The Role of the Toxin-Coregulated Pilus in the Pathogenesis of *Vibrio cholerae* O1 Biotype El Tor

Elena Voss

Microbial Pathogenesis Unit

Department of Microbiology and Immunology

University of Adelaide

Adelaide 5005

Australia

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Abstract

As part of the development of an effective cholera vaccine, much work has been done to identify and characterise the protective antigens of *Vibrio cholerae* O1. In recent years the toxin-coregulated pilus (TCP) has been demonstrated to be essential for the colonisation of the human gut by *V. cholerae* O1 of classical biotype and to be a protective antigen of such strains in the infant mouse cholera model. The major aim of the studies presented in this thesis has been to establish whether TCP is significant in the pathogenesis of *V. cholerae* strains of the alternative El Tor biotype.

When this study was commenced El Tor strains had not previously been demonstrated to assemble surface TCP. It was known that El Tor isolates have DNA with homology to the classical *tcp* operon (which encodes the genes for TCP biogenesis) and produce TcpA, the pilin monomer, under appropriate culture conditions. A modification of these culture conditions resulted in improved production of TcpA by El Tor strains. The production of antisera specific for the (unprocessed forms of the) two biotypic variants of TcpA confirmed that the classical and El Tor proteins have biotype-specific epitopes. Subsequently a serum was raised against native (processed) El Tor TcpA; when used for immuno-electron microscopy (IEM), this reagent allowed the first demonstration of the presence of typical TCP bundles on the surface of several El Tor strains. The same antiserum protected infant mice against challenge with *V. cholerae* O1 El Tor, demonstrating TCP to be a protective antigen of this biotype. Sera containing antibodies to classical or El Tor TCP only protected against challenge with strains of the homologous biotype, suggesting that antibodies specific for shared epitopes are not protective. Finally, antibodies to El Tor TCP were shown to be sufficient to protect mice from challenge with strains of the novel O139 serovar.

To determine the significance of TCP in the pathogenesis of strains of El Tor biotype, *tcpA* mutants were constructed in four strains. Initially this involved insertional inactivation of the *tcpA* gene but subsequently similar results were obtained using in-frame deletion mutants. When assessed in the infant mouse cholera model, the *tcpA* mutants were severely attenuated and displayed dramatically reduced colonising capacities compared with their wild-type parents. These results indicate TCP is an essential colonisation factor of *V. cholerae* El Tor.

The El Tor strain O17 does not normally produce TcpA *in vitro*, but assembles TCP when carrying of a cosmid encoding the classical *tcp* operon. Western blot and IEM analyses of the O17 cosmid clones using the biotype-specific anti-TcpA reagents demonstrated that both classical and El Tor TcpA were assembled into TCP. Furthermore GM_1-ELISA revealed elevated cholera toxin production in the cosmid clones compared with the parent strain. Clearly factors encoded by the cosmid are capable of activating the O17 *tcp* and *ctx* operons. Subsequent studies indicated that the cosmid-encoded *toxT* gene was responsible for activating these operons in O17, but further study is required to determine why regulation of virulence gene expression differs between the biotypes of *V. cholerae* O1.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Elena Voss

I dedicate this thesis to my husband and to my parents

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Abbreviations

Ace	accessory cholera enterotoxin
ACF	accessory colonisation factor
Ap	ampicillin
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CEP	core encoded pilin
CIP	calf intestinal phosphatase
Cm	chloramphenicol
CTP	cytosine 5'-triphosphate
GTP	guanosine 5'-triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra-acetic-acid, disodium salt
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FSHA	fucose-sensitive haemagglutinin
Gm	gentamycin
HA	haemagglutinin
HRP	horse-radish peroxidase
IEM	immuno-electron microscopy
IMCM	infant mouse cholera model
kb	kilobase
kDa	kilodalton
Km	kanamycin
kV	kilovolts
LB	Luria broth

LD_{50}	dose of organisms capable of killing 50% of infant mice within 48 hours
LPS	lipopolysaccharide
MAb	monoclonal antibody
mg	milligram
MFRHA	mannose-fucose-resistant haemagglutinin
ml	millilitre
mM	millimolar
MSHA	D-mannose-sensitive haemagglutinin
NA	nutrient agar
NB	nutrient broth
OD	optical density
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD_{50}	dilution of serum capable of protecting 50% of infant mice challenged
pmol	picomoles
Pmx	polymyxin B
Rif	rifampicin
RNA	ribonucleic acid
RPCR	recombination polymerase chain reaction
RT	room temperature
R	resistant
S	sensitive
sc	subcutaneous
SDS	sodium dodecyl sulphate
Sm	streptomycin
Sp	spectinomycin
TBS	Tris-buffered saline
Tc	tetracycline
TCP	toxin-coregulated pilus
TTBS	Tris-buffered saline with Tween-20 added

TTP	thymine 5'-triphosphate
UTP	uridine 5'triphosphate
µF	microFarad
µg	microgram
µl	microlitre
µm	micrometre
UV	ultraviolet
V. cholerae	*Vibrio cholerae*
v/v	volume per volume
w/v	weight per volume
Zot	zonnula occludens toxin

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Bibliography

Chapter 1 Introduction

In 1994, the powerful television and newspaper images of Rwandan refugees dead or dying of cholera were a stark reminder to the world community that this disease has the potential to swiftly re-emerge as a significant killer. Despite the many advances in our understanding of the pathogenesis, transmission and treatment of cholera, medical science has been unable to arrest the spread of this disease, or prevent its occurrence during periods of overcrowding caused by natural disasters or military conflicts.

1.1 The causative agent

Vibrio cholerae of the O1 serogroup was until 1992 the only serogroup known to cause epidemics of the diarrhoeal disease Asiatic cholera. The organism is a comma-shaped, Gram-negative bacterium with a single polar flagellum. *V. cholerae* strains are categorised as either O1 or non-O1 on the basis of the O (polysaccharide) antigens of the lipopolysaccharide (LPS). To date there are 140 recognised O-serogroups (Shimada *et al*, 1994) but only O1 and more recently O139 have been associated with epidemics of cholera. Strains belonging to the O1 serogroup may be divided into two biotypes - classical and El Tor - on the basis of various biological properties. The most reliable of these differential characteristics has been the susceptibility of classical strains and the resistance of El Tor *V. cholerae* to Mukerjee's type IV phage (Mukerjee, 1963). The O139 serovar is closely related to the El Tor biotype; it is thought to have arisen from the pre-existing O1 El Tor by gene transfer from other non-O1 *V. cholerae* (Manning *et al*, 1994).

1.2 The modern history of cholera

It is thought that, prior to 1817, cholera was almost exclusively confined to the deltas of the Ganges and Brahmaputra rivers. There are ancient accounts from Asia, China and Europe which describe patients with cholera-like disease, but there is of course, no way of confirming that these outbreaks were indeed cholera.

In 1817, cholera began to spread from the Indian subcontinent to virtually all over the world in what some say was a series of six pandemics (Pollitzer, 1959). The start and finish dates of the six pandemics have been debated and indeed it has been suggested that there was one continuous pandemic caused by classical *V. cholerae* O1 (Blake, 1994). The causative agent is known to have been classical *V. cholerae* for the fifth and sixth pandemics (Blake, 1994). Although it is assumed that the classical biotype was predominant from 1817 to 1961, the possibility cannot be excluded that an alternative serovar or biotype of *Vibrio cholerae* was responsible for any of the pandemics before the fifth (Blake, 1994).

It was Robert Koch in 1883, who identified the infectious agent of cholera to be the comma-shaped bacteria found in the gut contents of cholera victims (Koch, 1884 a and b). This was confirmed when the doubting Professor von Pettenkofer and his student Rudolph Emmerich, attempting to disprove Koch, swallowed pure cultures of vibrios and both subsequently became ill with cholera (Feachem, 1982).

In 1961, the seventh pandemic of cholera began, originating in Celebes, Indonesia and rapidly spreading through Asia and the Middle East. Unlike the previous pandemic, *V. cholerae* O1 El Tor was responsible. The El Tor biotype (named after the quarantine station in Sinai where it was first discovered in 1906) was not previously considered to have pandemic potential, causing only occasional outbreaks of mild cholera (Barua, 1992). Within ten years El Tor cholera had spread to Africa and southern Europe and more recently to South America. From

Peru, its initial point of introduction in 1991, it has advanced inland with stunning rapidity and now affects virtually all the countries of South America (Tauxe *et al*, 1994).

1.3 The epidemiology of cholera

1.3.1 The work of Snow and Koch

Perhaps the most important and influential work on cholera epidemiology in the last century was that of John Snow. His major work, *On the Mode of Communication of Cholera* was published in 1855 and was the result of many years of careful study of cholera outbreaks in various parts of both England and Scotland (reviewed by Feachem, 1982). Snow hypothesized that cholera was the result of a proliferation of "morbid material" or "cholera poison" in the intestines of infected individuals and transmission was either waterborne or via the faecal-oral route.

Although Snow is mostly remembered for clearly demonstrating the connection between contaminated water and cholera, he made regular observations of the socio-economic factors that pre-disposed sections of the community to a greater risk of infection. He recognised that where waterborne transmission could not explain an epidemic, poverty and all its trappings was inevitably to blame. Despite constantly emphasising the importance of person-to-person and contaminated food-to-person transmission, it took more than forty years after his death in 1858 for the faecal - oral route to be accepted as more than simply waterborne transmission.

Thirty years after John Snow's death, Robert Koch presented an address to the Imperial German Board of Health in Berlin in which he discussed his findings in both Egypt and India. Koch's work led not only to the identification of the cholera vibrio but provided some insight into the epidemiology of the disease. He noted that humans were the sole host and that infection could result in a range of outcomes from asymptomatic to severe purging. Like

Snow, Koch had ample evidence to demonstrate that waterborne transmission of the disease was only one mode of infection, even observing that ingestion of contaminated food led to a greater risk of disease if "lumps of undigested food entered the intestine" thereby providing "comma bacilli" with protection from stomach acidity. He believed cholera vibrios could survive outside the human body in environmental niches such as stagnant water or slow moving streams and rivers where the organisms could persist if there was a supply of nutrients from decomposing vegetable or animal matter (Koch, 1884 a and b). He also found that cholera vibrios were killed if dessicated, providing him with an explanation for why outbreaks were never traced to imported dried foods from cholera endemic places. Furthermore Koch recognised the epidemiological significance of improved travel, predicting shorter travelling times would increase the potential for direct and rapid spread of cholera to Europe from endemic countries.

The work of John Snow and Robert Koch provided much information about the cause and epidemiology of cholera. Unfortunately it took decades for their work to become widely accepted and acted upon.

1.3.2 Survival of *V. cholerae* in the environment

The likelihood that *V.cholerae* O1 could survive long periods in the environment was disregarded for almost a century after Robert Koch alluded to the possibilty. Until the 1970's the dogma had been that toxigenic *V.cholerae* O1 could not persist for more than a few hours or days outside the human host. For this reason *V. cholerae* O1 had also been considered as a separate species from non-O1 *V. cholerae* and non-agglutinable vibrios found in the aquatic environments. Much work done in the last twenty years has disproved these beliefs. The

studies of Colwell and co-workers provided evidence that both O1 and non-O1 *V. cholerae* are embraced in a single species (Citarella and Colwell,1970; Colwell and Spira, 1992).

The next important advance was the discovery that *V. cholerae*, along with a number of other entero-pathogenic bacteria such as *E. coli*, *Salmonella* and *Shigella* species, can enter a "dormant" state otherwise referred to as "viable but non-culturable" (Colwell *et al*, 1985). The organisms are alive as assessed by metabolic criteria but are incapable of growth in routine culture media. This is distinct from starved cells which are morphologically similar to dormant vibrios i.e reduced in size and ovoid, but which are still culturable. Colwell and co-workers performed direct viable counts on acridine orange-stained or fluorescent-antibody tagged cells to accurately determine the number of vibrios present in water samples and the length of time these organisms could persist in microcosms which simulated environmental conditions. "Viability" of *V. cholerae* O1 (as judged by growth on conventional laboratory media) was lost within a day in the microcosm but epifluorescent microscopy revealed the concentration of vibrios remained virtually the same throughout the duration of the experiment (eight days). Environmental conditions which favour the "viable but non-culturable" state appear to be low nutrient concentration, elevated salinity and/or reduced temperature (Colwell *et al*, 1985). It was shown in rabbit ligated ileal loop assays that inoculation with 10^6 dormant vibrios from a microcosm produced a similar degree of fluid accumulation and haemorrhage as the control loop inoculated with a pure culture of 10^6 *V. cholerae*, thus confirming that dormant organisms retain the capacity to rapidly express their pathogenic potential (Colwell *et al*, 1985).

Since the finding that *V. cholerae* O1 can persist in a dormant state, it has been proposed that *V. cholerae* is indigenous in brackish water and estuarine surroundings and can readily adapt to the tidal and seasonal changes that occur in these environments (Hood and Ness, 1983; Colwell *et al*, 1985). The seasonality associated with increased recovery of vibrios from

riverine or estuarine waters is also related to the presence of certain algae and zooplankton (or copepods). A laboratory study of *V. cholerae* attached to cyanobacteria (blue-green algae) found that the vibrios were maintained in a culturable state for over 15 months (Islam *et al*, 1990). Huq and coworkers (1984) have demonstrated in laboratory microcosms that *V. cholerae* associated with live planktonic copepods remain culturable for longer periods than if associated with dead copepods. In a subsequent field survey of plankton samples collected in Bangladesh over a three year period, *V. cholerae* O1 was detected in 63% of samples but the bacteria were non-culturable. The results suggested that when attached to plankton *V. cholerae* remain dormant until proliferation of copepods occurs, generally during the warmer months.

In an aquatic environment, the ability of *V. cholerae* to attach to various surfaces such as chitin, the main component of crustacean shells (Nalin *et al*, 1979), probably increases their chance of survival. The association with copepods, algae or shellfish such as crabs may provide vibrios with substrate to survive unfavourable conditions and in some cases there may be a symbiotic relationship (Huq *et al*, 1983). Adherence to inert, sedimenting material deposits the organisms in surroundings with potentially higher nutrient levels than in the water column; alternatively, if nutrient levels are low the bacteria can become dormant (Grimes *et al*, 1986). What is clear is that *V. cholerae* O1 has a life-cycle outside the human gut. The public health implications are obvious and screening of water from rivers and estuaries must take into account that conventional culture conditions may not detect the presence of potentially pathogenic organisms.

In the Riverine district of Queensland, Australia, eleven rivers were found to have toxigenic *V. cholerae* O1 El Tor Inaba. The numbers of vibrios fluctuated according to the season, peaking during the summer months (Bourke *et al*, 1986). Cases of cholera acquired as a result

of drinking or bathing in these waters is rare and the surrounding area is generally sparsely populated making it highly unlikely that contamination via human infection maintains the levels of cholera vibrios. It appears the Australian El Tor strains, which are probably clonal, have adapted to the freshwater environment and may have been there for thousands of years (Bourke *et al*, 1986).

In contrast, samples of the Gulf Coast waters of the United States have often undetectable levels of toxigenic *V. cholerae* O1 El Tor but have an environmental reservoir in the crabs and bivalve shellfish. Sporadic outbreaks of cholera occur in most cases as a result of eating cooked crabs (25min of steaming was found to be insufficient to kill the vibrios!) and occasionally from raw oysters. Seasonal variations are similar to those seen in Australia with most cholera cases occurring in the summer months (Miller *et al*, 1985). In Africa, cholera has become endemic in areas that are far from coastal waters indicating *V. cholerae* has the ability to adapt to a much wider range of environments than has traditionally been thought (Goodgame and Greenough, 1975).

1.3.3 Endemic cholera

Endemic cholera refers to a recurrent pattern of cholera infection, and was for some time thought to be due to the constant seeding of the environment by infected human faeces. Typical endemic environments are considered to be estuarine deltas in tropical to semi-tropical climates. This situation clearly applies to Bangladesh and India where there is abundant opportunity for the continuing contamination of water sources. However as mentioned above, studies carried out in the USA (Blake *et al*, 1980) and Australia (Bourke *et al*, 1986) have demonstrated that endemicity does not require constant human seeding into a water source. Instead, in these regions *V. cholerae* O1 has aquired an environmental niche.

In developing countries with endemic cholera the relative incidence of severe disease is low compared to epidemic situations. When the population is divided into subgroups it becomes clear that young children between the ages of 2 and 5 years are at most risk of developing severe disease (Mosley, 1969). If these children survive their first infection with *V. cholerae* O1 they rarely succumb to the disease again. From this it was inferred that the disease is an immunising event and clinical studies have since demonstrated that infection with *V. cholerae* O1 induces long-lasting immunity (Levine *et al*, 1981). Studies in Bangladesh have demonstrated an inverse relationship exists between the incidence of cholera and age, and also between the former and serum vibriocidal titres (Mosley, 1969). Presumably within communities of cholera-endemic regions, immunity is naturally acquired by repeated exposure to vibrios (McCormack *et al*, 1969). What must also be considered is that some infections will be asymptomatic making these individuals an ideal source of infection amongst family contacts and the wider community if hygiene standards are low (Feachem, 1982).

Infection with the El Tor biotype has a lower case/infection ratio and longer carriage time than classical *V. cholerae* (Bart *et al*, 1970; Woodward and Mosley, 1972). The spread of cholera, particularly El Tor infection, via non-waterborne routes must be as significant as waterborne transmission. Several workers have in the past dismissed person-to-person transmission as being of little importance (Gangarosa and Mosley, 1974; Merson *et al*, 1980), their main argument being the large inoculum required for infection. However Feachem (1982) argued strongly that often the evidence presented for waterborne transmission could just as easily apply to non-waterborne routes such as person-to-person contact. Snow commented on the incidence of people becoming ill with cholera after handling the bedding and clothing of cholera victims and then eating without washing their hands. Many cultures in developing countries have the custom of eating with their fingers and often from a communal

plate. Burial ceremonies of cholera victims have been implicated in transmission because of the practice of relatives of the deceased cleaning the body (inside and out!) prior to burial followed by providing meals in the family home (Glass and Black, 1992). In the same communities mothers of small children with cholera are at risk of infection through constant exposure to soiled clothing and bedding which when damp can retain very high numbers of viable vibrios.

1.3.4 Epidemic cholera

When cholera is introduced into a new region, the lack of pre-existing immunity means all age groups are equally susceptible to infection. in addition, proportionally more of those infected experience a severe form of disease (Clemens *et al*, 1994). Unlike the endemic situation, there is no local reservoir and so entry of the disease can usually be traced to a single source such as food, water or infected individuals coming from an endemic area. The spread of cholera across the globe has historically been via trade routes, pilgrimages and travellers.

In developed countries the high standards of hygiene and public health measures usually ensures secondary spread is rare. With the exception of travellers returning from cholera-endemic regions, the source of the infection is often via contaminated shellfish or seafood taken from waters where *V. cholerae* has established an environmental niche (Glass and Black, 1992). While it is much easier to stem the spread of cholera and prevent it from becoming endemic in developed countries, it is exceedingly difficult in the developing world. In Africa, the 1970 cholera epidemic was devastating to many communities with inadequate resources and limited access to health care. As a consequence high mortality rates occurred (Swerdlow and Isaacson, 1994). The Latin American epidemic by contrast has had a very low mortality rate principally due to the open communication and surveillance between and within countries,

as well as rapid mobilization of health workers and highly effective use of oral rehydration therapy (Tauxe *et al*, 1994).

1.3.5 The emergence of *V. cholerae* O139

Commencing in October 1992, explosive outbreaks of cholera-like illness occurred across the Indian subcontinent (Ramamurthy *et al*, 1993) and Bangladesh (Albert *et al*, 1993). The causative agents were found to be strains of toxigenic *V. cholerae* serologically distinct from the 138 known serogroups (Cholera working group, 1993). The novel serovar was named O139 or Bengal as it was first isolated from coastal areas surrounding the Bay of Bengal (Shimada *et al*, 1994). Since 1992, the spread of O139 cholera to neighbouring countries has been swift with cases now documented in Nepal, Thailand, Malaysia, Pakistan, China and Saudi Arabia (Albert, 1993). In the past, non-O1 strains have never demonstrated epidemic potential but there is little doubt that *V. cholerae* O139 is an exception and we are witnessing the start of the eighth pandemic.

A great deal of work has already been done to characterise the O139 serovar. Its considerable similarity to O1 strains of the El Tor biotype is well documented (Johnson *et al*, 1994; Berche *et al*, 1994; Calia *et al*, 1994; Hall *et al*, 1994). The principal difference is the unusual LPS structure of O139 isolates; when analysed by SDS-PAGE, these strains were found to have a truncated (semi-rough) LPS (Manning *et al*, 1994). The other distinguishing characteristic of O139 strains is the production of a capsule which by analogy with other encapsulated non-O1 vibrios may be associated with increased virulence (Weintraub *et al*, 1994). There is mounting evidence to suggest that the O139 O-antigen and capsular polysaccharide biosynthesis genes are closely linked and have only recently been acquired, probably through horizontal transfer from a non-O1 strain to an O1-El Tor strain (Waldor *et*

al, 1994; Comstock *et al*, 1995; Bik *et al*, 1995). The incoming DNA has evidently displaced many of the genes involved in O-antigen biosynthesis in O1 strains (Manning, 1994).

The startling feature of this serovar is its ability to afflict all age groups within cholera endemic areas. Evidently pre-existing immunity to *V. cholerae* O1 is not protective against challenge with O139 strains. This may be due to the capsule masking underlying antigens preventing (otherwise) protective antibodies from binding or it may indicate that antibodies to non-LPS antigens are insignificant in the protective immune response resulting from naturally acquired infection. Either way the emergence of the O139 serovar has serious implications for cholera vaccine design. The dissemination of O139 strains across the world is likely to parallel the speed with which El Tor cholera spread through the immunologically naive communities of Latin America.

1.4 Susceptibility to cholera

Environmental and social factors which govern the incidence and persistence of cholera in whole communities are well understood. An area which has received relatively little attention is the study of innate factors which dictate an individual's susceptibility to cholera.

1.4.1 Infectious dose and gastric acidity

Stomach acidity provides a formidable barrier to cholera infection as indicated by the high infectious dose required to produce disease in normal individuals. In a volunteer study of healthy adult males, 10^8 classical *V. cholerae* were required to produce diarrhoea in 50% of the participants (ID_{50}) and 10^{11} vibrios to produce cholera-like disease. If sodium bicarbonate was administered prior to challenge the ID_{50} dropped to 10^4 vibrios to produce diarrhoea and 10^8 vibrios for disease (Hornick *et al*, 1971). Epidemiological studies of a cholera epidemic in

Italy revealed attack rates were higher amongst people with low gastric acidity (hypochlorhydria) due to surgical resection of the stomach (Baine *et al*, 1974). Furthermore medications or drugs such as cannabis, that neutralise or reduce gastric acid, have been shown in clinical studies to pre-dispose individuals to enteric infections (Ruddell and Losowsky, 1980; Nalin *et al*, 1978). Ingestion of vibrios with food may lower the infectious dose, by either buffering the stomach pH or protecting the vibrios from exposure to acid.

Chronic colonisation of the stomach lining by *H. pylori* is known to cause irreversible damage to the gastric mucosa ultimately resulting in permanent hypochlorhydria. This damage is aggravated by nicotine and it is interesting to note that heavy smoking is common in developing countries where cholera is endemic (Cover *et al*, 1992). Furthermore like *V. cholerae*, transmission of *H. pylori* is probably via the faecal-oral route. Although there has not yet been a definitive study, the evidence available suggests that chronic *H. pylori* infection may be a predisposing factor in cholera infection (Richardson, 1994).

Other enteropathogenic bacteria such as *E. coli* (Goodson and Rowbury, 1989) and *Salmonella typhimurium* (Foster and Hall, 1990) display an inducible acid tolerance response (ATR). Exposure to a moderately acidic environment induces an ATR-specific pH homeostasis system which helps maintain the intracellular pH as the external pH drops, thus enabling the bacterium to survive acid conditions below pH 4 (Foster, 1993). There is no evidence to suggest vibrios display a similar adaptive response to low pH, nor is it known whether such exposure triggers or indeed represses the expression of *V. cholerae* virulence factors.

1.4.2 O blood group and cholera

In 1977 Barua and Paguio published the results of a small study of host susceptibilty to cholera with respect to the patient's ABO blood group. The authors compared 87 bacteriologically proven cases of cholera with 33 cholera-like diarrhoea cases in the Philippines. Although their results were not statistically significant it was found that a relatively larger group of cholera cases occurred in group O individuals but the disease was seen infrequently in group A individuals. The authors concluded that the possible link between ABO group and susceptiblity to cholera was worthy of further investigation. In the same year Chaudhuri and De published the results of a survey of 200 cholera patients during the 1976 cholera season in Calcutta. Group O was found to be the commonest blood group among these patients, the frequency being almost double that of the controls and the difference statistically significant (Barua and Paguio, 1977).

In 1979 Glass *et al* (1985) began a much larger study of patients hospitalised at Matlab hospital (ICDDR, Bangladesh), for a variety of diarrhoeal diseases due to bacterial or viral agents. A significant association between ABO blood group and susceptibility to diarrhoeal disease was only found for cholera. Consistent with the findings of Chaudhuri and De (1977) cholera patients were twice as likely to be blood group O. A follow-up study of family contacts of cholera patients revealed blood group was unrelated to the risk of infection with strains of *V. cholerae* O1 of either biotype (Glass *et al*, 1985). Furthermore, no association between blood group and the severity of disease following classical infection was found. However the severity of disease was directly related to the blood group of patients with El Tor infection; severe diarrhoea occurred more often in blood group O individuals and less often in blood group AB patients when compared to those with asymptomatic infection. These results led Glass and co-workers to speculate that the very low prevalence of group O genes and the

high prevalence of group B genes found among people of the Ganges Delta was partly the result of the constant evolutionary selective pressure imposed by cholera. In another study, Clemens and coworkers (1989) analysed the results from field trials of a killed whole cell-B subunit vaccine. Blood group O patients had a higher risk of severe disease due to El Tor *V. cholerae*. More surprising (and still unexplained) was the finding that type O individuals vaccinated with the killed whole-cell vaccine (with or without B subunit) were not as well protected against severe cholera.

The basis for the relationship between ABO blood group and susceptibility to cholera is not understood but there are currently two theories. There is some evidence from studies in pigs that blood group substances secreted into the gastrointestinal tract are capable of interacting with cholera toxin, preventing it from binding to membrane receptors (Bennun *et al*, 1989; Monferran *et al*, 1990). A neutral glycosphingolipid containing fucose was found in the intestinal mucosa of pigs secreting blood group A-active substances. This "fucolipid" was able to bind cholera toxin and be recognised by anti-human blood group A antiserum. There was a clear correlation between the occurrence of certain blood group substances and the presence and absence of glycosphingolipid that interacts with cholera toxin. It has been estimated that 80% of the population are secretors i.e. they secrete blood group substances consisting of glycosphingolipids and glycoproteins (mucins) into tissue fluids and the gastrointestinal tract. The hypothesis of Bennun *et al* (1989) and Monferran *et al* (1990) is that individuals with blood group O, and those of any ABO blood type who are non-secretors, show a pre-disposition to severe cholera due to the absence in the gut of specific compounds with antigenic properties of blood group A or B which can interact with cholera toxin and thereby neutralise it.

Another hypothesis is that there is an increased availability of receptors for vibrios to bind in people of O blood group. Histo-blood group antigens are abundantly expressed in the small intestinal epithelium and the H-antigen (L-fucose $\alpha 1 \rightarrow 2$D-gal$\beta 1 \rightarrow$R) forms the backbone of these structures. Residues that occur on the H antigen form the A and B antigens (N-acetylgalactosamine and D-galactose respectively). It has been speculated that the H-antigen may be a receptor for vibrios based on analogy with observed fucose-sensitive haemagglutination of human O erythrocytes by both biotypes of *V. cholerae* (Lagos *et al*, 1995). In an O blood group background, the availability of the H antigen as a potential receptor would be greater than in any other blood group. The intriguing observation of Lagos and co-workers (1995) that the live attenuated vaccine strain CVD103-HgR induced a significantly higher serum vibriocidal response in blood group O recipients contrasts the findings of Clemens *et al* (1989) that a killed whole cell vaccine was less effective in recipients of this blood group. The former finding is consistent with the live attenuated vaccine organisms having better adherence to the intestinal epithelium. Recently it has been shown that adherence of *Helicobacter pylori* to gastric mucosa is greater in individuals who express the histo-blood group antigen Le^b, which like the H-antigen has a terminal L-fucose in its structure (Boren *et al*,1994).

1.4.3 Other factors

Breast-feeding of infants has been identified as a major factor in reducing the risk of cholera. In cholera-endemic areas, the high level of protection provided by breast-feeding is probably due to passive immunisation of the infants from antibodies present in the mother's milk. Clemens *et al* (1990) compared Bangladeshi children under the age of three who were breast-fed to those that were not. Children who were not breast-fed had a 70% increased risk of

cholera. There may also be non-immunoglobulin factors in milk that provide protection against cholera by competing with receptors on target cells. Holmgren and co-workers (1983) demonstrated an inhibitory effect of human breast milk on agglutination of chicken erythrocytes by *V. cholerae*. Haemagglutination-inhibition was associated with glycoproteins and free oligosaccharides present in the milk, raising the possibility that these components might also interfere with attachment of *V. cholerae* to the gut mucosa (Holmgren *et al*, 1983). Later studies of lactating rats fed cholera toxin prior to gestation found these animals (but not control lactating rats) produced a non-immunoglobulin antisecretory factor that could protect infant rats from challenge with cholera toxin (Lange *et al*, 1986); it remains to be demonstrated whether the same factor is present in the breast milk of mothers living in cholera endemic areas.

1.5 Diagnosis and treatment of cholera

1.5.1 Clinical Manifestations

The incubation period for naturally occurring cholera can vary from a few hours to five days but is usually 2-3 days (Oseasohn *et al*, 1966; Hornick *et al*, 1971). The outcome of infection can also range from asymptomatic to severe diarrhoea. The ratio of patients with mild disease compared with those requiring medical attention is ca. 1:1 for infection with classical *V. cholerae* O1 and ca.7:1 for El Tor infection (Bart *et al*, 1970). Mild disease is characterised by a few episodes of stools with no mucous or blood and no significant nausea or vomiting. In severe disease there is vomiting and voluminous watery diarrhoea. The onset of diarrhoea can be sudden or slow but is usually painless, with faecal material initially present but eventually replaced by fluid with the characteristic appearance of "rice-water". If untreated, severe cholera can result in death within 24hr of onset due to hypovolemic shock and acidosis.

1.5.2 Diagnosis

There is no need to have a laboratory confirmation in order to treat a patient with suspected cholera. A clinical diagnosis of dehydration and watery diarrhoea should indicate fluid replacement is the main objective regardless of the pathogen. Laboratory confirmation is however essential for epidemiological studies and surveillance as part of cholera control programs. Feacal specimens should be collected as soon as possible from patients presenting with cholera-like illness, preferably pior to antibiotic therapy. There are several rapid diagnostic tests which have been developed that are particularly useful for the identification of cholera in remote field situations. However traditional laboratory culture and analysis techniques are necessary to characterise isolates (~~Bradford~~ Kay *et al*, 1994).

1.5.3 Treatment.

It is well documented that during the 19th century treatment of cholera contributed to the high mortality rates. Contra-indicated practices such as purging with calomel and blood-letting were common-place. In the mid-1800s, William Brooke O'Shaughnessy and Thomas Latta advocated the use of intravenous fluid therapy but their advice went largely unheeded until the early 20th century (Carpenter, 1992). In the past thirty years the treatment of cholera has been refined to such an extent that providing the facilities are available the mortality rates are minimal. This is exemplified by the less than 1% mortality rate observed in the 1991 cholera epidemic in Peru (~~Waehsmuth *et al*, 1993~~ Tauxe et al, 1994).

1.5.3.1 Rehydration therapy

Dehydration, acidosis and potassium depletion of cholera patients occurs as a result of excessive diarrhoea. Rehydration therapy involves replacing the fluid and salts in the correct proportions. The World Health Organisation (WHO) have developed an oral rehydration salts (ORS) solution that approximates the water and salt content of the diarrhoeal stool. The standard WHO ORS formula is not optimal for cholera as it was designed to be suitable for use with other milder diarrhoeas as well (Nalin, 1994). Despite this, 80-90% of cholera patients will be adequately treated through oral rehydration and not require intra-venous fluid replacement. The latter (preferably using Ringer's lactate solution with 5% dextrose) is only recommended for patients presenting with severe dehydration and should only be continued until the patient is able to drink (WHO, 1993; Bennish, 1994).

1.5.3.2 Antibiotic therapy

Antibiotic therapy alone is inadequate for the treatment of cholera and is best used in conjunction with rehydration therapy to assist in the reduction of the volume and duration of diarrhoea and excretion of vibrios. Oral antibiotics are usually commenced once initial rehydration has been achieved. Tetracycline or a long-acting derivative, Doxycycline, are the antibiotics of choice, although Trimethoprim-sulfamethoxazole, Furazolidone, Norfloxacin, Erythromycin and Chloramphenicol are alternatives (WHO, 1993). In choosing the antibiotic therapy the regional pattern of antibiotic resistance must be considered as in the past twenty years there has been a notable increase in *V. cholerae* strains with multiple resistances to the commonly used antimicrobials (Glass *et al*, 1980; Morris *et al*, 1985).

Prophylactic antibiotic therapy is not recommended on a community basis due to the obvious potential for selection of antibiotic resistant strains. It may be considered as a method of

preventing secondary cases within family contacts during a cholera epidemic but in general prophylactic therapy is considered to be impractical, expensive and of dubious value (WHO, 1993).

1.5.3.3 Feeding

Maintaining feeding during treatment has been shown to assist nutritional recovery in cholera patients; in particular breast-feeding of infants should be continued. There is no evidence to suggest that the bowels should be rested and nutrients are still absorbed during episodes of cholera (Molla *et al*, 1982). Feeding may initially lead to an increase in the volume of diarrhoea but has also been observed to decrease its duration. Overall, continued feeding during treatment is thought to increase the chance of an uneventful recovery and promote better weight gain once the disease has been cured (Molla et al, 1982; Molla et al, 1983; WHO, 1993).

1.6 Pathogenesis

For those vibrios which have survived passage through the stomach, the small intestine presents a number of formidable barriers to the establishment of infection. Intestinal peristalsis, mucous secretion and epithelial cell desquamation are just a few of the non-specific host defense mechanisms *V. cholerae* must contend with (Rabbani, 1986). Having penetrated the mucous layer, organisms must adhere and multiply to prevent being cleared from the gut. Several factors produced by *V. cholerae* O1 have been implicated in attachment to the mucosal surface

1.6.1 Colonisation of the mucosal surface

1.6.1.1 Motility, chemotaxis and penetration of the mucosal layer.

V. cholerae strains are motile via a single polar sheathed flagellum. *In vitro* and *in vivo* studies have shown that motility is important for virulence by promoting colonisation of the intestine (Guentzel and Berry, 1975; Guentzel *et al*, 1977, Yancey *et al*, 1978). There are two possible mechanisms by which the flagellum might assist colonisation. The first is by promoting interactions between the vibrio and the intestinal mucosa by enabling the organism to move rapidly towards chemotactic stimuli. The second mechanism involves adherence factors on the flagellum which assist in attachment of the organism to the mucosa (Jones and Freter, 1976; Attridge and Rowley, 1983a). More recently Richardson (1991) examined the roles of motility and flagellar structure in pathogenicity of both classical and El Tor *V. cholerae*. Three groups of motility mutants were isolated using transposon and chemical mutagenesis -non-motile and flagellate; non-motile and aflagellate; non-motile and aflagellate but with a sheath-like structure. All of these mutants were assessed in three animal models (rabbit ileal loop model, the removable intestinal tie adult rabbit diarrhoea model and the suckling mouse model). In the three animal models motility was determined to be the principal factor contributing to pathogenesis; in addition, the flagellar structure was found to play a role in colonisation in the RITARD model (Richardson, 1991).

The capacity of vibrios to respond to chemotactic stimuli is of great importance as chemotaxis promotes interaction with the mucosal epithelium (Freter *et al*, 1981). Adult rabbit intestinal loop studies have demonstrated that non-chemotactic mutants are at a competitive disadvantage to chemotactic parent strains. This was correlated with a greater association with the mucosa by the latter (Freter *et al*, 1981). Chemotaxis-driven migration may be along stress

channels which form in the mucous gel although this phenomenon has only been observed *in vitro* and it is unknown whether the same occurs *in vivo* (Freter *et al*, 1981).

Little is known about the existence of factors other than ~~motiltiy~~ motility and chemotaxis which assist the passage of vibrios through the intestinal mucous. Burnet and Stone (1947) first identified a "mucinase" which they surmised might aid vibrio penetration of the mucous layer by degrading the mucous (Finkelstein *et al*, 1983). *V. cholerae* also secretes DNases which may have a role in degrading the large amount of DNA present in the mucous, thereby reducing viscosity and assisting passage of vibrios towards the mucosal epithelium (Focareta and Manning, 1991).

1.6.1.2 Adherence factors

1.6.1.2.1 Haemagglutinins (HAs)

The agglutination of a variety of erythrocytes by cholera vibrios has been associated with the ability of these organisms to adhere to the microvilli of brush border membranes (Jones *et al*, 1976). Sugars such as L-fucose and D-mannose can inhibit haemagglutination and adherence *in vitro* lending support to the idea that the HAs act as colonisation factors *in vivo* (Bhattacharjee and Srivastava, 1978; Jones *et al*, 1976; Jonson *et al*, 1989a). However to date there is no strong evidence that any of the HAs plays an indispensable role in the pathogenesis of *V. cholerae* O1. These HAs can be divided into two groups, soluble and cell-associated (Hanne and Finkelstein, 1982).

The soluble HA/protease

The soluble HA/protease (also known as the cholera lectin and soluble haemagglutinin) is a zinc- and calcium-dependent metalloprotease closely related to *Pseudomonas aeruginosa* elastase (Booth *et al*, 1983; Häse and Finkelstein, 1990). The protease has several functions

attributed to it including the activation of cholera toxin by nicking the A subunit (Booth *et al*, 1984) and hydrolysis of fibronectin, mucin and lactoferrin - factors which may form part of the host defense mechanism (Finkelstein *et al*, 1983). Despite evidence that specific antibodies to the HA/protease can inhibit attachment of vibrios to intestinal epithelium *in vitro* (Finkelstein and Hanne, 1982), HA/protease mutants are fully virulent in infant rabbits (Finkelstein *et al*, 1992) and so this protein is unlikely to be a major virulence determinant. It is possible that the main function of the HA/protease is to facilitate release of vibrios back into the environment by acting as a "detachase" by destroying receptors for attachment (Finkelstein *et al*, 1992).

The fucose-sensitive haemagglutinin (FSHA)

Expression of the FSHA is growth-phase dependent (Hanne and Finkelstein, 1982). This HA is better expressed by classical strains than El Tor strains (Booth and Finkelstein, 1986; Jonson *et al*, 1989a), but is often difficult to detect particularly in recent isolates. It is thought that long term culturing may change the vibrio's surface structure, leading to better exposure of the FSHA (Jonson *et al*, 1989a). Recently Nakasone and Iwanaga (1993) described inhibiting the adherence of a classical strain to rabbit intestinal epithelium in the presence of L-fucose but not D-mannose. No specific structure has been ascribed to FSHA and its role in pathogenesis remains speculative.

The mannose-sensitive haemagglutinin (MSHA)

Unlike FSHA, MSHA production is not growth phase dependent and is more commonly detected in El Tor *V. cholerae* than classical strains. Under *in vitro* growth conditions MSHA can sometimes mask the expression of other cell-associated HAs (Hanne and Finkelstein, 1982; Jonson *et al*, 1991b). The MSHA is produced as a pilus (Jonson *et al*, 1991b). The N-

terminal amino acid sequence of the pilin subunit, MshA, is highly homologous to the N-terminal sequence of various type 4 (NMePhe) pilins and interestingly *mshA* is found within a cluster of type 4 pilin genes (Jonson *et al*, 1994). As will be discussed later, type 4 pilins have been implicated in colonisation of various other pathogenic Gram-negative organisms.

The role of MSHA in colonisation of the human intestine is unclear. Finn *et al* (1987) isolated MSHA-negative strains of the toxin-deficient strain JBK70. One of these mutants was evaluated in an adult rabbit model and found to be defective in colonisation of the ileum compared with its parent strain. However the MSHA mutant was not well characterised and was potentially defective in the production of other factors. Further investigation is required to establish the role of MSHA in pathogenesis of *V. cholerae*.

Mannose-fucose-resistant haemagglutinin (MFRHA)

Hanne and Finkelstein (1982) identified a third cell-associated HA which was not inhibited by either mannose or fucose. The MFRHA was most readily detected in late exponential to stationary phase cultures. This HA has been implicated as a virulence determinant (Franzon *et al*, 1993) but its role in colonisation has not been demonstrated.

Other haemagglutinins

A novel non-fimbrial haemagglutinin was isolated from a non-O1 *V. cholerae* strain and subsequently found to be present in *V. cholerae* O1 strains (Banerjee *et al*, 1990). Distinct from the soluble HA/protease, this 62kDa HA is found in culture supernatants but its presence there is thought to be in the form of vesicles released as a result of the formation of outer membrane blebs during bacterial degeneration. The haemagglutinating activity of this protein is inhibited by glycoproteins, fetuin, asialofetuin and mucin but not by ovalbumin and simple

sugars, suggesting a requirement of complex carbohydrates for binding (Banerjee *et al*, 1990). The mode of binding of the 62kDa HA is similar to that proposed for cholera toxin and shiga toxin to glycolipids and neoglycoproteins (Nayanendu and Banerjee, 1995). The role of the 62kDa HA has yet to be determined.

1.6.1.2.2 Lipopolysaccharide (LPS)

The lipopolysaccharide has been implicated as an adhesin of *V. cholerae*. Freter and Jones (1976) demonstrated that adherence of vibrios to rabbit intestinal slices could be inhibited by antibody to LPS. Chitnis and co-workers (1982b) were also able to prevent the adhesion of three *V. cholerae* strains to rabbit intestinal mucosa using anti-LPS antibodies. The same antiserum had no effect on the adhesion of two strains of non-agglutinating vibrios. Similarly, Attridge and Rowley (1983b) found attachment of vibrios to segments of mouse intestine was inhibited by anti-LPS antibody. Finally, Booth *et al* (1986) prepared monoclonal antibodies directed against O-antigenic determinants and showed that these could block binding to erythrocytes. Collectively these studies are consistent with a direct role for LPS in vibrio adherence, but other explanations are possible. For example, antibodies to LPS can inhibit the motility of *V. cholerae* (Attridge and Rowley, 1983a). Since non-motile vibrios colonise much less efficiently than their motile counterparts (Section 1.6.1.1), antibody-mediated immobilization might dramatically reduce attachment. This explanation would not account for the ability of isolated LPS to inhibit the adherence of *V. cholerae* to intestinal mucosa *in vitro* (Chitnis *et al*, 1982b)

The role of LPS as an adhesin can not be easily assessed. Mutations in the *rfb* region - encoding products for LPS biosynthesis - give rise to markedly attenuated strains with complex phenotypes, resulting from improper localisation of outer membrane proteins as well as

impaired biogenesis of secreted factors such as pili (Manning *et al*, 1994). Furthermore *rfb* mutants have abnormal flagella and therefore impaired motility (Manning *et al*, 1994). With such a diversity of defects it is impossible to ascribe the attenuation of such mutants to the loss of a putative O-antigen adhesin. The significance of LPS as a protective antigen will be discussed later.

