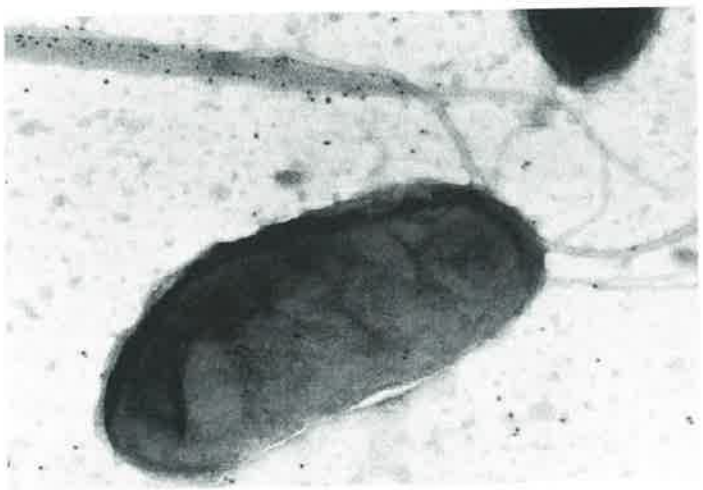
Molecular export and pilin assembly: TCP biogenesis in *Vibrio cholerae*



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Submitted for the degree of Doctor of Philosophy
University of Adelaide
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NAME: JON IREDELL **COURSE:** PhD

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Contents - abbreviated:

Corrigenda:

The following corrections are to be inserted into the thesis *Molecular export and pilin assembly: TCP biogenesis in Vibrio cholerae*, submitted for the award of Ph. D. at the University of Adelaide 1997.

1. page 19, Fig S1 and page 151, Fig. 4.9 and text: Western immunoblots are essentially qualitative in nature.
2. page 22, lines 1-2: the "elements" referred to are probably type-4 pili
3. page 22, para 1: add Russell and Darzins reference appended below
4. page 40, section 1.6.2, line 5: replace (give referenced example) with (von Heijne, 1992)
5. page 70, 5 lines from bottom: It should be pointed out that PulD is a *K. oxytoca* protein, here expressed in *E. coli*
6. page 74, end para 1, should read: Fig. 1.10, overleaf
7. page 81, end para 1: While Glu:Lys substitution at +5 of *P.aeruginosa* pilin subunit allows functional assembly, as stated p.78 (Pasloske *et al.*, 1989), it is not clear whether adhesion is solely or primarily affected.
8. page 83, para 2; add introductory sentence: *TcpB* is predicted to encode a protein with the prepilin leader sequence and N-terminal region of a type-4 pilin or pseudopilin (Table 1.2, Fig. 1.10).
9. page 89, line 2 to read: ... mutations lead to retention of TCP expression under some conditions that normally repress it (Taylor *et al.*, 1988).
10. Work in Chapter 2 was completed and accepted for publication (see publications arising, p.iv) prior to awareness and availability to the author of the important publication by Rhine and Taylor, 1994. This latter paper reports the El Tor biotype *tcpA* sequence with comparison to classical biotype *tcpA* sequence, and has not been duly acknowledged in Chapter 2. The author would like to highlight this and express deep regret at the unintentional omission of reference to this work, as the Chapter is largely drawn from the publication cited on p.iv. and should have been more correctly updated prior to inclusion in the thesis.
11. page 95: primers 525 contains a *Sac*I site, and 526 a *Bam*HI site, not both *Bgl*II sites as shown in Table 2.1.
12. page 102, section 2.6: The 1.1 kb product from primers 525:526 was a *Sac*I *Bam*HI fragment, not a *Bgl*II fragment as stated. Chromosomal DNA or a 4.8kb *Bgl*II fragment of the *tcpA*-containing region (as cloned into pPM4101-4) are suitable as template.
13. page 106, Fig. 2.4 and related text: Note that it is not possible to conclude a *tcpA* origin for the PCR-derived bands in the absence of a stringent *tcpA*-specific Southern blot.
14. page 113, Fig. 3.2: pPM4130 contains the entire 20.5 kb *Sac*I fragment
15. page 117, Fig. 3.4 and legend; delete from under the figure: (ii) TcpA immunoblot; add to first sentence; ...and V669, using polyclonal antisera to TcpA
16. page 122, Fig. 3.7 legend: Lanes 1 and 2 contain 569B, lanes 3 and 4 contain V663.
17. page 146, Fig. 4.6: pPM4110 begins at *tcpT* start site (as indicated on page 177), and not at the *Eco*RI site
18. page 159, Fig. 4.14 title, add: with respect to TCP localisation
19. page 159, line 3: JRI3 is the H1 *tcpT* mutant, and JRI4 the V663-derived strain
20. pages 162, 163, Fig. 4.16 needs panels labelled (a) and (b)
21. page 164, line 1: 569B and V663 data previously described (p.128) - delete reference here
22. page 170, line 4 from bottom: replace TcpT with EpsE
23. page 184, line 9 from bottom should read: Fig. 5.7 panel b
24. page 191, Fig. 5.10 legend: Lane 3: Z17561 (no plasmid). Note that the M2 antibody detects a full-length FLAG-tagged protein (c), and that a higher than predicted MW band is also detected for $TcpT^{F20}$. Taken together, the data suggests the possibility of conformational variation for TcpT, $TcpT^{F}$, and $TcpT^{F20}$ when expressed in TCP-expressing Z17561.
25. page 192, Fig. 5.12 legend: Note the presence of higher than predicted MW bands as well as lower MW bands. This could relate to imperfect specificity of the polyclonal antisera, or to conformational variation and degradation products as suggested by previous blots (Figs. 5.7, 5.9-11).
26. page 197, Table 5.2 legend: Note the lack of negative transdominance of the $TcpT^{20}$ proteins. This is discussed below (p. 203)
27. page 203: The failure of the $TcpT^{F20}$ and $TcpT^{Q20}$ proteins to interefere with endogenous TcpT cannot be explained by instability of the mutant protein (see Fig. 5.10 and text), suggesting a transient association or that the loss of the Walker motif and minor disruption of the preceding N-terminal α-helix was sufficient to abolish effective interaction. Previous work has highlighted the importance of the N-terminal region for such associations in closely related proteins PulE (Possot and Pugsley, 1994) and EpsE (Sandkvist *et al.*, 1995).

Corrigenda:

28. other important typographical or contextual errors:

p1	references x2	replace Stroeher *et al.*, 1995 with Stroeher *et al.*, 1995b
p9	line 3rd from bottom	replace *toxI* with *toxT*
p11	line 2nd from bottom	sensitive
p12	line 10	insert adjacent to *Attridge* ref, Thelin and Taylor, 1996
p15	line 16	insert adjacent to *Attridge* ref, Rhine and Taylor, 1994
p17		Jonson *et al.*, 1992 (not 1991b)
p18	line 12	(see Section 1.3 and Finkelstein *et al.*, 1992)
p21	line 2	specific antisera directed against HAP
p71	line 5 from bottom	replace Lindeberg *et al.*, 1996 with Lindberg *et al.*, 1996
p74	line 11	replace Fig 1.9 with Fig. 1.10
p93	title	replace Tcpa with TcpA
p94	line 5	replace Faast *et al.*, 1987 with Faast *et al.*, 1989
p102	title	tcpA should be *tcpA*
p114	line 2 from bottom	replace Stroeher *et al.*, 1996 with Stroeher *et al.*, 1995b
p209	line 9	replace type-4 pilins with type-4 like pseudopilins
p221	line 11	replace ideal with ileal
p232	section 7.2.1	thiamine 50 µg/ mL
p233	antibiotic concentrations	µg/mL, not mg/mL
p236		resuspended in 100µg/mL, and 10µg loaded on a gel
p241	section 7.5.1	incubation for 30 min; last line: replace TcpA with protein of interest
p269	reference	replace Marquez, G.G., 1989 with Marquez, G.G., 1984

amendments to references:

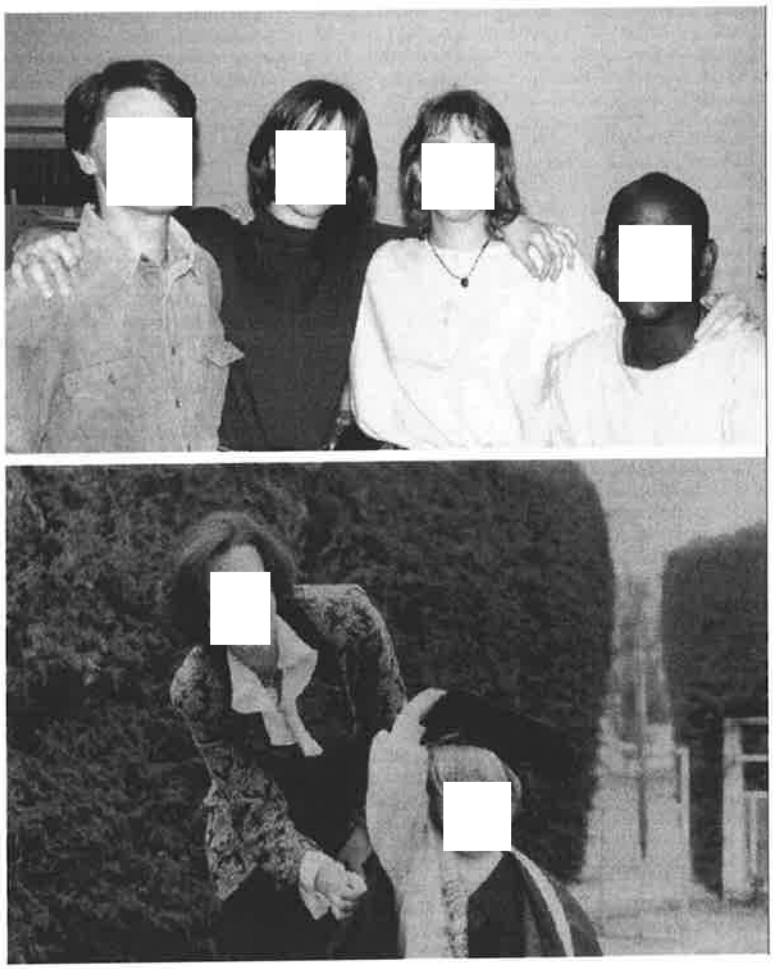
I. *add*: 353a. Russell, M.A. and Darzins, A. 1994. The *pilE* gene of Pseudomonas aeruginosa, required for pilus biogenesis, shares amino acid sequence identity with the N-termini of type 4 prepilin proteins. Mol. Microbiol. 11:137-154

II. *delete* Reference #72 (d'Enfert *et al.*, 1989). Reference 71 is correct.

III. *add*: 336a. Rhine, J.A., and Taylor, R.K. 1996. TcpA pilin sequences and colonization requirements for O1 and O139 *Vibrio cholerae*. Mol. Microbiol. 13:1013-1020

IV. *add*: 406a. Thelin, K.H., and Taylor, R.K. 1996. Toxin-coregulated pilus, but not mannose-sensitive haemagglutinin, is required for colonization by *Vibrio cholerae* O1 El Tor biotype and O139 strains. Infect. Immun. 64: 2853-2856

ACKNOWLEDGMENTS



The burden of this work was greatly lightened by sharing it with friends and colleagues in arms, Maria Mavris (left), Monica Ogierman (centre) and David Ogunniyi (right), who kept me from taking it all too seriously. This is dedicated to Melissa O'Connor in thanks for her love and smiling support, keeping the home fires burning while doing all her own work, and to our daughters Mebh (right) and Sigrid (centre, hidden).

Thanks to Paul Manning for encouraging me to find my own feet, for his generous support both here and overseas, and for the hospitality of the household which he so frequently extended to all of us.

Special thanks also to Uwe Stroeher, my chief instructor in the early days, for his personal friendship at home and his cheerful guidance at work. Thanks to Elena Voss, Sue Williams, Tony Focareta, Uwe Stroeher, Kathy Jedani, and Renato Morona for their unselfish donations of time in discussions of papers and endless drafts of thesis material. Thanks to Steve Attridge for his cheerful commonsense approach to it all, and his willingness to lend an ear. Chris Cursaro (and Terry Proctor) and the team of technical wizards past and present made it all so much easier. Marilyn Henderson (CEMSSA, Adelaide) is gratefully acknowledged for her endeavours with the frozen sections, as is Jorg Stierhof (Max Planck Institut, Tübingen) for his good advice. The hospitality of Thomas Meyer and his family is warmly remembered, as is the help and friendship extended by members of his laboratory during a 2 month stay at the Max Planck Institut in Tübingen. Thanks to Gunhild Jonson for her cheerful companionship in the lab. and her friendship outside it during her stay with us in Adelaide, and for the memories she leaves us all. (I am also indebted to Monica Ogierman for performing the haemolysin assays, and to Gunhild Jonson for performing the cholera toxin assays described in Section 4.2.6)

The work described herein was supported by Postgraduate Scholarships from the National Health and Medical Research Council (2/92-8/95) and from the Microbial Pathogenesis Unit of the University of Adelaide Department of Microbiology and Immunology (9/95-12/95). Work at the Max-Planck Institute was supported by a Max-Planck Research Prize to Paul A. Manning and Thomas F. Meyer.

Publications arising from this thesis:

1. **Iredell, J., Manning P.A. 1994.** The Toxin-coregulated Pilus of *Vibrio cholerae*: a model for the biogenesis of type IV pilins ? Trends in Microbiol. **2**:187-192

2. **Iredell, J., Manning P.A. 1994.** Biotype-specific *tcpA* genes in *Vibrio cholerae* FEMS Microbiol. Lett. **121**: 47-54

3. **Iredell, J., Stroeher, U., Ward, H., Manning, P.A. 1997.** Characterization of *rfb* mutants *in Vibrio cholerae O1.* FEMS Microbiol. Lett. - *in press*

4. **Iredell, J., Manning P.A. 1997.** Outer membrane translocation arrest of the TcpA pilin subunit in *rfb* mutants of *Vibrio cholerae* 569B. J. Bacteriol. 179: 2038-2046

5. **Iredell, J., Manning P.A.** The action of TcpT in the cytoplasmic membrane of *Vibrio cholerae* defines an early step in type-4 pilin biogenesis. (*submitted*: Mol. Microbiol)

6. **Iredell, J., Manning P.A. 1997.** Translocation failure in a type 4 pilin operon: *rfb* and *tcpT* mutants in *Vibrio cholerae.* Gene - *in press*

7. **Iredell, J., Manning P.A.** Degradation of TCP/ TcpA suggests a role for the soluble haemagglutinin/ protease of *Vibrio cholerae. (in preparation)*

Abstract

TcpA is the pilin subunit protein of the Toxin-Coregulated Pilus (TCP), an essential fimbrial adhesin of *Vibrio cholerae.* The export of TcpA and assembly of the TCP is explored in this thesis as a paradigm of macromolecular export in Gram negative bacteria. TcpA is examined in detail in an attempt to define strictly conserved regions between species, but is found to be strongly conserved within the three main biotypes. The TCP of the recently emergent O139 (Bengal) serotype is demonstrated to be of El Tor type. The possibility that proteases such as the soluble haemagglutinin (SHA) may have a detachase role centring on TCP dispersal/ TcpA degradation is also discussed.

TCP is a type-4-related fimbrial adhesin, and a useful model for the study of type 4 pilus biogenesis. Homologies to other bacterial macromolecular transport pathways give additional significance to such studies. Transposon mutagenesis of the putative perosamine biosynthesis genes in the *rfb* operon of *V. cholerae* strain 569B eliminates LPS O-antigen biosynthesis but also leads to a specific defect in TCP export. This defect is corrected by complementation of the *rfb* mutation alone. Localization of TcpA is made difficult by the hydrophobic nature of this bundle-forming pilin, which floats anomolously in sucrose density gradients, but the processed form of TcpA can be found in membrane and periplasmic fractions prepared from these strains. While TcpA cannot be detected by surface immunogold labelling in Transmission Electron Microscope (TEM) preparations, EDTA pretreatment facilitates immunofluorescent (IF) antibody-labelling of whole cells, and ultrathin cryosectioning techniques confirm membrane and periplasmic accumulation of TcpA. Salt and detergent extraction, protease accessibility, and chemical crosslinking experiments suggest that although TcpA has not been assembled on the cell surface, subunit interactions are otherwise identical to those within TCP. In addition, TcpA-mediated fucose-resistant hemagglutination of murine erythrocytes is preserved in whole cell lysates, suggesting that TcpA has obtained its mature conformation. These data localize a stage of type-4 pilin translocation to the outer membrane, at which stage export failure leads to the accumulation of pilin subunits in a configuration similar to that within the mature fibre. Possible candidates for the outer membrane defect are discussed.

The highly conserved TcpT protein, encoded from within the promotor-distal region of the *tcp* operon, also receives special attention. Normally present in small amounts and tightly associated with the inner membrane *in vivo*, overexpression *in vitro* is greatly enhanced by substitutions within the ribosome binding site and a fully functional TcpT fusion protein can be purified from the cytoplasm. TcpT is a cytoplasmic membrane-associated ATP-binding protein, homologues of which are essential in type 4 pilin assembly and a number of outer membrane translocation systems in Gram-negative bacteria. A functional analysis of this protein has not been previously reported, and this constitutes the first description of the translocation defect consequent upon mutation of this class of protein in a pilin system. TcpT, and a highly conserved ATP-binding motif within it, are shown to be absolutely and specifically required for export of the type 4 pilin subunit and assembly of TCP. Export-arrested TcpA subunit is localised to the cytoplasmic membrane in *tcpT* mutants. The data suggest that fundamental subunit-subunit interactions occur immediately after processing by the unique prepilin peptidase common to all type 4 systems, and prior to the action of cytoplasmic membrane proteins homologous to TcpT. TcpT does not appear to introduce major changes in subunit interrelationships, although cytoplasmic membrane-arrested subunit may be degraded in *tcpT* mutants. The action of TcpT confers stability upon TcpA in isogenic *tcpT rfb* double mutants during a stage in translocation occurring before the periplasmic face of the outer membrane. The chromosomal *tcpT* mutation can be complementated by *tcpT* cloned into promotorless vectors, the complementing activity of such constructs being most influenced by co-transcription of TcpE (a putative cytoplasmic membrane protein encoded in the *tcp* operon, by a gene immediately downstream of *tcpT*) and by alterations in the hydrophilic N-terminal region of the complementing TcpT-encoding constructs. Other data is presented to suggest that differences in the activity of TcpT fusion proteins may be due to increased stability of a membrane-associated complex in which both TcpT and TcpE participate.

Abbreviations used in this thesis

1. Text

bp	Base Pair
cfu	Colony - Forming Unit
Ctx	Cholera (Entero)Toxin
DSP	Dithiobis Succinimidyl Propionate
DTSSP	3,3'-Dithiobis Sulphonyl Succinimidyl Propionate
EDTA	Ethylene Diamine Tetraacetic Acid
km^R	Kanamycin resistant/ resistance
MCS	Multiple Cloning Site
Nt	Nucleotide
PBS	Phosphate - Buffered Saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethyl Sulphonyl Fluoride
RBP	Ribulose Binding Protein
RBS	Ribosome Binding Site
RCPCR	Reverse Circle PCR
TAE	(Tris) Acetic acid - EDTA
TBE	(Tris) Boric acid - EDTA
tcp, TCP	Toxin - Coregulated Pilus

2. Amino Acids

Acidic			
	D	Asp	Aspartate
	E	Glu	Glutamate
	Y	Tyr	Tyrosine
	C	Cys	Cystine
Basic			
	K	Lys	Lysine
	R	Arg	Arginine
	H	His	Histidine
Polar non-ionic			
	N	Asn	Asparagine
	Q	Gln	Glutamine
	S	Ser	Serine
	T	Thr	Threonine

Non-polar			
	G	Gly	Glycine
	A	Ala	Alanine
	V	Val	Valine
	L	Leu	Leucine
	I	Ile	Isoleucine
	M	Met	Methionine
	P	Pro	Proline
	F	Phe	Phenylalanine
	W	Trp	Tryptophan

3. Measures

Standard (metric) nomenclature and abbreviations are used throughout.

Contents - detail:

Chapter One - Introduction

Chapter Three - Outer Membrane Translocation Arrest of the TcpA Pilin Subunit in *rfb* Mutants of *Vibrio cholerae* O1 strain 569B

Chapter Four - TcpT is essential for translocation of the TcpA subunit beyond the cytoplasmic membrane

Chapter Five - Cytoplasmic Membrane-Association of TcpT *in vivo*

Chapter 6 - General Discussion

Chapter Seven - Materials and Methods

Chapter One

Introduction

Infectious disease remains the most important of all causes of human morbidity and mortality worldwide. It is the terminal event in most lives, either as a primary episode in an otherwise well person or as the final crisis in a setting of lowered resistance from other causes. The Gram negative human pathogens have a wide and varied virulence repertoire and it is incumbent upon those who wish to better understand their parasitism to study these mechanisms in detail. This thesis examines an aspect of the pathogenesis of a model extracellular enteric pathogen, the causative agent of human cholera.

1.1 Structure and function; conserved elements of phenotype

Ultimate biological success of an organism depends on the establishment of a secure ecological niche and/ or the ability to adapt to new ones. The flexibility of the genome is an important element in the latter. Internal gene rearrangements may occur (Meyer *et al.,* 1990; Pierce *et al.,* 1985), as well as stable genomic integration of foreign genetic material (Collis and Hall, 1992; Silver and Bostian, 1993; Stroeher *et al.,* 1995). Elements that might promote genetic mobility or exchange have been described in *Vibrio cholerae* (Pearson *et al.,* 1993; Barker *et al.,* 1995), and the acquisition of new genetic material *via* a recently recognised insertion sequence (Stroeher *et al.,* 1995, Bik *et al.,* 1995) has given rise to a recent epidemic wave of cholera (Albert *et al.,* 1993). The ability to harbour and exchange novel self-replicating genetic material such as

antibiotic-resistance plasmids is even more common (Glass *et al.,* 1980, 1983; Morris *et al.,* 1985; Silver and Bostian, 1993).

The archetypal enteric pathogen is a motile, double membraned, bacilliform organism, the biochemical and physical characteristics of which are further defined largely by the intracellular or extracellular nature of the parasitic lifestyle. An extracellular pathogen is usually obliged to secrete a number of virulence factors, to exhibit diversity of nutrient usage, and is often better adapted to survival outside the host. Secreted factors serve to localise the pathogen within a specific environmental niche by elaborating a specific adesin and/ or modifying the local environment to better suit the parasite (for review, see Finlay and Falkow, 1989) or to specifically sequester scarce elements such as cobalamins and iron (Postle, 1990; Kadner, 1990; Webster, 1991). The Gram negative envelope is a protective barrier against the outside world, but also complicates macromolecular export and the assembly of surface structures. In sections that follow, comments on the putative pathogenic mechanisms of *V. cholerae* will be followed by a discussion of the well-characterized transport systems in (Gram-negative) bacteria, with particular reference to type-4 pilin biogenesis, the ultimate subject of this thesis.

1.1.1 Toxigenic illness: accident or design ?

It would seem that the cholera poison, when reproduced in sufficient quantity, acts as an irritant on the surface of the stomach and intestine, or what is still more probable, it withdraws fluid from the blood circulating in the capillaries, by a power analagous to that by which epithelial cells of the various organs abstract the different secretions of the healthy body.

(Snow, 1855)

Cholera is probably one of the best known of human plagues, the name alone still sufficiently evocative to appear in titles of contemporary popular fiction (Marquez, 1984). It stands in the public imagination alongside the dread diseases of Tuberculosis (the "consumption" of Hippocrates: Lloyd, 1983) and the "Black Death" of medieval Europe or Defoe's 17th century London (Defoe, 1928). Comprehensive reviews of the clinical illness and current epidemiological status are readily available (Greenough, 1990; Blake, 1994), and it is not my intention to describe the disease in detail in these pages.

It suffices for the purposes of our discussion to state that cholera is a dehydrating diarrhoeal catharsis which is variably accompanied by nonspecific constitutional and gastrointestinal symptoms such as fever, headache, colic and vomiting. Severe illness may result in purging of the entire body weight equivalent over four to seven days (Hirschhorn *et al.,* 1968), in a high volume stool with an electrolyte composition similar to that of plasma. A fatal outcome may follow in as little as two or three hours after the

first onset of symptoms (Greenough, 1990). The basic arsenal of the cholera pathogen appears to consist of toxic (particularly the cholera toxin, Ctx) and adhesive elements (particularly the toxin-coregulated pilus, TCP), augmented to an uncertain degree by a number of other factors which are discussed below.

The cholera enterotoxin is both characteristic of (Keasler and Hall, 1993) and essential for (Herrington *et al.,* 1988) the pathogenesis of *V. cholerae*. It therefore follows that the associated phenotype is likely to be no accident, but rather a reflection of the lifecycle of this organism (Manning, 1994). The chief enterotoxic principle of the pathogenic cholera organism is fully characterised and is one of the best understood of such elements (Mekalanos, 1985; Finkelstein, 1990; Kaper *et al.,* 1995).

Ctx appears to promote colonisation of the small gut in a rabbit model, perhaps because of the ideal liquor in which the organisms are bathed and disseminated inside the toxin-affected gut lumen (Pierce *et al.,* 1985). This is reflected in the observed *in vivo* selection of hypertoxigenic strains (Pierce *et al.,* 1985).

The epithelial cell receptors for cholera toxin (Ctx) in the gut mucosa are GM_1 gangliosides (King and van Heyningen, 1973), to which holotoxin binds tightly, exerting its effect for many hours. Assembled and exported as a multimeric holotoxin from the periplasm (Hirst and Holmgren, 1987a, 1987b) before activation by proteolytic 'nicking', the mature toxin consists of five binding (B) subunits and an active (A_1) adenylate-cyclase stimulating subunit, disulphide-bonded to the linking A_2 subunit (Mekalanos *et al.,* 1979; Booth *et al.,* 1984). The A_1 fragment is translocated through

the target membrane by conformational change resulting from binding of the B subunits, mediating ADP-ribosylation of host cell proteins which result in elevated cAMP levels and altered ion transport in mucosal cells (for review, see Mekalanos, 1985; Finkelstein, 1990; Kaper *et al.*, 1995). The cholera stool is thus primarily (but not solely; see also Peterson and Ochoa, 1989) the net result of non-specific adenylate cyclase activation in all epithelial cells.

V. cholerae O1 secrete a neuraminidase, which is predicted to perform the task of reduction of higher order gangliosides to the monosialyl form suitable for binding of the cholera toxin B subunit (Kabir *et al.,* 1984). Contributions from accessory toxins such as the accessory cholera enterotoxin (ACE) and the zonula occludens toxin (ZOT) are undefined at present, but the expression of ZOT and ACE is probably independent of that of Ctx (Baudry *et al.,* 1992; Trucksis *et al.,* 1993). The action of ZOT to loosen intercellular tight junctions (Fasano *et al.,* 1991) may nevertheless contribute significantly to the overall 'leakiness' of the mucosa. ACE appears to be a membrane-active toxin, able to increase fluid secretion in the ligated ileal loop infection model (Trucksis *et al.,* 1993).

1.1.2 Carriage of El Tor strains

Disease is generally attributable to the overwhelming proliferation of organisms, with the accompanying elaboration of host-toxic elements. 10^6 cfu of cholera organisms in volunteers soon becomes 3 x 10^7 cfu/ml of stool, of average total volume over 4 litres (Levine *et al.,* 1988). It might therefore be reasonably argued that host defenses need only be adequate to arrest proliferation and ameliorate symptoms in an intralumenal extracellular infection such as human cholera, thus allowing returning normal flora and concentrating stool to act against the remainder as numbers fell. Cells capable of competing more effectively in the gut lumen would be in a position to benefit from the biodiversity and relatively sheltered environment offered within, after the clinical 'infection' is resolved. The 1:30-100 case-carriage ratio of El Tor compared to 1:2-4 for classical biotype (Woodward and Mosley, 1971; Gerichter *et al.,* 1973) suggests that El Tor strains are better equipped to survive in the gut lumen than those of classical biotype. To explain this, El Tor strains need differ from the classical biotype only in the capacity to exploit the host as a niche for base-level survival ("carriage"). Apart from subtle differences in regulation of cholera toxin (CTX) and TCP suggested by *in vivo* experiments (Voss and Attridge, 1993; Voss *et al.,* 1996; Thomas *et al.,* 1996), El Tor strains also possess the additional cytolytic capacity encoded by the complete *hlyA* gene.

1.1.3 A conserved cytolysin is characteristic of El Tor strains.

Iron limitation is a major barrier to growth of bacterial parasites in the host, and specific energy-expensive uptake systems have evolved to deal with this (Bagg and Neilands, 1987; Postle, 1990). Most iron within mammalian hosts is tightly complexed within molecules such as transferrin, lactoferrin, and ferritin. While less important for organisms living in the relatively iron-enriched intracellular milieu, iron limitation is an important antibacterial defence against extracellular parasites such as *V. cholerae* (reviewed in Bagg and Neilands, 1987 and Finkelstein *et al.,* 1983). The *V. cholerae* El Tor *hlyA* gene encodes an 82 kDa haemolytic protein which is transported into the periplasm as a protoxin, and cleaved after export into 65 kDa and 15 kDa peptides (Honda and Finkelstein, 1979; Goldberg and Murphy, 1985; Rader and Murphy, 1988; Yamamoto *et al.,* 1990; Alm *et al.,* 1988; 1991). This mediates lysis of sheep red blood cells *in vitro* by most El Tor strains (Pollitzer, 1959), and is subject to regulation by an Fe-stress response (Stoebner and Payne, 1988; Williams and Manning, 1991). In an El Tor *wt* strain, deletion of the *hlyA* gene leads to a 100-fold diminution in LD_{50} for suckling mice (Williams *et al.,* 1993). Classical strains have a deletion within hlyA, leading to a truncated product of 27 kDa which appears to have enterotoxic but not cytolytic activity (Alm *et al.,* 1988; Alm *et al.,* 1991). Presence of the entire gene is characteristic of El Tor pathogenic isolates, and has been proposed as a biotype-specific marker (Alm and Manning, 1990). It begs the question as to why such an apparently valuable virulence factor should be biotype-specific. The cytolytic moiety of the pore-forming toxin might increase access to host haem and non-haem iron sources (and

perhaps also facilitated by secreted proteases), permitting coregulated iron-uptake by specific system/s (Stoebner and Payne, 1988; Goldberg *et al.,* 1990, 1991).

