). However, subsequent testing showed that the oral route of administration was not as effective as parenteral vaccination in conferring resistance to a clinical infection (Cash et al. 1974b).

1.10.1.2 Toxoid vaccines

The recognition of the fact that cholera is a toxin-mediated disease (De 1959; Finkelstein & LoSpalluto 1969), suggested the feasibility of developing protective toxoid vaccines, an approach which had proved successful in combatting diphtheria and tetanus. As discussed above (Section 1.8.3), data from various animal models indicated that antibodies to the toxin could mediate protection against experimental cholera, prompting the development of toxoid vaccines for clinical application. A formalized toxoid (which was stabilized with glycine to prevent partial reversion to toxicity) was prepared (Ohtomo 1977) and tested in the Philippines (Noriki 1977) but had no impact on the incidence of infection. A glutaraldehyde toxoid prepared by Rappaport and colleagues (1974) was tested in Bangladesh. Protection was observed up to 24 weeks, but the cumulative protection over a one-year period was marginal (Curlin et al. 1976).

Volunteer studies suggest that resistance to clinical infection is provided by antibacterial rather than antitoxic immunity (Cash *et al.* 1974a; Levine *et al.* 1979). Oral administration of three doses of cholera toxoid failed to provide any protection against cholera challenge, whereas previous clinical infection conferred enduring resistance to re-challenge with strains

of homologous or heterologous serotype (Levine *et al.* 1979; 1981a). More recently, Levine *et al.* (1988b) have shown unequivocally that resistance can be expressed in the absence of antibodies to cholera toxin.

1.10.2 Current approaches to vaccination

The disappointing results obtained from field trials designed to evaluate the efficacy of inactivated parenteral vaccines contrasted with the realization that clinical infection is itself a very effective immunizing process. Epidemiologic findings (Mosley *et al.* 1968; Glass *et al.* 1982) and experimental challenge studies in volunteers (Cash *et al.* 1974a; Levine *et al.* 1981a) have demonstrated that cholera infection is followed by potent, long-lasting immunity for at least three years (Levine *et al.* 1981a). Current knowledge about intestinal immunology (Section 1.9) indicates that the oral route is superior to the parenteral route for stimulating the formation of sIgA antibody in the gut mucosa. Indeed, this knowledge, coupled with the collective failure of parenteral vaccines, provided a strong impetus for the development of cholera vaccines suitable for oral administration. Two main approaches have been followed over the past 10-15 years, with a third option being developed more recently.

1.10.2.1 Live attenuated oral vaccines

The basic approach employed in the development of these vaccines has been to inactivate the genes for toxin production, leaving intact those bacterial components required for gut colonization. The prototype vaccine was the *V. cholerae* strain Texas Star-SR which was attenuated by chemical mutagenesis (Honda & Finkelstein 1979). This mutant produces the B but not the A subunit (A^-B^+) of cholera toxin. In volunteer studies, it was found to colonize and proliferate in the gut and to elicit serum vibriocidal antibody responses comparable to those observed following natural infection. Production of sIgA against several vibrio antigens

was demonstrated (Levine *et al.* 1984). However, in spite of the presence of the B subunit, antitoxin responses were poor (Levine *et al.* 1983, 1984). Although the strain was well tolerated by a majority of volunteers, 24% experienced mild diarrhea, a side effect which precluded further work with this strain (Levine *et al.* 1984). It did play a role, however, in exploring the feasibility of mimicking infection-derived immunity by means of an oral attenuated vaccine (Levine *et al.* 1984), thus paving the way for the genetically engineered strains to be discussed below.

The first genetically engineered candidate vaccine strains were described in 1984 (Kaper *et al.* 1984a, 1984b). Strains of *V. cholerae* known to cause disease and to give rise to protection in volunteers (Levine *et al.* 1979; Levine 1980; Levine *et al.* 1981a) were used to prepare a series of derivatives from which the genes encoding both the subunits of the toxin (A^-B^-) or just the A subunit (A^-B^+) were deleted by site-directed mutagenesis (Kaper *et al.* 1984a, 184b).

Volunteer studies with JBK 70 (A^-B^-) and CVD 101 (A^-B^+) showed that these strains colonized well and elicited good vibriocidal and antitoxic (CVD 101) responses. Furthermore, JBK 70 (A^-B^-) vaccine strain gave significant protection (89% efficacy), illustrating that resistance is not dependent upon the generation of antitoxic immunity. However, both strains were reactogenic (Levine *et al.* 1988b), causing mild diarrhea similar to that seen with the prototype Texas Star-SR described above. One possibility is that the process of colonization *per se* provides sufficient irritation to the epithelial surface that mild diarrhea will inevitably occur in a proportion of recipients (Smith & Linggood 1971; Finkelstein 1984; Wanke & Guerrant 1987). Alternatively, *V. cholerae* might secrete multiple toxin molecules, so that deletion of the genes encoding the well-recognized cholera toxin might not completely abrogate diarrheagenic potential (Levine *et al.* 1988b).

Levine *et al.* (1988a, 1988b) have performed further volunteer trials to address this issue. Deletion of the El Tor hemolysin/cytotoxin genes did not decrease reactogenicity of JBK70 or CVD101 (Levine *et al.* 1988b). In addition, studies with CVD103, an A^-B^+ derivative of a Classical Inaba strain 569B which lacks the genes for the El Tor hemolysin, confirmed that this molecule was not the cause of diarrheagenic activity (Levine *et al.* 1988a). However, although CVD103 caused diarrhea in 12% of volunteers, a mercury-resistant derivative appears more promising. CVD103-HgR was well tolerated by 24 of 25 recipients, while retaining the immunogenicity and protective efficacy of CVD103 (Levine *et al.* 1988a). Further clinical evaluation of this strain is clearly warranted. At this stage the precise mechanism responsible for the reactogenicity of these derivatives remains unclear (Kaper 1989).

1.10.2.2 Non-viable oral vaccines

Evidence that antitoxic and antibacterial immunity act synergistically to protect against experimental cholera (Svennerholm & Holmgren 1976) provided the impetus for the development of a combined bacterial-toxoid vaccine. Although protective antitoxic immunity has been shown to be directed primarily against determinants of the B subunit (Holmgren *et al.* 1977; Holmgren & Svennerholm 1985), the targets of protective antibacterial antibodies remain to be elucidated particularly with respect to clinical infection (see Section 1.8). Accordingly the combined vaccine comprises killed whole bacteria and the isolated B subunit of the cholera toxin (Clemens *et al.* 1986).

This vaccines has now been extensively evaluated. Administration has been shown to stimulate local sIgA antitoxin and anti-LPS responses in volunteers in both endemic (Svennerholm *et al.* 1984a) and non-endemic areas (Jertborn *et al.* 1984). No side-effects were observed in studies with several population groups (Black *et al.* 1987; Svennerholm *et al.* 1984a; Migasena *et al.* 1989), or in a Bangladesh field trial (Clemens *et al.* 1987). Vibriocidal serum

responses were low (Black *et al.* 1987; Clemens *et al.* 1987; Migasena *et al.* 1989) when compared with that induced with the attenuated vaccine strains discussed above (Levine *et al.* 1988b). Nevertheless, it has proved to be protective in volunteers (Black *et al.* 1987) and in a recent field trial in Bangladesh (Clemens *et al.* 1988, 1990). To confer a 70% protection rate, it had to be given in two doses to adults in Bangladesh over a three year observation period (Holmgren *et al.* 1989; Clemens *et al.* 1990).

The combined bacterial-toxoid vaccine clearly represents a major advance over the killed parenteral vaccines, in that the protection conferred is enduring. As a vaccine it is not yet ideal, however; its main deficiency is that protection in young children waned rapidly with time after vaccination (Clemens *et al.* 1988, 1990; Holmgren *et al.* 1989). It is also debatable as to whether the inclusion of the B subunit is justifiable from the viewpoint of vaccine production cost. A comparison of the protective efficacies of the combined vaccine and a vaccine comprising just the bacterial component indicates that the advantages of the former are transient (Kaper 1989).

1.10.2.3 Live oral Cholera/Typhoid hybrid vaccine

In an attempt to combine the superior immunogenicity of a live vaccine with the safety of an inactivated formulation, an attempt is now underway to clone a protective antigen from *V. cholerae* into the attenuated, live oral typhoid vaccine *Salmonella typhi* Ty21a (Germanier 1984). The latter is now accepted as an effective typhoid vaccine, the safety of which has been demonstrated in numerous volunteer studies and field trials (Germanier 1984). Although the protective antigens of *V. cholerae* remained undefined with regard to clinical infection, circumstantial evidence suggested that antibodies to LPS could confer immunity (Section 1.8.1). Accordingly the genes responsible for O-antigen synthesis in *V. cholerae* were cloned (Manning *et al.* 1986) and transferred into Ty21a (Morona *et al.* 1988).

One of the resulting clones, EX645, has now been shown to be safe and immunogenic in man (Forrest *et al.* 1989). In a recent cholera challenge trial, volunteers immunized with EX645 and then challenged with pathogenic *V. cholerae* excreted significantly fewer bacteria and suffered significantly less diarrhea than unvaccinated controls (Tacket *et al.*, In press). Although this study showed that antibodies to the Oantigen of *V. cholerae* are sufficient to confer protection against clinical disease, a greater consistency of immune responses will be required before such a vaccine warrants extended evaluation.

1.10.4 Conclusion

To achieve the greatest public health effect in endemic areas an ideal cholera vaccine should be (a) safe without side effects, (b) easy to administer, (c) inexpensive in view of the target population and (d) highly protective preferably after one dose. Clearly none of the vaccine candidates just discussed meet all of these criteria. Because of its safety and long-lasting protective efficacy, the combined inactivated vaccine is the only suitable formulation for widespread use in endemic areas. Unfortunately, however, although safe, the cost of this vaccine, and the fact that multiple doses are required, limits its utility. A greater drawback is the fact that it provides only short term protection in young children, the group with the greatest risk of infection. Although the attenuated live oral vaccine being developed by Levine's group elicits stronger immune responses and would be much cheaper to produce, two problems remain. First, the issue of residual reactogenicity is not yet satisfactorily resolved. Second, the safety and efficacy of such a vaccine remains to be demonstrated in young children. As pointed out by Kaper (1989), rapid progress in vaccine research makes it unlikely that another 100 years will pass without a successful vaccine against cholera.

1.11 Aims of Thesis

At the commencement of these studies the protective antigens of *V. cholerae* remained undefined. Although circumstantial evidence suggested that antibodies to LPS are protective against human disease, the inactivated whole cell vaccine possesses these antigens yet remains inadequate. The presence of non-LPS protective antigens on at least one *V. cholerae* strain (569B) appeared to merit further investigation, to at least ascertain the distribution of these components among recent field isolates. Such a study represents the major aim of Chapter 3 of this thesis. Clearly, if such strains share non-LPS protective antigens, further study of these antigens might eventually identify new components of vaccine significance. During the course of these initial experiments, studies by Mekalanos' group (Taylor *et al.* 1987) resolved the controversy surrounding pilus production by *V. cholerae*. The critical involvement of TCP in infant mouse pathogenesis offered a new approach to cholera vaccine development. It seemed important to confirm these findings, and to determine whether TCP represents a protective antigen in this model. This, together with other attempts to identify non-LPS protective antigens of *V. cholerae*, is the focus of Chapter 4.

The aim of the final experimental chapter of this thesis is to clone genes involved in the biosynthesis of TCP. The derivation of clones expressing TCP would allow unequivocal confirmation of initial findings concerning the significance of these pili as virulence determinants and protective antigens in the infant mouse model.

Chapter 2

Materials and Methods

2.1 Bacterial strains

Most of the strains used are shown in Table 3.1, which lists biotype, serotype, virulence, source and year of isolation (wherever possible). All are resistant to streptomycin (100 μg/ml); when necessary, such resistance was selected by growth on streptomycin-containing (100 μg/ml) nutrient agar (NA; Difco, Detroit) plates. Other strains used but not listed in Table 3.1 are described and discussed in Chapters 4 and 5.

2.2 Maintenance and growth conditions

Permanent stocks were stored in the lyophilized state in 10% skim milk at 4 C, and also frozen at -70 C in a mixture of 0.4 ml 80% (v/v) glycerol plus 0.6 ml 1% peptone (Difco, Bacto-peptone). Strains were reconstituted with nutrient broth (NB; Difco, Detroit) as required. Working stock cultures were maintained on NA stabs stored at room temperature for periods of up to eight weeks. For routine use, the stabs were streaked onto NA plates which were then incubated at 37 C overnight. Several of the resulting colonies were used to inoculate 10-ml aliquots of NB, which were then shaken with vigorous aeration at 37 C unless otherwise

specified. Such cultures typically attained a concentration of 1-2 x 10^9 bacteria per ml after 3-4 hours.

A sloppy agar overlay technique (Stocker 1949) was employed to ensure that the strains remained highly motile because motile strains have been shown to be more virulent in infant mice (Guentzel & Berry 1975; Attridge & Rowley 1983a). A NA plate with 100-200 isolated colonies was overlaid with 10-12 mls of sloppy agar (2 parts NB to 1 part melted 2% NA, prewarmed to 45 C). The plates were left at room temperature for 15 minutes to allow the overlay to set, and then incubated at 37 C. Those colonies comprised of motile bacteria gradually developed a halo as the organisms swam into the sloppy agar. Microscopic observation of broth cultures seeded with "haloed" or "non-haloed" colonies confirmed the reliability of this technique.

2.3 Specialized growth media

Water used to prepare media was deionised and filtered, using the Milli RO60 system (Millipore Corporation, U.S.A.). Media used to study the production of pili in *V. cholerae* strains were the following:

Tryptone water (Tweedy *et al.* 1968): 1g Bacto tryptone and 0.5g NaCl were dissolved in 100 ml of water.

Trypticase soy broth (Faris *et al.* 1982): obtained from Baltimore Biological Laboratories (BBL), Baltimore.

Peptone saline broth (Evans *et al.* 1978): 2g Bacto peptone and 0.5g NaCl dissolved in 100 mls of water.

Peptone saline agar (Evans *et al.* 1978): Peptone saline broth to which 2g of Agar (Difco) was added.

Colonization factor broth (CFB; Evans *et al.* 1979): consisted of 10g casamino acids (Difco), 1.5g yeast extract (Difco), 0.05g $MgSO_4$ and 0.005g $MnCl_2$ in 1 liter of water.

Colonization factor agar (CFA; Evans *et al.* 1979): prepared by adding Bacto agar (2% w/v) to CFB medium.

TCG Medium (Ehara *et al.* 1986): consisted of 10g Bacto tryptone, 2g yeast extract (Difco), 5g NaCl, 3g $NaHCO_3$, 2g monosodium-L-glutamate, 0.2g thioproline (Sigma), 1mM EGTA (Sigma) and 20g Bacto agar in 1 liter of water.

LB Medium (Miller 1972): was prepared by adding 10g tryptone (Difco), 5g yeast extract (Difco) and 10g NaCl to 1 liter of water with adjustment of pH to 6.5.

V. cholerae strains were inoculated into the above media and incubated at different temperatures for varying periods (see Chapter 4).

When appropriate, antibiotics were added to broth and solid media at the following final concentrations: ampicillin (Ap; Sigma) 100 μg/ml; kanamycin (Km; Sigma) 50 μg/ml; tetracycline (Tc; Calbiochem), 10 μg/ml for *E. coli* and 4 μg/ml for *V. cholerae* (Chapter 5); streptomycin (Sm; Glaxo), 100 μg/ml.

2.4 Chemicals, reagents and buffers

Reagents were prepared with deionised and filtered Milli Q water as described above. Chemicals were analytical grade. Phenol, sodium dodecyl sulphate (SDS) and sucrose were from BDH chemicals. Tris was Trizma base from Sigma. EDTA was from Sigma. Bovine serum albumin (BSA; fraction V) was from Flow Laboratories, Rockville, Md.). Cesium chloride (technical grade) was from May & Baker.

The following electrophoresis grade reagents were obtained from the sources indicated: acrylamide and ammonium persulphate (Bio-rad), ultra pure N,N'-methylene-bisacrylamide

from Boehringer Mannheim, West Germany. The substrate for immunoblotting was 4-chloro-1-naphthol (Sigma).

Buffers and solutions were also prepared with Milli Q deionised water. Saline was 0.85% (w/v) NaCl in water, while phosphate buffered saline (PBS; pH 7.2) comprised of 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4 and 0.2g KH_2PO_4 in 1 liter of water.

2.4.1 Enzyme-linked immunosorbent assay (ELISA) Buffers

25mM Tris-HCl (pH 7.5): was made by dissolving 3.03g Tris base in 900 ml of water and adjusting the pH to 7.5 with 1M HCl before making the volume up to 1 liter.

Tris-Saline-Azide (TSA, Coating buffer): prepared by dissolving 7.72g NaCl (132 mM), 3.03g Tris (25 mM) pH 7.5 and 0.5g NaN_3 in 1 liter of water.

Washing buffer with Triton X-100: prepared by dissolving 43.83g NaCl, 3.03g Tris, 5ml Triton X-100 in 4000 ml of water and adjusting the pH to 7.6 with approximately 18 ml of 1M HCl before making the volume to 5 liters.

0.25 M Triethanolamine: 37.5g triethanolamine was neutralised with 2.5M HCl (ca 60 mls) to pH 7.6; 1g NaN_3 was added and the volume made up to 1 liter with water.

BSA/Tween: 16g NaCl, 2g NaN_3, 0.4g BSA, 100 mls of O.25 M triethanolamine, and 10 mls of 10% Tween 20 in 2 liters of water.

Enzyme Diluent (for diluting enzyme/antibody conjugate): 8g NaCl was dissolved in 800 ml water, followed by the addition of 50 ml of 0.25M triethanolamine pH 7.6 and 2mls of 500X Mg^{++}/Zn^{++} (500 mM $MgCl_2$/1.25 mM $ZnCl_2$), 0.2g BSA and 0.2g NaN_3; the volume was made up to 1 liter.

2.4.2 Detergents

The following detergents were used in an attempt to solubilise outer membrane preparations of *V. cholerae*:

ionic: Sodium deoxycholate (BDH) & Sarkosyl (Ciba-Geigy);

non-ionic: Triton X-100 (BDH), Triton X-114 (Serva, Feinbiochemica, Heidelberg), Tween 20 (Sigma), Brij 58 (Serva), Nonidet P40 (BDH), Urea (BDH-Analar), detergent test kit No 263450 (Calbiochem Behring Diagnostics, La Jolla, California);

zwitterionic: Zwittergent detergent test kit No. 693030 (Calbiochem).

2.4.3 Enzymes and immunoconjugates

The following enzymes were obtained from Sigma: deoxyribonuclease I (DNAse I), ribonuclease I (RNAse I), lysozyme and hyaluronidase. Pronase and Proteinase K were from Boehringer-Mannheim.

Restriction endonucleases *Bam*HI, *Eco*R1, *Sau*3A and *Xba*I were purchased from Boehringer-Mannheim. DNA modifying enzymes were purchased from New England Biolabs (T4 DNA ligase) and Boehringer-Mannheim (DNA polymerase I, Klenow fragment of DNA polymerase I and molecular biology grade, calf intestinal alkaline phosphatase).

Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Nordic Immunological Reagents (Tilburg, the Netherlands).

2.5 Animals

2.5.1 Adult mice

Adult female closed-colony inbred outbred LAC mice were obtained from the Central Animal House of the University of Adelaide. These mice were bred under specific pathogen-

free conditions and subsequently moved to a conventional animal room These mice were used for preparation of antisera and as a source of enterocytes for *in vitro* studies of bacterial adherence.

2.5.2 Infant mice

A breeding colony of conventionalized LAC mice was maintained in the Medical School Animal House of the University of Adelaide. Each breeding cage contained four female mice and one male, and the breeding stock was replaced at six-monthly intervals. The infant mice were used at about 5 days of age (weight range restricted to 2.4-2.75g) for studies with the infant mouse cholera model (see below).

2.6 Preparation of boiled and formalin-killed organisms

(i) **Boiling.** Bacteria were grown in NB, washed twice in saline, then resuspended to a concentration of approximately 10^{10} per ml. A viable count was performed, following which the suspension was heated to 100 C for 2 hours. The organisms were then washed three times in saline and resuspended in saline to the original volume; aliquots were plated onto NA to ensure that no viable organisms remained. 0.1% (w/v) NaN_3 was added to the suspension which was then stored at 4 C.

(ii) **Formalin-inactivation.** An equal volume of 1% (v/v) formalin was added to a suspension of *V. cholerae* (at approximately 10^{10} per ml in PBS pH 7.2). After incubation at 37 C for 60 minutes the bacteria were washed three times with PBS and resuspended in saline to the original volume; aliquots were plated on NA to ensure that no viable organisms remained. The suspension was stored at 4 C with 0.1% (w/v) NaN_3.

2.7 Isolation of LPS from V. cholerae

LPS was isolated from several strains during the course of these studies for the production and absorption of antisera, and sensitization of RBCs for hemagglutination assays. LPS was extracted from cells with hot 90% (w/v) phenol/water using the procedure of Westphal & Jann (1965). Briefly, a 100 ml bacterial saline suspension (20 mg/ml dry weight) was heated to 68 C. An equal volume of 90% phenol/water (prewarmed to 68 C) was mixed into the cell suspension and stirred continuously at 68 C for 30 min. The mixture was allowed to cool and the resulting phases separated by centrifugation in glass buckets (1500 rpm, 20 min, Coolspin, MSE). The aqueous phase was collected and stored at 4 C. The phenol phase was re-extracted with half the original volume of prewarmed water (68 C) for 30 min. with stirring, and the phases again separated by centrifugation. The aqueous phases were combined and dialysed against 5 liters of water, overnight at 4 C. Insoluble material was pelleted by centrifugation (500g for 5 min at 4 C in an SS34 rotor, Sorvall). The LPS was precipitated from the supernatant with 5-6 volumes of cold ethanol containing 250 mg of sodium acetate powder. The precipitate was collected by centrifugation (4000g for 30 min. at 4 C in a Sorvall GSA rotor) and resuspended in 25 ml of distilled water containing 5 mM $MgCl_2$. A small quantity (approximately 1 mg) of DNAse I and RNAse I was added, and incubated for 60 min. at room temperature. This was followed by the addition of pronase (1-2 mg) and further incubation for 30 min. The mixture was again centrifuged at low speed, (3000g for 10 min. at 4 C in an SS34 rotor), before deposition of LPS by ultracentrifugation (100,000g for 2 hr at 4 C; 60 Ti rotor in a Beckman L8-80). Each pellet was then suspended in 5 ml distilled water, of which 0.25 ml was used for dry weight and 0.1 ml for estimation of protein by Lowry's method (Lowry *et al.* 1951) using BSA as standard. LPS preparations obtained in this manner usually contained more than 1% contaminating protein and were therefore subjected to a second phenol

extraction. This then reduced the level of protein contamination to < 1%. All preparations were stored at 4 C in the presence of 0.1% (w/v) NaN_3.

2.8 Preparation of OMPs

Preparation of OMPs was carried out as previously described by Manning *et al.* (1982). Bacteria were cultured in NB at 37 C for 18 hr, harvested by centrifugation in a Sorvall GS3 rotor at 8,000g for 15 min, and resuspended in 50 ml of PBS, pH 7.2. After disruption in a French pressure cell (Aminco; Silverspring, MD), the lysate was clarified by centrifugation at 10,000g for 20 min, and the OMP in the supernatant was recovered by ultracentrifugation at 100,000g for 90 min at 4 C (45Ti rotor; Beckman). Each of the resulting pellets was resuspended in 5 ml of distilled water; after pooling, protein content was determined using the method of Lowry *et al.* (1951).

For solubilization of OMPs, 1 ml of OMP (2 mg/ml) and 1 ml of 50 mM Tris-HCl (pH 7.5) with or without detergent were incubated at 37 C for 1 hr. After incubation, the mixture was spun at 100,000g for 30 min at 4 C. The pellet was washed twice with Tris-HCl and finally resuspended in 1 ml of 1x Lug (sample) buffer, aliquotted and frozen at -20 C.

2.9 Production of antisera to whole cells and to OMPs of V. cholerae

2.9.1 Preparation of mouse antisera

Hyperimmune antisera to whole cells were prepared by intraperitoneal immunization of mice with increasing doses (either 10^5-10^7 or 10^7-10^9) of live bacteria; animals were given fortnightly injections for 10 weeks. Blood was withdrawn from the retro-orbital plexus under ether anesthesia 10-12 days after the last immunization and allowed to clot; sera were

separated, filtered (pore size, 0.22 μm; Millipore Corp, Bedford, Mass), and stored at 4 C or at -20 C in aliquots containing 0.02% (w/v) NaN_3.

For the preparation of antisera to OMPs mice were sequentially immunized intraperitoneally on days 0, 14, 21 and 28 with 10, 30, 100 and 120 μg of OMP extracted with buffer alone (control) or with 2% Sarkosyl (Sark-Omp, see Section 4.2.2) respectively. Mice were bled 10 days after the last immunization and sera collected as described above.

2.9.2 Preparation of rabbit antisera

Adult rabbits were obtained from the Central Animal House of this University for production of antisera to whole cells by a combined subcutaneous/intravenous immunization protocol. Equal volumes of 4% sodium alginate and washed cholera vibrios (10^7/ml in normal saline) were mixed and 0.25 ml of the mixture was deposited subcutaneously; without removing the needle, but using a new syringe, 0.15 ml of 0.4% $CaCl_2$ was injected into the same site. This procedure was repeated at three other sites. At the same time 0.5 ml of the washed bacterial suspension was administered intravenously. At 3-4 days intervals thereafter, increasing doses of live *V. cholerae* (10^7-10^9/ml in 0.5 ml saline) were given intravenously. The rabbits were bled by cardiac puncture under anesthesia 10 days after the last immunization. The serum was collected, pooled, filtered and stored as described above.

The method used to prepare antisera to OMPs was essentially that of Boulard & Lecroisey (1982). 569B OMP was electrophoresed in all 16 tracks of an 11-20% SDS-PAGE. After destaining, the gel was immediately cut into individual tracks containing all the proteins and kept at 20 C. The rabbit was immunized without adjuvant by sc injections of the gel homogenates of one track on day 0, three tracks on day 10 and 6 tracks on day 17. The rabbit was bled 10 days after the last injection as described above.

2.10 Absorption of antisera

Antisera were variously absorbed with LPS or OMP isolated from *V. cholerae*, or with intact bacteria, as described below.

2.10.1 Absorption with LPS or OMP

Antisera were absorbed three or four times with LPS (0.1 mg/ml) or OMP (1.0 mg/ml) as required. Alternate absorptions were incubated at 37 C for 2 hr and at 4 C overnight, the latter to allow the removal of less avid antibodies. After the first of each absorption series the suspension was spun at low speed to remove the visible precipitate; with subsequent absorptions, LPS or OMP were removed by ultracentrifugation at 100,000g (Beckman LT80).

2.10.2 Absorption with bacteria

Unless specified otherwise, antisera were absorbed three or four times with live or boiled *V. cholerae* (5×10^{10}/ml) with alternating incubation conditions as described above. After each absorption, bacteria were removed by low speed centrifugation (4,000g), and at the end of the absorption series the serum was sterilized by passage through a 0.22 μm filter.

2.11 In vitro assays of V. cholerae adherence: attachment to isolated murine intestinal epithelial cells (enterocytes).

2.11.1 Preparation of enterocytes

The method used for enterocyte preparation is a modification of that described by Curman *et al.* (1979). The mice were killed by cervical dislocation, and the entire small intestines excised and flushed with 50-100 ml of ice-cold PBS (pH 7.2). After trimming away

adhering mesentery and Peyer's patches, each intestine was slit longitudinally and rinsed in PBS before being cut into 1-2 cm strips; these were incubated in ice-cold PBS (containing 5mM EDTA) with mild agitation for 15 min. The strips were then washed in cold PBS and incubated in prewarmed PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg BSA and 2 mg/ml hyaluronidase for 50 min at 37 C with intermittent shaking.