1.6.2.1.3 Toxin co-regulated pilus (TCP)

Identified in 1987 by Taylor and co-workers, TCP is the most well characterized pilus of *V. cholerae*. The name toxin co-regulated pilus came from the observation that TCP were produced under conditions favourable for expression of cholera toxin (Taylor *et al*, 1987). It's expression is positively controlled by the global regulator ToxR which also modulates production of cholera toxin. Mutations in *tcpA*, the gene encoding the structural subunit of TCP, result in severely attenuated strains unable to colonise either the infant mouse or human small intestine (Taylor *et al*, 1987; Herrington *et al*, 1988). The highly hydrophobic nature of TCP causes the pili to aggregate in large bundles which in the case of classical strains leads to visible clumping and sedimentation of the bacteria in liquid culture (Taylor *et al*, 1987; unpublished observations).

Amino acid sequence analysis of the pilin subunit TcpA found it to have significant homology to the type IV pilins. Type IV pili have been linked with the virulence of a number of Gram negative bacterial pathogens such as *Pseudomonas* sp., *Neisseria* sp., *Moraxella* sp., and *Dichelobacter* sp. The Type IV pilins were once referred to as the NMePhe pilins because of the N-terminal N-methylphenylalanine residue. However TcpA and a number of other type IV pilins identified since have alternative modified N-terminal amino acids instead of phenylalanine and so the name NMePhe pilins has been dropped. Produced as a 23kDa pre-pilin, the 25

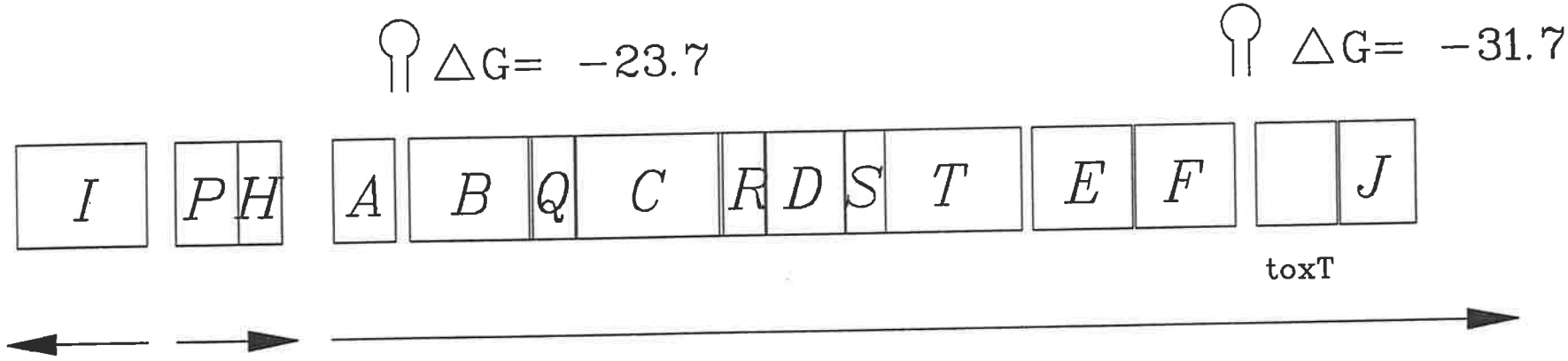
amino acid leader peptide of TcpA is unusually long for a type IV pilin (Faast *et al*, 1989; Shaw and Taylor, 1990). Another feature common amongst type IV pilins is the hydrophobic nature of the amino terminus. This is thought to allow the mature pilin of 20.5kDa to be translocated across the cell envelope and to promote subunit-subunit interactions during pilus assembly (Shaw and Taylor, 1990).

There are 15 genes grouped together to form the *tcp* gene cluster (Figure 1.1) and several of these are transcriptionally and translationally coupled (Brown and Taylor, 1995). Mutations in *tcpBCDEF* lead to reduced levels of pilin and an absence of TCP on the cell surface which implies these genes are required for pilus biogenesis (Kaufman and Taylor, 1994). In addition, other unlinked genes are needed for TCP biogenesis, such as *toxR* and *tcpG*. The TcpG protein catalyses disulphide bond formation in a number of exported proteins including cholera toxin and TcpA, indicating this protein has a general housekeeping role (Peek and Taylor, 1992; Yu *et al*, 1992). Characterisation of the functions of the transcriptionally coupled genes in the *tcp* operon has been hampered by the fact that transposon insertions have polar effects on the expression of downstream genes (Ogierman *et al*, 1993). Consequently the function of only very few *tcp* genes is known; although putative roles in pilus assembly and transport have been assigned to many of the genes on the basis of predicted amino acid sequence homologies with secretion proteins of other Gram-negative bacteria (Ogierman *et al*, 1993; Kaufman *et al*, 1993; reviewed by Iredell and Manning, 1994). Apart from *tcpA*, the two best characterised genes of the operon are *tcpJ* and *toxT*.

TcpJ is the pre-pilin peptidase which cleaves the TcpA leader peptide and amino-methylates the mature pilin (Kaufman *et al*, 1991). Located in the inner membrane, the highly hydrophobic nature of TcpJ suggests it spans the cytoplasmic membrane and it is thought that cleavage of the leader peptide occurs on the cytoplasmic side of the membrane (Kaufman *et al*,

Figure 1.1 Map of the *tcp* gene cluster

Arrows indicate the direction of transcription.


△G= −23.7
△G= −31.7
I
P
H
A
B
Q
C
R
D
S
T
E
F
J
toxT

1991). TcpJ has significant homology with the PilD protein of *P.aeruginosa* which also acts as a pre-pilin peptidase and is located in the cytoplasmic membrane. This type of peptidase appears to be common in type IV pilin export (Kaufman *et al*, 1991).

Situated between *tcpF* and *tcpJ* is the *toxT* gene whose product is essential for *tcpA* expression (Higgins *et al*, 1992; Ogierman and Manning, 1992). ToxT is a member of the AraC family of transcriptional activators and was recognised by its ability to activate *tcpA* expression in an *E.coli* background (DiRita *et al*, 1991). Expression of ToxT is directly controlled by ToxR; in turn ToxT can activate the *ctx* and *tcp* operons (DiRita *et al*, 1991; Brown and Taylor, 1995).

1.6.1.2.4 Accessory colonisation factor (ACF)

Four closely linked ToxR-regulated genes were found to encode factors that promote colonisation of the infant mouse gut. A mutation in any of the four genes in the classical strain 395 produced an ca. ten-fold reduction in both colonisation and LD_{50} relative to the parent strain (Peterson and Mekalanos, 1988). The colonisation defect of the *acf*::Tn*phoA* mutants was not as severe as that caused by a *tcpA*::Tn*phoA* mutation which led to the proposal that these genes are required for the production of an accessory colonisation factor (ACF). The four genes were designated *acfA*, *acfB*, *acfC* and *acfD* (Peterson and Mekalanos, 1988). The presence of the *acf* genes immediately downstream of the *tcp* operon suggested their function may be to assist TCP in colonisation of the small intestine (Peterson and Mekalanos, 1988), but the mechanism by which this is achieved is unclear at present.

The roles of the *acfA* and *acfC* gene products are not known. AcfB is related to HlyB and TcpI of *V. cholerae*, sharing amino acid sequence similarity in a region highly conserved among bacterial methyl-accepting chemotaxis proteins. It has been proposed that AcfB acts as

an environmental sensor which interacts with *V. cholerae* chemotaxis machinery (Everiss *et al*, 1994). Parsot *et al* (1991) demonstrated AcfD to be a lipoprotein with possibly a minor role in serum resistance. An open reading frame designated *orfZ* overlaps the 3' end of *acfD*. The *orfZ* predicted protein is likely to be a secreted product and has significant sequence similarity to various flagella biosynthetic genes of *Salmonella* sp. (Hughes *et al*, 1994). When grown under conditions optimal for ToxR-activated gene expression, *acfD* mutants have reduced motility and an altered morphology but this effect is diminshed under ToxR-repressive conditions. Whether the altered phenotype of *acfD* mutants is due to polar effects on *orfZ* is still under investigation (Hughes *et al*, 1994).

1.6.1.2.5 Core encoded pilin (CEP)

The *ctx* genetic element of *V. cholerae* O1 comprises a core region of genes encoding cholera toxin (*ctxAB*), Zot and Ace toxins. This region is flanked by two or more copies of a repeated sequence known as RS1. Deletion of the entire *ctx* element reduces colonisation of strains previously rendered non-toxinogenic, implying a product which enhances colonization is encoded within the element. A gene with a predicted pilin-like product has been identified and named *cep* (core encoded pilin). The predicted amino acid sequence of CEP has significant similarity with the *fxp* gene product of *Aeromonas hydrophila*, a pilin subunit implicated in adherence of (Pearson *et al*, 1993). Although not essential for colonisation, the colonisation-enhancing properties of *cep* may promote the acquisition of the *ctx* element by non-toxinogenic strains (Peterson *et al*, 1993).

1.6.2 Multiplication

One of the essential requirements of a pathogen is an ability to grow in the host. The faster the pathogen can multiply the more likely it is to successfully overwhelm the non-specific host defense mechanisms and cause disease (Smith, 1989). It is interesting to speculate whether the infectious dose varies depending on the source of the inoculum. Conceivably bacteria derived from an environmental niche have a lag phase before multiplication begins *in vivo* making them more susceptible to non-specific host defenses whereas epidemic spread from patient to patient may require a lower dose because these vibrios are primed for multiplication in the human gut (Freter, 1980). There is no doubt that vibrios undergo extensive multiplication *in vivo* as indicated by the large numbers of vibrios excreted in the cholera stool. Volunteers fed ca.10^6 CFU of the El Tor strain N16961 subsequently excreted ca. 3 x 10^7 vibrios /g of stool in an average volume of 4.2l (Levine *et al*, 1988a).

1.6.3 Cholera toxin

The elaboration of cholera toxin is accepted to be the major cause of diarrhoea in cholera although it is by no means the only diarrhoeagenic factor secreted by *V. cholerae* O1. There have been several reviews on the structure, function and expression of cholera toxin (Mekalanos, 1985; Spangler, 1992; Kaper *et al*, 1994a) which reflect the attention this factor has received since its discovery (De, 1959; Dutta *et al*, 1959). Cholera toxin was first demonstrated to be a secreted factor by Finkelstein and co-workers (1964) who showed that culture supernatants contained far greater toxinogenic activity than vibrio cell lysates. Soon after Finkelstein and Lo Spalluto (1969) purified cholera toxin and showed that it stimulated fluid accumulation in isolated ileal loops of guinea pigs.

Secreted as a holotoxin, cholera toxin forms a ring configuration in which the A subunit (ca. 27kDa) is surrounded by five B subunits (ca. 11.7kDa each; Gill, 1976). The structural genes *ctxA* and *ctxB* are overlapping but are not translationally coupled. Instead, *ctxB* is translated at a much higher efficiency than *ctxA* resulting in a ratio of A to B subunits of at least 1:5 (Mekalanos *et al*, 1983). The *ctx* operon forms part of a 4.5kb region known as the "core region" which is flanked by at least one copy of a 2.7kb sequence known as RS1. Recombination between RS1 sequences can lead to tandem duplication and amplification of the core region in a *recA*-independent manner particularly after *in vivo* passage of strains (Mekalanos *et al*, 1983; Pearson *et al*, 1993). RS1 also encodes a site-specific recombination system implicated in the conversion of non-toxinogenic *V. cholerae* to toxigenic strains by enabling the integration of the core region at an 18bp sequence known as *att*RS1 (Pearson *et al*, 1993).

The epithelial cell receptor for cholera toxin was first identified as GM1-ganglioside by King and Van Heyningen, (1973). To be enzymatically active the A subunit is cleaved to produce two disulphide-linked fragments, A1 and A2. Binding of the holotoxin to GM1-ganglioside via the B subunits induces translocation of the A1 fragment through the cell membrane; this then catalyses ADP-ribosylation of a G protein that regulates adenylate cyclase activity. Interference with adenylate cyclase activity leads to elevated cAMP in the cell and by a mechanism which is still not understood alters ion transport in villous and crypt cells. Chloride secretion is increased and sodium absorption decreased, providing an osmotic force which drives water from the tissues into the gut lumen and manifests itself as diarrhoea (Mekalanos, 1985). There is mounting evidence that the massive fluid secretion caused by cholera toxin is in part the result of elevated prostaglandin release into the gut lumen, in particular release of prostaglandin E2 (Peterson and Ochoa,1989; Peterson *et al*, 1991; Hill and Ebersole, 1991).

The effects of cholera toxin appear to vary along the length of the small intestine with most fluid secretion in the duodenum and jejunum and least in the ileum (Banwell *et al*, 1970). It is also possible that cholera toxin acts on the colon. Speelman *et al* (1985) found colonic water absorption was decreased and potassium secretion elevated in cholera patients but could only speculate as to the mechanism that might be involved.

V. cholerae secretes several toxins and many theories exist as to the purpose of producing these factors in infection. Clearly the toxins ensure dispersal of the pathogen back into the environment via the purging of large volumes of heavily contaminated diarrhoea (Finkelstein, 1973). Another role may be to promote bacterial multiplication in the gut by providing vibrios with essential nutrients such as trace elements that would not otherwise be available (Freter *et al*, 1981; Pierce *et al*, 1985). In this context a study by Pierce *et al* (1985) demonstrated that cholera toxin promotes colonisation of the small intestine of adult rabbits; mutants unable to produce the holotoxin or synthesize the A subunit colonised less efficiently than the fully toxinogenic parent strains. The mechanism by which cholera toxin promotes colonisation is not known although it was speculated that the toxin-induced fluid secretion provides a better environment for vibrio multiplication (Pierce *et al*, 1985). It was also hypothesized that the colonization advantage associated with cholera toxin production leads to *in vivo* selection of hyper-toxinogenic variants. This would explain the observation of toxin gene amplification in *V. cholerae* following *in vivo* passage (~~Pierce *et al*~~ Mekalanos, 1985).

V. cholerae produces a neuraminidase (NANase) which catalyses the conversion of higher order gangliosides to GM1, the receptor for cholera toxin. It has been hypothesized that the neuraminidase may enhance the effect of cholera toxin by increasing the number of receptors on the cell surface (Kabir *et al*, 1984). To address this, Galen *et al* (1992) compared isogenic NANase$^-$ mutants with their wild-type parent strain *in vitro* and *in vivo*. In the presence of

neuraminidase, increased binding and uptake of cholera toxin by mouse fibroblasts was observed but *in vivo* the effect of neuraminidase was less obvious. Fluid accumulation in nonfasted suckling mice challenged with 10^9 CFU of NANase$^-$ strains was reduced by 18% compared with wild-type strains. At lower doses no difference in fluid accumulation was detected leading the authors to conclude the NANase has "a subtle but significant role" in promoting cholera toxin binding and uptake by cells (Galen *et al*, 1992).

1.6.4 Zonula occludens toxin (ZOT) and accessory cholera enterotoxin (Ace)

The observation that attenuated vaccine strains carrying mutated *ctxAB* genes are still able to produce mild to moderate diarrhoea in recipients led to the search for other toxic factors (Levine and Pierce, 1992). It had been hypothesized that the residual diarrhoea caused by the live attenuated vaccine strain JBK70 (from which *ctxAB* has been deleted) may be caused by a Shiga-like toxin (Pearson *et al*, 1990). Shiga-like toxin activity has been detected *in vitro* in both human and environmental isolates of *V. cholerae* (O'Brien *et al*, 1984) but the *in vivo* significance of the Shiga-like toxin is unknown (Pearson *et al*, 1990).

By examining the effects of *ctx*-positive and *ctx*-negative *V. cholerae* strains on rabbit intestinal tissue mounted in Ussing chambers, a toxin increasing intestinal tissue conductance was identified (Fasano *et al*, 1991). The toxin acts by altering the structure of the intercellular tight junctions or zonula occludens and hence was named zonula occludens toxin (Zot). The gene encoding Zot was found immediately upstream of the *ctx* operon. Although the *zot* gene encodes an ca. 45kDa polypeptide (Baudry *et al*, 1992), the active protein was previously predicted to be less than 30kDa in size (Fasano *et al*, 1991) and this suggests that Zot undergoes post-translational processing (Baudry *et al*, 1992). The preliminary data of Baudry

et al (1992) indicates expression of *zot* is independent of the ToxR regulatory protein (see Section 1.6.6.1).

Together, *zot* and the *ctx* genes comprise only 55% of a 4.5kb core region. While investigating the pathogenic significance of the remaining portion of this region, Trucksis *et al* (1993) identified a third toxin encoded immediately upstream of *zot*. Named accessory cholera enterotoxin (Ace), this toxin increases the potential difference across epithelial membranes and induces increased fluid secretion in ligated rabbit ileal loops (Trucksis *et al*, 1993). A small (11.3kDa), predominantly hydrophobic protein, Ace has similarities to a family of ion-transporting ATPases, but this is the only clue as to its mechanism of action.

1.6.5 Haemolysin

One of the characteristics which has traditionally been used to distinguish strains of El Tor biotype from classical *V. cholerae* is production and secretion of a haemolysin (Pollitzer, 1959). The practice of using haemolysis of sheep red blood cells as a criterion for biotyping is now of dubious value, since recent El Tor isolates are weakly or non-haemolytic. However, isolates can be classified into biotypes using probes specific for the classical and El Tor haemolysin genes (Alm *et al*, 1990b).

The El Tor haemolysin has been shown to be both cytotoxic and enterotoxic (Honda and Finkelstein, 1979; Ichinose *et al*, 1987, Hall and Drasar, 1990; Alm *et al*, 1991). The product of the El Tor *hlyA* gene, HlyA, is produced as an 82kDa pre-pro-toxin. On secretion through the cell envelope a 79kDa inactive pro-toxin is formed following cleavage of the signal peptide. The mature and active 65kDa protein is then released into the culture medium by proteolytic cleavage of a 15kDa fragment (Goldberg and Murphy, 1985; Rader and Murphy, 1988; Alm *et al*, 1988; Hall and Drasar, 1990; Yamamoto *et al*, 1990).

Classical biotype strains are non-haemolytic. This is due to an eleven base pair deletion in *hlyA* found in all classical strains examined to date. The deletion causes a frameshift mutation leading to premature polypeptide chain termination and the formation of a truncated 27kDa product known as HlyA* (Alm *et al*, 1988; Rader and Murphy, 1988). HlyA* is reported to have enterotoxic activity in rabbit ileal loop assays (Alm *et al*, 1991) but to be non-haemolytic on sheep erythrocytes (Richardson *et al*, 1986). It is therefore proposed that the two activities of the El Tor haemolysin reside at either end of the protein with N-terminal enterotoxic activity and C-terminal haemolytic/cytolytic activity (Alm *et al*, 1991).

The haemolysin appears to contribute to *V. cholerae* pathogenesis. Williams *et al* (1993) assessed the virulence of an El Tor *hlyA* mutant in the infant mouse cholera model (IMCM) and found it to be 100-fold reduced in virulence compared with its parent strain. Although the *in vivo* role of the haemolysin is poorly understood, a possible function is the provision of iron (Sciortino and Finkelstein, 1983). The haemolysin may have a cytotoxic effect on intestinal epithelial cells resulting in the release of intracellular iron-containing compounds which can be scavenged by *V. cholerae* (Stoebner and Payne, 1988). El Tor infections sometimes result in a bloody diarrhoea and this has been attributed to the haemolysin. Whether the enterotoxic activity of HlyA contributes to the diarrhoea seen in human infection is unclear. Diarrhoea occurred in volunteers who ingested Δ*ctx* strains JBK70 and CVD101 or their isogenic Δ*hlyA* derivatives (Levine *et al*, 1988a). In contrast, Alm *et al* (1991) reported that a mutation that abolished the production of HlyA and HlyA* removed the residual toxicity and diarrhoea of JBK70 in a rabbit ileal loop model. These workers claimed that the *hlyA* deletion used by Levine *et al* (1988a) was not extensive enough, leaving the capacity to produce a functional (enterotoxic) HlyA* molecule.

1.6.6 Regulation of virulence gene expression

V. cholerae pathogenesis is an extremely complex process involving a number of regulatory systems which coordinate gene expression in response to a wide variety of environmental signals. In this way virulence determinants are not constitutively expressed but are only produced when required, and only at appropriate anatomical sites. Coordinate regulation of genes and operons encoding bacterial virulence factors is a common feature amongst Gram-negative pathogens such as *Agrobacterium tumefaciens* (Stachel and Zambryski, 1986), *Bordetella pertussis* (Weiss and Falkow, 1984) and *Salmonella typhimurium* (S.I Miller *et al*, 1989).

1.6.6.1 The ToxR regulon

The master regulator of the most studied coordinate regulatory system of *V. cholerae* O1 is the ToxR protein. First discovered by its ability to trans-activate a *ctx-lacZ* fusion in *E. coli* (Miller and Mekalanos, 1984), ToxR has since been found to be essential for the expression of at least 17 other genes in addition to those encoding the cholera toxin subunits. These include genes required for the synthesis of TCP (Section 1.6.2.1.3; Taylor *et al*, 1987), ACF (Section 1.6.2.1.4; Peterson *et al*, 1988), OmpT and OmpU (Miller and Mekalanos, 1988). Not all the genes controlled by ToxR are virulence determinants *per se*. For example, *aldA* the gene encoding aldehyde dehydrogenase, is positively regulated by ToxR but *aldA* mutants are not attenuated in the IMCM (Parsot and Mekalanos, 1991) and it can only be speculated whether these mutants are attenuated in their natural host (Parsot and Mekalanos, 1991). Expression of the ToxR regulon is essential for the pathogenesis of *V. cholerae* as *toxR* mutants are avirulent in both the IMCM and humans (Taylor *et al*, 1987; Herrington *et al*, 1988).

ToxR is a 32.5kDa trans-cytoplasmic membrane protein with a large N-terminal domain in the cytoplasm and a smaller C-terminal portion in the periplasm (Miller *et al*, 1987). The N-terminal portion harbours the DNA-binding (transcriptional activation) domain whereas the periplasmic portion of ToxR acts as an environmental sensor (Miller *et al*, 1987). The DNA-binding domain of ToxR shares similarity with regulatory proteins belonging to the two-component family and mutations at conserved residues within the region of homology abolish DNA-binding activity and transcriptional activation (Otteman *et al*, 1992).

Only two genes are known to be directly activated by ToxR. Gel-retardation studies revealed expression of the *ctxAB* operon occurs as a result of ToxR binding to tandem repeats of a specific element (TTTGAT) in the promoter region upstream of *ctxAB* (Miller *et al*, 1987). The number of repeats varies between strains and is subject to amplification during intestinal passage (Miller *et al*, 1987). Expression of the *ctxAB* operon requires the presence of another regulatory protein, ToxS. The *toxS* gene is located immediately downstream of, and is co-transcribed with, *toxR* (Miller *et al*, 1989). The 19kDa ToxS protein is membrane associated but is predominantly located in the periplasm where it interacts with the periplasmic, C-terminal portion of ToxR (DiRita and Mekalanos, 1991). The interaction of ToxS with ToxR is thought to stabilize the spontaneous formation of ToxR dimers which may be a critical requirement for activation of the DNA-binding activity of ToxR (Miller *et al*, 1987; DiRita and Mekalanos, 1991; Dziejman and Mekalanos, 1994).

The only other gene known to be directly activated by ToxR is *toxT*. Located within the *tcp* operon between *tcpF* and *tcpJ*, *toxT* encodes a 32kDa regulatory protein belonging to the AraC family of transciptional activators widespread among Gram-negative bacteria. A common feature of these regulators is that they control gene transcription under specific environmental conditions in response to particular effectors (Higgins *et al*, 1992). A ToxR-

binding site and a ToxR-dependent promoter have been identified within the pre-coding region of *toxT*. Unlike the *ctxAB* promoter the TTTGAT repeat elements are not present in the *toxT* promoter region (Higgins *et al*, 1992; Ogierman and Manning, 1992) indicating ToxR has the capacity to bind to more than one specific site. Some transcription of *toxT* occurs as a result of read-through from the *tcpF-toxT* intergenic region although how significant this source of transcript is compared to the level resulting from the ToxR-dependent *toxT* promoter remains to be determined (Higgins and DiRita, 1994). ToxT does not appear to control its own expression in *E. coli* but whether the same applies in *V. cholerae* is also unknown (Higgins and DiRita, 1994). Although *toxT* has a ToxR-dependent promoter, ToxR alone is insufficient for activation of a *toxT-lacZ* fusion in *E. coli* which suggests transcription requires another *V. cholerae* protein factor or perhaps a specific DNA topology. A less likely hypothesis is that activation does not occur in *E. coli* because ToxR is unable to interact effectively with the *E. coli* RNA polymerase (Higgins and DiRita, 1994).

Several of the genes regulated by ToxR including *tcpI*, *tcpA*, *tcpC*, *aldA* and *tagA* as well as *ctxAB* are directly activated by ToxT (DiRita *et al*, 1991). Furthermore if ToxT is constitutively expressed from a plasmid promoter under certain conditions the requirement for ToxR is relieved (DiRita *et al*, 1991).

Several environmental parameters influence expression of the ToxR regulon. Osmolarity, pH and amino acid concentrations have been found to modulate ToxR function. Interestingly the control of ToxR-regulated genes is not necessarily at the level of ToxR production. Miller and coworkers (1987) observed cholera toxin production could be restored in a *toxR* mutant by providing ToxR constitutively *in trans*. However *ctxAB* expression in the complemented *toxR* mutant was still regulated by osmolarity and amino acid concentration in the same manner as the wild-type parent. Temperature control of ToxR has been found to be mediated, at least in

part, at the level of transcription. Directly upstream of and divergently transcribed from *toxR* is the *htpG* gene, the product of which belongs to the *hsp*90 family of heat shock proteins (Parsot and Mekalanos, 1990). At 37°C *htpG-lacZ* fusions show elevated transcription whereas *toxR-lacZ* fusions were reduced in expression relative to transcription at 22°C. Parsot and Mekalanos (1990) have proposed that heat-shock control of *toxR* expression at 37°C results from exclusion of the σ-70 RNA polymerase (required for *toxR* expression) by an alternative σ-32 (RpoH) polymerase which recognises the *htpG* promoter. The *in vitro* finding that at 37°C *toxR* expression is down-regulated is at odds with the fact that the ToxR regulon is clearly expressed at 37°C *in vivo*. Parsot and Mekalanos (1990) suggest other environmental signals modulate the effect of temperature and the heat-shock response in a finely controlled manner to ensure the synthesis of virulence determinants occurs at the appropriate anatomical sites.

1.6.6.2 Control of virulence gene expression by iron and the Fur system

A source of iron is essential for bacterial growth but *in vivo* the availability of free Fe^{3+} is often limiting. In humans, proteins such as lactoferrin and transferrin efficiently chelate free ferric ions; therefore in order to compete with the host, bacteria must produce their own high affinity iron-binding ligands (siderophores). A number of Gram-negative pathogens including *V. cholerae* O1 produce a ferric iron uptake regulator (Fur) protein which coordinately regulates the expression of genes required for iron acquisition. In response to high iron conditions, Fur binds to a specific sequence known as the Fur box in the promoter of iron-regulated genes and represses their transcription (Litwin *et al*, 1992).

The studies of Sciortino and Finkelstein (1983) have demonstrated the expression of iron-regulated outer membrane proteins of *V. cholerae in vivo*, indicating low iron levels in the

intestine. Regulation of gene expression by iron is extremely complex. Recently Litwin and Calderwood (1994) examined the protein profiles of a non-revertible *fur* mutant and its wild-type parent following growth in the presence of high and low concentrations of iron, and identified fifty six proteins that were regulated by iron and Fur. At least one of the proteins is a virulence determinant. IrgA is a 77kDa iron-regulated, major outer membrane protein and *irgA* mutants are attenuated at least 100-fold in an animal model (Goldberg *et al*, 1990a). IrgA has significant homology to the entire class of TonB-dependent proteins and may therefore be involved in TonB-dependent transport across the *V. cholerae* outer membrane (Goldberg *et al*, 1992). It had previously been suggested that IrgA may be the receptor for iron-vibriobactin complexes based on it's similarity to the *E. coli* ferri-enterochelin receptor, FepA (Goldberg *et al*, 1990b). However *irgA* mutants are unaltered in their ability to utilize iron or transport vibriobactin (Goldberg *et al*, 1992).

The *irgB* gene is adjacent to *irgA* but transcribed in the opposite direction. Fur repression of *irgB* maintains low levels of IrgA but when iron is limiting, *irgB* is de-repressed and activates strong transcription of *irgA* (Litwin and Calderwood, 1993). Preliminary data suggest IrgB controls the expression of other iron-regulated genes (DiRita, 1994).

1.6.6.3 Regulation of virulence gene expression by HlyU

Production of the El Tor haemolysin HlyA is increased in response to iron stress (Stoebner and Payne, 1988) and is also upregulated by the HlyR protein (von Mechow *et al*, 1985). More recently a novel regulatory locus, *hlyU* has been found to positively regulate *hlyA* expression (Williams and Manning, 1991). HlyU belongs to a family of small regulatory proteins with a characteristic helix-turn-helix domain common to a number of DNA-binding proteins (Williams *et al*,1993). Furthermore studies in the IMCM suggest HlyU promotes the

expression of virulence determinants *in vivo* (Williams *et al*, 1993). HlyU is known to regulate the expression of at least one other protein. A 28kDa secreted protein, unrelated to HlyA, is dependent on HlyU for expression, but at present nothing is known about the function of this protein (Williams *et al*, 1993).

1.7 Potential protective antigens of *V. cholerae* O1

1.7.1 Cholera toxin

Cholera toxin is a potent immunogen and induces a strong humoral and intestinal antibody response. The immune response is almost entirely directed against the B-subunit (Holmgren, 1991). Despite this there is no evidence that antibodies to toxin are protective against human infection. Field trials of parenteral toxoid vaccines failed to demonstrate protection against cholera (Noriki, 1976). Similarly in a volunteer study, oral or parenteral administration of gluteraldehyde treated cholera toxin was not protective against experimental cholera challenge (Levine *et al*, 1979).

The results of studies performed using the rabbit ligated ileal loop model have suggested that a combined antibacterial and antitoxin response acts synergistically to confer protection (Svennerholm and Holmgren, 1976). This finding combined with the observation that clinical cholera evokes a strong antitoxic response in addition to an antibacterial response prompted the addition of the B-subunit to an oral killed whole vibrio vaccine (B-WCV; Svennerholm *et al*, 1984). Data from a field trial of the B-WCV demonstrated it had significantly improved protective efficacy compared with the WCV component alone but only for the first six months following vaccination. No improvement in long-term efficacy was found (Clemens *et al*, 1990).

The insignificance of an anti-toxic response in protective immunity to cholera is further illustrated by the ability of live attenuated vaccines lacking toxin genes to elicit protective immune responses (Levine *et al*, 1988b). Evidently anti-toxic immunity is not essential for protection. In summary, it seems that although cholera toxin can elicit a strong immune response, anti-toxin immunity is neither necessary nor sufficient for protection against cholera (Cash *et al*, 1974; Clemens *et al*, 1990).

1.7.2 Lipopolysaccharide

LPS is composed of three distinct regions. The lipid A anchors the molecule to the outer-membrane while the core oligosaccharide links the lipid A to the outermost region, the O antigen. The O antigen consists of repeat units of the polysaccharide perosamine acetylated with 3-deoxy-L-glycero-tetronic acid. Quinovosamine is also known to form part of the O antigen but has yet to be localised. Substitutions which occur on the perosamine give rise to the serotypes Inaba and Ogawa (Manning *et al*, 1994). Covering the entire surface of the bacterial cell and present in the flagellar sheath, LPS is not surprisingly a major immunogen.

Antibodies against LPS are protective in several animal models (Neoh and Rowley, 1972; Chitnis *et al*, 1982a; Jansen *et al*, 1988; Svennerholm and Holmgren, 1975). Based on studies in the rabbit ileal loop model Holmgren and Svennerholm (1976) found that the anti-bacterial activity of immune sera was almost entirely directed against LPS and could be removed by absorption with purified LPS. Attridge and Rowley (1983b) showed that antibodies to LPS were sufficient to mediate protection in the widely-used IMCM; the key protective function of these antibodies was the capacity to inhibit vibrio attachment.

Serum vibriocidal antibody levels are used as a guide to the stimulation of anti-bacterial immunity and LPS is considered to be the major target antigen in vibriocidal assays (Neoh and

Rowley, 1970 and 1972; Svennerholm and Holmgren, 1976). Studies in Bangladesh have shown that the prevalence and titre of naturally acquired, vibriocidal antibody increases with age, presumably as a result of repeated natural exposure to cholera (Mosley, 1969). Associated with the increasing serum level of vibriocidal antibody is a declining incidence of disease in older age-groups (Mosley, 1969). Cholera convalescents develop significant serum antitoxic and vibriocidal antibody levels (Svennerholm *et al*, 1984). Studies with live, attenuated *V. cholerae* vaccine candidates have shown that a strain's potential to elicit strong vibriocidal antibody responses provides the best correlate of its protective efficacy (Migasena *et al*, 1989). Although serum vibriocidal antibodies are not considered to contribute directly to enteric defence, their presence is thought to reflect the generation of an intestinal sIgA response (Attridge, 1991; Levine and Pierce, 1992).

The protective significance of the immune response against LPS was illustrated by sero-epidemiologic data obtained in field trials of parenterally administered vaccine preparations. In the 1968-1969 vaccine field trial performed in rural Bangladesh, participants received either monovalent, classical Inaba or Ogawa whole cell vaccines or an El Tor Inaba cell wall fraction. Both the Inaba vaccine formulations provided significant protection (90% for three months) against classical Inaba infection but the Ogawa vaccine was ineffective (Mosley *et al*, 1970). The only known serotype-restricted antigen is the O-antigen of LPS which suggests that the protective immune response observed in this field trial was primarily directed against LPS.

The recent *V. cholerae* O139 epidemics provide further evidence of the significance of LPS as a protective antigen. The O139 serotype strains have caused outbreaks of disease in cholera-endemic regions of India and Bangladesh. Despite the fact that the adult population within these regions has long-standing, pre-existing immunity to *V. cholerae* O1 strains, all age groups were susceptible to O139 cholera. Recent studies indicate O139 strains are closely

related to O1 strains of El Tor biotype, the major difference being the O-antigen which is biochemically and antigenically unrelated (Waldor *et al*, 1994; Bik *et al*, 1995; Comstock *et al*, 1995). Evidently antibodies to other shared virulence determinants are collectively insufficient to protect against infection implying LPS is the most important protective antigen in natural infection.

1.7.3 Non-LPS protective antigens

The O-antigen of LPS may be the dominant immunogen on the vibrio cell surface but there are other non-LPS antigens which can elicit a protective response. Studies in the IMCM demonstrated the protective efficacy of antibodies to undefined non-LPS determinants (Neoh and Rowley, 1970; Attridge and Rowley, 1983b), one of which was subsequently shown to be TCP (Sharma *et al*, 1989a).

1.7.3.1 Flagellar proteins

Animal studies have suggested the existence of a flagellum-associated protective antigen. Eubanks *et al* (1977) found an antigen present in a crude flagella preparation induced antibodies that were protective in a suckling mouse model. Later Yancey *et al* (1979) demonstrated the same antigen was protective in the rabbit ligated ileal loop model. However, the flagellar preparations of Eubanks and Yancey were not purified, raising the possibility of contaminating non-flagellar components inducing protective antibodies. There are no data available as to whether antibodies to the flagellar antigens are protective in humans.

1.7.3.2 Haemagglutinins

The only *V. cholerae* HA to be evaluated as a protective antigen is the mannose-sensitive haemagglutinin (MSHA). MSHA pili appear to be immunogenic in humans as Svennerholm *et al* (1994) detected antibody to MSHA in 60% of cholera patients infected with El Tor strains. Recent work has shown that antibodies to MSHA provide some protection against challenge with El Tor strains but not against classical strains in the IMCM and rabbit intestinal loop model (Osek *et al*, 1992; Osek *et al*, 1994). It has been proposed that the MSHA is an adhesin for the El Tor biotype, assuming a function equivalent to that of TCP in colonisation by classical *V. cholerae* (Osek *et al*, 1994). Unlike TCP, MSHA has yet to be demonstrated to be an essential colonisation factor for either biotype in human infection.

1.7.3.3 Outer membrane proteins (OMPs)

There are five major OMPs between 25-45kDa in size, commonly found in *V. cholerae*. OmpS (43kDa), similar to the LamB protein of *E. coli*, is a maltoporin whose expression *in vitro* is growth-phase dependent but is known to be expressed *in vivo* (Lång *et al*, 1988; Lång and Palva, 1993). OmpT and OmpU (42kDa and 40kDa respectively) are outer membrane porins whose expression is downregulated under conditions where OmpS is upregulated (Lång and Palva, 1993); unlike OmpS, they are also regulated by ToxR (Miller *et al*, 1987). Two other proteins, the OmpA-like protein (35kDa) and OmpV (25kDa) are not controlled by ToxR or maltose. The OmpA-like protein appears to be an analogue of the *E. coli* protein OmpA. OmpA acts as a phage receptor, is required for the action of colicin K and L and for conjugation with F-like donors (Alm *et al*, 1986). The OmpV protein is known as the common immunogenic protein as it is highly immunogenic and appears to be common amongst

Vibrio strains (Manning and Haynes, 1984). The roles of these proteins in stimulating protective immune responses remains unknown.

The outer membrane profile of *V. cholerae* changes depending on the culture conditions used (Sciortino and Finkelstein, 1983; Jonson *et al*, 1990). Antibody responses to *V. cholerae* OMPs have been studied in volunteers following experimentally induced cholera (Richardson *et al*, 1989). Seroconversion to OMPs ranging in size from 22-38kDa was detected in approximately half the volunteers but sIgA recovered from jejunal fluid was found to react with proteins less than 25kDa in size, some of which were unique to *in vivo* grown organisms (Richardson *et al*, 1989). Sciortino (1993a) has described an 18kDa OMP which induces a significant immune response in humans. This protein is present in outer membrane preparations from vibrios grown in low-iron conditions, which show an OMP profile similar to that of vibrios recovered from *in vivo* (Sciortino, 1993b). The 18kDa OMP was named the cholera protective antigen (CPA) since monoclonal antibodies to this protein protect suckling rabbits from lethal challenge with *V. cholerae* (Sciortino, 1989). The structure and function of the CPA is currently being investigated and it will be of great interest to know if CPA is a protective antigen in human infection.

Sengupta *et al* (1992) assessed the potential of the major OMPs of *V. cholerae* to induce protective immunity in the suckling mouse model. Antibodies to OMPs ranging in size from 40- to 43kDa and a 20kDa protein were shown to provide significant protection against *V. cholerae* challenge. To a lesser extent antibodies to 27- to 28kDa OMP were also protective. The 20kDa protein was later identified as TcpA however the other proteins are thought to be porins (Sengupta *et al*, 1992). Recently it was found that pre-treatment of *V. cholerae* strain 395 with antibodies raised against the 53kDa OMP prevented colonisation of the infant mouse gut (Singh *et al*, 1994). Preliminary studies suggest that expression of the 53kDa OMP is co-

ordinately regulated with OmpU and TcpA (Singh *et al*, 1994). It is possible the 53kDa protein corresponds to the 58kDa protein identified by Taylor *et al* (1987) whose expression is regulated by ToxR.

1.7.3.4 Toxin-coregulated pilus

TCP has been shown to be a protective antigen for *V. cholerae* O1 of the classical biotype in the IMCM (Sharma *et al*, 1989a and b; Sun *et al*, 1990a and b). The same animal studies failed to demonstrate TcpA to be a protective antigen of the El Tor biotype, a finding which is addressed by the studies in this thesis. Whether TcpA is a protective antigen in human infection is still under investigation. Hall *et al* (1991) found no evidence of an immune response to TCP in either the serum or jejunal fluid of volunteers challenged with classical *V. cholerae*. Furthermore, a slight rise in anti-TCP titer from acute to convalescent phase was detected in only three of six patients who contracted El Tor cholera. However as will be discussed later in this thesis the results of this study are not conclusive.

1.8 Human immune response to cholera

1.8.1 The mucosal immune system

The largest mammalian lymphoid organ is the gut associated lymphoid tissue (GALT). GALT has features which distinguish it from other lymphoid tissue and it functions independently of the systemic immune mechanism (Czerkinsky *et al*, 1993). When a pathogen is ingested the host immune response begins at the site of gut lymphoid follicles known as Peyer's patches. Covering the surface or dome of the follicles are microfold (M) cells, specialised epithelial cells which sample the gut contents by phagocytosis. Antigens taken up

by M cells are delivered intact to antigen-presenting cells (parenchymal intestinal macrophages and dendritic cells) which in turn process and present antigens to B and T cells. Once sensitised the lymphocytes migrate through the lymphatics enter systemic circulation before migrating to tissues underlying the intestinal (and other) mucosal surface(s) (Holmgren, 1991; Czerkinsky *et al*, 1991; McGhee *et al*, 1992; Czerkinsky *et al*, 1993). Here B cells differentiate into plasma cells and begin producing predominantly sIgA class antibody. This antibody makes up ca.80% of all isotypes produced in mucosal tissues (McGhee *et al*, 1992; Czerkinsky *et al*, 1993) and is ideally suited for enteric defence as it is resistant to normal intestinal proteases. sIgA has also been reported to interfere with the scavenging of growth factors such as iron by potential pathogens, potentiate the function of some non-specific host defence mechanisms and to mediate antibody-dependent T cell-mediated cytotoxicity (Czerkinsky *et al*, 1993). Furthermore IgA limits uptake of previously encountered antigens (McGhee *et al*, 1992). In circumstances where the intestinal immune system has prolonged exposure to an immunogen, a portion of the sIgA molecules make their way into systemic circulation where they are extracted by the liver and then excreted into the gut lumen via bile. This additional route by which SIgA can enter the small intestinal lumen may represent an important means of preventing colonisation (Levine and Pierce, 1992).

The mucosal immune system encounters a myriad of antigens daily but responds to only a limited number. Antigens which stimulate mucosal immunity must have survived degradation in the gut environment (thus excluding most food antigens) and must be able to closely associate with the mucosal epithelium. Bacteria and viruses which can enter and multiply within GALT are particularly good at stimulating a local immune response. Other strong immunogens include bacterial toxins and adhesins such as pili that bind bacteria to the mucosal surface (Holmgren, 1991).