1.1.4 The soluble haemagglutinin: a conserved protease without a specific role ?

The soluble haemagglutinin/ protease (SHA/ protease or HAP) is conserved in all pathogenic isolates of *Vibrio cholerae* (Hanne and Finkelstein, 1982; Svennerholm *et al.,*1983; Booth and Finkelstein, 1986), including O139 strains (Jonson, pers. comm.). It is a zinc-dependent metalloprotease (Booth *et al.,* 1983), which may be only one of a number of *V. cholerae* proteases (Young and Broadbent, 1982; Ogierman and Manning, in preparation) and is secreted by the *eps* system along with cholera toxin (Overbye *et al.,* 1993). The HAP cleaves a number of substrates *in vitro* including the iron-binding protein, lactoferrin (Finkelstein *et al.,* 1983), and activates the A subunit of cholera toxin (Booth *et al.,* 1984). However, cholera toxin can apparently also be activated by a number of other proteases normally present in the gut, since virulence in infant rabbits is unaffected by a *hap* mutation (Finkelstein *et al.,* 1992). Liquefaction of proteins or mucin in the gut lumen, or hydrolysis of an iron-binding protein such as lactoferrin, would surely confer biological advantage in parasitism of the human host, but might be undetectable in animal models with usual endpoints such as ID_{50}, LD_{50}, and in competition studies against *wt* strains (since, pertinent to the latter case, HAP is a secreted protein). Redundancy of HAP is inconsistent with its wide conservation in pathogenic strains. However, *hap*-mutant strains have been observed to more readily detach from isolated rabbit gut mucosa *in vitro*, and a role as a 'detachase' has therefore been proposed (Finkelstein *et al.,* 1992).

1.1.5 Regulation systems: cholera toxin and the coregulated adhesin, TCP

The ToxR protein has been proposed to be a global virulence regulator (Miller and Mekalanos, 1988) which controls the expression of the gene cluster encoding biogenesis of the Toxin-Coregulated Pilus (Taylor *et al.,* 1987), as well as expression of the *acf* genes (Peterson *et al.,* 1988), and expression of a number of other genes which may not be directly involved in virulence (Miller and Mekalanos, 1988; Parsot and Mekalanos, 1988). The ToxS/ ToxR sensor/ transcriptional activator pairing (Miller *et al.,* 1987; DiRita and Mekalanos, 1991; Dziejman and Mekalanos, 1994) transduces environmental signals based on temperature, pH and osmolarity (DiRita *et al.,* 1991).

ToxT is an AraC-type transcriptional activator subject to ToxR control (Ogierman and Manning, 1992; Higgins *et al.,* 1992), and is found immediately upstream of the specific peptidase, TcpJ (Kaufman *et al.,* 1991). ToxT supplied *in trans* can bypass the need for ToxR activation, in those genes (*tcp*) involved in the ToxR-ToxT regulatory cascade (DiRita *et al.,* 1991). Situated at the end of the transcriptionally linked *tcp* operon (Ogierman *et al.,* 1993), *toxT* may be weakly transcribed by readthrough of the *tcpA-F* message, but extensive transcriptional attenuation in the *tcpF-toxI* intergenic region and the identification of ToxR-dependent (and lesser ToxR-independent) promotors as well as specific DNA binding of ToxR in this region has been demonstrated (Higgins and Di Rita, 1994).

1.1.6 Regulation systems: iron and the haemolysin

Iron stress and regulation of specific iron uptake systems are common themes in bacterial enteropathogens. A large number of iron-regulated outer membrane proteins have been identified in *V. cholerae* (Sciortino and Finkelstein, 1983; Litwin and Calderwood, 1994), including a homologue to members of the TonB-dependent iron uptake system (Goldberg *et al.,* 1990b; Goldberg *et al.,* 1992), and an adjacent activator (Litwin and Calderwood, 1993; DiRita, 1994). Iron stress upregulates the gene encoding the HlyA protein (Stoebner and Payne, 1988) (discussed in section 1.1.3). A connection of uncertain import at present is the finding of the independent HlyU regulator of *hlyA* (Williams and Manning, 1991), which upregulates *hlyA* and at least one other gene (Williams *et al.,* 1993).

1.2 Of Haemagglutinins, Adhesins, and Sticky Bullets...

An invasive organism may reach its goal in a number of ways. At one extreme, passive uptake by macrophages implies the risk of destruction, or of not being taken up at all and therefore failing to enter the intracellular stage of the life cycle. Cellular resources must therefore be devoted to survival in the harsh outside milieu (until an opportunity arises) and then to survival inside a professional killer cell when the opportunity is presented (i.e. when consumed by a macrophage). At the other extreme, specific targeting of certain host cells dimishes the risk that organisms will fail to enter and

increases the likelihood that the host will be ideal. Organisms adopting such a strategy can therefore afford to be relatively more fastidious and fragile.

V. cholerae is not a fastidious organism, and lives an entirely extracellular lifestyle. It therefore has arguably different requirements of an adhesin than those of an organism for which accurate targeting and secure adhesion are simply strategies to promote efficient invasion, and for which the intracellular phase is integral to the lifecycle.

1.2.1 Haemagglutinins

In vitro agglutination of red blood cells is a convenient laboratory phenotype, and many secreted and surface elements of the cholera organism were initially defined by this means (Jones and Freter, 1976; Hanne and Finkelstein, 1982; Holmgren *et al.,* 1983). Fucose-sensitive adhesion to rabbit epithelium *in vitro* has been described in a classical strain (Nakasone and Iwanaga, 1993), and a growth-phase dependent fucose-sensitive agglutinin of human red blood cells has been identified (FSHA: Hanne and Finkelstein, 1982; Holmgren *et al.,* 1983) which is generally better expressed by strains of classical than El Tor biotype (Jonson *et al.,* 1994). A mannose-fucose-resistant haemagglutinin (MFRHA) has also been described (Franzon and Manning, 1986) which appears to be important in the infant mouse model (Franzon *et al.,* 1993) but has proven difficult to further characterise (Barker, 1995), and is of unknown relevance in human infection.

Better understood is a type-4 pilus initially discovered as a mannose-senstive agglutinin of chicken red blood cells (Holmgren *et al.,* 1983; Jonson *et al.,* 1991). MSHA is not a

bundle forming semi-rigid pilus like the TCP. It can be detected as a laterally extruded fibre (Jonson, 1991), and is readily entangled in the powerful polar flagellum (Fig. 1.1). While MSHA production has been clearly demonstrated in El Tor strains *in vitro* (Jonson *et al.,* 1991), and antibodies directed against MSHA have been reported after natural human infection (Jonson, 1991), the significance of this adhesin in human infection remains to be demonstrated. Initial experiments showing that MSHA is required for virulence in animal models (Finn *et al.,* 1987; Osek *et al.,* 1992) could not be reproduced in another laboratory. Careful studies in sets of isogenic *mshA* and *tcpA* mutants conclusively ruled out a virulence role for the MSHA in the infant mouse cholera model (Attridge *et al.,* 1996). Its ubiquity in strains of El Tor biotype (Barua, 1974; Holmgren and Svennerholm, 1983; Jonson *et al.,* 1989) argues for a significant role, perhaps outside the human host. If, for example, environmental isolates of virulent *V. cholerae* El Tor normally express the MSHA, ingested organisms in an endemic area would bring the externally presented protein into contact with the mucosal immune system. This would occur in the pharynx rather than in the gut, where the organism is greatly reduced in number by gastric acidity, and may provide one possible explanation for an immune response to the MSHA after 'natural' infection, since only successful infections would generate TCP, which would then encounter the gut mucosal immune system. Background MSHA-specific seropositivity would therefore be higher than that for TCP in an endemic area in these circumstances, and should be age (and therefore exposure) - related. It is equally plausible that MSHA is in fact important to virulence in humans, but is not adequately evaluated in the extant animal models. Only appropriate human studies would resolve this apparent dilemma.

The soluble haemagglutinin has been mentioned (Section 1.1.4), and will be referred to again in the Supplement (below) and in Section 1.3.

1.2.2 Adhesins other than TCP that may be coregulated with Cholera Toxin

The putatively mobile 'core' *ctx* element contains the genes encoding cholera toxin, and two accessory toxins (see below). In addition to various toxin genes in this region, a pilin-like protein is encoded which is not essential for, but may enhance, gut colonisation (Pearson *et al.,* 1993). Co-regulated with the cholera toxin, in the ToxR regulon, are *acfABCD*, Tn*phoA* insertion in any of which leads to a modest colonisation defect (Peterson and Mekalanos, 1988). Their position downstream of the *tcp* operon is of unknown significance and their biological function/s unclear, although *acfB* probably encodes a methyl-accepting chemotaxis protein (Everiss *et al.,* 1994), and a reading frame that overlaps *acfD* is predicted to encode a protein with homology to flagellar biosynthesis proteins (Hughes *et al.,* 1994). A variety of other pilins and haemagglutinins have also been described (Tweedy *et al.,* 1968; Hall *et al.,* 1988; Yamamoto *et al.,* 1986; Iwanaga *et al.,* 1989; Ehara *et al.,* 1991), but are of unknown relevance in human disease.

Figure 1.1 MSHA and TCP: two examples of *V. cholerae* type-4 fimbriae

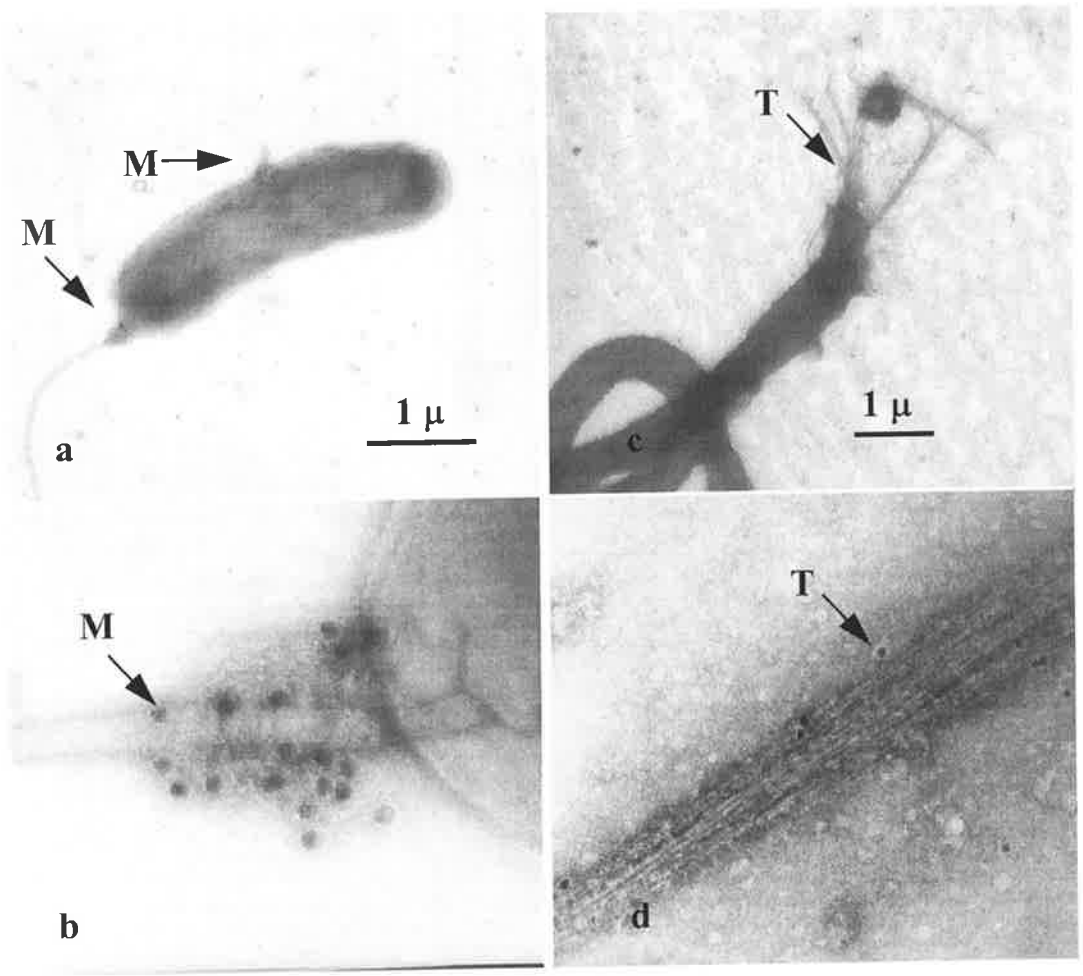


Fig. 1.1 Immune electron micrographs of *Vibrio cholerae* strains grown under conditions to promote the expression of MSHA (M) and TCP (T). The MSHA (M) is laterally extruded from the organism (panel a) and is a relatively flexible fibre which may become entangled in the polar flagellum (panel b). By contrast, the TCP (T) is found in a polar location when still attached end-on (panel c), but is usually found in clumped bundles of parallel fibres (panel d) which may be cell-associated or completely free in the medium. Upper panels also contain 1 μ size markers (horizontal bars). Labelling by specific antisera is detected with secondary antibodies conjugated to 10 nm gold particles.

1.2.3 TCP is the only adhesin of proven significance *in vivo*

TCP was first recognised as a virulence determinant controlled by the same transcriptional regulator as cholera toxin (Taylor *et al.,* 1987; Miller and Mekalanos, 1988). Thought to be a homopolymeric adhesin akin to the type-4 pilins found in many Gram negative bacteria (Taylor *et al.,* 1987; Faast *et al.,* 1989; Shaw and Taylor, 1990), TCP can be found as cell-associated pili, but more commonly as bundles of rigid filaments. Hard evidence for exclusively polar expression of TCP is lacking but is generally assumed (Sharma *et al,* 1989), and while this truism has been applied to all type-4 pili (Tennent and Mattick, 1994) it is clearly not valid for the MSHA (Fig. 1.1). The 'autoagglutination' mediated by these pili *in vitro* is common to a number of type-4 pili (Henriksen *et al.,* 1975; Love *et al.,* 1984; Todd *et al.,* 1984; Taylor *et al.,* 1987; Girón *et al.,* 1991). The pili of *V. cholerae* and of enteropathogenic *E. coli* form large bundles of aggregated extracellular fibres *in vitro,* and it has been suggested that these should be regarded as a subclass of type 4 pili (Tennent and Mattick, 1994).

TCP is the only fimbrial adhesin known to be essential for cholera pathogenesis, and this has been shown for both biotypes in an animal infection model (Taylor *et al.,* 1987; Attridge *et al.,* 1993) and, for the classical biotype, in the human host (Herrington *et al.,* 1988). The colonisation defect in the infant mouse cholera model consequent upon specific TCP knockouts in strains of either classical or El Tor biotype is several orders of magnitude greater than any other putative adhesin (Peterson and Mekalanos, 1988; Pearson *et al.,* 1993; Attridge *et al.,* 1993; Attridge *et al.,* 1996).

1.2.4 Adhesion, chemotaxis and motility: the cholera organism as a sticky bullet

Flagellar motility is vital to virulence in the infant mouse cholera model (Attridge and Rowley, 1983) and the flagellar structure appears to promote colonisation *per se* in the removable intestinal tie adult rabbit diarrhoea (RITARD) model (Richardson, 1991). The polar flagellum imparts a forward motility to *V. cholerae* that is so striking as to be a diagnostic marker in the presence of typical clinical illness (Greenough, 1990), and chemotaxis-driven motility along stress lines in the mucous gel *in vitro* (Freter *et al.*, 1981) may provide us with a glimpse of *in vivo* behaviour. The presence of a mucinase (SHA/ protease: Hanne and Finkelstein, 1982) and DNAse (Focareta and Manning, 1991) may also aid in motility through the mucus, and the notion of a flagella-driven chemotactically-guided projectile (Fig.1.2) may be supported by reports of initial association to rabbit mucosa by the non-flagellar pole (Nelson *et al.*, 1976; Teppema *et al.*, 1987). The combination of a 'sticky' forward pole and a powerful flagellar motor, at opposite ends, may allow the organism to push through the mucus to a specific target site, as a 'sticky bullet'. It should be emphasised that expression of 'stickiness' is temporally and situationally specific, being appropriately co-ordinated with that of other virulence factors. This is not a novel concept of cholera pathogenesis, and readers are referred to the 'swim to arrive, stop to kill' paradigm previously outlined (Gardel and Mekalanos, 1994).

1.2.5 Extracellular bundles: incompatible with the sticky bullet model ?

TCP is seen both as intimately cell-associated parallel fibre bundles, and as copious cell-free bundles, when grown *in vitro* (Jonson et al 1991a). These bundles are also seen in stool from infected animal models and human clinical specimens (Jonson *et al.,* 1991b), and therefore cannot be easily dismissed as *in vitro* artefact. A functional homology may exist in the closely related type-4 bundle-forming pilus (BFP) of Enteropathogenic *E. coli* (EPEC), the localised adhesion phenotype in cell culture (Skaletsky *et al.,* 1984) giving us perhaps a glimpse of the pathogenic strategy employed by *V. cholerae.*

Supplement: TcpA is subject to degradation in vitro by a co-secreted protease

In contrast to conventional presentation of the Introductory section, some experimental data is included here in the form of a Supplement, presented in this manner because it contains data which do not otherwise appear amidst the body of experimental work described in Chapters Two to Five, inclusive. This section does not bear directly upon the issue of pilin translocation, but is important contextually and facilitates the discussion of pathogenesis at this point.

In the course of the work done for this thesis, it was noted that early logarithmic phase classical cultures of *V. cholerae* seemed to have amounts of TCP comparable with those of late logarithmic phase cultures, and further exploration of this phenomenon suggested that a cosecreted protease degrades TCP and TcpA *in vitro*. The evidence for this is presented here, and it is argued that the data is consistent with the 'detachase' model previously proposed (see Section

TCP is present in comparable amounts in early and late cultures

Divided cultures of Z17561 cells grown for optimal TCP expression were harvested at 4 and 16 hours and subjected to SDS-PAGE and immunoblot for TcpA. Equal volumes of culture were pelleted and serially diluted (Fig. S1, overleaf). Estimates of cell number were made by direct counting in a Nebauer chamber, and by serial dilutions onto plain nutrient agar plates. The apparent doubling in levels of immunoreactive TcpA as

estimated by serial dilutions of immunoblots is much less than expected for the approximately 10-fold increase in viable cell count.

Figure S1. Serial dilutions of TcpA after *in vitro* culture

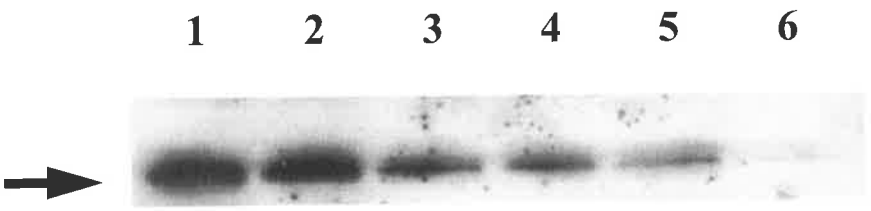


Figure S1. Serial twofold dilutions of Z17561 whole cells from 4 hours (lanes 1,3,5) and 16 hours (lanes 2,4,6). Starting volumes contained approximately 9 x 10^8 cfu/ ml (lane 1) and 1.2 x 10^{10} cfu/ ml, with 95 - 100% viability when Nebauer counts were compared with plating.

Clumped appearance of Z17561 cultures grown for TCP expression in vitro is lost on standing at 37°C, and is accompanied by proteolytic degradation of TcpA

The clumping or autoagglutination characteristic of TCP expressing classical strains of *V. cholerae* (Taylor *et al.*, 1987) is seen to disappear gradually over a period of $3^1/_2$ - 4 hours from AKI cultures that were moved after 4 hours of growth (ca. 5 x 10^8 cfu/ml) to stand at 37°C. TcpA is still detectable by immunoblot at this point, but is undetectable after 16 hours (Table S1). This loss of clumping does not occur at 4°C, and is also preventable by the addition of the broad spectrum protease inhibitor, phenylmethyl sulphonylfluoride (PMSF, 1.0 mM final concentration). In order to determine whether

this effect was dependent upon cellular activity, and to see whether excess of an alternative substrate could 'protect' TcpA, cells were washed in PBS before resuspension in fresh nutrient broth for holding at 37°C in the presence of of Na azide (final concentration 0.5%), or bovine serum albumin (BSA; final concentration 2.5% w/v). The apparent requirement for cell viability is consistent with protein secretion, and the protective effect of BSA is consistent with a broad spectrum protease activity (Table S1).

Table S1: Macroscopic agglutination of TCP-expressing Z17561 grown *in vitro* is lost after shift to 37°C

	1hr	1½	2 hrs	2½	3 hrs	3½	4 hrs	16 hrs
37°C shift (AKI)	+++	+++	+++	+++	++	+	(+)	-
PMSF (AKI)	+++	+++	+++	+++	+++	+++	+++	-
4°C shift (AKI)	+++	+++	+++	+++	+++	+++	+++	+++
37°C shift (NB)	+++	+++	+++	+++	++	+	(+)	-
0.5% azide (NB)	+++	+++	+++	+++	+++	+++	+++	+++
2.5% BSA (NB)	+++	+++	+++	+++	+++	+++	+++	+

Table S1. +++ indicates coarse agglutination (*wt* levels) with clearing of cultures on standing for 10 minutes at 25°C; ++ indicates reduced agglutination that is still easily visible; +: fine agglutination only; (+) indicates the absence of visible agglutination, but with TcpA still detectable by immunoblot; -: TcpA is not detectable by immunoblot. Cultures were all grown in AKI medium for 4 hours for TCP induction, and transferred directly (AKI) or with a change of medium to Difco nutrient broth (NB) to stand at 37°C

The soluble haemagglutinin/ protease may be the active element

Cells grown for *tcp* induction were washed in PBS and resuspended in fresh nutrient broth (at a final dilution of 3 x 10^8 cfu/ml). After addition of specific antisera at 1:100, cells held at 37°C overnight were still visibly clumped, and were positive for TcpA by immunoblot. Polyclonal rabbit antisera raised against the cytoplasmic membrane protein TcpT (expressed in *E. coli*), and absorbed against *tcpT*-delete strains (JRI1 and JRI2; see Section 4.2), failed to prevent the loss of clumping or of immunoreactive TcpA from immunoblots of whole cell preparations run on SDS-PAGE (Table S2).

Table S2. Inhibition of TcpA degradation by antisera to the SHA

	Visible clumping		**TcpA immunoblot**
	(0 hours)	(16 hours)	
No additives	+++	-	**neg**
αHAP 1:100	+++	++	**pos**
αTcpT 1:100	+++	-	**neg**

Table S2. TcpA and TCP and preserved in the presence of antisera against HAP. Z17561 cells were grown at a final dilution of ca. 3 x 10^8 cfu/mL before division into 1 mL aliquots for the experiment. Onservations are reported after brief pelleting (10 sec at 15000 rpm in Heraeus bench microfuge) and resuspension in fresh nutrient broth (0 hours) and after holding overnight at 37°C (16hrs). Scoring of TCP production is described in Section 7.2.3.

1.3 Putting it all together: a current model for pathogenesis ?

Sensing of arrival in appropriate conditions is transduced into biogenesis of these elements (reviewed in DiRita, 1992; discussed in section 1.2.3). It is not inconceivable that signals are also transduced into altered flagellar activity. While there is no direct evidence for this, it is interesting to note the presence of homologues to flagellar biosynthesis genes in the *acf* region, and of altered motility in *acfD* mutants which is apparently more prominent under conditions optimal for *tcp* and *ctx* (and *acfABCD*) expression (Hughes *et al.,* 1994). Both the *tcp* and *acf* gene clusters encode apparent methyl-accepting chemotaxis proteins (Harkey *et al.,* 1994; Everiss *et al.,* 1992). This association between adhesins and chemotaxis in *Vibrio cholerae* has been pointed out before (Gardel and Mekalanos, 1994), and is also found in *Pseudomonas aeruginosa*, which has a closely related type-4 pilin adhesin (Darzins, 1993).

TCP is seen both as intimately cell-associated parallel fibre bundles, and as copious cell-free bundles, when grown *in vitro* (Jonson *et al.,* 1991a). These bundles are also seen in stool from infected animal models and human clinical specimens (Jonson *et al.,* 1992), and therefore cannot be easily dismissed as *in vitro* artefact. The data presented here show that TCP bundles are dispersed and immunoreactive TcpA degraded *in vitro*, and that this is prevented by the addition of a broad spectrum protease inhibitor, by shift to 4°C, or addition of an alternative protease substrate (bovine serum albumin, BSA). The soluble haemagglutinin/ protease is a likely candidate for this activity. The addition of a polyclonal antiserum raised against the HAP, but not against another Vibrio

protein, is able to block this activity *in vitro* and suggests the HAP as a candidate. The difficulty of evaluating a 'detachase' role in animal models has been ably demonstrated (Finkelstein *et al.,* 1992), and it should be remembered that while TCP is the only adhesin of proven pathogenic significance (see Section 1.2.3), it is only one of many that have been identified and the HAP is only one of many proteases that may be significant *in vivo* (for overview, see Kaper *et al.,* 1995). Discrete in-frame deletions of the HAP in Z17561 and/ or 569B to explore this phenomenon further in isogenic mutant sets, and the addition of radiolabelled TcpA to purified HAP *in vitro*, would help to finalize the issue.

Fig. 1.2 outlines a proposed model of cholera pathogenesis, based on all that has been described above. Microcolonies of *V. cholerae* form adjacent or adherent to the gut mucosa and grow quickly, secreting cholera toxin and the soluble protease/ haemagglutinin (among others). TCP mediates intercellular association perhaps more between the bacteria themselves than between the bacteria and mucosal cells, akin to the 'non-intimate' attachment of the first (BFP-mediated) phase of EPEC colonisation (Skaletsky *et al.,* 1984). As the local concentration of cholera toxin rises, the mucosa becomes increasingly a secretory surface. The SHA/ protease activates those A subunits which have not been nicked by gut proteases, and may perform other functions such as release of iron sequestered in chelating proteins (eg. lactoferrin, transferrin) and haem proteins (porphyrins, etc.). The complete El Tor 'haemolysin' may even promote this by direct cell toxicity. DNAses and proteases promote liquefaction of the mucous gel and facilitate motility. Early hypersecretion from the gut promotes dissemination of vibrios

within the gut lumen to promote colonisation of available surfaces, and dilutes toxic elements within the stool.

Once massive solute flux begins, organisms are washed out by bulk action. As free swimmers, they are able to rapidly disperse into environmental niches and/ or new hosts. The large volume diarrhoea facilitates the delivery of pathogens into groundwater, and protects them briefly from drying. Any organisms entrapped in microcolonies by the hydrophobic TCP would be less able to take advantage of rapid dissemination into the environment and, if host-specific, pili would be of no value outside the host. Ongoing rapid multiplication of the organism would become quickly nutrient-limited in the environment, and a large amount of daughter cells produced in the absence of TCP unlikely to eventuate.

Dispersal, degradation, or shedding of pili as the concentration of CTX and protease rose in the gut would simultaneously free more organisms and facilitate their dispersal in the high volume emissions of clinical cholera. If a detachase role exists for the soluble haemagglutinin or other proteases, it should operate on interbacterial associations as well as on bacteria-mucosa associations. Figure 1.2 is a simplistic representation of the pathogenesis of *V. cholerae,* intended as a simple working model to illustrate a putattive role for a "detachase", if such a function operates *in vivo*.

Figure 1.2 The role of a detachase in *V. cholerae* pathogenesis

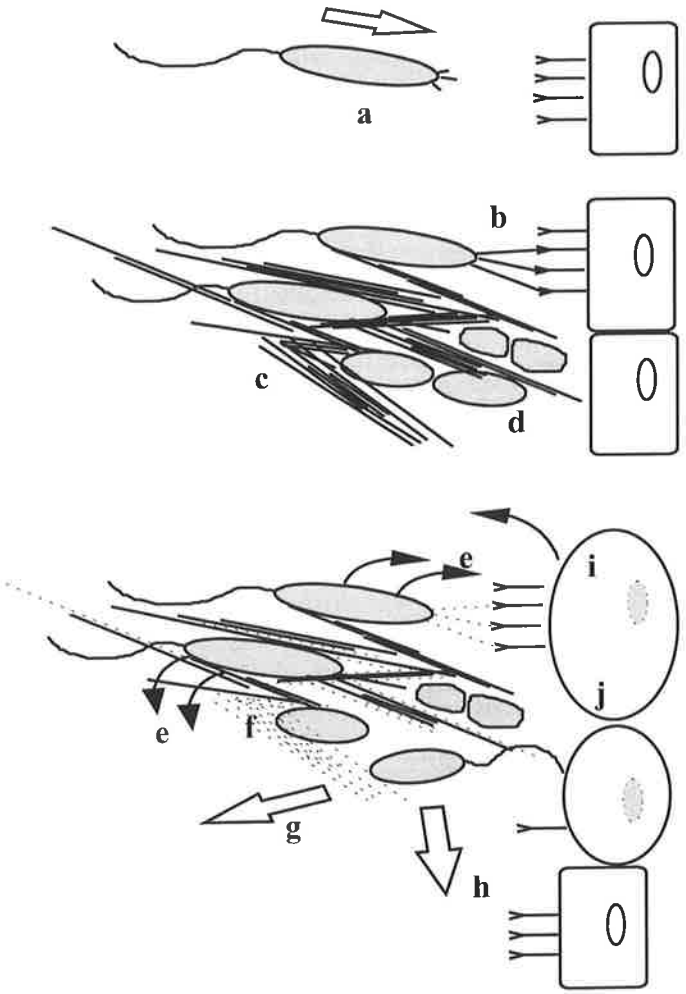


Fig.1.2 *Vibrio cholerae* uses its powerful polar flagellum to approach target adhesion sites on cells (a). Once the appropriate niche is sensed (b), production of TCP (a and c) promotes microcolony formation, in which daughter cells (d) are held in an ideal environment. With continued production of cholera toxin and other secreted virulence factors (e), decreased production, dispersal, and/ or active degradation of the tangling TCP bundles by endogenous or exogenous proteases (f) would permit departure of daughter cells into the environment via the stool (g), or onto new targets (h). The dramatic cellular dysfunction and sublethal insult consequent upon the action of cholera toxin (i), accompanied by the possible disruption of tight junctions by the zonula occludens toxin (j), may lead to premature cell death and increased epithelial shedding.

1.4 Energy transduction and bacterial membranes: general considerations

Armed with an insight into the pathogenesis and lifestyle of *V. cholerae*, it is now appropriate to deal with issues pertinent to the export and assembly of the essential adhesin, TCP. The ultimate purpose of this next section is to provide a background for interpretation of experimental results in the TCP biogenesis system. Homologies within type-4 pilin export pathways have been recognized for some time to extend into systems dedicated to the transport of substrates as diverse as proteins, naked DNA, and bacteriophage (Pugsley, 1993a; Hobbs and Mattick, 1993). The problems of membrane passage and energy transduction are common to all of these, and useful insights can be gleaned from a range of systems in bacteria and higher organisms. The discussion is therefore organised along thematic rather than systematic lines, and is not intended to be a catalogue of protein transport mechanisms.