The resulting enzymatic digest was filtered through a nylon sieve to separate the epithelial cells from tissue segments and large cell aggregates. The filtered cells were sedimented by centrifugation at 100g for 3 min at 4 C, washed twice in PBS and resuspended in 2 ml of 30% (v/v) Percoll (Pharmacia, Uppsala, Sweden) containing 3 mM dithiothreitol (Sigma). The suspension was then layered over a discontinuous Percoll gradient (1 ml layers of 70%, 60% and 50% and a 2-ml layer of 40% [v/v]) in screw-capped siliconized glass tubes. The cells were centrifuged at 100g for 10 min at 4 C in a bench centrifuge (MSE) with a swing-out rotor for separation of enterocytes from lymphocytes. The purified enterocytes were washed twice with PBS and resuspended in 1 ml of RPMI 1640 medium (Flow). The total cell count was determined in a hemocytometer, and the concentration was adjusted to 1-2 x 10^5 cells/ml by addition of RPMI 1640 medium. Viability was assessed by exclusion of 0.2% (w/v) trypan blue and was generally 60%.

2.11.2 In vitro enterocyte adherence assay

For each assay 0.1 ml of bacterial suspension (1-3 x 10^7/ml in PBS) was added to 1 ml of freshly prepared enterocytes (1-2 x 10^5/ml in RPMI 1640 medium) in disposable plastic centrifuge tubes (Sterilin, Middlesex,England). Preliminary experiments had indicated that a ratio of 10 bacteria per enterocyte was optimal for assessment of bacterial adherence. After incubation at 37 C for 15 min, the enterocytes were sedimented by centrifugation at 100g for 3 min, and nonadherent bacteria removed by three 10ml washings with PBS. The pellet was

resuspended in 1 ml of incubation medium and homogenized at full speed for 15 sec (Ultra Turrax, Janke & Kunkel, Staufen, Federal Republic of Germany). Suitable dilutions of the homogenate were made in normal saline and plated on NA plates containing Sm. Percentage adherence was calculated by relating the total number of bacteria adhering to the enterocytes to the number added initially.

2.11.3 Inhibition of adherence.

An assay was developed to assess the capacities of various antisera to inhibit the attachment of vibrios to enterocytes. Bacteria were pretreated with the test antiserum at various dilutions or with normal mouse serum at a final dilution of 1:10 for 10 min at 37 C. They were then added to the enterocyte suspension for determination of residual adherence capacity as described above.

2.12 In vivo assays with infant mice

The infant mouse cholera model was first described by Ujiiye *et al.* (1968) and subsequently extended by Chaicumpa & Rowley (1972) who demonstrated that specific antisera could protect mice from experimental cholera. This model has been used in these studies to assess the virulence of different strains of *V. cholerae* and to determine the efficiencies with which various antisera are able to provide protection against experimental cholera (Attridge & Rowley 1983a, 1983c).

The infant mice used in these assays (Section 2.5.2) were removed from their parents about 5-6 hours before use, to permit emptying of stomach contents. Unless stated otherwise, the challenge strain was grown in NB at 37 C to a concentration of approximatelt 10^9/ml. Depending upon the challenge dose, the organisms were either simply diluted or alternatively

spun out of growth medium and resuspended in peptone saline (PS, a 0.1% w/v solution of proteose-peptone [Difco] in saline). After appropriate dilution, 0.1 ml aliquots were administered orally to infant mice using a smooth-tipped hypodermic needle (22g). After challenge the mice were not returned to their mothers but were kept in tissue-lined plastic containers in a laboratory incubator at 25 C.

2.12.1 Virulence assays

Serial five-fold dilutions were prepared from a PS suspension of the test strain of *V. cholerae*, and each was used to feed one group of 8-12 mice. Forty-eight hours after challenge, the survival of mice within each group was noted, and these data used to construct a plot of cumulative percentage mortality versus $\log_{10}$ challenge dose (using the method of Reed & Muench 1938). By interpolation it was then possible to determine the (48 hour) LD_{50} dose for a given strain - that is, the number of organisms capable of killing 50% of the mice to which it is administered.

2.12.2 Protection assays

Five-fold dilutions of the test serum were used to pre-treat separate aliquots of a PS suspension of the challenge organisms. After incubation at 37 C for 15 minutes, these mixtures were fed to different groups of infant mice (8-12 per group). The bacterial concentration in the original suspension had been arranged such that each mouse would receive 20 LD_{50} doses in the 0.1 ml challenge inoculum. Control mice received organisms which had been incubated in PS alone, or in the presence of normal mouse serum. The survival of all groups was then followed until all of the control mice had died; generally this occurred 42-52 hours after challenge (whereas mice given PS alone consistently survived for 72 hours).

Following the plotting of cumulative percentage mortality against $\log_{10}$ serum dilution (Reed & Muench 1938), the protective endpoint of the serum was obtained by interpolation as that (theoretical) dilution which could protect 50% of the mice from death. The reciprocal of this dilution was referred to as the protective titer of the serum, expressed in PD_{50} units per ml.

2.13 Isolation of V. cholerae pili

Glass trays (30.5 x 30.5 cm) containing 500 ml of CFA were seeded with 10^9 (nonmotile) *V. cholerae* 569B and incubated for 36 hours at 25 C. The bacteria were harvested and resuspended in 12.5 mM Tris-HCl, pH 7.0 containing 25mM NaCl, 4mM $MgCl_2$ and 4 mM $CaCl_2$ (Taylor *et al.* 1987) or in 10 mM Tris buffer, pH 7.5, (Korhonen *et al.* 1980) or in PBS pH 7.2 (Al-kaissi & Mostratos 1985), to a final cell concentration of 0.14 g/ml. Cells were sheared by a 5 min treatment in a Sorvall Omnimixer (at half speed) on ice. The suspension was clarified by centrifugation at 2600g and the pellet resuspended to its original volume and further sheared by two passages through a 21-gauge needle. After another clarifying spin, the two supernatants were pooled, treated with 50% saturated ammonium sulphate and left overnight at 4 C. The resulting precipitate was centrifuged (10,000g for 30 min at 4 C) and the free pili recovered from the supernatant by ultracentrifugation (100,000g for 1 hr at 4 C) and suspended in 2 ml of PBS. This suspension was applied to a self-generating isopycnic cesium chloride gradient. After ultracentrifugation (150,000g for 18 hours at 4 C) three distinct bands were observed and these were collected by puncturing the side of the tube with a 19-gauge needle and aspirating with a syringe. After removal of cesium chloride by dialysis against PBS, the three fractions were analysed by SDS-PAGE and by transmission electron microscopy.

2.14 In vitro titration of antibodies to V. cholerae

2.14.1 Hemagglutination

For efficient sensitization of sheep RBCs (SRBCs) with LPS, it was necessary to pre-treat the LPS with alkali. LPS was diluted to a final concentration of 2 mg/ml and treated with 0.02N NaOH (using 1N NaOH). After overnight stirring at room temperature the pH was restored to neutrality (by addition of concentrated HCl) and the material stored at 4 C with 0.1% (w/v) NaN_3.

SRBCs were washed three times and resuspended to a concentraion of 2.5% (v/v) in saline. Alkali-treated LPS was added to a final concentration of 50 μg/ml, and the mixture incubated with continuous rolling at 37 C for 90 minutes. The SRBCs were again washed three times, and finally resuspended to 1% (v/v) in saline. Sensitized SRBCs were always freshly prepared on the day of use.

Serial two-fold dilutions of the test sera or normal serum were made in saline in plastic microtiter trays (Disposable Products, Australia). A standard serum of known hemagglutinating capacity was included to check the efficiency of erythrocyte sensitization. Following the addition of an equal volume (25μl) of a 1% (v/v) suspension of sensitized SRBCs to each well, the trays were shaken briefly and incubated at 37 C for 60 minutes. The hemagglutination titer was expressed as the reciprocal of the highest serum dilution effecting an RBC settling pattern clearly different from that seen in control wells (lacking serum).

2.14.2 ELISA

ELISA was performed in round-bottomed vinyl microtiter plates (Costar, Cambridge, Mass) using buffers and solutions described in Section 2.4.1. Trays were sensitized with OMPs of *V. cholerae* (see section 2.8) at a concentration of 10 μg/ml in TSA buffer (coating buffer);

100 μl of antigen was added to each well and incubated at 37 C for 2 hours followed by a further incubation overnight at 4 C. After washing three times with washing buffer, the wells were blocked with 120 μl of BSA-Tween for 6-8 hours at 4 C, and then washed again as before. To assay antibodies in serum, duplicate 4-fold serial dilutions were prepared using BSA-Tween and incubated overnight at 4 C. The trays were then washed four times and bound antibody was detected by application of a goat anti-rabbit globulin alkaline-phosphatase conjugate (kindly supplied by Dr. Peter Ey of this Department; optimal dilution of 1:1500) in enzyme diluent (100 μl per well). After overnight incubation at 4 C, the trays were again washed four times and 100 μl of 1 mg/ml substrate solution (p-nitrophenyl phosphate disodium [Sigma] in 10% diethanolamine buffer) was added to each well. After incubation for 3 hours at 37 C, the resulting color development was read at 405nm using a Titertek Multiscan automated spectrophotometer, with reference to control wells which had received diluent instead of antibody dilution. The antibody titer of any given sample was defined as the reciprocal of the dilution which produced a mean OD of 0.150. Normal and positive sera were always included as controls.

2.15 SDS-PAGE and immunoblotting.

2.15.1 SDS-PAGE

SDS-PAGE of OMP, whole cell lysates and other protein fractions were performed on 11-20% polyacrylamide gradients or straight 12% polyacrylamide gels using a modification of the procedure of Lugtenberg *et al.* (1975) as described by Achtman *et al.* (1978). Samples were resuspended in 1x Lug (sample) buffer and heated at 100 C for 3 min prior to loading. Gels were generally electrophoresed at 100 V for 4 hours (11-20% gradient gels) or 25 mA constant current (12% PAGE gels). Protein staining was achieved by incubation overnight with gentle agitation with Coomassie Brilliant Blue G250 in 50% (v/v) methanol and 10% (v/v) acetic acid.

Destaining was achieved using several changes of 7.5% (v/v) acetic acid, 10% (v/v) methanol and 10% (v/v) ethanol over 24 hours.

Molecular weight markers (Sigma) were α-lactalbumin (14,200), ß-lactoglobulin (bovine milk; 18,400), trypsin inhibitor, soybean (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000), bovine serum albumin (66,000), phosphorylase B (97,400), ß-galactosidase (116,000) and myosin (205,000).

2.15.2 Immunoblotting (Western blotting)

After SDS-PAGE the proteins were electrophoretically transferred to nitrocellulose (Schleicher & Schüll, Dassel) for 2 hours at 200 mA in a transblot cell (Bio-rad). The transfer buffer used was 25mM Tris-HCl, pH 8.3, containing 192 mM glycine and 5% (v/v) methanol. After transfer, the nitrocellulose sheet was incubated for 30 minutes in 5% (w/v) skim milk powder in Tween-Tris-buffered saline (TTBS; 0.05% [v/v] Tween 20, 20 mM Tris-HCl, 0.9% [w/v] NaCl) to block non-specific protein binding sites. The nitrocellulose was then reacted with antiserum by the method of Towbin *et al.* (1979). This was then followed by overnight incubation of the nitrocellulose at room temperature in a 1:2,000 dilution of a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG. Binding of primary antibody was detected by 5-10 minutes incubation at room temperature with the substrate (10 mg 4-chloro-1-napthol [Sigma] dissolved in 3.3 mls 99.5% methanol and mixed with 16.5 mls of Tris-buffered saline (TBS; 20 mM Tris-HCl, 0.9% [w/v] NaCl) containing 30 μl H_2O_2) as described by Hawkes *et al.* (1982).

2.16 Colony blotting

A nitrocellulose disc (9 cm diameter, Schleicher & Schüll, Dassel) was placed onto agar plates containing the colonies to be screened. Once the colonies had adhered to the disc (1 min), it was removed and placed, colony side up, on a piece of Whatman 3 MM paper; bacteria were lysed *in situ* with 0.5 M HCl and left in the dark for 30 min, according to Hawkes *et al.* (1982). The cell debris was removed from the nitrocellulose with a jet of normal saline. The method used for antigen detection was the same as that used following Western transfer.

2.17 Construction of V. cholerae gene bank

2.17.1 Preparation of V. cholerae genomic DNA

This was essentially as described by Manning *et al.* (1986). Cells were grown overnight with aeration at 37 C in 100 ml of brain heart infusion broth (BHI, Difco). Bacteria were centrifuged at room temperature and resuspended in 2 mls cold 25% (w/v) sucrose in 50 mM Tris-HCl (pH 8.0). To this was added 1 ml lysozyme (5 mg in 0.25 M EDTA, pH 8.0) and the mixture incubated on ice for 10 min. After the addition of pronase (10 mg in 1 ml of TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), the mixture was incubated for 10 minutes at 37 C followed by the addition of 0.25 ml of lysis solution (5% Sarkosyl in 50 mM TrisHCl, 0.25 M EDTA, pH 8.0). This was incubated at 56 C for 60 min with occasional gentle swirling. The lysate was then gently extracted three times with TE buffer saturated with phenol (prepared by adding 1g phenol per ml of TE buffer). Residual phenol was removed by extracting twice with diethyl ether, and the DNA solution was then dialysed overnight against 5 liters of TE buffer at 4 C. The DNA yield was routinely 800 to 1,300 μg/ml per 100 ml cultures.

2.17.2 DNA quantitation

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

2.17.3 End filling with Klenow fragment

Protruding ends created by the cleavage with *Eco*R1 and *Bam*HI were filled using the Klenow fragment of *E. coli* DNA polymerase I. Typically 1 μg of digested DNA, 2 μl 10x nick translation buffer (Maniatis *et al.* 1982), 1 μl of each dNTP at 2 mM concentration, and 1 unit Klenow fragment were mixed and incubated at 37 C for 30 minutes. The reaction was stopped by heating at 60 C for 10 minutes, followed by removal of unincorporated dNTP and enzyme by centrifugation through a 1 ml Sepharose CL-6B column.

2.17.4 Construction of pPM2101

Plasmid pPM2101 was constructed as follows (Figure 5.2). Approximately 1 μl of DNA of the plasmid pHC79 (obtained from Dr. J. Collins; Hohn & Collins 1980) was digested with the restriction endonuclease *Eco*R1. The protruding ends produced by cleavage with *Eco*R1 were filled using Klenow fragment of *E. coli* DNA polymerase I. The plasmid pSUP201-1 (obtained from Dr. A. Pühler; Simon *et al.* 1983) was digested with the restriction endonuclease *Bam*HI to yield a 1.6kb fragment containing the mobilization region of the plasmid RP4 from pSUP201-1. This digest was similarly treated with the Klenow fragment of *E. coli* DNA polymerase I. Treated pHC79 was ligated overnight to treated pSUP201-1 using a 1:10 ratio and transformed into *E. coli* S17-1 (*pro, hsdR, recA*; and integrated plasmid RP4-2-Tc::Mu-Km::Tn7) selecting for Ap^R and Tc^R. The cloning of the 1.64kb mobilization region was selected for by looking for mobilization into *E. coli* K12 Sm10 (*thi, thr, leu,* Sup^E; Km^R;

and integrated plasmid RP4-2-Tc::Mu), selecting for Ap^R, Tc^R and Km^R. Any positive clones were further screened by making DNA preparations using the three-step alkali lysis method described by Garger *et al.* (1983) and digested with the restriction endonucleases *Pst*I and *Bam*HI to show 1.6kb increase in this fragment of positive clones.

2.17.5 Construction of cosmid gene bank

Genomic fragments of approximately 40 kb were generated by controlled partial digestion of *V. cholerae* Z17561 genomic DNA with the restriction endonuclease *Sau*3A. The cosmid vector pPM2101 was cleaved with *Bam*HI and treated with molecular biology grade alkaline phosphatase (Boehringer Mannheim Biochemicals, Sydney) 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 recombination deficient (*rec*A) *E. coli* K12 S17-1. Cells harboring cosmid clones were detected by plating onto nutrient media containing ampicillin. Greater than 90% of the colonies were Tc^S and could be assumed to contain *V. cholerae* DNA.

2.17.6 Mobilization of cosmid bank

The cosmid bank of Z17561 in *E. coli* K12 S17-1 was mobilized into the *V. cholerae* strain O17 (El Tor Ogawa, Sm^R). This was done by replica-plating the cosmid bank onto a lawn of O17 cells (200 μl of log phase culture) on nutrient medium and incubating at 37 C for 4 hours. These conjugation plates were then replicated onto nutrient medium containing Ap (50 μg/ml) and Sm (200 μg/ml). After overnight incubation the colonies were re-patched onto CFA agar containing Ap and screened.

2.17.7 Transformation

This is basically a modification of Brown *et al.* (1979b). *E. coli* K12 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_{650} of 0.6. The cells were chilled on ice for 20 min., pelleted at 4 C in a bench centrifuge, resuspended in half volume of cold 100 mM $MgCl_2$, centrifuged again, and resuspended in a tenth volume of cold 100 mM $CaCl_2$. This was allowed to stand for 60 min. on ice, after which the cells were deemed to be competent, and 0.2 ml were mixed with DNA (volume made to 0.1 ml with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and left on ice for a further 30 min. The cell/DNA mixture was heated at 42 C for 2 min, then 3 ml BHI was added and the mixture incubated with shaking for 1-2hr at 37C. The culture was plated onto selection plates directly or concentrated by centrifugation before plating. Cells with sterile buffer were run as a control.

2.18 EM

2.18.1 Transmission EM

For negative staining, strains of *V. cholerae* were suspended in PBS to 10^9 cells/ml. One drop of this suspension was applied to a Formvar-coated copper grid (300 mesh, Graticules Ltd., Tonbridge, Eng.) for one minute, washed once with distilled water, then stained with 1% uranyl acetate for 1 minute. Excess fluid was removed by filter paper. Grids were then examined in a Jeol JEM 100S electron microscope operated at an accelerating voltage of 80 KV.

2.18.2 Immunoelectron microscopy (IEM)

Colloidal gold particles (ca. 15-20 nm) were prepared using the citrate method as described by de Mey & Moermans (1986) and conjugated with Protein A (Pharmacia).

V. cholerae cells grown on NA or CFA plates were harvested and washed once in PBS (pH 7.2) containing 1% BSA (PBS-BSA) A drop (35 μl) of cell suspension was placed on a sheet of parafilm. A poly-L-lysine treated formvar electron microscope grid was then placed plastic side down on the surface of this drop for 2 min (Mazia *et al.* 1978). The grid was then successively transferred to drops of the following reagents placed on the same parafilm sheet: PBS-BSA, twice (1 min each); antibody (1:10 dilution in PBS-BSA containing 0.05% Tween 20) for 15 min; PBS-BSA, three times (1 min each); Protein A-gold (1:50 dilution in PBS-BSA containing 0.05% Tween 20) for 15 min; distilled water, three times (1 min each). Excess fluid was removed from the grid using filter paper and the grid allowed to air dry without negative staining. In some experiments, immuno-gold labelled preparations were negatively stained with a solution of 2% w/v uranyl acetate for 1 min at room temperature. Grids were examined in a Jeol JEM 100S transmission electron microscope operated with an accelerating voltage of 80 kV.

Chapter 3

Non-LPS Protective Antigens shared by Classical and El Tor biotypes of V. cholerae

3.1 Introduction

Studies in various model systems have shown that antibodies to the LPS determinants of *V. cholerae* can mediate protection against experimental cholera (Watanabe & Verwey 1965; Neoh & Rowley 1972; Svennerholm 1975; Holmgren *et al.* 1977; Attridge & Rowley 1983c). In consequence the possible prophylactic potential of the non-LPS components has been largely ignored. As discussed in Section 1.10, conventional cholera vaccines generally comprise preserved suspensions of vibrios inactivated by treatment with phenol, formalin or heat; despite the presence of LPS antigens, such vaccines have conferred only short-term protection in the field (Joo 1974). The realization that other bacterial enteropathogens possess labile surface components that can function as protective antigens (Rutter & Jones 1973; Morgan *et al.* 1978) prompted the evaluation of the prophylactic significance of the non-LPS determinants of *V. cholerae*.

In 1970, Feeley had shown that live vibrios were more effective than heated ones in the active mouse protection test, raising the possibility of heat-labile protective determinants (Feeley 1970). Subsequently, Neoh & Rowley (1972) showed that a mouse antiserum prepared against *V. cholerae* 569B, and then repeatedly absorbed with highly purified homologous LPS,

retained significant protective activity. Bellamy *et al.* (1975) went further to show that antibodies present in an IgG fraction of a similar rabbit antiserum were protective and directed against heat-labile non-LPS determinants. Finally, more recent studies compared the protective activities of antibodies to the LPS or non-LPS antigens of *V. cholerae* 569B and concluded that the latter more efficiently protected infant mice from challenge with this strain (Attridge & Rowley 1983c). The same workers also showed that the protective activities of various antibody preparations correlated with their capacities to inhibit vibrio attachment *in vitro*, suggesting that at least some of the non-LPS protective antigens may play a role in adherence. This finding provides an encouraging analogy with studies of enterotoxigenic *E. coli* in which isolated colonization factors have been successfully used as prophylactic immunogens (Rutter & Jones 1973; Morgan *et al.* 1978).

The studies outlined above clearly demonstrated the existence of non-LPS protective antigens in *V. cholerae*, at least in the Classical Inaba strain, 569B. Further investigation of the vaccine potential of such components seemed warranted. The immunoprophylactic significance of the non-LPS antigens of *V. cholerae* obviously depends on the frequency of their distribution and the extent to which they are shared or strain specific. In a previous study, Attridge & Rowley (1983c) were unable to detect such determinants in the O17 and T50 strains, both of which are of El Tor biotype and Ogawa serotype. It was clearly essential to begin by examining other strains for the presence of non-LPS protective antigens, as the existence of shared components of this type might offer a new approach to vaccine development.

3.2 Characterization of V. cholerae strains

For this study, a total of 17 strains was used (Table 3.1). *V. cholerae* strains isolated in 1985 are referred to as recent field isolates, in contrast to the old laboratory strains which were first isolated 20-40 years ago. The significance of this distinction will become apparent later.

Table 3.1 Characteristics of *V. cholerae* strains

Strain	Biotype	Serotype	Virulence[1]	Source (year of isolation)[2]
569B	Classical	Inaba	$5.5 \pm 0.66 \times 10^5$	a(1946)
CA401	Classical	Inaba	$2.6 \pm 0.26 \times 10^3$	b(1953)
Z17561	Classical	Inaba	$9.3 \pm 0.46 \times 10^2$	c(1985)
CA411	Classical	Ogawa	$1.1 \pm 0.01 \times 10^3$	b(1953)
AA14041	Classical	Ogawa	$9.4 \pm 0.08 \times 10^3$	c(1985)
NIH41	Classical	Ogawa	$1.2 \pm 0.01 \times 10^3$	b(1941)
8233	El Tor	Inaba	$5.7 \pm 0.68 \times 10^5$	b(pre-1975)[3]
V86	El Tor	Inaba	$8.7 \pm 3.40 \times 10^5$	a(1961)
AA13993	El Tor	Inaba	$2.4 \pm 2.40 \times 10^4$	c(1985)
BM69	El Tor	Inaba	$1.0 \pm 0.23 \times 10^4$	d(1985)
358	El Tor	Inaba	$5.5 \pm 0.65 \times 10^4$	d(1985)
O17	El Tor	Ogawa	$9.4 \pm 0.18 \times 10^5$	a(pre-1965)
T50	El Tor	Ogawa	$8.0 \pm 2.80 \times 10^5$	a(pre-1962)
AA14073	El Tor	Ogawa	$4.1 \pm 1.24 \times 10^4$	c(1985)
H-1	El Tor	Ogawa	$4.8 \pm 2.40 \times 10^3$	d(1985)
64	El Tor	Ogawa	$1.8 \pm 0.31 \times 10^3$	d(1985)
569B/165	Classical Inaba x non-O1 Vibrio		$> 10^9$	a(1972)

[1] Expressed as LD_{50} values, calculated as described in Section 2.12.1. Data are mean ±SE values for two to four determinations

[2] a = Dr. K. Bhaskaran (Central Drug Institute, Lucknow, India); b = Dr. J. Berry (University of Texas, Austin); c = Dr. B. Kay (International Center for Diarrheal Disease Research, Dhaka, Bangladesh); d = Dr. S.C. Pal (National Institute of Cholera and Enteric Diseases, Calcutta, India).

[3] See Guentzel & Berry (1975).

The 569B/165 hybrid *Vibrio* was prepared by conjugation between *V. cholerae* strain 569B and the non-cholera vibrio strain 165 (Bhaskaran 1971), and carries the LPS determinants of the 165 strain on the non-LPS antigenic background of the 569B strain (Bhaskaran 1971; Attridge & Rowley 1983c).

Biotype was confirmed by assessing sensitivity to the antibiotic polymixin B (50 units/ml) and by typing with biotype-specific phages. Serotype was confirmed by slide agglutination using specific typing sera. Virulence was determined by the infant mouse cholera model (see Section 2.12.1). The data in Table 3.1 show that 16 of the 17 strains were pathogenic in this model, although strains O17 and 8233 had to be passaged in infant mice to increase their virulence to the levels shown. The 569B/165 hybrid was non-pathogenic.

3.3 The existence of non-LPS protective antigens in strains CA401 and CA411

3.3.1 Production of antisera to 569B/165, CA401 and CA411 strains

To study the distribution of non-LPS protective antigens, it was decided to prepare antisera to three strains of *V. cholerae*: 569B/165, CA401 and CA411. The first antiserum was prepared by immunizing mice with live vibrios of the hybrid strain 569B/165 as described in Section 2.9.1. In a previous study, Attridge & Rowley (1983c) have confirmed the antigenic relationship between strain 569B/165 and its *V. cholerae* parent, in particular the absence of 569B LPS antigens from the hybrid strain. An anti-569B/165 serum was found to protect infant mice from challenge with 569B, demonstrating the existence of non-LPS protective antigens in this strain (Attridge & Rowley 1983c). Such a serum, therefore, provided a convenient means of surveying other strains for the presence of such determinants.

Antisera were also prepared against the CA401 (Classical Inaba) and CA411 (Classical Ogawa) strains, but these sera had to be absorbed with homologous LPS (Section 2.10.1) so that potentially protective anti-LPS antibodies were removed. HA assays performed on the LPS-absorbed antisera showed a titer of < 1:2, indicating the efficacy of this procedure.

3.3.2 Protective activities of antisera

The infant mouse model was used for assessment of the protective capacities of the antisera. Initial assays showed that the anti-569B/165 serum was indeed able to protect against challenge with the parental 569B strain. Furthermore, the LPS-absorbed anti-CA401 and anti-CA411 sera were also protective against homologous challenge, suggesting the existence of non-LPS protective antigens in these two strains. To eliminate the possibility that in the latter instances protection was due to sub-agglutinating concentrations of anti-LPS antibodies in the absorbed sera, aliquots of each serum were further absorbed with live or boiled homologous vibrios. Absorption with live organisms reduced the protective titers of the LPS-absorbed sera by 97% or 98%, whereas absorption with boiled vibrios reduced the titer by only 14% or 3.5% (Table 3.2). It was concluded that strains CA401 and CA411 do possess non-LPS protective antigens.