1.8.2 Infection-derived immunity to cholera

There are conflicting reports in the literature as to whether naturally acquired infection with *V. cholerae* O1 induces long-lasting immunity to re-infection. Woodward (1971) found no association between previous cholera infection and protection against secondary infection in rural Bangladesh. However Glass *et al* (1982) repeated the study in the same area and concluded that an initial episode of treated cholera provided 90% protection against a future episode of clinical illness. More recently Clemens *et al* (1991) also undertook a study in rural Bangladesh and found that a primary episode of classical cholera afforded complete protection (in agreement with Glass and co-workers) whereas an initial episode of El Tor cholera provided negligable protection.

Despite the conflicting data obtained in field studies, volunteer studies have clearly demonstrated that clinical cholera is an immunising event and results in long-term protection against re-infection (Cash *et al*, 1974; Levine *et al*, 1981). Cash and co-workers (1974) observed complete protection against re-challenge with a homologous strain of *V. cholerae* four to twelve months after the initial episode of cholera. Subsequently Levine *et al* (1983a) were able to demonstrate that protective immunity evoked by an initial clinical infection with classical *V. cholerae* persisted for at least three years. The finding of Clemens *et al* (1991) that an initial infection with *V. cholerae* El Tor provides no protection against re-infection is in sharp contrast to the results of volunteer studies with the El Tor vaccine strain JBK70. Oral administration of JBK70 resulted in colonisation of the intestine, evoked high-levels of vibriocidal antibody and provided significant protection against subsequent challenge with wild-type *V. cholerae* O1 (Levine *et al*, 1988a).

As *V. cholerae* O1 is a non-invasive organism it is assumed that protection against this enteropathogen is primarily mediated by sIgA. In cholera-endemic areas, patients convalescing from clinical cholera have high levels of intestinal sIgA directed against cholera toxin and LPS (Svennerholm *et al*, 1984). Additionally sIgA antibodies against *V. cholerae* are detectable in breast milk (Majumdar *et al*, 1981; Jertborn *et al*, 1984). Importantly long-lasting mucosal immunity is provided by the strong immunological memory in the sIgA system (Holmgren, 1991).

1.9 Cholera vaccine development

1.9.1 Past approaches to cholera vaccination: parenteral killed whole cell and toxoid vaccines

From the turn of the century until the 1970's, the conventional approach to cholera vaccination was parenteral administration of a vaccine preparation. During the 1960's proper evaluation of the effectiveness of parenteral killed whole cell cholera vaccines began with well organised field trials (Mosley *et al*, 1973; Philippines cholera commitee, 1973). It became obvious from the data collected that these vaccines provided moderate but short-lived protection of only a few months. The duration of protection could be extended to 12-18 months if an adjuvant was used however this often led to severe reactions at the site of injection. In endemic populations protection was only observed in older children and adults and this was interpreted to mean the vaccine was merely boosting pre-existing, naturally acquired immunity to cholera. Consistent with this interpretation, parenteral vaccination failed to prevent infection and disease in epidemic situations (Sommer, 1972).

The development of toxoid vaccines was prompted by the realization that the manifestations of cholera are a result of toxin secretion, together with the success of other toxoid-based vaccines for example against diphtheria and tetanus. Parenteral administration of formaldehyde- and gluteraldehyde-treated cholera toxin preparations failed to protect against disease in the Philippines and Bangladesh respectively (Noriki, 1977; Rappaport *et al*, 1974; Curlin *et al*, 1976). It was becoming clear at this time that an anti-bacterial rather than anti-toxin response provided immunity to *V. cholerae* O1 infection (Cash *et al*,1974) and so development of toxoid vaccines was largely abandoned.

1.9.2 Current approaches to vaccination

With greater understanding of gut immunity and the recognition that immunity to cholera resides within the intestinal mucosa oral cholera vaccine preparations were developed. The oral delivery of immunogens would hopefully mimic natural infection and provide long-lasting immunity to re-infection (Holmgren and Svennerholm, 1983). Most importantly for a non-invasive organism such as *V. cholerae* O1 prevention of disease depends on the induction of a sIgA response. Oral cholera vaccines currently being developed fall into two groups. Preparations consist of either killed whole cells with or without the addition of the purified B-subunit of cholera toxin, or live attenuated recombinant strains. The results from field-trials of these oral vaccines indicate significant long-term protection against cholera is achievable.

1.9.2.1 Oral inactivated vaccines

The combined B-subunit killed whole cell vaccine developed by Holmgren and co-workers is the most extensively tested oral cholera vaccine preparation. The addition of the B-subunit to the inactivated bacteria was based on the observation of a synergistic effect between anti-

bacterial and anti-toxin immunity in an animal model (Svennerholm and Holmgren, 1976; Holmgren *et al*, 1977). Each dose of vaccine is composed of 1mg purified B-subunit and 10^{11} heat- or formalin-killed *V .cholerae* O1 in phosphate buffered saline. The three strains are used in manufacture of the vaccine, ensuring both classical and El Tor biotypes and Inaba and Ogawa serotypes are represented. The vaccine is administered with an alkaline buffer to protect it from gastric acidity.

Clinical trials in Swedish, Bangladeshi and American volunteers showed that the B-subunit whole cell vaccine did not produce adverse side reactions, and that after two or three doses it stimulated sIgA anti-toxin and anti-bacterial responses comparable to those induced by natural infection (Svennerholm *et al*, 1984; Jertborn *et al*, 1984; Black *et al*, 1987). Furthermore American volunteers given three doses of the B-subunit whole cell vaccine (or the whole cell component alone) were protected against challenge with an inoculum of *V. cholerae* that caused disease in 90-100% of unvaccinated controls (Black *et al*, 1987).

In 1985 the whole cell vaccine (with or without the addition of B-subunit) was evaluated in a large, randomised placebo-controlled field trial involving 89,596 Bangladeshi adults and children. In the first 6 months following vaccination, the efficacy of the B-subunit whole cell vaccine was 85%, and of the whole cell component alone 56%. During this initial period children less than 6 years of age were as well protected as adults but unfortunately the protective efficacy in young children rapidly diminished thereafter. After three years, vaccination with either preparation gave moderate protection (51% for the combined vaccine and 52% for the whole cell component only). For reason(s) which remain unknown, immunisation provided better protection against classical than El Tor infection. An unexpected benefit provided by the B-subunit was cross-protection against LT-producing enterotoxinogenic *E. coli*. Cross-protection peaked at two months post-vaccination at 75%

but dropped to 37% within a year. Additionally and unexpectedly, a reduction in mortality rates due to all diarrhoeal disease was also observed (Clemens *et al*, 1990).

The moderate long-term protection afforded by the B-subunit whole cell vaccine is encouraging and it has the advantage of being completely safe. The major disadvantage is the lack of long-term protection in very young children - the group most at risk of developing life-threatening illness. The requirement for multiple doses is an additional drawback as this necessitates repeated visits to or by health care workers. This vaccine is also expensive to produce particularly with the addition of the B-subunit. Finally, in an effort to improve vaccine efficacy other antigens may soon be included. Candidate antigens include TCP and MSHA pili, both of which have been implicated in the pathogenesis of *V. cholerae* O1 .

1.9.2.2 Live oral cholera vaccines

Prior to the advent of recombinant DNA technology, live attenuated *V. cholerae* strains were isolated as naturally occurring or selected following chemical mutagenesis. Neither approach was satisfactory as the mutation(s) were undefined and there was always the potential for reversion to fully virulent strains. Recombinant DNA technology provided the tools whereby genes encoding virulence determinants non-essential for the induction of protective immunity could be eliminated.

JBK70 was one of the first gene-deleted vaccine strains to be ~~trialled~~ tested in humans. Derived from the El Tor strain N16961, JBK70 is unable to produce either the A or B-subunit of cholera toxin. Volunteers receiving a single oral dose of JBK70 ranging from 10^6 to 10^{10} were well protected upon challenge with 10^6 wild-type N16961 (89% vaccine efficacy) and had a significantly lower excretion rate of the challenge strain compared with controls. Unfortunately JBK70 produced an unacceptable level of adverse reactions in 6 of 14 recipients

the most common being residual mild diarrhoea accompanied by malaise, abdominal cramps, nausea and headache (Levine *et al*, 1988a). CVD101, an A^-B^+ derivative of the classical strain 395, produced similar side effects and so a challenge trial was not performed (Levine *et al*, 1988a).

The cause of the residual reactogenicity in these strains was unknown. The only other toxic factor known to be produced by *V. cholerae* O1 at this time was a shiga-like toxin and so a parent strain which did not produce this toxin was selected for the construction of other vaccine candidates. CVD103 is an A-B+ derivative of the classical strain 569B; when administered at a dose of 10^8 organisms only five of 46 volunteers developed mild diarrhoea but no other adverse reactions were recorded. Furthermore vibriocidal and anti-toxin responses induced by CVD103 were nearly the same as those recorded in volunteers after challenge with the parent strain 569B (Levine *et al*, 1988b). CVD103 vaccinees were subsequently challenged with toxinogenic *V. cholerae* O1 of classical (Inaba and Ogawa) and El Tor (Inaba) biotype. The protective efficacy against classical Inaba and Ogawa was 87% and 82% respectively, and against El Tor Inaba challenge was 67% (Levine *et al*, 1988b).

The results of clinical trials with CVD103 were sufficiently encouraging to merit field trials to determine the protective efficacy among inhabitants of cholera endemic areas. To enable the unequivocal identification of the vaccine strain in the field, CVD103 was genetically tagged by the introduction of a mercury-resistance gene into the *hlyA* locus. The presence of the HgR marker would facilitate the assessment of the persistence of CVD103-HgR in the environment and the rate of secondary transmission to close contacts of vaccinees. An unexpected bonus of this genetic manipulation was an absence of diarrhoea in any of the 18 volunteers given a single oral dose of CVD103-HgR (5×10^8 viable organisms) and an even lower excretion rate than CVD103. Most importantly, the serum vibriocidal and anti-toxin responses elicited by

CVD103-HgR were equivalent to those seen with CVD103. Its protective efficacy against challenge with El Tor biotype matched that of CVD103.

As a prelude to large scale field trials, an assessment of the safety and immunogenicity of CVD103-HgR was performed in adults and children residing in cholera endemic areas. Safety and immunogenicity studies in Thai (Migasena *et al*, 1989) and Swiss adults (Cryz *et al*, 1990) corroborated the findings of Levine *et al* (1988b). Analysis of the data from a more recent study of 324 Thai soldiers and civilians given different immunisation regimens of CVD103-HgR suggests that in endemic areas a higher dose of vaccine (5 $X10^9$ cfu) is required to consistently stimulate an adequate response. Similarly an oral dose of 5 X 10^9 cfu was required to achieve a suitable level of seroconversion in 79% of the 5-9 year old Indonesian children (Su-Arehawaratana *et al*,1992). Fortunately even at the elevated dose the vaccine was well tolerated. A study of the safety, immunogenicity, excretion and transmissibility of CVD103-HgR in pre-school age Indonesian children has since been performed. A single dose of 5 X 10^9 organisms induced a significant rise in vibriocidal antibody in 75% of vaccinees. Vaccine excretion was minimal as was secondary transmission. Furthermore there was no evidence of persistence of CVD103-HgR in the environment (Simanjuntak *et al*, 1993). In 1993 a large-scale randomised placebo-controlled field trial involving 68,000 Indonesians aged between 2 and 42 years was begun; surveillance will be maintained for a three year period.

A live attenuated El Tor vaccine strain is currently under developement. Obtaining a suitable strain lacking reactogenicity has proved difficult. A recent vaccine strain derived fron JBK70 lacks Zot and Ace toxins as well as the haemolysin/cytolysin. When tested in volunteers, CVD110 induced a strong immune response but produced diarrhoea in almost all recipients which precludes its use as a vaccine (Tacket *et al*, 1993).

The clinical data suggest CVD103-HgR affords greater protection against challenge with classical than El Tor strains (Levine et al, 1988b). For this reason, and since strains of El Tor biotype are the agents of the current cholera pandemic, attempts have been made to construct an El Tor analogue of CVD103-HgR. Obtaining a suitable strain lacking reactogenicity has proved difficult. A recent vaccine strain derived from JBK70 lacks Zot and Ace toxins as well as the haemolysin/cytolysin. When tested in volunteers, CVD110 induced a strong immune response but produced diarrhoea in almost all recipients which precludes its use as a vaccine (Tacket et al, 1993).

Taylor and co-workers (1994) have described the construction of the vaccine prototype Peru-14, derived from a Peruvian El Tor Inaba strain. Peru-14 was constructed by deletion of the *ctx* genetic element (encoding *ctxAB*, *zot*, *ace* and *cep*) as well as a site-specific toxin acquisition cassette (RS1). The deletion of the attRS1 site removes a hot spot for recombination, reducing the potential for Peru-14 to regain toxin genes. A deletion in *recA* was also made to further ensure that the vaccine strain will not reaquire virulence determinants. [This precaution may have been unnecessary as a recent study of the likelihood of CVD103-HgR reaquiring toxin genes suggests the probability of such an event is minimal (Kaper *et al*, 1994b)]. At doses less than 10^9, Peru-14 did not cause diarrhoea or other side effects but elicited a significant rise in vibriocidal antibody titre in all vaccinees. In an albeit small experimental challenge study, the protective efficacy of Peru-14 against challenge with a wild-type El Tor Inaba was 80% (Taylor *et al*, 1994).

Another prototype recombinant vaccine has recently been described. Häse and co-workers (1994) have created recombinant strains which secrete inactive cholera toxin analogs. It has previously been shown that the holotoxin is more immunogenic than the B-subunit alone (Pierce *et al*, 1983) and so it was hoped that the presence of inactive toxin analogs would

improve the efficacy and duration of protection (Häse *et al*, 1994). Site-directed mutagenesis of the A subunit was used to remove its ADP-ribosyltransferase activity and an inactivated zot gene was also introduced by homologous recombination. The recombinant strains were found to be avirulent in the infant rabbit model however they have yet to be tested in humans. The cholera toxin analogs alone have the potential to be used as oral or parenteral conjugate vaccines but nonetheless will be useful in studying the role of ADP-ribosyltransferase activity in the immunogenicity and adjuvant effect of cholera toxin (Häse *et al*, 1994).

In summary, live attenuated vaccines have the advantages of being cheaper to produce and of reliably inducing a significant immune response after a single dose. The latter would considerably expedite mass immunisation programmes. Residual diarrhoea and other side effects have precluded several candidate strains from use in humans although suitably non-reactogenic vaccine strains have now been constructed.

1.9.2.3 Attenuated *Salmonella typhi* expressing *V. cholerae* antigens

The attenuated *S. typhi* strain Ty21a has been successfully used as a safe, effective, oral typhoid vaccine which stimulates both humoral and cell-mediated immunity (Germanier, 1984). It has previously been shown to have the capacity to express protective antigens of *Shigella sonnei* and *entero-toxinogenic E. coli* (Formal *et al*, 1981; Yamamoto *et al*, 1985) and was therefore considered a potential carrier of *V. cholerae* antigens. It was hoped that this approach would produce a bivalent vaccine able to confer resistance to both cholera and typhoid.

A hybrid Ty21a/Inaba organism was constructed by introducing a plasmid encoding the genes for Inaba O-antigen synthesis into a rifampicin-resistant, thymine-dependent derivative of Ty21a. Genes complementing thymine auxotrophy were present on the plasmid to stabilise its

presence in the vaccine strain. The resultant strain EX645 stimulated significant antibody responses to both *S. typhi* and *V. cholerae* O-antigens in a small clinical trial (Forrest *et al*, 1989). When assessed for its efficacy against *V. cholerae* O1 challenge, EX645 proved to be disappointing in that widely varying immune responses were elicited among the eight volunteers. Of the eight vaccine recipients, only two (who displayed the best vibriocidal responses) were protected from subsequent cholera challenge. However the vaccine did diminish the severity of illness and reduce excretion of the challenge organisms (Tacket *et al*, 1990).

In an attempt to improve the consistency of anti-*V. cholerae* immune responses, EX645 was superceded by EX880. The latter is unable to synthesize *S. typhi* O-antigen and as a result *V. cholerae* O-antigen is always detectable on the cell surface, regardless of the culture conditions (Attridge *et al*, 1991). Although immunogenicity trials confirmed that EX880 was more immunogenic than EX645, the strain was not tested for protective efficacy.

1.10 Aims of this thesis

TCP has been found to be critical for the colonisation of classical biotype strains in the IMCM and in humans. The obvious potential of this pilus as a protective antigen led to the suggestion that it be included in cholera vaccine preparations to improve their protective efficacy (Taylor et al, 1987). At the time this work was commenced there were no data available to demonstrate that TCP are equally important in the pathogenesis of *V. cholerae* O1 El Tor. As the current cholera pandemic is due to El Tor strains it seemed necessary to resolve whether TCP was an essential colonisation factor and protective antigen of this biotype.

The major aim of Chapter 3 is to establish whether TCP are assembled by El Tor strains during growth *in vitro*. Chapter 4 aims to evaluate the *in vivo* significance of TCP by constructing *tcpA* mutants of several El Tor strains and comparing their *in vivo* behaviour with wild-type. In addition, studies in the IMCM focus upon the protective potential of antibodies to El Tor TCP.

The El Tor strain O17 fails to synthesize TcpA during *in vitro* culture, but does so when carrying a cosmid encoding the classical *tcp* operon. The aim of Chapter 5 is to determine whether the TCP produced by the O17 cosmid clones is solely cosmid-derived or whether the chromosomal *tcp* operon is also expressed. If the latter situation pertains, it will be of interest to identify the cosmid-encoded factor(s) capable of trans-activating expression of the O17 *tcpA* gene.

In summary, the major aim of this thesis is to determine the significance of TCP in the pathogenesis of *V. cholerae* O1 strains of El Tor biotype.

Chapter 2 Materials and Methods

2.1 Bacterial strains and plasmids

V. cholerae and *E.coli* strains used are listed in Tables 3.1 and 2.1 respectively. Plasmids are listed in Table 2.2.

2.2 Maintenance and growth conditions

Strains were stored lyophilized or as working stocks which were maintained at -70°C in 1% Bactopeptone (Difco) containing 30%(v/v) glycerol. Strains were streaked from glycerols onto nutrient agar (Oxoid) and incubated overnight at 37°C. Unless indicated, bacteria were cultured in nutrient broth (Oxoid) with aeration at 37°C.

To assess TcpA/TCP and cholera toxin production strains were cultured using a modification of the AKI-method described by Jonson *et al* (1991a). AKI medium (0.3% $NaHCO_3$, 0.5% NaCl, 1.5% Bactopeptone [Difco], 0.4% yeast extract [Difco]; Iwanaga and Yamamoto (1985) was prepared fresh for each experiment. A filter-sterilised solution of $NaHCO_3$ (7.5mg/ml) was prepared and added to the medium immediately prior to use. Unless otherwise indicated, 100ml flasks containing 5ml AKI broth cultures were inoculated with a single colony from a fresh overnight plate, gassed with 5%CO_2 for 30sec and incubated at 3O°C for 3.5-4hr without shaking; they were then incubated with vigorous shaking (250 oscillations/min; Orbital shaking water bath, Paton industries) at the same temperature for 17-19hr.

Terrific broth (Maniatis *et al*, 1982) consisted of 1.2% (w/v) tryptone (Difco), 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 0.017M KH_2PO_4 and 0.072M K_2HPO_4.

Table 2.1 *E. coli* strains used in this study.

Strain	Description	Reference/Source
DH5α	F⁻*supE*44 Δ*lacU*169 (ϕ80*lacZ* ΔM15) *hsdR*17 *recA*1*endA*1 *deoR gyrA*96 *thi*-1 *relA*1.	J. Pohlner[a]
S17-1	*pro hsdR* RP4-2-Tc::Mu Km::Tn7	U. Priefer[a]
S17-1λ*pir*	A lysogen of S17-1[*pro hsdR* RP4-2-Tc::Mu Km::Tn7] used for conjugal transfer of plasmids with R6K replicon	de Lorenzo *et al* (1990)
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu	U. Priefer[a]
SM10λ*pir*	A lysogen of SM10 [*thi thr leu tonA lacY supE supE recA*::RP4-2-Tc::Mu] used for conjugal transfer of plasmids with R6K replicon.	Kaniga *et al* (1991)
SY327λ*pir*	A lysogen of SY327 [Δ(*lac pro*) *argE rif nal A recA*56]used for maintenance of plasmids with R6K replicon.	J. B. Kaper[b]

[a] Max Planck-Institut fur Biologie, Tübingen, FRG.
[b] Center for Vaccine Development, University of Maryland, Baltimore, USA

Table 2.2 Plasmids used in this study

Plasmid	Description	Reference or source
pUC18/19	Ap^R	Vieira and Messing (1982)
pBluescript S/K	Ap^R	Stratagene
pGP1-2	Km^R	Tabor and Richardson (1985)
pJ225	Ap^R, Km^R	J. Hackett (unpublished)
pME305	Tc^R	Rella *et al* (1985)
pH1JI	Gm^R, Sp^R, Sm^R	Beringer *et al* (1985)
pACYC184	Tc^R, Cm^R	Chang and Cohen (1978)
pCACTUS	Temperature sensitive suicide vector contains *sacB* gene for positive selection of recombinants. Tc^R	C. Clark[a]
pCVD442	Suicide vector with Pir-dependent R6K replicon. Contains *sacB* gene for positive selection of recombinants. Ap^R	Donnenberg and Kaper (1991)
pPM3290	5kb *Xba1* fragment harbouring *tcpA* gene of H1 (El Tor) in pUC18. Ap^R	E. Voss (1990)
pPM2103	pHC79 harbouring ca.30kb DNA from H1 (El Tor) encompassing *tcp* operon. Ap^R	Sharma *et al* 1989a

[a] University of Adelaide, Adelaide, South Australia.

LB medium (Miller, 1972) consisted of 1% tryptone (Difco), 5% (w/v) yeast extract (Difco) and 10% (w/v) NaCl. The pH was adjusted to 6.5 prior to autoclaving. LB agar was prepared by the addition of 7.5% Bacto-agar (Difco) to LB medium. LB agar with 6 or 10% (w/v) sucrose (but no added NaCl) was prepared by the addition of an appropriate volume of a filter-sterilised solution of sucrose (50% w/v).

SOC medium consisted of 2% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose.

M9 minimal medium consisted of M9 salts (0.48M Na_2HPO_4, 22mM KH_2PO_4, 19mM NH_4Cl, 8.6mM NaCl), 0.4% (v/v) glycerol, pH 6.5.

When appropriate, antibiotics were added to media at the following final concentrations: ampicillin (Ap; Sigma) 100μg/ml; kanamycin (Km; Sigma) 50μg/ml; gentamycin (Gm; Sigma) 40 μg/ml; rifampicin (Rif; Sigma) 200μg/ml; tetracycline (Tc; Calbiochem, prepared in 50% ethanol) 10μg/ml for *E. coli* and 4μg/ml for *V. cholerae*; polymyxin B (Pmx, Sigma) 100U/ml; spectinomycin (Sp; Sigma) 80μg/ml; chloramphenicol (Cm; Calbiochem, prepared in 100% ethanol) 25μg/ml.

2.3 Animals

Swiss infant mice weighing between 2.4-2.7g were used for *in vivo* studies of *V. cholerae* O1 strains. Antisera were raised in adult New Zealand white or outbred rabbits.

2.4 Chemicals and reagents

All chemicals used were analytical grade. Ethanol, methanol, propan-2-ol, iso-amyl-alcohol, hydrochloric acid, glycerol, phenol, sodium dodecyl sulphate (SDS), sodium chloride,

ammonium acetate and sucrose were from BDH Chemicals. The four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), Trisma base (Tris) and glycine were purchased from Boehringer Mannheim. Caesium chloride (Cabot) was technical grade. Ethylene-diamine-tetra-acetic-acid, disodium salt (EDTA), calcium chloride, magnesium sulphate, magnesium chloride, potassium chloride, potassium di-hydrogen orthophosphate, di-potassium hydrogen orthophosphate, di-sodium hydrogen orthophosphate, sodium citrate, sodium hydrogen carbonate, sodium hydroxide, Triton X-100, chloroform and formaldehyde were from Ajax Chemicals. Triethanolamine, Tween20, polyvinylpyrollidone, cobalt chloride, dithiothreitol (DTT), Adenosine-5'-triphosphate (ATP), Herring sperm DNA and bovine serum albumin (fraction V) were obtained from Sigma. Sarkosyl was purchased from Ciba-Geigy. Ficoll was obtained from Pharmacia.

Digoxigenin (DIG) DNA labelling and detection kits were purchased from Boehringer-Mannheim.

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

Sterile, deionised and filtered water was used to prepare all buffers and reagents for DNA and RNA manipulation, otherwise reagents and buffers were prepared with deionised water.

2.5 Enzymes and immunoconjugates

DeoxyribonucleaseI was obtained from Bresatec, Lysozyme from Sigma and Pronase from Boehringer-Mannheim. All restriction endonucleases were purchased from either Boehringer-Mannheim, New England Biolabs, Pharmacia or Amersham and used according to the suppliers instructions.

Other DNA modifying enzymes were purchased from the following suppliers: Amersham (T4 DNA ligase, calf intestinal phosphatase) and Boehringer-Mannheim (DNA polymeraseI, Klenow fragment of DNA polymeraseI, molecular biology grade alkaline phosphatase and terminal transferase). Taq polymerase (Ampli Taq) was purchased from Perkin Elmer Cetus Corp.

Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Kirkegaard and Perry Laboratories Inc. Anti-digoxigenin-POD (Fab fragments) was from Boehringer Mannheim.

2.6 Preparation of *V. cholerae* cell fractions

The cell fractionation procedure was a modification of that described by Osborn *et al.* (1972). *V. cholerae* strains were grown using the AKI method as described in section 2.2. 20ml cultures were centrifuged in a Beckman JA20 rotor (10,000 rpm, 10 min, 4°C) and the pellets resuspended in 1ml of 20%(w/v) sucrose, 30mM Tris-HCl pH 8.1 and chilled on ice. Bacterial cells were converted to sphaeroplasts by the addition of 0.1ml of 1 mg/ml lysozyme in 0.1M EDTA pH 7.3 and incubated on ice for 30min; they were then centrifuged as above and the supernatant collected (periplasmic fraction). The cell pellet was frozen in an ethanol dry ice bath for 30min, thawed and dispersed vigorously in 3ml 3mM EDTA, pH 7.3; cells were then lysed by 60 x 1 sec bursts with a Branson Ultrasonifier. Unlysed cells and large cell debris were removed by low speed centrifugation (5,000rpm, 5min, 4°C). The supernatant containing the membranes and the cytoplasm was centrifuged at 35,000 rpm in a 50Ti rotor for 60min at 4°C in a Beckman L8-80 ultracentrifuge. The supernatant (cytoplasmic fraction) was collected and the membrane pellet resuspended in 1ml of 2% (v/v) Triton X-100, 25% sucrose, 10mM Tris-HCl pH 7.8, 1mM EDTA. After incubating the membrane fraction for 15min at

room temperature (RT) the insoluble (outer membrane) fraction was separated from the soluble (cytoplasmic membrane) fraction by centrifugation at 35,000rpm as described above. The triton-insoluble fraction (pellet) was resuspended in 1ml of 10mM Tris-HCl pH7.8, 1mM EDTA.

2.7 Isolation of unprocessed TcpA from *E. coli*

2.7.1 Construction of plasmids and *E. coli* clones for over-expression of *tcpA*

A 1.0kb *Dra*1 fragment spanning El Tor *tcpA* was subcloned from pPM3290 (Table 2.2) into the *Sma*I site of pBluescript-SK. The resultant plasmid pPM3352 contained the *tcpA* gene in the correct orientation for expression from the T7 promoter. Plasmid pPM3282 contains the classical *tcpA* gene in pBluescript-SK in the correct orientation for expression from the T7 promoter and was constructed in a similar manner to pPM3352 (C. Meaney, personal communication). pPM3352 and pGP1-2 (which encodes the T7 RNA polymerase) were sequentially transformed into the *E. coli* strain DH5α to produce the strain SA42. The *E. coli* strain DH1 was first transformed with pPM3282 and then with pGP1-2 to produce the strain E1838 (C. Meaney, personal communication).

2.7.2 Over-expression of *tcpA*

The over-expression of *tcpA* genes under the control of the T7 RNA polymerase promoter was performed using a modification of the method described by Tabor and Richardson (1985). E1838 and SA42 were streaked onto nutrient agar plates containing kanamycin (50μg/ml) and ampicillin (50μg/ml) and incubated at 30°C overnight. A single colony was used to inoculate 10ml of Terrific broth (Section 2.2) containing kanamycin and ampicillin and incubated overnight at 30°C with shaking. This culture was diluted 1 in 20 into fresh Terrific broth with

kanamycin and ampicillin and incubated at 30°C with shaking to an OD_{650} of ca. 0.6. The flasks were then shifted to 42°C with shaking for 30-45min to induce expression of the T7 RNA polymerase. Rifampicin (4mg/ml freshly prepared stock in methanol) was then added to a final concentration of 200µg/ml and the culture maintained at 42°C for a further 30min before being transferred to 37°C and incubated with shaking overnight.

2.7.3 Outer membrane preparations

The bacterial suspensions were centrifuged at 10,000rpm for 10min and the supernatants discarded. Pelleted cells were washed once with 50mM Tris, pH7.5, centrifuged again resuspended to ca. 10^{11}/ml in 10mM HEPES, pH7.5 and kept on ice prior to being passed through a French pressure cell (Amicon) six times at 50,000 Psi. Intact cells were subsequently removed from the suspensions by centrifugation at 7000rpm for 10min. To pellet the cell membranes the supernatants were centrifuged at 42,000rpm for 40min (Beckman L8-80 Ultra-centrifuge). Membrane pellets were extracted twice with 2% Triton X-100 in 10mM HEPES, pH7.5 for 15min at RT. To collect the outer membranes (Triton insoluble material) the suspensions were centrifuged at 35,000rpm for 60min. The resultant pellets were resuspended in 10mM Tris, pH7.5 and stored frozen at -20°C as 4mg/ml stocks of outer membrane preparations (OMPs).

2.8 *V. cholerae* "pili preparations"

This procedure is a modification of that described by Cowell *et al* (1987) for the purification of pili from *Bordetella*. A 100ml AKI broth culture was centrifuged at 10,000rpm for 10min. The cell pellet was washed once with PBS before being resuspended in 10ml PBS (0.14M NaCl, 27mM KCl, 1.5mM K_2HPO_4, 8mM Na_2HPO_4), 10mM EDTA. To shear pili from the

vibrios, the bacterial suspension was passed through a 22 gauge needle twice and then through a 25 gauge four times. Bacterial cells were pelleted by centrifugation at 7,500rpm for 7min and the supernatant retained while the cell pellet was resuspended as before and sheared again. The supernatants were pooled and centrifuged to remove remaining cells. Ethanolamine was added to a final concentration of 0.125M and the suspension allowed to stir slowly at 4°C for 16-18hr. To pellet the pili the suspension was centrifuged at 24,000rpm (Beckman L8-80 ultracentrifuge, ti50 rotor) for 20min. The pellet was resuspended in 0.5ml PBS. Ethanolamine was removed by dialysis against PBS. Total protein was determined using the BCA protein assay (Pierce) according to the manufacturer's protocol. The preparation was stored at -20°C as a ca. 1mg/ml stock.

2.9 Production of antisera against unprocessed and native TcpA

2.9.1 Preparation of antiserum to classical TCP

Anti-TCP serum was raised against a crude pili preparation of the classical x non-O1 hybrid strain 569B/165 (also known as V9) which was grown on CFA agar (25°C for 40hr; Sharma *et al*, 1989a). After scraping the culture from the plates and resuspending in PBS, pili were sheared from the vibrios as described in section 2.8. An antiserum was raised by sub-cutaneous (sc) immunisation of a rabbit with 100µg of pili preparation (emulsified in 1.0ml Freunds complete adjuvant and injected at four sites). Subsequently the animal received four similar doses (with the antigen emulsified in Freunds incomplete adjuvant) at 14-day intervals. Ten days after the final immunisation the rabbit was exsanguinated and the serum stored at -20°C. A working stock of the antiserum was kept at 4°C with 0.05% (w/v) azide. The antiserum was absorbed four times with live 017 and four times with live V779 (see Table 3.1; 5 X 10^{10} bacteria/ml per absorption). Alternating absorptions were incubated at 37°C for 4hr

or overnight at 4°C. Following each absorption the serum was clarified by centrifugation and after the final absorption passed through a 0.2μm millipore filter. This serum is hereafter referred to as anti-V9 TCP serum.

2.9.2 Preparation of antisera to unprocessed classical and El Tor TcpA

Antisera to unprocessed TcpA proteins of classical and El Tor biotype were raised by immunising rabbits with outer membrane preparations (OMPs) from *E. coli* clones SA42 and E1838 (Section 2.7). Rabbits were immunised using the same protocol as described for anti-V9 TCP. Aliquots of the resultant sera were absorbed eight times with OMP prepared from the *E. coli* vector strain (2mg/ml per absorption) alternating the absorptions between 4h at 37°C and overnight at 4°C. To make the sera biotype-specific the serum raised against classical TcpA was absorbed six times with O17 followed by six absorptions with SA42 OMP (2mg/ml). The serum prepared against El Tor TcpA was absorbed six times with 10^{10}/ml TCP-positive Z17561 followed by six absorptions with E1838 OMP (2mg/ml). After each absorption the sera were centrifuged to pellet the bacteria (4000g, Beckman JA-20 rotor) or OMP (100,000g, Beckman Ti-65 rotor).

2.9.3 Preparation of antisera to native El Tor TcpA

Antisera to processed El Tor TcpA were raised by immunising rabbits with "pili preparations" (Section 2.8) from the strains H1 or EV37 (Table 3.1), using the immunisation protocol described above. The resultant sera were made TcpA-specific by absorbing 18 times with cell envelope fractions (4mg/ml per absorption) prepared from the appropriate AKI-grown isogenic *tcpA*::Km^R mutant (Table 3.1). The absorptions alternated between 4h at 37°C and overnight

at 4°C. The sera were clarified after each absorption by centrifugation at 100,000g (Beckman Ti-65 rotor).

2.9.4 Isolation of the IgG fraction of antisera

The IgG fractions of an antisera were isolated using a modification of the procedure described by Steinbuch and Andran (1969). A 10ml aliquot of serum was diluted with 20ml of acetate buffer (0.06M, pH4.0) after which 7.5ml of octanoic acid (Density 0.91, BDH) was added dropwise with vigorous stirring. The mixture was left stirring vigorously for 60min at RT and then centrifuged using a Beckman JA20 rotor at 12,000rpm for 30min. The supernatant was retained and 0.4ml of 1M Tris-HCl, pH9.0 and 2.6gm ammonium sulphate was added per 10ml of supernatant. After stirring at 4°C for 1-2h the solution was again centrifuged as described above. The supernatant was decanted and the pellet resuspended to 25% of the original volume of serum with TESA (0.025M Tris-HCl pH8.0, 0.13mM EDTA, 0.133M NaCl, 0.05% NaN_3). The IgG fraction was then dialysed against TESA to remove residual ammonium sulphate and then stored at 4°C in the presence of 0.05% (w/v) sodium azide.

2.10 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western transfer

2.10.1 SDS-PAGE

For analysis by SDS-PAGE, bacterial suspensions (ca. 10^{11}/ml) were prepared by resuspending bacterial pellets in saline and then adding an equal volume of 2x sample buffer (0.25mM Tris-HCl pH 6.8, 2%(w/v) SDS, 10%(v/v) glycerol, 5%(v/v) β-mercaptoethanol,

15% (w/v) bromophenol blue) prior to loading. Samples were heated at 100°C for 3min before loading 10µl/well onto a 15% polyacrylamide gel.

SDS-PAGE was performed using a modification of the procedure described by Lugtenberg *et al* (1975). Samples were electrophoresed through the gel at 150V for 2-3hr. Gels were stained overnight with Coomassie Brilliant Blue G250 in 50% (v/v) methanol and 10% (v/v) acetic acid. To remove excess stain, the gel was washed with several changes of 7.5% (v/v) acetic acid, 10% (v/v) methanol and 10% (v/v) ethanol over 24h.

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

2.10.2 Western transfer and detection

Proteins were transferred to nitrocellulose (Schleicher and Schuell) at 200mA for 2hr in a trans-blot cell (Bio-Rad). The transfer buffer used consisted of 25mM Tris-HCl, pH 8.3, 192mM glycine and 20% (v/v) methanol (Towbin *et al*, 1979). The blot was incubated for 1hr in blotto (5% skim milk powder in TTBS [0.05% (v/v) Tween 20, 20mM Tris-HCl, pH 7.4, 0.9%(w/v) NaCl]) to block non-specific binding sites before incubating in anti-TCP serum (diluted in blotto) for 2-16hr at RT. Unbound antibody was removed by washing the filter (3x 10min) in TTBS before incubation with HRP-conjugated goat anti-rabbit IgG (diluted 1:40,000 in blotto) for 90min at RT. Prior to detection, the filter was washed (4x 5min) with TTBS and then (2x 5min) with TBS [20mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl].

Enhanced chemiluminescence (ECL) detection was used to visualize antigen-antibody complexes. ECL detection reagent was prepared according to the manufacturer's directions

(Amersham); the filter was incubated in the reagent for 1min, drained, covered with clear plastic and exposed to X-ray film (Kodak X-Omat) for 5min.

2.11 Electron microscopy (EM) and immuno EM (IEM)

For EM, bacterial suspensions (ca. 10^{10}/ml) were prepared and 40µl aliquots spotted onto Parafilm "M" laboratory film (American National Can). Colloidin-coated copper grids (300 mesh, TAAB) were placed coated side down on the bacterial suspensions for 5min. Excess suspension was removed by blotting the grid with Whatmann 3MM paper. The grid was then transferred onto a 40µl drop of 1% uranyl acetate for 10sec, blotted and stored in a dust free environment. For IEM, colloidin coated nickel grids (200 mesh, TAAB) were treated with poly-L-lysine (0.1mg/ml; Sigma) for 5min before being transferred to the bacterial suspension. The grids were blotted as described above and then successively transferred onto drops of the following solutions: 3% BSA-PBS for 3min, anti-TCP serum (diluted 1:50 in 3% BSA-PBS) for 15min, two saline washes, Protein-A-gold (Amersham; diluted 1:100 in distilled water) for 10min, two distilled water washes and 1.0% uranyl acetate for 1min. All grids were examined using a Phillips EM300 transmission electron microscope at an accelerating voltage of 80kV.

2.12 DNA extraction procedures

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

Genomic DNA from either *V. cholerae* or *E. coli* was prepared according to Manning *et al.* (1986). Cells from a 20 ml shaken overnight culture were pelleted in a bench centrifuge for 10 min and washed once with TES buffer (50mM Tris-HCl, pH 8.0, 5mM EDTA, 50mM NaCl). The pellet was resuspended in 2ml of 25% (w/v) sucrose, 50mM Tris-HCl, pH 8.0 and 1ml of lysozyme (10mg/ml in 0.25mM EDTA, pH 8.0) was added and the mixture incubated on ice

for 20min. 0.75ml TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0) and 0.25ml of lysis solution (5%(w/v) sarkosyl, 50mM Tris-HCl, pH 8.0, 0.25mM EDTA, pH 8.0) were added, together with 2mg solid pronase. The mixture was gently mixed and incubated at 56°C for 60min. This was followed by three extractions with TE-saturated phenol and two extractions with diethyl-ether. The genomic DNA was either precipitated with four volumes of 100% ethanol and resuspended in 1ml of TE buffer or transferred to a dialysis bag and dialysed against TE buffer for ca. 48hr.

2.12.2 Plasmid extraction procedures

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

Method 1: Triton X-100 cleared bacterial lysates were prepared from 10 ml overnight cultures by a modification of the procedure of Kahn *et al.* (1979). Cells were resuspended in 0.4 ml 25% (w/v) sucrose in 50mM Tris-HCl, pH 8.0 and transferred to reaction tubes (1.5ml). 0.5ml lysozyme (10 mg/ml, freshly prepared) and 0.5ml of 0.25M EDTA, pH 8.0 were added to the cells, which were incubated on ice for 15 min before the addition of 0.5 ml TET buffer (50mM Tris-HCl, 66mM EDTA, pH 8.0, 0.4% Triton X-100). Chromosomal DNA was pelleted by centrifugation (20 min, 4°C, Eppendorf) and discarded. The supernatant was extracted twice with TE-saturated phenol (pH 7.5) and twice with diethyl-ether. Plasmid DNA was precipitated by the addition of 0.6 volumes of propan-2-ol and allowed to stand at -70°C for 30 min. The precipitated DNA was collected by centrifugation (10 min, Heraeus Biofuge 15), washed once with 1 ml 70% (v/v) ethanol, dried *in vacuo* and resuspended in 50ml TE buffer.

Method 2: Large scale plasmid purification was performed by the three step alkali lysis method (Garger *et al.*, 1983). Cells from a one litre culture were harvested (6,000 rpm, 15

min, 4°C, GS-3, Sorvall) and resuspended in 24ml of solution 1 (50mM glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA). Freshly prepared lysozyme (4ml of 20 mg/ml in solution 1) was mixed with the cell suspension and incubated at RT for 10 min. Total cell lysis was achieved by the addition of 55ml of solution 2 (0.2M NaOH, 1% (w/v) SDS) followed by a 5 min incubation on ice. Following the addition of 28ml solution 3 (50mM potassium acetate, 11.5% glacial acetic acid) the mixture was incubated on ice for 15 min, and then centrifuged at 4°C, 8000 rpm for 20 min (GSA rotor, Sorvall). The supernatant was extracted with an equal volume of a (TE-saturated) phenol, chloroform, isoamyl alcohol mixture (25:24:1). Plasmid DNA from the aqueous phase was precipitated with 0.6 volumes propan-2-ol for 10 min at RT and collected by centrifugation (10,000 rpm at 4°C, 35 min, GSA, Sorvall). The DNA pellet was washed with 70% (v/v) ethanol, dried *in vacuo* and resuspended in 4.8ml TE. Plasmid DNA was further centrifuged through a two step CsCl-ethidium bromide gradient according to Garger *et al.* (1983). The DNA band was removed by side puncture of the tube with a 19 gauge needle attached to a 1 ml syringe. The ethidium bromide was extracted using isoamyl alcohol, while CsCl was removed by dialysis against TE at 4°C. DNA was stored at 4°C.