The cytoplasmic (inner) membrane (CM) is an energised barrier with an actively maintained proton gradient ($\Delta\mu_H+$) providing the electron-motive force with a ready source of phosphate bond-derived energy (ATP) in the cytoplasm. Adjacent to both protein manufacturing machinery and periplasmic sensing systems, inner membrane proteins are readily maintained in forms appropriate for their required biological functions, and thus ideally situated to participate in complex biological machines such as those required to transport hydrophilic substances across these hydrophobic bilayers. The approximately 6nm bilayer structure of the CM has about equal amounts of protein

and lipid, the former probably distributed through the latter in a fluid mosaic, in which 'tethering' of certain proteins may occur by association with internal, external or transmembrane structures, while others 'float' in the bilayer.

By contrast, the Gram-negative bacterial outer membrane is non-energised and structurally quite different, primarily due to the presence of the characteristic lipopolysaccharide. The large diglucosamine backbone of Lipid A, tight packing of esterified saturated fatty acyl chains, and the network of calcium salt bridges amongst the phosphate headgroups and core oligosaccharides on adjacent molecules makes for limited fluidity and low penetrability by non-polar compounds - a perfect barrier in an aqueous environment (reviewed in Nikaido and Vaara, 1985; Kadner, 1990). Passage of small molecules is typically effected by passive diffusion through trimeric β-barrel porins or by facilitated diffusion via specific importers (see Benz, 1988 and Nikaido, 1992 for reviews). Neither of these systems require specific energy sources, and illustrate transmembrane passage in which the inherent kinetic energy of a given substrate can drive a biased reaction to effect translocation. Such a concept has been extended to protein export, and is presented below (Figure 1.3).

Fig. 1.3 The Brownian ratchet model of protein translocation

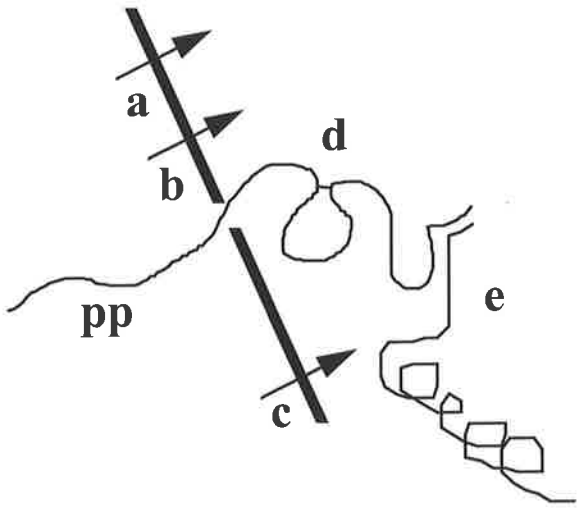


Fig. 1.3 (after Simon *et al.,* 1992). The translocating polypeptide (pp) enters an aqueous transporter channel in the hydrophobic bilayer, and oscillates bidirectionally in Brownian motion. Experimental evidence for bidirectional oscillation of translocating peptides in the translocation pore (Ungermann *et al.,* 1994) supports such a notion. Biasing factors such as electrochemical gradients (a), pH gradients (b), and concentration gradients (c) promote one direction over another, so that forward motion is not equally opposed by reverse. This is further enhanced by the development of protein secondary structure in the new environment (d) or association with other proteins (e). Specific factors that maintain export competence, facilitate delivery to the translocation site, and actively move substrate through the pore are all strategies that have evolved to increase specificity and efficiency, and will be discussed in detail in the sections that follow. Many of these processes require specific energy sources.

1.5 Interdependence of substrate competence and initiation of translocation in biological transport pathways

1.5.1 Protein folding in the cytosol and periplasm: the enzymatic refoldases

In eukaryotic systems, the formation of correct disulphide bonds and the *cis-trans* isomerisation of peptide bonds preceding proline residues are rate-determining processes in the correct folding of many proteins. Protein disulphide isomerases (Freedman, 1989) and peptidyl-prolyl *cis-trans* isomerases (Harding *et al.,* 1989) act independently as well as cooperatively in mammalian cells (Schönbrunner and Schmid, 1992) to promote correct folding. Thioredoxin is the cytoplasmic protein disulphide isomerase of *Escherichia coli* (Edman *et al.,* 1985) and is represented in all classes of organisms from bacteria to higher eukaryotes (Holmgren, 1985), as are the peptidyl-prolyl *cis-trans* isomerases (Fischer and Schmid, 1990). The fact that disulphide bond oxidoreductase activity is apparently also required on the periplasmic side for exported proteins in *E. coli* (Bardwell *et al.,* 1991) and *V. cholerae* (Yu *et al.,* 1992; Peek and Taylor, 1992) points to an unfolded intermediate state as the principle configuration for substrates newly translocated across the cytoplasmic membrane. Such enzymes simply recognise and reopen kinetic dead-end folding structures (Höj *et al.,* 1991) and are not capable of influencing the three-dimensional conformation of the nascent peptide, in the manner of the classic 'chaperone'.

1.5.2 Molecular chaperones and substrate competence

The 'molecular chaperones' constitute a heterologous group of proteins, which function to promote proper assembly of oligomers, to optimise correct folding and refolding of cytosolic proteins, and to stabilise precursor forms of secreted and membrane proteins. Filling a key biological role, they are found in prokaryotic and eukaryotic systems and may be divided into several subclasses. The term was coined to define the role of nucleoplasmin in histone formation, in which nucleoplasmin in essential for, but not included in, the final complex (Laskey *et al.,* 1978). Recognised as inducible proteins capable of preventing insoluble aggregates under stress conditions such as heat shock (reviewed in Rothman, 1989), many chaperones still carry the prefix *hsp* (**h**eat **s**hock **p**roteins). For the purposes of this discussion, the most important subclasses are the secretion-targeting chaperones (particularly SecB: see section 1.6.5) and other cytosolic chaperones which, in the case of GroEL in *E. coli* at least, promote a dynamic ('molten intermediate') state (Martin *et al.,* 1991), which is stable, protected, and competent for subsequent translocation or folding into a mature protein. A parallel may exist in the pH-mediated conformation-driven unfolding/ insertion of diphtheria toxin into endosome membranes. In that model, changes in charge and thus conformation are triggered by low pH (5-5.5) in the endocytic vacuole. Energy for translocation, stored in the molecular structure, is thus transduced into work (reviewed in London, 1992).

Space does not permit a full discussion of structure and function of all subclasses of molecular chaperones, but several excellent reviews exist (Rothman, 1989; Höj *et al.,* 1991; Gething and Sambrook, 1992; Craig *et al.,* 1993). It is worth emphasising the

distinction however, between the DnaK (hsp70 subclass) and GroEL (hsp60 subclass) chaperones widely represented in nature (Gatenby and Ellis, 1990), and the 'delivery' chaperones such as SecB, which introduce and (to extend the simile) facilitate the engagement of substrate and transporter (Section 1.6.3). Trigger factor is a 62 kD ribosomal protein which maintains proOmpA in 1:1 complex in an assembly competent state (Crooke *et al.,* 1988). The Syc proteins of *Yersinia spp.* may serve a similar function to SecB (Section 1.7.5).

1.5.3 Molecular chaperones: active protein folding

Protein transport across the inner membrane takes several seconds (translocation $t_{1/2}$ of 3-12 seconds: Randall, 1983). The development of secondary structure in milliseconds (Kim and Baldwin, 1982; Jaenicke, 1991), and tertiary structure in seconds (Kim and Baldwin, 1982), means that in the absence of systems to promote correct folding tangled knots of misfolded proteins would surely result (Kim and Baldwin, 1982). The delivery of nascent peptide to translocase is facilitated by the binding of various cytosolic chaperones. In bacteria, these include the general-purpose (Hsp60-class) peptide GroEL, the more specific delivery system, SecB, and the ribosomal chaperone protein ('trigger factor'). All of these form soluble complexes with cytosolic precursor proteins *in vitro* (Lecker *et al.,* 1989). GroEL is an Hsp60 cytosolic chaperone, forming a 14-mer of 65 kDa subunits (Weiss *et al.,* 1988; Kumamoto *et al.,* 1989), and its interaction with DnaK in the cytoplasm of *E. coli* will now be considered as an instructive paradigm.

Ordered protein folding in *E. coli* is initiated by DnaK, a protein of the Hsp70 class, which binds the extended nascent chain in an ATP-dependent manner (Buchberger *et al.,* 1994) and cooperates with other cytosol proteins to pass the peptide on to the catalytically active Hsp60 protein, GroEL. This protein promotes folding into the native state (Langer *et al.,* 1992). The intermediate proteins required for the function of Hsp70-type chaperones, DnaJ and GrpE, are required for the ATP hydrolysing activity and subsequent nucleotide release that powers this part of the system (Langer *et al.,* 1992; Hendrick *et al.,* 1993; Fig. 1.6), and homologues of these proteins can be found in mammalian cells (Cheetham *et al.,* 1992).

The Hsp60 (GroEL) class of molecular chaperones is required in *E. coli* for a large range of cellular functions. Mutants grow poorly, are temperature-restricted in growth, and have reduced rates of DNA and RNA synthesis (Fayet *et al.,* 1989; Gething and Sambrook, 1992). Present in large amounts in the cell, and induced by cell stress, these ATP-binding chaperonins can suppress temperature-sensitive mutations in a variety of genes, suggesting an active 'refoldase' role (van Dyk *et al.,* 1989). This 'refoldase' role extends to at least some secretory proteins, since secretion of β-lactamase requires GroEL *in vivo* (Kusakawa *et al.,* 1987), but the export-dedicated chaperones (SecB and related proteins) appear to suffice for most other secretory proteins (Lecker *et al.,* 1989).

A cascade model of the folding of nascent peptide, as proposed by Langer *et al.* (1992) is simplified as follows, and presented in Fig. 1.4:

1. Cytosolic Hsp70 protein (DnaK) binds nascent chains on ribosomes, in an ATP-requiring step (Buchberger *et al.,* 1994).

2. DnaJ stimulates ATP hydrolysis. The ADP-bound DnaK has increased affinity for the unfolded protein (Liberek *et al.,* 1991; Palleros *et al.,* 1991), and GrpE is required for efficient release of the bound nucleotide. These interact successively with folding peptide, apparently by binding specificities for structural elements that are sequentially exposed during folding (Langer *et al.,* 1992).

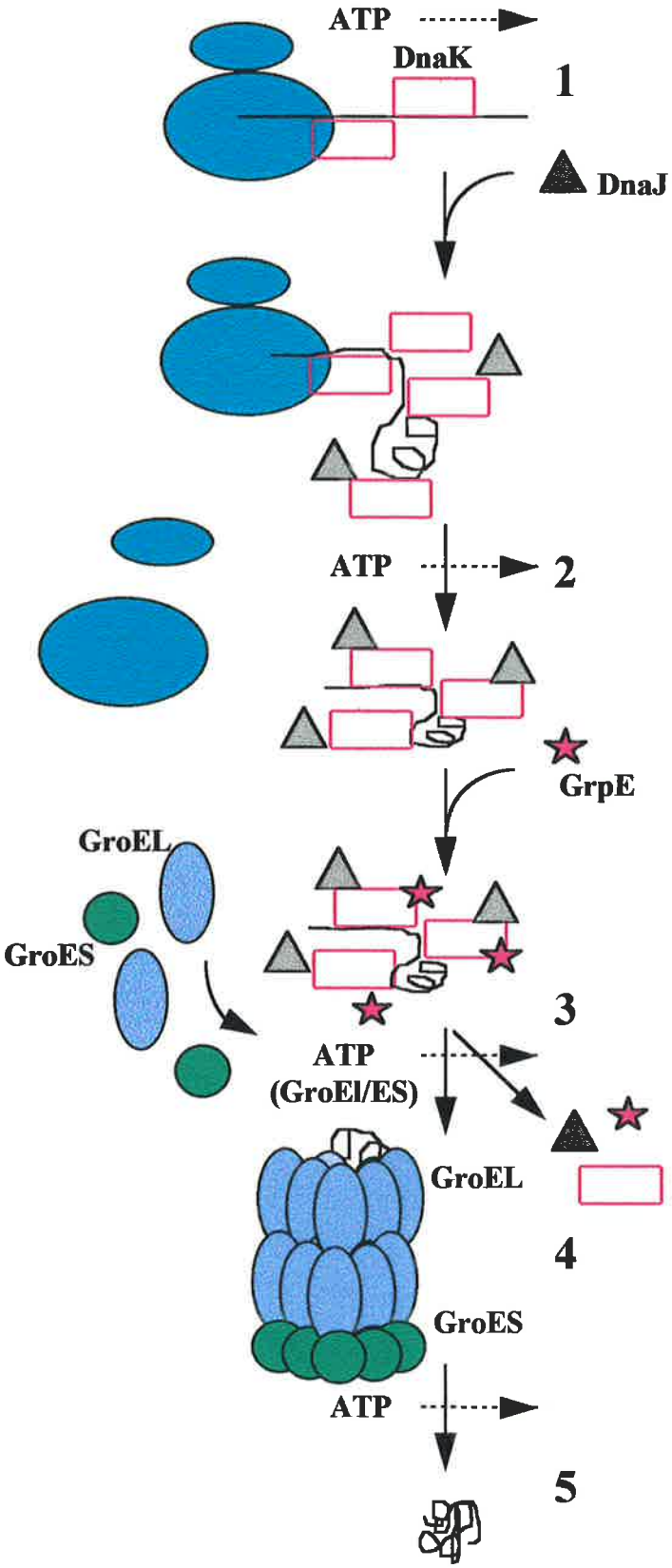
3. GroEL (Hsp60 class) binds the DnaK/ DnaJ complex in a 14-mer of two rings of seven. Peptide can then be released to GroEL, these processes being coupled by GrpE and requiring ATP hydrolysis.

4. GroES binds the GroEL-peptide complex.

5. Folded protein is released, with hydrolysis of ATP

In this model, disulphide interchange proteins and peptidyl-prolyl isomerases are facilitators, promoting formation and breakage of otherwise rate-limiting bonds, and may be excluded from participation only in the GroEL/GroES complex.

Fig. 1.4 Protein folding mediated by the hsp70:hsp60 interactions



1.6 The Sec cytoplasmic membrane export pathway in bacteria

The best understood and most important of the bacterial cytoplasmic translocases is essentially comprised of the six *sec*-encoded (**sec**retion) proteins (Table 1.1), three of which have also been identified by suppressor mutation analysis of signal sequence defects. The three *sec* mutations for which suppressor alleles (**pr**otein **l**ocalization: *prl*) of this type have not been identified (*secB, D,* and *F*) were presumed not to directly interact with the signal sequence.

Table 1.1 The Sec proteins

SecA	102 kDa multimer; cytoplasm or cytoplasmic membrane	widespread ATP-binding protein in Gram negative bacteria; also found in *B. subtilis*
SecB	18 kDa tetramer; cytoplasmic	widespread in Gram-negative bacteria; secretory chaperone
SecD	67 kDa; cytoplasmic membrane	integral inner membrane protein; may be involved in release of translocated chain
SecE	14 kDa; cytoplasmic membrane	participates with SecY in channel formation
SecY	48 kDa; cytoplasmic membrane	participates with SecE in channel formation
SecF	39 kDa; cytoplasmic membrane	integral inner membrane protein; may be involved in energy transduction in the ATP-independent phase of translocation
SecG	(Band 1 or p12); cytoplasmic membrane	formation of the transmembrane channel; copurifies with Sec Y and SecE.

Other alleles which suppress the negative transdominant effect on export of a *secY* mutant gene in the presence of a normal *secY* gene have been identified (Shimolke *et al.,* 1992), but are of uncertain role at present.

1.6.1 Bacterial signal peptides and the Sec system: targeting and insertion sequences

The highly conserved secondary structure of the signal peptide dictates insertion into the membrane and membrane translocase, sufficiently homologous in targeting within both gram negative bacterial cytoplasmic membrane and mammalian endolasmic reticulum (ER) that the signal peptides from these different systems are highly interchangeable. Gram negative signal peptides can also direct secretion through the Gram positive bacterial secretion apparatus (Wang *et al.,* 1993). Despite the presence of a SecA protein in *Bacillus subtilis* which can bind *E. coli* inner membrane vesicles with similar affinity to *wt* SecA (van der Wolk *et al.,* 1993), there are clear differences in the Gram positive signal peptide (von Heijne and Abrahamsen, 1989) and the Gram negative signal peptide appears to be generally inefficient in *B. subtilis* (Collier, 1994). The role of the signal peptide in both mammalian ER and bacterial cytoplasmic membrane (CM) is to open protein conducting aqueous channels, independent of the translocating chain that follows (Simon and Blobel, 1992). Thus, the opening of a gated channel is followed by the translocating peptide which then occupies the aqueous pore.

The conserved features of the 'typical' or 'classical' bacterial signal peptide are dictated by the secondary structure. These features have been recently reviewed (Izard and Kendall, 1994). The stereotypical signal peptide in *E. coli* can be divided into three regions (Fig. 1.5). The N-terminus is five or six residues with a couple of positively charged amino acids such as lysine or arginine. It is thought that the main role of this region is to create the appropriate dipole, the 'positive inside' rule ensuring correct

orientation (von Heijne, 1986, 1992). Net negative or neutral charge in this region leads to great reductions in export efficiency (Inouye *et al.*, 1982; Puziss *et al.*, 1989), and substitution of a histidine residue in this region is tolerated functionally only if the pH is adjusted to maintain a positive charge (Sasaki *et al.*, 1990). The net positive charge may promote association with inner membrane phospholipids by simple electrostatic attraction (Keller *et al.*, 1992), and may also promote loop formation by interaction with negatively charged residues near the cleavage site (Batenburg *et al.*, 1988).

Fig. 1.5 Features of the 'typical' bacterial signal peptide

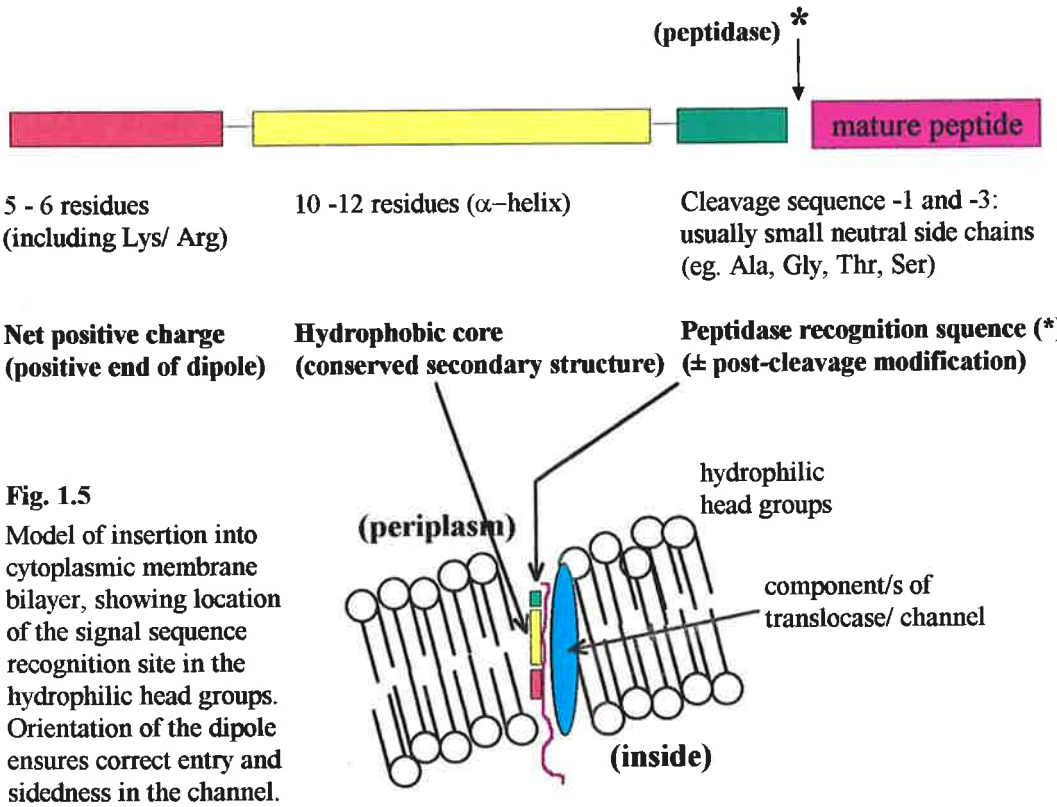


Fig. 1.5
Model of insertion into cytoplasmic membrane bilayer, showing location of the signal sequence recognition site in the hydrophilic head groups. Orientation of the dipole ensures correct entry and sidedness in the channel.

The hydrophobic core of about twelve amino acids, usually rich in leucine and alanine, probably forms an α-helix in hydrophobic environments. This region has upper and lower limits on length, a definable threshold level of hydrophobicity for efficiency, and is intolerant of charged residues (Izard and Kendall, 1994). Suppressor mutations of signal peptide core mutants found in SecA, E, and Y suggest that there may be some direct interaction of the hydrophobic core with the cytoplasmic membrane translocase (Fikes and Bassford, 1989; Stader *et al.,* 1989; Osborne and Silhavy, 1993). The evidence that engineered signal peptide mutants of greater than natural efficiency (Rusch *et al.,* 1994) are not mirrored in nature is presented as evidence of the fine tuning of a multicomponent multiple-use exporter (Izard and Kendall, 1994).

The hydrophobic core is followed by a more polar region which contains the signals for specific cleavage by an endopeptidase. Although it seems likely that the primary role of the core is that of a membrane-spanning hydrophobic segment, a ten residue α helix with a six residue N-terminal positively charged region would not span the ca. 6nm inner membrane bilayer. It is suggested that anchoring of the N-terminal charged group on the cytoplasmic side, with placement of the cleavage site in the hydrophilic headgroups of the bilayer would necessitate conformational change to bring the active site for the appropriate signal peptidase into this region. The alternative possibility put by the same authors (Izard and Kendall, 1994), that the charged N-terminus is drawn into the membrane to the level of the head groups itself, is perhaps supported by evidence that SecA is inserted along with the translocating peptide in the transport channel (Kim *et al.,* 1994; Economou and Wickner, 1994). Sequential ATP-driven insertion-release of 20 residue lengths of substrate, controlled by other *sec* products

could conceivably pull a correctly oriented signal sequence through to the periplasmic face for cleavage. Parallels have been drawn, with respect to topological effects of the transmembrane electrochemical potential ($\Delta\mu_H+$), between *sec*-independent translocation of short loops of integral inner membrane proteins and insertion of the signal sequence prior to *sec*-dependent translocation (Andersson and von Heijne, 1994). Although probably absent from the eukaryotic ER, the transmembrane potential of the bacterial CM (von Heijne *et al.,* 1986) and the mitochondrial inner membrane from the matrix side (Gavel and von Heijne, 1992) have been thought to underly the "positive inside" rule. The behaviour of specific peptides in the presence and absence of the proton ionophore CCCP (to collapse the transmembrane gradient), with facilitated translocation of negatively charged residues and opposed translocation of positively charged residues (Andersson and von Heijne,1994), suggest that the dipolar effect of the signal sequence is dependent upon an intact $\Delta\mu_H+$. Indeed, initial cleavage and translocation of a typical signal peptide can be *sec*-independent and may be uncoupled from *sec*-dependent translocation of the following polypeptide chain (Nilsson *et al.,* 1993).

It is no surprise that the cleavage recognition site exhibits greatest specificity in terms of primary amino acid sequence, as defined for the LepB or the LspA peptidases in *E. coli*. These proteins catalyse the cleavage of the signal prepeptide from the 'mature' protein moiety on the periplasmic face of the CM. The -1 and -3 residues upstream of the LepB cleavage point are typically amino acids with small neutral side chains, often glycine, serine, or threonine, but most frequently alanine (von Heijne, 1984). These residues are also required to be precisely located with respect to the core region (von

Heijne, 1984; Jain *et al.,* 1994). Cleavage specificity is for either the LepB-type or the LspA-type peptidases, the latter associated with acylation the +1 cys of the mature peptide (see Pugsley, 1993a for review).

1.6.2 Sec-independent translocation across the cytoplasmic membrane

Most inner membrane integral proteins have only short periplasmic loops that are probably translocated in a *sec*-independent manner (Rohrer and Kuhn, 1990; Andersson and von Heijne, 1993). Periplasmic loops of greater than ca. 60 amino acids in length appear to be relatively independent of the 'positive inside' rule, perhaps because they must utilize the *sec* system (give referenced examples). Short hydrophobic peptides of up to 38 residues may be translocated in a SecA- and SecY-independent manner that requires a membrane $\Delta\mu_H+$ (Cao and Dalbey, 1994), and translocation of the 100 residue periplasmic N-terminal tail of the *E. coli* integral inner membrane protein ProW also appears to be dependent upon $\Delta\mu_H+$ (Whitley *et al.,* 1994). Interestingly, $\Delta\mu_H+$ does not seem to be required for insertion of a smaller (18-23 residue) hydrophobic segment, suggesting that the conformational energy gain of insertion of the very small hydrophobic peptide into the relatively apolar bilayer is sufficient to drive the reaction (Cao and Dalbey, 1994). This may be an important parallel to the hydrophobic core of the typical bacterial signal sequences.

1.6.3 The transmembrane potential and ATP hydrolysis by SecA power the active export of peptide segments through the cytoplasmic membrane translocase

First described in 1981, SecA was found in a genetic screening of pleiotropic secretion-defective temperature-sensitive mutants in *E. coli* (Oliver and Beckwith, 1981). SecA may recognize and bind substrate in the cytosol initially, since they can be co-immunoprecipitated from the cytosol (Chun and Randall, 1994), and free SecA binds an OmpA hybrid in the absence of membrane (Akita *et al.,* 1990). The ability of SecA to also bind the signal peptide and mature components of the substrate peptide, the SecB preprotein chaperone (Hartl *et al.,* 1990), inner membrane anionic phospholipids (Lill *et al.,* 1990, Cabelli *et al.,* 1991), and the channel-defining integral inner membrane proteins SecY/E (Hartl *et al.,* 1990) strongly suggests that SecA is central to the initiation of translocation at the membrane itself. After recognition of peptide and chaperone, and targeting to the plasma membrane translocase in this manner (Hartl *et al.,* 1990), substrate-binding SecA hydrolyzes ATP (Lill *et al.,* 1989). ATP-requiring membrane insertion of SecA requires association with the preprotein and with membrane proteins SecY/E (Lill *et al.,* 1989; Economou and Wickner 1994).

SecA binds ATP, but hydrolysis requires binding of the preprotein (Lill *et al.,* 1989). ATP-binding by SecA directs a conformational change which drives the translocation of a short segment of the preprotein into the membrane channel formed by SecY/E/G (Economou *et al.,* 1995), this cycle of insertion and release being regulated by the high affinity ATP-binding site at the amino-terminus of SecA (Rajapandi and Oliver, 1996). The substrate is driven into the channel in segments of approximately 20 residues

(Scheibel *et al.,* 1991; Arkowitz *et al.,* 1993), consistent with a transmembrane span of 6nm. A 30kDa arm of the hydrophilic SecA protein travels through the membrane pore with the translocating segment, becoming protease susceptible from the periplasmic side at that point (Kim *et al.,* 1994; Economou and Wickner 1994). This piston-like action of SecA is followed by release of the substrate after ATP hydrolysis, permitting the next ($\Delta\mu_H$+ -driven) phase of translocation, which requires an intact transmembrane potential but not ATP (Schiebel *et al.,* 1991). This second phase may be much less important once the signal peptide has travelled through, and other elements biasing the reaction (such as periplasmic secondary and tertiary structure) prevent 'slide-back' between the cycles of SecA-driven transport. The demonstration in sphaeroplasts of protease accessibility of the ATP-binding hydrophilic HisP subunit of the *Salmonella typhimurium* histidine permease suggests that this may not be an unusual strategy in analogous proteins (Baichwal *et al.,* 1993).

1.6.4 SecA directly interacts with integral inner membrane proteins in a complex that is recruited by appropriately presented competent substrate

SecE is another CM protein with an essential role in the Sec translocase. SecA and SecY can be crosslinked to translocating peptide, shielding it from phospholipids in the bilayer (Joly and Wickner, 1993), and there is ample evidence for the interaction of SecE and SecY at both genetic (Bieker and Silhavy, 1990; Bieker-Brady and Silhavy, 1992; Flower *et al.,* 1995) and biochemical levels (Brundage *et al.,* 1990, 1992; Akimaru *et al.,* 1991). These three integral inner membrane proteins are believed to be cyclically assembled and disassembled in a process which is triggered by the arrival of

the preprotein signal peptide/ SecA/ SecB complex. (Bieker-Brady and Silhavy, 1992; Schekman, 1994). The "Band 1" protein (SecG) copurifies with SecY-E and has been recognised as an integral inner membrane protein which is involved in the membrane channel (Douville *et al.,* 1994; Economou *et al.,* 1995), and can be reconstituted into an efficient protein translocase with SecA, Y and E *in vitro* (Hanada *et al.,* 1994).

1.6.5 Delivering competent substrate to the translocase: SecB as a targeting system and chaperone

The SecB chaperone has been shown to associate with a number of exported protein precursors *in* vivo (Kusters *et al.,* 1989), and to prevent the folding of maltose binding protein and pre-PhoE into compact (translocation-incompetent) structures (Collier *et al.,* 1988; Kumamoto, 1989). Interaction with the chaperone to maintain translocation competence must therefore be virtually immediate and/ or further unfolding must occur at the translocation port. Indeed, in studies of the translocation of a proOmpA-Dihydrofolate reductase fusion protein via the Sec system in *E. coli* (Arkowitz *et al.,* 1993), unfolding of peptide during translocation occurs as a part of the translocation cycle in the absence of $\Delta\mu_H+$ or ATP.