To assess the vaccine significance of these components, it was important to look at their distribution among other *V. cholerae* strains, and to determine the extent to which they were shared or strain-specific. At this stage it seemed possible that possession of such antigens might be restricted to strains of the Classical biotype.

Table 3.2 Protective activities of LPS-absorbed antisera to CA401 and CA411: effect of further absorptions with live homologous vibrios[a]

Serum	Protective Activity		
	Before further absorption	After absorption with	
		Boiled vibrios	Live vibrios
LPS-absorbed anti-CA401	1980	1710 (14%)[b]	55 (97%)
LPS-absorbed anti-CA411	2000	1930 (3.5%)	40 (98%)

[a] Antiserum to CA401 which had a protective titer of 1:7480 (see Table 3.3) was diluted 3.7 times to give an equivalent titer of 1:2000. Each antiserum was tested for its capacity to mediate protection against challenge with the homologous strain.

[b] Figures in parentheses refer to percentage reduction of protective titers.

3.4 Distribution of non-LPS protective antigens among V. cholerae strains

3.4.1 Old laboratory strains

In the first series of experiments, the antiserum to strain 569B/165 and the LPS-absorbed antisera to strains CA401 and CA411 were tested for their capacities to protect infant mice from challenge with eight old laboratory strains of *V. cholerae*. The same spectrum of activity was seen with each antiserum; protection was mediated against isolates of the Classical biotype but not against those of the El Tor biotype (Table 3.3). The protection observed was not serotype specific. These data were encouraging, for they raised the possibility that all strains of Classical biotype might possess common non-LPS protective antigens. It seemed important, however, to check this finding using *V. cholerae* strains recently isolated from the field.

3.4.2 Recent field isolates

A second series of protection experiments was performed using eight isolates obtained from India and Bangladesh. In contrast to the previous finding, both of the sera tested protected not only against the two Classical strains but also against the six of El Tor biotype (Table 3.3). Again, however, protection extended to isolates of heterologous serotype, although the titers obtained were consistently higher in cases of homologous challenge. These results suggested a difference with respect to the expression of non-LPS protective antigens by old and recent El Tor isolates. To confirm this apparent dichotomy, antisera were prepared against two of the latter strains, absorbed with homologous LPS as before, and then surveyed for protective capacities against a range of challenge strains. The absorbed sera retained significant protective activities against recently isolated strains of the four common biotype-

Table 3.3 Distribution of shared non-LPS protective antigens among sixteen strains of *V. cholerae*

Strain	Antiserum to				
	569B/165	CA401[a]	CA411	AA13993	H-1
Old Isolates					
569B	687 ± 50	1780	1330 ± 147	ND[b]	ND
CA401	433 ± 31	2500	1240 ± 124	ND	ND
CA411	333 ± 38	1310	2000 ± 135	ND	ND
NIH41	393 ± 22	1250	1690 ± 190	ND	ND
8233	< 10	< 10	< 10	< 10	< 10
V86	< 10	< 10	< 10	ND	ND
O17	< 10	< 10	< 10	< 10	< 10
T50	< 10	< 10	< 10	ND	ND
Recent Isolates					
Z17561	ND	3480	590 ± 95	2510 ± 192	1160 ± 7
AA14041	ND	2570	1630 ± 130	2190 ± 368	2260 ± 227
AA13993	ND	5590	573 ± 40	3770 ± 226	1350 ± 109
BM69	ND	4450	543 ± 84	ND	ND
358	ND	3990	520 ± 12	ND	ND
AA14073	ND	1150	1200 ± 86	ND	ND
H-1	ND	1290	1390 ± 86	1830 ± 336	3560 ± 88
64	ND	1830	1270 ± 112	ND	ND

NOTE. Data show protective activities (mean ±SE for multiple estimates) of antisera, expressed as 50% protective endpoints as described in Section 2.12.2. Antisera to strains 569B/165 and CA411 were titrated three times against each strain, antisera to strains AA13993 and H-1 were titrated twice, and the antiserum to strain CA401 was titrated once.

[a]Separate batches of antiserum to strain CA401 were tested against the two sets of isolates. The serum assayed against the recent isolates had a protective titer of 1:7480 against homologous vibrios.

[b] ND = Not determined.

serotype combinations (Table 3.3). As with the three sera tested earlier, no protection was evident when the challenge strain were old isolates (8233 and O17) of El Tor biotype (Table 3.3).

3.4.3 Conclusion

Collectively, these data indicate that all of the Classical strains examined, as well as recent isolates of El Tor biotype, share components which function as protective antigens in the infant mouse cholera model. Analogous components were not detected on any of four old laboratory El Tor strains. The possibility that the latter might possess the common antigens but that the corresponding antibodies are not sufficient to confer protection against such strains was eliminated by the finding that absorption of an anti-569B/165 serum with live O17 vibrios did not lead to a reduction in protective activity (data not shown, but see Section 5.2).

3.5 Subsets of non-LPS protective antigens are serotype-restricted

3.5.1 Demonstration by protection assays

The data in Table 3.3 show that, on average, the protective titer of an antiserum was two-fold higher when the challenge strain was of homologous rather than heterologous serotype. This finding suggested the existence of serotypically restricted non-LPS protective antigens in addition to the shared components discussed above. To examine this point more directly, two sera were extensively absorbed with strains of heterologous serotype in an attempt to prepare sera which would protect mice against challenge with strains of homologous serotype only.

The sera selected for these absorptions were rabbit antisera prepared against CA411 or 569B/165; the former was extensively absorbed with homologous LPS as described in

Section 2.10.1. Both sera were protective against challenge with either serotype, although as noted previously, each was more active against homologous challenge (Table 3.4). Further absorption of the LPS-absorbed anti-CA411 serum with vibrios of heterologous Inaba serotype (strain 569B) effectively removed protective activity against this serotype, but the residual serum retained significant protective activity against each of five Ogawa challenge strains (Table 3.4).

Similar absorptions of the anti-569B/165 serum with vibrios of heterologous Ogawa serotype (strain CA411) failed to remove all protective activity against challenge with the absorbing strain, and this situation still pertained even after further absorptions with the same and subsequently a different Ogawa strain (AA14041). However, the residual protective potential against strains of Ogawa serotype was considerably less than that observed against homologous Inaba challenge strains (Table 3.4). Furthermore, these protection assays revealed a biotype-associated difference between the various Ogawa challenge strains used, a point which was clarified by subsequent studies (see Section 4.6).

3.5.2 Demonstration by IEM

These experiments suggested the existence of serotype-restricted non-LPS protective antigens. The extensively-absorbed anti-CA411 serum was tested for its content of antibodies capable of binding to strains of homologous (CA411) or heterologous (569B) serotype by IEM. In line with the protection data, significant numbers of gold particles were observed in close association with CA411 vibrios, whereas little binding was evident with 569B bacteria (Figure 3.1).

Table 3.4 The existence of serotype-restricted non-LPS protective antigens in *V. cholerae*[a]

	Challenge Strains									
	Ogawa serotype					Inaba serotype				
Antiserum	CA411	NIH41	AA14041	H-1	64	569B	CA401	Z17561	AA13993	BM69
LPS-absorbed anti-CA411	4210	3320	ND	ND	ND	1200	1080	ND	ND	ND
569B-absorbed[b]	3320	2500	2890	2950	2810	< 20	< 20	< 20	< 20	< 20
Unabsorbed anti-569B/165[c]	1370	2210	ND	ND	ND	6110	5000	ND	ND	ND
CA411/AA14041 absorbed[d]	260[e]	420	290[e]	< 20	< 20	3340	2580	2550	2240	2780

[a] Antisera were titrated only once for protective activities.

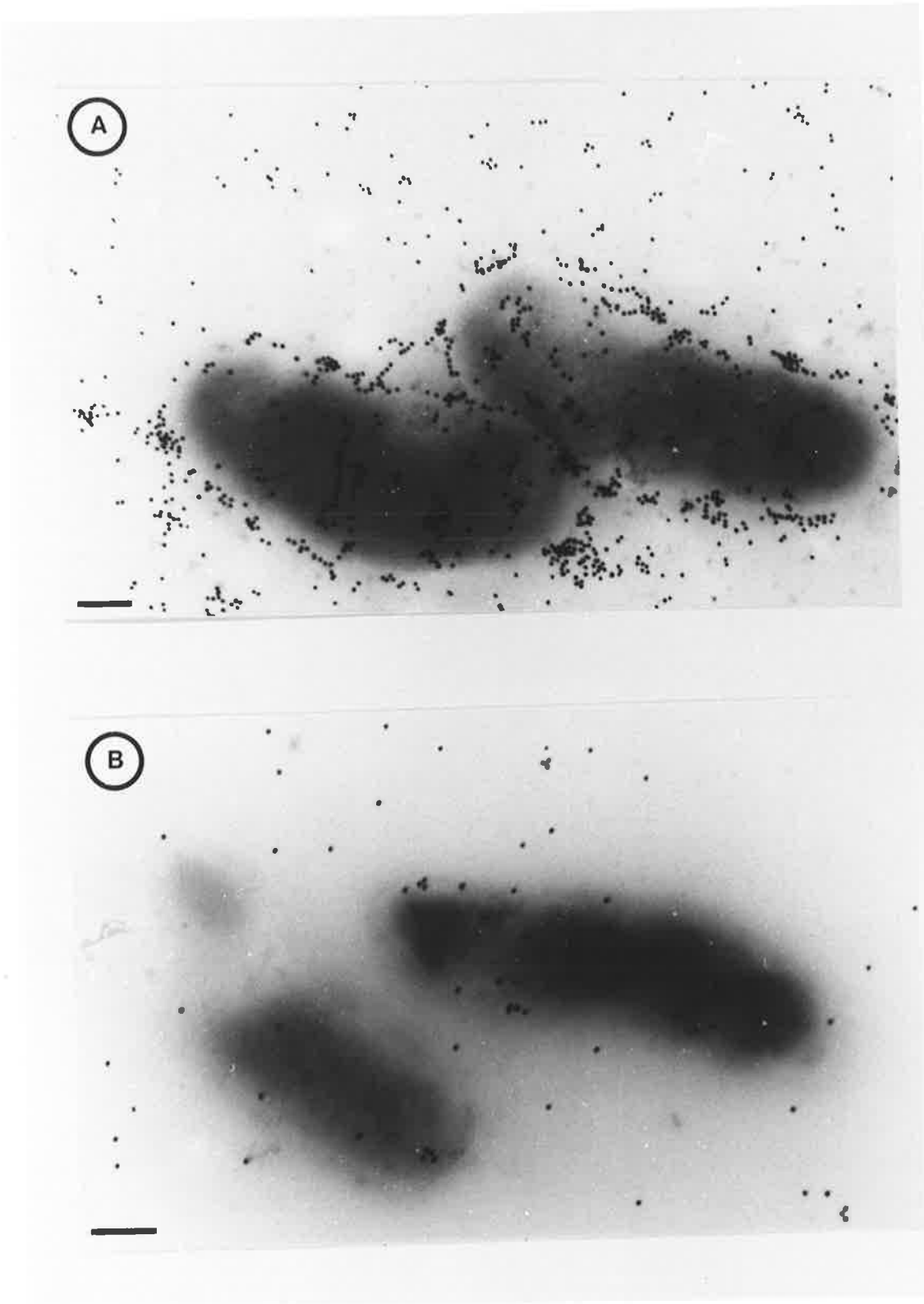
[b] LPS-absorbed anti-CA411 was further absorbed five times (thrice at 37 C, 2 hr, and twice at 4 C, overnight) with live 569B.

[c] Anti-569B/165 tested unabsorbed as it contains only antibodies to non-LPS protective antigens (see Section 3.2).

[d] Absorbing anti-569B/165 seven times with live CA411 did not remove all protective activity against strains of Ogawa serotype; it was further absorbed three times with live AA14041.

[e] PD_{50} of 260 and 290 was observed when the challenge strains CA411 and AA14041 were grown under routine cultural conditions (NB at 37 C). However, when they were grown on CFA at 25 C the PD_{50} values were 970 and 1380, respectively (see Section 4.6 for details).

Figure 3.1 Demonstration of (Ogawa) serotype-specific non-LPS determinants . An extensively absorbed anti-CA411 serum (see text) was used for IEM examination of *V. cholerae* strains CA411 (A) and 569B (B). Following growth on NA plates (37 C for 24h), bacteria were harvested, incubated with 1:10 dilution of absorbed anti-CA411 serum, followed by 1:50 dilution of protein A-colloidal gold particles at room temperature. Bars represent 200nm.


A
B

3.6 Correlation between protective activities of antisera and their capacities to inhibit in vitro attachment

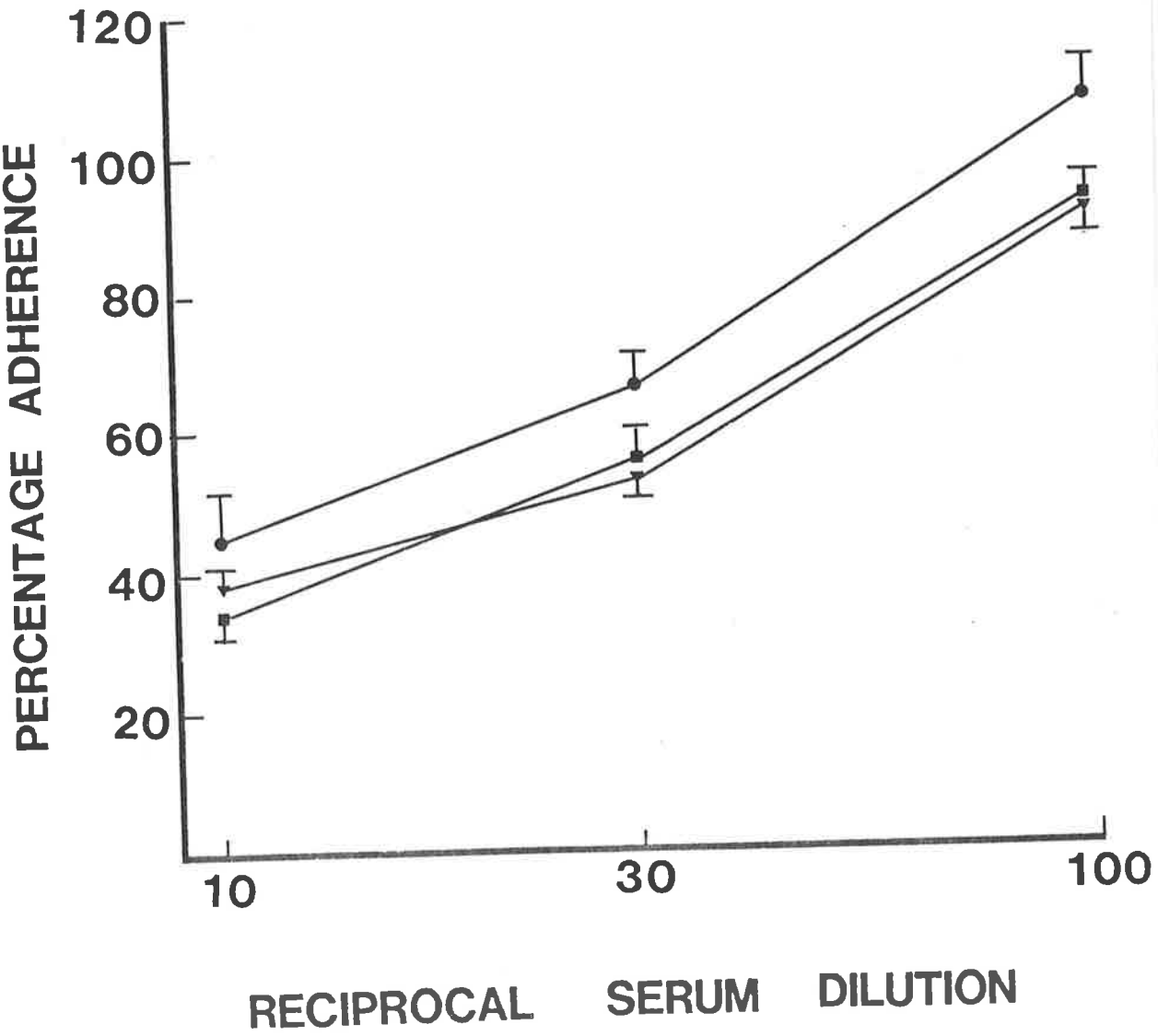
3.6.1 In vitro enterocyte adherence assay

Adhesion of *V. cholerae* to the mucosa of the small intestine is now recognized as an important early event in colonization. In an attempt to confirm the previously reported link between the capacities of a serum to protect infant mice from choleraic infection and to inhibit the *in vitro* attachment of bacteria of the challenge strain (Attridge & Rowley 1983c), an adherence assay was developed using enterocytes isolated from the adult mouse small intestine (as described in Section 2.11). Phase-contrast microscopy showed that the enterocyte isolation procedure yielded populations of epithelial cells, which retain their characteristic columnar morphology and have clearly defined apical brush borders (not shown). On the average, 60% viability was indicated by Trypan Blue exclusion. Preliminary experiments demonstrated that a ratio of 10 bacteria per enterocyte was optimal for assessment of bacterial adherence.

3.6.2 Inhibition of adherence

Three of the antisera described above were tested for their capacities to inhibit the *in vitro* attachment of *V. cholerae* to isolated murine enterocytes. The antiserum to strain 569B/165 was initially tested against the 569B parent strain, and the LPS-absorbed antisera to strains CA401 and CA411 were tested against homologous vibrios. Each serum produced a concentration-dependent inhibition of attachment (Figure 3.2), confirming a previous finding by Attridge & Rowley (1983c) that antibodies to non-LPS determinants can inhibit the *in vitro* binding of sub-agglutinating concentrations of vibrios. Each serum was then tested for its capacity to inhibit the adherence of the seven other old isolates used in this study (final serum dilution, 1:10). All three antisera consistently inhibited the attachment of each of the four

Figure 3.2 Antibody-mediated inhibition of *V. cholerae* adherence *in vitro*. Antisera were tested for their capacities to inhibit bacterial attachment to isolated murine enterocytes, as described in Material and Methods (Section 2.11.3). Residual percentage adherence after pretreatment with antiserum is shown relative to adherence observed after preincubation with a 1:10 dilution of normal mouse serum (100%). Graphs show binding of strain CA411 in the presence of LPS-absorbed antiserum to strain CA411 (●), binding of strain CA401 in the presence of LPS-absorbed antiserum to strain CA401 (■), and binding of strain 569B in the presence of antiserum to strain 569B/165 (▼). Data are mean ±SE (bars) attachment values from three determinations.


120
100
80
60
40
20
PERCENTAGE ADHERENCE
10
30
100
RECIPROCAL SERUM DILUTION

strains of Classical biotype, whereas the binding of the El Tor strains was not reduced (Figure 3.3; results with V86 and T50 strains are not shown but were similar to those obtained with strains O17 and 8233).

Since there was a consistent correlation between the presence of the non-LPS protective antigens and susceptibility to antibody-mediated inhibition of adherence, it was predicted that the binding of the recent field isolates would also be reduced in the presence of such antisera. Figure 3.4 shows that this is indeed the case. These results strongly reinforce the previous demonstration (Attridge & Rowley 1983c) of a correlation between the capacities of antisera to mediate protection and to inhibit *in vitro* attachment.

3.7 Discussion

The major finding described in this chapter is that each of eight recent field isolates of *V. cholerae* bear common non-LPS protective antigens, despite differences in biotype, serotype, and geographic origin. Of the eight older isolates of *V. cholerae*, only those of Classical biotype express these antigens (Table 3.3); the basis for this restricted distribution remains uncertain. It seems unlikely that the old El Tor strains have lost such components during prolonged storage in the laboratory, since similar treatment has not affected the old Classical *V. cholerae* strains. Perhaps the expression of non-LPS protective antigens by recent field El Tor isolates results from interaction between the two biotypes in the environment. Epidemiological studies have revealed that the biotype most commonly associated with clinical cholera cases can change over a period of time in a given location (Samadi *et al.* 1983). Although the factors responsible for such biotype switching remain undefined, there would seem to be ample opportunity for interaction between vibrios of the two common biotypes in the field. It is therefore conceivable that vibrios of El Tor biotype have, comparatively recently, acquired the genetic potential to express non-LPS protective antigens. Since at least some of these components may play a role

Figure 3.3 Capacities of three antisera to inhibit the *in vitro* attachment of *V. cholerae* strains of Classical or El Tor biotype. Residual adherence capacities of six *V. cholerae* strains (A, 569B; B, CA401; C, CA411; D, NIH41; E, 8233; and F, O17) are shown after preincubation with each of three antisera: (top) LPS-absorbed antiserum to strain CA411, (middle) LPS-absorbed antiserum to strain CA401, and (bottom) antiserum to strain 569B/165. Percentage adherence is relative to that displayed by control organisms preincubated with a 1:10 dilution of normal mouse serum (100%). Histograms show mean residual adherence and bars represent the SE (from three determinations).

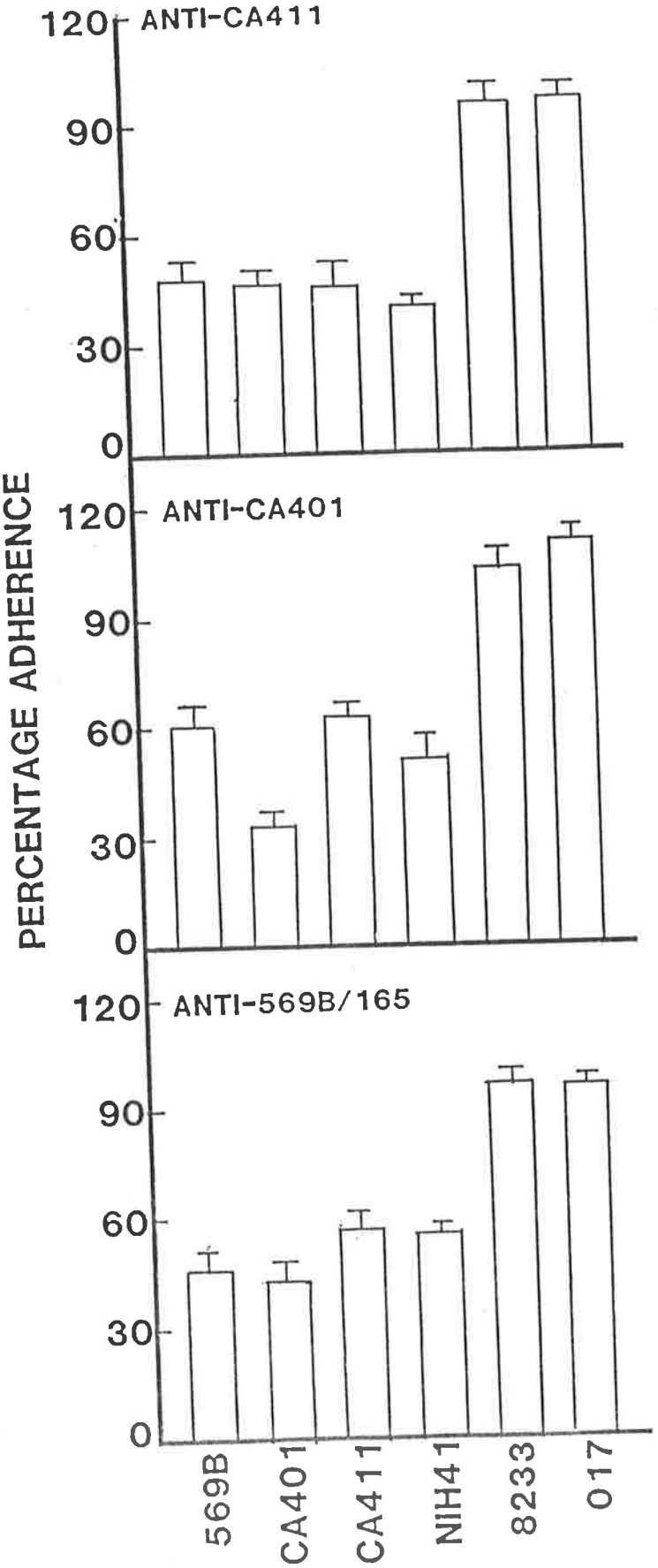
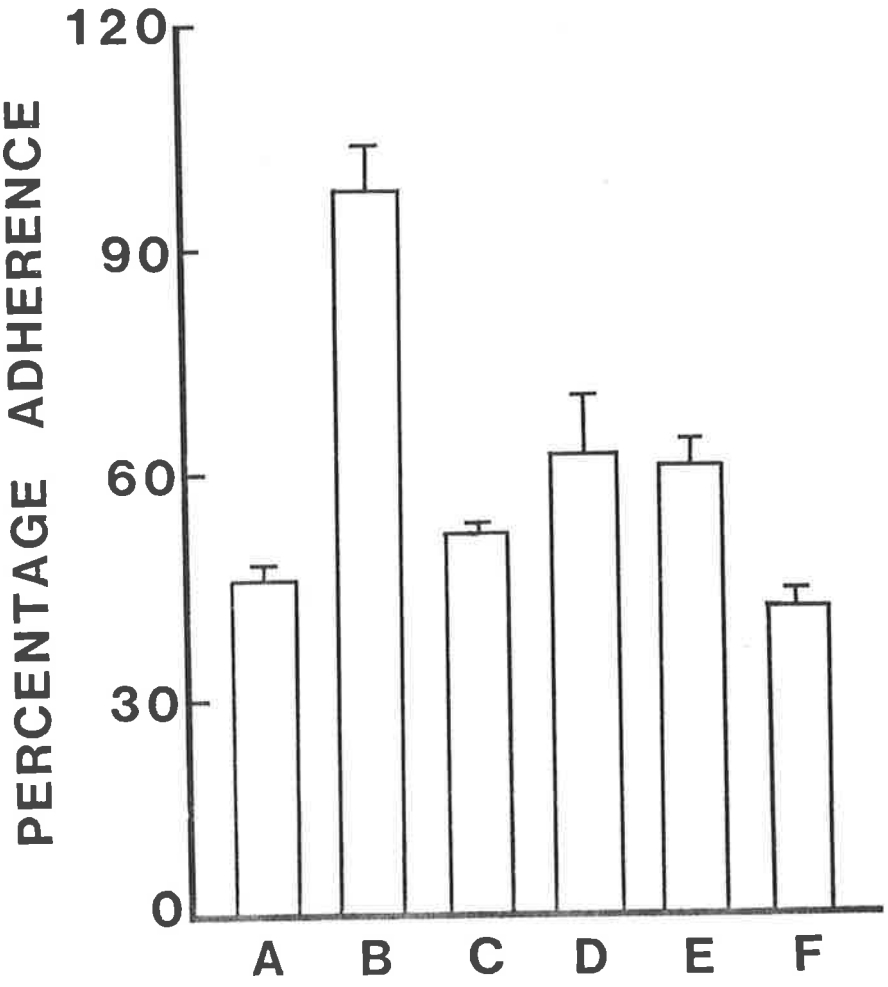

ANTI-CA411
ANTI-CA401
ANTI-569B/165
PERCENTAGE ADHERENCE
120
90
60
30
0
569B
CA401
CA411
NIH41
8233
017

Figure 3.4 Capacity of antiserum to strain CA411 to inhibit the *in vitro* attachment of recent *V. cholerae* field isolates of El Tor biotype. Residual adherence capacities of six *V. cholerae* strains (A, 569B; B, O17; C, AA13993; D, BM69; E, H-1; and F, 64) are shown after preincubation with antiserum to strain CA411 (absorbed with homologous LPS). Percentage adherence is relative to that shown by control organisms preincubated with a 1:10 dilution of normal mouse serum (100%). Histograms show mean residual adherence, and bars represent the SE (from two determinations).