Method 3: Small scale plasmid purification was performed using a modification of the three step alkali lysis method of Garger *et al.* (1983). Overnight bacterial cultures (1.5ml) were transferred to a microfuge tube, harvested by centrifugation (3min, Heraeus Biofuge 15), and resuspended in 0.1ml of solution 1. The cells were lysed by the addition of 0.2ml of solution 2, followed by a 5min incubation on ice. After the addition 0.15ml of solution 3 and a further 5min incubation on ice, the mixture was briefly centrifuged (90sec, Heraeus Biofuge 15). The supernatant was transferred to a fresh tube and extracted once with TE-equilibrated phenol and once with diethyl ether. Plasmid DNA was precipitated by the addition of 0.6 volumes of propan-2-ol and a 10min incubation at RT. The DNA was collected by centrifugation (15min,

Heraeus Biofuge 15), washed with 70% (v/v) ethanol and dried *in vacuo*. The pellet was resuspended in 50µl of TE and stored at 4°C.

2.13 Analysis and manipulation of DNA

2.13.1 DNA quantitation

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

2.13.2 Restriction endonuclease digestion of DNA

Most cleavage reactions were done using the restriction enzyme buffer specified and supplied by the manufacturer of the enzyme. 0.1-0.5µg of DNA or purified restriction fragment was incubated with 2 units of each restriction enzyme in a final volume of 30µl, at 37°C, for 2hr. The reactions were terminated by heating at 65°C for 10 min. Prior to loading onto a gel, a one tenth volume of tracking dye (15% (w/v) Ficoll, 0.1% (w/v) bromophenol blue, 0.1 mg/ml RNase A) was added.

2.13.3 Analytical and preparative separation of restriction fragments

Electrophoresis of digested DNA was carried out at room temperature on horizontal, 0.6%, 0.8% or 1% (w/v) agarose gels (Seakem HGT). Gels were run at 100V in either TBE buffer (67mM Tris base, 22mM boric acid and 2mM EDTA, final pH 8.8), or TAE buffer (40mM Tris acetate and 2mM EDTA). After electrophoresis the gels were stained in distilled water containing 2 mg/ml ethidium bromide. DNA bands were visualized by trans-illumination with UV light and photographed using 665 negative film (Kodak).

The sizes of restriction enzyme fragments were calculated by comparing their relative mobility with that of *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA. The sizes used were: 8.37kb; 7.2kb; 6.05kb; 4.9 kb; 3.55 kb; 2.68 kb; 1.73 kb; 1.61 kb; 1.29 kb; 1.19 kb; 0.99 kb; 0.86 kb; 0.63 kb; 0.48 kb; 0.38 kb (Franzon and Manning, 1986).

To extract a DNA fragment from an agarose gel the required DNA band was excised and the gel slice placed inside dialysis tubing with ca. 700µl of sterile TAE. The gel slice was then positioned in an electrophoretic tank containing TAE buffer and electrophoresed for ca. 1-2h at 100V. The buffer was transferred from the dialysis bag to a sterile reaction tube (1.5ml) and the DNA precipitated with two volumes of ethanol and one tenth volume of 3M sodium acetate, pH 5.0 for ca. 2h at -20°C. The precipitated DNA was collected by centrifugation (15 min, Heraeus Biofuge 15), washed once with 1ml 70% (v/v) ethanol, dried *in vacuo* and dissolved in TE buffer.

Alternatively DNA was extracted from a gel slice using QIAEX DNA extraction kit (QIAGEN) according to the manufacturer's instructions.

2.13.4 Dephosphorylation of DNA using alkaline phosphatase

Restriction enzyme-digested DNA was treated with alkaline phosphatase by the following method. 0.1-0.5mg DNA was incubated with 1 unit of alkaline phosphatase (calf intestinal [CIP]), for 30 min at 37°C. The reaction was terminated by the addition of EDTA, pH 8.0 to a final concentration of 3mM followed by heating at 65°C for 10min. The reaction mix was then extracted with TE-saturated phenol and the DNA precipitated and collected as described in Section 2.13.3.

2.13.5 End-filling with Klenow fragment

Protruding ends created by cleavage with restriction endonucleases were end-filled using the Klenow fragment of *E. coli* DNA polymerase I. In a final volume of 20µl, 1mg of digested DNA or purified DNA fragments was mixed with 2µl 10 x nick-translation buffer (0.5M Tris-HCl pH7.2, 0.1M $MgSO_4$, 1mM DTT, 0.05% BSA), 1µl of each dNTP (2mM) and 1 unit of Klenow fragment. After incubation at 30min at 37°C, samples were extracted twice with an equal volume of phenol:chloroform (1:1) and the DNA precipitated and collected as described in Section 2.13.3.

2.13.6 *In vitro* cloning

DNA to be subcloned was combined with appropriately cleaved vector DNA at a ratio of insert to vector of ca. 3:1. Together with the DNA to be ligated the reaction mix contained in a total volume of 20µl, T4 DNA ligase (2U) and a final buffer concentration of 20mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM dithiothreitol (DTT), 0.6mM ATP for 16 hr at RT. The ligated DNA was then used directly for transformation of *E. coli* strains. If the ligated DNA was to be electroporated into *E. coli* strains, the DNA was precipitated as described in Section 2.13.3.

2.13.7 Dye-terminator sequencing

Plasmid DNA was purified on a Qiagen column prior to performing dye terminator sequencing. Kits for dye-terminator sequencing were purchased from Boehringer Mannheim. In a Gene Amp™ reaction tube (0.5ml, Perkin Elmer) 9.5µl of "Go" pre-mix (Boehringer Mannheim) was mixed with 1-2 µg template DNA and 3.2pmol primer; this was made up to a final volume of 20µl with sterile Milli-Q water and then overlaid with ca. 40µl of mineral oil (Nujol, Perkin

Elmer). The reaction mixture was subjected to 25 cycles of 96°C x 30sec, 50°C x 15sec, 60°C x 4min. Once the reaction cycles were completed, 80µl of sterile Milli-Q water was added to the reaction tube. The aqueous phase was transferred to a sterile microfuge tube and 100µl of phenol/chlorofrom/water (70:20:10) added. The tube was vortexed and the phases separated by centrifuging at 12,000 rpm for 1min (Heraeus Biofuge 15). The organic phase was discarded and the aqueous phase re-extracted twice more. DNA was precipitated at -20°C for 16-18hr by the addition of 10µl 3M Na Actetate pH 5.2 and 300µl ice-cold 100% ethanol, DNA was then collected by centrifuging for 30min at 15,000rpm at 4°C; the pellet was washed with 70% ethanol then dried before being resuspended in 4.5µl loading buffer (83% deionised formamide, 8.3mM EDTA pH8.0) and loaded onto a 6% polyacrylamide-8M urea gel. The gel was run on a DNA sequencer (Applied Biosystems 373A) and the data analysed by Analysis™ programme 1.2.1 (Applied Biosystems).

2.14 Bacterial transformation, electroporation and conjugation.

2.14.1 Bacterial transformation

Transformation was performed essentially according to the method described by Brown *et al* (1979). *E. coli* strains were made competent for transformation with plasmid DNA as follows: an overnight shaken culture (in NB) was diluted 1:20 into NB and incubated with shaking to 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 0.5 volume of ice-cold 100mM $MgCl_2$, centrifuged again and resuspended in 0.1 volume of ice-cold 100mM $CaCl_2$. After standing on ice for 60 min, competent cells (0.2ml) were mixed with DNA (1-2µg), left on ice for a further 30 min and then heat-shocked at 42°C for 90sec. 1ml NB was added to the transformation mixture which was then incubated at 37°C for 1-2 hr, before plating onto selection plates.

2.14.2 Preparation and electroporation of *E. coli* and *V. cholerae*

Electrocompetent DH5α cells were freshly prepared according to the Bio-Rad protocol. Briefly, 0.5ml of an overnight broth of DH5α was inoculated into 100ml Luria broth and incubated at 37°C with shaking until the cells reached an OD_{600} 0.5-0.8. The cells were transferred to sterile SS-34 tubes (Nalgene) and allowed to cool on ice for 30min before centrifugation at 10,000rpm for 7min at 2°C (JA-20 rotor, Beckman). The supernatant was discarded and the cells gently resuspended in 100ml of ice-cold, sterile, distilled water (dH_20). The cells were subjected to two more washes; the first in 50ml ice-cold dH_20 and then a final wash in 5ml ice-cold, sterile 10% glycerol. Finally, the cell pellet was resuspended to a tenth of the original culture volume with 10% glycerol and kept on ice.

Preparation of electrocompetent *V. cholerae* cells was essentially the same as that described for *E. coli* with the exception that the cells were first washed with an equal volume of ice-cold sterile 1mM $MgCl_2$, 1mM $CaCl_2$ solution followed by a wash with a sterile, ice-cold 0.5mM $MgCl_2$, 0.5mM $CaCl_2$ solution. *V. cholerae* cells were finally resuspended in 1ml of the latter solution and kept on ice.

In a sterile microfuge tube on ice, ca. 1-2µl PCR product or plasmid DNA (in TE or sterile Milli-Q water) were mixed with 40µl of electrocompetent *E. coli* or *V. cholerae* and then transferred to an ice-cold sterile *E. coli* Pulser™ cuvette (0.2cm electrode gap, Bio-Rad). The gene pulser (Bio-Rad) was set at 25µF and the pulse controller at 200Ω. The *E. coli* and *V. cholerae* cells were pulsed at 2.5kV and 2.0kV respectively with time-constants of 4.6-4.7msec. Immediately after electroporation, 1ml of SOC medium (section 2.2) was added to the cuvette, the contents mixed and transferred to a sterile microfuge tube, and then incubated at 37°C for 60-90min. After centrifugation at 15,000 rpm for 1-2min, the supernatant was

discarded and the cells gently resuspended in 400µl of sterile saline and plated onto NA containing appropriate antibiotic.

2.14.3 Bacterial conjugation

Overnight broth cultures grown in NB or LB were diluted 1:20 and grown to early exponential phase. Donor and recipient bacteria were mixed at a ratio of 1:10 and the cells pelleted by centrifugation (5000 rpm, 5 min, bench centrifuge). The pellet was gently resuspended in 200ml of broth and spread onto a cellulose acetate membrane filter (0.45mm, type HA, Millipore Corp.) on a NA plate. This plate was incubated for 4hr at 37°C, when the bacteria on the filter were collected by vortexing the filter in 10 ml NB. After removing the filter the cell suspension was centrifuged as described above, the cell pellet resuspended in 400µl of sterile saline, and samples plated onto selective agar.

2.15 Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides (oligos) were synthesized on an Applied Biosystems 381A DNA synthesizer in the trityl-off mode and butanol extracted prior to use. Reagents were purchased from Applied Biosystems or Ajax Chemicals.

2.16 Polymerase chain reaction (PCR) and recombination PCR (RPCR).

2.16.1 PCR

The protocol used for PCR is that described by Delidow (1993) for the generation of PCR products with cohesive ends. The PCR reaction was performed in GENE AMP™ reaction tubes (0.5ml, Perkin Elmer) in a 100µl volume containing Taq buffer (50mM KCl; 10mM Tris-HCl pH8.3, 1.1mM $MgCl_2$, 0.01% (w/v) gelatin), 200µM each deoxynucleoside triphosphate

(dNTP), 100pmol each restriction site-tagged primer, 200ng of plasmid template or genomic DNA and 2.5U Taq polymerase (Perkin Elmer). The reaction was overlaid with a drop of light mineral oil (Nujol, Perkin Elmer) and following an initial denaturation period of 3min at 95°C, was subjected to 20 cycles of amplification (94°C x 1min, 55°C x 2min, 72°C x 1min) using a DNA thermal cycler (Perkin Elmer). Following PCR, 200µl of chloroform was added to the reaction tubes to cause the mineral oil to fall to the bottom of the tube. After briefly centrifuging to ensure the phases have separated, the upper phase was transferred to a clean microfuge tube. DNA was precipitated by the addition of 0.5 volumes of 7.5M ammonium acetate plus 2.5 volumes of 95% ethanol and incubated at RT for 10min. The DNA was collected by centrifuging at 12000 rpm for 15min (Heraeus Biofuge 15), washed with 70% ethanol, dried *in vacuo* and resuspended in 20µl of sterile water.

2.16.2 RPCR

To create an in-frame deletion in the H1 *tcpA* gene the technique of RPCR was employed. The protocol used was that described by Jones and Howard (1991). Plasmid DNA was collected and purified using a QIAGEN mini-column Kit according to the manufacturer's protocol. The PCR reaction was performed in GENE AMP™ reaction tubes (O.5ml, Perkin Elmer) in a volume of 50µl containing Taq buffer, 200µM each dNTP, 25pmol each primer, 2ng of template and 1.25U Taq polymerase (Perkin Elmer). The reaction samples were overlaid with 1-2 drops of light mineral oil (Nujol, Perkin Elmer) and following initial denaturation at 94°C for 1min, were subjected to 25 cycles of amplification (94°C x 30sec, 50°C x 30sec and 72°C x 1min/kilobase of PCR product) using a DNA thermal cycler (Perkin Elmer). Following PCR, 200µl of chloroform was added to the reaction tubes to cause the mineral oil to fall to the bottom of the tube. After briefly centrifuging to ensure the phases

were separated, the upper phase was transferred to a clean microfuge tube. DNA was then collected as described.

2.17 Preparation of DNA probes and oligonucleotide probes

DNA fragment probes and oligonucleotide probes were labelled with digoxigenin-11-dUTP (DIG-11-dUTP; Boehringer Mannheim) according to the manufacturer's protocol. DNA fragment probes were labelled using random-primed labelling. DNA to be labelled (10ng-3µg) was denatured at 95°C for 10min, then chilled on ice for 3min before the addition of 2µl Hexanucleotide mix, 2µl dNTP labelling mix and 2U Klenow fragment. The reaction volume was made up to 20µl with sterile Milli-Q water, and incubated at 37°C for 20hr. The reaction was stopped by the addition of 2µl of 0.2M EDTA, pH 8.0. DNA was precipitated by the addition of 2.5µl 4M LiCl and 75µl of pre-chilled 100% ethanol. After a minimum of 2hr at -20°C, DNA was collected by centrifugation at 12,000g for 10min. The DNA pellet was washed with 50µl 70% ethanol (-20°C), dried *in vacuo* and resuspended in 20µl 10mM Tris-HCl, 1mM EDTA, pH8.0.

Oligonucleotide probes were labelled using 3'-end labelling. In a reaction volume of 25µl, ca.200ng of oligonucleotide was mixed with 2.5µl 10x tailing buffer (1.4M potassium cacodylate, 300mM Tris, pH7.2, 1mM DTT), 2.5µl DIG-11-dUTP, 1µl terminal transferase and 1µl 400mM $CoCl_2$. The reaction was incubated at 37°C for 60min. All probes were stored at -20°C until required.

2.18 Southern transfer and hybridisation

Chromosomal DNA was transferred onto nitrocellulose (Schleicher and Schuell) as described by Maniatis *et al* (1982). Pre-hybridisation buffer for DNA fragment probes consisted of 5x SSC (0.15M NaCl, 0.015M sodium citrate), 50mM sodium phosphate buffer pH6.4, 50% formamide, 5X Denhardt's reagent (0.1% Ficoll, 0.1% polyvinylpyrollidone, 0.1% Fraction V BSA) and 0.1mg/ml herring sperm DNA. Pre-hybridisation buffer for oligonucleotide probes consisted of 1M NaCl, 0.1M Tris-HCl pH7.6, 5x Denhardt's reagent, 0.05% SDS, 0.025M EDTA and 0.1mg/ml herring sperm DNA. Following hybridisation with oligonucleotide probes, stringency washes (3x 10min) were performed at 5°C below the melting temperature (Tm) of the oligonucleotide with 5x SSC, 0.1% SDS. Filters hybridised with fragment probes were washed (2x 5min) with 2x SSC, 0.1% SDS at RT, then with (2x 15min) 0.2x SSC, 0.1% SDS at 65°C. Filters were incubated in blocking reagent (5% Skim milk in Buffer 1 [0.1MTris-HCl, 0.15M NaCl pH7.5]) for 1hr before being incubated with anti-digoxigenin-POD Fab fragments (1/10K dilution in Buffer 1, Boehringer Mannheim) for at least 30min. Unbound antibody-conjugate was removed by washing the filters (4x 5min) in Buffer 1 and then PBS (2x 5min). Detection of target DNA was performed using ECL (Amersham or Boehringer Mannheim) as described previously for Western blot detection.

2.19 DNA colony blot and hybridisation

This procedure is a modification of the method described by Grunstein and Hagness (1975). Bacterial colonies were transferred onto a nitrocellulose filter (Schleicher and Schuell) either by overlaying the colonies after overnight incubation or by placing the filter over the agar prior to overnight incubation. The filter was gently lifted off the plate and transferred colony-side up Whatmann 3mm paper soaked with lysis solution (10% SDS). After 5min at RT, the filter was

transferred to 3mm paper soaked with denaturation solution (0.5M NaOH, 1.5M NaCl) for a further 5min. After transfer to 3mm paper soaked with neutralisation solution (1.5M NaCl, 0.5M Tris-HCl pH 8.0) for 5min, the filter was air-dried before being baked at 80°C *in vacuo* for 60-90min. Cell debris was removed from the filter by placing it in 0.1% SDS and gently scrubbing the surface. Prehybridisation, hybridisation and detection were performed as described above for Southern transfers.

2.20 Preparation of RNA

Total cellular RNA was collected using the hot-phenol method described by Aiba *et al* (1981). 5ml AKI broth cultures were centrifuged at 7000rpm for 10min to pellet bacteria. The cell pellet was resuspended in 0.5ml lysis buffer (20mM NaOAc, 1mM EDTA, 0.5% SDS, pH5.5) and immediately transferred to a microfuge tube containing 0.5ml phenol (equilibrated against 20mM NaOAc, 1mM EDTA, pH5.5) at 65°C. After vortexing to mix the phases and incubating at 65°C for 5min, the microfuge tube was centrifuged to separate the phases. The aqueous phase was then re-extracted three times with hot phenol. RNA was precipitated by the addition of 2.5 volumes of 100% ethanol at (-20°C) and then resuspended in 50µl 20mM Tris, 5mM $MgCl_2$ pH 7.6. To remove any remaining DNA the samples were treated with DNase I at 37°C for 20min, re-precipitated, resuspended in 50µl RNase-free water and stored at -70°C.

2.21 RNA analysis

2.21.1 RNA quantitation

The concentration of RNA in solutions was determined by measurement of absorption at 260nm, assuming an A_{260} of 1.0 is equal to 40mg RNA/ml (Miller, 1972).

2.21.2 RNA slot blot and hybridisation

RNA samples were denatured by treatment with formaldehdye (200µl sample: 600µl 50% (v/v) formaldehyde in 10x SSC for 10min at 65°C). DNA controls were treated with NaOH (final conc. 0.3M; 65°C for 20min) then neutralised by adding an equal volume of 20x SSC. Samples (200µl per well) were applied to nitrocellulose (pre-soaked in 10x SSC) using a slot blot apparatus (Hoefer Scientific, CA). The filter was then baked at 80°C for 60min before being placed in pre-hybridisation buffer for DNA fragment probes (section 2.18) for 2h at 42°C followed by overnight hybridisation with the probe of interest. RNA slot blots were subsequently treated in an identical manner to that described for Southern blots (2.18).

2.22 *In vitro* characterisation of mutant strains

2.22.1 Growth rate

The growth rates of *tcpA* mutants were compared with those of the corresponding wild-type strains by performing *in vitro* competition experiments in both nutrient and M9-minimal media. Mixed inocula of ca. 5x 10^3 of each strain were prepared by dilution of overnight or early log-phase cultures. The mixed cultures were incubated at 37°C with shaking for 7-8h (ca.10^8 bacteria/ml). To prevent the NB cultures from reaching stationary phase during this period, they were sub-cultured after ca.4hr. The initial ratio of the two strains in the mixed culture was determined by plating dilutions of culture onto NA and selective media; this ratio was compared with that present in the culture after 7-8hr incubation at 37 °C.

2.22.2 Motility

Motility was assessed using the sloppy-agar overlay method (Attridge and Rowley, 1983a). Cultures were grown in nutrient broth to an OD_{650} of ca. 1.0 then diluted and spread onto nutrient agar plates to give between 100-200 bacteria per plate. After overnight incubation at 37°C, the resulting colonies were overlaid with sloppy agar (1 part nutrient agar to 2 parts nutrient broth at 45°C; 10ml per plate). When the overlay had set the plates were incubated at 37°C for 30min. The percentage of colonies surrounded by a halo - indicating the presence of motile organisms - was then noted.

2.22.3 Mannose-sensitive haemagglutination

Haemagglutination assays were performed using chicken erythrocytes and in the presence or absence of mannose, as described by Svennerholm *et al* (1991). Strains were cultured in trypticase soy broth (Difco) without glucose at 37°C for 4-6hrs (ca. 3-5x 10^9 bacteria/ml). Cultures were centrifuged and the bacterial pellet resuspended in modified Krebs Ringer Tris solution (KRT; NaCl 7.5g/L, KCl 383mg/L, $MgSO_4.7H_20$ 318mg/L, $CaCl_2.2H_20$, pH 7.4) to approximately 10^{10}/ml. Two-fold serial dilutions (in KRT, 25µl/well) were prepared for each bacterial suspension in one row of a plastic microtitre tray (Costar); 25µl of erythrocyte suspension (1% (v/v) in KRT) was added to each well and the tray incubated at RT for 30-60min. For haemagglutination inhibition assays, 50µl of KRT solution containing 0.1% D-mannose was added to each well after serially diluting the bacterial suspensions. The trays were incubated at RT for 15min before adding 25µl/well of erythrocyte suspension and incubating at RT for 30-60min.

2.22.4 Haemolysin production

Haemolytic activity was assayed using a modification of a procedure described by Alm *et al* (1990a). Strains were cultured in nutrient broth to an OD_{650} of ca. 1.0. After centrifugation 0.5ml of culture supernatant was mixed with an equal volume of 5% sheep red blood cells (in PBS) and incubated at 37°C. 200µl aliquots were collected every 30min for a period of 4hrs; bacterial cells were removed by centrifugation and the amount of haemoglobin released was measured at A_{414}.

2.22.5 Cholera toxin production

Strains were grown in CO_2-gassed AKI cultures as described in Section 2.1 and the levels of cholera toxin present in the supernatant estimated using a modification of the original procedure described by Holmgren (1973). Microtitre trays (96 well; Costar or Nunc polysorp) were coated with 2µg/ml monosialoganglioside-GM1 (Sigma) diluted in PBS. Following overnight incubation at 4°C trays were washed 4 times with PBS containing 0.05% Tween (PBS-Tween). Non-specific binding sites were blocked by incubating for 1hr with BSA-Tween [20%(w/v) bovine serum albumin, 0.05% Tween 20, 0.0125M triethanolamine, 0.14M NaCl]. Replicate serial dilutions of a standard cholera toxin preparation (4µg/ml; Sigma) and appropriate dilutions of culture supernatants were prepared across rows of the trays which were then incubated for 2hr at 37°C. The trays were washed 5 times with PBS-Tween and then incubated with the IgG fraction of a polyclonal anti-cholera toxin serum (diluted 1:70,000). Following 4 washes with PBS-Tween to remove unbound antibody the trays were incubated overnight at 4°C with sheep anti-rabbit IgG conjugated with alkaline phosphatase (diluted 1:1000) in enzyme diluent [0.14M NaCl, 0.25mM triethanolamine, 0.002% (w/v)

BSA, 0.5mM $MgCl_2$ and 1.25μM $ZnCl_2$]. Unbound conjugate was removed by washing 5 times with BSA-Tween before adding substrate [1mg/ml pNitrophenylphosphate, di-sodium (Sigma) in 10.5% diethanolamine, 1mM $MgCl_2$, pH9.8] and incubating at 37°C for 90min. The OD_{405} was measured using a Titertek Multiscan ELISA tray reader, and the cholera toxin concentration in each sample estimated by interpolation from the standard curve.

2.23 *In vivo* studies in the infant mouse cholera model

2.23.1 LD_{50} studies

The virulence of a given strain was assessed by determining the 48hr LD_{50} value (Attridge and Rowley, 1983b) - that is, the number of organisms capable of killing 50% of the infant mice within 48hr. Serial dilutions of the strain of interest are made in peptone-saline (0.1% proteose-peptone (Difco) in saline) and each dilution is then orally administered to groups of 5-6 mice (100μl dose per mouse, delivered via a smooth tipped hypodermic needle). The mice are then kept at 25°C for 48hr after which time the number of survivors in each group is noted. Using these data, a plot of cumulative percentage mortality versus $\log_{10}$ challenge dose (Reed and Muench, 1938) is then plotted and the 48hr LD_{50} dose determined by interpolation.

2.23.2 Competition experiments

Competition experiments were performed by feeding the infant mice a mixed inoculum comprising approximately equal numbers of parent and mutant organisms; the dose of each strain equal to ca. $10LD_{50}$ of the parent strain (Attridge, 1979). The mice were maintained at 25°C for 22-24hr before being killed and the small intestine removed; this was then homogenised in 2ml saline using a homogeniser (Ultraturrax). To determine the ratio of parent and mutant organisms present at the time of sacrifice, dilutions of each gut homogenate were

spread onto NA and selective agar. From the number of colonies present on these plates the ratio of parent to mutant was calculated.

2.23.3 Passive immunisation assays

The protective titres of antisera are expressed as PD_{50} values and were determined essentially as described by Attridge and Rowley (1983b) with the exception that the challenge organisms were centrifuged and suspended in fresh culture medium. The PD_{50} represents the (theoretical) dilution of serum capable of protecting 50% of the mice challenged. Aliquots of vibrio suspensions were mixed with various dilutions of the test anti-TcpA reagent and incubated at 37°C for 15min, before being orally administered to infant mice. Groups of 5-6 infant mice were each fed a 100µl inoculum containing 10-20LD_{50} of the *V. cholerae* strain mixed with antibody; a control group received an inoculum containing only bacteria. The mice were maintained at 25°C until the last control mouse died (usually after 42-48hrs). The PD_{50} value is estimated by interpolation from a plot of cumulative percentage mortality vs. serum dilution (Reed and Muench, 1938).

Chapter 3 *In vitro* production of TcpA/TCP by *V. cholerae* O1 El Tor

3.1 Introduction

The toxin co-regulated pilus (TCP) was initially found on a strain of classical biotype (Taylor *et al*, 1987) and most classical strains produce TCP in a number of media providing the incubation temperature is 30°C. In 1988, Taylor *et al* suggested TCP be included in cholera vaccine preparations to improve their efficacy as their work and that of Herrington *et al* (1988) had demonstrated TCP was essential for colonisation of infant mice and humans. Subsequently antibodies to (classical) TCP were found to be protective in the infant mouse cholera model (Sharma *et al*, 1989a and b).

At the time our studies were commenced (1991), the world was still experiencing the seventh cholera pandemic - the first to be caused by *V. cholerae* El Tor. TCP assembly by El Tor strains had not been demonstrated but it was known that at least some El Tor strains possessed DNA with homology to the classical TCP operon (Taylor *et al*, 1988). Soon after, Shaw and co-workers (1989) demonstrated *in vitro* TcpA production by an El Tor strain. The *tcpA* gene from this strain was cloned and sequenced, revealing a protein of similar molecular weight and approximately 80% homologous to classical TcpA. Nevertheless, other studies failed to demonstrate polymerized TCP on the surface of El Tor vibrios under conditions conducive to TCP production by strains of classical biotype (Hall *et al*, 1988; Sharma *et al*, 1989a). Evidently the conditions required for El Tor *tcpA* expression are more stringent than those suitable for classical *V. cholerae*. Subsequently Jonson and coworkers (1990) reported some success in this respect, using a modification of a culture method originally developed by

Iwanaga and Yamamoto (1985) to maximally stimulate cholera toxin production by El Tor strains. Iwanaga's AKI-SW method involves switching the AKI broth culture from stationary to shaking incubation at the end of the exponential growth phase. The initial period of growth in poorly aerobic conditions was found to be essential for optimum cholera toxin production. Jonson's modification was to change the incubation temperature to 30°C (see section 2.2). The AKI-SW/ 30°C method induced TcpA production in many El Tor strains. Furthermore, when rabbits were inoculated with El Tor organisms grown under conditions unfavourable to *tcpA* expression, bacteria recovered following *in vivo* growth were positive for TcpA by Western blot analysis (Jonson *et al*, 1990). The results suggested that El Tor TcpA production may be better *in vivo* than *in vitro*, although no attempt was made to determine if surface assembly of TcpA into pili occurred.

Using an antiserum raised against purified classical TCP, Sun and associates (1990a) were able to demonstrate passive protection of infant mice against challenge with an El Tor strain grown under *tcpA* inducing conditions. However protection was assessed using the antiserum at only a 1:2 dilution. Their claim that this serum reacted only with TcpA was based on an IEM study of the classical strain O395 in which the antiserum was diluted 1:100, leaving the possibility that antibodies to contaminants present in the immunising preparation may have contributed to the protective activity of the serum. Furthermore, using an *in vitro* inhibition of adherence assay, the antiserum was much less effective at blocking the binding of TcpA-positive El Tor vibrios than TCP-positive classical vibrios to immobilised epithelial cells. Sun *et al* (1990a) concluded that El Tor strains produce less TCP than classical *V. cholerae* and possibly produce other factors which promote adherence. This confirmed doubts about the likely benefits of supplementing vaccine formulations with (classical) TCP, given that the current pandemic is caused by El Tor *V. cholerae*.

In the absence of any conclusive proof of El Tor TCP assembly or of the protective efficacy of antibodies to TCP against El Tor infection, it seemed important to establish the role of TCP in the pathogenesis of El Tor biotype strains. Preliminary Southern hybridisation studies had revealed differences between the carboxy-terminal portions of the classical and El Tor *tcpA* genes (Voss, 1990). This is the region which shows least homology between the Type 4 pilins (Shaw and Taylor, 1990) and which probably encodes the functional or adhesive domains of the protein (Irvin *et al*, 1989). Sun *et al* (1991) generated a panel of monoclonal antibodies against classical TCP and demonstrated that the protective epitopes of TcpA are in the carboxy-terminal portion of the pilin (Sun *et al*, 1990b). Monoclonal antibodies (MAbs) to the protective epitopes of classical TcpA failed to provide significant protection against challenge with El Tor strains in the infant mouse model. This raised the possibility that the two TcpA pilins are sufficiently antigenically dissimilar to make antibodies to classical TcpA/TCP inefficient at detecting El Tor TcpA/TCP. If in addition El Tor vibrios express less surface TcpA, detection of any polymerisation of TcpA into TCP might only be possible using a serum specific for the El Tor protein.

This chapter describes the results of *in vitro* studies of El Tor TcpA/TCP production, in which antisera raised against either classical or El Tor TcpA are used in immunoblotting and IEM analyses.

3.2 The effect of CO_2 on TcpA expression in classical and El Tor *V. cholerae*.

Several media will support TcpA/TCP production by classical *V. cholerae* but the same is not true for El Tor strains. Prior to commencing the work for this thesis, TcpA production by strains of El Tor and classical biotype was assessed following growth in a variety of media and

culture conditions. These included CFA agar, 25°C/40hr; DSM agar, 30°C /24hr; AKI agar, 30°C/5%CO_2/24hr and AKI broth, 30°C/ 4hr standing, 16-18hr shaking. Of these only the AKI broth method supported TcpA production by El Tor strains (Voss, 1990), confirming the findings of Jonson *et al* (1990). We became interested in the possible enhancement of TcpA production by adding CO_2 to the culture environment as there was some indication that CO_2 affected TcpA production (Peterson, cited by Sun *et al* 1990a). In addition, Shimamura *et al* (1985) had reported that cholera toxin production - which would be expected to reflect TCP production (Taylor *et al*, 1987) - was increased by the presence of CO_2.

Two El Tor strains (H1 and N16961) and two classical strains (11441 and 11966) were selected for this study. A bacterial suspension of each strain was prepared by inoculating a single colony into 10ml AKI; this was dispensed into two flasks, one of which was gassed with 5% CO_2. Both flasks were then incubated at 30°C for 3.5hr standing followed by 18hr shaking. A dramatic effect on TcpA production was found; Western blotting revealed marked increases in TcpA synthesis when the strains were cultured in the presence of CO_2 (Figure 3.1).

A less dramatic effect on cholera toxin expression was observed. Supernatants of gassed and ungassed cultures were assayed for toxin content by GM1-ELISA and up to 3-fold higher concentrations of toxin were present in gassed cultures (data not shown). Based on these results, all AKI broth cultures were subsequently gassed with 5% CO_2.

3.3 Assessment of El Tor TcpA/TCP expression using a polyclonal antiserum raised against classical TCP.

3.3.1. Production of antiserum to classical TCP.

A rabbit polyclonal antiserum was raised against a crude TCP preparation isolated from the (classical x non-O1) hybrid strain, 569B/165, also known and hereafter referred to as V9. This

Figure 3.1 The effect of CO_2 on TcpA expression by strains of El Tor and classical biotype during growth in AKI medium.

Samples: **1**, E1838 OMP

2, H1 (El Tor) +CO_2

3, H1 -CO_2

4, N16961 (El Tor) +CO_2

5, N16961 -CO_2

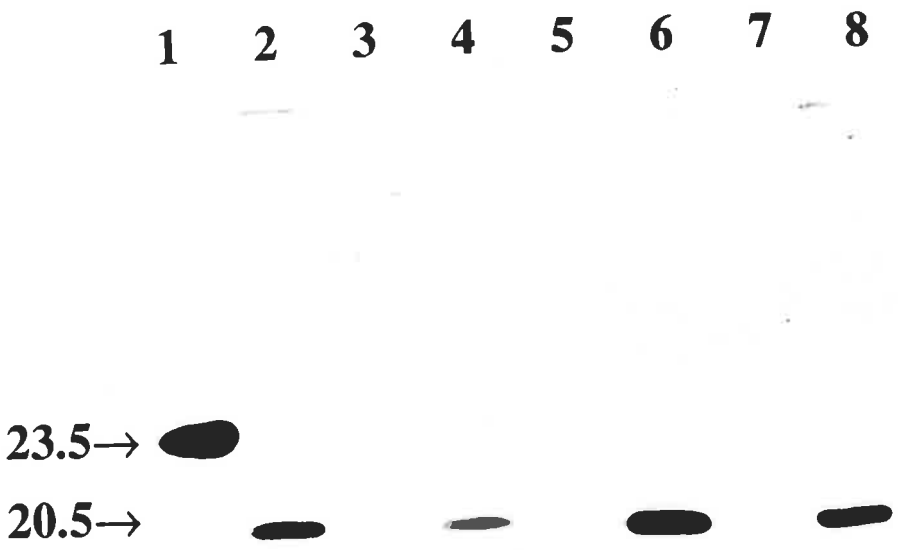
6, 11441 (classical) +CO_2

7, 11441 -CO_2

8, 11966 (classical) +CO_2

9, 11966 -CO_2

All tracks were loaded with ca. 10^9 bacteria. The blot was detected using anti-V9 TCP serum (diluted 1:1500). Arrows on the left indicate the position of unprocessed TcpA (23kDa) and processed TcpA (20.5kDa).


1 2 3 4 5 6 7 8 9
23.5→
20.5→

strain was chosen because of its ability to produce large amounts of TCP. Furthermore antibodies to V9 LPS do not react with *V. cholerae* O1 LPS, eliminating any concern that contaminating anti-LPS antibodies might interfere with the interpretation of results. The method used to prepare the crude pili fraction used for immunisation is described in section 2.8 and the immunisation protocol is described in section 2.9.1. The resultant antiserum, anti-V9 TCP, was extensively absorbed to be TcpA-specific by absorbing several times each with an isogenic *tcpA* mutant of V9, the El Tor strain O17 and an *E. coli* strain. Specificity for TcpA was confirmed by immunoblotting: Western blots revealed reactivity against TcpA- expressing strains of both classical and El Tor biotype but not against isogenic *tcpA* mutants (Figure 3.2).

3.3.2. Strain Survey of El Tor strains using anti-V9 TCP.

Twenty one El Tor strains isolated at various times and from various locations were assessed for TcpA production by Western blotting and for assembly of TCP by IEM (Table 3.1). A range of isolates was selected to determine whether the ability to produce TcpA/TCP related to the time and/or place of isolation. Four classical strains (also listed in Table 3.1) were included for comparison. The strains were grown using AKI broth at 30°C with 5% CO_2 atmosphere (3-4hr standing followed by 16-18hr shaking).

Using the anti-V9 TCP reagent Western blot analysis showed that the four classical strains produced TcpA and IEM examination revealed large TCP bundles abundantly labelled with gold particles (data not shown). The AKI broth method resulted in TcpA production by some, but not all, of the El Tor strains examined; of the 21 El Tor strains surveyed, Western blotting showed only seven strains produced detectable amounts of this protein (Figure 3.3). TcpA expression did not appear to relate to the time or place of isolation. Culture supernatants from the strains surveyed for TcpA production were assayed for cholera toxin production by GM_1-

Figure 3.2 Immunoblots demonstrating the specificity of the antisera to TCP or TcpA.

Samples: **1**, SA42 OMP (El Tor)

2, E1838 OMP (classical)

3, V781 (Z17561 *tcpA* isogenic mutant)

4, Z17561 (classical)

5, EV23 (H1 *tcpA* isogenic mutant)

6, H1 (El Tor).

All tracks were loaded with ca. 10^9 bacteria (harvested from AKI cultures incubated in the presence of CO_2) with the exception of the OMP tracks which were loaded with 30μg of protein. Blot A was detected using anti-V9 TCP serum (diluted 1:1500), blot B was detected using the anti-E1838 OMP (diluted 1:1500) and blot C was detected using anti-SA42 OMP (diluted 1:500).

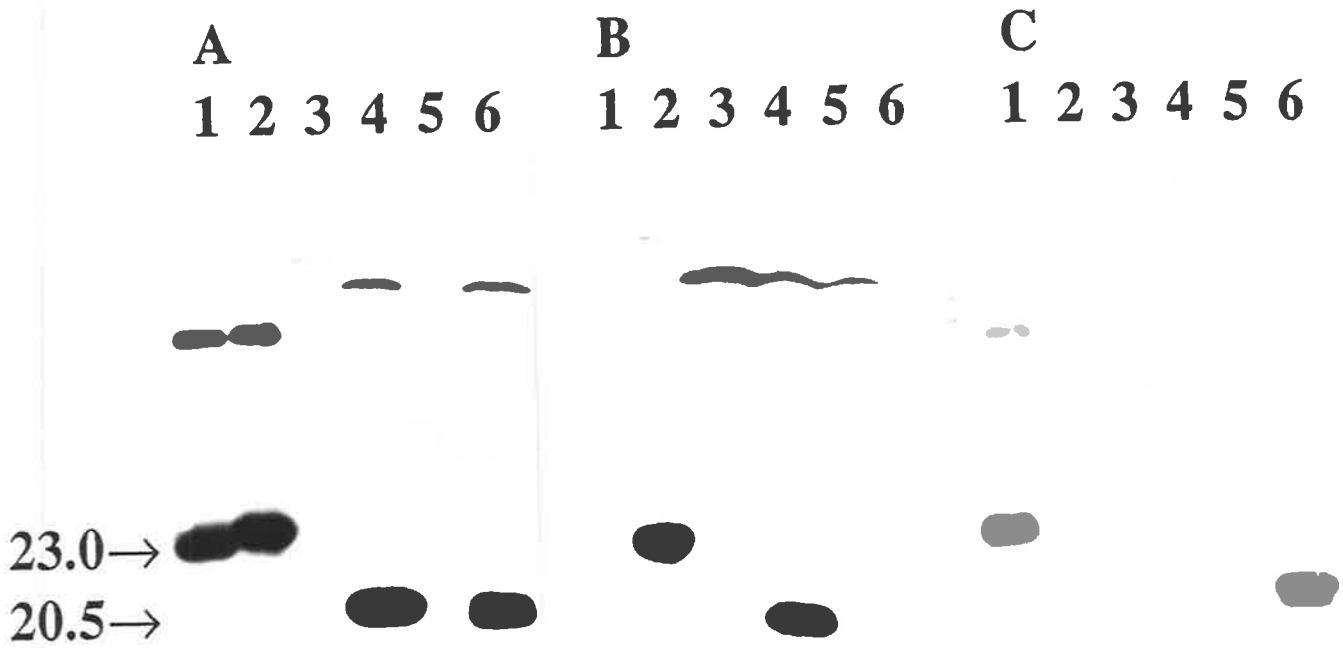

A
1 2 3 4 5 6
B
1 2 3 4 5 6
C
1 2 3 4 5 6
23.0→
20.5→

Table 3.1 Characteristics of *V. cholerae* strains

Strain	Biotype/Serotype	Year isolated
V. cholerae O1		
569B	classical/Inaba	1946[a]
569B/165	classical/Inaba x Non-O1 vibrio	1972[k]
Z17561	classical/Inaba	1985[b]
11441	classical/Inaba	1987[c]
11966	classical/Inaba	1987[c]
O17	El Tor/Ogawa	pre-1965[a]
AV86	El Tor/Inaba	1961[a]
T50	El Tor/Ogawa	pre-1962[a]
R1MD	El Tor/Ogawa	1980[g]
8233 B4	El Tor/Inaba	pre-1975[e]
NSW1	El Tor/Ogawa	1982[f]
13149	El Tor/Inaba	1982[f]
3083-10BT	El Tor/Ogawa	1983[b]
AA13993	El Tor/Inaba	1985[c]
H1	El Tor/Ogawa	1985[d]
20871	El Tor/Ogawa	1989[c]
21108	El Tor/Inaba	1989[c]
21161	El Tor/Inaba	1989[c]
14188	El Tor/Ogawa	1989[c]
V84	El Tor/Inaba	1990[d]
V87	El Tor/Ogawa	1990[d]
V90	El Tor/Inaba	1990[d]
V96	El Tor/Ogawa	1990[d]
N16961	El Tor/Inaba	pre-1988[i]
T19479	El Tor/Inaba	1979[i]
1621	El Tor/Ogawa	pre-1978[h]
V779	Z17561 *tcpA*::Km^R	1989[l]
V. cholerae O139		
AI-1837		1993[i]
AI-1838		1993[i]
AI-4450		1993[i]
AI-1841		1993[i]
AI-1852		1993[i]
AI-1854		1993[i]
AI-1855		1993[i]
AI-4260		1993[i]

[a] Strain obtained from Dr K. Bhaskaran, Central Drug Institute, Lucknow, India.
[b] Strain from Dr R. Finkelstein.
[c] Strain obtained from Dr B. Kay, International Centre for Diarrhoeal Diseases Research, Dhaka, Bangladesh.
[d] Strain obtained from Dr S.C. Pal, National Institute of Cholera and Enteric Diseases, Calcutta, India.
[e] Strain obtained from Dr J. Berry, University of Texas, Austin, USA
[f] Strain obtained from CIH, Australia.
[g] Source unknown.
[h] Strain obtained from Dr J.E. Ogg.
[i] Strain obtained from Prof J. Holmgren, University of Göteborg, Göteborg, Sweden.
[j] Dr J. Albert, International Centre for Diarrhoeal Diseases Research, Dhaka, Bangladesh.
[k] The construction of the 569B/165 hybrid strain is described in Sharma *et al* (1989a).
[l] Strain constructed by R. Faast, University of Adelaide, Adelaide, South Australia.