SecB is required for normal protein export in *E. coli* (Kumamoto and Beckwith, 1983), and binds with high affinity to a variety of nascent export precursors as a homotetramer (Lecker *et al.,* 1989; Hardy and Randall, 1991; Kumamoto and Francetic, 1993). SecB may bind the signal sequence of translocating peptide (Watanabe and Blobel, 1989a,b; Altman *et al.,* 1990a), as well as the mature part of the protein (Hardy and Randall,

1991; Altman *et al.,* 1990b; Kim *et al.,* 1992). While not all SecA-dependent substrates are also SecB-dependent, hybrids of (SecB-independent) ribose-binding protein (RBP) and (SecB-dependent) OmpA show that SecB-dependence is encoded within the amino-terminal third of the OmpA export precursor (Strobel *et al.,* 1993). SecB binding probably occurs on multiple regions of the substrate prepeptide (Altman *et al.,* 1990a), and it may be that the apparent requirement for the presence of signal peptide reflects the retardation of folding rather than direct signal sequence binding (see Kumamoto, 1991 and Pugsley, 1993a for discussion).

Other chaperonins can substitute for SecB in Sec-dependent export, and SecB is not required for cell viability, but the relative specificity of SecB for presecretory proteins indicates that this chaperone is rightly seen as a member of the Sec translocase (reviewed in Kumamoto, 1991). SecB-independent proteins (eg. RBP and β-lactamase) probably rely on alternative chaperones, although they still weakly associate with SecB *in vitro* (Hardy and Randall, 1991) and are partially affected *in vivo* by SecB mutations (Kumamoto and Beckwith, 1983; Kim *et al.,* 1992). Ffh, a homologue of a mammalian chaperone-like protein (Romisch *et al.,* 1989), sequesters pre-β-lactamase in the cell when overexpressed (Ribes *et al.,* 1990), and is required for SecB-independent SecA-dependent translocation of such proteins (Philips and Silhavy, 1992).

It has been suggested that a role of these cytosolic factors is to act as traffic police to some extent, with changes in their relative amount fine-tuning the fate of nascent peptides (Kim *et al.,* 1992). The relatively broad specificity of the chaperones is cited in support of this (Gething and Sambrook, 1992), as well as evidence that overproduction

of GroEL promotes export of an ordinarily translocation-incompetent LamB-LacZ fusion protein in *E. coli* (Philips and Silhavy, 1990). An unusual internal duplication in SecA leads to partial suppression of a *secB* phenotype as well as suppression of point mutations in the signal sequence of pre-maltose binding protein (preMBP), a common experimental substrate (McFarland *et al.,* 1993). Translocation initiation by SecA binding of SecB-protein complex (Kumamoto and Beckwith, 1985; Hartl *et al.,* 1990), and perhaps also Ffh-protein complex, is an obvious targeting mechanism.

1.6.6 SecD and SecF: Integral inner membrane proteins in the translocase

Although they are not required for translocation in artificial systems *in vitro* (Bieker-Brady and Silhavy, 1992), deletions of *secD* and *secF* are severely export deficient and barely viable at 37°C (Pogliano and Beckwith, 1994a). Specific antibody bound to SecD interferes with release of translocated secretory proteins, leading to 'stacking' of non-released mature protein and accumulation of precursor (Matsuyama *et al.,* 1993). SecD and SecF are integral membrane proteins with large periplasmic domains present in about 3-60 copies per cell (Pogliano and Beckwith, 1994b). They facilitate protein translocation in the *wt* when overexpressed, suppress signal sequence mutations as strongly as overexpressed SecA, and more strongly than any *secE* suppressor (*prlG*) identified (Fikes and Bassford 1989; Pogliano and Beckwith 1994a). This is consistent with a suggested role for SecD and SecF in transduction of the transmembrane potential, critical to the second (ATP-independent) phase of translocation initiation (Arkowitz and Wickner, 1994).

Fig. 1.6 The Sec cytoplasmic translocase

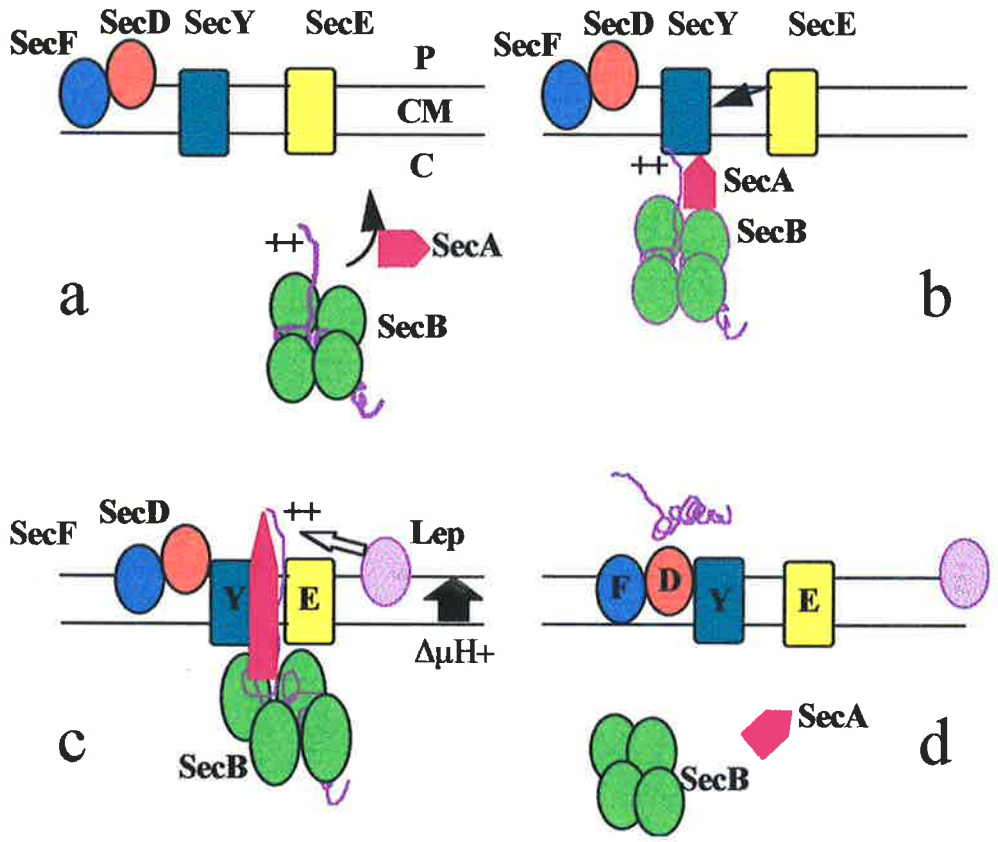


Fig.1.6 The SecB-signal sequence-SecA complex forms (panel a) and is thought to interact with SecY, with recruitment of SecE (panel b) followed by extension of an arm of SecA with the 20 aa translocating segment through the channel (panel c). SecA hydrolysis of ATP is needed for release of substrate to allow the $\Delta\mu_H+$ -driven phase of translocation to proceed (panel d). Processing by Leader peptidase (Lep) is shown. The complex dissociates after successive rounds complete the translocation, and is free to form again with appropriate signals. SecD is required to release mature translocated peptide, and SecD and F may well be more important in the $\Delta\mu_H+$ -driven second stage, thus most critical in translocation initiation by signal peptide. SecG is not included in the simplified diagram, but is likely to be intimately associated with SecE (see text).

1.7 Single step translocation in Gram-negative bacteria

Members of a large family of one-step macromolecular exporters with a common ATP-binding domain are often grouped together as the ABC (**A**TP-**b**inding **c**assette) transporters, and include the periplasmic permeases (importers), multidrug resistance (MDR) exporters, and the α_1-haemolysin (Hly) pathway of *E. coli* (reviewed in Blight and Holland, 1990; Higgins, 1992; Braun *et al.,* 1993; Fath and Kolter, 1993). More examples of single-step export may yet be demonstrated, as at least one fully exported protein in *V. cholerae* is not a typical substrate for any of the currently described systems (Williams *et al.,* 1996).

1.7.1 Export targeting is encoded in the C-terminal region of HlyA

The large (110 kDa) HlyA protein is exported across both membranes of *E. coli* without a detectable periplasmic intermediate (Felmlee *et al.,* 1985), independent of SecA (Mackman *et al.,* 1985), and dependent upon an uncleaved C-terminal signal sequence (Koronakis *et al.,* 1989). The Hly transporter can export related fusion proteins and chimeras (Nicaud *et al.,* 1986; Koronakis *et al.,* 1987) and exhibits a high level of competence for heterologous substrates (Delepelaire and Wandersman, 1990; Fath *et al.,* 1991). Despite expectations of a largely conformational C-terminal recognition sequence due to the apparent tolerance of mutations and alterations in this region (Koronakis *et al.,* 1987), site-directed mutagenesis points to specific contact residues

(Stanley *et al.,* 1991; Kenny *et al.*, 1992; Chervaux and Holland, 1996), suggesting that secondary structure may be less important than originally supposed.

1.7.2 An ATP-binding protein provides a common link

The conserved ATP-binding cassette region consists of a relatively conserved stretch of about 200 amino acids which includes an ATP-binding pocket with a GXGKST ("Walker A motif") turn at the end of a region of α helix that brings the ATP-binding lysine residue into proximity with an aspartate in the less-conserved "Walker B motif" (Walker *et al.,* 1982). The role of a negatively charged residue in the latter region may centre on an interaction with Mg^{2+} in the Mg^{2+}-ATP (Walker *et al.,* 1982). This pairing of motifs is usually found in bacterial permeases and exporters such as HlyB of the α-haemolysin export pathway (Felmlee *et al.,* 1985), and KpsT of the capsular (Group II) polysaccharide export pathway (Smith *et al.,* 1990), both to be found in *Escherichia coli* (see also later, section 1.12.5) The periplasmic permeases are structurally similar to the group as a whole in having the ATP cassette in a hydrophilic cytoplasmic membrane-associated protein. This binds with hydrophobic proteins in the membrane, and incoming substrate is presented to this complex by a periplasmic accessory factor (Kerpolla *et al.,* 1991). Importers such as these differ from the so-called type I exporters (eg. α-haemolysin in *E. coli*) in their separation of the membrane-spanning domains from the ATP cassette as distinct proteins. The eukaryotic ABC transporters, like bacterial ABC exporters, have the ATP binding and transmembrane elements on the same peptide. Mutations in highly invariant residues in the HlyB ATP-binding fold abolishes exporter function and ATPase activity, even while ATP-binding and

consequent conformational change still proceeded, indicating that ATP hydrolysis itself is essential (Koronakis *et al.,* 1995).

1.7.3 Accessory proteins in the periplasm and outer membrane

HlyB, the ABC-cassette protein that places the Hly machinery in this class, is encoded contiguously with another membrane protein, HlyD (Wagner *et al.,* 1983). These two membrane proteins suffice for specific export of varied fusion proteins (Nicaud *et al.,* 1986). However, TolC, an outer membrane protein initially identified by its role in the bi-membrane spanning *tol* import system of Gram negative bacteria (reviewed in Webster, 1991; Koronakis and Hughes, 1996), is also absolutely required for export beyond the outer membrane in this and in related systems (Wandersman and Delepelaire, 1990).

While accessory factors, including homologues of TolC, are required in systems where export to the medium is required (Létoffé *et al.,* 1993; Fath and Kolter, 1993), the transcytoplasmic membrane passage and periplasmic assembly of Group II polysaccharides is promoted by an ATP-cassette protein in the apparent absence of any such additional elements (Fath and Kolter, 1993). Representatives of the key ABC-cassette protein include the KpsT protein of *E. coli* K5 (Smith *et al.,* 1990), the BexA protein of *Haemophilus influenzae* (Kroll *et al.,* 1988), and RfbE of *Salmonella typhimurium* (Zhang *et al.,* 1993), all of which are dedicated to polysaccharide transport. In some of these, potentially interacting integral cytoplasmic membrane proteins have been identified which are highly homologous (Smith *et al.,* 1990; Zhang

et al., 1993), and it is likely that these are part of a multiprotein complex (Zhang *et al.,* 1993) in these and in other *rfb* clusters (Kido *et al.,* 1995), although no direct association has been demonstrated.

1.7.4 The "type III" secretion system is a widely represented *sec*-independent translocation mechanism, exporting substrates from the cytoplasm

The term "type III" is applied to that group of systems exemplified by the Yop (**Y**ersinia **o**uter **p**rotein) secretion apparatus in *Yersinia spp.* (Michiels *et al.,* 1991, Haddix and Straley 1992, Bergman *et al.,* 1994), and also represented at least to some extent in *Shigella flexneri* (Vankatesan *et al.,* 1992; Alloui *et al.,* 1992, 1993; Bergman *et al.,* 1994) and *Salmonella typhimurium* (Galan *et al.,* 1992; Bergman *et al.,* 1994). Yops are secreted in a *sec*-independent manner, without a cleaved signal sequence. While the N-terminal region contains the elements essential for translocation, there is no striking amino acid homology in the secretion domains (Michiels *et al.,* 1990; Michiels and Cornelis, 1991). YopH fusion proteins are efficiently secreted (Michiels and Cornelis, 1991), and YopE N-terminal fusions of adenylate cyclase are transported into HeLa cells without internalization of the infecting bacterium (Sory and Cornelis, 1994).

1.7.5 Yops are targeted to the transporter by specific cytosolic chaperones

There is no detectable similarity in the N-terminal regions essential for the export of different Yops by the same apparatus, suggesting that targeting is highly individual or is

conformational. The identification of cytosolic 'chaperones' for YopE, YopH, and YopD (Wattiau *et al.,* 1993, 1994), has helped to resolve this question. These small cytosolic proteins (SycE, H and D) are required for export, specifically binding their respective Yops in the amino-terminal secretion-targeting region (Wattiau and Cornelis, 1993). Sequence similarities between the Syc and related chaperones are weak, excepting the presence of a small highly conserved C-terminal α-helical motif present in SycE, SycH, and in apparently related proteins from *P. aeruginosa* and enteropathogenic *E. coli* (Wattiau *et al.,* 1996). While the mechanism of action of these proteins is as yet undefined, they illustrate a recurrent theme in protein transport.

1.7.6 ATP-binding proteins and outer membrane channels are common themes

Two more important features stand out in the Yop and related systems. One is the presence of a protein with an essential ATP-binding motif which is required for Yop secretion (Woestyn *et al.,* 1994). This protein, YscN, has homology to the β subunit of the F_0F_1 proton-translocating ATPase of *Escherichia coli* (Kanazawa *et al.,* 1982) and to putative ATPases in protein transport and flagellar assembly machinery in a number of organisms (van Gijsegem *et al.,* 1993, Eichelberg *et al.,* 1994; Bergman *et al.,* 1994).

The second is the presence of a member of the pIV/ PulD superfamily of outer membrane proteins, YscC (Genin and Boucher, 1994), which is essential for Yop secretion (Plano and Straley, 1995). MxiD in *Shigella flexneri* is homologous to the YscC protein in relative entirety, but only to the C-terminal region of the PulD gene (Allaoui *et al.,* 1993). Since the pIV protein, more closely related to PulD, appears to

have a globular periplasmic N-terminal domain and an outer membrane-inserted C-terminus (Russel, 1994a), it is perhaps predictable that this would be the case, since system specificity and chaperone-like interactions appear to be encoded within the N-terminal region of PulD, at least (Hardie *et al.,* 1996; discussed in more detail in section 1.11.2).

1.8 Mitochondrial protein import as a paradigm of energised passage across two membranes

1.8.1 Co-operation between independent transporters in the inner (energised) and outer (non-energised) membranes.

Useful parallels exist between the import to the matrix of proteins across the outer and inner mitochondrial membranes and the export of proteins from the cytoplasm of Gram negative bacteria. The inner membrane of mitochondria is evolutionarily related to the bacterial plasma membrane, and the outer membrane is unenergised. Protein import into mitochondria is performed by different translocation machineries for the inner and outer membranes (Hwang *et al.,* 1989; Mayer *et al.,* 1993), acting independently but co-operatively (Baker and Schatz, 1991; Pfanner *et al.,* 1992). Preprotein binds the outer membrane import complex, which serves as a general insertion pore and comprises at least eight proteins (Söllner *et al.,* 1992) including a specific receptor for the signal sequence (Murakami *et al.,* 1993). Contact with the inner membrane at membrane contact sites permits further translocation (Pfanner *et al.,* 1992). Once translocation through the inner membrane translocase has begun, the N-terminal signal peptide is

cleaved in the matrix by a specific peptidase, and association with other energy-requiring matrix proteins drives the process to completion (see below, and Fig. 1.7). Essential components of the mitochondrial import machinery consist of the prepeptide signal sequence, cytosolic cofactors, the outer and inner membrane import protein complexes, the matrix-associated peptidase, and the matrix Hsp60 and Hsp70 chaperonins (Fig. 1.7; reviewed in Pfanner *et al.,* 1994; Glick, 1995).

1.8.2 Translocation and substrate competence are interdependent

An important premise of the Brownian ratchet model is that translocation competence maintained by cytosolic chaperones, or by direct delivery from ribosomes, involves the presentation of unfolded peptide. The spontaneous unfolding of many proteins is in the order of hours to days (Creighton *et al.,* 1993), and would therefore be rate-limiting unless directly facilitated. Artificial mitochondrial precursor proteins form folded complexes even when produced in the presence of cytosolic chaperones *in vitro* however (Wachter *et al.,* 1994), and the state of the Hsp60 chaperonin-bound molecule in *E. coli* is that of a loosely folded intermediate rather than a fully unfolded form (Martin *et al.,* 1991). In mitochondria, the stably folded cytochrome b_2 haem-binding domain is rapidly imported and requires Hsp70 (which firmly binds incoming peptide; Scherer *et al.,* 1990) and ATP in the matrix (Glick *et al.,* 1993). In any case, it is clear that transport of appropriately targeted proteins with cleavable N-terminal presequences across the outer membrane into the mitochondrion requires coupling with transport across the energised inner membrane (Hwang *et al.,* 1991; Rassow and Pfanner, 1991).

Fig. 1.6 Mitochondrial import is energised from within

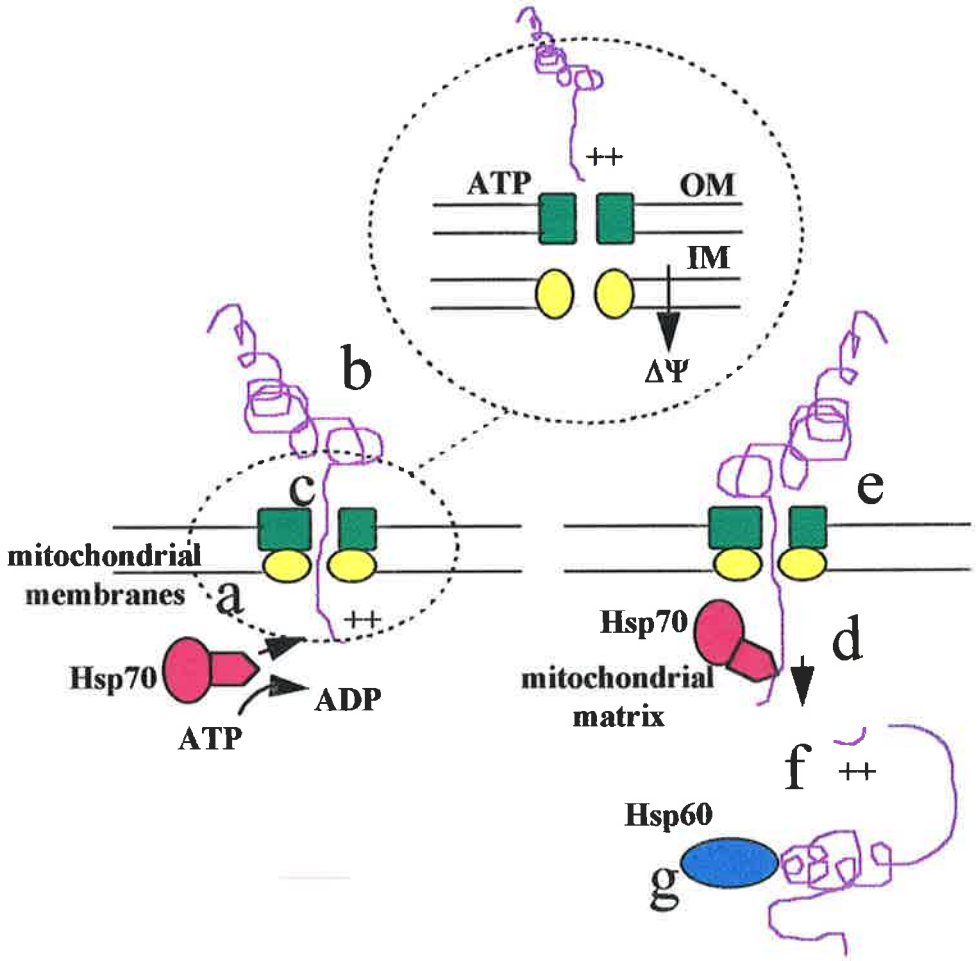


Fig. 1.7 After Pfanner *et al.*, 1994, Glick, 1995, and Mayer *et al.*, 1995: Imported substrate crosses the mitochondrial membranes by coupled transport. Positively charged signal peptide (++) is specifically recognised at the OM transporter (inset). Translocation proceeds no further than signal peptide insertion without engagement of the IM translocase, and requires energy, including an intact transmembrane potential (ΔΨ). Unfolding is facilitated by binding of presequence to both translocases. Hsp70 in the mitochondrial matrix transduces ATP-derived energy into work (a), pulling the folded translocating chain (b) through the channel (c). Completion of translocation (d) requires ATP, Hsp70, and the transmembrane potential. Cleavage of the signal peptide occurs on the matrix side (f) by a specific peptidase. Interactions with cytosolic cofactors (b), and Hsp60 chaperonins (g), facilitate these processes.

1.8.3 Mitochondrial import is energised from within

A model of mitochondrial import is presented above. Energy for transport is derived from the transmembrane potential of the inner membrane, and from ATP within the matrix, both of which are essential. The key to the process is the recognition/ initiation role of the signal peptide. This role will be further discussed in detail, in a later section.

1.8.4 Targeting by the prepeptide 'signal' sequence is an essential initiation step

No sequence homology has been identified between components of the secretory pathway of Gram negative bacteria and the import pathway of mitochondria, but analogous processes can be argued and both systems rely on targeting of substrates by direct binding of the substrate itself to transporter proteins (Akita *et al.*,1990; Murakami *et al.*,1993). Both bacterial and non-bacterial signal peptides have been shown to open protein conducting channels in artifical membranes (Simon and Blobel, 1992). Isolated mitochondrial outer membrane vesicles prepared with enclosed matrix-processing peptidase cleave externally added precursor proteins, but the mature form remains externally accessible to protease, indicating that the signal sequence suffices to cross the OM but can go no further. The presequence binds on the *trans* side, and contact with the inner membrane translocase permits release from here and drives partial translocation and unfolding (Mayer *et al.*, 1993, 1995). The bias of this interaction can be fitted into the ratchet picture of continued transport across a membrane (Simon *et al.*, 1992). The ATP-driven pulling activity attributed to Hsp70 proteins in the lumen of mitochondria and endoplasmic reticulum (Glick, 1995) might well be compared to the

ATP-driven pushing of substrate through the bacterial plasma membrane translocase by SecA protein (Kim *et al.,* 1994, Economou and Wickner, 1994, reviewed in Schekman, 1994; see Section 1.6.3).

While the signal sequence typical of incoming proteins to the mitochondrial matrix is quite different to the typical bacterial signal peptide, the signal sequences of outgoing proteins from the mitochondrial matrix are amongst a number of similarities between the export of proteins across the evolutionarily related bacterial plasma membrane (Hartl and Neupert, 1990; Baker and Schatz, 1991).

1.9 Outer membrane translocation competence

In considering fidelity of transport systems, it has been argued that since all exported bacterial proteins must pass the cytoplasmic membrane, a highly specific recognition apparatus is not required (Schekman 1994). If initial trans-cytoplasmic membrane passage to a periplasmic intermediate pool is followed by a second system which exports or surface assembles the substrate, then a general system with broad recognition followed by a series of specific systems for final handling would be logical. Certain well-characterised importers simply bridge the gap directly to transduce the energy required from the cytoplasmic to the outer membrane (Postle, 1990; Kadner, 1990; Webster, 1991; Fath and Kolter, 1993), and we have already discussed one-step exporters of very large molecules such as the Yops and the *E. coli* α-haemolysin.

Examples of highly specific second membrane transporters, and systems in which the whole is self-contained, are discussed in the following sections.

1.9.1 A periplasmic intermediate capable of autosecretion: the IgA protease

The IgA protease of pathogenic *Neisseria spp.* provides a useful model of outer membrane transport, its apparent simplicity telling us something of the minimum requirements for such a process. This model of autosecretion (Pohlner *et al.,* 1987) is mirrored in the IgA protease of *Haemophilus influenzae* (Grundy *et al.,* 1987) and in a serine protease of *Serratia marcescens* (Mizayuki *et al.,* 1989). A 30 kDa core region in the C-terminus of the IgA_{β} moiety assembles into the outer membrane in a β–barrel structure which then serves as a pore for the translocation of following peptide via a small linker region (Klauser *et al.,* 1992) (Fig. 1.8).

The translocating substrate is present in the periplasm as a partially folded intermediate, and translocation of a Ctx-B subunit fusion to the IgA_{β} is prevented by the formation of disulphide bonds in the periplasm (Klauser *et al.,* 1992). The translocated protein arrives in the periplasm via the conventional *sec*-dependent route, and is translocated from here as a linear peptide before cleavage on the external face of the outer membrane in an autoproteolytic fashion, which can be mimicked *in vitro* by other proteases (Pohlner *et al.,* 1987, Klauser *et al.,* 1990, 1992).

This system demonstrates a need for a small and simple outer membrane channel, but is permissive only of linear translocation. In the absence of a demonstrable energy supply,

one must presume that a simple Brownian ratchet mechanism applies, with random movement through the pore biased primarily by the development of secondary structure in the external milieu, as has been proposed (Simon *et al.*, 1992). Initiation of the process is presumably consequent upon conformationally-directed assembly into the outer membrane of the IgA_β core, which process places the linker region externally, thus drawing the N-terminally connected linear peptide into the translocation channel so formed (Fig. 1.8; Pohlner *et al.*, 1987, Klauser *et al.*, 1992) and obviating the need for a transmembrane potential to insert a signal peptide.

Fig. 1.8 IgA protease: autosecretion through a β-barrel outer membrane channel

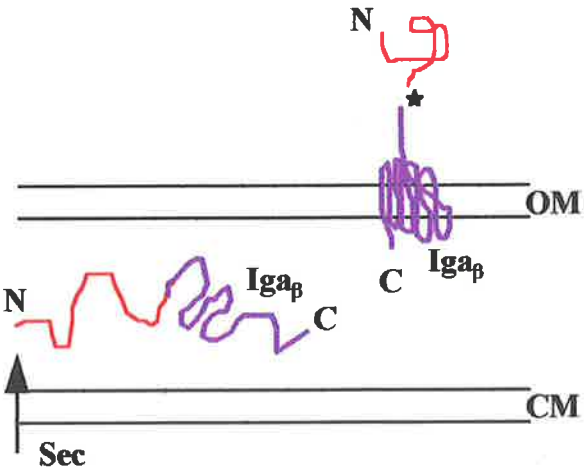


Fig. 1.8 IgA protease crosses the cytoplasmic membrane *via* the Sec system, with the concomitant loss of the signal peptide. Outer membrane passage follows insertion of the Iga_β domain, which forms a porin-like channel for passage of the linear N-terminal domain. Acquisition of secondary structure (eg. disulphide bonds) in the periplasm prevents passage (Jose *et al.*, 1996). Translocation through the channel is probably initiated by the folding of the Iga_β domain, which pulls a significant section of the N-terminal tail through to the external face of the outer membrane. Biasing factors such as secondary structure formation would suffice to promote further translocation (recall the Brownian ratchet, Fig. 1.3), and the competely translocated structure is cleaved by the mature enzyme (★ in Fig. 1.8).

1.9.2 Periplasmic folding: martialling outer membrane traffic and maintaining competence for export and/ or assembly

Periplasmic chaperones are seen in at least two well-understood circumstances in Gram-negative export. The first is similar to the role performed by SecB in delivery to the Sec cytoplasmic transporter, and the archetype (PapD) will be discussed below in the context of biogenesis of the P pilus of uropathogenic *E. coli.* The second is the chaperoning of outer membrane proteins to their final destination. The recently described PulS-PulD interaction in the main terminal branch of the General Secretory Pathway (GSP) of *E. coli/ K. oxytoca* (Hardie *et al.,* 1996) will be described in the context of that important system, but a similar example is also seen in the related T-DNA exporter in *Agrobacterium tumefaciens* (Jones *et al.,* 1994). These proteins fulfill the primary criterion for true chaperones in that they are essential for stability and development of correct secondary or tertiary structure of their client peptides. The second criterion, that they do not themselves participate in the final structure, is true of PapD but uncertain in the case of PulS, as we will see below. The enzymatic refoldase activity so important in the cytosol is also reflected in the periplasm, and this will be discussed with the main terminal branch of the General Secretory Pathway.

1.10 Surface assembly of a complex heteropolymer: P pilus biogenesis in uropathogenic *E. coli.*

1.10.1 Pilus morphology

Adhesive pili in Gram negative bacteria of the type I class are traditionally divided into a couple of different groups on morphological grounds. One is represented by the ca. 7nm diameter rod-like fibres of typical "type I" class such as the P pilus (Gong and Makowski, 1992), and the other is the open helical fibril of 2-5nm typified by K99 (de Graaf *et al.,* 1984; de Graaf and Mooi, 1986). This latter is visibly more flexible and less rigid, but it is now apparent that the rigid helical P pili are tightly wound fibres tolerant of plastic and elastic deformation without depolymerisation (Bullitt and Makowski, 1995). This class of fibrillar adhesin, whatever its morphology, appears to be assembled and exported in a similar manner by a discriminative outer membrane assembly complex (Dodson *et al.,* 1993; Hultgren *et al.,* 1993; Fig. 1.9).

Fig. 1.9 Assembly of the P pilus at the outer membrane.