PERCENTAGE ADHERENCE
120
90
60
30
0
A
B
C
D
E
F

in adherence (see below), it is possible that they contribute to pathogenic potential. In this context, it is interesting to note that the present pandemic is the first to have been caused by vibrios of El Tor biotype.

In addition to the non-LPS protective antigens that are shared by strains of either serotype, the data suggest the existence of additional serotype-restricted components. Thus the protective titers of the five antisera used in this study were always greater against strains of homologous serotype (Table 3.3). The data presented in Table 3.4 provide more direct evidence for the existence of serotype-restricted components. Absorption of LPS-absorbed anti-Ogawa serum with vibrios of Inaba serotype effectively removed protective activity against this serotype, but the residual serum retained significant protective activity against Ogawa challenge strains. Similar data were obtained in a second experiment in which anti-569B/165 serum was absorbed with strains of Ogawa serotype. Collectively these experiments confirm the existence of serotype-restricted non-LPS protective antigens.

As noted previously by Attridge & Rowley (1983c), a consistent correlation was found between the capacity of an antiserum to protect infant mice and its capacity to inhibit the *in vitro* attachment of the challenge strain. The use of sub-agglutinating concentrations of vibrios in the attachment studies, and the fact that many strains are virulent when administered in low doses (i.e. sub-agglutinating concentrations), would argue against antibody-mediated bacterial agglutination as a critical mechanism of protection in this model. The blocking of vibrio adhesins is a more likely mechanism (Freter & Jones 1976; Attridge & Rowley 1983c). The correlation observed between these two activities of the non-LPS antibodies (protective capacity and inhibition of adherence) is consistent with a role for at least some of these components in colonization.

The failure to remove the residual protective antibodies from LPS-absorbed anti-CA401 and anti-CA411 sera by absorption with boiled vibrios confirms the heat-labile nature

of these antigens (Watanabe *et al.* 1969; Attridge 1979). It seems unlikely that relatively labile non-LPS protective antigens would escape denaturation during the preparation of conventional killed whole cell vaccines. Studies by Cryz and colleagues (1982) have shown that phenol- or heat-inactivation drastically reduces the immunogenicity of cholera vibrios, although formalin treatment appears less deleterious. The recent demonstration of the clinical significance of at least one non-LPS component (see Chapter 5) will necessitate a more careful approach to the problem of vaccine inactivation. Alternatively, should such components acquire the status of protective antigens, it could be argued that they would be more effectively presented to the immune system by an attenuated live oral vaccine (see Section 1.10.2.1).

Chapter 4

Role of TCP as a Colonization Factor and a Non-LPS Protective Antigen in the Infant Mouse Cholera Model

4.1 Introduction

The results of Chapter 3 have clearly indicated that common non-LPS protective antigens are expressed by four old strains of Classical biotype and eight recent field isolates of both biotypes, but that such determinants are not detectable on four old El Tor strains. Although these antigens remain undefined, their relative lability (Attridge 1979) suggests that they might be protein in nature (Cryz *et al.* 1982; Attridge & Rowley 1983c). Candidate proteins would be those associated with flagellar or pilus structures, or located elsewhere in the outer membrane (Section 1.8.2).

Several studies implicate the flagellum as a determinant of virulence in *V. cholerae*, though there has been debate as to whether this structure functions solely by providing motility (Guentzel & Berry 1975), or whether it also plays a role in attachment (Attridge & Rowley 1983a). In the present context, Eubanks *et al.* (1977) have described a non-LPS protective antigen associated with the vibrio flagellum which was detected in both Inaba and Ogawa strains of the Classical biotype. Yancey *et al.* (1979) also described a similar heat-labile non-LPS flagellar-associated antigen.

V. cholerae interacts intimately with the mucosal surface of the intestine, the initial event in the pathogenesis of cholera. For some other bacterial enteropathogens, this colonization has been shown to depend on the presence of pili which mediate adherence to receptors on host cells (Jones 1980; Gaastra & de Graaf 1982). For example, K88 pili have been shown to be critical for the *in vitro* attachment and *in vivo* colonization of ETEC strains of porcine origin (Smith & Linggood 1971; Jones & Rutter 1972). Subsequent studies showed that the (heat-labile) K88 protein could be used as a protective immunogen, eliciting the production of antibodies which confer protection by inhibiting colonization (Rutter & Jones 1973; Rutter *et al.* 1976).

As has already been pointed out (Section 1.3.3.4), early attempts to detect pili on *V. cholerae* met with variable success (Finkelstein & Mukerjee 1963; Barua & Chatterjee 1964; Tweedy *et al.* 1968; Adhikari & Chatterjee 1969). Until very recently, there was no evidence to suggest that such structures played any role in vibrio adherence (reviewed by Jones 1980). During the course of these studies, however, reports from Ehara *et al.* (1986) and Taylor *et al.* (1987) revived interest in the possible involvement of pili in the pathogenesis of cholera. There is now no doubt that TCP are required for efficient colonization of both the human (Herrington *et al.* 1988) and infant mouse gut (Taylor *et al.* 1987).

The experiments to be described in this chapter were aimed at elucidating the nature of the non-LPS protective antigens of *V. cholerae*. Comparisons of OMP preparations (by SDS-PAGE and immunoblotting) failed to reveal any consistent difference which could be correlated with the presence of non-LPS protective antigens, and subsequent experiments with detergent-treated OMPs also failed to implicate any particular protein band. Later experiments were designed to evaluate the importance of TCP as a protective antigen in the infant mouse model.

4.2 Studies of V. cholerae OMPs

4.2.1 Analysis by SDS-PAGE and immunoblotting

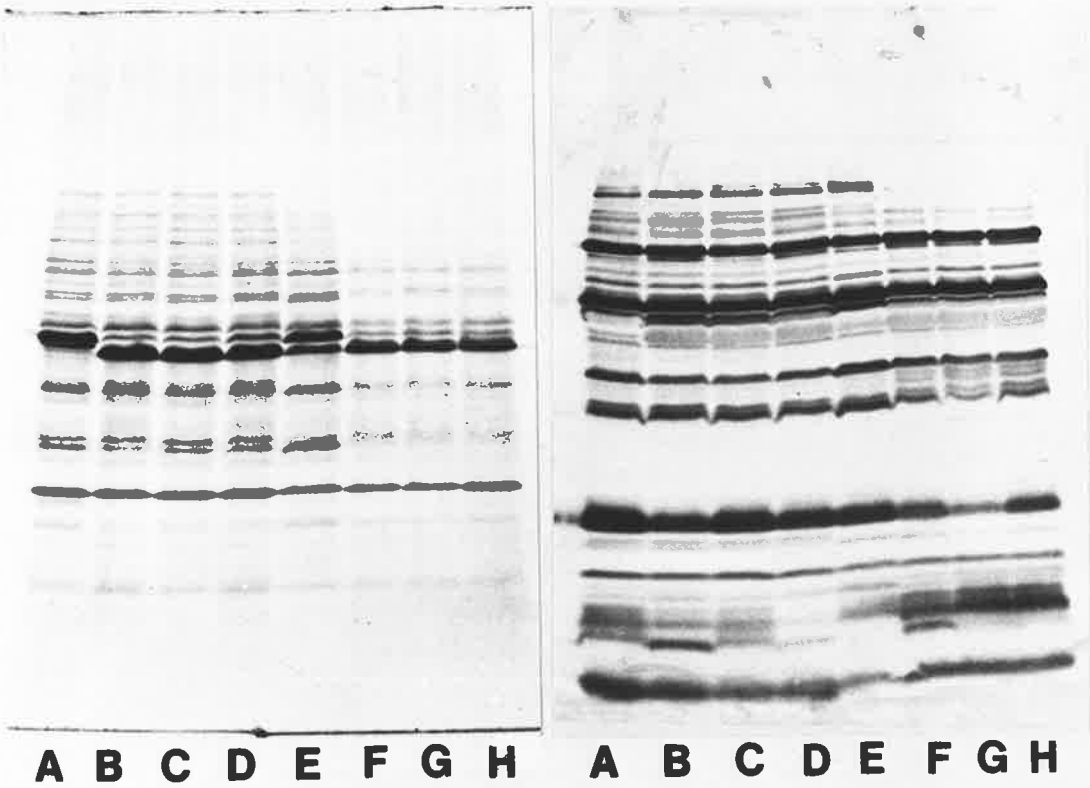
Preliminary experiments were carried out with the *V. cholerae* 569B strain. Both OMP and flagellar preparations of this strain were compared. EM examination of the latter confirmed the presence of flagellar structures, although contamination with membrane vesicles was apparent (data not shown). SDS-PAGE analysis (as described in Section 2.15.1) of the two preparations resulted in identical banding patterns (data not shown). As there was no obvious difference between them, OMP rather than flagellar preparations of other *V. cholerae* strains were used, mainly because of ease of preparation (Section 2.8).

In order to determine whether any consistent difference could be detected at the protein level between *V. cholerae* strains possessing or lacking non-LPS protective antigens, the protein profiles of OMP preparations of 8 *V. cholerae* strains were examined by SDS-PAGE. A representative experiment is shown in Figure 4.1, (left). None of the protein bands could be correlated with the previously determined distribution of non-LPS protective antigens. To determine whether such a correlation might be revealed by immunoblotting (Section 2.15.2), OMP preparations of the 8 strains were run on SDS-PAGE, transferred to nitrocellulose and incubated with the protective serum (anti-CA411; Section 3.3.2). As shown in Figure 4.1, (right), however, this approach also failed to implicate any protein as a candidate protective antigen.

4.2.2 Detergent extraction of OMP preparations

Because of the large number of bands seen in SDS-PAGE and immunoblotting analyses, it was decided to treat OMP preparations with a variety of detergents (see Section 2.4.2) to selectively solubilize some of the proteins and thereby facilitate further experimentation. A

Figure 4.1. SDS-PAGE and immunoblotting of *V. cholerae* OMPs. Lane A, O17; lane B, 8233; lane C, H-1; lane D, BM69; lane E, 569B; lane F, CA411; lane G, AA14041; and lane H, Z17561. OMPs were prepared from various strains, and 20 μg-quantities were analyzed by SDS-PAGE. Protein staining (left) or immunoblotting (with protective LPS-absorbed antiserum to strain CA411; right) followed.


A B C D E F G H
A B C D E F G H

comparison of the protein profiles by SDS-PAGE of the insoluble OMPs (pellets after ultracentrifugation, Section 2.8) with those of the untreated OMPs showed that Sarkosyl appeared the most promising of the ten detergents examined, in that it selectively extracted proteins from 569B OMP (Figure 4.2, lanes C & D). The number of protein bands was reduced to six (M_r values ranging from 21-45 kDa) (Figure 4.2, lanes C & D). By comparing the capacities of detergent-treated or control (treated with buffer only) OMP preparations to remove protective antibodies by absorption, it was possible to assess the protective potential of antibodies directed against proteins present in the insoluble pellet. In one such experiment, Sarkosyl-insoluble 569B OMP (Sark-OMP) removed 79% protective activity from a rabbit anti-569B/165 serum, whereas the control OMP removed 100%. This suggested that the proteins solubilized by this detergent were not the major non-LPS protective antigens but were retained in the insoluble fraction.

This tentative conclusion was strengthened by a second experiment in which groups of mice were immunized with Sark-OMP or control OMP prepared from the 569B strain (see Section 2.9.1). The resulting sera were absorbed with 569B LPS (as described in Section 2.10.1) to remove potentially protective antibodies specific for LPS determinants, and then compared for protective activity against 569B challenge. The similarity of the protective titers obtained (PD_{50} 2000 of the anti-Sark-OMP versus 2500 of the anti-control-OMP sera) indicates that the major non-LPS protective antigens are retained in the Sarkosyl-insoluble OMP fraction.

Immunoblotting analysis using the LPS-absorbed antiserum prepared against Sark-OMP failed to reveal any difference in the protein profiles of strains possessing or lacking non-LPS protective antigens (Figure 4.3, lanes J, K & L). In addition, this serum was further absorbed with boiled 569B four times (to remove antibodies to heat-stable antigens) and with live O17 four times (to remove antibodies to non-protective heat-labile antigens) and analyzed

Figure 4.2 SDS-PAGE analysis of detergent treated *V. cholerae* 569B OMP preparations. Lane A, 2% Nonidet P40; lane B, 3% Nonidet P40; lane C, 1% Sarkosyl; lane D, 2% Sarkosyl; lane E, 2% sodium deoxycholate; lane F, 1M urea; lane G, 2 M urea; lane H, 2% Tween 20; lane I, control OMP; lane J, control OMP; lane K, 2 % Brij 58; lane L, 1% Brij 58; lane M, 1% Triton X-114; lane N, 0.5% Triton X-114; lane O, 20 mM EDTA; lane P, 10 mM EDTA (Final concentrations). Arrows on the left point to molecular weights of protein bands seen in lane C after 1% Sarkosyl treatment. These are, top to bottom, 45 kDa, 42 kDa, 38 kDa, 32 kDa, 25 kDa and 21 kDa. Arrows on the right indicate standard molecular weight markers (lane M): 205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20.1 kDa and 14.2 kDa.

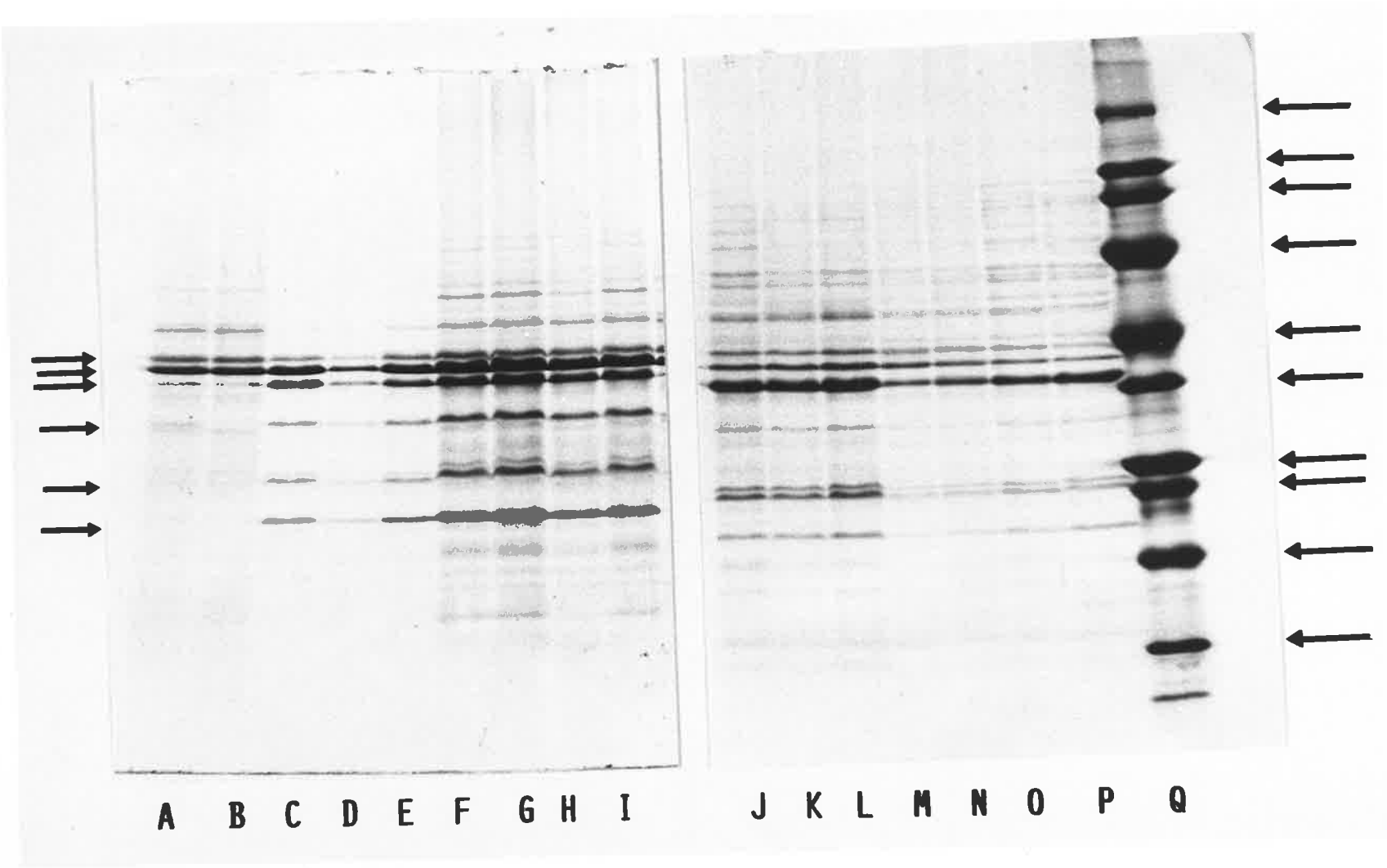
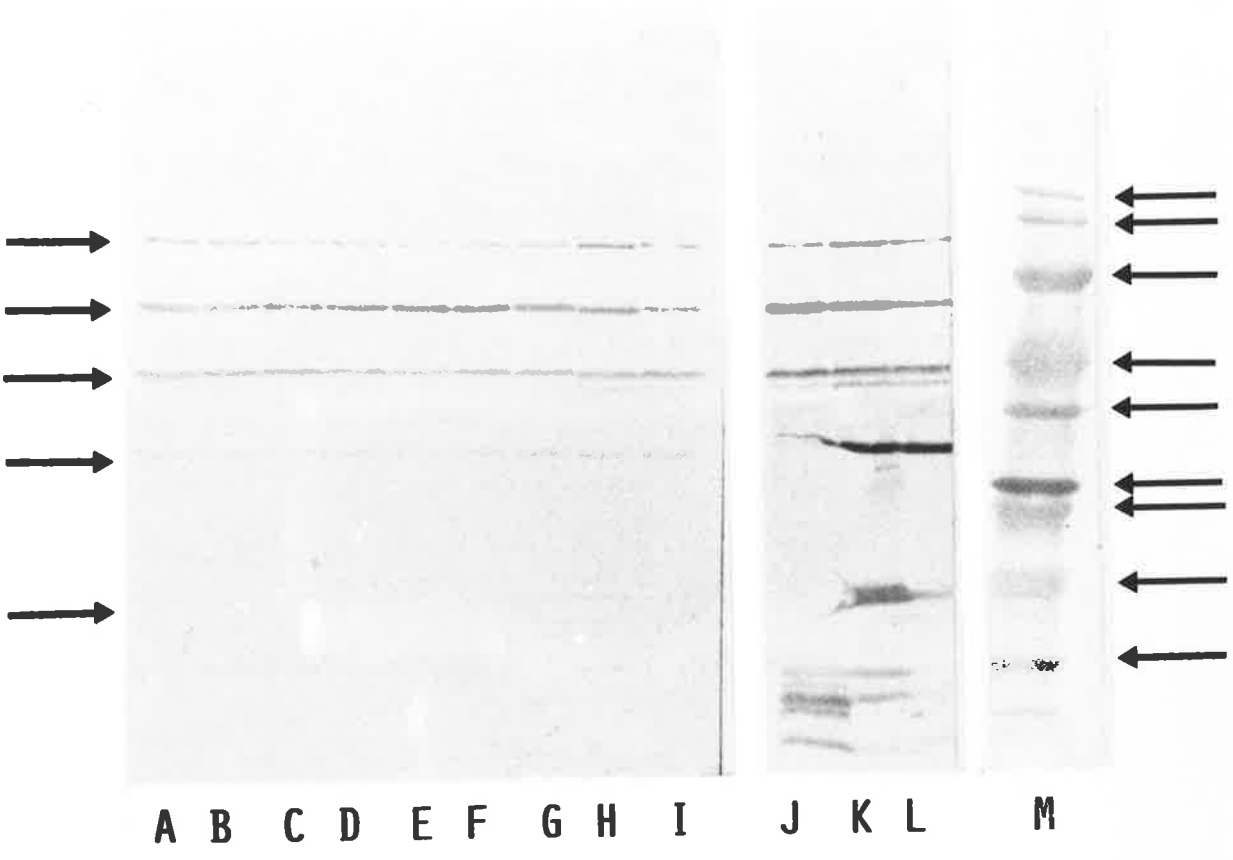

A B C D E F G H I
J K L M N O P Q

Figure 4.3 Immunoblot analysis of *V. cholerae* OMPs with anti-Sark-OMP antisera. Lane A, H-1; lane B, BM69; lane C, 8233; lane D, AA14041; lane E, Z17561; lane F, CA411; lane G & J, O17; lanes H & K, 569B; lanes I & L, 569B Sarkosyl-treated OMP pellet; lane M, standard molecular weight markers: arrows indicate positions - 116 kDa, 97.4 kDa, 65 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20.1 kDa and 14.2 kDa. Arrows on the left indicate positions of specific proteins. These are from top to bottom - 78 kDa, 58.5 kDa, 45 kDa, 38 kDa and 18 kDa. Lanes J-L tested with LPS-absorbed anti-Sark-OMP while lanes A-I tested with LPS-absorbed anti-Sark-OMP further absorbed with boiled 569B (four times) and live O17 (four times).


A B C D E F G H I J K L M

again by immunoblotting (Figure 4.3, lanes A-I). In both instances three proteins with M_r values of 78, 58.5 and 18 kDa not evident by SDS-PAGE analysis (Figure 4.2) reacted with the antisera (Figure 4.3). After additional exhaustive absorptions with live O17, the antibodies still reacted with O17 OMPs (Figure 4.3, lane G); However, when attempts were made to excise the relevant protein bands from SDS-PAGE gels and to use the (denatured) proteins to immunize mice, no protective antibodies were generated; the antisera, nevertheless reacted with 569B OMP in an ELISA (6400), showing the proteins to be immunogenic.

4.2.3 OmpV

As mentioned in Section 1.8.2.1, a 25 kDa protein (OmpV) is a major outer membrane protein of *V. cholerae* 569B (Stevenson *et al.* 1985; Pohlner *et al.* 1986a,1986b). This protein is found in a variety of *V. cholerae* strains including 569B/165 and 1074 (Manning *et al.* 1982), the latter a nontoxigenic Brazilian environmental isolate (Levine *et al.* 1982). Since a 25 kDa protein is present in 569B OMP preparations (Figure 4.2), experiments were carried out to determine whether this might represent a non-LPS protective antigen. The structural gene for the OmpV protein had been cloned (Stevenson *et al.* 1985) and an OmpV clone was kindly provided by Dr. P. Manning. When aliquots of a (protective) anti-569B/165 serum were absorbed with the OmpV-expressing clones or with the vector strain as a control, no significant reduction in protective activity were observed. In addition, antiserum raised against the clone expressing OmpV was not protective. These experiments indicated that OmpV is not a protective antigen in this model.

4.3 Expression of pili by V. cholerae strains

4.3.1 Preliminary experiments

During the course of these studies, Ehara *et al.* (1986) described culture conditions suitable for the production of pili by *V. cholerae*. These growth conditions, and others previously reported to promote the expression of pili *in vitro* (Tweedy *et al.* 1968; Faris *et al.* 1982; Ehara *et al.* 1986), were therefore used for an EM study of pilus production by four strains: 569B (the strain most commonly used to challenge infant mice), 64 (a recent field isolate of El Tor biotype), H-1 (a recent field isolate of El Tor biotype) (see Table 3.1) and K23-7 (a non-motile El Tor strain used by, and obtained from, Ehara *et al.* 1986). Liquid cultures were incubated at both 25 C and 37 C in shaking or stationary conditions, with varying incubation periods (16-72 hrs). When the bacteria were examined by EM, a maximum of 1-2 pili per cell were observed on a small minority (approximatley 5%) of vibrios.

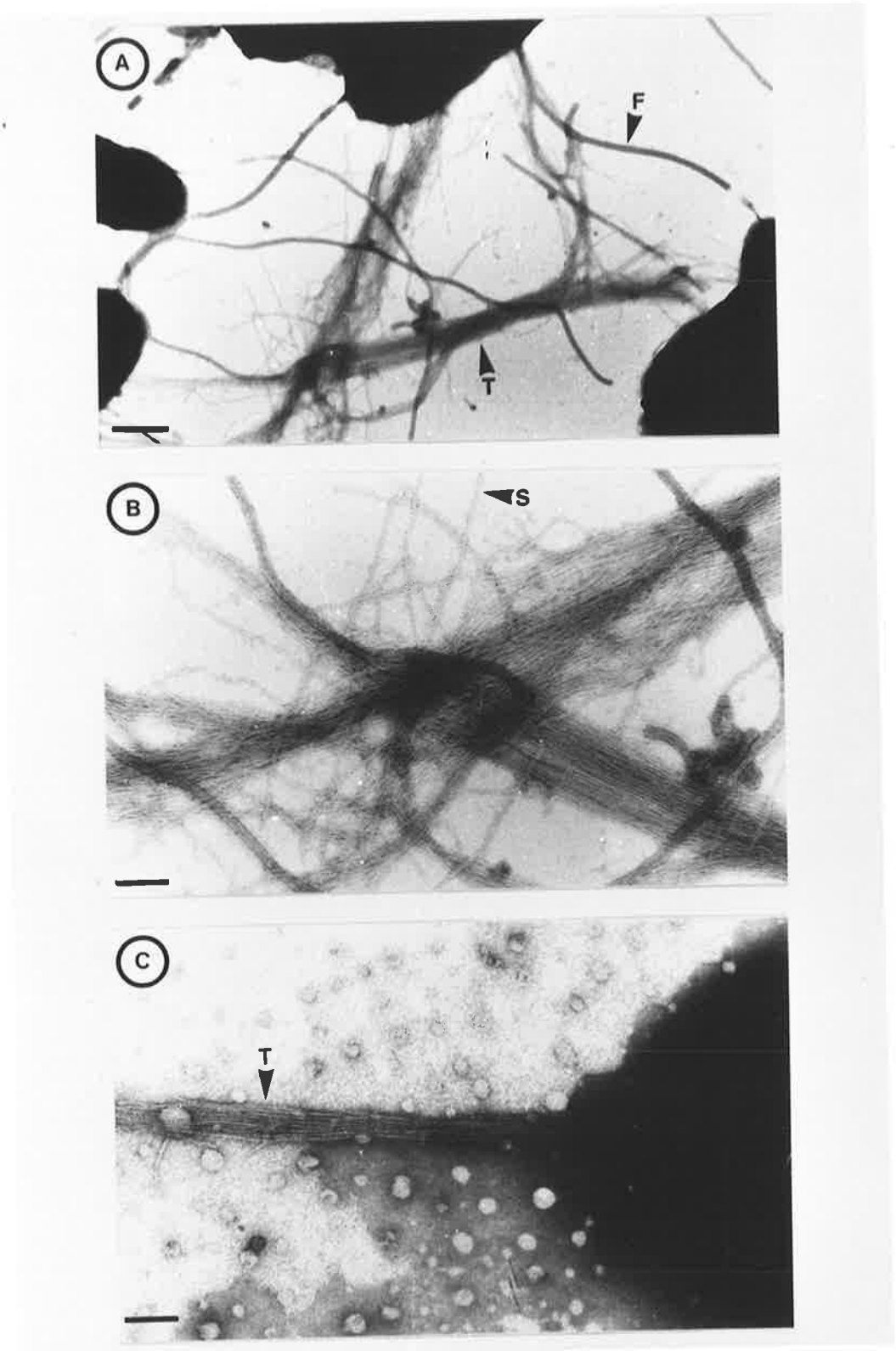
In an attempt to select for a piliated subpopulation of organisms, sequential subcultures were performed; medium containing a sterile glass slide was inoculated with *V. cholerae* and after 24 hrs incubation, the slide was transferred to fresh medium and the procedure repeated twice. However, no significant enrichment for piliated bacteria was achieved. Next, El Tor strains 64 and K23-7 were inoculated onto the TCG agar medium used by Ehara *et al.* (1986) and incubated at 30 C or 37 C overnight. A few flexible pili (5-7 nm in width) were seen on the surfaces of 5-10% of the cells, but again the degree of piliation was inadequate, not only for an attempt to isolate these structures, but also for studies designed to examine their role in pathogenesis.