Figure 3.3 Immunoblots of El Tor strains cultured using the AKI method.

Samples: **A**, Z17561 (classical)

B, O17

C, V86

D, H1

E, AA13993

F, V84

G, V87

H, V90

I, V96

J, 14188

K, 20871

L, 21108

M, 21161

N, T19479

O, N16961

Each track is loaded with ca. 10^9 bacteria. Blot I was detected using the anti-V9 TCP serum (diluted 1:1500) and blot II was detected using the anti-SA42 OMP (diluted 1:500).

I

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

II

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

ELISA. Toxin production by El Tor biotype strains was lower than that attainable with classical strains and varied considerably between isolates (data not shown).

When the seven TcpA-producing strains were examined by IEM using anti-V9 TCP, no gold labelling of any surface structures was observed and no TCP-like bundles were seen. These results were supported by the findings of Jonson *et al* (1992) however the possibility remained that the inability to detect El Tor TCP was due to use of inappropriate antibodies; to this point, all the studies of El Tor TCP production involved the use of antisera or monoclonal antibodies raised to classical TCP. We knew from earlier work that there are biotype-related differences between the *tcpA* genes of classical and El Tor isolates (Voss, 1990) which could result in antigenically dissimilar pilins (Sun *et al*, 1990b). Consequently, antibodies to classical TCP may have a lower affinity for the El Tor protein which, in an assay as insensitive as IEM, might result in a failure to detect El Tor TCP. For this reason we sought to raise an antiserum specific for the El Tor protein.

3.4 Assessment of El Tor TcpA/TCP expression using biotype-specific anti-TcpA reagents.

3.4.1 Production of antisera to unprocessed TcpA.

During the course of these studies Jonson *et al* (1992) reported an unsuccessful attempt to produce an antiserum to El Tor TcpA. Rabbits immunised with viable El Tor vibrios failed to produce antibodies able to recognise El Tor TcpA in immunoblots. This suggested that El Tor vibrios produce little if any surface TcpA/TCP and prompted us to use a different approach.

Inducible expression vectors enable expression of a gene at high levels. The temperature-inducible T7 expression system (Tabor and Richardson, 1985) was used to over-express the El Tor and classical *tcpA* genes. The *E. coli* clones, SA42 and E1838, carrying plasmids

encoding El Tor and classical *tcpA* respectively, were constructed as described in Materials and Methods. The TcpA proteins produced in SA42 and E1838 are in their unprocessed form of 23.0kDa as *E. coli* lacks the appropriate signal peptidase (TcpJ; Kaufman *et al*, 1991) which in *V. cholerae* processes the pilin to its mature form of 20.5kDa. When over-expressed using the T7 system, most of the TcpA formed inclusion bodies. Several attempts were made to solubilize the protein from the inclusion bodies but none was successful. Fractionation studies of SA42 and E1838 revealed TcpA was present in the outer membrane of the *E. coli* clones (not shown) and so outer membrane preparations (OMPs) were used to immunise rabbits. The protocol used for immunisation is described in section 2.9.2.

The resultant antisera were absorbed with *E. coli* OMPs containing TcpA protein of the heterologous biotype to eliminate antibodies which recognise shared epitopes, thereby creating biotype-specific anti-TcpA reagents. The specificity of the reagents was checked by Western blot; as shown in Figure 3.2, anti-E1838 OMP and anti-SA42 OMP were specific for TcpA proteins of classical and El Tor biotype, respectively.

3.4.2 Strain survey of El Tor strains using biotype-specific anti-TcpA reagents.

The 21 El Tor strains were re-assessed for TcpA production using the anti-SA42 OMP serum, initially by Western blot and then by IEM. Comparison of identical blots detected with anti-V9 TCP and anti-SA42 OMP revealed that the intensity of the TcpA bands seen with strains of the El Tor biotype is greater when detected using the El Tor TcpA-specific serum (Figure 3.3). The increased sensitivity of the anti-SA42 OMP resulted in another three strains being scored as TcpA-producing, in addition to the seven already identified using anti-V9 TCP. These ten strains were subsequently examined by IEM using both anti-SA42 OMP and anti-V9 TCP. Surprisingly, no gold binding was observed on the surface of any of the El Tor strains.

These findings were consistent with Jonson's suggestion that El Tor strains do not assemble TcpA into TCP, at least *in vitro* (Jonson *et al*, 1992). However, an alternative explanation would be that the anti-SA42 OMP does not detect native pilin. Although it was not possible to test the latter hypothesis directly, it was possible to examine whether the analogous anti-E1838 OMP serum (specific for classical TcpA) could bind to native TCP present on Z17561. IEM studies showed that this serum did not react with TCP, indicating that the sera prepared against unprocessed TcpA did not recognise native pilin.

3.5 Fractionation studies.

Although the biotype-specific reagents anti-SA42 OMP and Anti-E1838 OMP did not enable us to directly determine the presence or absence of TCP on the surface of TcpA-producing El Tor strains, cell fractionation studies of these strains could elucidate where the TcpA pilin remains in the bacterium if it is not polymerised. AKI-grown classical strain Z17561 and El Tor strain H1 were fractionated according to the method described in section 2.6. Western blot analysis of the cell fractions using anti-V9 TCP revealed an identical pattern of distribution of TcpA between Z17561 and H1 (Figure 3.4). Although present in varying amounts in all fractions most of the TcpA produced appeared to localise to the cell envelope. To determine whether the pilin was in the cytoplasmic or outer membrane, the cell envelopes of both strains were treated with Triton X-100. Analysis of the soluble (cytoplasmic membrane) and insoluble (outer membrane) fractions showed most of the TcpA was in the cytoplasmic membrane. Unexpectedly, this was the same for both biotypes. However it must be remembered that solubilization of membranes using detergents such as Triton X-100 can sometimes give misleading results as not all outer membrane proteins may be insoluble and conversely not all cytoplasmic membrane proteins may be soluble in Triton X-100. As an alternative to using

Figure 3.4 Immunoblot of whole cells and cell envelope fraction of classical and El Tor *V. cholerae* O1.

Samples: **1**, Z17561 (classical)

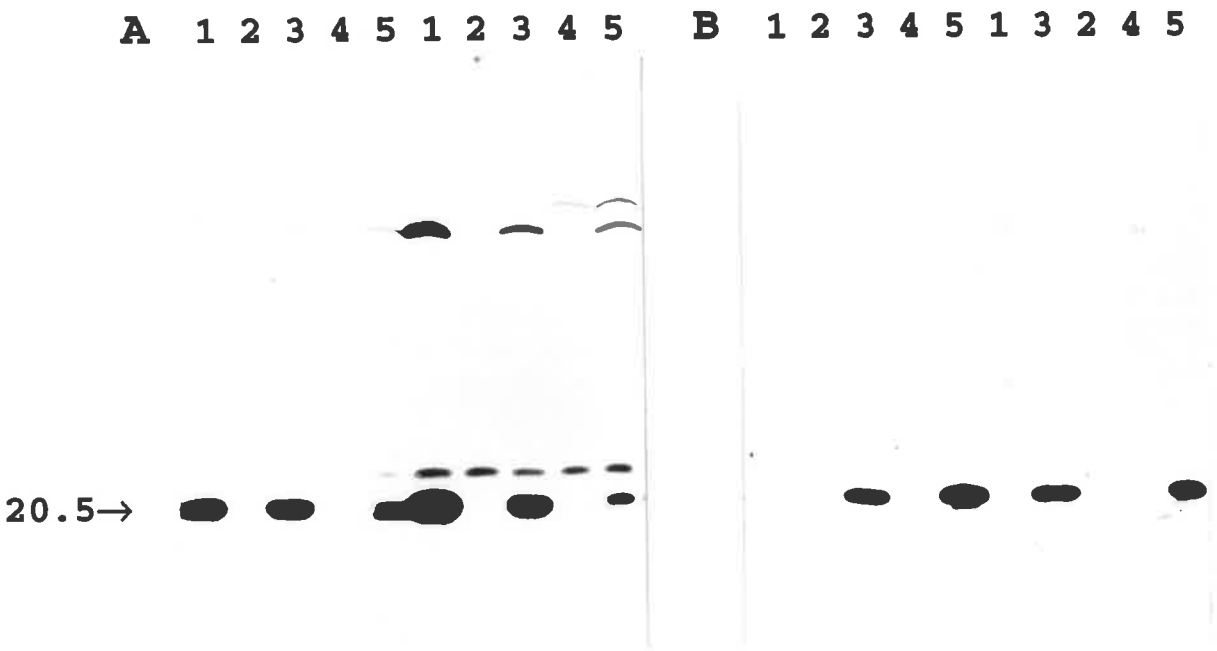
2, O17 (El Tor)

3, DS2 (O17[pPM2103])

4, EV23 (H1 *tcpA* isogenic mutant)

5, H1 (El Tor)

The first five tracks in both blots are loaded with whole cells (ca. 10^9 bacteria) and the remaining five tracks are loaded with cell envelope fraction (ca. 10μg protein). Immunoblots of the periplasmic and cytoplasmic fractions are not shown but no TcpA was detected in these fractions. Blot A was detected using the anti-V9 TCP serum (diluted 1:1500) and blot B was detected using the anti-SA42 OMP (diluted 1:500).


A 1 2 3 4 5 1 2 3 4 5
B 1 2 3 4 5 1 3 2 4 5
20.5→

detergents, attempts were made to separate the membranes by sucrose density gradient centrifugation. However very poor separation of the membranes made interpretation of the results difficult. It was therefore assumed based on the results of Triton X-100 solubilization that TcpA accumulates in the cytoplasmic membrane of both classical and El Tor organisms prior to assembly.

3.6 Production of antisera to native El Tor TcpA

TCP are easily collected from the surface of classical vibrios by shearing the pili from the cells followed by differential centrifugation (see 2.8). If El Tor strains assemble TCP, even in reduced amounts compared to classical strains, it might be possible to isolate these pili using the same procedure. Crude "pili preparations" were subsequently isolated from Z17561, H1 and EV37, an O17 cosmid-bearing derivative which produces El Tor TcpA (described in section 5.2). Western blotting (using the anti-SA42 OMP) confirmed the presence of TcpA in all three preparations (Figure 3.5), but IEM revealed characteristic TCP bundles only in the Z17561 preparation (not shown). Although TCP were not observed in the H1 and EV37 "pili preparations", there were many membrane vesicles which from earlier fractionation studies would be expected to contain TcpA.

In a further attempt to raise an antiserum which would react with native El Tor TcpA, the crude H1 and EV37 "pili preparations" were used to immunise rabbits. The resultant antisera, referred to as anti-H1 TcpA and anti-EV37 TcpA, were absorbed with cell envelope preparations from isogenic *tcpA* mutants of H1 and O17, in order to make them TcpA-specific. As expected, the residual sera were not biotype-specific, but it was interesting to note that they displayed only weak reactivity against classical TcpA on Western blots (Figure 3.6).

Figure 3.5 Immunoblot of classical and El Tor "pili preparations".

Samples: **1**, E1838 OMP in blots A and B, SA42 OMP in blot C

2, Z17561 (classical)

3, O17 (El Tor)

4, DS2 (O17 [pPM2103])

5, EV36 (O17 [pPM2103])

6, EV37 (O17 [pPM2103])

7, H1 (El Tor)

8, EV35 (H1 [pPM2103])

Each track was loaded with ca. 10μg protein with the exception of track 1 which was loaded with ca. 30μg protein. Blot A was detected using the anti-V9 TCP serum (diluted 1:1500), blot B was detected using the anti-E1838 OMP (diluted 1:1500) and blot C was detected using the anti-SA42 OMP (diluted 1:500).

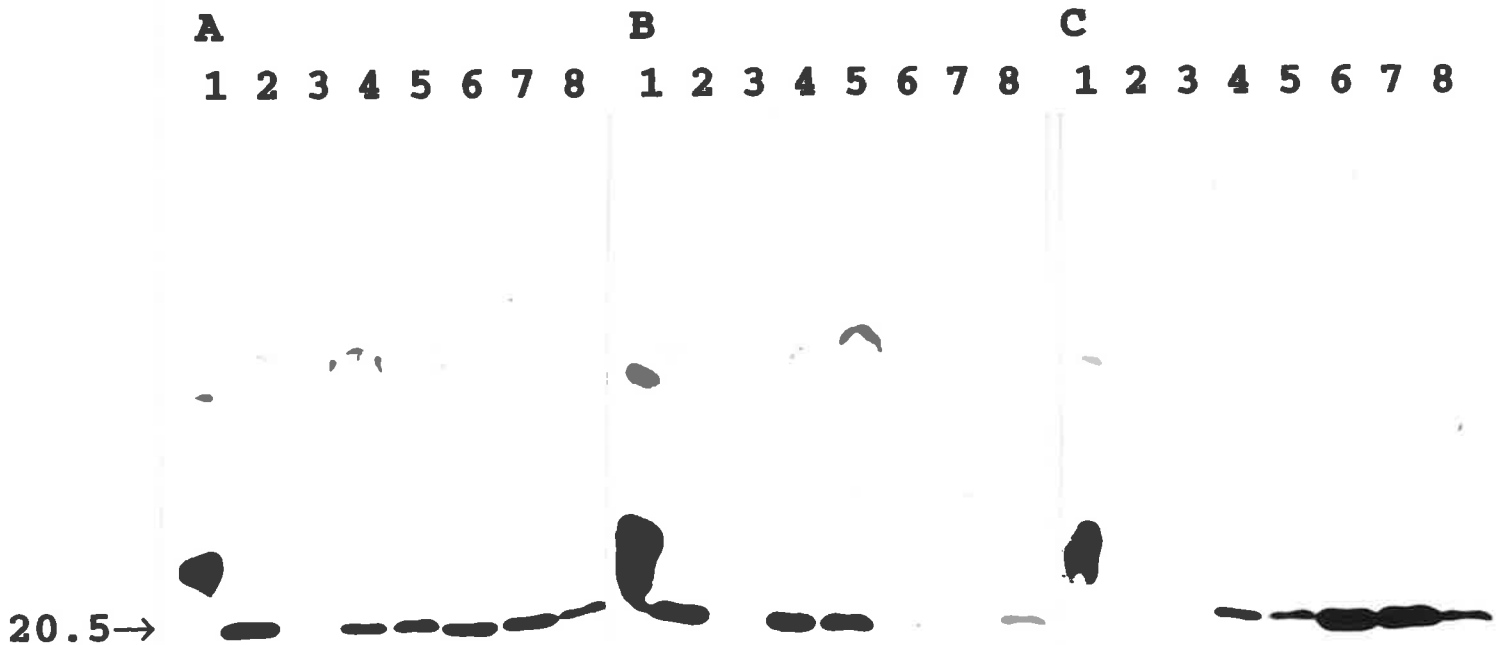

A
B
C
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
20.5→

Figure 3.6 Immunoblot demonstrating the reactivity of an antiserum raised against native El Tor TcpA (anti-H1 TcpA).

Samples: **1**, SA42 OMP

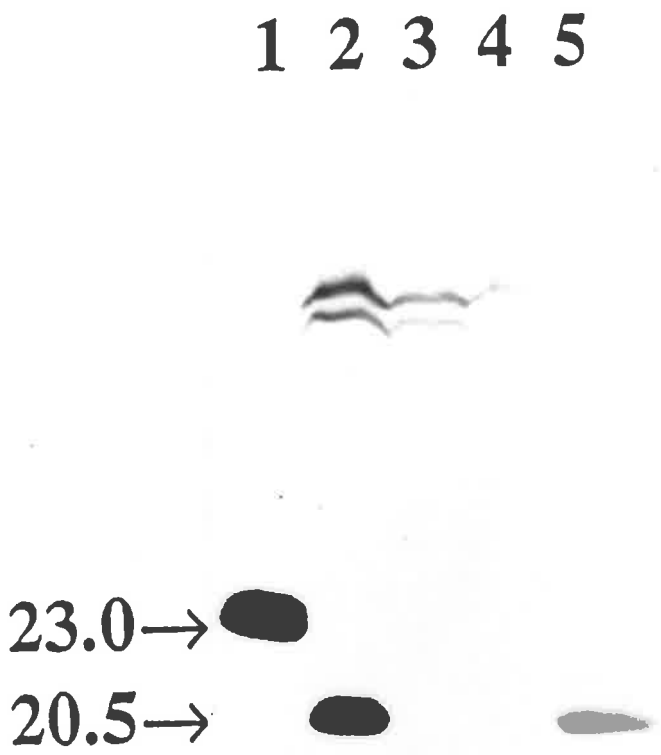
2, H1 (El Tor)

3, EV50 (H1 *tcpA* isogenic mutant)

4, O17 (El Tor)

5, Z17561 (classical).

All tracks were loaded with ca. 10^9 bacteria with the exception of track 1 which was loaded with ca. 30μg of protein. The blot was detected using anti-H1 TcpA (diluted 1:5000).


1 2 3 4 5
23.0→
20.5→

Five of the TcpA-producing El Tor strains and the classical strain, Z17561, were grown using the AKI broth method and examined for TCP expression by IEM using the anti-H1 TcpA serum and anti-V9 TCP. As expected, abundant gold-labelling of Z17561 TCP bundles was evident when grids were treated with anti-V9 TCP but only very poor labelling of classical TCP was observed using anti-H1 TcpA, in line with the weaker immunoblot reactivity of this serum to classical TCP (Figure 3.6). As noted previously, no gold-labelling of any structures was seen when the five El Tor strains were probed with anti-V9 TCP. However when the same strains were examined using anti-H1 TcpA, bundles of TCP covered with gold particles were found in all cases (Figure 3.7). Identical results were obtained with the anti-EV37 TcpA reagent (not shown). These results showed for the first time that El Tor strains can assemble TcpA into TCP during *in vitro* growth.

3.7 TcpA and cholera toxin production in O139 "Bengal" strains.

An epidemic of "non-O1" cholera caused by the O139 or "Bengal" serotype recently began sweeping through parts of Asia and has already spread into South America (Section 1.3.4). The properties of this new serotype suggest that it has arisen from a strain of El Tor biotype which has acquired genes which specify the production of a capsule and an altered lipopolysaccharide (Manning *et al*, 1994; Waldor *et al*, 1994; Comstock *et al*, 1995; Bik *et al*, 1995). To examine whether the O139 serotype is otherwise "El Tor-like" with respect to TcpA/TCP production, eight O139 isolates from Bangladesh were assessed by Western blotting and IEM for TcpA/TCP production.

Duplicate Western blots were probed with either the anti-E1838 OMP or anti-SA42 OMP. Figure 3.8 demonstrates that some but not all of the O139 isolates express TcpA *in vitro* and the pilin is only recognised by the reagent specific for the El Tor monomer. All TcpA-positive

Figure 3.7 Electron micrographs of TCP bundles detected by anti-H1 TcpA (diluted 1:20) demonstrating very weak immuno-gold labelling of the classical TCP bundles in panel A compared with the abundant gold-labelling of the El Tor TCP bundles in panels B, C and D.

Panel **A**, Z17561 (classical) mag. 5.5×10^4

Panel **B**, N16961 (El Tor) mag. 7×10^4

Panel **C**, H1 (El Tor) mag. 2.7×10^4

Panel **D**, V84 (El Tor) mag. 2.7×10^4.

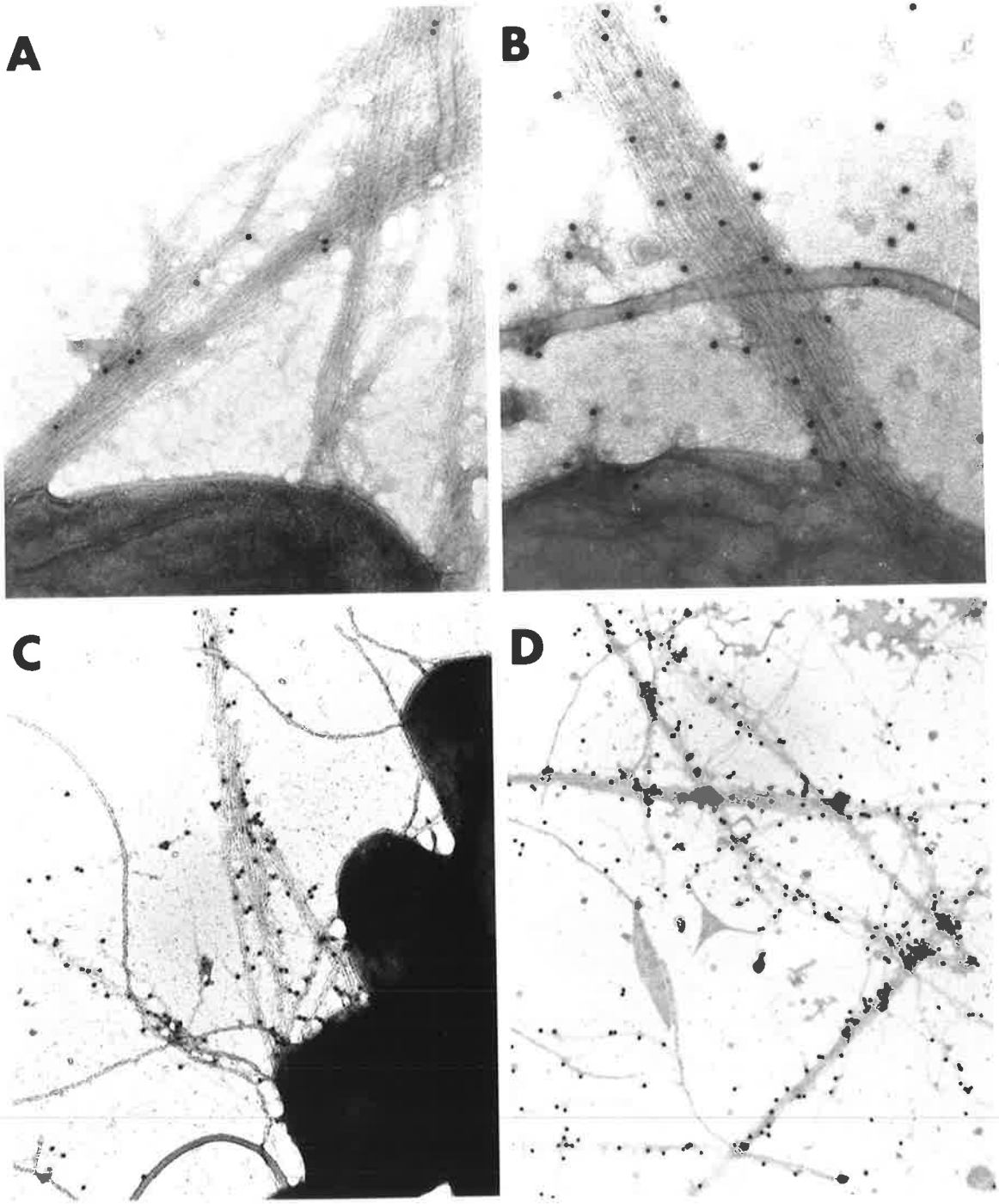

A
B
C
D

Figure 3.8 Immunoblot of *V. cholerae* O139 isolates.

Samples: **A**, H1 (El Tor)

B, O17 (El Tor)

C, AI-1837

D, AI-1838

E, AI-4450

F, AI-1841

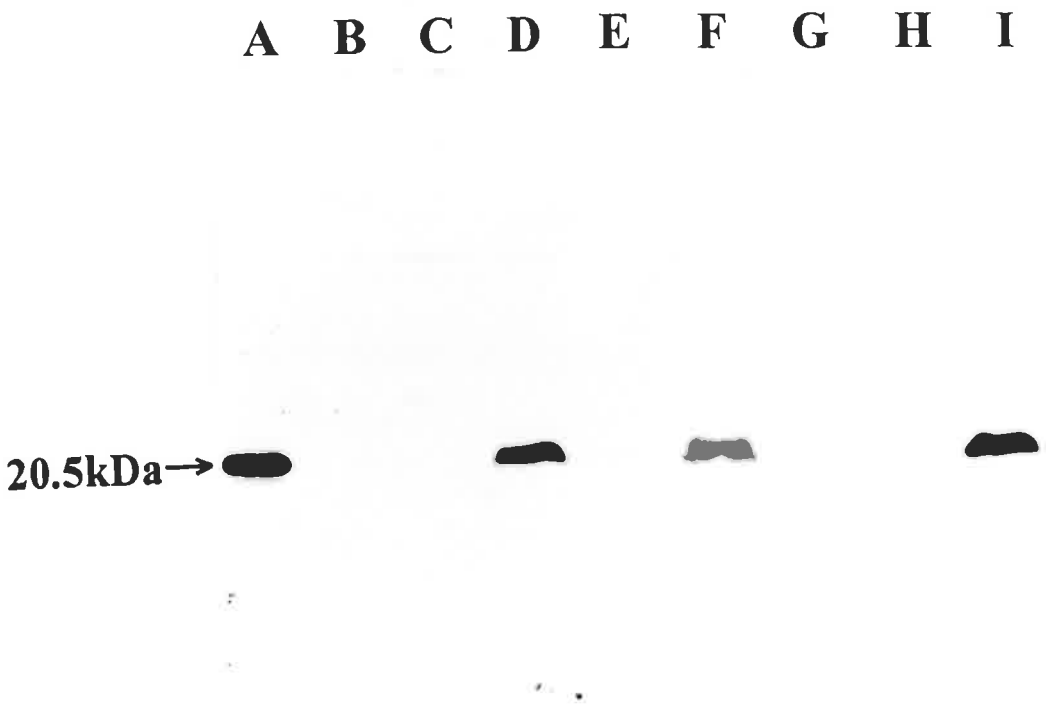
G, AI-1852

H, AI-1854

I, AI-1855

J, AI-4260

All tracks were loaded with ca. 10^9 bacteria. The blot was detected using anti-SA42 OMP (diluted 1:500).


A B C D E F G H I J
20.5kDa→

strains were examined by IEM using the anti-H1 TcpA reagent; gold-labelled TCP bundles were observed for each isolate (data not shown). The level of TCP expression varied considerably, but was in keeping with the size of the band detected by Western blotting.

Supernatants from the AKI broth cultures used to assess TcpA/TCP production were assayed for the presence of cholera toxin. There was considerable variation in the levels of toxin detected between strains but the range of concentrations was typical of O1 El Tor strains (data not shown).

3.8 Discussion

Increasing the carbon dioxide tension of culture environments has been demonstrated to affect the expression of virulence determinants of various Gram-negative and Gram-positive bacteria (Shimamura *et al*, 1985; Makino *et al*, 1988; Caparon *et al*, 1992; Austin, 1993). The addition of 5% CO_2 to the modified AKI-SW culture method of Jonson *et al* (1990) resulted in a dramatic increase in TcpA production, as well as increased cholera toxin secretion, in strains of both biotypes (Figure 3.1). The mechanism by which CO_2 exerts this effect is not known, although a pH-mediated effect would seem likely, given that pH can influence the expression of ToxR-regulated genes (Peterson *et al*, 1988b). Recent studies of regulation of the protective antigen gene (*pag*) of *Bacillus anthracis* have shown that the level of bicarbonate and CO_2 in the culture environment have a direct effect on transcription of *pag* which is unrelated to anaerobiosis or pH changes (Bartkus and Leppla, 1989; Koehler *et al*, 1994). Whether CO_2 has a direct effect in *V. cholerae* remains to be demonstrated.

The assembly of TcpA into TCP by strains of El Tor biotype has never previously been demonstrated. Infant mouse protection studies by Sun *et al* (1990b) are consistent with the *in vivo* assembly of El Tor TcpA, as monoclonal antibody to classical TCP provided marginal

protection against challenge with El Tor vibrios. The authors concluded that the weak protection observed may reflect poorer TCP expression on the surface of El Tor strains compared with classical *V. cholerae*. Jonson and associates (1992) assessed the *in vivo* and *in vitro* expression of TCP by strains of classical and El Tor biotype, reporting that levels of TcpA attainable *in vitro* were comparable to those found after *in vivo* growth. When nine *in vivo*-grown El Tor strains were tested by inhibition ELISA only one had detectable surface TcpA. No TCP were identified by IEM of *in vivo* grown El Tor strains. TCP-like structures were observed in EM preparations of cholera stool from one of four infected individuals but failed to be identified by IEM. Based on the above studies both Sun and coworkers (1990b) and Jonson *et al* (1992) have suggested that, unlike classical strains, TCP may not be an essential colonisation factor for the El Tor biotype.

Until now, studies of TcpA/TCP expression by El Tor strains have been performed using antibody raised to classical TCP. Since the biotypic forms of TcpA are antigenically different, and given the likelihood that El Tor strains show reduced surface expression of TcpA, it seemed worthwhile to attempt to prepare an antiserum specific for El Tor TcpA before accepting that El Tor strains cannot polymerize this protein into pili.

Initially *E. coli* clones were constructed in which the *tcpA* genes of both biotypes were over-expressed. Outer membrane preparations of these clones, containing unprocessed TcpA, were used to immunise rabbits and the resultant antisera extensively absorbed to be not only TcpA-specific but also biotype-specific. The El Tor-specific reagent detected TcpA production in ten of the 21 El Tor strains examined by Western blotting - three more than were detected by the anti-V9 TCP serum (Figure 3.3). Still, however, eleven isolates did not produce detectable amounts of TcpA *in vitro*, raising the possibility that there are two subgroups of El Tor strains. This would imply that regulation of *tcpA* expression differs not only between biotypes but also

within the El Tor biotype. Alternatively within the El Tor biotype there might exist a continuum from high to low (*in vitro*) TcpA expression, so that detection of TcpA would depend on the sensitivity of the anti-TcpA reagent used.

Although the biotype-specific anti-TcpA reagents were very sensitive indicators of protein production when used in Western blots, the failure of the anti-E1838 serum to recognise TCP bundles on Z17561 suggested that they did not react with native TCP. The sera were raised against the unprocessed form of TcpA and presumably this lacks the conformational epitopes of the processed, native pilin. Fractionation studies performed with these sera gave no indication that TcpA is distributed differently within El Tor and classical vibrios;. in both H1 and Z17561, TcpA localised to the cell envelope and principally to the cytoplasmic (Triton-soluble) membrane. Using immuno-gold labelling of ultra-thin sections, Kaufman *et al* (1991) have recently demonstated that TcpA is distributed equally between the inner and outer membranes of wildtype *V. cholerae*. This supports our finding that TcpA is membrane-associated but also suggests that preferential localization to the cytoplasmic membrane may be an artefact of Triton solubilization.

Subsequently, a crude "pili preparation" from the El Tor strain H1 was used to raise an antiserum to native TcpA. After extensive absorption this serum was TcpA-specific, and was used to examine five TcpA-producing El Tor strains by IEM. TCP bundles were found in all the strains examined (Figure 3.7), showing for the first time that El Tor TcpA is assembled into TCP. There is little doubt that the anti-H1 TcpA serum was essential for detecting TCP bundles on El Tor vibrios. When the same strains were subjected to IEM using anti-V9 TCP, no gold-labelling was observed, despite the fact that this reagent reacts with (denatured) El Tor TcpA in Western blots. Evidently the anti-V9 TCP serum lacks antibodies which recognise conformational epitopes (if these exist) associated with the N-terminal (conserved)

region of the pilin monomer. The classical strain Z17561, included in the IEM studies for comparison, is a strong producer of TCP and TCP bundles can be identified even in the absence of antibody-mediated gold-labelling. In contrast however, no structures resembling TCP were apparent when El Tor strains were examined by (non-immuno) EM. Apart from the possibility that strains of the El Tor biotype may produce less TCP than classical strains (as previously suggested by Sun *et al*, 1990b; Jonson *et al*, 1992), perhaps El Tor TCP less efficiently bind the uranyl acetate used as the negative stain in EM, making identification without immuno-probes more difficult.

Eight O139 or "Bengal" serotype strains from Bangladesh were cultured for TcpA/TCP production and assessed by Western blotting using the biotype-specific antisera (Figure 3.8). Five of the eight strains produce detectable amounts of TcpA *in vitro* and furthermore the pilin produced reacts only with the El Tor-specific anti-TcpA serum. IEM studies of the five TcpA-positive strains using the anti-H1 TcpA confirmed the presence of TCP bundles. In agreement with the Western blot results, no gold labelling of bundles occurred when the anti-V9 TCP was used. Production of cholera toxin was found to vary between the O139 strains, none of which produced levels comparable to those commonly found with classical *V. cholerae*. These results support the theory that the O139 serotype derives from an El Tor strain which has acquired new genes specifying the synthesis of novel lipopolysaccharide (Manning *et al*, 1994).

Chapter 4 The role of TCP in the pathogenesis of *V. cholerae* O1 El Tor

4.1 Introduction

Studies in the infant mouse cholera model showed TCP to be essential for colonisation by classical *V. cholerae* (Taylor *et al*, 1987) and later studies demonstrated the same also applies to human infection (Herrington *et al*, 1988). When this work was commenced however, there was no evidence to implicate TCP as an important colonisation factor for strains of the El Tor biotype. Indeed, other workers had suggested there may be alternative adherence factors utilized by El Tor strains (Sun *et al*, 1990a, Jonson *et al*, 1991b). Since the seventh pandemic is caused by *V. cholerae* El Tor, it was clearly important to clarify this issue.

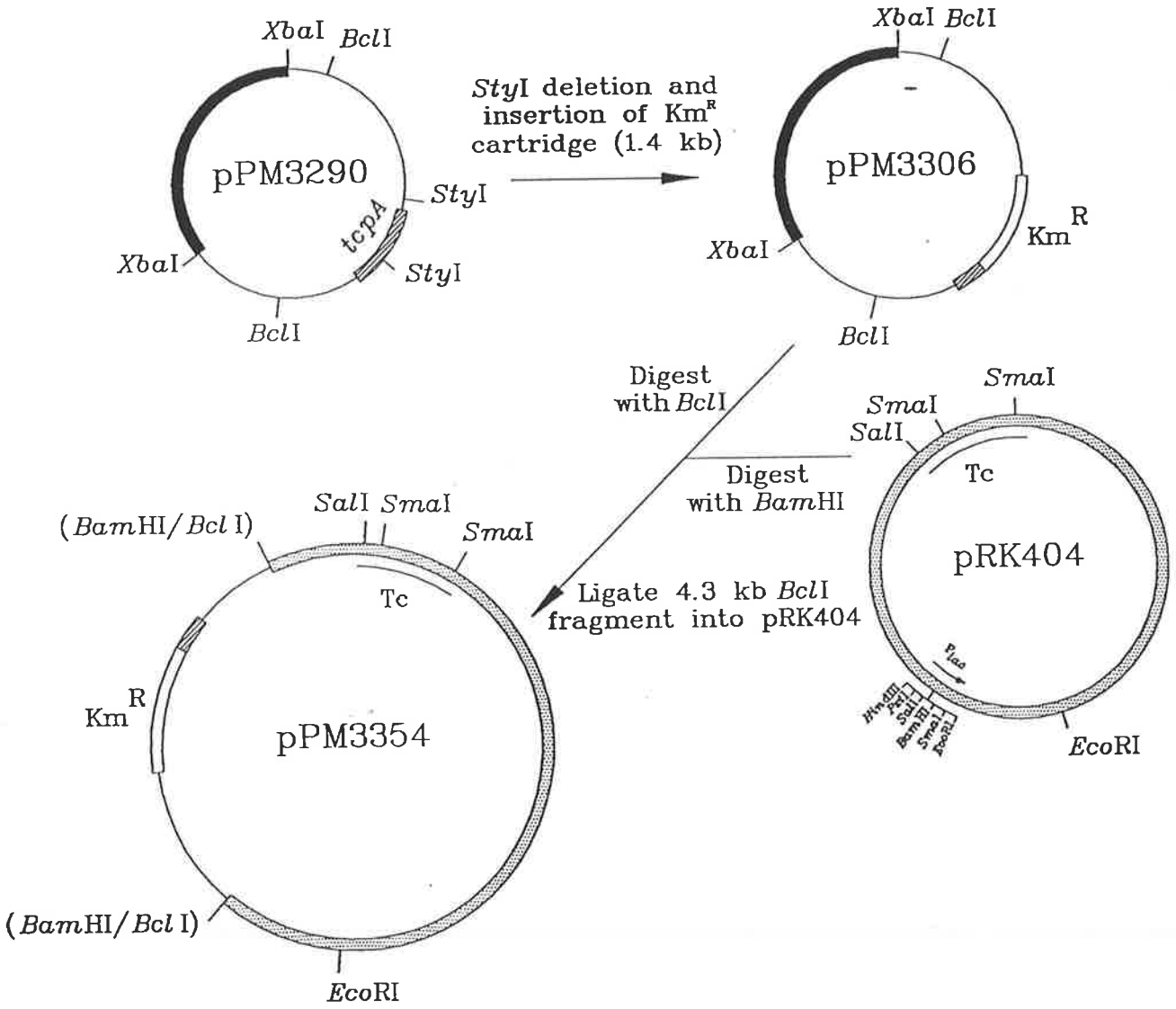
As described in the previous chapter, an antiserum specific for native El Tor TcpA allowed the first demonstration of TCP bundles on the surface of *V. cholerae* El Tor. To investigate the role of TCP in the pathogenesis of El Tor strains, *tcpA* mutants were constructed to compare their *in vivo* behaviour with that of their wild-type parents. The results of these studies are presented in this chapter. Initially *tcpA* mutants were obtained by insertional mutagenesis, inactivating the gene by introduction of an antibiotic resistance cartridge. Although this approach might also result in reduced expression of downstream genes, such mutants are comparatively easy to obtain. Subsequently, encouraged by results with these strains, more refined *tcpA* mutants were constructed by incorporation of an in-frame deletion into the target gene. It was expected that studies with the latter strains would allow an unequivocal evaluation of the role of TCP in El Tor infection.

4.2 Construction of El Tor *tcpA*::Km^R mutants

The approach used to construct El Tor *tcpA*::Km^R mutants was a modification of that described by Manning (1992) and is outlined in Figure 4.1. The cosmid pPM2608 encodes the *tcp* operon of the El Tor strain H1, and also carries ca. 20kb DNA of unknown function. pPM2608 was digested with *Xba*I and a 5kb fragment harbouring the *tcpA* gene was cloned into pUC18 to produce plasmid pPM3290. pPM3290 was subsequently digested with *Sty*I, which results in the deletion of approximately 370bp of the carboxy-terminal portion of *tcpA* as well as 41bp of the intergenic region between *tcpA* and *tcpB*. A Km^R cartridge from pUC4-K (Vieira and Messing, 1982) was inserted at the site of the deletion to create plasmid pPM3306. A 4.3kb *Bcl*I fragment harbouring the mutated *tcpA* gene was then cloned into the mobilizable vector pRK404 (Ditta *et al*, 1980). Construction of the resultant plasmid, pPM3354 is outlined in Figure 4.1.

The resultant plasmid, pPM3354, was conjugated via an *E. coli* donor into four El Tor strains. To determine whether the *in vivo* consequences of a mutation in *tcpA* might relate to the *in vitro* capacity of a strain to produce TcpA/TCP, two strains which produce TcpA *in vitro* (H1 and N16961) and two which do not (O17 and AA13993) were chosen for this study. Transconjugants were selected as Pmx^R, Tc^R and Km^R. By a process of homologous recombination, the mutated *tcpA* gene would replace the chromosomal *tcpA* gene. The residual plasmid bearing the functional copy of *tcpA* was chased out using the incompatible plasmid pH1JI. Putative *tcpA*::Km^R mutants carrying pH1JI were selected as Km^R, Gm^R and Tc^S. Strains were cured of pH1JI by overnight culture in nutrient broth without added Gm, followed by growth on Km agar. Two Km^R, Gm^S, Tc^S colonies were selected for each strain and analysed by Southern blotting using probes to both the Km^R cartridge and *tcpA*. The presence of the Km^R cartridge was thus confirmed and the *tcpA* probe demonstrated its

Figure 4.1 Construction of pPM3354.


XbaI BclI
pPM3290
StyI
tcpA
StyI
XbaI
BclI
StyI deletion and insertion of KmR cartridge (1.4 kb)
XbaI BclI
pPM3306
KmR
XbaI
BclI
Digest with BclI
Digest with BamHI
SmaI
SmaI
SalI
Tc
pRK404
Plac
HindIII
PstI
SalI
BamHI
SmaI
EcoRI
EcoRI
Ligate 4.3 kb BclI fragment into pRK404
SalI SmaI
SmaI
(BamHI/BclI)
Tc
KmR
pPM3354
(BamHI/BclI)
EcoRI

location within the *tcpA* gene, since the band detected in the mutants showed the expected size increase (Figure 4.2). Western blotting confirmed the loss of TcpA production in the mutants (Figure 4.3).

It seemed likely that insertion of the Km^R cartridge would have polar effects, disrupting the expression of downstream genes. This would make complementation with a minimal fragment implausible, and so to complement the effects of the mutation the cosmid pPM2608 was conjugated into one of the *tcpA* mutants of each strain. The cosmid restored the ability of the H1 and N16961 mutants to produce TcpA/TCP when cultured appropriately; moreover pPM2608 converted the O17 and AA13993 *tcpA* mutants to TcpA/TCP-producing strains *in vitro*, even though their parent strains do not display this capacity (Figure 4.3).

4.3 Characterization of *tcpA*::Km^R mutants

Prior to their assessment in the infant mouse cholera model (IMCM), it was necessary to characterise the *tcpA* mutants with respect to a number of other properties which might contribute to the pathogenicity of the parent strains. This was done to ensure that, to the best of our knowledge, any effect on colonising capacity or virulence could be attributed to the mutation introduced into *tcpA*.

The mutants and their cosmid-complemented variants were found to have similar growth rates (in minimal medium and nutrient broth) to those of the wild-type strains. Sloppy agar overlays did not show any difference in motility between the parent strains and their isogenic mutants. Haemolytic activity was also assayed; although O17 was the only parental strain to display any significant haemolysis, no detectable differences in haemolytic activity was found between the parent strains and their respective mutants. Recently the MSHA has been shown to be a pilus and there is some suggestion that it may play a role in colonisation of the gut by

Figure 4.2 Southern blots of El Tor parent strains and *tcpA*::Km^R mutants.