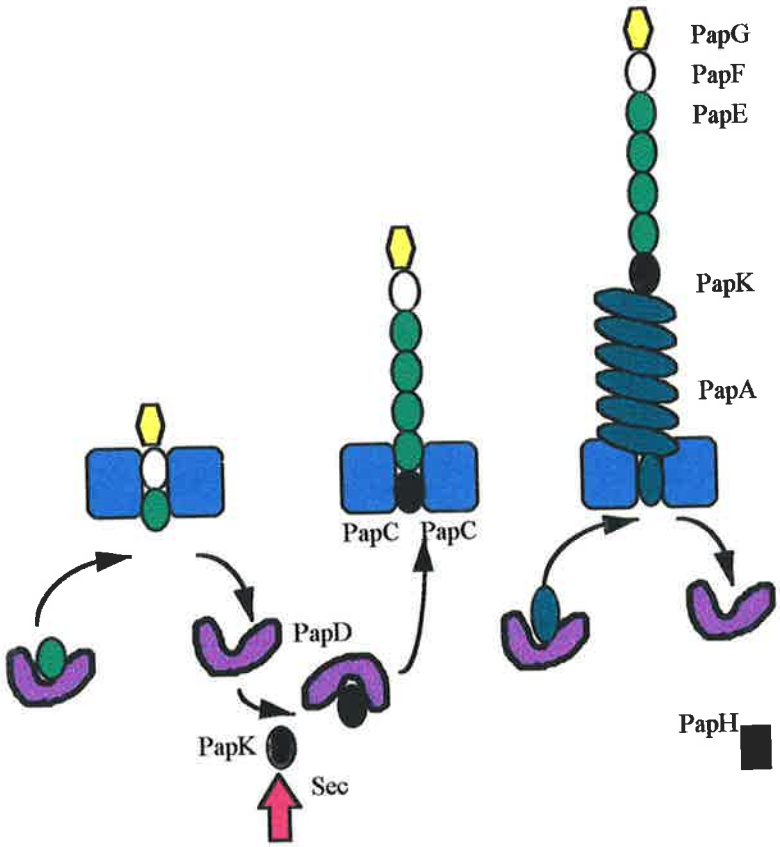


Fig. 1.9 P pilus assembly is illustrated, after Kuehn *et al.*, 1991. The PapD chaperone delivers pilin subunits to the PapC usher protein, after cytoplasmic translocation in the ususal Sec-dependent fashion (heavy arrow). In the absence of PapD, subunits form disordered aggregates in the periplasm. The chaperone acts as a periplasmic shuttle, with differential avidity for substrate.

1.10.2 Binding specificity

P pili are specifically required to bind target ligand on uroepithelial cells. A tip-presented adhesin, PapG (Lindberg *et al.,* 1987), binds the Gal-α(1-4)-Gal moiety (α-D-galactopyranosyl-(1-4)-β-D galactopyranoside) of glycosphingolipids (Bock *et al.,* 1985) in a conformation-specific manner which determines the high affinity recognition of three different isoreceptors, and accounts for specific tissue tropisms exhibited *in vivo* (Strömberg *et al.,* 1990; Strömberg *et al.,* 1991; reviewed in Hultgren *et al.,* 1993). The distinguishing feature of the P pili from other subunits such as K99, is the tight right-handed helical packing of the former, determined by the bonding of the main structural subunit (PapA) to other PapA subunits in adjacent turns of the helix. This results in a 6.8 nm diameter 1μ fibre as the main body of the pilus, with 3.3 subunits per turn and a helical symmetry identical to that of other type I pili from *E. coli* (Gong and Makowski, 1992; Bullitt and Makowski, 1995). The rigidity so imposed can be disrupted by mechanical stress to 'break' without separating, or may 'unravel' as a loose open helix to five times its original length (Bullitt and Makowski, 1995).

1.10.3 The P pilus is a complex heteropolymer

This rigid but deformable helical rod is attached at the outer membrane by an 'anchor' protein, PapH (Båga *et al.,* 1987). This protein appears to terminate the growing pilus as an ultimate addition to the structure. The pilus rod supports a flexible tip fibrillum composed predominantly of a 'minor' pilin (PapE) which is virtually a separate structure. This presents the PapG tip adhesin, linking it to the fibrillar region *via* PapF,

which appears to be required for growth of the PapE tip fibrillum (Lindberg *et al.,* 1986). The open helix of the PapE tip fibrillum is similar structurally to the mechanically disrupted (plastically stressed) form of the PapA shaft (Bullitt and Makowski, 1995), as might be predicted from the biological role of the pilus adhesin. PapK appears to be a linker protein that terminates the PapEFG structure (Kuehn *et al.,* 1992; Jacob-Dubuisson *et al.,* 1993) and firmly fixes it to the main body of the PapA coiled fibre. *PapK* mutants are able to assemble functional pili, but have a reduced number of tip adhesins, consistent with a loss of stability at the junction of the two regions of the shaft structure, and *papF,papK* double mutants are completely non-piliated (Jacob-Dubuison *et al.,* 1993).

1.10.4 Assembly of the ordered P pilus heteropolymer is orchestrated by the chaperone-usher interaction at the outer membrane.

The interdependent assembly of the heteropolymeric P pilin is largely orchestrated by the interaction between an outer membrane 'usher' protein (PapC) and a periplasmic 'chaperone' (PapD) (Dodson *et al.,* 1993). Both of these proteins are essential for assembly, are highly conserved in critical regions, and are widely represented in related systems (Bakker *et al.,* 1991; Hultgren *et al.,* 1991; Holmgren *et al.,* 1992). Subunits are exported into the periplasm across the cytoplasmic membrane in a Sec-dependent manner, after processing by the classical type I signal prepeptidase associated with substrates handled by this system (Dodd and Eisenstein, 1984; Pugsley 1993a). PapD binds in a stable bimolecular complex in the periplasm with substrates such as the tip adhesin PapG, which is maintained in mature configuration (Kuehn *et al.,* 1991). The

PapD-PapG complex is resistant to 6M urea, and probably held by ionic bonds. PapG can be freely exchanged between PapD molecules, the role of PapD being to cap and uncap the interactive surfaces of subunits immediately after translocation (Kuehn *et al.,* 1991), so as to facilitate ATP-independent delivery to PapC for assembly (Hultgren *et al.,* 1991; Dodson *et al.,* 1993). Binding of the subunit occurs within a region comprised of two β-barrel globular domains oriented toward each other in a boomerang shape, each fomed by two antiparallel β–pleated sheets and topologically identical to an immunoglobulin fold (Holmgren and Brändén, 1989; Holmgren *et al.,* 1992). The general scheme of paired globular domains separated by a ligand-binding hinge region, with closure of the cleft to bury the bound ligand, is common to a wide variety of periplasmic ligand-binding proteins for which the crystal structure has been solved (Quiocho and Ledvina, 1996). Specific mutagenesis has demonstrated the essential nature of certain solvent-exposed residues in the PapD cleft, abolishing binding to pilin subunits and to the PapK 'adaptor' or 'linker' protein (Slonim *et al.,* 1992). Binding probably centres on such highly conserved residues, which are concentrated within regions of β strand in the cleft region between the two globular domains, while variable residues are found in the loop region between the β strands (Holmgren *et al.,* 1992).

Assembly occurs in a specific order at the outer membrane. PapC is an 86 kDa outer membrane protein essential to this process that may form a porin-like channel (Norgren *et al.,* 1987), and is a member of a large family of outer membrane 'usher' proteins with similar functions (Mooi *et al.,* 1986; Roosendaal and de Graaf, 1989; Jalajakumari *et al.,* 1989; Allen *et al.,* 1991; Holmgen *et al.,* 1992; reviewed in Pugsley 1993a). Apart from key roles played by minor 'linker' pilins such as PapF, PapK, and the 'anchor'

PapH in ensuring correct order of assembly, differential affinity of the PapC 'usher' protein for chaperone(PapD)-subunit complexes greatly increases the efficiency of pilin assembly. The three tip fibrillar proteins (PapE, F, and G) bind PapC when complexed to PapD, with the most distally located pilin subunit having the highest affinity in complex and PapD alone having relatively poor affinity (Dodson *et al.,* 1993). Many *E. coli* strains co-express more than one representative of this class of pilus, with close relatedness in genetic organization (Hacker and Morschhäuser, 1994). Investigation of the interchangeability of these highly homologous biogenesis proteins has shown that system specificity is conferred by the chaperone-usher interaction, with the chaperone and usher acting efficiently in natural pairs (Klemm *et al.,* 1995).

1.11 Assembly in and passage through the outer membrane

This next section continues with a discussion of the major terminal branch of the General Secretory Pathway (GSP) for exported bacterial proteins, and of the biogenesis of type-4 pili, of which TCP is an example. Little is known about the fate of pilin subunits between processing at the cytoplasmic face of the inner membrane, and hence the focus will be on structural aspects and data from apparently related systems. The recently solved crystal structure of the type-4 pilin subunit of *Neisseria gonorrhoeae* MS11 (Parge *et al.,* 1995) is especially valuable because of the degree of relatedness between the type-4 pilin subunits generally (Dalrymple and Mattick, 1987). Data from the main terminal branch of the General Secretory Pathway (GSP), and the *pul* system in particular, are particularly useful in terms of the extent of functional analysis available. Outer membrane transport of Sec-dependent substrate in such systems should be regarded as fundamentally different from the assembly of the type-4 pilin however, as the latter is only indirectly Sec-dependent (apparently relying on Sec-dependent transport of certain elements consituting the putative transporter/s, but not of the substrate itself).

1.11.1 The type II branch of the GSP exports substrate from a pooled periplasmic intermediate state

The most important outer membrane export system for virulence factors in Gram-negative bacteria appears to be that described as the main terminal branch of the

General Secretory Pathway. Widely represented in Gram negative bacteria, the best characterised of these pathways is that responsible for the export of pullulanase across the outer membrane in *Klebsiella oxytoca* (reviewed in Pugsley, 1993a). Homologues have been identified in a number of key members of the translocase, and are clearly able to handle a range of substrates (Overbye *et al.,* 1991; Nunn and Lory, 1992). The nomenclature is confusing at present, but attempts to organise this may result in some useful clarification in the near future. The Gsp (General Secretory Pathway) acronym favoured by Pugsley (Pugsley 1993; Hardie *et al.,* 1996), will be used to refer to apparent archetypal representatives from the *pul* pathway, unless otherwise specified. Some of the most important Gsp (Pul) proteins are presented in Table 1.2, with a few of the best-characterised homologues in related pilin and non-pilin systems.

The typical substrate of the 'main' terminal branch of the General Secretory Pathway (GSP) is pooled in the periplasm as a stable intermediate (Hirst and Holmgren, 1987a and 1987b; Poquet et al 1992) after Sec-dependent export into the periplasm (Pugsley *et al.,* 1991). The presence of proteins in the periplasm to promote correct folding or to directly chaperone, emphasizes the need for presentation of substrate in a competent state for export and/ or assembly into a final structure. A disulphide bond oxidoreductase protein (DsbA) is important to a range of basic functions including protein export in many organisms (Bardwell *et al.,* 1991; Kamitani *et al.,*1992; Yu *et al.,* 1992; Peek and Taylor, 1992; section 1.5.3), the pleiotropic nature of mutations in this gene contrasting with the system specificity exhibited by the product of the *papD* gene, the periplasmic chaperone of the (type 1) P pilus sytem (section 1.10.4).

Table 1.2 Homologies within pilin and non-pilin transport systems

organism	substrates	peptidase	pseudopilins	ATPase	CM	OM
main branch of the GSP[1]		**GspO**	**GspGHIJ**	**GspE**	**GspF**	**GspD**
K. oxytoca	pullulanase	PulO	PulGHIJ	PulE	PulF	PulD
P. aeruginosa	multiple	PilD	XcpTUVW[2]	XcpR	XcpS	XcpQ
V. cholerae	multiple	3	EpsGHIJ	EpsE	EpsL[4]	5
type-4 pilin export						
P. aeruginosa	PilA	PilD	PilV, PilG	PilB[6]	PilC	PilQ
V. cholerae	TcpA	TcpJ	TcpB	TcpT	TcpE,R,D[7]	TcpF,C[7]
DNA uptake						
B. subtilis[6]	DNA	ComC	ComG-3,4,5[8]	ComG-1	ComG-2	-

notes:

1. Gsp nomenclature is shown, and the lettering system is being applied to the more recent pathways (eg. *out, exe*) as they are being reported (Pugsley, 1993a, for review). Related proteins have been discovered in *E. coli* (reviewed in Hobbs and Mattick, 1993), and illustrate the inherent difficulty of defining a system by reconstitution in a 'heterologous' host.

2. This nomenclature (Bally *et al.*, 1992) appears to be widely used, but these were also independently recognised and described as PddABCD (Nunn and Lory, 1992).

3. Secretion of Ctx is normal in the presence of a TcpJ mutation (G. Jonson, J.R.I., and P.A.M., unpublished data).

4. EpsL is not a homologue of PilC, but is shown to interact with EpsE (Sandkvist *et al.*, 1995). There is no evidence that GspF/ PilC homologues and GspE/ PilB homologues interact.

5. No GspD homologues have been described to date in this system.

6. PilT and PilU are homologues of PilB which are essential for twitching motility but do not abolish fimbriation (Whitchurch *et al.*, 1990; Whitchurch and Mattick, 1994)

7. TcpE, R, and D are the only Tcp proteins predicted to be in the cytoplasmic membrane (section 1.12.6). No close homologue of PilC/ GspF exists. There is no GspD homologue in the Tcp system, but TcpC is a lipoprotein and TcpF is possibly a porin-like protein (Parsot and Mekalanos, 1988; Kaufman *et al.*, 1993; Ogierman and Manning, 1992; Ogierman *et al.*, 1993, 1996).

8. DNA uptake in a Gram-positive organism: a GspD homologue is probably unnecessary (see Dubnau, 1991).

The manner of delivery of export-competent substrate to outer membrane components of the GSP is at present unknown. Other than broadly specific protein folding enzymes such as DsbA, no true substrate chaperone has been identified in this system. Specific signal sequences have not been identified, and the permissive substrate feature/s may be conformational (Kornacker and Pugsley, 1990; Connell *et al.,* 1995). Nevertheless, and reminiscent of the specific competence region of *E. coli* HlyA and related proteins (Section 1.7.1), specific residues may have key roles (Connell *et al.,* 1995) - perhaps simply related to their influence on secondary structure.

The role of the +2 Asp residue (following the post-cleavage modification of the mature N-terminal cys residue by the Lsp peptidase) in localising lipoproteins to the outer membrane (Ghrayeb and Inouye, 1984; Yamaguchi *et al.,* 1988; Poquet *et al.,* 1993) has been further elucidated by the recent demonstration of a 20 kDa carrier protein which is specifically involved (Matsuyama *et al.,* 1995; see also Section 1.11.2). The pattern of conformational targeting and limited numbers of key (usually charged) residues appears to be common in many of the systems we have discussed. Accordingly, one would predict a carrier-type chaperone to be associated with cholera toxin, pullulanase, and like substrates which pool as partially folded intermediates in the periplasm. The 'pseudopilins' are less likely candidates for a 'shuttle' role, but could conceivably supplant it in some way. Perhaps other periplasmic carriers remain to be found for Sec-dependent proteins destined for or beyond the outer membrane, such as OmpA or Ctx. If the system specificity resides primarily within the interaction between substrate and outer membrane translocase, such carriers may be of broad specificity, and might then

lend themselves more readily to physicochemical (eg. co-purification) rather than genetic searches.

1.11.2 A specific outer membrane translocation step in Gram-negative bacteria is characterised by a conserved superfamily of outer membrane proteins

The most important class of outer membrane proteins for the purposes of this discussion is represented by GspD in the Gsp translocase (d'Enfert *et al.,* 1989). The significance of this protein superfamily owes to its representation in a wide range of export pathways (Genin and Boucher, 1994), including those for non-Sec dependent (type III) outer membrane secretion (Michiels *et al.,* 1991; Allaoui *et al.,* 1993), type IV pilin export (Martin *et al.,* 1993), and the export of filamentous phage (Russel, 1994b). A GspD homologue is also essential for natural competence in *Haemophilus influenzae* (Tomb *et al.,* 1991). The likely connection between the *Neisseria gonorrhoeae* homologue *omc* and piliation (T. Rudel and T.F. Meyer, pers. comm.) in the light of the known relationship between piliation and DNA uptake in that organism (Biswas *et al.,* 1989) suggests a possible role for *omc* in the natural transformability of *Neisseria gonorrhoeae* as well.

PulD is a multimeric protein in the outer membrane of *Escherichia coli*, which forms high molecular weight detergent-resistant complexes essential to export in that system (Hardie *et al.,* 1996). The presence of a small peripherally associated outer membrane protein, PulS (d'Enfert and Pugsley, 1989), for which at least one homologue may be identifiable in *Erwinia chrysanthemi* (Condemine *et al.,* 1992; Hardie *et al.,* 1996),

prevents the breakdown of complexed and monomeric forms of PulD (Hardie *et al.,* 1996). These two proteins co-associate to the outer membrane in flotation gradients, and the stoichometry of the PulS protective effect suggests a chaperone-like association. PulS appears to protect the C-terminal part of the protein (Hardie *et al.,* 1996), which is periplasmically located (d'Enfert *et al.,* 1989). This conflicts somewhat with data from the well-characterised pIV protein of filamentous phage (below) which suggests that the C-terminal region of these proteins is permissive for membrane localisation (Russel, 1994b), but may be reconciled by the suggestion that premature C-terminal aggregation prior to outer membrane insertion is prevented by the chaperoning of PulS (Hardie *et al.,* 1996). The highly conserved 60 amino acids of the C-terminal region of this class of proteins (Genin and Boucher, 1994) contains a useful identifying motif (Huang *et al.,* 1992) in these proteins. N-terminal halves that are conserved within identifiable subfamilies (Martin *et al.,* 1993; Genin and Boucher, 1994) may define the necessary system specificity. Since pIV can form heterologous multimers with other GspD-like proteins (Kasmierczak *et al.,* 1994), the C-terminal region is relatively tightly conserved (Genin and Boucher, 1994), and Gsp(Pul)S is apparently acting here (Hardie *et al.,* 1996), it would be interesting to see the effect of Gsp(Pul)S on the fractionation of co-expressed PilQ (Martin *et al.,* 1993) and pIV in *E. coli.* In fact, the recent demonstration of species specificity of GspD homologues within very closely related systems (Lindeberg *et al.,* 1996) supports the notion of specific recognition of competent substrate by outer membrane translocase components. Efficient cross-complementation is not therefore an inevitable corollary of functional similarities in the C-terminal regions as demonstrated by the formation of mixed multimers *in vitro* (Kazmierczak *et al.,* 1994).

1.11.3 PilC is a highly conserved protein common to pilin and non-pilin operons

Homologues of PulF in the pullulanase-dedicated export system (Possot *et al.,* 1992), are found in other protein export pathways (Lindeberg and Collmer, 1992; Howard *et al.,* 1993), as well as in type-4 pilin systems and other more distantly related pathways (Hobbs and Mattick, 1993; Tønjum *et al.,* 1995; Table 1.2). These proteins are apparently integral inner membrane proteins, are highly similar between related systems, and are required for normal functioning of the pathways in which this has been tested (Lindeberg and Collmer, 1992; Howard *et al.,* 1993; Tønjum *et al.,* 1995).

1.11.4 Filamentous phage export: an efficient gateway from the cytoplasm

The assembly and export of the *E. coli* bacteriophages f1, fd and M13 have been proposed as useful paradigms for the main terminal branch of the GSP (Russel, 1994a) and, by extrapolation, type IV pilin export. The most important protein in this regard is the pIV protein, a homologue of the outer membrane PulD and PilQ proteins in type II secretion and type IV pilin systems respectively (Genin and Boucher, 1994). Filamentous phage particles of 65 x 9000 Å are composed of five different proteins, each of which is an integral inner-membrane protein in *E. coli* before their assembly. A helical array of the major coat protein pVIII forms a tubular shell for the genome within (Day *et al.,* 1988), and contributes to its packing (Hunter *et al.,* 1987). Minor capsid proteins at the particle ends are surface exposed in the mature phage (Endemann and Model, 1995) and are essential for phage assembly (Webster and Lopez, 1985). Proteins

I/XI and IV are apparently essential for and dedicated to the assembly process, but do not participate in the final structure.

The pIV protein is a multimeric protein in the outer membrane, probably forming a dodecameric channel through which the 65 Å assembled phage can pass (Kasmierczak *et al.,* 1994). One would expect that such a channel would be poorly tolerated if left open (Killman *et al.,* 1993), but *wt* pIV does not increase the sensitivity of *E. coli* to membrane perturbants. Mutation of the highly conserved Gly355 residue has this effect but does not prevent normal multimerisation of pIV, suggesting that gating is disrupted (Russel, 1994b). The pI protein has an essential ATP-binding motif, and a model of conformational change of pI followed by periplasmic interaction with the globular N-terminal third of pIV has been proposed (Russel, 1994c). The formation of mixed multimers of pIV and some of its bacterial homologues (Kasmierczak *et al.,* 1994) underlines the relatedness of the GspD proteins, but it is noteworthy that the distantly related *P. aeruginosa* filamentous phage Pf3 contains no direct pIV homologue, and that the ATP-binding motif of pI is the only shared element between analogous *E. coli* and *P. aeruginosa* filamentous phage assembly pathways (Russel, 1997).

1.12 The type-4 pilin as an export substrate

A recurring theme and a defining element of relatedness in type-4 pilin systems and the main terminal branch of the General Secretory Pathway is the presence of a highly conserved hydrophobic region at the amino-terminal third of type-4 pilin subunits

(Dalrymple and Mattick, 1987; Parge *et al.,* 1995). An unique peptidase (Kaufman *et al.,* 1991; Nunn and Lory, 1991) cleaves these and closely related non-pilin substrates. Amino-terminal sequence analysis of TcpA provided the first evidence that TCP might be related to type-4 pilins (Taylor *et al.,* 1987). This was subsequently confirmed and extended when the nucleotide sequence data became available (Faast *et al.,* 1989; Shaw and Taylor, 1990). In contrast to the methylated phenylalanine residue commonly found at the extreme amino-terminus of mature type-4 pilins, TcpA has a methylated methionine residue (Kaufman *et al.,* 1991). In fact, conservative substitutions in this position are tolerated to some degree in the related PilA of *P. aeruginosa* (Strom and Lory, 1991), and natural variations are not restricted to *V. cholerae* (Donnenberg *et al.,* 1992; Fig. 1.9, overleaf).

The precursors of the type-4 pilins generally have a short hydrophilic prepeptide sequence (Dalrymple and Mattick, 1987), in contrast to the 25 residue prepeptide region of TcpA (Faast *et al.,* 1989; Taylor *et al.,* 1990). The functional significance of this difference remains to be determined. These prepeptides are probably cleaved on the cytoplasmic face of the inner membrane (Kaufman *et al.,* 1991; Strom *et al.,* 1993), and the role of the typical (Sec-dependent) signal peptide hydrophobic region is thus arguably usurped by the mature amino-terminal hydrophobic region. This region of close homology (up to the turning glycine residue at position 54 or 55) in type-4 pilins generally (Dalrymple and Mattick, 1987; Figs. 1.10, 1.11), may be important in translocation across the outer membrane and/or subunit-subunit interaction in assembly of the final structure.

Figure 1.10 Type-4 pilins and related proteins in non-pilin systems

```
                                  5
1              MKSLQKG  FTLIELMI VVAIIGILAAFAIPAYNEYIAR
2                MNAQKG  FTLIELMI VIAIIGILAAIALPAYQDYISK
3              MNTLQKG  FTLIELMI VIAIVGILAAVALPAYNEYTAR
4                MKAQKG  FTLIELMI VVAIIGILAAIAIPQYQNYVAR

5     VSKIMNKKYEKG  LSLIESAMVLALAATVTAGVMFYYQSASDS
6   M              QEG  MTLLEVII VLGIMGVVSAGVVTLAQRAIDS
  (QLLKQLFKKKFVKEEHDKKTG)
7              MRKYQQG  VGLLEAILASAV    LG  MALVA
8  MLLKSRHRSLHQSG  FSMIEVLVALLLISIGILAAIA
9              MQRQRG  FTLLEIMVVIVI    LGVLASLV
10           LQRRQQSG  FTLIEIMVVVVI    LGILAALV
11             MNEKG  FTLVEMLIVLFI    ISILLLIT
12             MERRQRG  FTLLEIMVVIVI    LGVLASLV
```

(Gaps are included in alignments to facilitate comparisons)

type-4 pilin proteins: major structural subunits

1 *Dichelobacter nododsus 265* (Elleman *et al.*, 1986)
2 *Moraxella bovis* β (Marrs *et al.*, 1985)
3 *Neisseria gonorrhoeae MS11* PilE (Meyer *et al.*, 1984)
4 *Pseudomonas aeruginosa PAK* PilA (Sastry *et al.*, 1983)
5 Bundle-forming pilus of enteropathogenic *E. coli* (Giron *et al.*, 1991)
6 *Vibrio cholerae* O1 TcpA (Faast *et al.*, 1989; Taylor *et al.*, 1990); most of the 25-residue signal sequence is compressed in parentheses

non-pilin peptidase substrates:

7 TcpB, encoded from within the *tcp* operon of *V. cholerae* (Ogierman and Manning, 1992)
8 PilV, required for type-4 pilin biogenesis in *P. aeruginosa* PAO (Alm and Mattick, 1995)
9 PulG, one of four "pseudopilins" in the *pul* operon of *K. oxytoca* (Reyss and Pugsley, 1990)
10 XcpT, one of four "pseudopilins" in the *xcp* operon of *P.aeruginosa* (Bally *et al.*, 1992)
11 ComG-3, one of three "pseudopilins" identified in the *Com-G* (DNA uptake) system of *B. subtilis* (Breitling and Dubnau, 1990)
12 OutG, one of four "pseudopilins" identified in the *out* operon of *Erwinia chrysanthemi* (He *et al.*, 1991; Condemine *et al.*, 1992)

1.12.1 Details of subunit structure suggest an assembly mechanism

The hydrophobic α_1 helical core ending with a sharp turn at Gly55 as identified in MS11 is highly conserved region in all the type IV pilins (Dalrymple and Mattick, 1987; Parge *et al.*, 1995). PhoA$^+$ fusions to the first 45 residues of the *P. aeruginosa*

PAK PilA subunit cross the cytoplasmic membrane, although a periplasmic pool is not detected in the wild type (Strom and Lory, 1987). The first half of this region (to Gln25 in Figs. 1.10 and 1.11) is more strictly conserved across species (Dalrymple and Mattick 1987; Shaw and Taylor, 1990).

Fig. 1.11 Type-4 pilin core structural features and critical residues

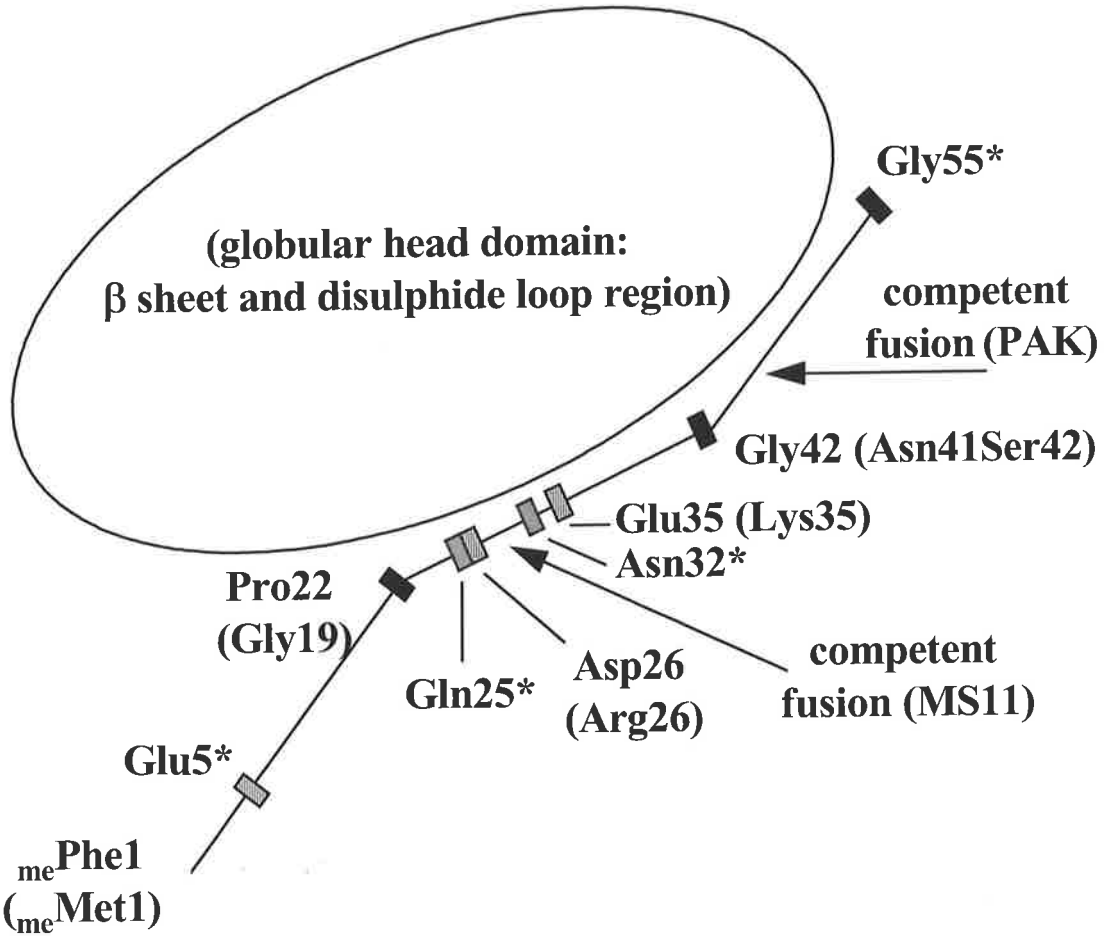


Fig. 1.11 A simplified schematic diagram of the processed type-4 pilin subunit, adapted from data for the recently solved MS11 PilE structure (Parge *et al.*, 1995) depicts the slightly sinuous α-β roll backbone (Met1-Gly55). Critical residues in MS11 PilE are indicated, and corresponding residues in TcpA included in brackets, unless identical (denoted by an asterisk). MS11 PilE C-terminal PhoA fusions after position 30 in PilE (Dupuy *et al.*, 1991) and PAK after position 45 (Strom and Lory, 1987) are apparently translocated across the cytoplasmic membrane. Gln25 marks the end of the most strictly conserved region

(Dalrymple and Mattick, 1987). Asp/Arg26 to Gly55 tethers the globular head domain in MS11, especially at Asn32 and Glu35. See text for details.

A PhoA fusion is fully functional when fused to the first 30 residues of MS11 (Dupuy *et al.,* 1991) and is localised to the cytoplasmic membrane in *E. coli*, suggesting that this region is important for translocation and serves as a hydrophobic anchor in the inner membrane. The second half of the α-helical core appears to tolerate some sequence variation and even some charged residues in both MS11 PilE and TcpA, but remains compatible with the coiled α-helical backbone. It has been suggested that this latter region (Asp/Arg26 to Gly55) may form salt bridges (Finlay *et al.,* 1986), and it has also been shown that monoclonal antibodies raised against epitopes here can prevent normal fimbrial assembly and function in *P. aeruginosa* (Rothbard *et al.,* 1984; 1985). The C-terminal portion of the α_1 helix interconnects with all four central β strands in the MS11 PilE subunit of *N. gonorrhoeae* to form a large hydrophobic core region within the globular head domain. A smaller hydrophobic core between the β sheet and the disulphide loop is stabilized in part by tethering to the α_1 region at residues Asn32 and Glu35 in MS11 PilE (Parge *et al.,* 1995).