4.3.2 Production of TCP

In 1987, investigators from the United States reported the expression of TCP by the Classical *V. cholerae* strain O395 (Taylor *et al.* 1987). To evaluate the significance of such pili in pathogenesis, these workers constructed a mutant of O395 which carried a deletion in the structural gene for the pilin subunit (TcpA). This mutant showed a 100,000-fold greater LD_{50} than its parent strain in the infant mouse model, and competition experiments indicated that TCP promoted colonization of the infant mouse intestine (Taylor *et al.* 1987). Although the protective activity of antibodies directed against TCP was not examined, these pili would be obvious candidates for non-LPS protective antigens. Accordingly, the Classical strains 569B and CA411 were cultured according to Taylor's protocol and examined by EM. However, no pili with the morphology of TCP were observed, although other morphological types were present (data not shown).

Next, the *colonization factor* media used extensively for the expression of pili by ETEC strains (Evans *et al.* 1979) were tested for their capacities to support pilus production by *V. cholerae*. Strain 569B was inoculated onto CFA and into CFB and incubated at 25 C or 37 C for 24 to 36 hrs. On examination by EM, thick aggregated bundles of pili similar to those described by Taylor *et al.* (1987) were observed on cells grown at 25 C. About 60% of the organisms harvested from CFA expressed these pili, and about 40% of those grown in the corresponding liquid medium. Figure 4.4 shows the morphology of the pili produced by two *V. cholerae* strains when grown on CFA at 25 C. The dimensions of the filaments were measured by the lattice spacing of crystalline catalase according to the method of Wrigley (1968). In support of the description given by Taylor *et al.* (1987), individual filaments were estimated to be about 7-10 nm wide. These filaments aggregated closely in parallel to form bundles of variable length and 0.1-0.3 μm in diameter.

Figure 4.4 Production of TCP on CFA. *V. cholerae* 569B (A, B) and Z17561 (C) grown on CFA at 25 C for 36 hours and negatively stained. F = flagellum; S = single TCP filament; T = bundles of TCP. Bar = 250 nm (A), 100 nm (B, C).


A
F
T
B
S
C
T

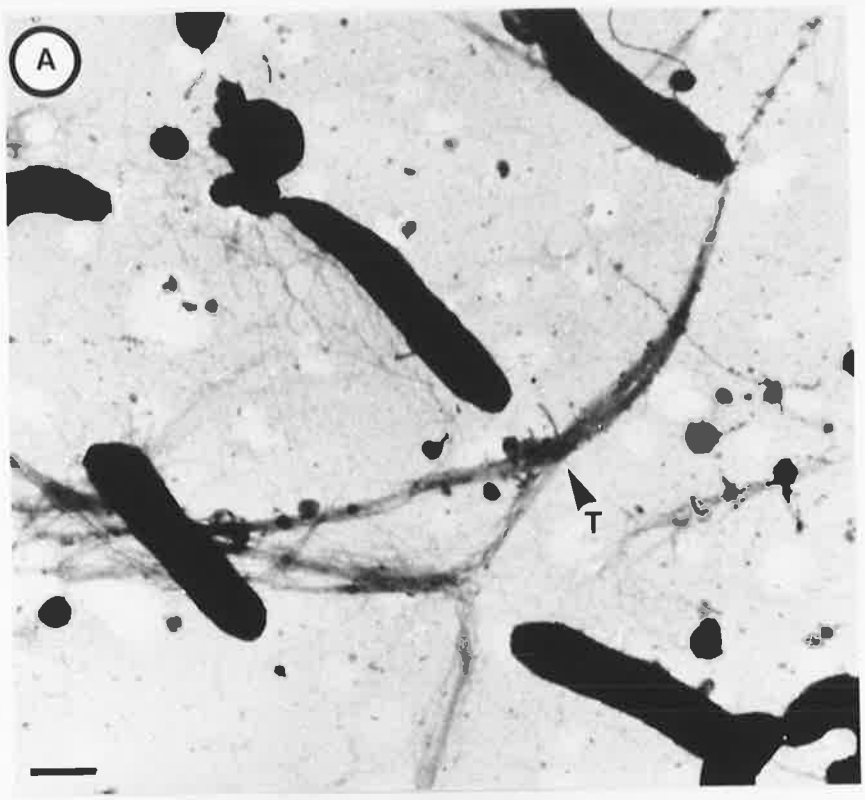
Further studies showed that the TCG medium described by Ehara *et al.* (1986) could also support TCP expression, although piliation was inconsistent (Figure 4.5). The culture conditions which most reliably promoted TCP production involved growth on NA at 37 C for 24 h, followed by growth on CFA (pH 6.5) for 24-36 hrs at 25 C. At this stage it was important to confirm that the pili expressed by these strains when grown in this manner were indeed TCP. To this end, a serum containing antibodies to TCP was obtained from Dr. R. Hall (Center for Vaccine Development, Baltimore), with the intention of examining the piliated bacteria by IEM. This serum had been prepared by immunization with TCP-expressing *V. cholerae* O395 (Hall *et al.* 1988) and upon receipt was absorbed four times with Ogawa LPS (Section 2.10.1) to remove anti-LPS antibodies, leaving behind antibodies exclusively to non-LPS determinants including those against TCP. IEM using this serum confirmed that the pili were indeed TCP (Figure 4.6).

4.3.3 Distribution of TCP

A number of *V. cholerae* strains were examined for their capacities to produce TCP under the growth conditions described above. In particular, a correlation was sought between the potential to express TCP and the previously determined distribution of non-LPS protective antigens. The results of an EM survey are presented in Table 4.1. Pili with the morphology of TCP were seen on all strains of Classical biotype examined, regardless of serotype, with the single exception of CA401; Taylor *et al.* (1987) also failed to detect significant TCP production by this strain. In contrast, however, TCP were not seen on any of 6 strains of El Tor biotype. Recent field isolates of the latter biotype, however, do possess non-LPS protective antigens (Section 3.4.2).

Immunoblotting studies confirmed the results of the EM survey. The serum used in these experiments was an anti-569B/165 serum which had been extensively absorbed to remove

Figure 4.5 Production of TCP on TCG. *V. cholerae* 569B grown in TCG agar at 25 C for 36 hours and negatively stained. T = bundle of TCP. Bar = 500 nm (A) 100 nm (B)


A
T

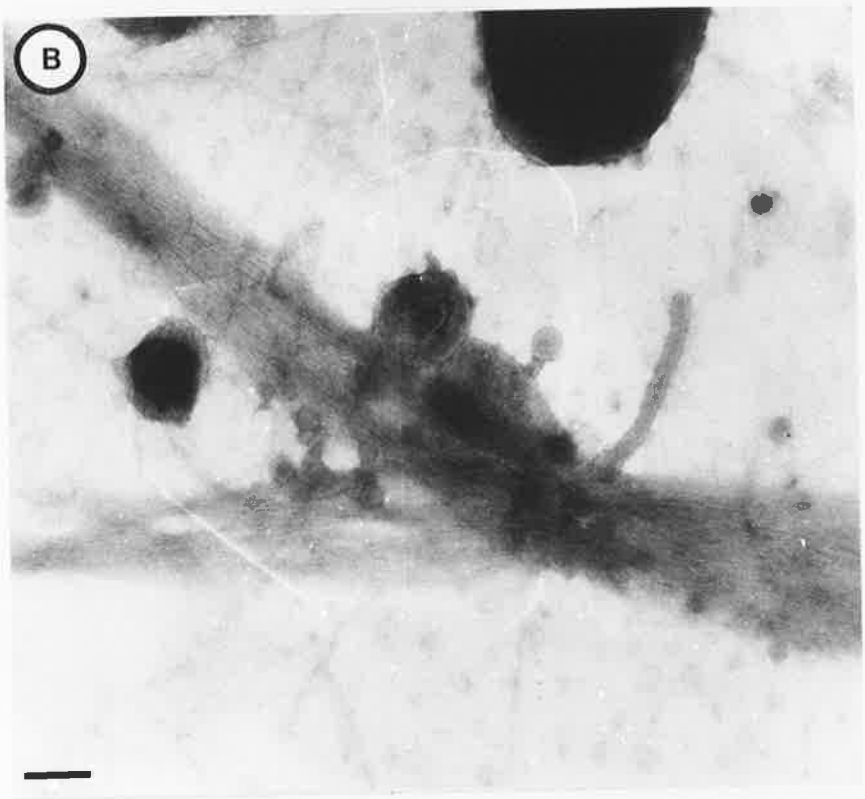

B

Figure 4.6 *V. cholerae* 569B grown on CFA at 25 C, reacted with LPS-absorbed anti-O395 antiserum, followed by Protein A colloidal gold conjugate, and negatively stained. T = bundle of TCP pili. Bar = 200 nm (A) and 100 nm (B)

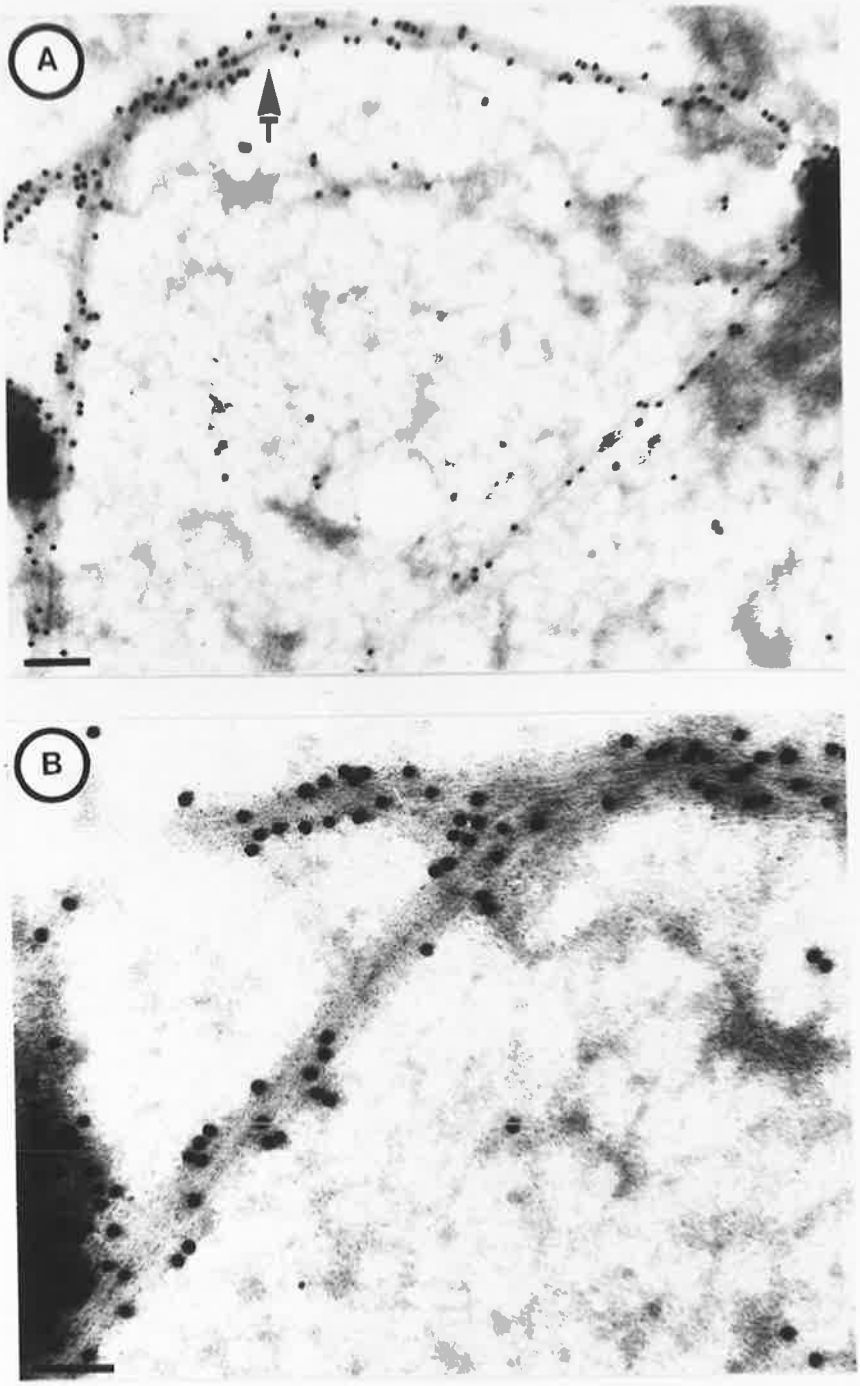

A
T
B

Table 4.1 Distribution of TCP

Strain	Expression[a]
Classical	
569B	+
CA401	-
CA411	+
Z17561	+
AA14041	+
569B/165	+
0395[b]	+
El Tor	
BM69	-
AA13993	-
H-1	-
64	-
K23[c]	-
O17	-

[a] Production of TCP on CFA at 25 C for 36 hrs.

[b] O395 is a Classical Ogawa kindly provided by Dr. R.H. Hall, Center for Vaccine Development, Baltimore, U.S.A.

[c] K23 is an El Tor Ogawa strain kindly provided by Dr. M. Ehara, Institute of Tropical Medicine, Nagasaki University, Japan.

antibodies to irrelevant bacterial components as a prelude to the attempted cloning of non-LPS protective antigens (see Section 5.2). IEM had confirmed that this serum contained antibodies to TCP (Section 4.6). The absorbed serum was initially tested against two Classical and four El Tor strains (two recent field isolates and two old laboratory strains) grown under conditions which promoted (CFA at 25 C) or repressed (NB at 37 C) TCP production. Figure 4.7 shows the results of subjecting the bacterial suspensions to SDS-PAGE and immunoblotting. When grown on CFA at 25 C both Classical strains produced a protein which could be detected by the antiserum. The molecular weight of this protein (20 kDa), and the the fact that it was not produced during growth in NB at 37 C (Figure 4.7), is consistent with it being the structural subunit of TCP. None of the El Tor strains synthesized this protein neither at 37 C nor at 25 C in any media.

In a second experiment, nine *V. cholerae* strains were cultured on CFA at 25 C and tested for TCP expression using the same antiserum. A crude TCP preparation (Section 2.13) was used as a positive control while 569B grown in NB at 37 C served as a negative control for immunoblotting (see Figure 4.7). As expected, the antiserum reacted with a 20 kDa protein expressed only by the Classical strains (with the exception of CA401) whereas no reaction was evident with the El Tor strains. The TCP preparation was strongly positive, supporting the conclusion that this protein represents the pilin subunit (Figure 4.8).

4.4 TCP is a virulence determinant in the infant mouse model

It was important to confirm the conclusion that TCP is a virulence determinant in the infant mouse model (Taylor *et al.* 1987). To this end the virulence of the non-motile variant of 569B (selected by the sloppy agar overlay technique as described in Section 2.2) was assessed following growth under conditions which supported or repressed TCP production. This variant

Figure 4.7 Immunoblotting analysis of *V. cholerae* strains for TCP expression. The follwoing strains were examined after culture on CFA at 25 C for 36 hours, (lanes A-F) and NB at 37 C (lanes G-L): H-1 (El Tor biotype, lanes A & G); AA13993 (El Tor, B & H); CA411 (Classical, C & I); 569B (Classical, D & J); O17 (El Tor, E & K); and 8233 (El Tor, F & L). They were resuspended in electrophoresis sample buffer and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose and the blots developed with 1:1000 rabbit anti-569B/165 CP serum (known to contain anti-TCP antibodies) followed by goat anti-rabbit IgG coupled with horse radish peroxidase and substrate. The arrow indicates the position of the molecular weight marker, ß-lactoglobulin (bovine milk), 18.4 kDa.

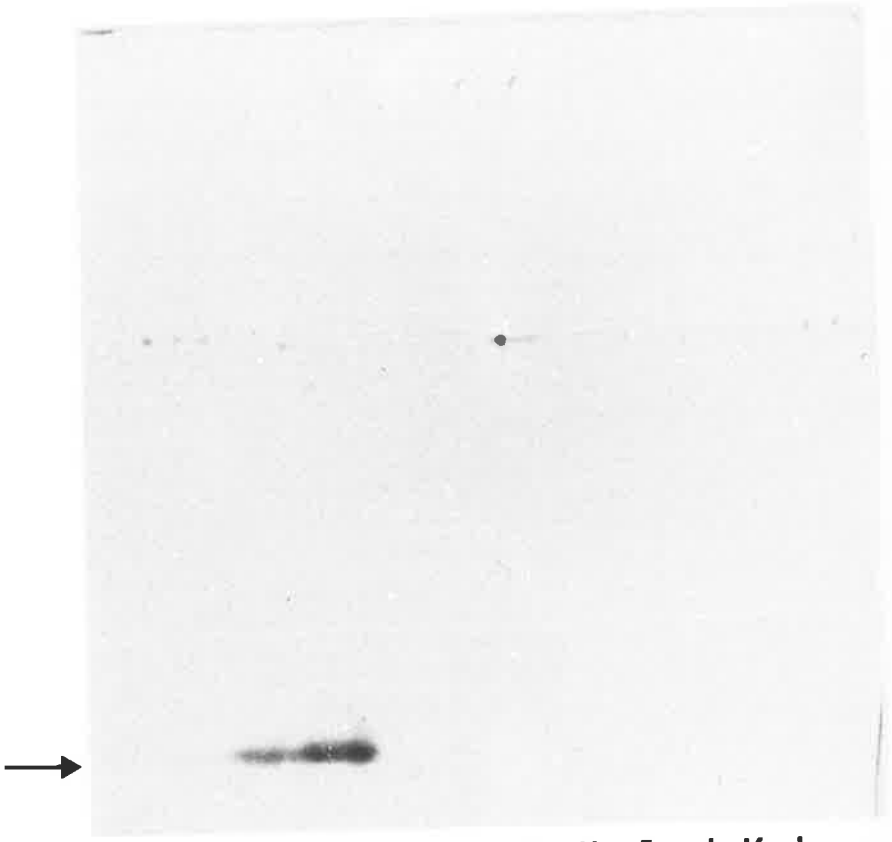
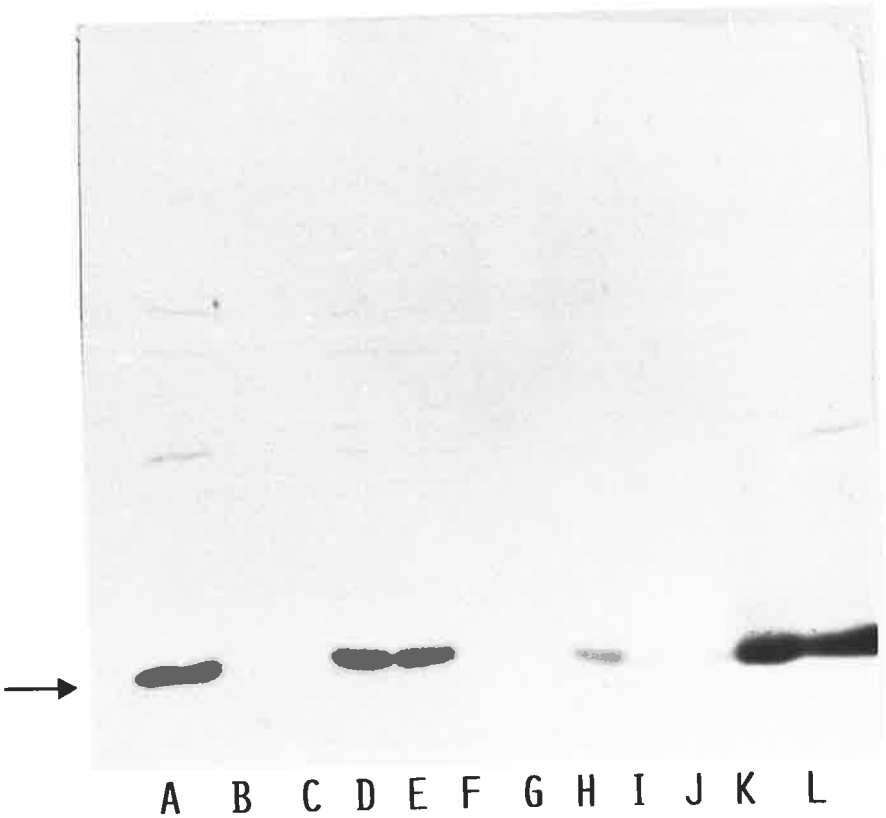

A B C D E F G H I J K L

Figure 4.8 Immunoblotting analysis of TCP expression by various strains of *V. cholerae*. A crude preparation of TCP (lanes A, L) or cell extracts from nine *V. cholerae* strains (lanes B-K) were immunoblotted using the serum described in the legend to Figure 4.7. Strains (biotype, serotype, lane position) tested were: H-1 (El Tor, Ogawa, B); AA13993 (El Tor, Inaba, C); AA14041 (Classical, Ogawa, D); Z17561 (Classical, Inaba, E); O17 (El Tor, Ogawa, F); 8233 (El Tor, Inaba, G); CA411 (Classical, Ogawa, H); CA401 (Classical, Inaba, I); 569B (Classical, Inaba, J & K). Strains were cultured on CFA at 25 C for 36 h and, in the case of 569B, also in NB at 37 C for purposes of comparison (Lane J). The arrow indicates the position of the molecular weight marker, ß-lactoglobulin (bovine milk, 18.4 kDa).


A B C D E F G H I J K L

was chosen in order to examine the effect ot TCP in the absence of the virulence-enhancing flagellar structure (Attridge & Rowley 1983a).

Expression of TCP by non-motile 569B led to a 100-fold increase in virulence; organisms grown on CFA at 25C had an LD_{50} of $1.27 \pm 0.4 \times 10^6$, compared with $2.25 \pm 0.11 \times 10^8$ for those grown in NB at 37 C.

4.5 Attempts to purify TCP

Given the pivotal role of TCP in colonization and virulence (Taylor *et al.* 1987; Herrington *et al.* 1988), and by analogy with the pilus colonization factors of ETEC, it seemed probable that TCP would represent a protective antigen of *V. cholerae*. Accordingly, attempts were made to purify these structures, in order to assess the protective activity of antibodies directed against them.

Following growth on CFA at 25 C, bacteria were resuspended in different solutions (see Section 2.13) prior to shearing to remove pili. Using PBS (AlKaissi & Mostratos 1985) or 12.5 mM Tris HCl (Taylor *et al.* 1987), the pili could be subsequently precipitated with ammonium sulphate and pelleted at low speed centrifugation, whereas with 10 mM Tris buffer (Korhonen *et al.* 1980), the pili resisted ammonium sulphate precipitation and could only be recovered by centrifugation at 100,000g for 1hr. Regardless of the method used, such pili preparations were always contaminated with other protein bands when examined by SDS-PAGE; when viewed by EM, LPS vesicles and flagella were evident (data not shown). In an attempt to remove these contaminants, the crude preparations were subjected to density gradient centrifugation following the method of Ehara *et al.* (1987), but EM examination revealed that this method was of little value.

Attempts were then made to purify the crude pili by adsorption onto guinea pig RBCs (Al-Kaissi & Mostratos 1985) but this too was unsuccessful. A final attempt was made to

separate pili from other cellular material using deoxycholate and concentrated urea according to the method of Korhonen *et al.* (1980), but this also proved futile. Since efforts to purify pili of *V. cholerae* had proved ineffective, this approach was abandoned in favor of less direct means of assessing the protective activity of anti-TCP antibodies.

4.6 TCP is a protective antigen in the infant mouse model

It was important to determine whether the protective anti-569B/165 serum (Section 3.5.1) contained antibodies to TCP and, if so, whether these antibodies contributed to the protective capacity of the serum. Although this serum was generated by immunization with organisms grown under conditions unfavorable for TCP expression (NB at 37 C), it seemed possible that a few TCP might have been produced under these conditions, or induced to do so following injection into the rabbit. The presence of anti-TCP antibodies was confirmed by IEM as shown in Figure 4.9 (A & B), which shows binding of antibody-conjugated colloidal gold particles to TCP. Subsequent work confirmed that, when grown on CFA at 25 C, the 569B/165 strain produces TCP as judged by EM, IEM and immunoblotting analyses (not shown).

Accordingly, the capacity of the anti-TCP antibodies to mediate protection against challenge with TCP-positive or TCP-negative organisms of the 569B strain (i.e., those grown on CFA at 25 C or in NB at 37 C, respectively) was assessed in the infant mouse model. The protective activity of the LPS-absorbed anti-TCP serum obtained from Dr. Hall (Section 4.3.2) was also assessed against both challenge inocula. The data are presented in Table 4.2. Anti-569B/165 showed a greater than fivefold PD_{50} when the challenge strain was allowed to express TCP pili *in vitro* (CFA, 25 C). A similar but greater effective rise in PD_{50} (ten-fold) was seen in anti-O395 LPS-absorbed serum when the same challenge strain was allowed to

Figure 4.9 Immunogold labelling of *V. cholerae* 569B. *V. cholerae* 569B was grown on CFA at 25 C for 36 hrs and the resulting organisms reacted with anti-569B/165 (A,B) or LPS-absorbed anti-CA411 (C,D) followed by Protein A-colloidal gold conjugate, and negatively stained. T = bundle of TCP pili. Bar = 200 nm (A), 100 nm (B), 100 nm (C) and 100 nm (D).

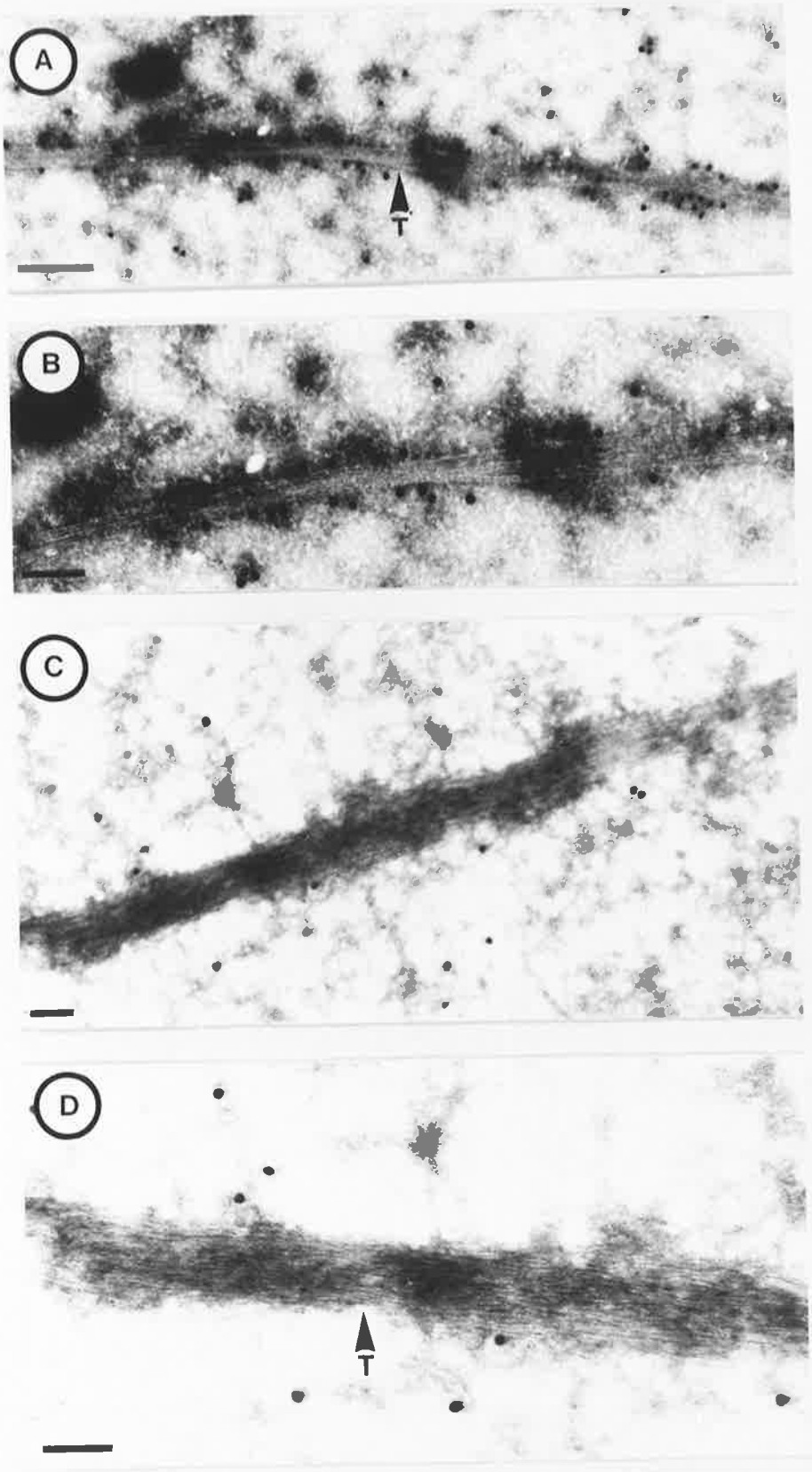

A
T
B
C
D
T

Table 4.2 Antibodies to TCP are protective in the infant mouse cholera model

	Protective activity against 569B[a]	
Antiserum to	TCP^{-}[b]	TCP^{+}[b]
569B/165	6,000 ± 330	34,000 ± 1,400
O395 absorbed with Ogawa LPS	23,000 ± 1,400	250,000 ± 14,000

[a] Protective activity in PD_{50} units/ml.