Samples: **1**, AA13993

2, AA13993 *tcpA*::Km^R #1

3, AA13993 *tcpA*::Km^R #2

4, O17

5, O17 *tcpA*::Km^R #1

6, O17 *tcpA*::Km^R #2

7, H1

8, H1 *tcpA*::Km^R #1

9, H1 *tcpA*::Km^R #2

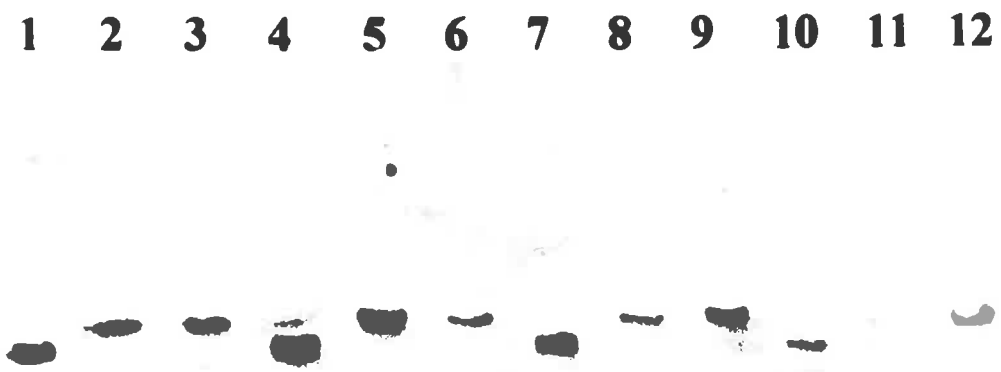
10, N16961

11, N16961 *tcpA*::Km^R #1

12, N16961 *tcpA*::Km^R #2.

Blot A was detected with the *tcpA* probe, oligonucleotide 193. Blot B was detected using the Km^R cartridge probe.

A



B

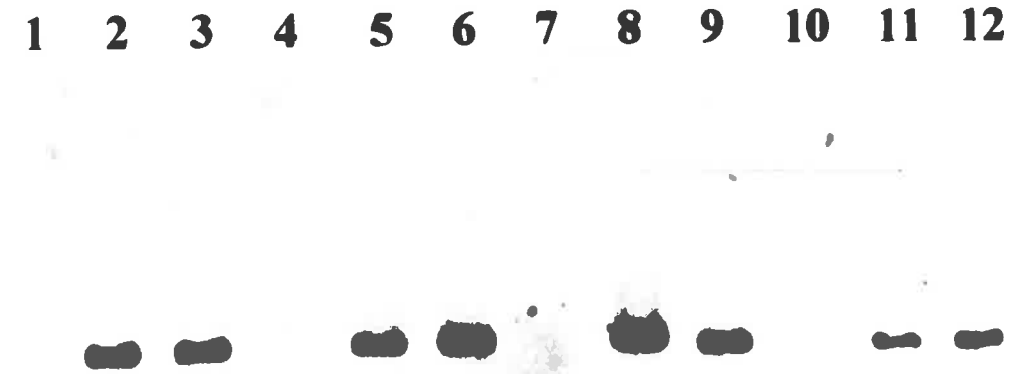


Figure 4.3 Immunoblot of El Tor parent strains, *tcpA* mutants and pPM2103-complemented *tcpA*::Km^R mutants.

Samples: **A**, SA42 OMP

B, O17

C, O17 *tcpA*::Km^R #1

D, O17 *tcpA*::Km^R #2

E, O17 *tcpA*::Km^R #1[pPM2103]

F, H1

G, H1 *tcpA*::Km^R #1

H, H1 *tcpA*::Km^R #2

I, H1 *tcpA*::Km^R #1[pPM2103]

J, AA13993

K, AA13993 *tcpA*::Km^R #1

L, AA13993 *tcpA*::Km^R #2

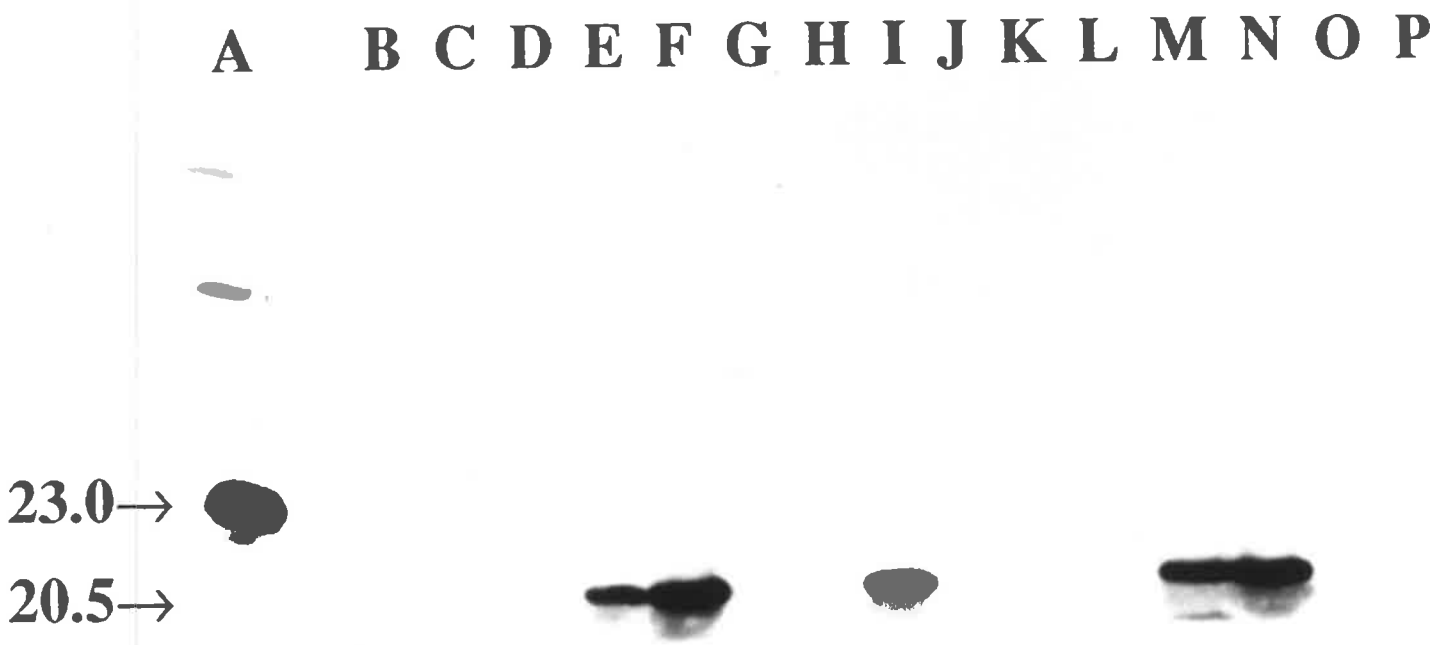
M, AA13993 *tcpA*::Km^R #1[pPM2103]

N, N16961

O, N16961 *tcpA*::Km^R #1

P, N16961 *tcpA*::Km^R #2.

N16961 *tcpA*::Km^R #1[pPM2103] produces TcpA/TCP but is not shown in this figure. All tracks are loaded with ca. 10^9 bacteria with the exception of track A which is loaded with ca. 30μg protein. Arrows indicate the position of unprocessed TcpA (23kDa) and processed TcpA (20.5kDa).


A B C D E F G H I J K L M N O P
23.0→
20.5→

El Tor strains (Jonson *et al*, 1992). Again, MSHA activity was found to vary between the wild-type strains, but within a strain the activities of *tcpA*::Km^R mutants, cosmid-complemented variants and parent strains were comparable (data not shown).

When AKI culture supernatants were assayed for cholera toxin production by GM1-ELISA, no significant difference between mutants and wild-type parent strains were detected. Cosmid-complemented *tcpA*::Km^R mutants of H1 and N16961 produced similar levels of toxin to their parent strains, but the cosmid-complemented mutants of O17 and AA13993 secreted significantly more toxin than wild-type (ca. 50-fold and 5-fold more respectively; ~~Table 4.1~~ data not shown). Subsequent studies revealed the enhanced toxin secretion found in these strains to be attributable to an activating factor(s) encoded by the cosmid, probably ToxT (see Sections 5.3 and 5.4).

4.4 *In vivo* behaviour of *tcpA*::Km^R mutants

4.4.1 Virulence studies

Virulence of the wild-type parent strains and *tcpA*::Km^R mutants was assessed in LD_{50} studies in infant mice. LD_{50} experiments (section 2.23.1) were performed in duplicate for each parent strain and its two isogenic *tcpA* mutants. The mutants were found to be dramatically decreased in virulence in the IMCM, with LD_{50} values increased at least 3000-fold (Table 4.2). The presence of cosmid pPM2608 (carrying the H1 *tcp* operon) essentially restored virulence to three of the four *tcpA* mutants; however the N16961 complemented mutant, although dramatically increased in virulence, was not restored to wild-type (Table 4.2). These data indicate that either TcpA, or another factor(s) whose production is affected by the *tcpA*::Km^R mutation but complemented by the presence of the H1 *tcp* operon *in trans*, is essential for the virulence of El Tor strains in this model.

Table 4.1 Concentrations of cholera toxin in AKI broth cultures of cosmid clones

Strain	Cholera Toxin Concentration[a]	
	Exp1	Exp2
Z17561	243	275
O17	4	2
V711	ND	135
EV36	270	ND
EV37	54	ND
H1	110	125
EV35	54	ND

[a] Concentration of cholera toxin is expressed as μg/ml.
ND = not done.

Table 4.2 Virulence of El Tor strains, *tcpA*::Km^R mutants and cosmid complemented mutants.

	LD_{50}[a]			
Strain	**Wildtype**	**Km#1**[b]	**Km#2**[c]	**Km#1[pPM2608]**[d]
O17	1.7×10^4	5.8×10^7	1.3×10^8	6.6×10^4
H1	1.4×10^4	8.1×10^7	2.1×10^8	1.2×10^4
AA13993	9.6×10^5	2.7×10^8	3.8×10^8	1.5×10^6
N16961	2.0×10^4	2.0×10^8	3.9×10^8	5.2×10^5

[a] Virulence is expressed as 48-hour LD_{50} dose.

[b,c] Two *tcpA*::km^R mutants of each El Tor strain were tested.

[d] Cosmid-complemented variant of *tcpA*::Km^R#1 mutant.

A small study was performed to determine the retention rate of the cosmid *in vivo*. Five mice were fed ca. 2 x 10^5 H1 *tcpA*::Km^R[pPM2608] and after 24h the small intestines were collected, homogenised and dilutions plated onto NA, Km and Ap plates. For each mouse the numbers of Ap^R and Km^R organisms recovered were ca.10^4-fold fewer than the NA counts (data not shown). It seemed that homologous recombination between the cosmid and the chromosome had occurred *in vivo*, restoring a functional chromosomal *tcpA* gene. Southern blot analysis of some of the Km^S, Ap^S organisms recovered confirmed loss of the Km^R cartridge and showed that the *tcpA* probe hybridised to a fragment identical in size to that detected in the wild-type parent (S.R. Attridge, personal communication).

4.4.2 Colonising capacity of *tcpA*::Km^R mutants

Competition experiments were performed to determine whether loss of TcpA/TCP production altered the ability of the four strains to colonise the small intestine. Infant mice were fed a mixed inoculum containing approximately equal doses of parent and isogenic *tcpA* mutant, the dose of each strain corresponding to about $10LD_{50}$ of the wild-type parent.

The results of the competition experiments complemented the findings of the virulence studies. For three of the four El Tor strains, the *tcpA*::Km^R mutants were found to have a dramatically reduced ability to colonise the infant mouse gut. For each mutant vibrio recovered from the small intestine 24h after dosing, 10^4-10^5 wild-type bacteria were present (Figure 4.4). These ratios were greater than that obtained for a classical strain (Z17561) and its isogenic *tcpA*::Km^R mutant which were included for comparison.

The effect of the *tcpA* mutation was less dramatic, although still significant, in the O17 strain. For reasons which are not yet understood O17 *tcpA* mutants were more able to persist in the

Figure 4.4 Comparison of the *in vivo* persistence of the *tcpA*::Km^R mutants and wild-type parents.

Data show the results of competition experiments performed between a parent strain and each of two isogenic *tcpA* mutants. Each dot represents the ratio of parent:mutant bacteria recovered from an individual mouse; circles show median values. Note that there are two different scales on the vertical axes. Input ratios were as follows: H1: *tcpA*::Km^R#1, 1:1

H1: *tcpA*::Km^R#2, 0.71:1

N16961: *tcpA*::Km^R#1, 0.83:1

N16961: *tcpA*::Km^R#2, 0.77:1

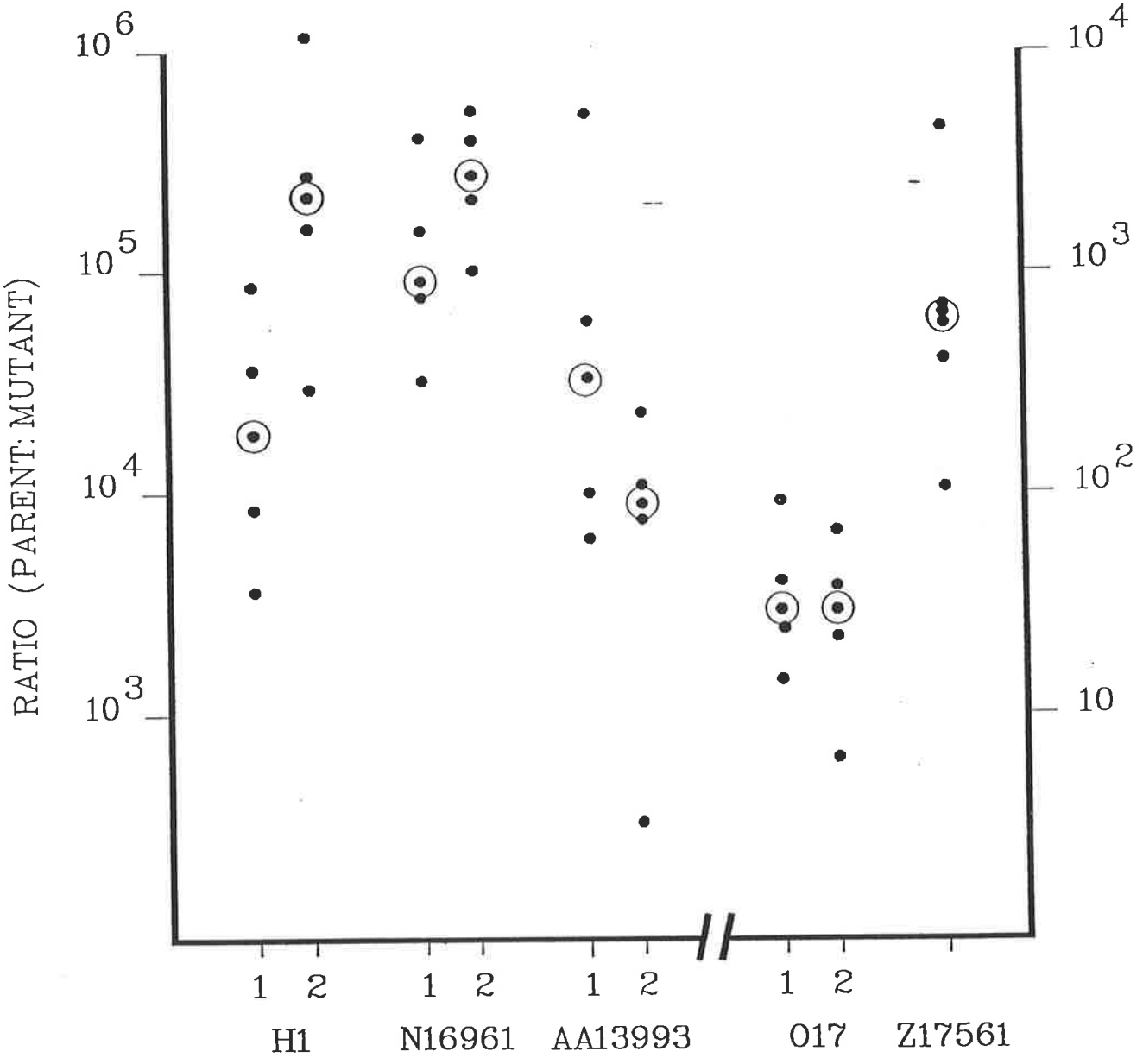
AA13993: *tcpA*::Km^R#1, 0.56:1

AA13993: *tcpA*::Km^R#2, 0.83:1

O17: *tcpA*::Km^R#1, 1.3:1

O17: *tcpA*::Km^R#2, 1.1:1

Z17561: *tcpA*::Km^R, 0.83:1


RATIO (PARENT: MUTANT)
10^6
10^5
10^4
10^3
10^4
10^3
10^2
10
1 2
1 2
1 2
1 2
H1
N16961
AA13993
017
Z17561

small intestine than mutants of the other three strains, with recovery ratios of wild-type:mutant being ca.30:1 (Figure 4.4).

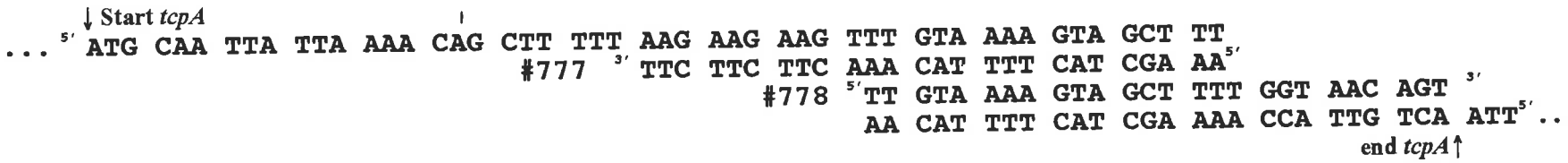
4.5 Construction of El Tor in-frame *tcpA* mutants

An in-frame deletion of the H1 *tcpA* gene (Δ*tcpA*) was created using the recombination polymerase chain reaction (RPCR) technique described by Coco *et al* (1993), which is a modification of the method described by Jones and Howard (1991). Two 26mer primers were made, which either bound within 42 base pairs of the 5' end (oligo 777) or within 18 base pairs of the 3' end of the *tcpA* gene (oligo 778, see Figure 4.5). The primers have complementary 5' ends, which following amplification create a product with ends that have a 16 base pair overlap. This overlap allows the linear PCR product to undergo recombination *in vivo* following electroporation into *E. coli*. The amplification reaction was carried out according to the protocol described in section 2.16.2 using as template for the reaction the ca. 5.9kb plasmid pPM3353, which carries a 3.27kb *Bcl*1 fragment harbouring the H1 *tcpA* gene. PCR amplification resulted in a ca. 5.3kb double-stranded linear product encoding all of pPM3353 except the ca. 600bp region between the primers (Figure 4.5a).

Following electroporation into DH5α, recircularised plasmid - pPM3360 - was recovered. Sequencing confirmed that the desired deletion had been made, leaving the nucleotide sequence in-frame. To overcome the possibility of having introduced mutations elsewhere in the (now 2.66kb) *Bcl*1 fragment during PCR amplification, a 0.253kb *Eco*RV fragment spanning only the deleted *tcpA* gene was subcloned from pPM3360 into *Eco*RV-digested pPM3353 to produce pPM3363. The 2.66kb *Bcl*1 fragment from pPM3363 was then cloned into the *Bam*H1 site of pBluescript-SK (pPM3367).

Figure 4.5a Construction of the in-frame deletion in the H1 *tcpA* gene using RPCR

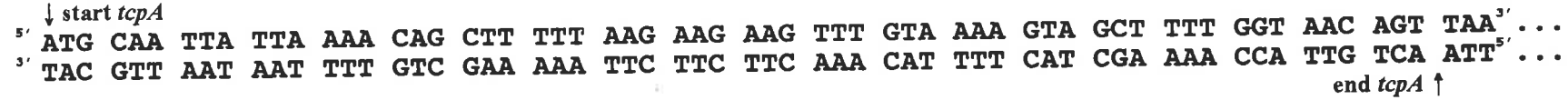
The diagram shows the remaining sequence of TcpA following PCR amplification using oligos #777 and #778. Recombination and repair *in vivo* via the homologous ends of the resultant linear ds DNA product completes the construction of the in-frame deletion within *tcpA*.



Transformation of linear ds DNA product into *E. coli*

↓

in vivo recombination and repair

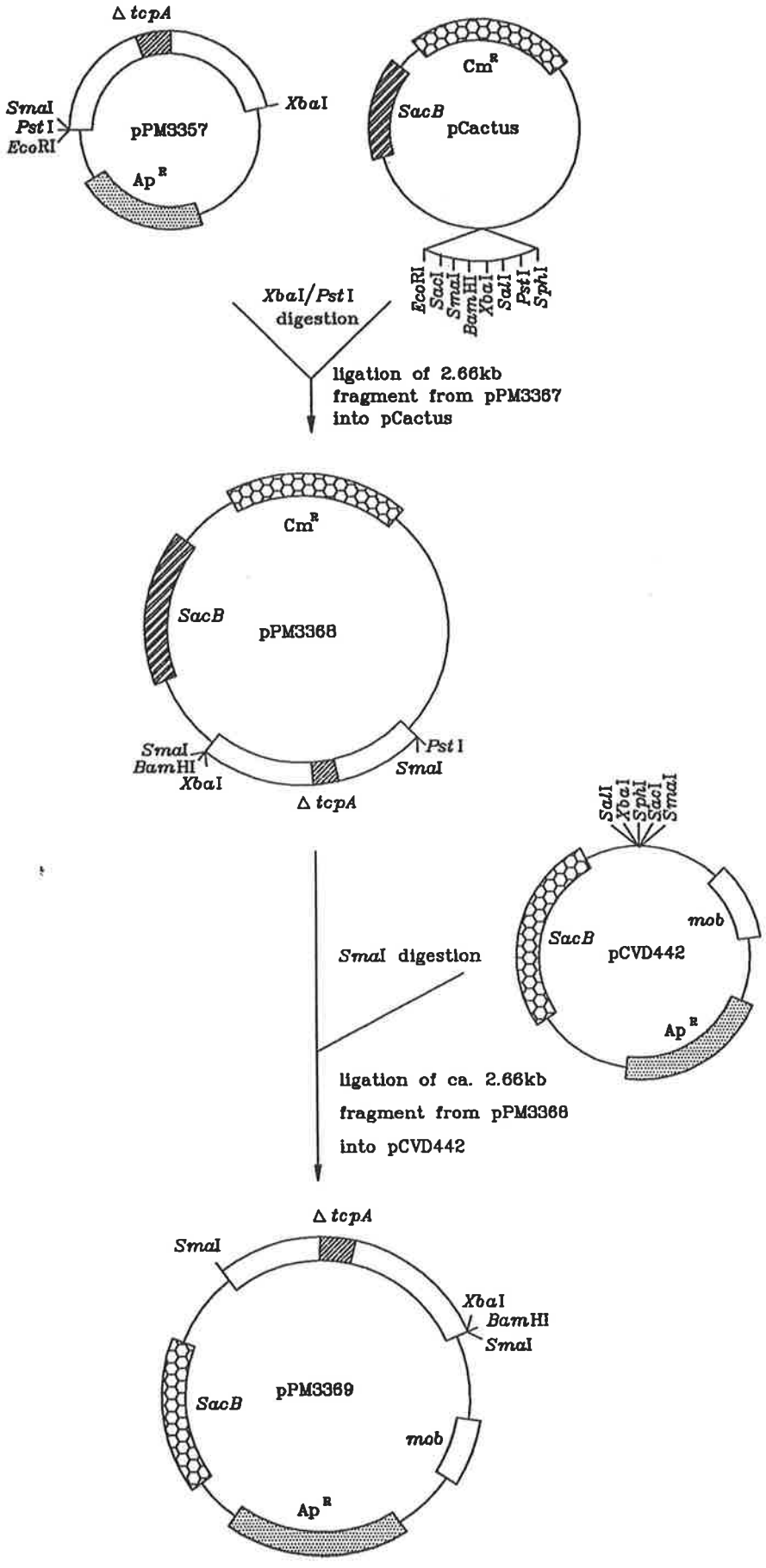


In-frame deletion of 612bp of *tcpA* leaving only 60bp

A 2.66kb *Pst*1-*Xba*1 fragment carrying the mutated *tcpA* gene from pPM3367 was cloned into the temperature-sensitive, suicide vector pCACTUS (C. Clark, personal communication) to yield plasmid pPM3368. When grown at non-permissive temperatures (≥37°C) pCACTUS is unable to replicate and can only persist if it can recombine into the host chromosome via the regions of homology provided by the insert. This vector also contains the *sacB* gene of *Bacillus subtilis* which allows positive selection for loss of the plasmid on exposure to sucrose. The *sacB* gene product converts sucrose to a lethal product, and so only those cells in which the vector sequences have been resolved from the chromosome by a double recombination event will survive growth at 37°C in the presence of sucrose (Blomfield *et al*, 1991). However, when pPM3368 was electroporated into *tcpA*::Km^R mutants of the four El Tor strains the electroporation efficiency was extremely low; in addition plasmid-bearing mutants grew very slowly, taking several days for colonies to become visible. After several unsuccessful attempts to isolate in-frame deletion mutants using pPM3368 this approach was abandoned.

Subsequently, an alternative suicide vector pCVD442 (Donnenberg and Kaper, 1991) was employed. pCVD442 has an R6K replicon which requires a helper protein, the pir protein, for replication. Consequently when introduced into a pir-negative strain the plasmid must integrate into the host chromosome via regions of homology provided by the insert or it will be lost. Like pCACTUS, pCVD442 has the *sacB* gene and also has the advantage of having the conjugal functions of plasmid RP4, making its transfer to *V. cholerae* more efficient than electroporation. A ca. 2.66kb *Sma*I fragment spanning the deleted *tcpA* gene was subcloned from pPM3368 into the *Sma*I site of the pCVD442 polylinker thus creating plasmid pPM3369 (Figure 4.5b). pPM3369 was conjugated via S17-1λ*pir* into one *tcpA*::Km^R mutant of each of the four El Tor strains. Exconjugants were selected on Pmx, Ap plates. For each strain, two

Figure 4.5b Construction of pPM3369


Δ tcpA
SmaI
PstI
EcoRI
pPM3357
XbaI
Ap R
Cm R
SacB
pCactus
EcoRI
SacI
SmaI
BamHI
XbaI
SalI
PstI
SphI
XbaI/PstI
digestion
ligation of 2.66kb
fragment from pPM3367
into pCactus
Cm R
SacB
pPM3368
SmaI
BamHI
XbaI
PstI
SmaI
Δ tcpA
SalI
XbaI
SphI
SacI
SmaI
mob
SacB
pCVD442
Ap R
SmaI digestion
ligation of ca. 2.66kb
fragment from pPM3368
into pCVD442
Δ tcpA
SmaI
XbaI
BamHI
SmaI
SacB
pPM3369
mob
Ap R

10ml Luria broths were inoculated with a single exconjugant and incubated overnight at 37°C in the absence of antibiotics. Dilutions of these cultures were plated onto Luria agar containing 6% sucrose but no added NaCl (which would otherwise protect the vibrios from the lethal effect of *sacB* expression; Blomfield *et al*, 1991). Comparison of cell counts on sucrose-containing and control plates revealed that ca. 97-99% of the bacteria were sucrose-sensitive and therefore had retained pCVD442 in the chromosome. For each strain, 100-200 sucrose-resistant colonies were patched onto nutrient agar and screened for sensitivity to Ap and Km. Four Km^S,Ap^S colonies were further screened by Southern blot to confirm insertion of the in-frame deletion and loss of the *tcpA*::Km^R mutation (Figure 4.6). Two Δ*tcpA* mutants of each strain were then selected for further study.

Prior to being assessed in the IMCM the Δ*tcpA* mutants were screened for *in vitro* production of virulence determinants as previously described for the *tcpA*::Km^R mutants (Section 4.3). The H1, N16961 and O17 Δ*tcpA* mutants showed no significant variation from their parent strains. However the AA13993 Δ*tcpA* mutants were found to be defective in the production of cholera toxin and therefore were omitted from the *in vivo* studies.

An attempt was made to complement the Δ*tcpA* mutation using a fragment encoding *tcpA* and its precoding region. Plasmid pPM3375 was constructed by cloning a 3.2kb *Bcl*1 fragment spanning the H1 *tcpA* gene (as well as *tcpH* and *tcpB*) into the *Bam*H1 site of pPM2182 (pGB2-*mob*; Williams *et al*, 1993). pPM3375 was mobilized into the Δ*tcpA* mutants of the H1, N16961 and O17 strains. Western blot analysis revealed TcpA was produced by N16961 Δ*tcpA*[pPM3375] and H1 Δ*tcpA*[pPM3375] strains but not by the O17 Δ*tcpA*[pPM3375] strains (Figure 4.7). The latter finding was not unexpected as the parent strain does not produce TcpA *in vitro* and expression of *tcpA* from pPM3375 was designed to be from its own promoter and under the control of the host cell *toxR* regulon.

Figure 4.6 Southern blot of El Tor parent strain, *tcpA*::Km^R mutants and Δ*tcpA* mutants.

Samples: **A**, H1

B, H1 *tcpA*::Km^R #1

C-F, H1 Δ*tcpA* #1-#4

G, O17

H, O17 *tcpA*::Km^R #1

I-L, O17 Δ*tcpA* #1-#4

M, AA13993

N, AA13993 *tcpA*::Km^R #1

O-R, AA13993 Δ*tcpA* #1-#4

S, N16961

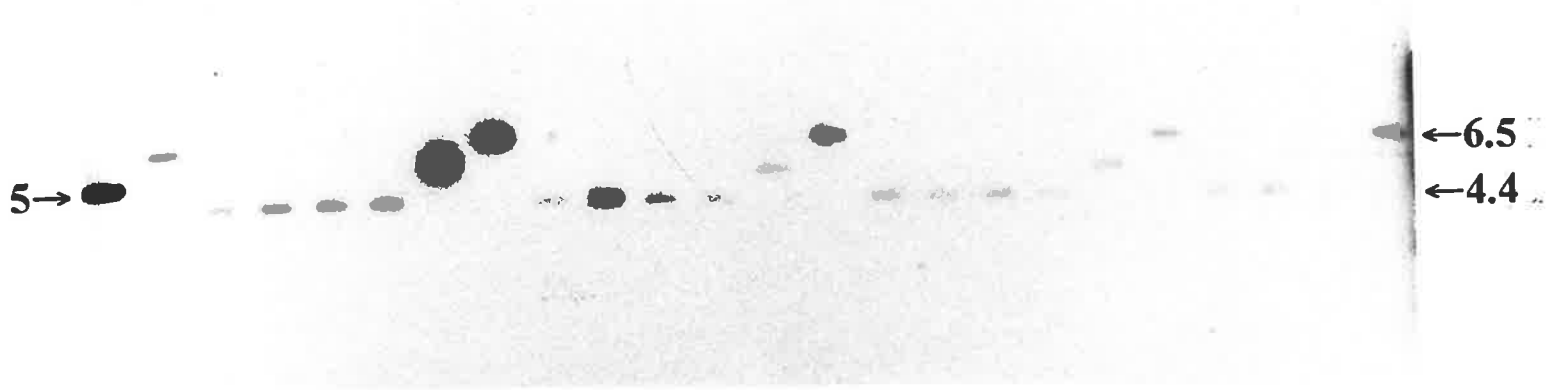
T, N16961 *tcpA*::Km^R #1

U-Y, N16961 Δ*tcpA* #1-#4.

Blot 1 was detected using *tcpA* fragment probe (5kb *Xba*I fragment harbouring H1(El Tor) *tcpA*). Blot 2 was detected using the Km^R cartridge probe. Arrows in blot 1 indicate the position of fragments detected in wild-type parents (5kb), Δ*tcpA* mutants (4.4kb) and *tcpA*::Km^R mutants (6.5kb).

1

A B C D E F G H I J K L M N O P Q R S T U V X Y



2

A B C D E F G H I J K L M N O P Q R S T U V X Y

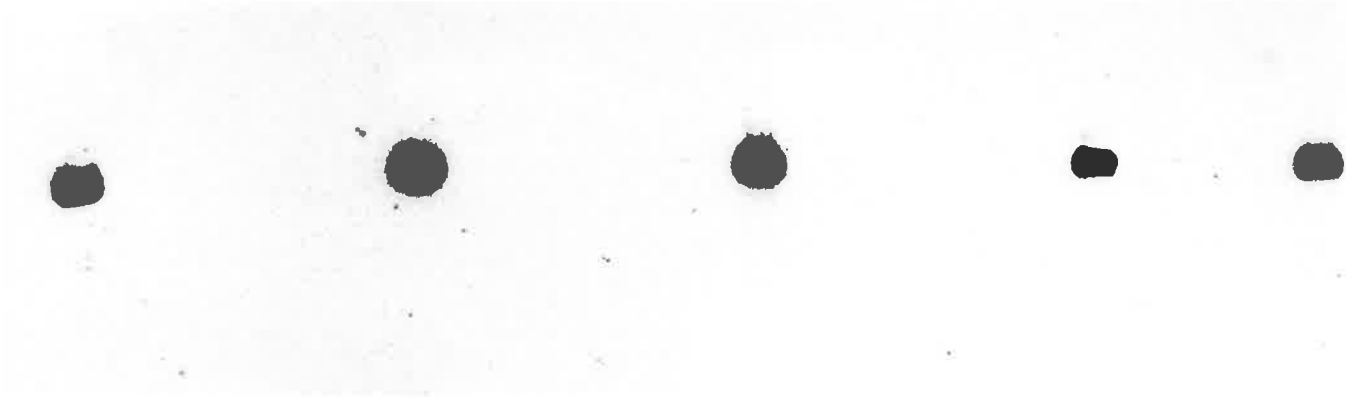


Figure 4.7 Immunoblot of El Tor parent strains, Δ*tcpA* mutants and Δ*tcpA*[pPM3375] strains.

Samples: **A**, SA42 OMP

B, H1

C, H1 Δ*tcpA* #1

D, H1 Δ*tcpA* #1[pPM3375]

E, N16961

F, N16961 Δ*tcpA* #1

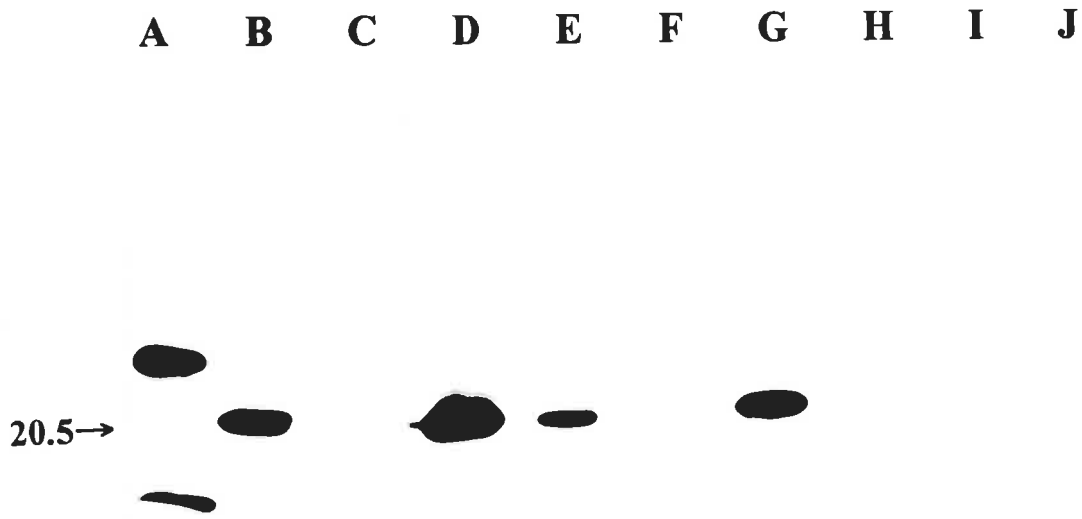
G, N16961 Δ*tcpA* #1[pPM3375]

H, O17

I, O17 Δ*tcpA* #1

J, O17 Δ*tcpA* #1[pPM3375]

All tracks were loaded with ca. 10^9 bacteria with the exception of track A which was loaded with ca. 30μg protein.


A B C D E F G H I J
20.5→

Prior to being assessed in the infant mouse cholera model the Δ*tcpA* mutants and their pPM3375-carrying derivatives (Δ*tcpA*[pPM3375]) were assayed for the same putative virulence determinants as mentioned previously in Section 4.3. No significant variation from wild-type was found.

4.6 *In vivo* behaviour of Δ*tcpA* mutants

4.6.1 Virulence studies

LD_{50} assays were performed to assess the virulence of the Δ*tcpA* mutants in the presence or absence of the pPM3375 plasmid. The Δ*tcpA* mutants were as attenuated as their isogenic *tcpA*::Km^R progenitors, showing an ca.10^4-fold increase in LD_{50} (Table 4.3). The attempt to complement the mutation with a *tcpHAB* fragment was only partially successful. pPM3375 increased the virulence of the N16961 Δ*tcpA* and H1 Δ*tcpA* mutants (80-fold and 12-fold respectively), however the O17 Δ*tcpA*[pPM3375] strain was only marginally less attenuated (Table 4.3).

pPM2182 (Table 2.2) was chosen as the vector for complementation because a previous study had suggested this plasmid was stably maintained *in vivo* (S.R. Attridge, personal communication). However it was necessary to ascertain if in this instance, poor complementation was related to plasmid instability. Five infant mice were fed an inoculum of each Δ*tcpA*[pPM3375] strain, using a dose equivalent to ca.10 LD_{50}s of the wild-type parent, and after 24h their small intestines were collected and homogenised. Dilutions of the homogenate were plated onto nutrient agar and Sp agar. The recovery of Sp^R organisms was lower than expected; only 10-15% of vibrios recovered from mice fed H1 Δ*tcpA*[pPM3375] or N16961 Δ*tcpA*[pPM3375] retained pPM3375 whereas 55% of the organisms recovered from mice inoculated with O17 Δ*tcpA*[pPM3375] carried pPM3375. So, while plasmid instability

Table 4.3 Virulence of El Tor strains, Δ*tcpA* mutants and Δ*tcpA*[pPM3375] mutants.

Strain	Wildtype	Δ*tcpA*	Δ*tcpA*[pPM3375]
O17	1.7×10^{4}[a]	$>2.5 \times 10^{8}$	6.0×10^{7}
H1	1.4×10^{4}	9.3×10^{7}	7.7×10^{6}
N16961	2.4×10^{4}	3.8×10^{8}	5.0×10^{6}

[a] virulence is expressed as 48-hour LD_{50} dose.

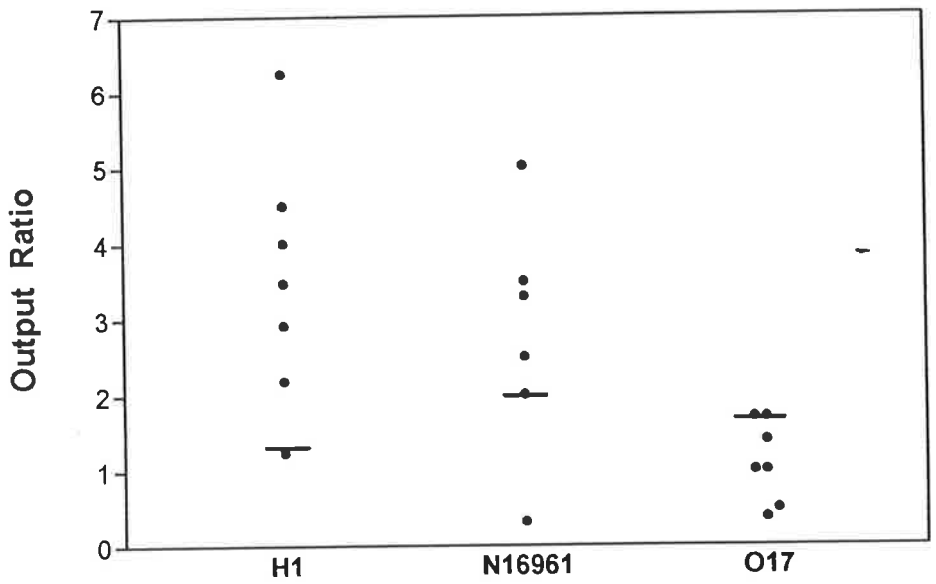
may be a (minor) contributing factor to the incomplete complementation of the H1 and N16961 Δ*tcpA* mutants, the mutant in which pPM3375 had the least effect (O17 Δ*tcpA*) paradoxically showed the highest retention rate.

4.6.2 Colonising capacity of Δ*tcpA* mutants

The ability of the *tcpA*::Km^R mutants to persist in the infant mouse gut had been drastically reduced by the disruption of the *tcp* operon. To ascertain if the effect of the in-frame mutation in *tcpA* was as severe, competition experiments were initially performed between the isogenic *tcpA*::Km^R and Δ*tcpA* mutant pairs. Differential counts were obtained by plating dilutions of gut homogenate on nutrient agar and Km agar. The H1 Δ*tcpA* mutant displayed a marginal advantage over its isogenic *tcpA*::Km^R counterpart (median ratios Δ*tcpA*:*tcpA*::Km^R were 0.77:1 input ratio; 3.5:1 output ratio) however the N16961 and O17 Δ*tcpA* mutants displayed no difference in colonising capacity to their isogenic *tcpA*::Km^R strains (Figure 4.8).

An attempt was made to differentiate Δ*tcpA* mutant vibrios from wild-type organisms by performing colony blots probed with an oligo to the region of *tcpA* deleted in the mutants. However this approach was found to be impractical and inappropriate due to the dramatically diminished colonising capacity of the Δ*tcpA* mutants. Therefore prior to comparing the Δ*tcpA* mutants with their wild-type parents in competition experiments, it was necessary to select antibiotic-resistant variants of the former to allow separate enumeration of the two strains. Spontaneous streptomycin-resistant (St^R) variants of the H1 and N16961 Δ*tcpA* mutants were isolated and screened for cholera toxin production prior to use in infant mice, as we have sometimes found defective toxin production in spontaneous antibiotic-resistant variants. The O17 strain is already St^R and so attempts were made to isolate spontaneous Sp^R variants of the

Figure 4.8 Comparison of the *in vivo* persistence of Δ*tcpA* and *tcpA*::Km^R mutants. Data show the results of competition experiments performed between Δ*tcpA* and isogenic *tcpA*::Km^R mutants. Each dot represents the ratio of Δ*tcpA*: *tcpA*::Km^R bacteria recovered from an individual mouse; bars indicate the input ratios for each strain pair.


Output Ratio
7
6
5
4
3
2
1
0
H1
N16961
O17

O17 Δ*tcpA* mutants. Unfortunately the variants selected were found to have altered *in vitro* growth rates and could not be used for this study.

The H1 and N16961 mutants were subsequently assessed in competition experiments with their wild-type parent strains. As expected on the basis of their marked attenuation, the colonising capacity of the Δ*tcpA*^{StR} variants was severely diminished (Figure 4.9).

4.7 Protective efficacy of antibodies raised against El Tor TcpA

The preparation of two antisera raised against native El Tor TcpA, which enabled the demonstration of TCP on the surface of El Tor strains, is described in Section 3.6. The availability of these reagents - anti-H1 TcpA and anti-EV37 TcpA sera - made it possible to determine whether antibodies to El Tor TcpA were protective in the infant mouse model. Although the sera were extensively absorbed to be TcpA-specific, they are not biotype-specific and so it was of interest to compare their protective activities against both classical and El Tor challenge strains. In addition, protection tests were performed using antibodies to classical TCP ('clone probing serum' (CPS); Sharma *et al*, 1989b).