Essential elements therefore appear to be the α_1 helical region extending to a highly conserved Gly55 turn, and a globular head formed by regions of β sheet which permit the presentation of hydrophilic epitopes and which is stabilised by salt bridges and hydrogen bonding to the C-terminal region of the α_1 region. A salt bridge involving the Glu5 which appears to be required in all type-4 pilin and related proteins (Hobbs and Mattick, 1993; Pugsley, 1993b) is thought to be a key to normal subunit interaction (Parge *et al.,* 1995) and a Glu:Lys substitution at this position in the PilA subunit of *P.*

aeruginosa results in the assembly of abnormal 'corkscrew' fibres (Pasloske *et al.*, 1989). In the presence of β-octylglucoside, the first 25 or so residues of subunits pack in unusual antiparallel dimers that serve to optimally reduce surface hydrophobicity *in vitro* (Parge *et al.*, 1990,1995). Minimisation of the hydrophobicity in the membrane is an appealing model for an intermediate stage, and other studies have also suggested a dimeric intermediate for type IV pilin assembly (Watts *et al.*, 1983). Nevertheless, computer modelling of the solved PilE crystal structure shows that dimer assembly produces a pilus structure incompatible with the physical character of the mature fibre, and predicts instead a monomeric assembly process (Parge *et al.*, 1995).

1.12.2 Secondary structure of TcpA: inferences from the PilE crystal structure

TcpA was examined in the light of this information and the data presented above, in terms of structure predictions (Figs. 1.11, 1.12). The criteria defined by Chou and Fasman (1978) and Rose (1978) were applied to TcpA, to predict regions of α helix and β sheet. As a test of validity, this method was applied also to the MS11 sequence. The large stretch of β sheet which comprise the known $\beta_{4,5,6}$ regions was correctly identified, and the β_1, β_2, and β_3 regions predicted to be mixtures of α and β. Since the prediction for the N-terminus to Gly55 in TcpA is the same as for MS11 PilE, we can be confident that this region is the highly conserved α_1 extended helical spine. Flexibility of the PilE spine is attributed to Pro22 and Gly42 (Parge *et al.*, 1995). TcpA has a Gly at position 19, and Asn41Ser42 are identified as helix-interrupting residues between the Leu40 and the Ile43-Gln49 region (Figs. 1.11, 1.12). The Asn32 of PilE is mirrored in TcpA, as is the charged residue at position 35 (Glu35/ Lys35), this region of

the α_1 spine holding, respectively, β strand and the externally presented 'sugar loop' of MS11 which precedes the β folds.

The globular head region of TcpA is similar in size to that of PilE up to the first cysteine (Shaw and Taylor, 1990). This region is predicted to be comprised of several β sheet regions: Leu69-Leu76; Phe88-Phe98; Phe108-Ile124; Thr125-Ile136; Thr159-Ile167. Ala103-Ala107 is possibly an α-helical β_2 to β_3 connection, and includes a lysine residue (Fig. 1.12). It is difficult to predict which residues would be bured and which exposed from the predicted structure alone, since charged residues forming salt bridges within the C-terminus of the α_1 chain might well be relatively internalized. The large disulphide loop Cys120-Cys186 includes a couple of predicted β strands (Fig. 1.12), and is presumably surface exposed since the important epitope/s of the O395 (classical biotype) of TcpA is localised to this region and the adjacent C-terminus by studies with monoclonal antisera (Sun *et al.,* 1991).

Fig. 1.12 TcpA : main structural elements

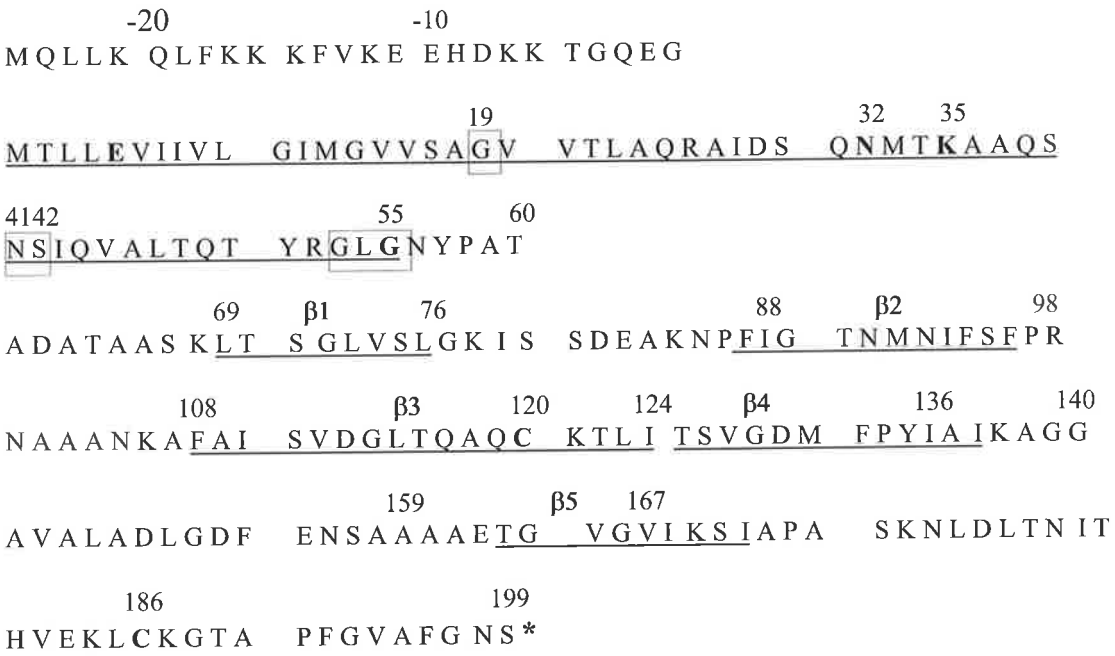


Fig. 1.12 Amino acid sequence of Z17561 TcpA. The top line shows the 25-residue prepeptide, and numbering restarts with the first residue of the mature structure. The helical backbone and helix-breaking residues within it are marked by underlining and boxing. Other significant residues mentioned in the main text are in bold, and the 5 regions of β sheet are indicated (underlines and italicised bold text).

1.12.3 Pseudopilins

The observation of a general secretion defect consequent upon a mutation in the type IV pilin-associated peptidase in *P. aeruginosa* (Nunn and Lory, 1991) brought with it the recognition of a shared pathway for type IV pilin export and protein secretion (Bally *et al.,* 1992; Nunn and Lory, 1992), which was confirmed in the *gsp* (*pul*) pathway of *Klebsiella oxytoca* (Pugsley and Dupuy, 1992). Proteins with close N-terminal homology to the type-4 pilins are now known to be common to the outer membrane (Gsp) translocases, DNA translocation systems, and the type IV pilin assembly

pathways (see Pugsley, 1993a). The substrates of this peptidase in non-pilin exporters are pilin-like in that they possess the specific cleavage site, and an amino-terminal region with close homology to that of the type-4 pilin subunit which probably forms a similar α-β helical spine (Figs. 1.10 and 1.11). Glu5 and Gly-1 (immediately before the cleavage site) are strictly conserved amongst the type IV pilins and pseudopilins and appear to be essential for assembly and cleavage respectively (Strom and Lory, 1991, 1993; Pugsley, 1993b; Hobbs and Mattick, 1993; Alm and Mattick, 1995). PulG pseudopilins in which Glu5:Ala or Glu5:Val substitutions have been performed are efficiently processed and methylated (Pugsley 1993b), while similar substitutions in the pilin subunit (PilA) of *P. aeruginosa* are processed but not normally methylated (Strom and Lory 1991). In both circumstances however, function is abolished.

The Gsp-type pseudopilins are typically present as a transcriptionally linked unit of four genes (Bally *et al.,* 1992; Lindeberg and Collmer, 1992; Howard *et al.,* 1993; Pugsley, 1993b). The degrees of homology of some well-characterised proteins to their Gsp (Pul) equivalents are listed below (Table 1.3).

Table 1.3

Overall degree of similarity to Gsp proteins at amino acid level:

		GspG	GspH	GspI	GspJ
Pseudomonas aeruginosa (Bally *et al.*, 1992)	**XcpT-W**	51%	23%	34%	36%
Aeromonas hydrophila (Howard *et al.*, 1993)	**ExeG-J**	71%	27%	50%	43%
Erwinia chrysanthemi (Lindeberg and Collmer, 1992)	**OutG-J**	34%	50%	52%	44%

A pilin-like scaffold formed by such proteins within the Gsp transporter apparatus has been proposed (Bally *et al.,* 1992; Nunn and Lory, 1992; Pugsley, 1993a; Hobbs and Mattick, 1993). Altered detergent solubility of one of four type 4 pilin-like proteins in the Xcp/Pdd secretion system of *P. aeruginosa* (XcpT) has been observed after processing (Bally *et al.,* 1992), and there is some evidence to support participation in a complex from studies of the PulG pseudopilin High level overexpression of this protein inhibits pullulanase secretion (Pugsley and Dupuy, 1992), but not by titration of peptidase activity (Pugsley, 1993b). In support of this, overexpression of pilin subunit in *P. aeruginosa* does not titrate out the activity of the shared peptidase, and has no effect on pilin or non-pilin secretion (Strom and Lory, 1992). Furthermore, a dominant negative effect is exhibited by normally processed C-terminal mutants of PulG expressed *in trans* (Pugsley, 1993b), and one is tempted to draw a parallel with the normally assembled but dysfunctional pilin fibres that result from disruption (Farinha *et al.,* 1994) or misfolding (Peek and Taylor, 1992) of the disulphide loop in *P. aeruginosa* and *V. cholerae,* respectively. Nevertheless, attempts to demonstrate participation of PulG in a multiprotein complex have been unsuccessful (Pugsley and Possot, 1993).

1.12.4 Type-4 pilin subunits in the cytoplasmic membrane

It remains to now consider translocation across the cytoplasmic membrane in the type-4 pilin pathways. TcpJ is the specific prepilin-peptidase required for the processing of TcpA (Kaufman *et al.,* 1991) and a member of a class of homologous proteins in type 4 pilin and non-pilin export systems (Hobbs and Mattick, 1993; Pugsley, 1993a; Fig.1.10). It appears to cleave and subsequently amino-methylate TcpA on the cytoplasmic face of the membrane in a Sec-independent fashion (Kaufman *et al.,* 1991), with the hydrophobic amino-terminus of the mature pilin probably remaining anchored in the cytoplasmic membrane until assembled into the pilus, as suggested for other type 4 pilins (Dupuy *et al.,* 1991; Hobbs and Mattick, 1993). This may obviate the need for a periplasmic chaperone such as PapD, no such protein having been identified in the TCP system or in other type 4 pilin systems. It may be that the highly conserved hydrophobic amino-terminal third of the type 4 and related proteins, such as TcpA, serves an anchoring and/or protein-protein interactive role in assembly and export, as discussed above.

It should be noted that the TcpB proteins is two to three times the molecular mass of other 'pseudopilins' in pilin operons (Alm and Mattick, 1995) and in the Gsp pathway (Pugsley, 1993b), although the N-terminal region remains a good fit (Fig.1.10). It is encoded by an open reading frame downstream and separated from *tcpA* by an attenuating stem loop structure (ΔG = -21.7 kcal/mol). Support for the predicted attenuation comes from Northern analyses which show that the level of mRNA downstream from *tcpA* is greatly reduced compared to that of *tcpA* itself (Thomas *et al.,*

1996), and from the observation that a *tcpB::*Tn*phoA* fusion is not as strongly expressed as a fusion with *tcpA* (Peterson and Mekalanos, 1988). TcpB has been proposed as a regulator of pilus length (Taylor *et al.,* 1988), analogous to PapH in the Pap pilus system (Hultgren *et al.,* 1993). As such, the stoichometric relationship of the length-regulating protein to the pilus subunit determines pilus length, presumably by acting as a "plugging" mechanism that prevents further elongation, and simultaneously acting as an anchor in the outer membrane (Parsot *et al.,* 1991). Such a stoichometry might suffice to regulate length, especially if such requirements are not particularly stringent, as might be expected in a bundle-forming pilus. However, there has been no clear demonstration of any protein fulfilling such a role in any of the type 4-related systems, and *tcpB::*Tn*phoA* mutants are reportedly TCP negative, as judged by autoagglutination and mouse colonisation assays (Peterson and Mekalanos, 1988). The *tcpB* phenotype is readily explained by transcriptional polarity, but the identification of a type 4 pilin-like leader sequence and amino-terminus on TcpB suggests that it may indeed be cytoplasmic membrane-anchored, and/or interact with TcpA via this domain.

1.12.5 ATP-binding motifs within a highly conserved cytoplasmic membrane-associated protein are essential in type-4 pilin exporters and related pathways.

A putative ATP-binding protein appears to be common to all the type-4 pilin and related systems. There are regions of strong homology (Fig. 1.13, overleaf) and even some interchangeability between proteins from different systems (Sandkvist *et al.,* 1995).

The Walker A consensus nucleotide binding site (Walker *et al.,* 1982) is highly conserved (see sections 1.7.2 and 1.12.5) and essential for activity of such proteins (Possot and Pugsley, 1994, Sandkvist *et al.,* 1995; Stephens *et al.,* 1995), although the Walker B motif (Walker *et al.,* 1982) is absent from some of these (Christie *et al.,* 1989, Turner *et al.,* 1993). Substitutions of some of the conserved residues of the PulE Walker B motif were well tolerated, although this did not include every charged residue in this region, including the Glu residue marked with an arrow in Fig. 1.13 (Possot and Pugsley, 1994). Binding of γ^{32}P-ATP to purified EpsE has been reported (Sandkvist *et al.,* 1995), and autophosphorylation and low-level ATPase activity of the VirB11 protein described (Christie *et al.,* 1989), albeit later somewhat contradicted (Stephens *et al.,* 1995). However, ATP could not be shown to bind PilB or XcpR (Turner *et al.,* 1993) *in vitro,* nor to PulE in cellular extracts (Possot and Pugsley, 1994).

A range of mutations and deletions that preserve the N-terminal region of PulE produce an interference effect when overexpressed *in trans.* PulE runs on SDS-PAGE at twice the expected molecular weight in non-reducing conditions, and is found equally with

unfractionated *E. coli* membranes in the presence or absence of other members of the *pul* operon (Possot and Pugsley, 1994).

Figure 1.13

		↓
TcpT	G T T G S G **K** T V	K G R L L L L **E** T L V P T
PilB	G P T G S G **K** T V	K G R V G I Y **E** V V K N T
EpsE	G P T G S G **K** S T	R G R T G I H **E** L L L V D
PulE	G P T G S G **K** S T	R G R T G I H **E** L L L V D

Fig. 1.13. Alignment of the Walker A and Walker B motifsof two pilin assembly-related and two protein export-related homologues (see text). The highly conserved Glu residue in the Walker B motif (arrow, E) is likely to be essential, but has not yet been shown to be so.

That the N-terminal region of PulE is not required for formation of heterodimers suggests that this region also interacts with another membrane component in *K. oxytoca*, perhaps something for which a sufficiently close homologue exists in *E. coli*. By contrast, EpsE (with the same highly conserved cysteine residues) is cytosolic when overproduced and runs as a monomer on a size separation column, but complexes stably within the membrane by the N-terminal region to another protein, EpsL (Sandkvist *et al.,* 1995). XcpR mutants are also able to exert transdominance, but this is not the case for the closely related PilB protein, responsible in the same organism for pilin export (Turner *et al.,* 1993).

Interestingly, more than one member of this class of protein has been found in association with the type-4 pili of *P. aeruginosa*. Twitching motility is a type-4 pilin-associated phenotype originally observed in *P. aeruginosa* (Bradley, 1972; Bradley,

1980), the physical basis for which remains obscure. Mutations in the *pilT* gene is associated with hyperfimbriation and loss of the twitching motility phenotype (Whitchurch *et al.,* 1991). An independently expressed gene in *P. aeruginosa* (*pilU*) encodes a closely related protein which is also required for twitching motility, and causes a hyperfimbriate phenotype (Whitchurch and Mattick, 1994). The twitching motility phenotype has not been described in *V. cholerae,* although a *pilT*-like gene has been found to co-exist with a *pilB* homologue in the operon associated with the phenotypically similar bundle-forming pilus of Enteropathogenic *E. coli* (Girón *et al.,* 1991; Sohel *et al.,* 1993; Stone *et al.,* 1996). The distinction between those proteins involved in the protein export systems, those involved in pilin systems, and those involved in twitching motility is not immediately apparent in structural terms, although minor variations in the predicted secondary structure of the C-terminus have been used to separate pilin system-associated proteins from other members of this superfamily (Possot and Pugsley, 1994).

1.12.6 The *tcp* operon of *Vibrio cholerae*: a model for type-4 pilin biogenesis ?

The biogenesis of *V. cholerae* TCP appears to be encoded by a cluster of 14 genes which includes proven (DiRita *et al.,* 1991) and putative (Thomas *et al.,* 1996) regulators, and the dedicated peptidase, TcpJ (Kaufman *et al.,* 1991) (Fig. 1.14).

Figure 1.14 The *tcp* operon

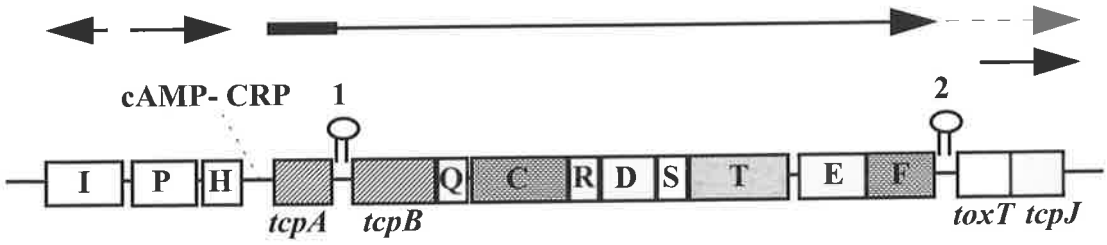


Fig. 1.14 The *tcp* operon is depicted schematically, with transcriptional orientation (arrows). Promotors have been identified in front of *tcpA* (Thomas *et al.*, 1996) and *toxT* (Higgins and DiRita, 1994). Primer extension studies (Thomas *et al.*, 1996) suggest that *tcpA* transcription is efficient, but that message is greatly diminished beyond the attenuating sequence at position 1 (Ogierman *et al.*, 1994). A putative cAMP-CRP binding site in front of *tcpA* is marked. Peptidase substrates (A and B) are distinguished by diagonal hatching, putative outer membrane proteins (C and F) by brick hatching, and CM proteins (R, D, and E) by dots. See Ogierman and Manning, 1992; Ogierman *et al.*, 1993, 1996).

Table 1.4 Tcp proteins

	size (and location)	homologues/ possible function	References (see below)
TcpI	42 kDa	chemotaxis signal transduction	3, 12
TcpP	26 kDa (? CM)	? regulatory	13, 14
TcpH	16 kDa	? regulatory	3, 12, 13
TcpA	23 kDa	type-4 pilin subunit	1, 4, 5
TcpB	47 kDa	? "pseudopilin"	2, 3
TcpQ	17 kDa (? periplasm)	unknown	13
TcpC	54 kDa (OM)	lipoprotein	3, 8, 9
TcpR	18 kDa (CM)	unknown	11
TcpD	32 kDa (CM)	unknown	11
TcpS	17 kDa (? periplasm)	unknown	11
TcpT	57.5 kDa (CM)	GspE homologue; ATPase	11, 12
TcpE	31.5 kDa (CM)	? PilC (*P. aeruginosa*) homologue	10, 11
TcpF	38 kDa (OM)	porin-like	10, 11
TcpJ	32 kDa (CM)	prepilin peptidase	6
ToxT	32 kda (cytoplasm)	transcriptional activator	7

Size in kiloDaltons (kDa) is given, with the predicted location (CM: cytoplasmic membrane; OM: outer membrane). ***References:*** 1. Taylor *et al.*, 1987; 2.Peterson and Mekalanos, 1988; 3 Taylor *et al.*, 1988; 4. Faast *et al.*, 1989; 5. Shaw and Taylor, 1990; 6. Kaufman *et al.*, 1991; 7. DiRita *et al.*, 1991.; 8. Parsot *et al.*, 1991; 9. Ogierman and Manning, 1992; 10. Kaufman *et al.*, 1993; 11. Ogierman *et al.*, 1993; 12. Harkey *et al.*, 1994; 13. Ogierman *et al.*, 1996; 14. Thomas *et al.*, 1996.

The TcpI protein is predicted to be a methyl-accepting chemotaxis protein (Harkey *et al.,* 1994), and Tn*phoA* mutations in this are TCP negative (Taylor *et al.,* 1988), suggesting a link between chemotaxis and TCP production (see section 1.2.4). The following predictions are based on the amino acids encoded by the DNA sequence determined in this laboratory, which was published during the course of these studies (Ogierman and Manning, 1992; Ogierman *et al.,* 1993; Ogierman *et al.,* 1996). TcpB and TcpA have been separately discussed (above).TcpP is hydrophilic protein with a 29 residue hydrophobic domain which may serve as a membrane anchor. TcpQ and TcpH have Sec-dependent leader sequences, and their hydrophilic nature suggests either periplasmic locations or export from the cell, but searches of the Genbank database do not reveal homologous proteins that might give a clue to its function.

TcpC is a ToxR regulated outer membrane lipoprotein (Parsot *et al.,* 1991), absent in *tcpA* and *tcpB::*Tn*phoA* mutant strains, presumably due to transcriptional polarity. More interestingly, it is undetectable in outer membrane preparations from *tcpD* or *tcpE* mutants, suggesting failure of correct localisation in these strains. An increase in complement sensitivity was detected in *tcpA*, *tcpB*, *tcpC*, *tcpD*, and *tcpE* mutants: it was suggested that this, along with the observed decrease in colonising ability, might be explained by lack of functional TcpC (Parsot *et al.,* 1991). This defect, along with the loss of TCP and a reduced ability to colonise infant mice, was common to all these mutants, but subsequent experiments with *tcpA* mutants in this laboratory have not been able to confirm the changes in complement sensitivity (Attridge *et al.,* 1993). TcpD lacks a signal sequence but has a typical membrane-spanning domain at residues 25 - 47. The distribution of positively charged amino acids suggests that the preceding

region is cytoplasmic and the carboxy-terminus periplasmic. This is consistent with the location of a PhoA$^+$ Tn*phoA* insertion with a fusion junction after residue 114 (R.K. Taylor, pers. commun.). TcpR has a markedly hydrophobic amino-terminus also capable of spanning the cytoplasmic membrane, although the bulk of the protein appears likely to be cytoplasmic. TcpS, like TcpQ, has a typical Sec-dependent signal sequence with no significant β-sheet domains in the relatively hydrophilic mature protein and it seems likely to be periplasmic (or exported from the cell). TcpE does not have a discernible signal sequence, but the region residues 108 to 131 may span the membrane. Taylor and coworkers (R.K. Taylor, pers. commun.) localised a PhoA$^+$ Tn*phoA* insertion to a fusion junction after residue 150, suggesting that TcpE is a trans-cytoplasmic membrane protein. It also has similarity with the *P. aeruginosa* PilC and related proteins (Tønjum *et al.,* 1995), and has been proposed as a participant in a cytoplasmic membrane-associated assembly complex (Kaufman *et al.,* 1993). The hydrophilic N-terminal region is a potential domain for protein-protein interactions in the cytoplasm or periplasm.

Although the lipoprotein TcpC is probably outer membrane-associated (Parsot *et al.,* 1991), there are no GspD homologues in the Tcp system. The 38 kDa TcpF protein is predicted to have a Sec-dependent signal sequence and porin-like β-sheet structure, and is a possible candidate for an outer membrane channel, perhaps even one through which the pilin homo-polymer is extruded. Neither this protein, nor indeed any of the Tcp proteins discussed other than TcpJ, TcpA, and ToxT, have been definitely localised or analysed functionally to a significant degree.

Aims of this thesis

The toxin-coregulated type-4-related pilus discussed herein is an essential virulence factor of *Vibrio cholerae* and a potential model for type-4 pilin biogenesis. The initial parts of this thesis aim to characterise the TcpA subunit protein itself, by examining both the *tcpA* gene and the expression of the protein *in vitro* as a prelude to exploration of TCP biogenesis. The *tcpA* gene was examined from a range of isolates in order to determine whether sequence conservation in the structural subunit would provide clues as to essential elements for biogenesis as well as information about the relatedness of different subunits that might have diagnostic and/ or vaccine relevance.

The fate of type-4 pilin subunits after processing and before assembly into an external fibre is unknown. Possessing a typical type-4 subunit, prepilin peptidase, a typical GspE homologue, and apparently being completely encoded in a single gene cluster, the *tcp* system lends itself to a genetic approach. Previously observed mutations in the *rfb* operon of the *Vibrio cholerae* O1 classical strain 569B which lead to failure of O-antigen biosynthesis (Ward and Manning, 1989) and a possible TCP assembly defect (Stroeher, 1992) provided a starting point. An initial objective was therefore to confirm and characterise the putative assembly defect in these strains in order to understand the contribution of outer membrane elements to the secretion and/ or assembly of the pilus structure.

A genetic approach to dissection of the transport system, targeting the highly conserved TcpT protein, required detailed analysis of the nature and function of TcpT by site-directed mutagenesis, and appropriate complementation studies. It was hoped that information about specific subcellular localisation and associations with other members of the Tcp complex might also be obtained. TcpT is predicted to operate at the level of the cytoplasmic membrane, although GspE homologues in the main terminal branch of the GSP are required for outer membrane transport. Examination of the TcpA subunit disposition in the setting of contrasting export defects at the inner and outer membrane in isogenic *rfb* and *tcpT* mutants, with appropriate genetic and physicochemical studies, should provide a glimpse of subunit trafficking between the membranes. The relatedness and ubiquity of outer membrane transporters in Gram-negative bacteria in which type-4 pilin-like proteins play an essential but presently obscure role, gives added significance to such investigations.

Chapter Two

Tcpa Is Strictly Conserved In Clinical Isolates Of *Vibrio Cholerae* O1 And O139

2.1 Introduction

The amino-terminal region of type-4 pilins has been shown to be critical for their cytoplasmic membrane translocation (Strom and Lory 1987, Dupuy *et al.,* 1991), and is conserved to such an extent that this region and the associated peptidase serve as defining elements in related pilin and non-pilin transport systems (Hobbs and Mattick, 1993). An important motivation for the initial study of subunit genes, and the main theme of the work that follows, is to identify clues to export and assembly. It was not known at the time this work was begun whether TCP was assembled in El Tor strains (Jonson *et al.,* 1992), but TcpA has since been shown to be an essential virulence factor in both biotypes of *Vibrio cholerae* O1 (Taylor *et al.,* 1987; Attridge *et al.,* 1993). In Western blotting experiments, there is great variation in subunit expression *in vitro* between classical and El Tor strains, and within the El Tor biotype itself (Voss and Attridge, 1993). Sequencing of the subunit gene has the potential to provide information about redundancy and conservation of essential structural elements within the encoded protein. Prediction of major antigenic differences are possible, as well as the development of diagnostic PCR primers based on what has since been shown to be an essential and highly conserved virulence factor. It was therefore decided to compare the

tcpA gene from a range of strains, including representatives of the newly recognised El Tor-like O139 (Bengal) serovar (Albert *et al.,* 1993).

2.2 Primer design and selection; amplification from chromosomal *tcpA* genes

The previously determined DNA sequence from classical strain Z17561 (Faast *et al.,* 1987) and El Tor strain H1 (Ogierman *et al.,* 1996) provided the necessary consensus sequence for the *tcpA* gene and adjacent regions. Existing oligonucleotide primers were selected and new ones designed from non-coding as well as internal regions of the open reading frame, so as to facilitate direct sequencing and cloning of PCR-amplified products (Figure 3.1; Table 3.1). Chromosomal DNA was prepared from a range of clinical cholera isolates selected for their biotypic, serotypic, temporal, and geographical differences (Table 3.2). El Tor strains were also chosen for their varying capacity to produce TcpA/TCP *in vitro* (Voss and Attridge 1993). Amplification of *tcpA* was performed by polymerase chain reaction (PCR) using annealing temperatures 2°C below that of predicted primer T_m. Primer pairs #525 and #568, and #567 and #568 (Table 2.1), produce products of 950 bp and 597 bp respectively in all clinical isolates of *V. cholerae* tested (Table 2.2).

Fig 2.1 Location of relevant *tcpA* restriction and amplification sequences

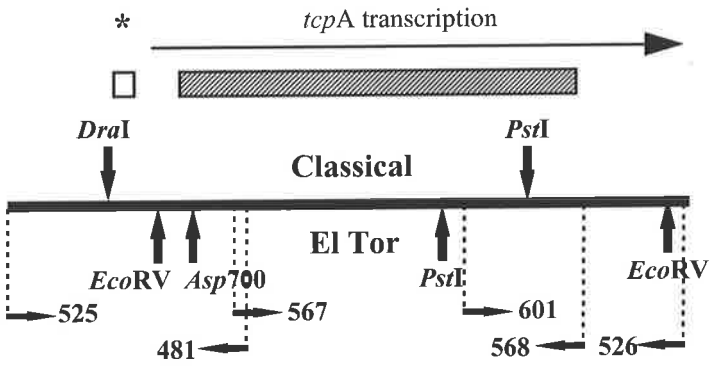


Fig. 2.1 *TcpA* transcriptional direction is shown, with relevant restriction sites and oligonucleotides, and the site of the cAMP-CRP sequence (box/ asterix) and open reading frame (hatched).