[b] The 569B challenge strain was grown under conditions which favored (CFA at 25 C) or repressed (NB at 37 C) the production of TCP (TCP^{+} or TCP^{-}, respectively.

express TCP *in vitro*. This clearly demonstrated that anti-TCP antibodies have protective potential. Later studies (Section 5.6.2) provided unequivocal confirmation that TCP is a protective antigen in this model. The appreciation of the protective potential of antibodies to TCP offered an explanation for previous data which had hitherto remained inexplicable. In Section 3.5.1 it was shown that, despite extensive absorptions with organisms of Ogawa serotype (strain CA411 seven times, AA14041 three times) to remove antibodies to antigens common to the two serotypes, the anti-569B/165 serum retained some protective activity against vibrios of the Ogawa serotype. This was indicated by the PD_{50} values obtained when mice were challenged with vibrios grown in NB at 37 C (PD_{50}s of 260 and 290 against CA411 and AA14041, respectively - Table 3.4). However, as mentioned in footnote (e) of this table, when these organisms were grown on CFA at 25 C and used as challenge strains, the PD_{50} values increased about four-fold (CA411, 970; AA14041, 1380). The higher protective endpoints obtained when the challenge organisms had been grown under conditions conducive to TCP expression suggested that antibodies to TCP had not been completely removed, and this was confirmed by IEM (not shown). In retrospect, this is not surprising, since the absorbing bacteria had not been cultured under conditions favourable for TCP expression.

In the reverse experiment, absorption of an LPS-absorbed anti-CA411 serum with bacteria of Inaba serotype (569B) successfully removed all protective activity against challenge organisms of the Inaba serotype (Section 3.5.1). In this instance, the initial anti-CA411 serum did not contain antibodies to TCP detectable by IEM (Figure 4.9, C & D). Hence, this explains why (as shown in Table 3.4) there was no residual activity left against the Inaba serotype (PD_{50} < 20).

4.7 Protection by anti-TCP antibodies is biotype restricted

EM and immunoblotting analyses had suggested that the capacity to express TCP was restricted to strains of the Classical biotype (Section 4.3.3). However, some strains of the El Tor biotype carry structural genes for TCP (Taylor *et al.* 1988a) and so it is possible that strains of this biotype can produce such pili *in vivo*. Therefore, it was imperative to determine the capacity of anti-TCP antibodies to protect infant mice from challenge with strains of Classical or El Tor biotype.

The anti-O395 supplied by Dr. Hall was chosen for the experiments to be described in this Section because of its greater protective efficacy against TCP-expressing challenge bacteria, compared to the anti-569B/165 serum (Table 4.2). This antiserum was first absorbed four times with live O395 (grown in NB at 37 C to inhibit expression of TCP) in order to remove potentially protective antibodies directed against LPS or non-TCP, non-LPS protective antigens.

Protection tests confirmed that the protective activity of the absorbed serum was primarily due to antibodies to TCP (Table 4.3). First, the serum was now only moderately protective against O395 or 569B vibrios cultured under conditions inhibitory to TCP production. In contrast, if these strains were grown on CFA at 25 C, the protective titers increased 20- or 300-fold, respectively (Table 4.3). Second, the absorbed serum was only weakly protective against CA401 (which, in this study, does not express TCP) challenge, despite the fact that this strain expresses non-LPS protective antigens (Section 3.4).

Consistent with the immunoblotting and EM analyses, when the challenge strains were of the El Tor biotype grown on CFA or in NB, the residual serum was unable to mediate protection (Table 4.3). These results clearly demonstrate that there is a correlation between

Table 4.3 Antibodies to TCP are protective only against challenge strains of Classical biotype[a]

Challenge strain	TCP status of challenge vibrios	
	TCP^-	TCP^+
O395	230 ± 50	5,000 ± 500
569B	430 ± 30	130,000 ± 11,000
O17 (PA^-)[b]	< 20	< 20
8233 (PA^-)	< 20	< 20
H-1 (PA^+)	< 20	< 20
AA13993 (PA^+)	< 20	< 20
CA401	70 ± 5	95 ± 8

[a] Figures show protective activity of absorbed anti-TCP O395 serum in PD_{50}s/ml. Challenge strains were grown to be quantitatively TCP^- or TCP^+ as described in legend to Table 4.2.

[b] PA^-/PA^+ refer to presence or absence of non-LPS protective antigens as described in Table 3.3.

the distribution of pili amongst *V. cholerae* strains and protective activity of anti-TCP antibodies and that it is biotype-restricted.

4.8 Discussion

The experiments described in this chapter were performed in the hope of elucidating the nature of the non-LPS protective antigens of *V. cholerae*. In an attempt to identify differences between the four strains lacking non-LPS protective antigens and the twelve strains which share such determinants (Section 3.4), OMP preparations were compared by SDS-PAGE and immunoblotting analyses. This approach was not helpful however, since the OMP profiles of the four old El Tor strains were not consistently different (Figure 4.1). Given the denaturing conditions required for such analyses, it is possible that none of the bands detected by immunoblotting involves the binding of protective antibodies. A number of PAGE systems and various detergent and solvent extractions of the OMP preparations were tested in order to obtain better resolution of the proteins but these did not prove fruitful.

Subsequent experiments were performed to test whether any of the non-LPS protective antigens might be associated with pilus structures produced by *V. cholerae*. Although experiments described here focussed upon the expression of TCP, other pili were seen by EM (data not shown). One of the other types seen, albeit in low numbers, was a flexible thin filament 5-7 nm in width. Ibrahim (1984) found slender flexible pili (3-4 nm in diameter) present in 1 of 3 El Tor strains tested as well as in one Classical strain. Al-Kaissi & Mostratos (1985) reported the presence of long twisted pili on some strains of the Classical biotype while Ehara *et al.* (1986) described the pili they observed as flexible, long fibres present on El Tor strains. It is possible the flexible pili seen on El Tor strains in this study are the same as those described by others (Ibrahim 1984; Ehara *et al.* 1986). Very recently, Hall *et al.* (1988) reported a

systematic EM study of the pilus types produced by *V. cholerae*. TCP were detected only on Classical strains, while two other types (B and C) were seen on all strains regardless of biotype.

Production of TCP by *V. cholerae* was first reported by Taylor *et al.* (1987) following growth of bacteria in LB medium at 30 C. Subsequent experiments by Hall *et al.* (1988) confirmed the existence of TCP and recommended growth on CFA at 25 C for optimal piliation. Initial experiments were designed to identify cultural conditions which would consistently support TCP production by *V. cholerae* strains used in this study. Inability to detect such appendages following growth of bacteria in LB medium suggests some difference in medium constituents. In agreement with Hall *et al.* (1988) however, TCP were observed when the bacteria were grown on CFA at 25 C. The width of the pilus filaments with TCP morphology was estimated to be 7-10 nm, similar to previous estimates (7nm, Taylor *et al.* 1987; 5-6nm, Hall *et al.* 1988).

EM and immunoblotting studies (Table 4.1, Figures 4.7 & 4.8) confirmed Hall's finding that, under the growth conditions adopted, production of TCP is restricted to strains of Classical biotype. In total, TCP have now been detected on - Classical strains (Taylor *et al.* 1987; Hall *et al.* 1988; this thesis) but on none of - El Tor biotype. The studies performed to date do not eliminate the possibility that strains of El Tor biotype will synthesize TCP under as yet undefined growth conditions *in vitro*, or perhaps only in response to environmental stimuli present in the gut. Infant mouse experiments designed to assess the capacity of anti-TCP antibodies to mediate protection against El Tor challenge strains failed to provide support for the latter (see below).

Various methods have been described for the isolation and purification of different pilus structures produced by *V. cholerae* (Korhonen *et al.* 1980; Al-Kaissi & Mostratos 1985; Ehara *et al.* 1987; Taylor *et al.* 1987). However, in my hands, none of these provided adequate purification of TCP, as all resulted in substantial protein and LPS contamination. It is known

that pili are extremely hydrophobic, consisting of a high proportion of nonpolar amino acids (Korhonen *et al.* 1980; Gaastra & de Graaf 1982) and TCP are no exception (Taylor *et al.* 1987). Accordingly pili tend to associate strongly with other bacterial surface components such as outer membrane vesicles and LPS. Attempted fractionation of pili on the basis of size and hydrophobicity using a sodium deoxycholate-urea buffer (Korhonen *et al.* 1980) proved futile. At least some of the protein contaminants seen in the TCP preparations could have been due to the presence of pilus-associated proteins as have been described for *Neisseria gonorrhoeae* (Muir *et al.* 1988). As time was limited, further attempts at purification were abandoned in favor of alternative approaches to the evaluation of the protective significance of TCP.

Pilus colonization factors have been recognized as virulence determinants not only for *E. coli* (Satterwhite *et al.* 1978; Gaastra & de Graaf 1982) but also for several other gram-negative bacterial pathogens (Kellogg *et al.* 1963; Duguid & Old 1980; Abraham & Beachy 1985). During the course of these studies TCP were shown to be essential for gut colonization in both infant mice (Taylor *et al.* 1987) and man (Herrington *et al.* 1988). In the former studies, mutant strains unable to produce TCP were shown to be unable to efficiently colonize the intestines of infant mice, and were consequently much less virulent than the wild-type *V. cholerae* (Taylor *et al.* 1987). Herrington *et al.* (1988) subsequently used such mutants to assess the importance of TCP for the colonization potential and vaccine efficacy of non-toxigenic *V. cholerae*. In contrast to the vaccine strain which could produce TCP, the mutants were found to be non-colonizing, non-immunogenic and non-protective.

Initial experiments defined growth conditions which consistently resulted in TCP production by *V. cholerae* 569B, the strain most commonly used in protection studies. After confirming the identity of the pili by IEM (Figure 4.6), infant mouse tests confirmed that TCP production significantly enhanced the virulence of the (non-motile) 569B strain. Although the 100-fold difference in virulence was less dramatic than that previously reported by Taylor *et al.*

(1987) for the O395 strain, this probably results from reliance upon a phenotypic rather than genotypic difference in TCP production. Thus a minority of the bacteria cultured under conditions unfavorable for TCP expression might have been TCP-positive; alternatively, the population might have been uniformly TCP-negative at the time of challenge, but synthesized these pili rapidly *in vivo*. In contrast, the mutants used by Taylor *et al.* (1987) were genetically incapable of TCP production.

Since virulence determinants such as colonization factors are prime candidates for protective antigens, it was of great interest to determine whether TCP might represent one of the non-LPS protective antigens of *V. cholerae*. In this respect it was encouraging to find that the protective anti-569B/165 serum (Section 3.5.1) contained antibodies to TCP, as judged by IEM (Figure 4.9) and immunoblotting analyses (Figure 4.7). The first evidence in support of the hypothesis that TCP can function as a protective antigen came from infant mouse assays designed to evaluate the protective potential of these anti-TCP antibodies. Sera containing such antibodies were markedly more effective in mediating protection if the challenge organisms were expressing TCP at the time of administration (Table 4.2).

Subsequent protection tests indicated that antibodies to TCP could mediate protection against challenge strains of Classical biotype only (Table 4.3), a finding consistent with the inability to detect the production of TCP by El Tor strains. This suggests that TCP is not the only non-LPS protective antigen in this model, since recent field isolates of El Tor biotype possess such components (Section 3.4). Attempts to identify non-LPS protective antigens by gene cloning are described in the next chapter.

Chapter 5

Molecular cloning of genes involved in biosynthesis of TCP

5.1 Introduction

When attempts to isolate non-LPS protective antigens from OMP preparations proved unsuccessful, it was necessary to devise alternative approaches to further characterize these components. To this end, the studies described in the previous chapter were performed to examine the protective significance of pilus structures produced by *V. cholerae*. Concurrently attempts were begun to isolate non-LPS protective antigens using the techniques of gene cloning, and these studies form the basis of the present chapter.

Initial experiments were designed to clone genes encoding non-LPS protective antigens of *V. cholerae* 569B and to express them in an *E. coli* recipient strain. As discussed below, this approach proved unsuccessful. At about that time, Taylor *et al.* (1987) reported their studies of TCP, from which it was clear that surface expression of TCP and other OMPs was dependent upon a functional *toxR* gene. Since the *E. coli* recipient strain used in this study lacked this gene an alternative cloning strategy was devised which allowed cloning of genes involved in TCP production. The availability of TCP-producing clones allowed unequivocal confirmation of the conclusions that TCP acts as both a virulence determinant and protective antigen in the infant mouse cholera model.

5.2 Preparation of clone probing serum.

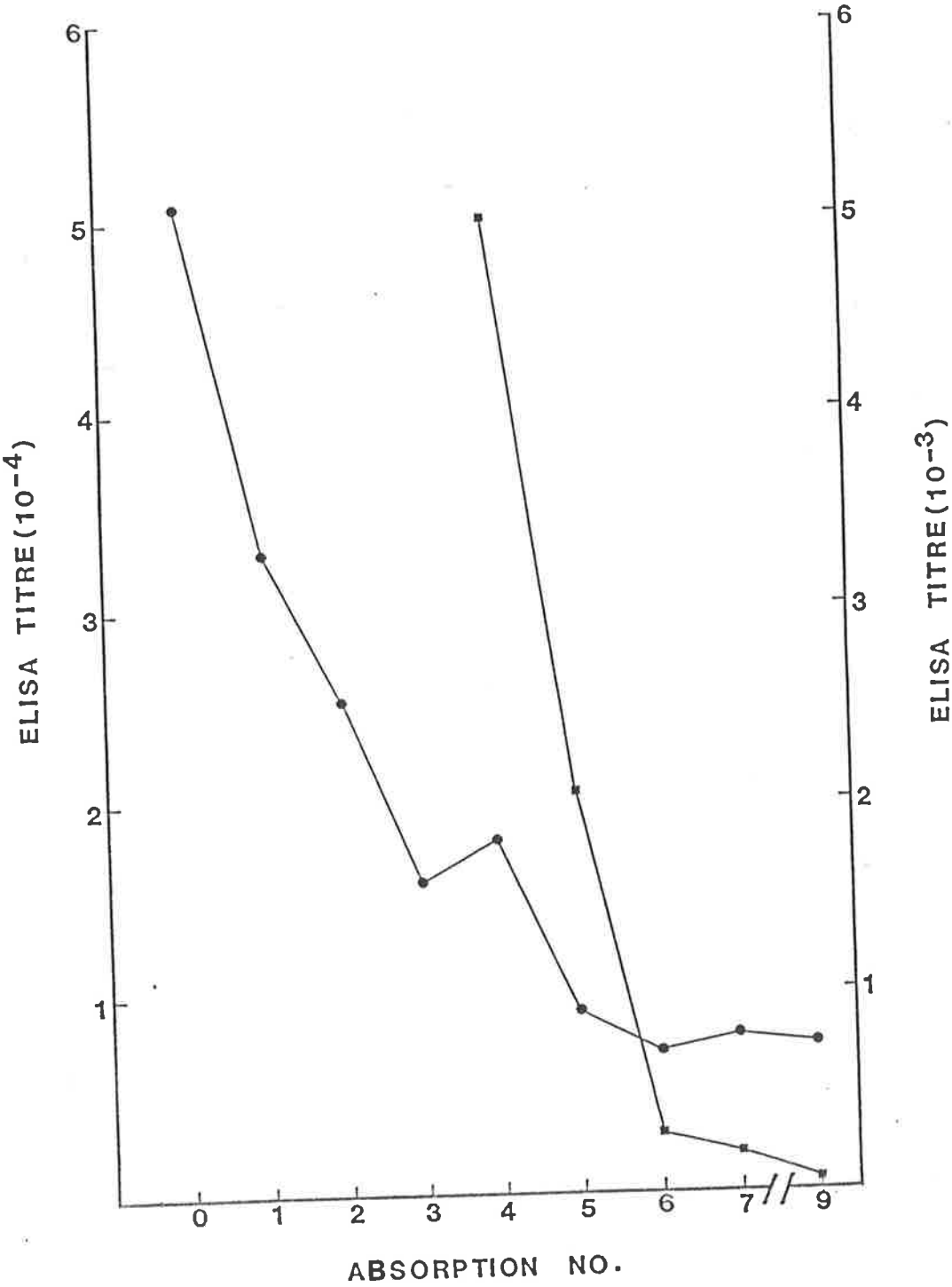
A clone-probing serum was required in order to detect by immunoblotting any clones expressing non-LPS protective antigens, and a protective rabbit anti-569B/165 serum (Section 3.5.1) was selected for this purpose. Before use the serum was extensively absorbed to remove antibodies to components other than non-LPS protective antigens, thereby reducing the likelihood of detecting "false-positive" clones during the screening process. Since non-LPS protective antigens are heat-labile and absent from old El Tor strains such as O17 (Section 3.4.1), the serum was absorbed four times with boiled 569B/165 (to remove antibodies to heat-stable antigens), three times with live O17 (to remove antibodies to non-protective heat-labile antigens), and finally two times with live *E. coli* K12 strain DH1 (to remove antibodies to the *E. coli* recipient strain).

Aliquots were kept after each absorption and tested in an ELISA using 569B/165 and O17 OMPs as antigens (Figure 5.1). The absorbed serum had ELISA titers of 7,300 and 49 against 569B/165 and O17 OMPs, respectively. This represents 14% and <0.10% of the original activity against both antigens, respectively (Figure 5.1). In contrast, the protective activity of the residual serum was virtually unchanged (protective titer against 569B challenge fell from 1260 to 1150), indicating selective retention of the antibodies of interest. The absorbed serum is henceforth referred to as anti-569B/165 CP (clone-probing) serum.

5.3 Preliminary cloning experiments

Initial attempts to clone non-LPS protective antigens involved construction of a 569B gene bank in *E. coli* strain DH1 using the cosmid vector pHC79. Details of this procedure are not given because these studies were not successful. A total of 400 colonies were screened by colony blotting (Section 2.16), using a 1:1000 dilution of anti-569B/165 CP serum. Sixteen of

Figure 5.1 Preparation of clone probing serum. An antiserum prepared in rabbits against live 569B/165 hybrid vibrios, and known to contain protective antibodies specific for TCP (Section 4.6), was extensively absorbed to remove antibodies of irrelevant specificities (Section 5.2). Absorptions 1-4 were with boiled 569B/165 vibrios, 5-7 with live O17 and 8,9 with live *E. coli* K12 Strain DH1. The unabsorbed and the aliquot of the absorbed sera at various stages were tested against 569B/165 OMP (left;) and O17 OMP (right;) in an ELISA assay. O represents unabsorbed serum. ELISA titer of unabsorbed serum against O17 OMP is 27,700 (not shown in Figure).


ELISA TITRE(10^{-4})
ELISA TITRE(10^{-3})
ABSORPTION NO.
0
1
2
3
4
5
6
7
9

the clones were positive and subjected to further analysis. Aliquots of unabsorbed anti-569B/165 serum were absorbed (as per Section 2.10.2) 4 times with each of the 16 positive clones or with positive (569B) or negative (*E. coli*) control strains and subsequently tested for removal of antibody activity by both ELISA and infant mouse protection tests. Although there was no correlation between reduction in ELISA titer and loss of protective activity (data not shown), 9 of the 16 clones reduced the protective titer by 75% or more. However, when mice were immunized with these nine clones none of the resulting antisera were protective (data not shown).

In an attempt to explain these disappointing results, the nine clones were again subjected to immunoblotting with CP serum, some twelve weeks after their original detection. Despite storage of the clones in glycerol at -70 C (Section 2.2), all were now negative and thus no longer expressing antigens of interest. This apparent instability was attributed to the fact that the *E. coli* recipient strain DH1 lacked a *recA*$^-$ background required for clone stability. However, subsequent studies by Mekalanos and colleagues made it clear that surface expression of TCP and other OMPs is dependent upon the *V. cholerae toxR* gene (Taylor *et al.* 1987; Miller & Mekalanos 1988). Since no such gene is present in *E. coli*, an alternative cloning strategy was devised.

5.4 Molecular cloning of genes involved in TCP production

5.4.1 Cloning strategy

The innovation that made possible the cloning of genes involved in TCP synthesis was the selection of a *V. cholerae* strain, O17, as the recipient of a cosmid gene bank initially constructed in *E. coli*. The O17 strain possesses the *toxR* gene required for expression of TCP (Manning, personal communication), but does not produce these pili under cultural conditions which promote TCP expression by strains of Classical biotype (Table 4.1; Hall *et al.* 1988).

Moreover O17 lacks non-LPS protective antigens in general (Attridge & Rowley 1983c; Section 3.4.1), facilitating detection of clones expressing such components.

V. cholerae Z17561 was selected as the donor strain since it expresses TCP well *in vitro* (Table 4.1) and, being a recent field isolate, it also offered the possibility of cloning any other non-LPS protective antigens it might possess (Section 3.4.2). Cosmid cloning and *in vitro* packaging of donor strain DNA into bacteriophage lambda (Simon *et al.* 1983) was used as a means of obtaining DNA fragments of sufficient size to clone the *tcp* operon.

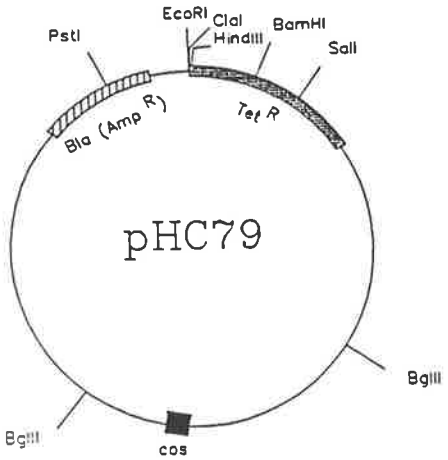
5.4.2 Construction of the mobilizable cosmid vector pPM2101

The cloning strategy adopted required a mobilizable vector, because of the need to reintroduce cloned *V. cholerae* genes via an *E. coli* intermediate recipient back into *V. cholerae*. Accordingly, the mobilizable (Mob) region of the broad host-range plasmid RP4 (from plasmid pSUP201-1) was introduced into the cosmid vector pHC79. This construction is shown in Figure 5.2; details of the construction are given in Section 2.17.4.

5.4.3 Cloning the TCP genes into V. cholerae O17

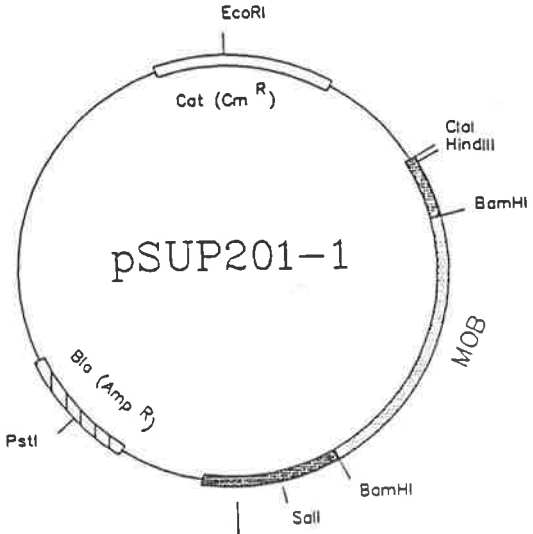
A gene bank was constructed by partially digesting the Z17561 DNA (prepared as described in Section 2.17.1) with the restriction endonuclease *Sau*3A to obtain fragments approximately 40 kb in size, which were then cloned into the *Bam*HI site of the vector pPM2101 (see Section 2.17.4). After overnight ligation at 4 C the DNA mixture was packaged into bacteriophage lamda (Section 2.17.5). The packaged phage were then used to infect *E. coli* K12 strains S17-1, from which the cloned DNA was mobilized into *V. cholerae* O17 by plate matings (Section 2.17.6). The resulting cosmid bank was stored on nutrient agar plates containing Ap at 4 C.

Figure 5.2 Construction of pPM2101. The cosmid pHC79 was cleaved with *Eco*RI and the protruding ends filled using Klenow fragments of DNA polymerase I. Plasmid SUP201-1 was cleaved with *Bam*HI, the fragment containing the RP4 Mob region was isolated and the protruding ends filled as above. The cleaved pHC79 and the Mob fragment was ligated overnight and the ligation transformed into strain S17-1 selecting for Tet^R. The correct constructs were then selected by their ability to be mobilized into strain SM10 selecting for Tet^R (pPM2101) Kan^R (counter-selection) against S17-1.



Cleave with EcoRI.

End fill with Klenow fragment.



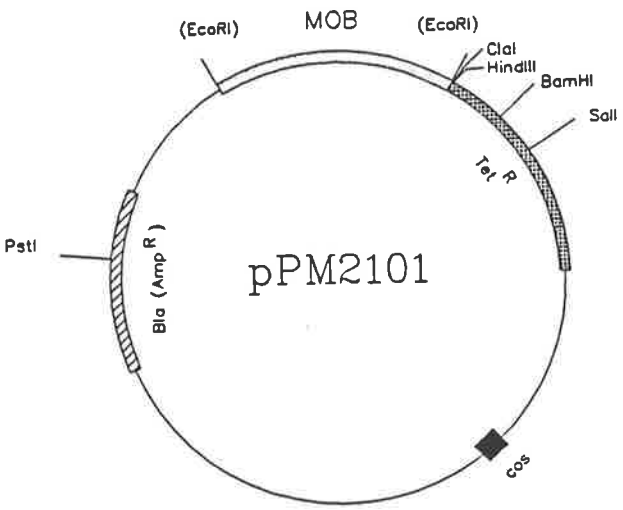
Cleave with BamHI.

End fill with Klenow fragment.

Ligate.

Transform into strain S17-1 and select for Tet^R.

Select for mobilization into strain SM10 screening for Tet^R and Kan^R.