Challenge organisms were grown under conditions which induce or repress TCP production as the studies of Sharma *et al* (1989a) had revealed that antibodies to TCP are more protective if the challenge organisms are expressing these pili. Aliquots of the challenge suspensions were pre-treated with various dilutions of the test sera and administered orally to different groups of mice; control mice received untreated bacteria. The protective efficacy of the anti-H1 TcpA and anti-EV37 TcpA reagents was dependent on the culture conditions and the biotype of the challenge strain; protection was only seen when using El Tor challenge organisms which had been grown under TCP-expressing conditions (Table 4.4). These sera were unable to protect

Figure 4.9 Comparison of the *in vivo* persistence of Δ*tcpA*^{StR} mutants and wildtype parents.

Data show the results of competition experiments performed between Δ*tcpA*^{StR} mutants and their wild-type parents. Each dot represents the ratio of parent:mutant bacteria recovered from an individual mouse. Input ratios were as follows:

H1 : H1Δ*tcpA*^{StR}, 1:1.3

N16961 : N16961 Δ*tcpA*^{StR}, 1:1.1

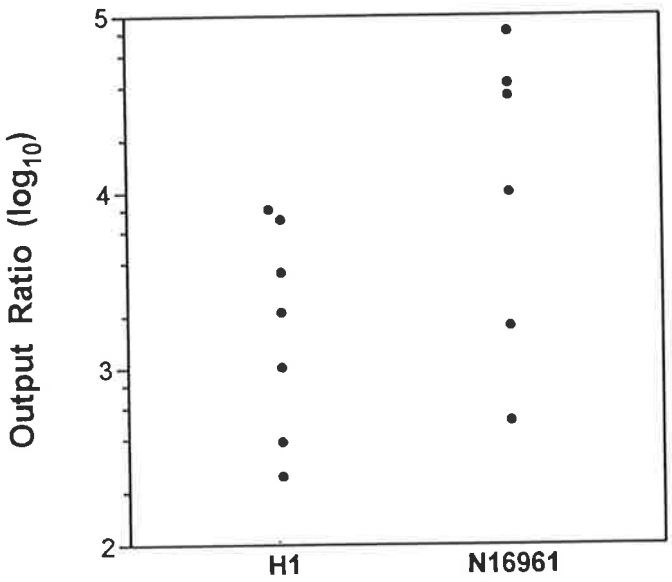

Output Ratio (log_{10})
5
4
3
2
H1
N16961

TABLE 4.4 Protective efficacies of antibodies to TCP

	Serum		
Challenge strain	**Anti-H1 TcpA**	**Anti-EV37 TcpA**	**CPS**
V. cholerae O1			
H1 (El Tor)	375	570	<20
N16961 (El Tor)	450	360	<20
569B (classical)	<20	<20	275
V.cholerae O139			
AI-1838	NT	340	NT
AI-1854	NT	195	NT

Figures show protectives titres (PD50 values) and represent the mean of 2 or 3 determinations, except that N16961 tests were performed only once. Challenge strains were cultured in CO_2-gassed AKI medium to promote TCP expression. None of the sera were protective against challenge with H1 or N16961 vibrios grown in NB (titres all <20). NT, not tested.

mice against challenge with classical O1 *V. cholerae* (strain 569B) or against TCP-negative El Tor vibrios (Table 4.4).

The CPS reagent had protective titres of 280 and 165 against challenge with TCP-positive or TCP-negative 569B respectively, but failed to protect against challenge with El Tor vibrios regardless of their TCP status.

The protective efficacy of the anti-EV37 TcpA reagent was also tested against challenge with three strains of the O139 serovar. In Section 3.7 it was reported that some O139 strains were found to produce TcpA/TCP recognised by antibodies raised against El Tor TcpA. The prospect that strains of the O139 serovar will be the agents of the eighth cholera pandemic made it of particular relevance to ascertain whether TcpA is a protective antigen of this serovar. When the O139 strains were cultured under conditions conducive to TCP expression, the protective titres (PD_{50}) of the anti-EV37 TcpA were similar to those seen with O1 El Tor challenge strains (Table 4.4).

4.8 Discussion

The construction of *tcpA* mutants in El Tor strains has permitted an evaluation of the importance of TCP in the pathogenesis of this biotype, at least in an animal model. The data presented in this chapter clearly indicate that TCP are critical for *in vivo* persistence and virulence of El Tor O1 strains.

Initially *tcpA*::Km^R mutants were constructed in four El Tor strains. Two of the strains selected (H1 and N16961) were chosen because they produce abundant TcpA/TCP *in vitro*, whereas the other two strains (O17 and AA13993) do not. When assessed in the IMCM each mutant was found to be dramatically attenuated; clearly the capacity to produce TcpA/TCP *in vitro* does not relate to the *in vivo* significance of the *tcp* operon. Subsequent competition

experiments revealed that the marked attenuation of the *tcpA*::Km^R strains was the result of their drastically reduced capacity to colonise the infant mouse gut. When compared with wild-type in mixed-infection competition experiments, the median recovery ratios of wild-type:mutant for the H1, N16961 and AA13993 strains ranged from 8,600:1 to 260,000:1. These ratios were far greater than that observed with a classical strain pair included for comparison. This was an unexpected finding for which there is currently no explanation.

Compared with the other *tcpA*::Km^R mutants, the two O17 mutants were better able to persist *in vivo* and yet displayed the same degree of attenuation as mutants in the other three El Tor strains. No explanation has yet been found for the atypical behaviour of the O17 mutants. It should be noted that of the four El Tor strains studied O17 is the only one which is not a recent field isolate and is unusual in that it auto-agglutinates in liquid culture. The agglutinin responsible for this phenomenon is unknown but could conceivably be an alternative colonisation factor which can compensate in part for the loss of TcpA. However, since the O17 mutants show the same dramatic attenuation as the other *tcpA*::Km^R mutants, it would be necessary to propose that the putative second colonization factor binds to different host receptors from TCP, leading to less efficient delivery of toxin. However at this time the existence of an additional colonisation factor in O17 is purely speculative. Although it has been proposed that MSHA may be an adhesin for strains of the El Tor biotype (Jonson *et al*, 1991b), there was no indication from the *in vitro* assays of MSHA activity that O17 is exceptional in this respect (data not shown).

Since the (probably) polar nature of the *tcpA*::Km^R mutation made it implausible to attempt complementation using a minimal-sized fragment encoding only *tcpA*, a cosmid encoding the entire El Tor *tcp* operon was mobilized into the mutants. pPM2608 restored the *tcpA*::Km^R mutants to virtually wild-type virulence. In ascertaining the retention rate of the cosmid *in*

vivo it was found that while large numbers of organisms were recovered from the mouse gut, only a very small proportion retained the Ap^R marker of the cosmid or were Km^R. This finding was interpreted as indicating recombination between the cosmid and the mutated host chromosome had occurred, restoring the strains to a wild-type phenotype/genotype. This was subsequently confirmed by Southern analysis using probes to *tcpA* and the Km^R cartridge (S. Attridge, personal communication).

Although these data showed that a mutation in *tcpA* could render El Tor strains virtually avirulent it was not possible to unequivocally attribute the attenuation of the *tcpA*::Km^R mutants to the defect in *tcpA* alone. It is thought that the major transcriptional unit within the *tcp* region begins with *tcpA* and continues through to *tcpJ* and probably into the *acf* gene cluster (Ogierman *et al*, 1993; Brown and Taylor, 1995). It has been suggested that *tcpA* is also expressed from an additional transcript beginning prior to *tcpA* and terminating at a putative RNA processing site immediately following *tcpA* (Ogierman *et al*, 1993). Therefore the probable disruption to the expression of downstream genes in the *tcp* operon could also have contributed to the marked attenuation observed.

In order to less ambiguously assess the effect of inactivating *tcpA*, strains in which the *tcpA* gene was inactivated by a large in-frame deletion (Δ*tcpA*) were constructed in the expectation that such a mutation would not affect expression of downstream genes in the *tcp* operon. Δ*tcpA* mutants were made from a *tcpA*::Km^R mutant of each of the four El Tor strains, but the AA13993 Δ*tcpA* mutants were subsequently excluded from the *in vivo* studies because of their defective *in vitro* production of cholera toxin. The remaining Δ*tcpA* mutants were found to be as severely attenuated as their isogenic *tcpA*::Km^R progenitors (Table 4.3). When the two types of mutants were compared in competition experiments neither mutant showed a significant advantage (Figure 4.8). The Δ*tcpA*^{StR} were subsequently also found to be unable to

compete with their wild-type parents with output ratios showing a 10^3-10^4-fold greater recovery of the latter (Figure 4.9).

In an attempt to complement the defect in the in-frame deletion mutants a fragment carrying the El Tor *tcpHAB* region (with the expression of *tcpA* under the control of its own promoter) was cloned into a low copy number vector (pPM2182) previously found to be well retained *in vivo* (S.R. Attridge, personal communication). The resultant plasmid pPM3375 complemented TcpA/TCP production in the H1 and N16961 Δ*tcpA* strains *in vitro*. Although not quantitative, Western blot analysis of these Δ*tcpA*[pPM3375] mutants suggested that they produced as much TcpA as their wild-type parents (Figure 4.7). Furthermore IEM analysis revealed TCP bundles on the surface of these strains (data not shown), again suggesting that the mutation is fully complemented *in vitro*. The O17Δ*tcpA*[pPM3375] strain did not produce TcpA however this was not unexpected, as O17 does not synthesize this protein *in vitro* and the plasmid-encoded *tcpA* gene is under the control of its own promoter and therefore dependent on the host cell *toxR* regulon.

pPM3375 partially complemented the virulence defect of two of the Δ*tcpA* mutant strains. The LD_{50} s of the H1 and N16961 Δ*tcpA*[pPM3375] mutants were 12-fold and 80-fold lower respectively. The reason for the incomplete restoration of virulence remains unclear, but it seems that plasmid instability *in vivo* was not a major contributing factor. Only 10-15% of organisms recovered from mice infected with the H1 and N16961Δ*tcpA*[pPM3375] strains retained the plasmid, yet even with this level of plasmid retention, greater restoration of virulence would be expected. Furthermore, O17 Δ*tcpA*[pPM3375] had the highest rate of plasmid retention yet showed the lowest increase in virulence. Recombination *in vivo* does not appear to have been a common event as the overall recovery of organisms from the intestines of mice infected with Δ*tcpA*[pPM3375] strains was very low compared with mice inoculated

with cosmid-complemented *tcpA*::Km^R mutants (data not shown). Presumably homologous recombination events between the host cell chromosome and pPM3375 were infrequent because of the much smaller region of homology (ca. 2kb) in comparison with the cosmid pPM2608, which has ca. 35kb of DNA with homology to the chromosome.

It is possible that the in-frame mutation in *tcpA* may have reduced the stability of the polycistronic mRNA transcript which begins from *tcpA* and ends probably continues through to the *acf* gene cluster (Brown and Taylor, 1995), thereby affecting the expression of factors required for TCP assembly. Parsot and Mekalanos (1991) suggested that decreased stability of the *tcp* polycistronic mRNA might explain the reduced expression of *tcpC* in *tcpD* and *tcpE* mutants. It is not possible on the basis of IEM to be confidant that TCP assembly is fully restored in the H1 and N16961 Δ*tcpA*[pPM3375] strains. A quantitative assay such as ELISA-inhibition (using whole bacteria) would be necessary to resolve this issue.

Alternatively, it is possible that TCP are expressed at normal (wild-type) levels on the surfaces of the H1 and N16961 Δ*tcpA*[pPM3375] strains, but that the colonisation potential of these pili is impaired in some way. A precedent for this exists in the work of Peek and Taylor (1992) in which the role of TcpG as a thiol-disulphide interchange protein was determined. *tcpG* mutants were found to assemble TCP, however these pili were non-functional. This was thought to result from the TcpA subunits assuming an incorrect conformation in the absence of TcpG, but still being polymerised into pili (Peek and Taylor, 1992). There is no reason to believe that expression of *tcpG* has been affected by our Δ*tcpA* mutation since the two genes are not linked. It also seems unlikely that *tcpT*, *tcpE* and *tcpF* expression has been disrupted as mutations in any of these genes results in no assembly of TcpA (J. Iredell, personal communication; Kaufman *et al*, 1993). However whether expression of *tcpBQCRD* has been

altered is unknown, nor is it known how disrupted expression of these genes might affect TCP function.

It was of great interest to evaluate the protective efficacy of antibodies to El Tor TCP. Several studies using the IMCM have demonstrated TCP to be a protective antigen of classical *V. cholerae* (Sharma *et al*, 1989a; Sun *et al*, 1990a and b; Osek *et al*, 1994), but at the commencement of these studies there were no strong data to indicate that TCP are also a protective antigen of El Tor strains. The failure of Sharma and co-workers (1989a) to demonstrate any protective effect of antibodies to TCP against El Tor strains can probably be ascribed to the use of inappropriate growth conditions and antiserum. At the time it was not known how to culture El Tor strains to induce TCP expression, nor had the epitope differences between the two biotypic forms of TcpA been described. Other studies which showed at best very marginal protection against *V. cholerae* O1 El Tor are subject to the same criticism concerning the use of inappropriate antibodies.

Sun *et al* (1990 a and b) demonstrated passive protection of infant mice against El Tor challenge organisms using both a polyclonal rabbit antiserum and a monoclonal antibody, MAb 169.1, both raised against classical TCP. At a dilution of 1:100 the polyclonal anti-TCP serum was protective against challenge with classical *V. cholerae*. The same serum was reported to be 100% protective against challenge with El Tor organisms but only at a dilution of 1:2. However, the protective efficacy of the pre-immune serum at a 1:2 dilution was 37% (Sun *et al*, 1989a), and so one must question the significance of the level of protection demonstrated against El Tor challenge. Similarly, 50% of infant mice survived an inoculum of El Tor organisms which had been pre-treated with MAb 169.1-containing ascitic fluid at a dilution of

only 1 in 2, compared with 11% of mice receiving bacteria pre-incubated with a control ascitic fluid. The authors suggested the reduced level of protection against El Tor strains (compared with classical strains) was probably due to lower surface expression of TCP by strains of this biotype and/or antigenic variation between the two biotypic forms of TcpA (Sun *et al*, 1990b).

More recently, Osek *et al* (1994) assessed the protective effect of antibodies to TCP against challenge with *V. cholerae* O1 in the IMCM. A polyclonal antiserum and the monoclonal antibody MAb 20:2 were raised against classical TCP; both these reagents provided solid protection against classical challenge organisms but little or no protection against (TcpA-positive) El Tor strains. The polyclonal antiserum and MAb 20:2 detected classical and El Tor TcpA on Western blots. The reactivity of the polyclonal antiserum with TCP of either biotype was not reported, but in a previous study Jonson *et al* (1992) had shown by IEM examination that MAb 20:2 binds classical TCP but does not detect El Tor TCP. Therefore it was not surprising that MAb 20:2 failed to protect against challenge with El Tor strains. Osek *et al* (1994) did acknowledge that the reactivity of their antibodies may be reduced against El Tor TCP but still considered poor surface expression of TCP by strains of this biotype as the most likely explanation for the lack of protection observed.

The anti-El Tor TcpA reagents prepared as described in Section 3.6 and the CPS reagent formerly raised against classical TCP (Sharma *et al*,1989a), were assessed for their protective efficacy in the IMCM against challenge organisms of both biotypes. The results presented in Table 4.4 demonstrate TcpA to be a protective antigen of strains of the El Tor biotype. Both anti-H1 TcpA and anti-EV37 TcpA demonstrated significant protective titres of between 350 to 570 when the El Tor challenge organisms were TCP-positive. The same reagents failed to protect against TCP-negative challenge strains, confirming that the protective activity was directed against TCP. Furthermore, the anti-El Tor TcpA reagents did not protect against

challenge with TCP-expressing classical vibrios (PD_{50} titres <20; Table 4.4). Similarly CPS provided significant protection against challenge with TCP-positive classical vibrios (PD_{50} titre 275) but not against El Tor strains, regardless of their TCP status (PD_{50} titre <20).

Clearly protection afforded by antibodies to TcpA is biotype-specific which strongly suggests that antibodies to epitopes shared by the two biotypic forms of TcpA are not protective. The demonstration that TcpA is a protective antigen of the El Tor biotype implies that the theory that El Tor strains assemble less TCP on their surface than classical strains is probably incorrect or otherwise of little significance *in vivo*.

Having shown in Section 3.7 that strains of *V. cholerae* O139 produce El Tor-like TcpA/TCP, it was of interest to determine whether TcpA is also a protective antigen of this serovar. The anti-EV37 TcpA reagent was tested for its protective efficacy against challenge with TCP-positive O139 vibrios. The PD_{50} titres of anti-EV37 TcpA against two O139 strains was found to be 340 and 195 (Table 4.4), indicating that TcpA is a protective antigen of O139 strains in the IMCM. The results presented in section 4.7 have important implications for cholera vaccine development, as will be further discussed in Chapter 6.

Chapter 5 Regulation of *tcpA* expression in the El Tor strain O17

5.1 Introduction

Regulation of *tcpA* expression is known to be under the control of the global regulator ToxR. This trans-membrane DNA-binding protein was first discovered when it was found to directly activate *ctx* gene expression in *E. coli* (Miller and Mekalanos, 1984). Since then ToxR has been found to co-ordinately regulate the expression of a number of virulence associated factors and outer membrane proteins (Taylor *et al*, 1987; Peterson *et al*, 1988b). Regulation of expression occurs at the transcriptional level (Peterson *et al*, 1988b) but with the exception of the *ctx* and *toxT* genes, ToxR does not directly activate the expression of genes in the ToxR regulon. Instead a regulatory cascade exists whereby ToxR as the master regulator induces the expression of the transcriptional activator ToxT, which in turn directly activates the expression of many ToxR-regulated genes (DiRita *et al*, 1991). During the course of these studies the *toxT* gene was found to be located within the *tcp* operon (Higgins *et al*, 1992; Ogierman and Manning, 1992).

Several environmental cues have been identified as important in ToxR regulation of virulence gene expression, including temperature, pH, osmolarity and amino acid concentration (Taylor *et al*, 1987; Peterson *et al*, 1988b; Miller and Mekalanos, 1988). There is some evidence to suggest the ToxR protein may be an environmental sensor, assuming an active state when the appropriate conditions are encountered (Miller *et al*, 1987). It is well documented that the *in vitro* conditions which stimulate cholera toxin and TCP production vary between classical and El Tor *V. cholerae* and even between strains within the El Tor biotype (Iwanaga and

Yamamoto, 1985; also see Section 3.4). The basis for this regulatory difference has not been elucidated.

Although *V. cholerae* O17 does not produce TcpA during growth *in vitro*, O17 derivatives carrying cosmids spanning the classical *tcp* gene cluster do and moreover they assemble surface TCP (Sharma *et al*, 1989b). When these studies were commenced O17 was known to have DNA with homology to the classical *tcp* region (Voss, 1990), but the reason(s) for its failure to produce TcpA *in vitro* was not known. It was of great interest to ascertain whether TcpA produced by the cosmid clones was exclusively of classical biotype or whether under the influence of classical genes expressed *in trans* the chromosomal El Tor gene is also expressed. The availability of the biotype-specific anti-TcpA sera, anti-E1838 OMP and anti-SA42 OMP (Section 3.4), provided a means of answering this question.

5.2 TcpA and cholera toxin production by cosmid clones

Sharma *et al* (1989b) constructed a gene bank from *V. cholerae* Z17561 DNA in *E. coli* using a mobilizable cosmid vector. Following transfer of the gene bank (by conjugation) into *V. cholerae* O17, four TCP-producing cosmid-bearing derivatives were isolated. One of these, DS2, carries the cosmid pPM2103 which was shown to have DNA harbouring the *tcp* operon of Z17561 (Sharma *et al*, 1989b; Faast *et al*, 1989). For the present study, new cosmid-carrying derivatives of O17 were constructed to compare with the original strain DS2. In addition, it was decided to mobilize the cosmid into the H1 strain because, unlike O17, this strain produces TcpA and assembles TCP *in vitro* and it was of interest to know whether the presence of pPM2103 would enhance TcpA/TCP expression.

The cosmid pPM2103 was conjugated via *E. coli* into H1 and O17. Ex-conjugants were selected for Amp^R, Pmx^R. One H1 cosmid clone, EV35, and two new O17 derivatives, EV36

and EV37, were selected for further study. These strains, together with the original strain DS2, were grown in AKI broth and TcpA production was assessed by Western blotting using the biotype-specific anti-TcpA sera. As shown in Figure 5.1 both biotypic forms of TcpA were detected in EV35, EV36 and DS2, but only El Tor TcpA was detected in EV37. The amount of El Tor TcpA produced by EV35 did not appear to be greater than that produced by the parent strain H1.

IEM examination of these clones was also performed, using both anti-V9 TCP serum and the anti-H1 TcpA reagent (section 3.6), to determine whether both classical and El Tor TcpA proteins were being assembled into pili. With the exception of EV37 which produced and polymerised only El Tor TcpA, the cosmid-carrying strains produced TCP containing both classical and El Tor TcpA, as evidenced by gold-labelling of the bundles by both anti-TcpA reagents (not shown). At present it is not possible to determine whether individual pili made by EV35, EV36 and DS2 are comprised solely of either classical or El Tor TcpA or are hybrids of both monomers.

AKI culture supernatants of H1, O17 and their cosmid-bearing derivatives were assayed for cholera toxin content by GM_1-ELISA. The presence of pPM2103 was found to result in greatly increased cholera toxin secretion in the O17 cosmid-bearing derivatives DS2, EV36 and EV37. DS2 and EV36 produced approximately 70-fold more toxin than O17; the effect in EV37 was less pronounced, with a ca. 14-fold enhancement in toxin production. H1 and its derivative EV35 produced comparable levels of cholera toxin, indicating that the presence of the cosmid did not affect the production of either TcpA or toxin in this strain.

Figure 5.1 TcpA expression in O17 and H1 in the presence of pPM2103.

Samples: **1**, EV35 (H1[pPM2103])

2, H1

3, EV36 (O17 [pPM2103])

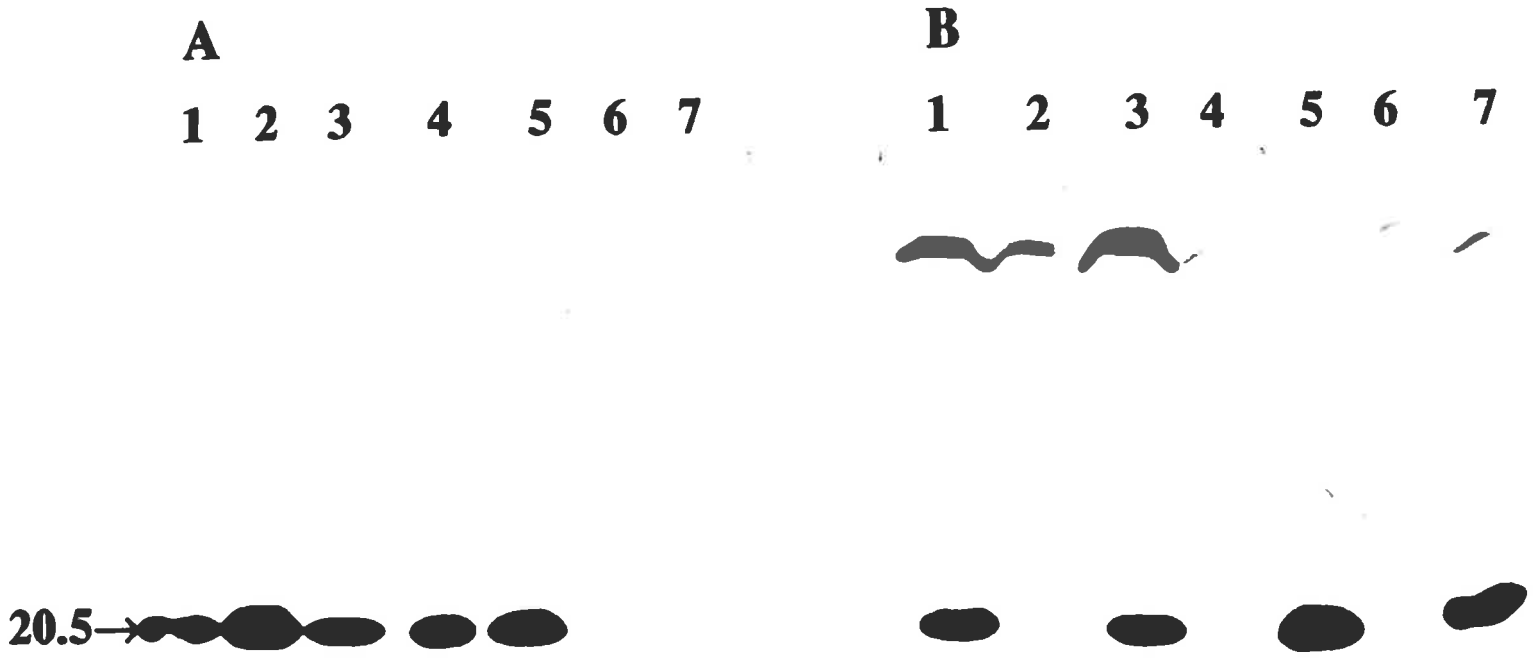
4, EV37 (O17 [pPM2103])

5, DS2 (O17 [pPM2103])

6, O17;

7, Z17561.

All tracks were loaded with ca. 10^9 bacteria. Blot A was detected with anti-SA42 OMP (diluted 1:500) and Blot B was detected with anti-E1838 OMP (diluted 1:1500). Arrow indicates the position of processed TcpA (20.5kDa).


A
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7
20.5→

5.3 Cosmid-encoded *toxT* activates TcpA and toxin expression in O17.

Western blot analysis of TcpA production by the O17 cosmid clones demonstrated that the host *tcpA* gene was being expressed. Additionally cholera toxin production was elevated in these clones compared with the parent strain. Clearly factor(s) encoded by the cosmid pPM2103 which are produced constitutively during *in vitro* growth in the presence of ampicillin, can activate chromosomal genes. Apart from ca.15kb of DNA which encompasses the *tcp* gene cluster, there are ca.20kb of cosmid DNA which have not been studied. Southern blot analysis showed neither *toxR* nor *ctx* genes were present on the cosmid (not shown). Anyway, O17 evidently has a functional *toxR* gene because O17 *toxR* mutants are attenuated in infant mice (S. Williams, personal communication) and secrete ca. 25-fold less cholera toxin than O17 *in vitro*. Further work suggested that expression of the cosmid-borne classical *tcp* region was also independent of chromosomal *toxR* function. When pPM2103 was conjugated into an O17 *toxR* mutant the resultant strains produced abundant TcpA (Figure 5.2) and showed ca.1000-fold enhancement of toxin production.

One candidate for a cosmid-borne activator of the O17 chromosomal *tcpA* gene would be *toxT*, which lies within the *tcp* gene cluster between *tcpF* and *tcpJ* (Higgins *et al*, 1992; Ogierman and Manning, 1992). Expression of *toxT* is regulated by ToxR but ToxT can directly activate both *tcpA* and *ctx* gene expression (Higgins *et al*, 1992). It therefore seemed feasible that the cosmid-encoded *toxT* gene was responsible for upregulating TcpA and cholera toxin production in the O17 cosmid-bearing derivatives. This possibility was examined by assessing the effect of the isolated *toxT* gene on *tcpA* and *ctx* expression in O17.

PCR amplification (Section 2.16.1) was employed to obtain a minimal DNA fragment encoding only *toxT* using the classical cosmid pPM2103 as template for the reaction. Primers 780 (5' **CGGGATCCCG**TATATATCTTCAGAGTAGAAC 3') and 781 (5'

Figure 5.2 TcpA expression in O17 *toxR* in the presence of pPM2103.

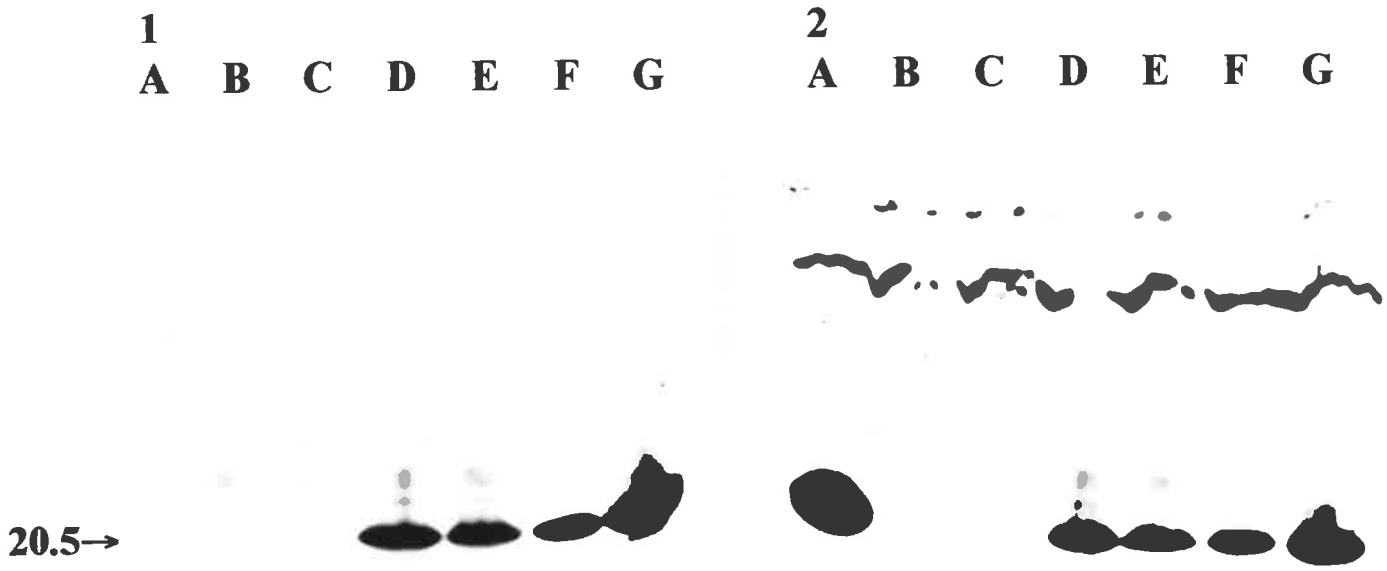
Samples: **A**, Z17561 (classical)

B, O17 *toxR*

C, O17

D-G, O17 *toxR* [pPM2103] clones 1-4.

All tracks were loaded with ca. 10^9 bacteria. Blot 1 was detected with anti-SA42 OMP (diluted 1:500) and Blot 2 was detected with anti-V9 TCP (diluted 1:1500).


1
A B C D E F G
2
A B C D E F G
20.5→

CGGGATCCCGTAGGATCAAGTAAACGTATTC 3'), which bind 5' and 3' of *toxT* respectively, were designed with *Bam*HI ends to facilitate cloning of the PCR product. As expected a ca. 0.9kb PCR product was obtained which was subsequently digested with *Bam*HI and cloned into the *Bam*HI site of the medium copy number vector pACYC184 (Chang and Cohen, 1978). Insertion of *toxT* at this site enabled expression from the promoter of the *tet* gene, encoding tetracycline resistance; the resultant plasmids, pPM3361 and pPM3362, carry *toxT* in the correct and incorrect orientation respectively for expression from this promoter (Figure 5.3). These plasmids were electroporated into O17 and two derivatives harbouring pPM3361 (EV67 A and B) and two harbouring pPM3362 (EV68 A and B) were selected for further study.

EV67 and EV68 were cultured using the AKI method and TcpA production assessed by Western blotting. TcpA was produced by EV67 and EV68 but not by O17 or O17[pACYC184] (Figure 5.4). This result demonstrates that the plasmid-encoded *toxT* gene product is sufficient to activate the chromosomal *tcp* region. Surprisingly, plasmid pPM3362 had a greater effect on O17 TcpA production than pPM3361.

Cholera toxin expression was increased in O17 in the presence of pPM3361 and pPM3362. Consistent with the differing amounts of TcpA found between clones carrying these plasmids, the level of cholera toxin in the supernatants of EV68 cultures was ca. 40-fold higher than that found in EV67 cultures.

5.4 Mutating the *toxT* gene of pPM2103

The finding that ToxT alone can activate O17 TcpA (and cholera toxin) production does not eliminate the possibility that other factors encoded by pPM2103 may also activate the chromosomal *tcp* gene cluster and the *ctx* operon. To examine this possibility it was necessary

Figure 5.3 Construction of plasmids pPM3361 and pPM3362

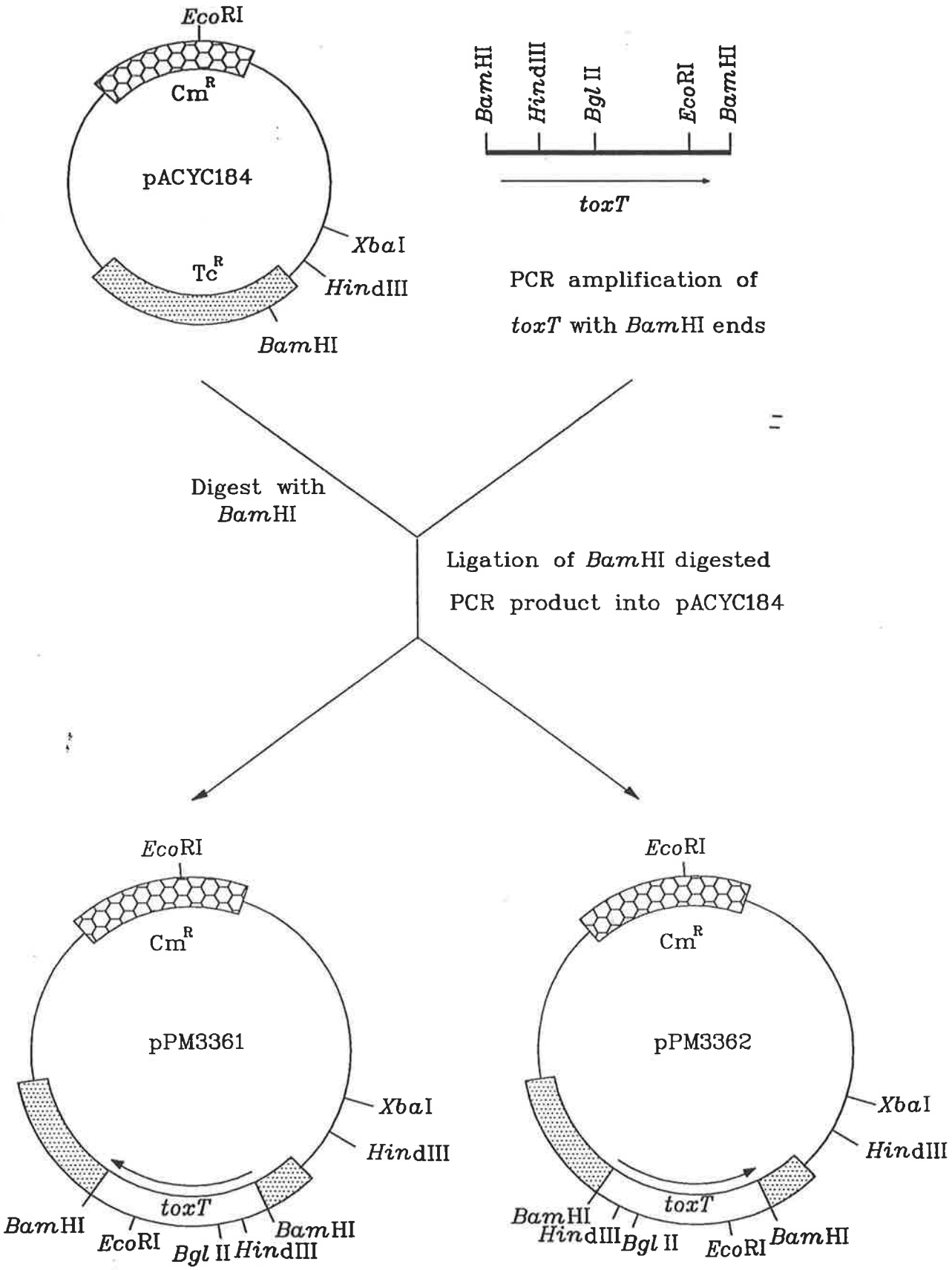

EcoRI
Cm^R
pACYC184
Tc^R
XbaI
HindIII
BamHI
BamHI
HindIII
Bgl II
EcoRI
BamHI
toxT
PCR amplification of
toxT with BamHI ends
Digest with
BamHI
Ligation of BamHI digested
PCR product into pACYC184
EcoRI
Cm^R
pPM3361
XbaI
HindIII
BamHI
toxT
BamHI
EcoRI
Bgl II
HindIII
EcoRI
Cm^R
pPM3362
XbaI
HindIII
BamHI
toxT
BamHI
HindIII
Bgl II
EcoRI
BamHI

Figure 5.4 TcpA production in O17 when *toxT* is provided *in trans*.

Samples: **A**, SA42 OMP

B, H1

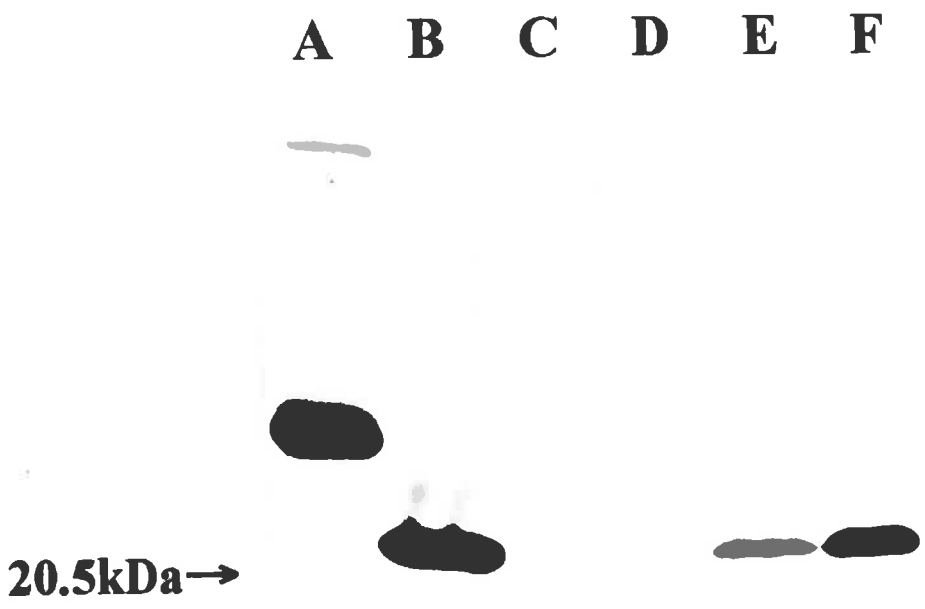
C, O17

D, O17[pACYC184]

E, O17[pPM3361]

F, O17[pPM3362]

All tracks were loaded with ca. 10^9 bacteria and track A was loaded with ca. 20μg of OMP. The blot was detected using the anti-SA42 OMP (diluted 1:500).


A B C D E F
20.5kDa→

to inactivate the cosmid-borne *toxT* gene and to then assess the effect of the modified cosmid on TcpA and cholera toxin expression in O17.

Inactivation of *toxT* was achieved by inserting a Km^R cartridge into the *Bgl*II site of *toxT* in pPM3362. The resultant plasmid pPM3370 was electroporated into EV33 (*E. coli* strain SM10 carrying pPM2103) with selection on agar containing both Km and Ap. It was expected that in some clones a double recombination event would occur whereby the *toxT*::Km^R gene would replace the functional *toxT* gene of pPM2103. Clones carrying both pPM3370 and pPM2103 were conjugated with O17 and the conjugation mix plated onto agar containing both Pmx and Km. As only the cosmid is mobilizable, the selection strategy would allow the growth of only those vibrios receiving a cosmid harbouring the mutated *toxT* gene. Several attempts to select for such a clone were unsuccessful, presumably due to the very low frequency at which the desired recombination event occurred.

An alternative strategy was subsequently devised. A 2.2kb *Bam*HI fragment bearing *toxT*::Km^R was subcloned from pPM3370 into the *Bam*H1 site of pCACTUS (Table 2.1) to produce plasmid pPM3372. pCACTUS has the temperature-sensitive form of the replicon of pSC101 (Blomfield *et al*, 1991). At permissive temperatures (<37°C) the plasmid can replicate but when shifted to non-permissive (≥37°C) temperatures the plasmid must integrate into the host cell chromosome or - in the present context - into another plasmid via regions of homology provided by the insert. In addition, the *sacB* gene present on pCACTUS provides a means of selecting against unresolved co-integrates (Blomfield *et al*, 1991).

pPM3372 was electroporated into EV33 and derivatives bearing both the cosmid and pPM3372 were selected on Km, Ap plates with overnight incubation at 30°C. Several of the resulting colonies were separately grown overnight at 30°C in Luria broth containing Km, Ap; various dilutions of these cultures were plated onto Luria plates with the same antibiotics and

incubated at 42°C overnight, to select for bacteria carrying recombinant plasmids. Subsequently, single colonies were picked from these plates and cultured with Km and Ap before spreading onto Luria agar plates (with no NaCl) containing Km, Ap and 6% sucrose. This enabled selection of organisms which had resolved pPM3372 and in which the mutated *toxT* gene had replaced the wild-type gene of pPM2103. After overnight incubation at 30°C, five sucrose-resistant, Km^R, Ap^R colonies were selected. Plasmid DNA was isolated and screened by restriction enzyme digestion and Southern blot analysis to confirm the presence of the mutated *toxT* (not shown). All five strains (EV94A-E) contained the desired cosmid (pPM3373).

pPM3373 was conjugated via EV94 into O17 and exconjugants selected on Pmx, Km plates. Eight Pmx^R, Ap^R, Km^R derivatives (EV105A-H) along with O17 and EV36 were then cultured using the AKI method. TcpA production was only detected in the positive control EV36 which carries pPM2103 (Figure 5.5). Cholera toxin levels in EV105 (A-H) culture supernatants were similar to O17. These results suggest that *toxT* is the cosmid-borne gene responsible for host *tcpA* and *ctx* expression.

5.5 Sequencing the O17 *toxT* gene

The DNA sequence of the classical *toxT* gene is known (Higgins *et al*, 1992; Ogierman and Manning, 1992), but the El Tor *toxT* gene has yet to be completely sequenced. The pre-coding region of the *toxT* gene from the El Tor strain H1 (which produces TCP and toxin when cultured in AKI medium *in vitro*) was sequenced and found to be identical to that of classical *toxT*. Sequence data of the carboxy-terminal 220 base pairs of H1 *toxT* revealed nine base changes, five of which result in a change in the size and charge of the amino acid residue

Figure 5.5 TcpA production in O17 [pPM3373] clones.