Table 2.1 Location of relevant *tcpA* restriction and amplification sequences

oligo-nucleotide	sequence (5' - 3')	+/-	site	comments
525	AAAGAGCTCGATCTCCACTCCGGAAATA	+	-203	*Bgl*II site
567	AAGAAAACCGGTCAAGAGGGTATGAC	+	+155	internal
601	AATAAAGCATT(T/C)GCATT	+	+479	internal
481	CCGGTTTTCTTATCGTGTTC	-	+121	internal
568	GAATGGAGCAG (T/A)CC(G/T)TTACA	-	+706	*tcpA* stop +677
526	AAAGGATACCGCCTCCAATAATCCGACAC	-	+850	*Bgl*II site

Classical		***El Tor***	
***Asp*700**	no site	***Asp*700**	+409
***Dra*I**	+44	***Dra*I**	-169
***Eco*RV**	no site	***Eco*RV**	+36; +820
***Pst* I**	+613	***Pst* I**	+459

Table 2.1 Primers are given in the 5'-3' orientation, and their sense with respect to direction of transcription given as + or -. The numbering used assigns the transcriptional start site to the +1 (nt) position, and denotes the position of the terminal 3' base of each oligonucleotide (site). Oligonucleotides #567, #601, and #481are internal to the *tcpA* coding sequence, and #568 is placed less than 30 nt after the stop codon for *tcpA*. The precise locations of important restriction sites in each biotype are also given, with numbers referring to the cleavage site.

Table 2.2 Strain table and *tcpA* PCR products

Strain; biotype and serotype	Origin	TCP (IEM)	PCR
a. *Vibrio cholerae* O1 clinical isolates			
AE21161; **O1 El Tor** Inaba	B'desh 1989	N	Y
H1;**O1 El Tor** Ogawa	B'desh 1985	+ (EV)	Y
GN9012 (V84); **O1 El Tor** Inaba	India 1990	+	Y
GN7007 (V87); **O1 El Tor** Ogawa	India 1990	nd	Y
O17; **O1 El Tor** Ogawa	India 1965	N (EV)	Y
GN9006 (V86); **O1 El Tor** Inaba	India 1961	N (EV)	Y
Z17561; **O1 Classical** Inaba	B'desh 1985	+	Y
AE10468; **O1 Classical** Ogawa	B'desh 1989	+	Y
569B; **O1 Classical** Inaba	India 1946	+	Y
CA 401; **O1 Classical** Inaba	India 1953	+	Y
b. *Vibrio cholerae* O139 clinical isolates			
AI-1837; **O139** "Bengal" strain	Dakka 1992	nd	Y
AI-1838; **O139** "Bengal" strain	Dakka 1992	nd	Y
AI-1841; **O139** "Bengal" strain	Dakka 1992	+	Y
AI-1852; **O139** "Bengal" strain	Dakka 1992	nd	Y
AI-1854; **O139** "Bengal" strain	Dakka 1992	nd	Y
AI-1855; **O139** "Bengal" strain	Dakka 1992	+	Y
AI-4260; **O139** "Bengal" strain	Dakka 1992	nd	Y
AI-4450; **O139** "Bengal" strain	Dakka 1992	nd	Y
c. *Vibrio cholerae* environmental isolates			
NSW 4: **O1** El Tor Ogawa; oysters	NSW, Australia	N	N
N 59: **non-O1**; river water	Qld., Australia	nd	N
N 120: **non-O1**; river water	NSW, Australia	nd	N
1074/78: **O1** El Tor*	Brazil 1978	N	N
1196/78: **O1** El Tor*	Brazil 1978	N	N
d. non-*Vibrio* isolates			
NCTC #10885; Kanagawa neg. ***Vibrio parahaemolyticus***		nd	N
NCTC #10884; Kanagawa pos. ***Vibrio parahaemolyticus***		nd	N

Table 2.2 The bacterial strains are listed with their biotype, serotype, and source of origin. Environmental isolates from Brazil are avirulent in infant mice (RK Taylor, pers. comm.). The result of examination by IEM for TCP is given: +: TCP found with biotype - specific antisera; N: negative; nd: not done; (EV): reported by E. Voss (Voss and Attridge, 1993; E.V., pers. comm.). Results of stringent PCR amplification of *tcpA* with #525/#568 and #567/#568 are given. Y: discrete products of 950 and 597 bp respectively from both reactions; N: no product from either reaction; nd: not done. DNA sequence was determined for the coding and preceding regions of all clinical O1 isolates and from O139 strains AI-1837 and AI-1855. Bracketed strain designations (V86) are laboratory strain collection numbers which have been used in other publications (Voss and Attridge, 1993; Voss , 1995).

2.3 Southern hybridisation patterns common to pathogenic isolates are reflected in the O139 (Bengal) serotype

Data localising protective epitopes to the C-terminus of TcpA (Sun *et al.,* 1990), and subsequently in the PAK and PAO type-4 pili of *P. aeruginosa* (Lee *et al.,* 1994), suggested that biotype specificity and/ or strain-strain variation could be found in the 3' region of *tcpA*. The predominance of the El Tor biotype epidemiologically, both in endemic (Bart *et al.,* 1970; Woodward and Mosley 1971; Gesichter *et al.,* 1973) and non-endemic regions (Desmarchelier *et al.,* 1989), made it the logical source for a probe. PCR primers 601 and 526 (Fig. 2.1; Table 2.1) were therefore used to manufacture a DNA probe specific for the 3' region of *tcpA* from strain H1 (El Tor).

After end-labelling with digoxigenin-UTP (Section 7.1.17), this probe detects a single 5.0 kb fragment in *Xba*I digests of chromosomal DNA from all clinical isolates of O1 (Table 3.2) and O139 (Table 2.2; Figure 2.3) when hybridised and washed at reduced stringency, in 50% formamide at 45°C. These data suggested genetic conservation in the *tcp* region of O139 strains that is in keeping with the known sequence of this region in Classical and El Tor biotypes. It can be seen that O139 isolates are more readily detected in Southern hybridization experiments under these conditions than the classical strain Z17561. Higher stringency washes with the same probe extinguishes reactivity altogether in classical strains (Voss, 1995), but not in O139 strains, suggesting that *tcpA* in these strains is uniformly closer to the El Tor sequence than that of classical *V. cholerae*.

Fig. 2.2: The 5.0 kb *Xba*I fragment containing *tcpA* is conserved in O139 strains

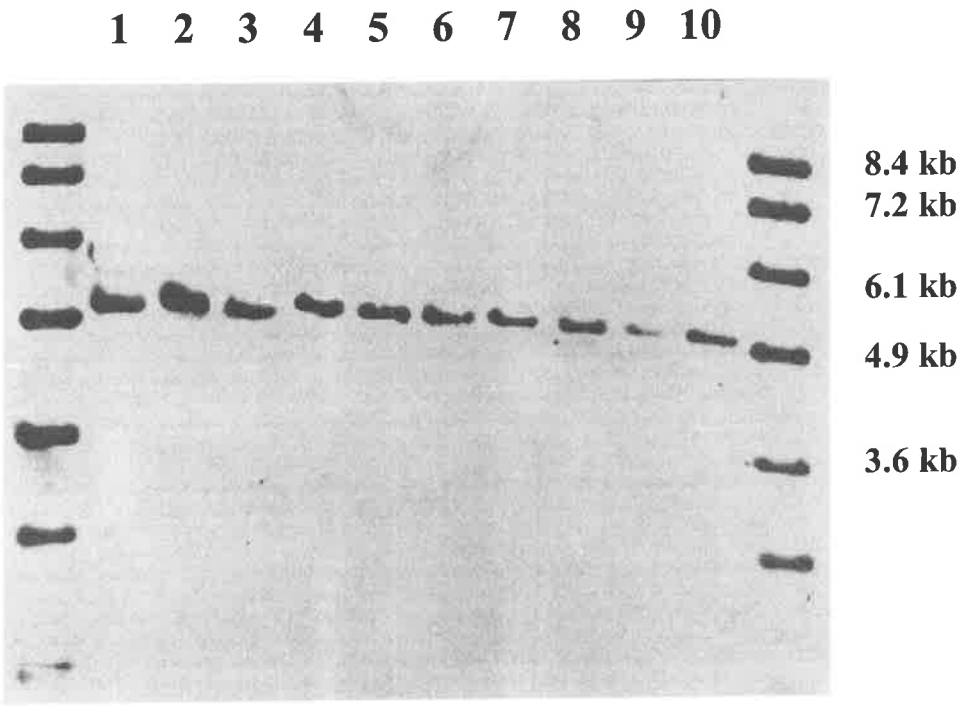


Fig. 2.2 The digoxigenin end-labelled H1 (El Tor) *tcpA* probe (product of primers #601/#256; see Fig. 2.1) detects a 5.0kb *Xba*I fragment in restriction digests of O139 chromosomal DNA. Lanes 1 to 8 are O139 (Bengal) clinical isolates, as listed in Table 3.3, and as follows: Lane 1: AI-1837; 2: AI-1838; 3: AI-1841; 4: AI-1852; 5: AI-1854; 6: AI-1855; 7: AI-4260; 8: AI-4450. Lane 9 contains Z17561 (classical biotype), and Lane 10 contains H1 (El Tor). All tracks were loaded with 10 µg of DNA and electrophoresed in TAE/ agarose (0.8%), before Southern transfer and hybridisation (Section 7.1.17).

2. 4 Restriction Fragment Length Polymorphism of PCR products

Amplification of the *tcpA* gene in the eight O139 isolates produced fragments of the expected size for the oligonucleotide pairings #525 and #568 (950 bp) and #567 and #568 (597 bp). The products of #525 and #568 were therefore subjected to restriction by different enzymes selected on the basis of the known sequences of Z17561 (Classical) and H1 (El Tor) strains (Fig. 2.3).

Fig. 2.3: Restriction fragment length polymorphism of *tcpA* genes in O139

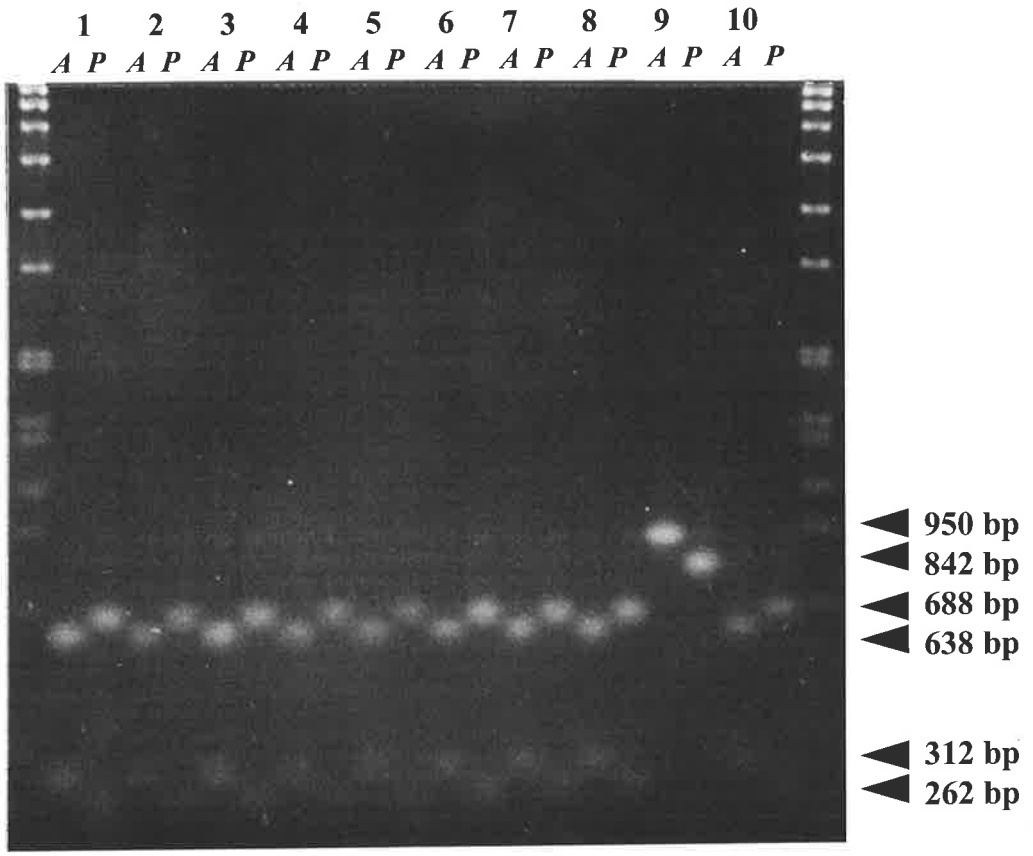


Fig. 2.3 Lane 1 - 8: O139 strains AI-1837, AI-1838, AI-1841, AI-1852, AI-1854, AI-1855, AI-4260, and AI-4450; 9: Z17561 (classical); 10: H1 (El Tor) Paired amplicons of *tcpA* (oligos #525 and #568; Fig. 2.1; Table 2.1) are digested with *Asp*700 (*A*) and *Pst*I (*P*). El Tor *tcpA* is cleaved by *Asp*700 and *Pst*I to

638 +312 bp and 688 + 262 bp, respectively. Classical *tcpA* contains no *Asp*700 site, and is cleaved by *Pst*I to 842 + 108 bp fragments.

Restriction fragment length polymorphisms (RFLP) were observed using *Asp*700 and *Pst*I. These were biotype-specific, with all eight O139 (Bengal) strains clearly identical in RFLP pattern to (El Tor) H1 and distinct from (Classical) Z17561.

2.5 Variable stringency PCR confirms discriminating power of primers

Using primer pairs #525/#568 and #567/#568, it was possible to PCR amplify *tcpA* with high stringency amplification (Section 7.1.13) from all clinical isolates, consistent with existing sequence data for Z17561, O395, and H1 and with the information gleaned from Southern blot analysis of O139 strains (Table 2.2). This technique may be a simple method to identify virulent *V. cholerae* strains in a biotype-specific manner, and therefore have useful applications in direct detection methods in environmental and clinical specimens. In order to further test the discriminatory power of the primer pairs described in Table 2.1, the stringency of PCR amplification was altered by lowering the annealing temperature to 40°C (Section 7.1.13), and tested against a number of O1 and non-O1 isolates (see Table 2.2) that had been identified as 'possible' cholera vibrios by conventional diagnostic methods. Low stringency amplification of *tcpA* from environmental *V. cholerae* isolates with the primer pair #525 and #526 (external to the coding region) produces no distinct product, but amplification of a number of different sized products occurs with the primer pair #567 and #568 (internal to the coding region). This includes products of the correct predicted length in two Brazilian O1 environmental isolates (Table 2.2), but attempts to sequence and subclone these by the

same methods applied to amplification products from clinical isolates were unsuccessful.

Fig. 2.4: Reduced stringency PCR with *tcpA*-specific oligomers

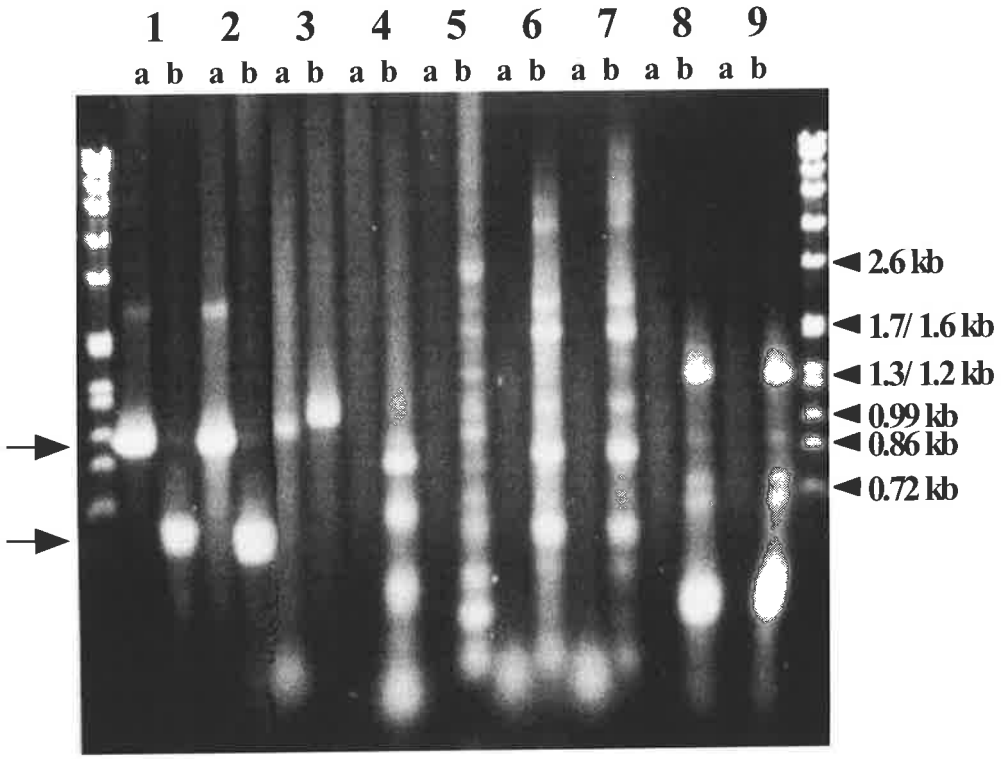


Fig. 2.4 Low stringency PCR amplification with oligonucleotide pairs #525 and #568 (a) and #567 and #568 (b) for each sample, giving products of 950 and 597 bp respectively (arrows, left margin). Lane 1: Z17561; 2: H1; 3: NSW4; 4: N59; 5: N120; 6: 1074/78; 7: 1196/78; 8: NCTC#10885; 9: NCTC#10884. The size marker tracks are in the outside lanes (SPP-1 digested with *Eco*RI), and some are marked in kilobases (kb) for ready reference (right). Close doublets (eg. 1.7/ 1.6 kb) are indicated with a single arrow.

The pattern of multiple bands produced from environmental *V. cholerae* isolates under low stringency PCR even with (internal) primer pair #567 and #568 was easily

distinguishable from the discrete product obtained with clinical isolates, while high temperature cycling gave product only from clinical isolates (Fig. 2.4).

2.6 Variation in the precoding region of tcpA does not explain previously observed variations in TCP and TcpA expression *in vitro*

The complete DNA sequences of the coding and precoding regions of *tcpA* were determined for four classical, six El Tor, and two O139 (Bengal) strains. This was achieved primarily by dye-terminator sequencing of PCR products. Oligonucleotides used have been described in Table 2.1 and Figure 2.1. As an adjunct to this method, subcloning of products allowed dye primer sequencing by M13 forward and reverse primers. The 1.1 kb *Bgl*II-ended product generated by primer pair #525 and #526 was subcloned into the *Bam*HI site of pBluescriptSK (Stratagene). Amplicons obtained with #525 and #568 were also digested with either *Eco*RV (El Tor and O139) or *Dra*I and *Pst*I (classical strains) for subcloning. A minimum of two cloned products were sequenced in both orientations from each strain in this manner, as an adjunct to the data obtained by direct sequencing.

The precoding regions varied little among classical strains and not at all in El Tor strains (Fig. 2.5), although there were regions of significant differences between the two biotypes. Overall, the DNA sequence identity between biotypes in the 264 bp precoding region is 86%. There is a region homologous to a cyclic AMP - cAMP-receptor Protein (cAMP-CRP) binding site with minimal inter-biotype variation, and with 75% identity to the cAMP-CRP consensus binding sequence (Valentin-Hensen *et al.*, 1982, 1991).

Furthermore, the transcription initiation site previously determined by primer extension in classical strains and only recently published (Thomas *et al.,* 1996), is optimally positioned for the function of such a site (Valentin-Hensen, 1991).

Fig. 2.5 Consensus precoding sequences from the Classical and El Tor biotypes

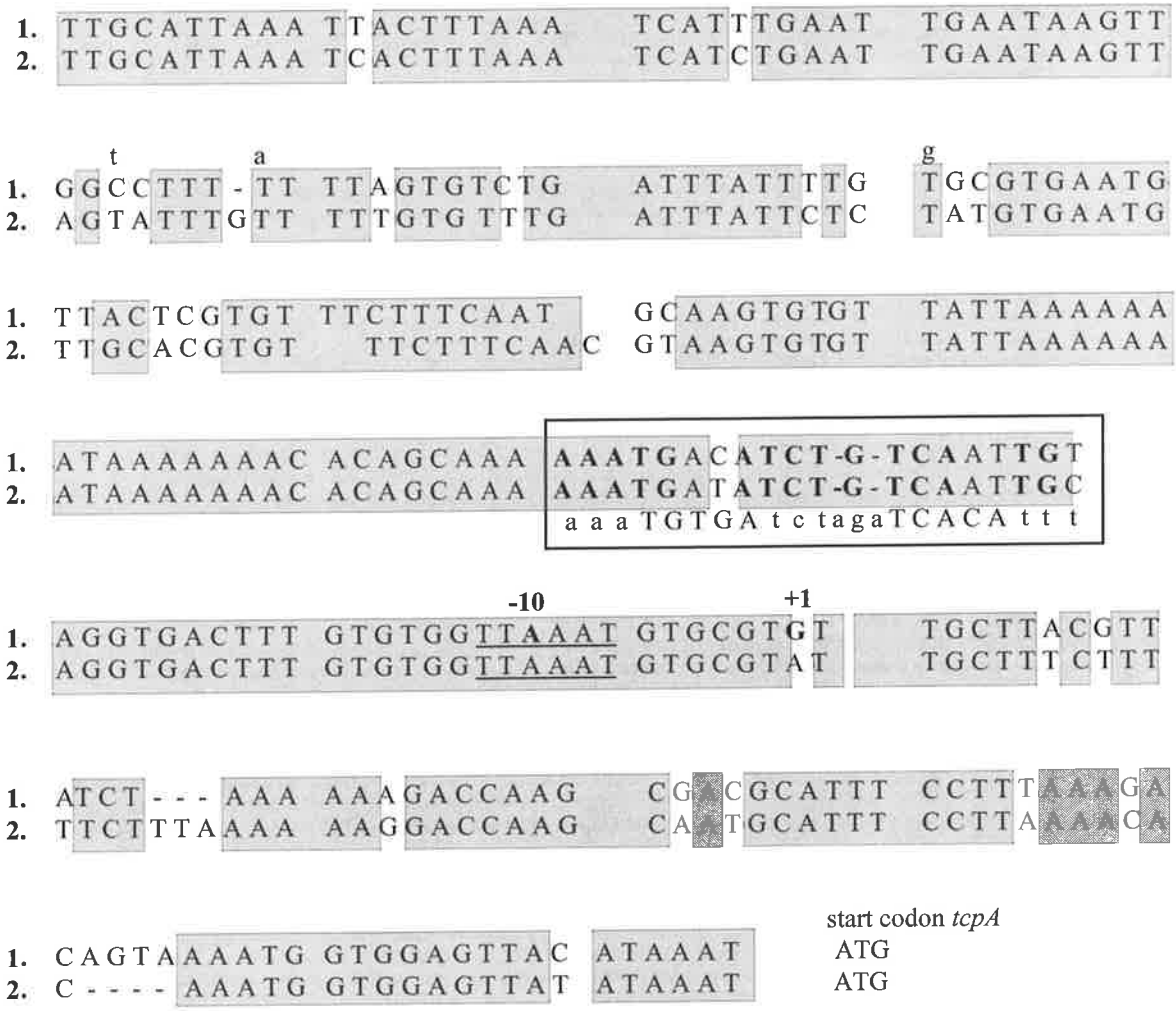


Fig. 2.5 The precoding region prior to *tcpA* is shown as the consensus for three classical (1) and six El Tor strains (2) of *V. cholerae* O1, with identical areas shaded. Two O139 strains were identical to the El Tor sequence (2). Variation found among the classical strains is entered in lower case above the site of variance. The +1 transcriptional start site determined for Z17561 (Thomas *et al.,* 1995) is marked, and the numbering is relative to this position. A putative cAMP-CRP binding site is boxed (large), with an ideal consensus sequence underneath. Highly conserved nucleotides are in upper case and less conserved nucleotides in lower case (Valentin-Hensen *et al.,* 1982, 1991). Matching nucleotides in *V. cholerae* are shown in bold.

2.7 Coding regions reflect strict conservation within biotype; *tcpA* genes from O139 (Bengal) strains are of El Tor type

Comparison of the predicted amino acid sequences of TcpA show strict conservation within biotype. The few DNA sequence variants detected within a biotype did not vary the encoded amino acids, nor select for rare codons of these residues in *V. cholerae* (Stroeher,1992) (Fig. 2.6). Significant amino acid differences between the biotypes were observed, however, with most substitutions occurring in the C-terminal one third of the protein (similarity 68%). Homology across the 42 residue C-terminal region, previously shown to harbour an important epitope or epitopes in classical strains (Sun *et al.,* 1990), is 76% identical at amino acid level, compared with 75% from the end of the highly conserved region at residue 55, and 91% for the mature protein overall. This contrasts with the sequence identity of the hydrophobic N-terminus of the mature protein that extends to the glycine residue at position 55, this region being characteristic of type-4 pilins (Dalrymple and Mattick, 1987), and defining the α–β rolled helical 'backbone' (Parge *et al.,* 1995). The *tcpA* genes of two O139 (Bengal) isolates sequenced are identical to those of El Tor strains.

Fig. 2.6 The amino acid sequence of TcpA is highly conserved and biotype-specific

signal sequence:

```
                -20                 -10
1.  MQLLK QLFKK KFVKE EHDKK TGQEG
2.  MQLLK QLFKK KFVKE EHDKK TGQEG
```

mature protein:

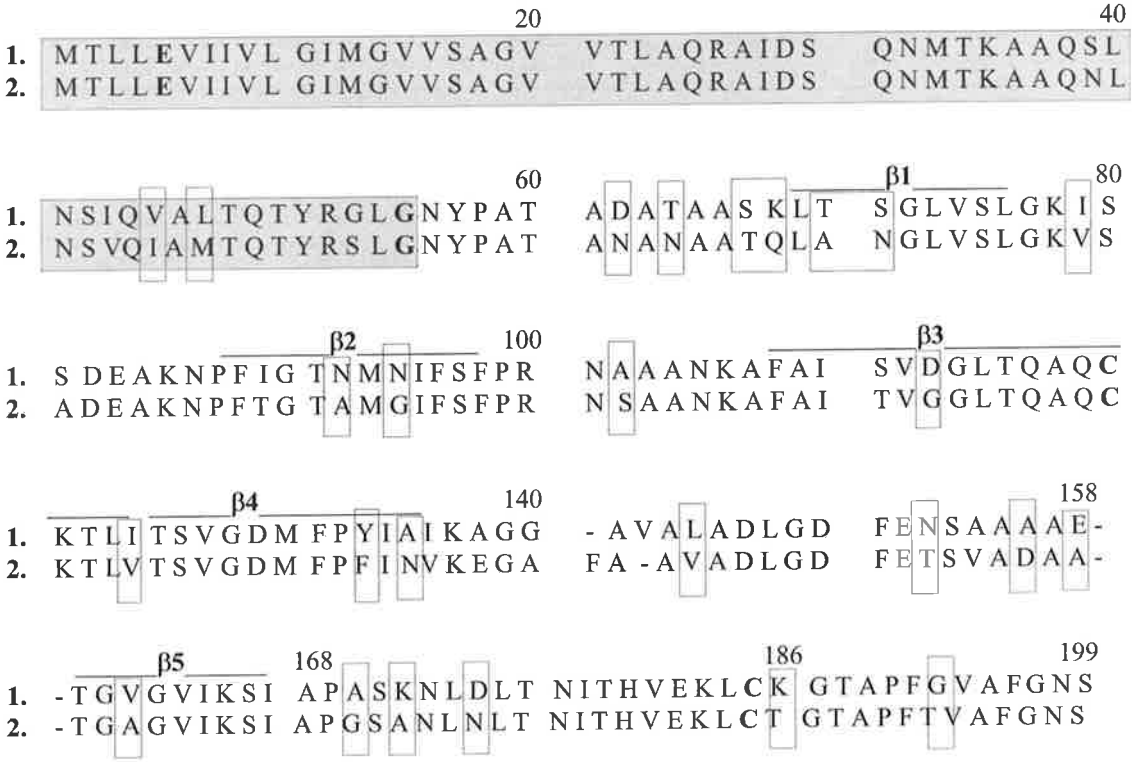


Fig. 2.6 The amino acid sequences predicted from DNA sequences derived for four classical (**1**) and six El Tor strains (**2**) of *V. cholerae* O1, and two strains of O139 (Bengal) serotype, AI-1837 and AI-1855, (identical to the sequence derived for El Tor H1), are presented in standard single letter code. In contrast to Fig. 2.5, all amino acid differences are boxed. The important Gly5, Gly55, Cys120, and Cys186 residues are in bold, and predicted regions of β sheet underlined (see also Fig. 1.11 and Section 1.12.2). It should be noted that the numbering is for the mature protein after cleavage of the 25 amino acid signal sequence, which is shown in the top two lines. The first 39 residues are absolutely conserved, and the hydrophobic backbone (from Met1 to Gly55, shaded; see Section 1.12.1) is only conservatively substituted between biotypes. The sequences were aligned by Clustal V, and spaces are inserted to facilitate this.

2.8 Concluding remarks

There is strict conservation of the *tcpA* precoding regions within the *V. cholerae* O1 biotypes which implies a biotype-specific requirement for this region and supports the notion of conserved regulatory differences affecting TCP exporession between the biotypes. It may be significant that most variation occurs in the AT-rich area downstream from the transcriptional start site identified in classical strains, suggestive of a post-translational regulatory mechanism (Jacques and Dreyfuss 1990). The invariant precoding regions found in El Tor strains which vary in the production of TcpA/TCP *in vitro* suggest regulatory influences from outside this region.

The demonstration of biotype-specific TcpA amino acid sequence data, and evidence adduced that place TcpA from O139 strains within the El Tor TcpA biotype, implies that the inclusion of the two antigenic forms should suffice in O1 and O139 vaccine preparations targeting TcpA. These sequence data also permit the ready development of PCR primers for diagnostic purposes. While the relationship between clinical and environmental isolates of *Vibrio cholerae* remains undefined, simple DNA-based methods such as PCR applied to a virulence gene (*tcpA*) allowed easy discrimination between the clinical isolates tested and a sample of environmental isolates in this study, and is consistent with previous findings (Keasler and Hall, 1993). While the detection of a virulence gene cannot be regarded as proof of pathogenic potential, DNA-based biotyping methods utilizing data such as those presented herein may prove to be valuable epidemiologic tools. Strict conservation in *tcpA* genes make it impossible to

comment on likely essential regions or motifs within TcpA, and denies us of easy targets for mutagenesis. However, significant biotype-specific variation in conserved residues identified in the globular domain after Gly55 (Sections 1.12.2, 1.12.3, and 2.7) may contribute to antigenic differences. Potentially important substitutions can be seen in the region between Gly55 and the first predicted β-strand (β1; refer to Section 1.12.3), inside the β3 strand (residue 113), and especially between β4 and β5 (in the disuphide loop region; refer to Figs 1.11 and 2.6). Mutagenesis studies in these regions may well prove to be rewarding.