5.4.4 Detection of positive clones

About 800 colonies were patched in duplicate onto CFA plates and incubated at 25 C for 36h to promote TCP expression (see Section 4.3.2). One set of colonies was transferred to nitrocellulose filters and lysed *in situ* using the method of Henning *et al.* (1979). Colony blotting (Section 2.16) with CP serum identified 4 strongly- and 4 moderately-positive clones from a bank of about 800 (Figure 5.3). These 8 clones and 2 negative clones were designated DS1-DS10 (containing plasmids pPM2102 to pPM2111, respectively) and subjected to various analyses.

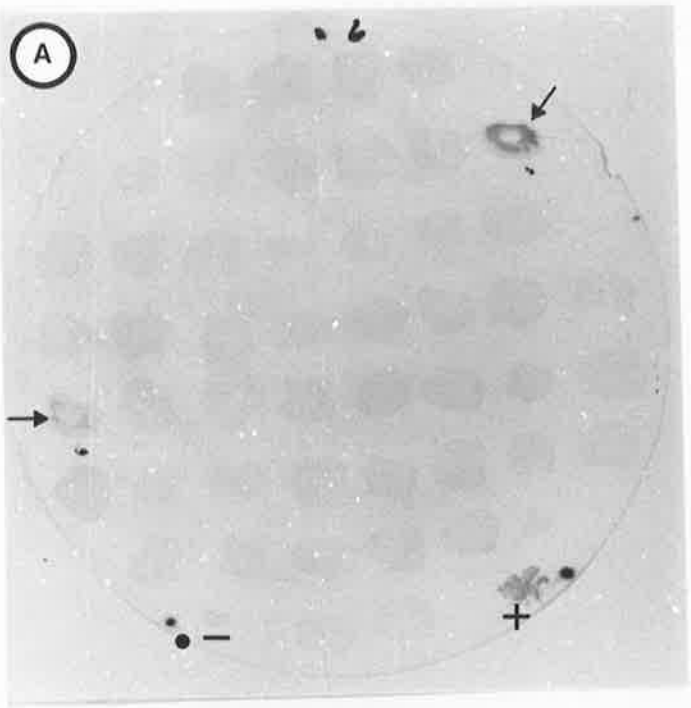
5.5 Analysis of the clones DS1-DS10

5.5.1 EM studies

Clones DS1-DS10, together with O17 (recipient strain, negative control) and Z17561 (donor strain, positive control) were inoculated onto CFA and incubated at 25 C for 36 hrs. EM examination of the harvested bacteria revealed that only the four strongly-positive clones (DS2, DS4, DS5, DS6) expressed pili with the morphology of TCP (Figures 5.4 & 5.5). Individual TCP filaments 7-10 nm in diameter were seen to aggregate closely in parallel to form bundles 100-200 nm in width. The remaining clones and the O17 recipient strain failed to produce such pili under these growth conditions. The donor strain Z17561 showed a higher level of TCP expression than the four O17 clones.

IEM analysis confirmed that the CP serum contained antibodies which bound to the pili produced by the four O17 clones, as visualized by the binding of protein A-colloidal gold particles (Figure 5.6 A & B). The pili produced by clones DS2, DS4, DS5 and DS6 were shown to be TCP by IEM using LPS-absorbed anti-O395 serum (Section 4.3.2; Figure 5.6 C & D). Neither serum significantly labelled O17 or the other 6 clones.

Figure 5.3 Colony blotting. Approximately 800 colonies of a cosmid bank derived from *V. cholerae* Z17561 (Classical Inaba) were screened for the presence of non-LPS antigens. Figure shows positively reacting clones (indicated by arrows) whilst + and - signs indicate positive (Z17561) and negative (O17) controls.


A
−
+

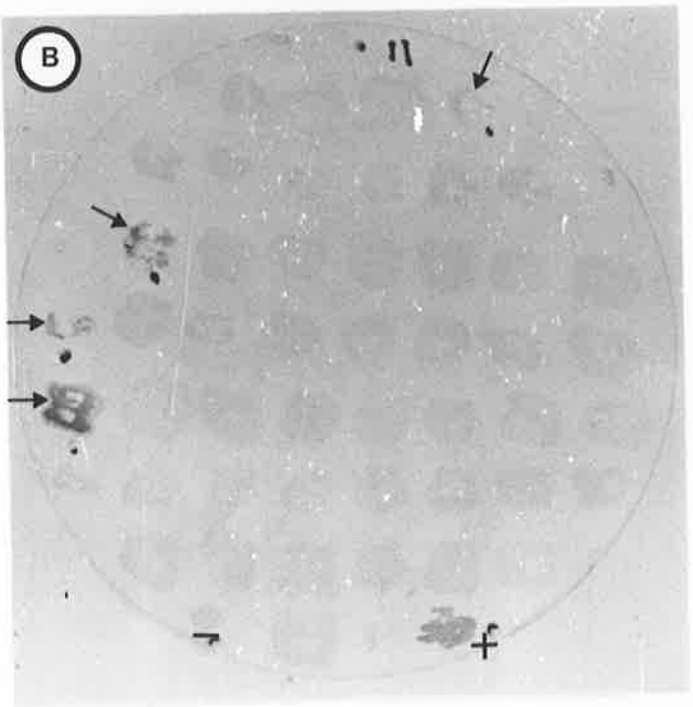

B
+

Figure 5.4 Production of TCP by clone DS2. Positive control (Z17561) and clone DS2 (O17[pPM2103]) were grown on CFA (with Ap for clone) at 25 C for 36 h, harvested in PBS and applied to grids for EM inspection (Section 2.18.1). A and B = Z17561; C and D = DS2. The bars in A and C = 200 nm and in B and D = 100 nm.

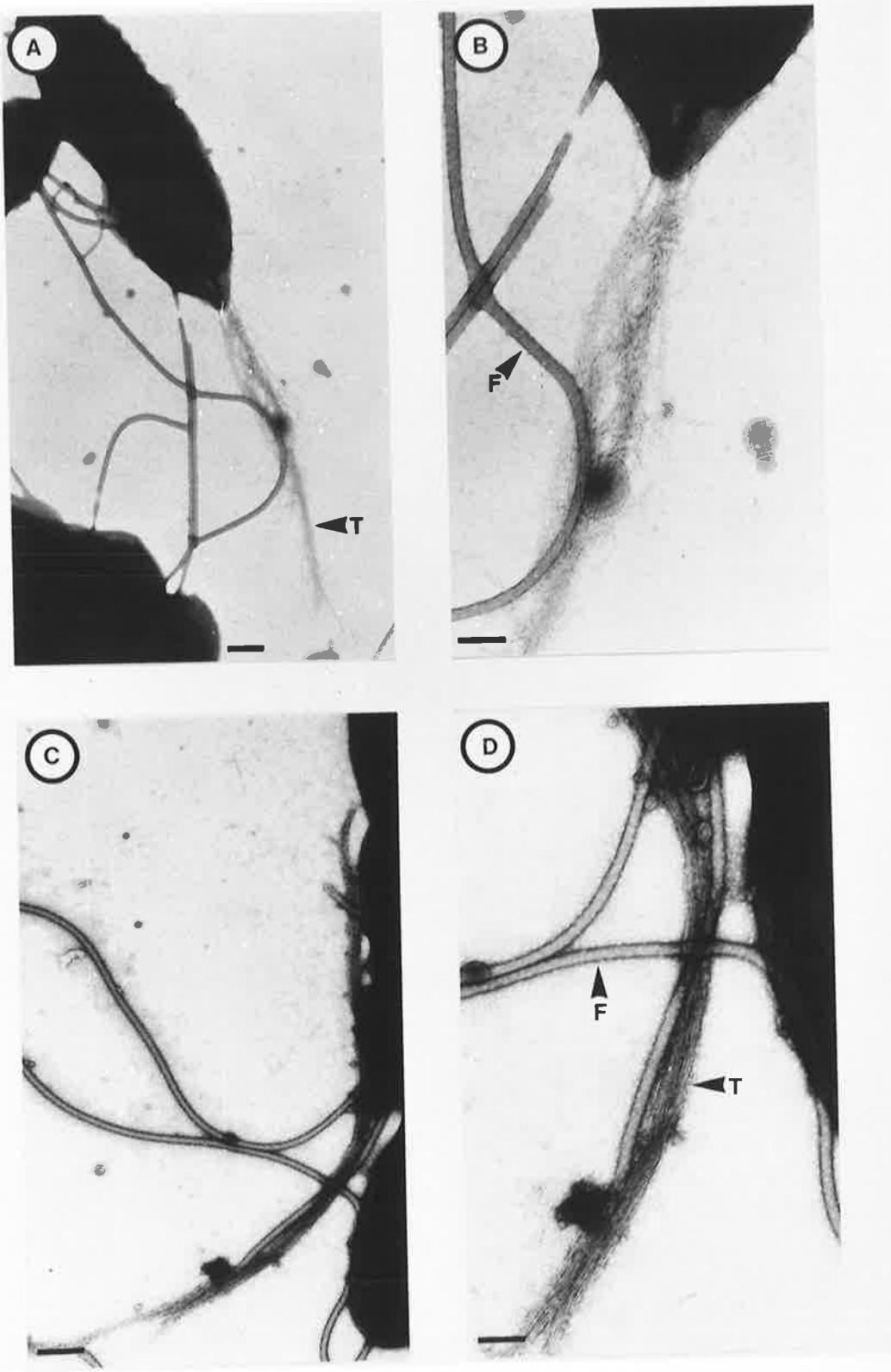

A
T
B
F
C
D
F
T

Figure 5.5 Production of TCP by clones DS5 and DS6. Clones DS5 (O17[pPM2106]) and DS6 (O17[pPM2107]) were grown on CFA (with Ap) at 25 C, harvested in PBS and transferred to grids for EM inspection (Section 2.18.1). A and B = DS5; C and D = DS6. The bars in A and C = 500 nm, and in B and D = 100 nm.

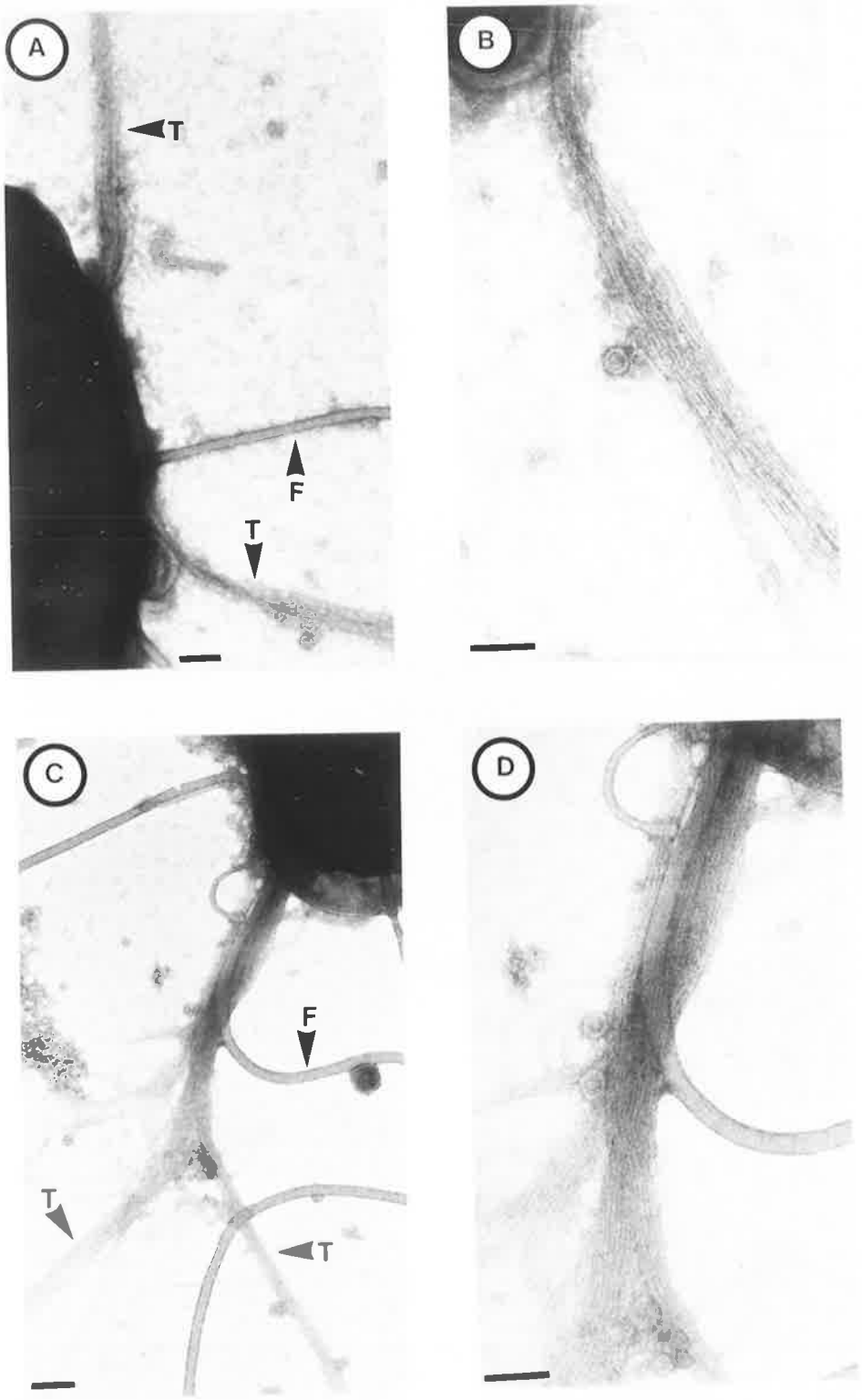
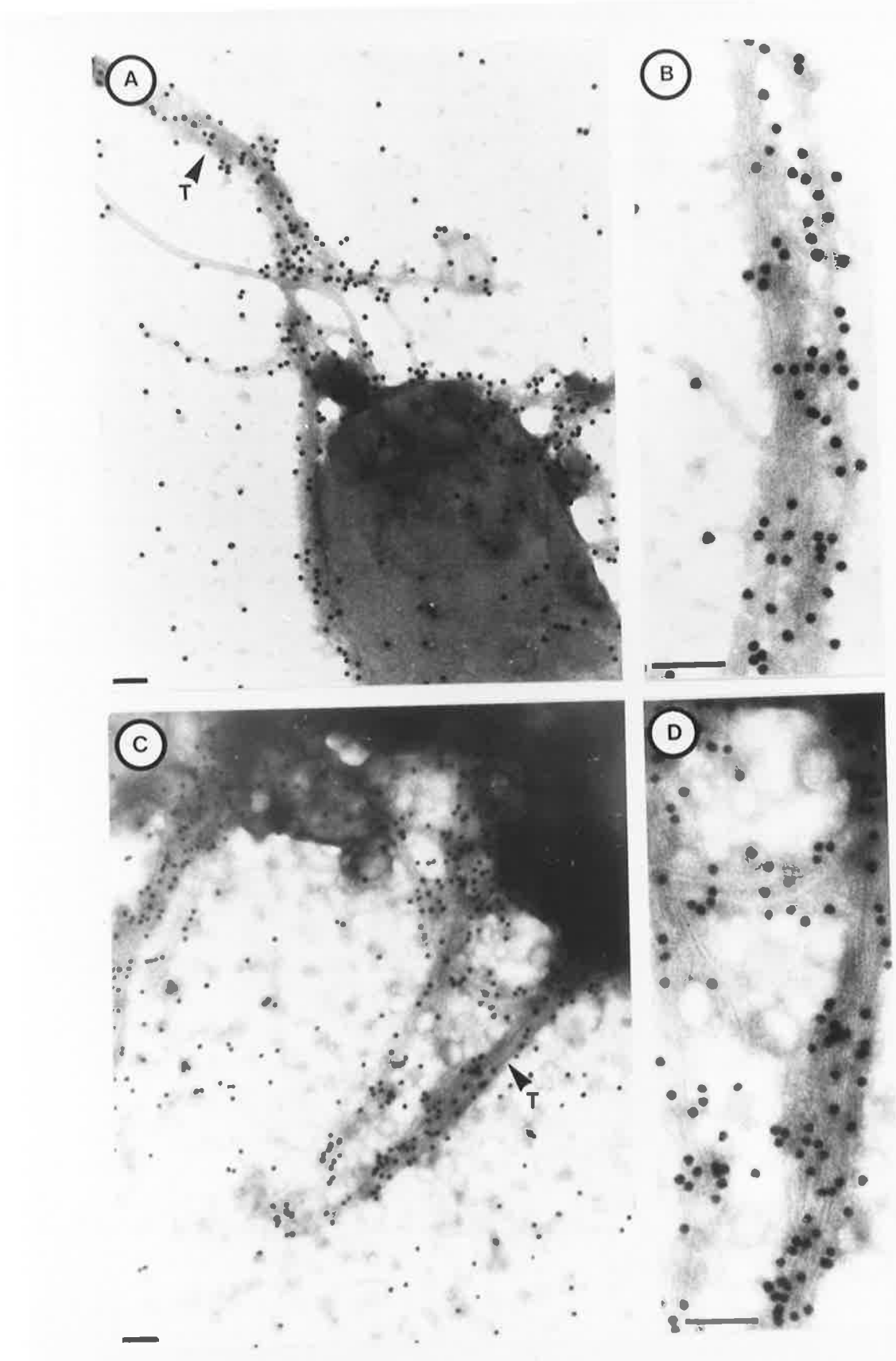

A
T
F
T
B
C
F
T
T
D

Figure 5.6 Immunogold electron microscopy of TCP produced by Clone DS5 (O17[pPM2106]). Clone DS5 was grown on CFA (with Ap) at 25 C, and harvested in PBS and transferred to grids for IEM. Bacteria were reacted with the antiserum (anti-569B/165 CP for A and B; LPS-absorbed anti-O395 for C and D) and then protein A-colloidal gold as described in Section 2.18.2. The bars correspond to 100 nm in each case.


A
T
B
C
T
D

5.5.2 Immunoblotting analysis

For immunoblotting analysis, the ten O17 clones, two positive controls (strains Z17561 and 569B) and one negative control (O17) were cultured on CFA at 25 C for 36 h. The resulting growth was harvested and the suspensions solubilized and subjected to SDS-PAGE prior to Western blotting with CP serum. In agreement with the EM observations, only the DS2, DS4, DS5 and DS6 clones, together with Z17561 and 569B, were positive by immunoblotting (Figure 5.7). In each case, the presence of a 20 kDa protein, corresponding to the TcpA pilin subunit (Taylor *et al.* 1987), was detected.

5.5.3 Molecular analysis of cloned DNA

In order to characterize the DNA carried by the 8 positive clones, crude plasmid preparations were transformed into *E. coli* K12 strain SM10 (see Section 2.17.7), whence plasmid DNA suitable for restriction analysis could be obtained (i.e. circumventing the problem of DNA-degrading enzymes produced by *V. cholerae*). This analysis revealed that each of the 4 TCP-producing clones carried DNA which included the *XbaI* fragments (Figure 5.8) characteristic of the region encoding TCP (Taylor *et al.* 1988a). This suggested that most of the genes required for biosynthesis of this pilus had been cloned into a single cosmid. Mobilization of the plasmids back into O17 confirmed that the cloned DNA conferred all of the properties originally associated with the positive clones.

Figure 5.7 Immunoblot analysis of whole cells. Whole cells of the various strains were grown on CFA (plus Ap for the clones) at 25 C for 36 h. They were resuspended in electrophoresis sample buffer and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose and the blots developed by incubation with 1:1000 rabbit anti-569B/165 CP serum, followed by goat anti-rabbit IgG coupled with horseradish peroxidase and finally substrate (Section 2.15.2). The samples are: A, O17; B, 569B; C, DS10; D, DS9; E, DS8; F, DS7; G, DS6; H, DS5; I, DS4; J, DS3; K, DS2; L, DS1; M, Z17561. The arrow indicates the position of the molecular weight marker ß-lactoglobulin (bovine milk, 18,400).

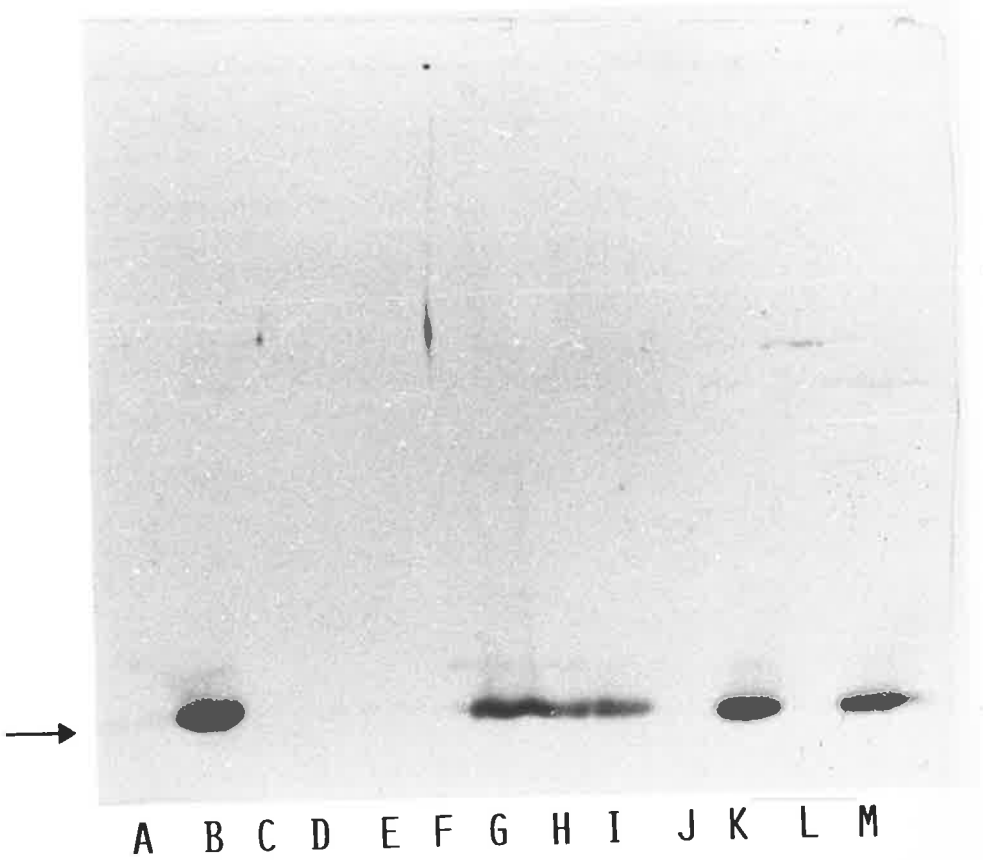
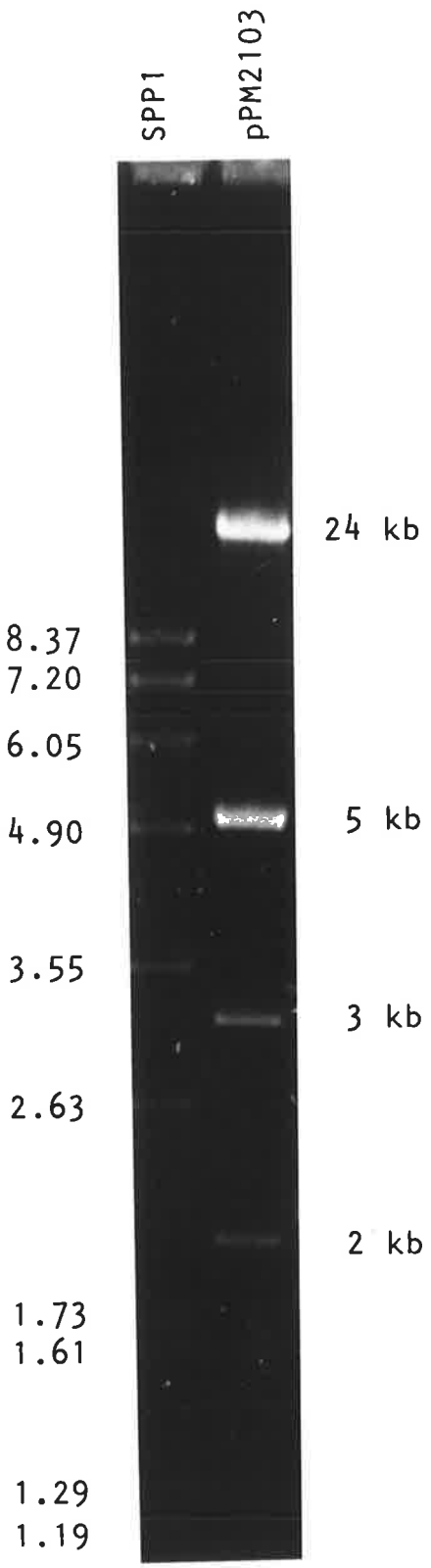

A B C D E F G H I J K L M

Figure 5.8 Restriction endonuclease analysis of pPM2103 (DS2 clone). 500 ng of DNA was digested with *Xba*I at 37 C for 1 hr. Electrophoresis of the digested DNA was carried out at room temperature on horizontal 0.8% agarose gel. The size of restriction fragments were calculated by comparing their relative mobility with that of *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA.


SPP1
pPM2103
24 kb
8.37
7.20
6.05
4.90
5 kb
3.55
3 kb
2.63
2 kb
1.73
1.61
1.29
1.19

5.6 Confirmation of TCP as a virulence determinant and non-LPS protective antigen of V. cholerae

5.6.1 TCP is a virulence determinant

The availability of TCP-expressing O17 clones provided an opportunity to confirm previous conclusions concerning the role of TCP as a virulence determinant and protective antigen in the infant mouse model (Section 4.4 & 4.6). Three such clones were found to be, on average, seventy times more virulent than the O17 recipient strain and a TCP-negative O17 clone (Table 5.1), confirming that production of TCP is sufficient to enhance virulence. In addition, protection tests indicated that antibodies to TCP are sufficient to confer protection in this model.

5.6.2 TCP is a protective antigen

The first experiment compared the capacities of TCP-positive and TCP-negative O17 clones to remove protective antibodies by absorption. Rabbit anti-569B/165 serum was absorbed four times with TCP-negative Z17561 (grown in NB shaking culture at 37 C) in an attempt to obtain a serum whose protective activity was primarily, if not exclusively, due to anti-TCP antibodies. Protection tests suggested that this aim had been achieved, since the absorbed serum had a PD_{50} of 155 against TCP-negative Z17561 but 4,290 against TCP-positive Z17561 (grown on CFA at 25 C). Moreover, the protective activity against the CA401 strain, which possesses non-LPS protective antigens (Section 3.4.1) but does not produce TCP (Table 4.1), fell from 5,000 to 110 as a result of these absorptions. Five 2-ml aliquots of the absorbed serum were then further absorbed with one of the O17 clones or with Z17561 as a positive control; the bacteria used for these absorptions were grown on CFA at 25 C to optimize TCP expression. Table 5.2 shows that Z17561 and each of three TCP-expressing O17 clones

Table 5.1 Production of TCP enhances the virulence of *V. cholerae* O17

Strain	Plasmid	TCP status[a]	Virulence[b]
O17	-	-	$2.82 \pm 0.10 \times 10^5$
DS2	pPM2103	+	$5.66 \pm 0.40 \times 10^3$
DS5	pPM2106	+	$4.38 \pm 0.13 \times 10^3$
DS6	pPM2107	+	$3.29 \pm 0.60 \times 10^3$
DS9	pPM2110	-	$3.61 \pm 0.73 \times 10^5$

[a] TCP status indicates the ability to produce TCP on CFA at 25 C in 24 h.