Samples: **A**, EV36

B, O17

C - J, O17 [pPM3373] clones A-H.

All tracks were loaded with ca. 10^9 bacteria. The blot was detected using the anti-SA42 OMP (diluted 1:500).

A B C D E F G H I J

20.5→ 

(Mourtzios, 1992). Whether these changes affect the activity of ToxT is unknown but it was of interest to ascertain if the H1 *toxT* gene could also activate *tcpA* and *ctx* expression in O17.

The H1 *toxT* gene was amplified from the cosmid pPM2608 in a PCR reaction using the primers 780 and 781 (described in Section 5.3). The product was cloned into pACYC184 in both orientations giving rise to plasmids pPM3365 and pPM3366 (opposite and correct orientation for expression from the *tet* promoter, respectively). Consistent with the earlier finding using the classical *toxT,* pPM3365 induced greater TcpA and cholera toxin production in O17 than pPM3366. The magnitude of the effect of these plasmids on TcpA and toxin production was similar to that of pPM3361 and pPM3362 (not shown).

The differing regulation of *tcp* gene cluster expression observed *in vitro* with strains of El Tor biotype such as H1 and O17 could be due to differences in the regulatory region (upstream) of *toxT* or differences within the gene. To ascertain if any differences do exist, the O17 *toxT* gene and its precoding region were isolated by preparing a subgenomic library in pBluescript-SK. Initially O17 chromosomal DNA was digested with *Eco*R1 and fragments between 2.8kb to 3.6kb isolated and ligated into *Eco*RI-cut pBluescript. After transformation into *E. coli*, DNA colony blots were performed to identify clones carrying plasmids of interest, using Dig-labelled classical *toxT* as the probe (section 2.17). Of the 100 colonies screened, three were positive; plasmid DNA from these clones was subjected to restriction enzyme analysis which revealed all three contained pBluescript-SK with an ca. 3.4kb *Eco*RI fragment (pPM3371).

Dye-terminator sequencing of pPM3371 using a primer which binds ca. 100 bases from the 3' end of *tcpF* demonstrated the region 5' of O17 *toxT* was identical to that of Z17561 and H1 (not shown). Further sequencing to the internal *Eco*R1 site of *toxT* revealed only one change at codon 193, changing the amino acid at that position from alanine to serine. The *Eco*R1 fragment of pPM3371 spans 75% of *toxT*, lacking the C-terminal portion which encodes the

helix-turn-helix domain. In order to sequence the 3'-end of the O17 *toxT* gene was amplified from O17 genomic DNA by PCR using primers 780 and 781 and cloned into the *Bam*H1 site of pUC19 to create plasmid pPM3377. The C-terminal encoding portion of O17 *toxT* was subsequently sequenced and found to be identical to the classical gene sequence.

5.6 Effect of constitutive *toxT* expression on TcpA and cholera toxin production in wild-type strains and their *toxR* mutants

The requirement for ToxR in the induction of TcpA synthesis can be bypassed by providing constitutive *toxT* expression *in trans*. Di Rita *et al* (1991) demonstrated this in a *toxR* mutant of the classical strain 395 and further showed that in this construct expression of ToxT-regulated genes was no longer dependent on the pH of the culture medium. To test whether *in vitro* TcpA and cholera toxin production are similarly independent of temperature in the presence of constitutive *toxT* function, the plasmids pPM3361 or pPM3362 (carrying a functional *toxT* gene) were electroporated into the El Tor strains O17 and H1, and the classical strain Z17561, as well as from their respective *toxR* mutants. Control and plasmid-bearing strains were then cultured in AKI broth at either 30°C or 37°C, and Western blot analysis and GM_1-ELISA performed to assess TcpA and cholera toxin production. The latter data are not shown but in all cases were consistent with the varying levels of TcpA detected by immunoblotting.

Neither pPM3361 nor pPM3362 altered TcpA or cholera toxin output by H1 and Z17561 under optimal *in vitro* growth conditions (AKI broth at 30°C) suggesting that maximal expression of these factors already occurs in these strains (Figure 5.6). As previously shown, greater activation of *tcpA* and *ctxAB* occurred in O17 in the presence of pPM3362 rather than pPM3361 (Figure 5.4, also Figure 5.7). The presence of pPM3361 induced better expression

Figure 5.6 The effect of pPM3361 on TcpA production by Z17561, H1 and their *toxR* mutants, at 30°C and 37°C.

Blot 1 samples: **A**, 569B/165 TCP preparation; **B**, Z17561 [pACYC184], 30°C; **C**, Z17561 [pACYC184], 37°C; **D**, Z17561 *toxR* [pACYC184], 30°C; **E**, Z17561 *toxR* [pACYC184], 37°C; **F**, Z17561 [pPM3361], 30°C; **G**, Z17561 [pPM3361], 37°C; **H**, Z17561 *toxR* [pPM3361], 30°C; **I**, Z17561 *toxR* [pPM3361], 37°C.

Blot 2 samples: **A**, SA42 OMP; **B**,; **C**, H1/pACYC184, 37°C; **D**, H1 *toxR* [pACYC184], 30°C; **E**, H1 *toxR* [pACYC184], 37°C; **F**, H1 [pPM3361], 30°C; **G**, H1 [pPM3361], 37°C; **H**, H1 *toxR* [pPM3361], 30°C; **I**, H1 *toxR* [pPM3361], 37°C.

All tracks were loaded with ca. 10^9 bacteria with the exception of track A which was loaded with ca. 10μg of protein in blot 1 and 20μg of protein in blot 2. Blot 1 was detected with anti-V9 TCP (diluted 1:1500) and blot 2 was detected using anti-SA42 OMP (diluted 1:500).

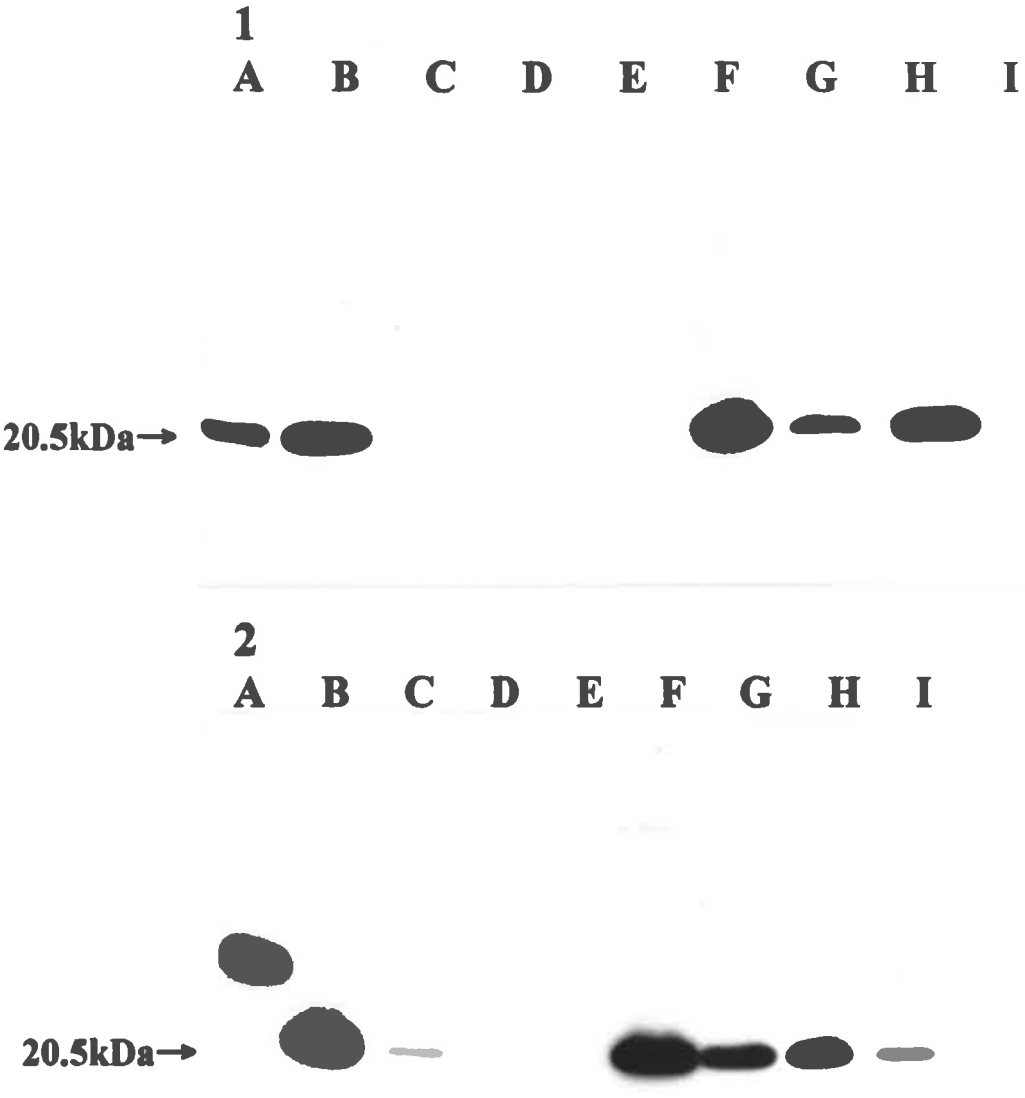

1
A B C D E F G H I
20.5kDa→
2
A B C D E F G H I
20.5kDa→

Figure 5.7 The effect of pPM3361 and pPM3362 on TcpA production by O17 and O17 *toxR*, at 30°C and 37°C.

Samples: **A**, SA42 OMP;

B, O17, 30°C

C, O17, 37°C

D, O17 toxR, 30°C

E, O17 toxR, 37°C

F, O17 [pPM3361], 30°C

G, O17 [pPM3361], 37°C

H, O17 *toxR* [pPM3361], 30°C

I, O17 *toxR* [pPM3361], 37°C

J, O17 [pACYC184], 30°C

K, O17 [pACYC184], 37°C

L, O17 [pPM3362], 30°C

M, O17 [pPM3362], 37°C

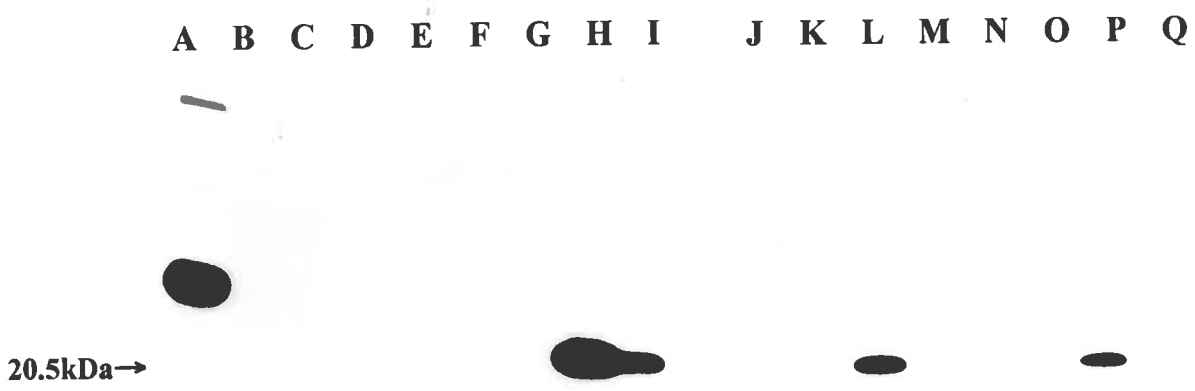
N, O17 *toxR* [pACYC184], 30°C

O, O17 *toxR* [pACYC184], 37°C

P, O17 *toxR* [pPM3362], 30°C

Q, O17 *toxR* [pPM3362], 37°C.

All tracks were loaded with 10^9 bacteria with the exception of track A which was loaded with 20µg protein. The blot was detected using anti-SA42 OMP (diluted 1:500).


A B C D E F G H I J K L M N O P Q
20.5kDa→

of TcpA and cholera toxin in all three *toxR* mutants at 30°C; little or no TcpA or toxin was detected in the presence of pPM3362 (Figures 5.6 and 5.7; data not shown).

Despite the provision of ToxT *in trans*, the three parent strains and their respective *toxR* mutants all produced significantly less TcpA and cholera toxin when grown at 37°C. TcpA and toxin production by all six strains was assessed at both temperatures in at least two separate experiments. Furthermore, plasmid DNA was recovered from pPM3361- and pPM3362-bearing isolates of O17 and O17 *toxR* and screened by restriction enzyme analysis to confirm the presence and identity of either plasmid.

5.7 Discussion

The presence of the cosmid pPM2103 allows the O17 strain to produce TcpA *in vitro* and also enhances the production of cholera toxin. The availability of the biotype-specific anti-TcpA reagents made it possible to determine if the TCP produced were derived exclusively from the cosmid-encoded classical *tcp* genes or whether the chromosomal gene cluster was also activated. Western blot analysis and IEM detected the presence of both biotypic forms of TcpA/TCP in O17 cosmid clones DS2 and EV36, indicating induction of the O17 *tcp* region (Figure 5.1). As expected the H1 cosmid derivative EV36 produced both biotypic forms of TcpA; however, it was interesting to note that the presence of pPM2103 did not increase the production of either cholera toxin or TCP in this strain. Presumably maximal activation of *tcp* and *ctxAB* genes occurs in H1 during growth in AKI at 30°C, so that the presence of additional activator has no effect.

For reasons which are unknown the EV37 derivative expressed only the chromosomal *tcpA* gene as no classical TcpA/TCP was detected; perhaps the cosmid carried by this strain has undergone a deletion spanning the classical *tcpA* gene. Alternatively, multiple recombination

events could have culminated in a strain in which the El Tor *tcpA* gene, in addition to being present in the chromosome, has replaced the pPM2103-encoded classical *tcpA* gene. Attempts were made to recover cosmid DNA from EV37 in order to study the nature of the cosmid in this strain, but unfortunately these proved unsuccessful. In retrospect the potential for such recombination could have been avoided by contructing cosmid-bearing derivatives in a *recA* O17 strain.

O17 *toxR* mutants carrying pPM2103 also expressed the chromosomally-encoded *tcpA* and *ctx* genes (Figure 5.2), demonstrating that activation of these genes was ToxR-independent in the presence of the cosmid (which does not carry *toxR*). Although ToxR is normally required for expression of the *tcp* region, it acts via an intermediate, ToxT. ToxT directly activates a number of ToxR-regulated genes in *E. coli* including *tcpA* and *ctxAB* (DiRita *et al*, 1991). As *toxT* is located within the *tcp* operon between *tcpF* and *tcpJ* (Higgins *et al*, 1992; Ogierman and Manning, 1992), it seemed a likely candidate as the cosmid-borne gene responsible for activating the host *tcp* and *ctx* regions in O17 and O17 *toxR* cosmid-bearing strains.

The classical *toxT* gene of pPM2103 was amplified by PCR and cloned into pACYC184. O17 derivatives carrying the resultant plasmids pPM3361 or pPM3362 were found to produce elevated levels of TcpA and cholera toxin compared with their wild-type parent. The level of TcpA and toxin production in O17 was clearly dependent on the orientation of *toxT* in pACYC184. As will be discussed later, better expression of these factors unexpectedly occurred in the presence of pPM3362 and not pPM3361. The data presented in Section 5.6 show that this (as yet unexplained) orientation effect was only observed in O17.

To ascertain whether there might be additional cosmid-encoded factors with the capacity to activate ToxR-regulated genes in O17, a cosmid carrying a mutated *toxT* gene (pPM3373) was derived from pPM2103. O17[pPM3373] strains did not produce TcpA and secreted wild-type

levels of cholera toxin, supporting the conclusion that the *toxT* gene on pPM2103 is the sole activator of chromosomal *tcp* and *ctxAB* operons. It is possible that the introduction of a Km^R cartridge into *toxT* has also affected the function of a downstream gene(s) which is also capable of trans-activation of the chromosomal *tcp* operon. This seems unlikely, however. The gene immediately downstream of *toxT* is *tcpJ* which encodes the TcpA signal peptidase. When Kaufman *et al* (1991) constructed *tcpJ* mutants by insertion of a Km^R marker into *tcpJ*, these mutants still produced TcpA (in its unprocessed form); there was no evidence that the polar mutation in *tcpJ* affected *tcpA* synthesis as a result of disrupted expression of downstream genes. It therefore seems reasonable to conclude that the pPM2103-encoded *toxT* gene is the sole activator of O17 *tcp* and *ctxAB* expression.

Despite this conclusion it appears highly unlikely that the failure of wild-type O17 to produce TCP *in vitro* is attributable to a defective *toxT* gene. O17 is as virulent in infant mice as any of the other El Tor strains used in this study and, as discussed in chapter 4, O17 requires TCP for colonization - from which it can be inferred that it expresses *toxT in vivo*. Nevertheless, the O17 *toxT* gene and the intergenic DNA between *tcpF* and *toxT* were sequenced to exclude the possibility that this region has significant differences to that of classical *V. cholerae* O1; none were found. The *toxR* gene of O17 has not been sequenced and it is unknown whether it differs from the *toxR* genes of other *V. cholerae* O1 strains. However O17 *toxR* mutants secrete less cholera toxin *in vitro* and are reduced in virulence (S. Williams, personal communication), which implies that the gene is functional in this strain. If it is therefore assumed that *toxR* and *toxT* are both functional, it seems reasonable to suggest that the environmental sensor(s) of O17, which modulates *toxR* and *toxT* expression, might somehow differ from that of other *V. cholerae* O1 strains which produce TcpA *in vitro*. This could

explain the failure of O17 to activate the ToxR regulon *in vitro*, whereas this evidently occurs *in vivo* in response to appropriate environmental stimuli.

DiRita and coworkers (1991) demonstrated in a *toxR* mutant that the requirement for ToxR in the induction of ToxR-regulated genes can be bypassed by providing constitutive *toxT* expression *in trans*. Furthermore expression of ToxT-regulated genes was no longer modulated by changes in the pH of the culture medium (DiRita *et al*, 1991). The aim of the study described in Section 5.6 was to determine the effect of temperature on cholera toxin and TcpA production in both classical (Z17561) and El Tor (H1 and O17) strains when ToxT is provided *in trans* to wild-type and *toxR* mutants.

The results obtained with Z17561 and H1 were very similar. Synthesis of TcpA and cholera toxin by H1 or Z17561 derivatives harbouring pPM3361 or pPM3362 was found to be identical to that of their wild-type parent strains following culture at 30°C; as expected, the strains showed undiminished production of both proteins. Moreover, like their parent strains, these *toxT* derivatives displayed dramatically reduced synthesis of both TcpA and cholera toxin at 37°C. As mentioned previously, *in trans* ToxT production activates expression of *tcpA* and *ctx* genes in O17 at 30°C but as found with H1 and Z17561, O17 *toxT* derivatives produced little or no TcpA and cholera toxin at 37°C. As expected the presence of pPM3361 (but not pPM3362) stimulated pilin and toxin production to wild-type levels in O17, H1 and Z17561 *toxR* mutants during culture at 30°C. However, when cultured at 37°C, TcpA and toxin production in these strains was negligible in the presence of either plasmid.

The orientation of *toxT* in pACYC184 influenced the level of host gene activation, reflecting stronger promoter activity and greater transcription of *toxT* in one orientation compared to the other. It was expected that transcription of *toxT* from pPM3361 would be greater than from pPM3362, as in pPM3361 the *toxT* gene is cloned in the same orientation as the *tet* promoter.

Although this was generally found to be the case, pPM3362 induced greater expression of TcpA and cholera toxin in wild-type O17. Since the identity of the plasmids carried by the two O17 *toxT* clones was verified by restriction enzyme analysis, this observation remains unexplained.

The inability of *toxT in trans* to relieve thermo-regulation of ToxR-dependent gene expression in wild-type or *toxR* mutants of O17, H1 and Z17561 is in contrast to the results of DiRita *et al* (1991), who found that pH-dependent expression of ToxR-regulated genes was abolished by providing ToxT constitutively. The mechanisms by which environmental signals activate genes of the ToxR regulon are not well understood, but it is evident that different stimuli exert their effects via different regulatory pathways (see Section 6.5).

Chapter 6 General Discussion

6.1 The antigenicity of El Tor TCP

When this study was commenced, *in vitro* expression of *tcpA* by El Tor strains had been demonstrated (Shaw *et al*, 1989) but despite attempts by other workers (Hall *et al*, 1988; Sharma *et al*, 1989a), there was no evidence for assembly of El Tor TcpA into surface pili. Indeed it was proposed that unlike classical *V. cholerae*, El Tor strains produce little or no TCP *in vitro* or *in vivo* and may use an alternative factor for adherence (Sun *et al*, 1989b and 1990a; Jonson *et al*, 1992). However it was known that there are significant differences between the carboxy-terminal sequences of the El Tor and classical *tcpA* genes (Shaw *et al*, 1989; Voss, 1990). Sun *et al* (1991) subsequently demonstrated that monoclonal antibodies directed against epitopes in this region of classical TcpA were protective. It therefore seemed possible that the differences between the two biotypic forms of the *tcpA* gene could result in antigenically dissimilar pilins. Earlier failures to detect surface pili on El Tor vibrios might then be explained by the fact that antibodies to classical TCP were used in attempts to detect El Tor TCP. To resolve this issue it was necessary to generate antisera to El Tor TcpA.

Initially, biotype-specific antisera raised against unprocessed TcpA were prepared. When used in Western blot analysis, these reagents clearly demonstrated the presence of biotype-specific epitopes and were more sensitive indicators of TcpA expression by strains of homologous biotype (Section 3.4). Epitopes common to the two biotypic forms of TcpA obviously exist, as indicated by the capacity of polyclonal antisera to detect TcpA of the alternative biotype on Western blots. However as the studies in Section 3.4 confirm, reactivity of a reagent on Western blot can not be used as an indication of the capacity of the reagent to detect native protein. For example, when Jonson *et al* (1991a) raised a panel of monoclonal

antibodies against classical TCP, one (MAb 20:2) was found to recognise both classical and El Tor TcpA by Western blot analysis. However in IEM studies MAb 20:2 detected classical TCP but failed to detect El Tor TCP. This suggests that while the epitope recognised by MAb 20:2 is shared by the two biotypic forms of TcpA, it is better exposed in classical than El Tor TCP. Perhaps the C-terminal sequence of the El Tor *tcpA* gene imposes an altered conformation on the pilin protein, in which the epitope defined by MAb 20:2 is no longer accessible to antibody.

Since the biotype-specific anti-TcpA reagents evidently failed to react with intact pili on the vibrio surface, antisera to native El Tor TcpA (anti-H1 TcpA and anti-EV37 TcpA) were generated. When used in IEM studies, these sera allowed the first demonstration of TCP on the surface of El Tor strains (Section 3.6). Both these sera had negligible reactivity with classical TCP, in line with the failure of the anti-V9 TCP reagent to detect El Tor TCP (Section 3.3). Since none of these reagents is biotype-specific, the IEM analysis indicated that detection of surface TCP requires the use of biotype-specific antibodies (Section 3.6). This suggests that once TcpA is assembled into TCP, the common epitopes are much less accessible to antibody than the biotype-specific determinants.

The El Tor-specific anti-TcpA serum (anti-SA42 OMP) was used to screen twenty-one El Tor strains for TcpA production following growth in conditions optimal for *tcp* expression. Although this reagent was the most sensitive indicator of TcpA synthesis by El Tor strains, eleven still scored as TcpA-negative. Evidently regulation of *tcpA* expression differs within the El Tor biotype. Perhaps there exist two subgroups of El Tor strains with distinct regulatory differences; alternatively isolates of this biotype might display a continuum of high to low TcpA expression *in vitro*. In the latter situation, detection of TcpA expression would only be limited by the sensitivity of the detection reagents and indeed this has been our previous

experience. For some time now, enhanced chemiluminescence (ECL) detection of immunoblots has been used in preference to colourimetric detection since it was found to be a (ca. 10-fold) more sensitive detection method (Voss, 1990). Furthermore, when the anti-V9 TCP serum was initially to survey the 21 El Tor strains, only seven were scored as TcpA-positive (Section 3.4). Clearly ECL detection combined with the use of the El Tor-specific antiserum (anti-SA42 OMP) has allowed visualization of TcpA production in El Tor strains which would otherwise have been scored as TcpA-negative. In either event, *in vitro* TcpA synthesis does not relate to the *in vivo* significance of TCP in El Tor pathogenesis (see below).

6.2 TCP is a colonisation factor of *V. cholerae* El Tor.

The major aim of this thesis was to determine the significance of TCP in the pathogenesis of the El Tor biotype. In order to do this El Tor *tcpA* mutants were constructed in strains which either do or do not express TcpA *in vitro* and their virulence and colonising capacity assessed in the IMCM. The data presented in Sections 4.4 and 4.6 demonstrate that, regardless of the capacity of the parent strains to produce TcpA/TCP *in vitro*, the mutants were uniformly avirulent and unable to effectively colonise the infant mouse gut.

Rhine and Taylor (1994) have recently described the construction and *in vivo* characterisation of an El Tor (N16961) *tcpA*::Km^R mutant. Comparison of their mutant with the N16961 *tcpA* mutants described in this study show all to be similarly reduced in virulence. The residual colonising capacity of Rhine and Taylor's mutant is however, much greater than that seen with our mutants. Whereas the output ratio of wild-type:*tcpA* mutant is ca. 40:1 in their study, it is ca. 10^3-10^4:1 with our mutants. The reason for this difference is unclear, but is possibly related to the (ca. 50-fold) lower virulence of their N16961 strain, which might reflect slower growth *in vivo*.

Attempts were made to complement the virulence defects of both the polar and in-frame *tcpA* mutants. The complementation strategy employed depended on the nature of the mutation. To complement the (polar) *tcpA*::Km^R mutants a cosmid encoding the El Tor *tcp* gene cluster was used, as polarity effects on *tcp* genes downstream of *tcpA* made complementation via the provision of only *tcpA in trans* implausible. This approach proved successful; cosmid-complemented *tcpA*::Km^R strains produced TCP *in vitro* and were restored to near wild-type virulence (Section 4.2). It was later found that a high frequency of *in vivo* recombination events had restored a wildtype-genotype to the *tcpA*::Km^R mutants.

It was expected that the provision of a functional *tcpA* gene would be sufficient to complement TCP production *in vitro* and restore virulence in the in-frame Δ*tcpA* mutants. Immunoblotting and IEM analysis demonstrated that the presence of a plasmid encoding *tcpHAB* (pPM3375) restored to these mutants the capacity to produce TcpA and assemble surface pili *in vitro.* It was therefore surprising to subsequently find that the virulence of the Δ*tcpA*[pPM3375] mutants was only partially and variably restored (Section 4.6). Recently Brown and Taylor (1995) reported that the provision of *tcpA in trans* restored *in vitro* TCP production in an in-frame *tcpA* mutant, but not in a polar *tcpA* mutant. EM examination revealed the TCP produced to be morphologically similar to wild-type pili. However the level of TCP produced by the complemented strain was significantly reduced compared to wild-type, such that the auto-agglutination characteristic of classical vibrios grown under conditions conducive to TCP synthesis was not observed. Unfortunately no information was provided concerning the *in vivo* behaviour of this strain. A quantitative assay of TCP production, such as an ELISA inhibition assay, would be required to determine whether reduced TCP assembly might be a factor in the partial complementation of our Δ*tcpA*[pPM3375] strains.

Reduced TCP production by the Δ*tcpA*[pPM3375] strains could be the result of decreased stability of the polycistronic mRNA which is thought to start at *tcpA* and terminate beyond *tcpJ* (Brown and Taylor, 1995). Based on Tn*pho*A mutagenesis studies, the *tcp* genes encoded by this transcript are predicted to be required for the transport and assembly of TcpA into TCP. For example, strains carrying mutations in *tcpB*, *tcpC*, *tcpD*, *tcpE* and *tcpF* all fail to synthesize surface TCP (Shaw *et al*, 1990). The fact that TCP are assembled by the Δ*tcpA*[pPM3375] mutants - notwithstanding the issue of quantitation - indirectly confirms that the *tcpA* mutation is non-polar. But it is possible that a deletion in *tcpA* reduces the stability of the polycistronic transcript thereby reducing TCP expression. This situation could conceivably be exacerbated *in vivo*, under the influence of additional regulatory networks induced in the gut environment.

There are other possible explanations for the failure of pPM3375 to completely complement the Δ*tcpA* mutants. Perhaps the presence of multiple copies of *tcpA* upsets a finely coordinated process of *tcp* assembly by creating a stoichiometric imbalance in the relative amounts of each factor required for TCP assembly. [This was clearly not a problem in the cosmid-complementation of the polar *tcpA* mutants. The provision of the entire *tcp* gene cluster , albeit in multiple copies, evidently ensured that the correct relative proportions of each factor were maintained.] Alternatively, the TCP produced by the Δ*tcpA*[pPM3375] strains may be non-functional *in vitro* or *in vivo*. The creation of a (polar or in-frame) mutation in *tcpA* may affect the expression of other *tcp* genes such that TCP are still assembled but are abnormal in function. Brown and Taylor (1995) observed that the their complemented *tcpA* mutant did not visibly clump in liquid culture. While the most likely explanation may simply be reduced TCP expression by the complemented mutant, the absence of clumping may reflect a loss of function. This possibility could be addressed through the design of an assay of TCP

function, for example adherence of TCP-producing strains to an intestinal epithelial cell line. However development of such an assay could be hampered by the presence of other *V. cholerae* adhesins capable of mediating attachment.

Workers in Sweden (Jonson *et al*, 1992; Osek *et al*, 1994) have proposed that MSHA-pili and not TCP may be important in pathogenesis of strains of the El Tor biotype. This theory was initially based on their observation that TcpA and MshA pilins are detectable in both classical and El Tor strains by Western blot analysis (Jonson *et al*, 1990). However TCP had at that time only been detected on the surfaces of classical strains, while MSHA seemed restricted to El Tor isolates (Jonson *et al*, 1991b and 1992). It was therefore suggested that in El Tor strains MSHA performs the same function as TCP does for classical *V. cholerae*. This hypothesis was apparently strengthened by later experiments which found biotype-associated differences in the protection afforded by antibody to either pilus type (Osek *et al*, 1994). However as discussed previously (Section 4.8), the failure of Osek and coworkers (1994) to detect El Tor TCP and to demonstrate protection against TcpA-positive El Tor vibrios can be attributed to their use of inappropriate antibodies. The results of our studies and those of Rhine and Taylor (1994) clearly demonstrate TCP to be a major colonisation factor which is essential for the pathogenesis of *V. cholerae* O1 El Tor, at least in the IMCM. Whether MSHA pili are an additional colonisation factor of *V. cholerae* O1 awaits the *in vivo* characterisation of *mshA* mutants.

6.3 TcpA is a protective antigen of *V. cholerae* O1 El Tor.

The preparation of anti-H1 TcpA and anti-EV37 TcpA (Section 3.6) enabled us to investigate the protective efficacy of antibodies to El Tor TCP. These antisera were extensively absorbed

with (whole cells and membrane fractions of) isogenic *tcpA* mutants grown under conditions conducive to the expression of ToxR-activated genes, in an attempt to make them specific for TCP. Passive immunisation studies in the IMCM demonstrated these reagents provided solid protection against TCP-positive El Tor strains. However, no protection against infection with TCP-negative El Tor strains or classical vibrios (regardless of TCP status) was observed. The converse occurred when antibodies to classical TCP were used. These results demonstrate that TCP is a protective antigen of classical and El Tor *V. cholerae* O1 (Section 4.7) but further show that the antibodies which mediate protection in this model are directed against the biotype-restricted epitopes of the proteins.

Despite the fact that TCP have been shown to be essential for colonisation of the human gut by classical *V. cholerae* (Herrington *et al*, 1988), there are no data available as to whether TcpA is a protective antigen in human infection. To date, only one study has examined the immune response to TCP following clinical cholera. Hall *et al* (1991) conducted a retrospective analysis of serum and intestinal secretory antibody responses to TCP and a number of other antigens in volunteers following infection with classical *V. cholerae*. Significant rises in antibody titres to all antigens with the exception of TCP (or a TcpA mimiotope) were found. Seroconversion to TCP was also assessed in six cholera patients following natural infection with El Tor *V. cholerae* O1. Three of the six patients demonstrated a meagre rise in anti-TCP titre from acute to convalescent phase (Hall et al, 1991). However, seroconversion may have been undetected or under-estimated during this study as the anti-TcpA titres were assessed in an ELISA assay in which classical TCP were used as the coating antigen. Based on the findings presented in Sections 3.6 and 4.7, it would have been more appropriate to have used El Tor TCP to coat the trays. The authors concluded that seroconversion to TCP can occur in natural cholera infection but that an anti-TCP response is

not necessary for solid, long-term protection. The results of Hall and coworkers (1991) are preliminary and a larger study of cholera patients is required to obtain a more definitive assessment of seroconversion to TcpA following cholera infection.

Taylor *et al* (1987) first suggested that TCP could be included in cholera vaccine preparations to improve their protective efficacy. Although at the time this proposal seemed premature , it has been vindicated by the studies described in this thesis. The experiments presented in Section 4.7 were performed in the IMCM and whether they apply to human infection remains to be demonstrated. It would seem worthwhile to at least show that El Tor TCP is a colonisation factor in human infection prior to its inclusion in cholera vaccine preparations. In the latter context, it would be essential to include both biotypic forms of TCP in future vaccines, as the biotype-specific epitopes would appear to be of greater vaccine significance. If as Hall *et al* (1991) suggest, TcpA is not adequately immunogenic during natural infection, then a major challenge will be to develop a means of presenting this antigen to the immune system such that a protective immune response is elicited. This could perhaps be achieved through the creation of hybrid vaccine strains such as an attenuated *Salmonella* vector which expresses TCP. Alternatively it may be possible to produce a conjugate vaccine in which TcpA is linked to a suitable carrier molecule (eg. B-subunit of cholera toxin).

6.4 *V. cholerae* O139

O139 strains have DNA with homology to the O1 El Tor *tcp* gene cluster and indeed sequencing studies have now shown that the *tcpA* genes are identical (Iredell and Manning, 1993; Rhine and Taylor, 1994). It was shown in section 3.7 that some O139 strains produce El Tor-like TcpA/TCP *in vitro*, and so it was of great interest to ascertain whether antibodies to El Tor TcpA could protect against O139 challenge. Subsequent passive immunisation

studies revealed these antibodies were indeed protective against TCP-positive O139 strains. This also suggests that TCP is a colonisation factor of O139 vibrios, although this remains to be demonstrated through the construction and *in vivo* assessment of O139 *tcpA* mutants.

During the initial epidemics of O139 cholera in India and Bangladesh, the majority of patients were adults (Nair *et al*, 1994). This is in contrast to the traditional pattern of disease in these areas, where the majority of cholera patients are children; adults have developed natural immunity to *V. cholerae* O1 through constant environmental exposure. The high attack rate of *V. cholerae* O139 was similar to that seen when epidemics of cholera caused by O1 El Tor strains occurred among the immunologically naive communities of South America. These epidemiological data suggest that pre-existing immunity to *V. cholerae* O1 does not protect against O139 infection, making it unlikely that current vaccines directed against *V. cholerae* O1 will be effective against the new serovar.

The rapid development of a vaccine directed against *V. cholerae* O139 is particularly important as strains of this serovar are predicted to be the agents of the next cholera pandemic. To this end, the same strategies used in the development of vaccines against O1 strains are applicable. A live attenuated O139 vaccine prototype strain has been described and is currently undergoing human trials (Waldor and Mekalanos, 1994). Past attempts at constructing an El Tor analogue of the classical live attenuated vaccine strain CVD103HgR have had limited success with unacceptable reactogenicity a frequent problem; since O139 strains are closely related to O1 El Tor strains (Johnson *et al*, 1994; Hall *et al*, 1994) it will be of interest to see whether a non-reactogenic vaccine strain can be constructed. Alternatively, an O139 equivalent of the B-WCV preparation could be quickly produced. The efficacy of such a formulation might be enhanced by the inclusion of O139 TCP, which might offer the additional advantage of inducing immunity to O1 *V. cholerae* El Tor.

6.5 Regulation of *tcpA* expression.

Regulation of TcpA expression differs between the biotypes and within the El Tor biotype. A major aim of Chapter 5 was to attempt to determine why the El Tor strain O17 does not produce TcpA *in vitro*. It was known that O17 derivatives bearing a cosmid encoding the classical *tcp* gene cluster, express the chromosomal pilin gene. Subsequent studies revealed that it was the product of the cosmid-encoded *toxT* gene which was solely responsible for the trans-activation of *tcpA* and *ctx* genes of O17. This was initially demonstrated by the finding that provision *in trans* of *toxT* alone was sufficient to induce both pilin and toxin synthesis by this strain (Section 5.3). Conversely, when the cosmid-encoded *toxT* gene was inactivated, trans-activation of O17 *tcpA* and *ctx* genes was not observed (Section 5.4). We have not investigated the possibility that the regulatory sequences of the O17 *tcpA* gene are less sensitive to ToxT and that activation requires high levels of ToxT production. Sequencing of the O17 *toxT* gene revealed it to be virtually identical to that present in the H1 strain, showing that the ability of O17 to produce TcpA/TCP *in vitro* cannot be ascribed to a defective ToxT. O17 would also appear to have a functional *toxR* gene, raising the possibility that the environmental sensor(s) are different in this strain. Southern hybridisation analysis has demonstrated the presence of the *toxS* gene in O17, but whether this gene is functional is unknown (Williams and Manning, unpublished observations). The "defect" in O17 could perhaps be identified by attempting to confer upon this strain the capacity to produce TCP *in vitro*. For example, a plasmid bank of H1 genomic DNA could be screened in an O17 strain carrying a promoterless reporter gene inserted immediately downstream of the regulatory sequences of *toxT*.

The mechanisms by which environmental signals activate genes of the ToxR regulon are not well understood, but it is evident that different stimuli exert their effects via different regulatory pathways. Expression of *toxR*-activated genes in *V. cholerae* O1 is affected by osmolarity, pH and amino acid concentration with optimum expression occurring in the normal physiological range (Miller *et al*, 1987). With the possible exception of osmolarity, there is no clear evidence that ToxR acts alone in sensing these environmental signals. *V. cholerae* containing *toxR-phoA* fusions on a plasmid and *toxR* null mutation on the chromosome are no longer responsive to osmolarity but still respond to changes in pH and amino acid concentrations (Miller *et al*, 1987).

Expression of ToxR-regulated genes in response to culture pH may be mediated through control over intracellular levels of ToxT, since wild-type *V. cholerae* 395 synthesizes *toxT* mRNA at the inductive pH of 6.5 but not at the repressive pH of 8.5 (DiRita *et al*, 1991). A *toxR* mutant provided with *toxT in trans* synthesized *toxT* mRNA and secreted cholera toxin regardless of the culture pH. In these studies toxT was expressed from the constitutive *tet* promoter of pBR327; the same promoter is present in the pACYC184 vector used in our experiments, which were designed to examine whether thermo-regulation of TcpA expression is similarly relieved by constitutive ToxT synthesis. The results suggest that the temperature-dependent expression of the ToxR regulon is not mediated at the level of ToxT synthesis. Provision of ToxT *in trans* induced TcpA and cholera toxin synthesis in three *toxR* strains grown in AKI broth at the permissive temperature of 30°C but when these strains were grown at 37°C pilin and toxin production were dramatically reduced. Similarly constitutive ToxT production failed to overcome thermo-regulation of TcpA and toxin synthesis in the wildtype parents (Section 5.6). Future experiments could analyse the levels of *toxT* and *toxR* mRNA

produced by these strains at the two incubation temperatures. Binding of ToxT to the *tcpA* promoter could also be examined using DNA mobility-shift assays.

There are several possibilities as to why constitutively-produced ToxT does not activate TcpA and cholera toxin production to wild-type levels in *V. cholerae* or *toxR* mutants during growth in AKI at 37°C. Other workers have shown that temperature can induce changes in DNA topology - in particular DNA supercoiling - via a histone-like protein which acts as a transcriptional silencer, thus altering gene expression in bacterial pathogens (Maurelli and Sansonetti, 1988; Dorman *et al*, 1990). It is conceivable that at 37°C the topology of the promoters of ToxT-activated genes such as *tcpA* and *ctxAB* either interferes with ToxT binding or alternatively prevents ToxT from making proper contact with the RNA polymerase to commence transcription. Studies with *V. cholerae* O1 have shown that the *acfA* and *acfD* genes are divergently transcribed and display ToxR/ToxT-dependent expression when chromosomally encoded. Furthermore, this activation is sensitive to inhibitors of DNA gyrase (Parsot and Mekalanos, 1992). When the *acfA-acfD* intergenic region was cloned into a low copy number plasmid, ToxR/ToxT-dependent regulation was virtually abolished (Parsot and Mekalanos, 1992). Another example of this type of regulation has been described in *Yersinia enterocolitica*. Transcription of the *Y. enterocolitica yop* genes requires VirF which (like ToxT) is an AraC-like protein. Activation of the *yop* promoters is thermo-regulated although binding of VirF is unaffected at 37°C, which led to the proposal that temperature-induced changes in DNA topology act together with VirF to initiate transcription (reviewed by Dorman and Bhriain, 1993).

Evidently thermo-regulation of virulence gene expression is modulated by other environmental factors *in vivo* For example, optimal toxin and TcpA/TCP synthesis by *V.*

cholerae O1 strains occurs at 30°C *in vitro* and yet the *in vivo* growth temperature is 37°C. Similarly, adherence and invasion of cultured mammalian cells by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* is more efficient at 26°C rather than 37°C and yet these organisms effectively adhere and invade cells *in vivo* at 37°C (Maurelli, 1989).

In this regard, Parsot and Mekalanos (1990) have proposed a model whereby *V. cholerae* O1 undergoes a "heat shock" or stress response during the early stages of infection. *htpG*, the gene encoding the bacterial homologue of the eukaryotic heat shock protein Hsp90, was shown to be located immediately adjacent to but divergently transcribed from *toxR*. Growth temperature was subsequently found to have a co-ordinate and reciprocal effect on expression from the *toxR* and *htpG* promoters. An increase in temperature from 22°C to 37°C induced ca. 6-fold greater expression from the *htpG* promoter and a corresponding ca. 5-fold decrease in expression of *toxR*. Regulation of expression from these promoters was found to be controlled by the level of σ-32 (RpoH) RNA polymerase (Parsot and Mekalanos, 1990). This response may assist survival of the organism during passage through hostile environments prior to reaching the mucosal surface of the small intestine. Furthermore by reducing *toxR* expression (and therefore ToxR-activated gene expression) the heat shock response would limit the production of virulence determinants at inappropriate anatomical sites. Parsot and Mekalanos (1990) further speculate that once the vibrios have reached the mucosal surface, the influence of other environmental parameters would lead to increased expression of ToxR-regulated genes.

Regulation of virulence gene expression is obviously very complex. *In vitro* studies of a single environmental parameter which influences expression of virulence determinants can be difficult to relate to the *in vivo* situation and must be interpreted with caution.

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