[b] Virulence is expressed as the LD_{50} for infant mice; figures show mean ± SE of 3 determinations

Table 5.2 Absorption with TCP-expressing O17 clones removes protective antibodies

Absorbing strain	TCP status	Protective activity vs Z17561*
Z17561	+	<20
DS2	+	<20
DS5	+	<20
DS6	+	<20
DS9	-	3980 ± 130

* The antiserum used was raised in rabbits against the hybrid strain 569B/165 and had been absorbed 4 times with Z1756ITCP$^-$ (grown in NB at 37 C) to leave residual protective antibodies which were directed primarily against TCP. Aliquots of this serum which has a protective titer of 4,290 ± 320 when assayed against Z17561 TCP$^+$ organisms, were then further absorbed with the strains shown. Residual protective activity against Z17561 TCP$^+$ was then determined.

removed all protective activity, whereas the negative clone was unable to do so. Since the O17 host strain has no non-LPS protective antigens, this result directly demonstrates the protective efficacy of antibodies to TCP.

In a second experiment, the anti-TCP serum obtained above was tested for protective activity against O17 (negative control), Z17561 (positive control) and four of the clones. Challenge bacteria were grown on CFA to promote TCP production and the results of the protection assays are shown in Table 5.3. The absorbed serum did not protect mice from challenge with O17 or the TCP-negative O17 clone, in support of previous results (Section 4.7) obtained using a different source of antibodies to TCP. Significantly, however, protection was observed against challenge with the TCP-producing O17 clones (Table 5.3), showing unequivocally that antibodies to TCP are sufficient for protection in this model.

5.6.3 TCP-producing O17 clones are immunogenic

A final experiment was designed to determine whether immunization with TCP-producing O17 clones would elicit the production of antibodies to TCP. Rabbits were immunized with three of the TCP-producing O17 clones (DS2, DS5 and DS6), one negative clone (DS9) or the Z17561 strain as a positive control. The immunizing bacteria were grown on CFA at 25 C for 36 hrs; after confirmation of TCP production by EM, the growth was harvested, suspended in PBS, and stored in aliquots at -20C.

Before assaying the resulting antisera for their capacities to protect infant mice from challenge with TCP-producing Z17561, it was first necessary to absorb them to remove potentially protective antibodies to Ogawa LPS. This was achieved by absorption with live O17 vibrios, and the success of the procedure is verified by the fact that the absorbed sera retained no protective activity against O17 challenge (Table 5.4). To determine whether the sera

Table 5.3 Antibodies to TCP are protective in the infant mouse cholera model

Challenge strain	TCP status[a]	Protective titer[b]
Z17561	+	4290 ± 320
Z17561 (NB)	-	155 ± 20
CA401	-	110 ± 10
O17	-	<20
DS2	+	400 ± 40
DS5	+	1030 ± 94
DS6	+	510 ± 45
DS9	-	<20

[a]Indicates the ability to produce TCP on CFA at 25 C for 24h.

[b]The protective titer is the PD_{50} against the various challenge strains. The antiserum used was raised in rabbits against the hybrid strain 569B/165 and had been absorbed 4 times with Z17561TCP$^-$ (grown in NB at 37 C) to leave residual protective antibodies which were directed primarily against TCP.

Table 5.4 TCP-producing clones are immunogenic and protective

Immunizing strain	Protective activity of O17-absorbed antisera[a] vs Z17561[c]	Protective activity of O17-absorbed antisera[a] vs O17[c]
Z17561	40,000 ± 450	<20
DS2	1,180 ± 120	<20
DS5	1,740 ± 240	<20
DS6	1,570 ± 110	<20
DS9[b]	<20	<20

[a] Rabbits were immunized with the strains listed, and the resulting antisera absorbed four times with live O17 (see text). When the anti-Z17561 was further absorbed with live Z17561 TCP$^-$ (grown in NB at 37 C) to remove antibodies to non-LPS protective antigens other than TCP, the PD_{50} was reduced to 25,900 ± 330.

[b] The PD_{50} of the unabsorbed anti-DS9 against O17 was 2360 ± 100.

[c] Challenge bacteria of the Z17561 or O17 strains were cultured on CFA at 25 C, and each protection test was performed twice.

contained protective antibodies directed against TCP, protection tests were next performed using TCP-positive Z17561 as the challenge bacteria.

The results of these assays (Table 5.4) indicate that the three TCP-producing O17 clones generated protective antibodies to TCP, whereas the TCP-negative clone did not. Thus TCP is immunogenic when presented on the O17 clones and antibodies directed against these pili are protective. The antiserum to Z17561 contained protective antibodies directed not only against TCP but also against other non-LPS antigens. Further absorption of the 017-absorbed anti-Z17561 serum with TCP-negative Z17561 reduced the protective titer by 35% (to 25,900). Even after this further absorption, the protective activity of the serum was much higher than those of the sera raised against the TCP-producing O17 clones, presumably reflecting the lower level of TCP expression by the latter.

5.7 Discussion

Initial attempts to clone non-LPS protective antigens from *V. cholerae* into *E. coli* were unsuccessful. In retrospect, the approach adopted was inappropriate for the cloning of TCP since it is now known that production of TCP requires a functional *toxR* gene (Taylor *et al.* 1987) which would not be present in *E. coli*. Accordingly, a new cloning strategy was devised which involved the chromosomal integration of a mobilizable plasmid. A cosmid gene bank prepared from *V. cholerae* Z17561 was mobilized into *V. cholerae* El Tor O17 via an *E. coli* intermediate recipient strain. This was achieved using a specially constructed mobilizable plasmid pPM2101 (Figure 5.2), which contains a 1.6kb *Bam*HI fragment encoding the *mob* region of RP4. Miller & Mekalanos (1988) recently employed a similar method to introduce DNA into bacterial cells in a nonreplicating form, to demonstrate that *toxR* regulates the expression of cholera toxin, TCP and some OMPs in response to certain environmental signals. *V. cholerae* strains Z17561

and O17 were selected as donor and recipient strains for the reasons outlined above (Section 5.4.1).

The plasmids carried by the four TCP-producing O17 clones contain the 2kb, 3kb, 5kb and 20 kb *XbaI* fragments which characterize (Taylor *et al.* 1988a) the *tcp* operon (Figure 5.8). In addition, when present in *E. coli*, these plasmids react with a *tcpA*-specific oligonucleotide probe, confirming the presence of this structural gene (R. Faast, U.H. Stroeher & P.A. Manning, unpublished data). Together these findings eliminate the possibility that what has been cloned is the *toxR* regulatory gene upon which TCP expression depends (Taylor *et al.* 1987). Moreover, the *toxR* gene has recently been cloned and shows a different restriction pattern (P.A. Manning, personal communication).

Although EM and immunoblotting analyses have failed to detect TCP expression by El Tor strains (Hall *et al.* 1988; Section 4.3.3), Taylor *et al.* (1988a) found that each of five clinical isolates of this biotype carried DNA to which a TCP probe could hybridize. Using pPm2103 as a probe for the *tcp* operon, it has recently been established that *V. cholerae* O17 and three other El Tor strains also possess homologous DNA (U.H. Stroeher & P.A. Manning, unpublished). Further study of the plasmids cloned here will be aimed at elucidating the interplay between plasmid and chromosomal genes which results in TCP production by the O17 strain. It remains feasible that the plasmids carry a functional regulatory element other than *toxR* such as *tcpH* (Taylor *et al.* 1988a) and that this complements a defective - or replaces a missing - controlling gene in *V. cholerae* O17. Alternatively, the "defect" which prevents TCP production by O17 (under growth conditions examined to date) might lie in a structural gene, though a simple gene mutation would not explain the failure of all El Tor strains to produce TCP. Finally it is possible that the TCP region can undergo rearrangements of the type recently observed with the genes specifying pilus production in *Moraxella bovis* (Marrs *et al.* 1988), and that El Tor strains have become locked into a non-expressing mode.

The TCP-producing O17 clones permitted further evaluation of the significance of TCP as a virulence determinant and protective antigen in the infant mouse model. The demonstration that TCP expression significantly increases the virulence of O17 (Table 5.1) confirms the original demonstration of the critical involvement of these pili in infant mouse pathogenesis (Taylor *et al.* 1987). Consistent with the role of TCP as a colonization factor (Taylor *et al.* 1987; Herrington *et al.* 1988) is the finding that antibodies to these pili are protective, presumably by inhibiting adherence to the intestinal epithelium (Attridge & Rowley 1983c). Thus only TCP-expressing O17 vibrios could remove protective antibodies by absorption (Table 5.2) and generate such antibodies upon immunization (Table 5.4). Antibodies to TCP are sufficient to mediate protection against challenge with TCP-positive Z17561 or O17 (Table 5.3), confirming previous results with the 569B and O395 strains of *V. cholerae* (Table 4.3). The protection observed against the TCP-positive O17 clones is particularly significant, as earlier experiments indicated that antibodies to TCP were not protective against challenge strains of El Tor biotype (Table 4.3), which fail to express TCP under the growth conditions examined to date (Table 4.1; Hall *et al.* 1988). Time did not permit a study of those moderately-positive O17 clones detected by colony blotting which did not produce TCP. Experiments are planned to determine whether these clones express non-TCP non-LPS protective antigens.

Chapter 6

General Discussion

6.1 Distribution of non-LPS protective antigens of V. cholerae

Previous studies with the infant mouse cholera model have demonstrated the existence of non-LPS protective antigens in *V. cholerae*. Antibodies to some of these determinants were shown to possess protective capacity (Neoh & Rowley 1970, 1972; Bellamy *et al.* 1975) and their protective efficacy was greater than that of anti-LPS antibodies (Attridge & Rowley 1983c). The presence of non-LPS protective antigens on at least one *V. cholerae* strain (569B; Attridge & Rowley 1983c), made it critical to determine the distribution of these components among recent field isolates. If such strains shared non-LPS protective antigens, it was felt that further study of these antigens might eventually identify new components of vaccine significance.

It was shown in Chapter 3 that *V. cholerae* of either biotype and of both common serotypes express common non-LPS antigens, although four old laboratory El Tor strains lack these determinants. The reason for this restricted distribution is unclear. It is unlikely that these determinants have been lost from El Tor strains during prolonged storage in the laboratory since Classical strains that were stored in the same manner still express them. Perhaps expression of non-LPS protective antigens by the new El Tor strains results from environmental interactions between the two biotypes (Section 3.7). Nevertheless, further

study of these antigens was clearly warranted. SDS-PAGE and immunoblotting analyses failed to identify a consistent difference in the OMP profiles of strains expressing or lacking non-LPS protective antigens and attempts to characterize these determinants were unsuccessful (Section 4.2).

6.2 Role of TCP as a colonization factor and a protective antigen

An earlier study of the antibacterial properties of polyclonal anti-*V. cholerae* sera sought to define a consistent *in vitro* correlate of antibody-mediated protection in the infant mouse cholera model. It was found that the capacity to directly block the *in vitro* attachment of *V. cholerae* provided such a correlate, whereas the capacities to immobilize or agglutinate the bacteria did not (Attridge & Rowley 1983c). Experiments in this thesis have confirmed and extended this finding. Each of three sources of antibodies to non-LPS protective antigens inhibited the *in vitro* adherence of strains possessing such determinants (as defined by protection assays). In contrast, these sera did not protect infant mice from challenge with, nor block the attachment of, old laboratory strains of El Tor biotype (Section 3.6.2). The implications from such data is that at least some of the non-LPS protective components may play a role in colonization *in vivo*.

Numerous studies attest to the significance of pili as colonization factors for a variety of bacterial pathogens, including noninvasive enteropathogens which establish toxin-mediated diarrheal syndromes similar to cholera (Section 1.3.3.4). Reports published during the course of this thesis resolved the controversial issue of of whether *V. cholerae* also produces pili (Ehara *et al.* 1986; Taylor *et al.* 1987; Hall *et al.* 1988). The experiments of Taylor *et al.* (1987) were of particular relevance, identifying one pilus type - TCP - a virulence determinant in the infant mouse cholera model. Attention therefore turned to assessing the significance of TCP.

Initial work involved optimizing growth conditions for TCP expression, and performing EM and immunoblotting analyses to determine whether the expression of TCP correlated with the expression of non-LPS protective antigens. These studies revealed that *in vitro* TCP production (following growth on CFA) was observed only with the Classical strains (with the exception of CA401) and not with the El Tor strains (Table 4.1; Figures 4.7 & 4.8). That is, whereas non-LPS protective antigens were detected on the new El Tor strains of *V. cholerae*, this was not the case with TCP expression. This suggests that TCP, although a major non-LPS antigen, is not the only protective non-LPS antigen. This point will be discussed further (Section 6.4).

Subsequent experiments confirmed the significance of TCP as a virulence determinant in this model. It was found that non-motile 569B vibrios were over 100-fold more virulent if cultured under conditions conducive to TCP expression (Section 4.4). Experiments described by Taylor *et al.* (1987) clearly showed that production of TCP conferred a significant advantage in terms of persistence in the infant mouse gut. Moreover, Herrington *et al.* (1988) demonstrated that TCP expression similarly promotes colonization in humans. When volunteers were challenged with virulent Classical O395 those who had been previously fed with a TCP-expressing strain were protected against illness whereas those who received the non-TCP expressing strain were not protected.

By analogy with the pilus colonization factors of ETEC strains (Levine *et al.* 1983), it was predicted that the pivotal role of TCP in vibrio colonization would similarly confer upon these pili the status of protective antigens. Although this remains to be demonstrated for human infection, the experiments described in this thesis show that this is indeed the case in the infant mouse cholera model. The protective efficacies of antisera shown to contain antibodies to TCP by IEM were dramatically increased if the challenge vibrios were TCP-positive (Table 4.2). One of these sera was then absorbed to obtain a serum whose protective

activity was primarily, if not exclusively, due to antibodies to TCP (Section 4.7). This residual serum was still highly protective against challenge strains of Classical biotype, but did not protect against El Tor vibrios which fail to produce TCP under the growth conditions tested to date (Table 4.3).

These initial experiments provided the first evidence that TCP is a protective antigen of *V. cholerae*. Unequivocal confirmation of this point came from subsequent studies which followed the cloning of genes involved in TCP production.

6.3 Molecular cloning of genes involved in the synthesis of TCP

An attempt to define the non-LPS protective antigens of *V. cholerae* involved the cosmid cloning of DNA prepared from a recent field isolate, using a cloning strategy discussed earlier (Section 5.4). This approach resulted in the cloning of genes involved in TCP production and expression of these genes in *V. cholerae* O17, a strain which normally lacks non-LPS protective antigens. TCP-producing O17 clones were, on average, seventy times more virulent than the unmodified recipient strain (Table 5.1) confirming earlier findings. Most significantly, a serum containing antibodies to TCP was shown to protect mice from challenge with TCP-positive 017 clones though not from challenge with (TCP-negative) O17 (Table 5.3). This provided unequivocal confirmation of previous experiments which had suggested that TCP is a protective antigen in this model. Not surprisingly, the TCP-producing O17 clones, but not O17, could remove protective antibodies by absorption (Table 5.2) and upon immunization elicit protective antibodies specific for non-LPS components (Table 5.4).

6.4 Is TCP the only non-LPS protective antigen of V. cholerae in the infant mouse model?

The protective efficacy of antibodies to TCP raised the question of whether this antigen is the only non-LPS protective component in this model. This was particularly important because of the finding that at least one of the antisera used to determine the distribution of such components among *V. cholerae* strains was found to contain antibodies to TCP, despite the fact that the bacteria used in the preparation of this serum had been grown under conditions unfavorable for TCP production (Section 3.5.1). Several lines of evidence suggests that *V. cholerae* possess additional non-LPS protective antigens, however.

First, the capacity to synthesize TCP does not correlate with the pattern of expression of non-LPS protective antigens. Although the latter components are found on recent field isolates of El Tor biotype, such strains fail to produce TCP as judged by EM, immunoblotting and protection experiments (Sections 4.3 & 4.6). These results are consistent with the presence of non-TCP, non-LPS protective antigens, at least on recently isolated El Tor strains. Second, the existence of serotype-restricted non-LPS protective components similarly argues against TCP being the only such antigen, since TCP synthesis is not serotype related. Third, the CA401 strain possesses non-LPS protective antigens (Table 3.3) yet is atypical among strains of Classical biotype in that it fails to produce TCP *in vitro* (Section 4.3.3). Finally, the Z17561 strain evidently possesses non-TCP, non-LPS protective antigens (Table 5.4).

6.5 Non-LPS protective antigens: relevant studies in other laboratories

The experiments described in this thesis have identified TCP as the first non-LPS protective antigen of *V. cholerae*, at least in the infant mouse model. Workers in several

laboratories are studying the nature of vibrio colonization, as colonization factors would be prime candidates for protective antigens.

The important contributions of Taylor, Mekalanos and colleagues have been referred to throughout this thesis, and represent a major step forward in the quest for an improved cholera vaccine. This group has recently purified TCP from a flagellum-negative derivative of *V. cholerae* O395 and shown that a resulting anti-TCP serum was highly protective in the infant mouse model, confirming results obtained in this thesis (Taylor *et al.* 1988b).

To date, TCP remains the only defined adhesin of *V. cholerae* (although there is some evidence to suggest that LPS can promote attachment - Section 1.3.3.2). Peterson & Mekalanos (1988) identified "accessory colonization factors" in the infant mouse model by studying *Tox*R-regulated genes in *V. cholerae*. Transposon insertions in any of a cluster of four genes resulted in a much reduced capacity to colonize the infant mouse gut, though the effect was less dramatic than that accompanying deletion of genes encoding TCP (Peterson & Mekalanos 1988). The nature of the accessory colonization factors remains to be defined.

Recent studies have investigated the role of HAs in *V. cholerae* colonization. Finn *et al.* (1987) constructed a mutant El Tor strain which lacked the mannose-sensitive cell-associated HA (Hanne & Finkelstein 1982) and found that this mutant showed a markedly impaired capacity to colonize the rabbit ileum. Nevertheless, oral immunization with the mutant protected rabbits from subsequent challenge with virulent *V. cholerae* (Finn *et al.* 1987). Although this would seem to indicate that antibodies to this HA are not necessary for protection in this system, further studies would be needed to eliminate the possibility that the mutant produces an inactive HA which retains the potential to elicit neutralizing antibodies.

Teppema *et al.* (1987) reported that a motile, (mannose-resistant) HApositive, El Tor strain and an aflagellate HA-negative, Classical strain showed similar capacities to colonize ligated ileal loops constructed in adult rabbits. On this basis it was concluded that factors other

than hemagglutinating activity or motility are involved in colonization in this model. This illustrated the importance of the experimental system in studies of vibrio pathogenesis, as the conclusion reached by Teppema *et al.* (1987) conflicts with the results of several earlier studies attesting to the significance of the flagellar structure in colonization (see Section 1.3.3.1). Attachment to the intestinal surface evidently occurs more readily in a static, closed system such as a ligated gut loop; indeed, some cholera models require a drug-mediated inhibition of peristalsis (Freter 1955a; Finn *et al.* 1987).

During the past decade, several groups have adopted the RITARD (removable intestinal tie - adult rabbit diarrhea) model developed by Spira *et al.* (1981) to study infection by, and immunity to, *V. cholerae*. The original procedure involved the temporary obstruction of the small bowel to promote bacterial colonization and the permanent ligation of the cecum to prevent fluid resorption, although modifications to this system have been described (Guinée *et al.* 1985). Colonization factors and protective antigens have not yet been defined for the RITARD model. Live *V. cholerae* immunize much more effectively against subsequent challenge than killed bacteria (Guinée *et al.* 1987; Rijpkema *et al.* 1987; Pierce *et al.* 1988), with the capacity for mucosal association providing the only correlate of protective efficacy (Pierce *et al.* 1988). Protection in this model is associated with a marked inhibition of intestinal colonization (Guinée *et al.* 1985; Lycke *et al.* 1986; Pierce *et al.* 1988), but the specificity of the protective antibodies remains unknown.

Svennerholm *et al.* have found that intestinal immunization with rough *V. cholerae* mutants can protect rabbits from subsequent challenge with fully virulent smooth cholera vibrios in the absence of any anti-LPS antibody response (cited by Jonson *et al.* 1989). This suggests a protective role for non-LPS antibodies. This is in contrast to their earlier observations indicating that protective capacity in the rabbit model resided exclusively in anti-LPS antibody fraction (Svennerholm 1980), a finding that seemed to have been corroborated by

Jansen *et al.* (1988). Nevertheless, this recent work by Svennerholm (cited by Jonson *et al.* 1989) extends the notion that non-LPS protective antigens might be important in other animal models besides the infant mouse model.

In 1983 Sciortino & Finkelstein reported that during growth in the intestines of infant rabbits, *V. cholerae* display a different pattern of OMPs from that characteristic of organisms cultured *in vitro*. This important finding focuses attention upon antigens expressed during growth *in vivo*, a potentially rewarding approach for investigators interested in vaccine development. Recently Jonson *et al.* (1989) confirmed that novel proteins are produced by *V. cholerae* during growth in the (adult) rabbit gut, and went on to show that at least some of these are immunogenic. Of interest was the finding that several of the proteins induced antibodies that were detected in convalescent phase sera from cholera patients (Jonson *et al.* 1989). However, none of these novel proteins appear to be TCP as *in vitro* growth under conditions conducive to TCP expression did not induce any of the *in vivo*-specific proteins nor did anti-TCP serum react with these proteins. Clearly it will be of great importance to define the antigens synthesized by *V. cholerae* during growth in the human gut.

6.6 Future studies

6.6.1 Non-LPS protective antigens of El Tor V. cholerae

The data presented in this thesis are consistent with a biotype-associated expression of TCP, but further studies in this area are required. Although EM and immunoblotting approaches failed to detect production of such pili by strains of El Tor biotype, this might merely reflect the fact that the stimuli for TCP synthesis differ between the biotypes. As discussed previously (Section 5.7) a number of El Tor strains have now been shown to carry DNA homologous to that encoding TCP. Taylor *et al.* (1988b) have recently reported 80% sequence homology between *tcp*A genes present in the Classical strain 569B and the El Tor E7946.

Potential antigenic epitopes were found to be highly conserved, suggesting that protective antigens might be shared between the pilus structures expressed by the two biotypes (Taylor *et al.* 1988b).

The protective efficacy of antibodies to TCP was evaluated against a panel of challenge strains to address the possibility that El Tor strains synthesize TCP *in vivo*. These experiments also showed a clear, biotype-related dichotomy however, in that such antibodies only conferred protection against bacteria of Classical biotype (Table 4.3). It should be noted that protective titers against Classical strains are low unless the challenge organisms are expressing TCP (as is necessarily the case with El Tor strains). An independent means of assessing the *in vivo* significance of TCP for El Tor *V. cholerae* would be to construct strains carrying inactivated *tcp*A genes (for example) and to compare the colonization of such strains with their corresponding wildtype parents. Such experiments are currently in progress.

In addition, an attempt will be made to clone the non-LPS protective antigens of recent El Tor isolates, using the approach which allowed cloning of genes involved in TCP expression. Any cloned components will be investigated for their capacities to promote *in vitro* attachment and *in vivo* colonization. IEM will be used to examine their distribution over the bacterial surface, with particular interest in the possibility that El Tor strains produce a biotype-associated pilus structure as suggested by Hall *et al.* (1988). As already discussed (Section 6.4), evidence suggests that *V. cholerae* express non-TCP, non-LPS protective antigens and this approach could lead to their identification. In this context the four positive 017 clones which were TCP-negative (Section 5.5.1) merit further study.

6.6.2 The nature of TCP-mediated attachment

The specificity of bacterial adherence is imparted by the recognition of target cell receptors by bacterial attachment factors. Evidence is accumulating to suggest that the actual

adhesins of gram-negative bacteria consist of minor protein molecules at the tip of the surface pili. There is now ample evidence that glycolipids can act as receptors for bacterial adhesins (reviewed by Leffler & Svanborg-Eden 1986). Adhesion of piliated *E. coli* to host cells has been shown to be mediated by pilus-associated adhesins of the Pap pilus not by pilin subunit *per se* (Uhlin *et al.* 1985). The protein associated with adhesion, has been found exclusively at the tip of the pilus and is a 35 kDa protein (PapG; Normark *et al.* 1986; Lindberg *et al.* 1987) that attaches to the globoseries of glycolipids with the recognition site being Galα1-4-Galß in human urinary tract epithelia (Leffler & Svanborg-Eden 1986). Other examples of lectin-like tip adhesins produced by *E. coli* are a 12 kDa protein of S pili that recognizes sialic acid (Moch *et al.* 1987) and a 29 kDa protein produced by type 1 fimbriae which mediates attachment to D-mannose (Abraham & Beachey 1988).

It is not known whether the attachment of *V. cholerae* to host cells is mediated by similar adhesins, which may be very minor components of the pilus. By analogy with *E. coli*, such proteins might have a critical role in adhesion of *V. cholerae* to host cells. Based on the organization of genes in the Pap operon (Lindberg *et al.* 1987), Taylor *et al.* (1988a) have proposed a model for the gene cluster involved in the biosynthesis of TCP (Figure 6.1). The *tcpA* gene produces the major pilin subunit, with *tcpG* representing a possible tip adhesin. Further molecular and biological analyses involving the construction of defined mutant strains is required to elucidate the mechanism of TCP-mediated attachment. Such studies will allow the design of vaccines aimed at inhibiting *V. cholerae* colonization by eliciting antibodies capable of neutralizing the binding potential of TCP.

The nature of host receptors involved in *V. cholerae* adherence remains undefined. Taylor *et al.* (1987) have shown that *V. cholerae* O395 expressing pili could hemagglutinate murine RBCs thus demonstrating that the pili apparently bind to receptors on the surface of host cells. The nature of the receptor was not identified but the agglutination was detected

Figure 6.1 Proposed organization of the genes associated with the production of the TCP pilus; (Taylor *et al.* 1988a). The *Xba*I fragment containing *tcp*G does not appear to be immediately contiguous with the remainder of the genes shown here.


Xbal
Xbal
Xbal
Xbal
Xbal
I H A B C D E F G
2kb

in the presence of L-fucose, a sugar that inhibits most of the hemagglutination mediated by *V. cholerae* O395 (Jones & Freter 1976; Jones 1980). One should be aware, however, that the characteristics of binding to RBCs might differ from the nature of the bacterial interaction with more relevant substrates (Attridge & Rowley 1983b).

6.6.3 Vaccine potential of the TCP

It seems probable that future studies will show TCP to be a protective antigen for human choleraic infection. Taylor *et al.* (1988a) have already suggested the incorporation of TCP into the oral killed whole cell vaccine developed by Holmgren's group (Section 1.10.2.2), which would presently contain little if any of this antigen. This might lead not only to an improvement in protective efficacy but also to a reduction in cost; if the presence of TCP permits at least a transient association of the killed bacteria with the intestinal surface it might be possible to reduce the number of bacteria per dose.

Other ways to exploit the putative protective potential of TCP include the development of a TCP-B subunit conjugate (Taylor *et al.* 1988a) or the delivery of TCP on an attenuated carrier strain such as *S. typhi* Ty21a. The latter could also express the protective O-antigens, as previously discussed (Section 1.10.2.3). Presumably TCP would be synthesized *in vivo* by attenuated live oral vaccine candidates, although the only data available to date suggest that such strains elicit anti-TCP responses in only a small minority of recipients (Levine *et al.* 1988b). It would be of interest to compare the immunogenicity, protective efficacy and reactogencity of various doses of TCP-positive and TCP-negative non-toxigenic *V. cholerae*.

The impact of incorporating TCP into potential cholera vaccine candidates is awaited with great interest. As already discussed, it is possible that any improvement in protective efficacy will be limited to infections caused by Classical *V. cholerae*. It would nevertheless appear that an effective cholera vaccine is considerably closer than it was just a few years ago.

Chapter 7

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