The TCP is a potential paradigm not only for type-4 pilin assembly but, because of apparent homologues in the systems (as detailed in Sections 1.11 and 1.12), for other aspects of the main terminal branch of the General Secretory Pathway. Considered as a secreted substrate, the type-4 pilin subunit is a useful model and TcpA is a good fit to the fully-defined structure of *Neisseria gonorrhoeae* MS11 PilE (Parge *et al.,* 1995). Further dissection of the TcpA translocation mechanism, centred on the analysis of genetically defined export defective phenotypes, will form the basis of chapters to follow.

Chapter Three

Outer Membrane Translocation Arrest of the TcpA Pilin Subunit in *rfb* Mutants of *Vibrio cholerae* O1 strain 569B

3.1. Introduction

Early studies in type-4 pilin biogenesis suggested cytoplasmic membrane assembly of pilus (Strom *et al.,* 1987; Dupuy *et al.,* 1991), but the fate of type-4 pilin subunits immediately after processing at the cytoplasmic membrane remains unknown. While the peptidase, its substrates, and a cytoplasmic membrane-associated ATP binding protein are almost universal partners in pilin and non-pilin pathways (Pugsley, 1993a;Hobbs and Mattick, 1993), the nature of other participating elements within the outer membrane is less clear. Members of the GspD superfamily are highly conserved and widely represented, and a representative is essential in the biogenesis of the type-4 pilin of *Pseudomonas aeruginosa* (Martin *et al.,* 1993). In the absence of a *tcp* homologue, one must propose either that a homologue exists outside the operon, that a structurally homologous protein exists which does not share amino acid sequence homology, or that an alternative mechanism or pathway exists for TCP. Potential candidates might be found in the related *eps* transport system for example, which is responsible for export of the co-regulated cholera toxin in *V. cholerae* (Overbye *et al.,* 1993). Within the *tcp* operon itself, *tcpC* and *tcpF* appear to encode, respectively, an outer membrane lipoprotein of unknown function and a putative porin-like protein that could conceivably

participate in the formation of a multimeric channel (Parsot *et al.,* 1991; Ogierman and Manning, 1992; Kaufman *et al.,* 1993).

Misassembly of outer membrane multimers in LPS mutants has been described (Sen and Nikaido, 1991; Laird *et al.,* 1994) and the significance of LPS to other transporter systems emphasized (Wandersman and Letoffé, 1993). Transposon-induced mutagenesis of the putative perosamine biosynthesis genes in *V. cholerae* 569B give rise to a rough phenotype in which the LPS lacks O-antigen (Ward and Manning, 1989), and preliminary observations suggested a TCP export-deficient phenotype in these stains (Stroeher, 1992). The disposition of TcpA pilin subunits in the 569B*rfb* mutant, V663, is examined further during the course of experimental work outlined in this chapter.

3.2. Perosamine biosynthesis mutants in *Vibrio cholerae* O1 strain 569B have a defect in TCP assembly

3.2.1. Previously described *rfb* strains are confirmed in their TCP phenotype

V663 and V669 are rough mutants of *V. cholerae* classical O1 strain 569B, in which Tn*5* (kanamycin resistant) insertions map to the putative perosamine biosynthesis genes of the *rfb* region (Ward and Manning, 1989). Using specific antisera to TcpA and optimal (AKI) growth conditions, no pilin structures could be detected in either V663 or V669 strains by IEM, nor was there any significant binding of immunogold label to the cell surface in grids prepared on several separate occasions (Fig. 3.1).

Fig. 3.1 Transmission EM of 569B and V663: immunogold labelling of TcpA

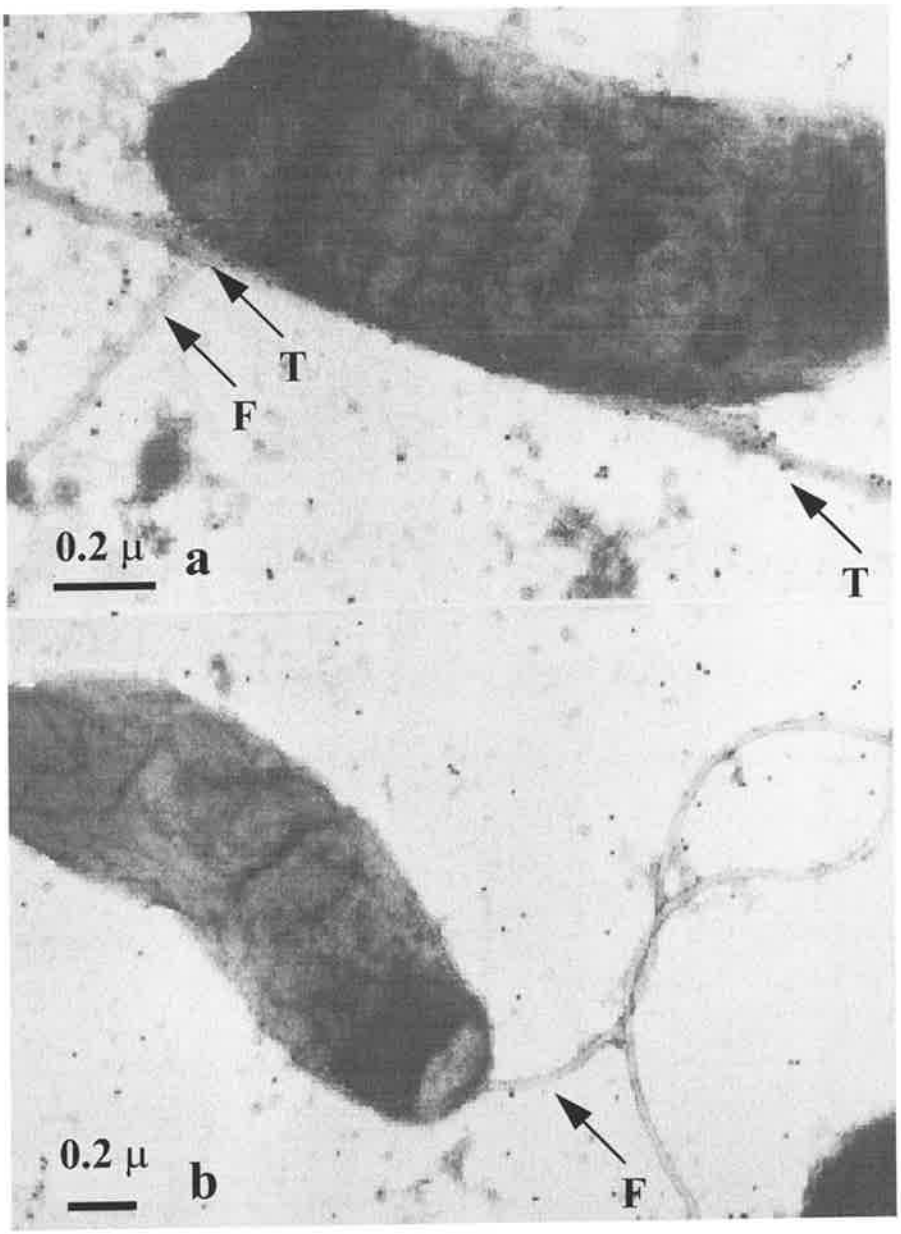


Fig. 3.1 Immunogold labelling in a transmission EM of 569B (panel a) and V663 (panel b) using polyclonal antisera to TcpA. A TCP bundle alonside the *wt* cell in the upper panel is indicated (T) and flagellae (F) are marked for comparison.

3.2.2. Specificity of the phenotype: motility, secretion and viability

Numerous surface-associated and secreted proteins of *V. cholerae* have been implicated in virulence (Manning, 1994), and preliminary observations had suggested that these strains might have a motility defect (Stroeher, 1992). Flagella were of normal morphology (Fig. 3.1), and motility was therefore examined and found to be normal in V663 and V669, in hanging drop preparations and in 0.3% agar overlays. Ctx, DNase and protease secretion were unaffected (Ward, 1990; Stroeher, 1992), as confirmed by GM_1-ELISA (for cholera toxin) and plate assay (for DNase and casein hydrolysis).

In order to examine the possibility that reduced viability in culture might contribute to phenotype differences between strains, comparisons of the viability (by serial dilution and counting; refer to Section 7.2.1) of early (4 hr) and late (16 hr) broth cultures were made. Estimates of viable counts, comparing V663 with 569B, are presented in Table 3.1. These show that doubling times of V663 and 569B are comparable, but longevity is greatly reduced in the *rfb* mutant V663. It was determined therefore that if populations of viable TCP-expressing cells were to be compared between strains, fractionation experiments should be done with early (4 hour) cultures. It also suggests a potential explanation for the previous impression of reduced motility in agar overlay assays, if cultures of *rfb* mutant strains were less viable than control cultures of isogenic smooth parent strains.

Table 3.1 Viable counts of *rfb* mutants

	A_{550}/ cell count (% viable): 4 hrs	**A_{550}/ cell count (% viable): 16 hrs**
569B (*wt* parent)	0.7/ 1.2 x 10^9 (100%)	2.39/ 1.5 x 10^{10} (73%)
V663 (569B*rfb*)	0.8/ 1.2 x 10^9 (76%)	2.71/ 7 x 10^8 (5%)

Table 3.1 Absorbance readings at 550nm (A_{550}) in AKI broth at 30°C and viable counts (Luria plates) give a guide to viability and growth at 4 and 16 hours under *tcp*-inducing conditions. Averages of two parallel observations are given for each strain. A_{550} is presented, followed by the calculated cell count (Neubauer chamber) after the backslash. The bracketed figure is the percentage of the counted cells (Neubauer chamber) which were viable, derived by colony counting after serial dilution onto AKI plates.

3.2.3 Complementation of the *rfb* defect restores LPS and TCP phenotypes

In oredre to confirm that the TCP expression defect in the described *rfb* strains was due to the mutation in LPS biosynthesis genes, complementation of the V663 *rfb* defect *in trans* had to be performed, and was achieved with some difficulty. pRMB2 is a pBR322-based plasmid which contains the 20.5 kb *Sac*I fragment of the *rfb* region of O17 (El Tor Inaba) depicted below (Fig. 3.2). This converts *E. coli* K12 to an Inaba serotype (Morona *et al.,* 1991), but had not previously been introduced into *V. cholerae*. Several attempts at electroporation of pRMB2 into V663 and 569B resulted in a few tetracycline resistant colonies that grew poorly even in subsequent broth culture.

Fig. 3.2 The 20.5 kb *Sac*I *rfb* region of pRMB2

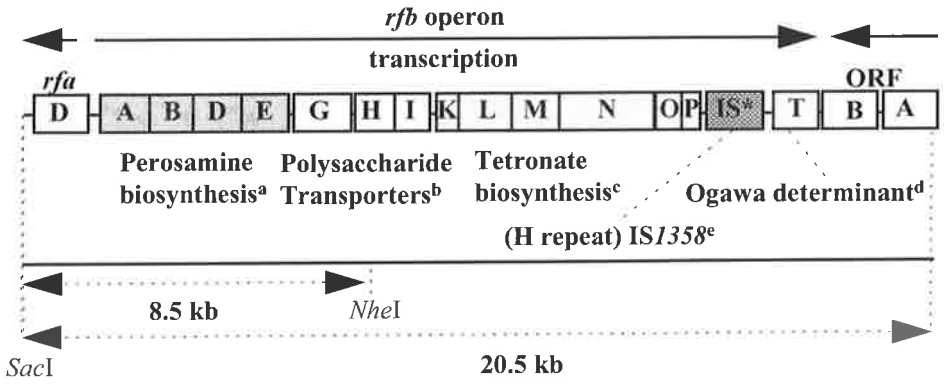


Fig. 3.2 The Tn*5* insertion in V663 maps to the perosamine biosynthesis genes (Ward, 1990). pRMB2 contains the 20.5 kb *Sac*I region from the *Vibrio cholerae* O17 (Inaba) *rfb* operon in a pBR322-derived plasmid (Morona *et al.*, 1991). pPM4130 is the 8.5 kb *Sac*I-*Nhe*I fragment, subcloned into pCACtus (*Sac*I-*Xba*I). Direction of transcription and putative functions are indicated (a-e: Stroeher *et al.*, 1995, Manning *et al.*, 1994, Morona *et al.*, 1995, Stroeher *et al.*, 1994, 1995). The incomplete insertion sequence characteristic of O1 strains, previously known as *rfbQRS* (Stroeher *et al.*, 1994; Manning *et al.*, 1994) is indicated. Nomenclature is *rfbA-P, T-V,* except for *rfaD.*

The apparent translational coupling of the operon meant that simple complementation of the perosamine biosynthesis gene cluster was unlikely to be effective. An 8.5 kb *Sac*I-*Nhe*I fragment of the *rfb* region (spanning the transposon insertion sites), cloned into the temperature-sensitive 'suicide' vector pCACtus to create pPM4130, failed to seroconvert V663 when introduced *in trans* at 30°C, and attempts to force homologous recombination (Section 7.1.12) yielded no seroconvertants among 600 colonies screened. The pCACtus vehicle was chosen for its low copy number (pSC101 replicon: one to six copies per cell) and the ultimate option of recombination into the chromosome (Clark *et al.*, in press). The entire *Sac*I region was therefore cloned into pCACtus to produce pPM4130. V663[pPM4130] has a reduced growth rate compared with either

V663 or 569B, the A_{550} reaching only 0.4 - 0.6 at 4 hours, and less than 2.0 at 16 hours with a viable count at that point of 37 % (see also Table 3.1). The higher copy number of the (pBR322-based) pRMB2 plasmid (Morona *et al.,* 1991) and the requirement for growth in tetracycline, which is poorly tolerated even at 4 μg/ml in *V. cholerae* (personal observations), may explain the toxicity of pRMB2 in V663.

V663 harbouring this large plasmid exhibited Inaba-specific agglutination and a normal LPS pattern (Fig. 3.3), and this was accompanied by the return of TCP bundles in IEM preparations under appropriate culture conditions. The O1 Inaba serotype, as well as the typical long-chain LPS pattern (Fig. 3.3), is also conferred upon *E. coli* DH5α by pPM4130. Interestingly however, pPM4130 had no effect on the phenotype of either a *wt* O139 *Vibrio cholerae* strain (AI-1837) or a transposon-mediated rough mutant of AI-1837 (Stroeher *et al.,* 1996) as judged by slide agglutination with an Inaba-specific antiserum.

Fig. 3.3 Complementation of the *rfb* defect in V663

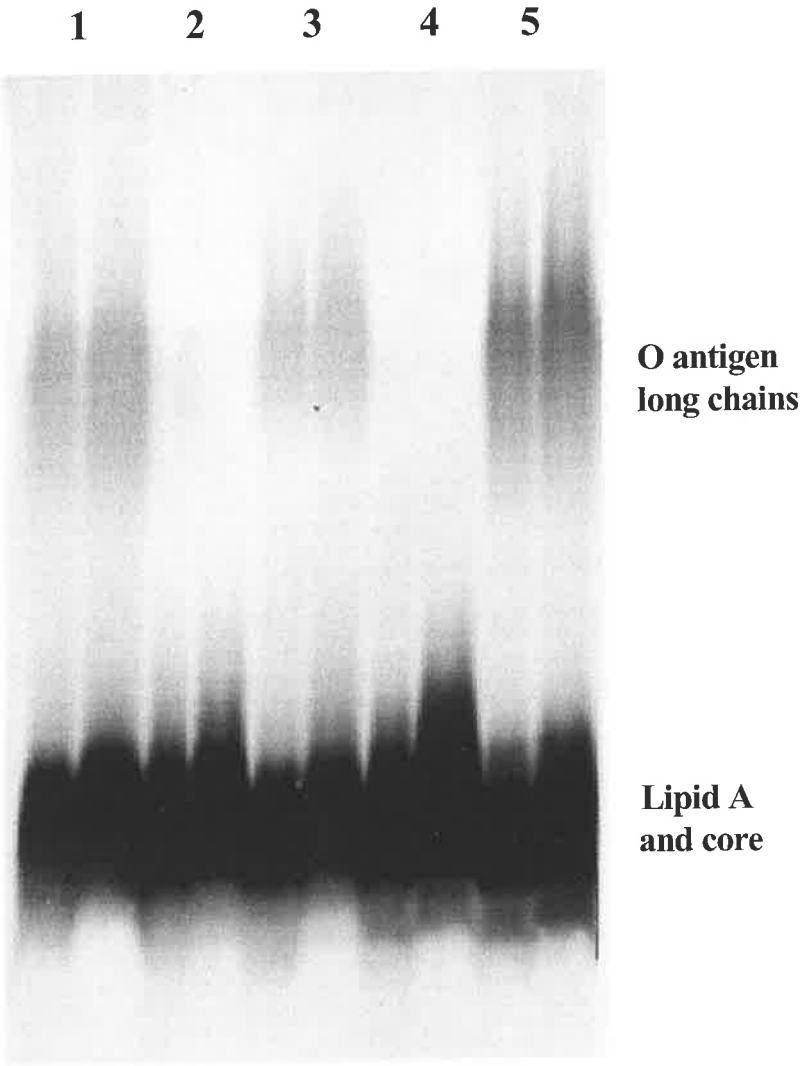


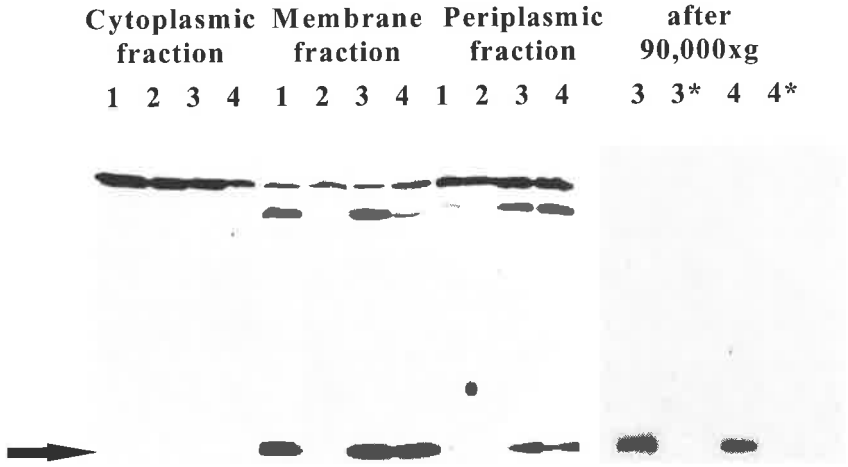
Fig. 3.3 Paired samples of whole cells were subjected to SDS-15% PAGE after Proteinase K treatment and boiling in sample buffer, with the first lane of each pair loaded containing $^1/_3$ the amount of the second lane. 1: 569B *wt*; 2: V663 (569B*rfb*); 3: V663[pPM4130]; 4: *E. coli* DH5α; 5: DH5α[pPM4130]. The *rfb* region on plasmid pPM4130 restores O-antigen long chains to V663, as well as to *E. coli* DH5α

3.3 TcpA is arrested at the level of the outer membrane

3.3.1 A periplasmic high-density TcpA pool in routine physical fractionation

Once it was established that the TCP defect specifically related to the LPS-biogenesis pathway mutation, it was necessary to explore whether TCP or TcpA was actually present and if so, where in the cell it was arrested in export. In order to conduct simple subcellular fractionations, cells were grown under TCP-inducing conditions, and then washed and resuspended in Tris/ EDTA (10mM)/ sucrose with lysozyme to release the periplasmic contents (Section 7.2.7). Whole cells and sphaeroplasts pelleted at 7000xg were separated from supernatant, which was taken as representative of the periplasmic fraction, and pelleted material was lysed in a French pressure cell. After another 7000xg clearing spin to remove unlysed cells, the membrane fraction was collected by centrifugation at 90,000xg for 1 hour at 4°C. The appearance of apparent periplasmic accumulation of TcpA in V663 and V669 prompted the question of whether this represented contamination from lysed cells (in view of the viability studies, summarised in Table 3.1). In fact, the periplasmic fractions were cleared of immunoreactive TcpA by centrifugation at forces required to pellet membranes but insufficient to pellet free pilin (Fig. 3.4).

Fig. 3.4 Immunoblot of TcpA in *rfb* mutants V663 and V669



(ii) TcpA immunoblot

Fig. 3.4 TcpA subunit is detected by immunoblot in periplasmic fractions of V663 and V669. Lane 1: 569B; 2: O17 (TcpA-negative control); 3: V663; 4: V669. The right hand panel shows periplasmic fractions after 90,000xg 60 mins. TcpA is found only in the pellets (3 and 4) of V663 and V669 respectively, but absent from the supernatant (3* and 4*) resuspended in equal volume.

The fact that TcpA-containing elements can be removed from the periplasmic fractions of V663 and V669 at 90,000xg might thus be explained simply by contamination of the periplasmic fraction with membrane material due to early lysis in *rfb* mutants, but nevertheless prompted further investigations.

3.3.2 Anomolous distribution of TcpA subunit in floatation gradients.

In order to better define the subcellular distribution of TcpA, whole cell lysates were underloaded into a sucrose floatation gradient. The location of major outer membrane

proteins and the cytoplasmic membrane-associated TcpT (by immunoblot) are indicated for comparison with the sucrose density of fractions (Fig. 3.5).

Figure 3. 5 TcpA floatation in sucrose density gradients

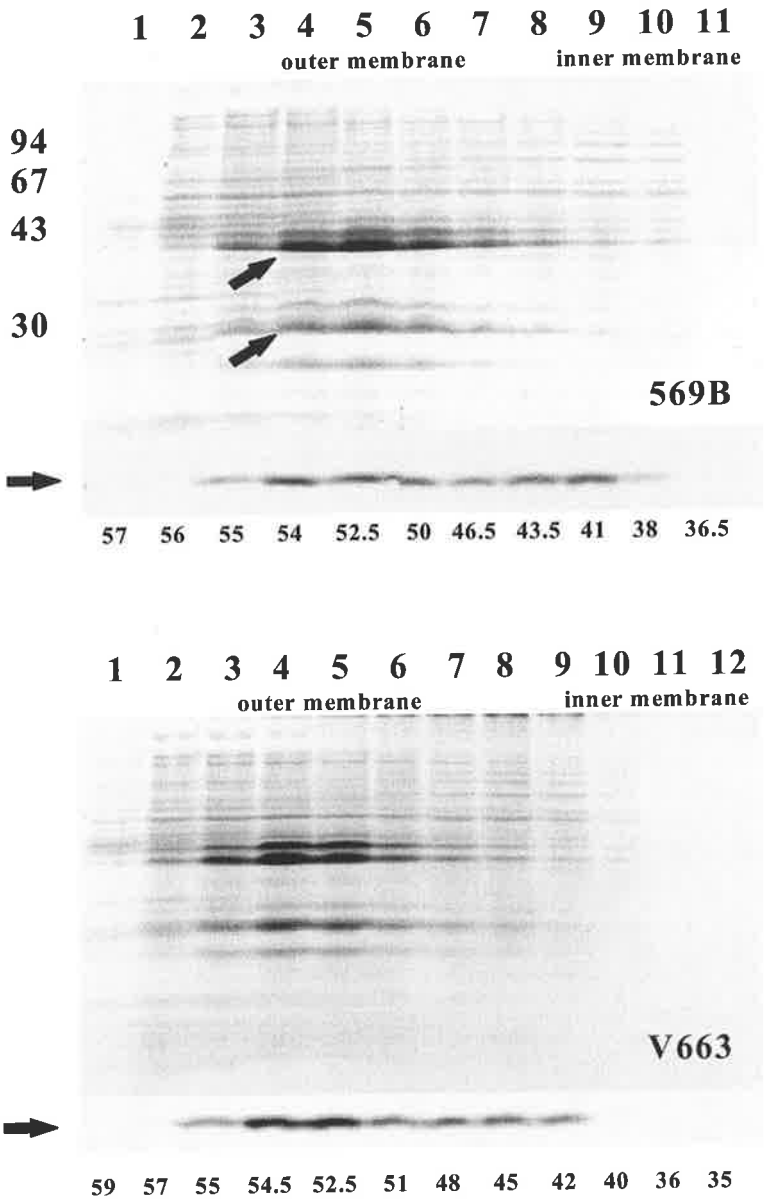


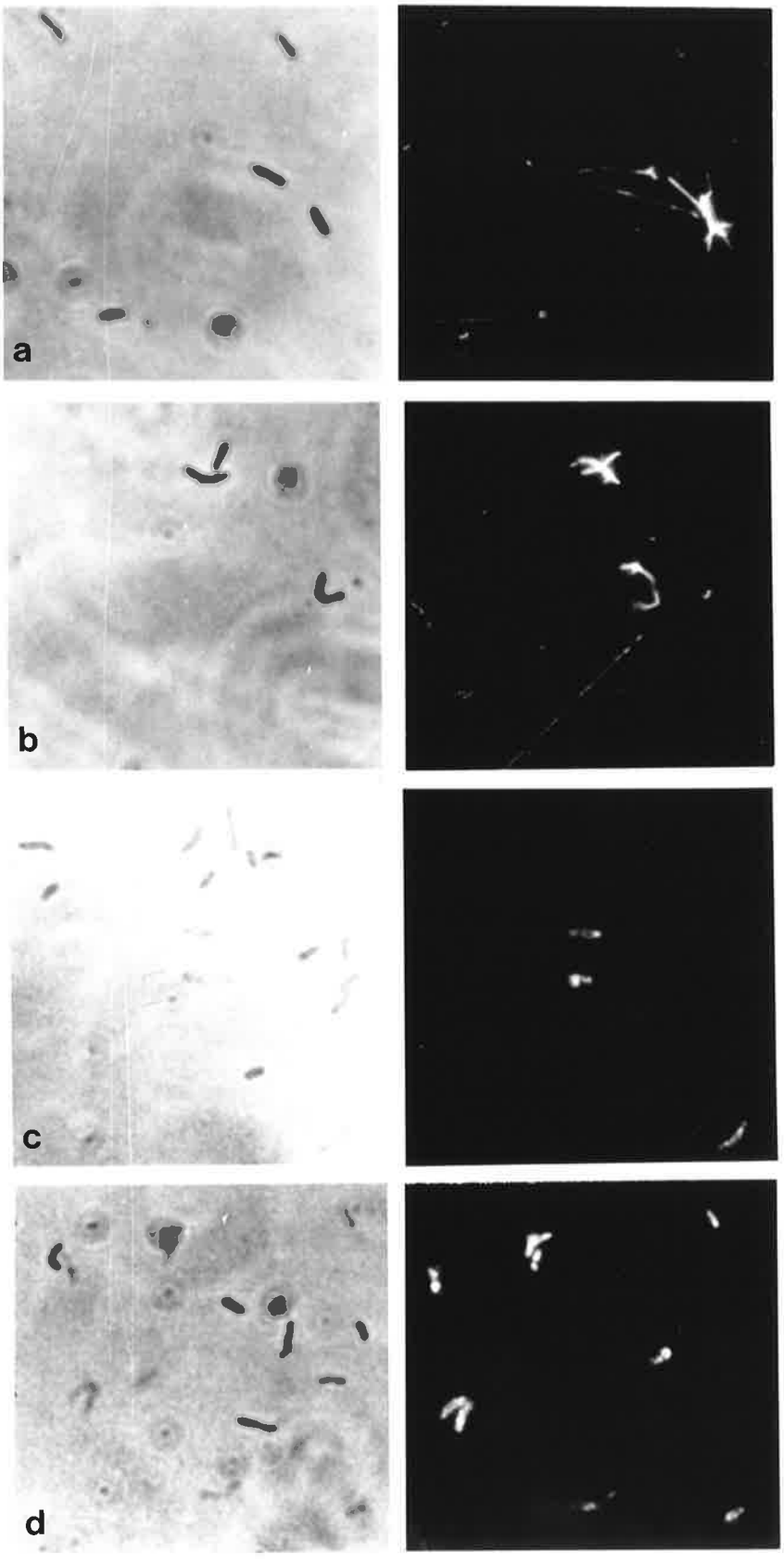
Fig. 3.5 Distribution of the TcpA subunit in sucrose floatation gradients. TcpA immunoblots (horizontal arrows) are presented under Coomassie-stained SDS-PAGE in each panel. Equal volumes of each fraction were loaded, and sucrose densities (% w/v) are given under each lane. Molecular weights (in kDa, along the left margin) and the location of the 42 and 25 kDa outer membrane proteins of 569B (Sengupta *et al.*, 1992) are indicated (arrows). TcpT, a cytoplasmic membrane-associated proteins (see Chapter 5), was detected in the last three fractions of both panels.

There is some predominance in outer membrane protein-containing fractions of V663, but the TcpA pilin subunit is seen to distribute widely throughout the gradients generally, nearly identically so for the *wt* parent and the isogenic *rfb* mutant.

3.3.3. EDTA pretreatment promotes fluorescent labelling of TcpA in V663.

In order to determine whether there was any surface or outer membrane presentation of TcpA, a monoclonal antibody specific for the pilin subunit (TcpA) was used in immunofluorescence (IF) studies. V663 (*rfb*::Tn*5*), and the isogenic parent strain 569B, were grown under TCP-inducing conditions, and prepared identically for IF labelling of whole cells either with or without prior exposure to EDTA. The cells were examined by phase contrast in each field, so that matching fluorescent cells were clearly identified. Three representative fields were examined for each specimen, and results were consistent on separate occasions. Approximately 70 - 90 % of V663 whole cells from 4 hour cultures were labelled after EDTA treatment, compared with only 10 - 30% of untreated V663 (Fig. 3.6). In the *wt* parent however, only external pilin bundles were labelled in each case. While release of cytoplasmic contents cannot be excluded, it is clear that TcpA can be readily detected in cells in which surface labelling is ordinarily absent by pretreatment with an agent that is known to disrupt the outer membrane.

Fig. 3.6 (overleaf) figure legend. Phase contrast (on the left) and corresponding immuno-fluorescent images (on the right) are presented for 569B and V663. Panels a and b show 569B without (a) and with (b) EDTA pretreatment. Panels c and d show V663 preparations without (c) and with (d) EDTA pretreatment. TcpA is detected with a monoclonal antibody, Tc20.2, and labelled with Texas Red-conjugated goat anti-mouse antisera.


a
b
c
d

3.3.4. Outer membrane blebs contain TcpA in both *wt* and *rfb* strains.

Conventional fractionation techniques had so far proved inconclusive, and in order to further determine whether TcpA could truly be found in a fraction highly enriched in material from the outer membrane, whole cells of 569B and V663 were incubated in LiAc/ LiCl/ EDTA as previously described (Pannekoek *et al.,* 1992). This method induces blebbing of the outer membrane and allows collection of a small amount of material predominantly from the outer membrane. As expected (from the published method), approximately 1% of total protein was harvested in this manner from both 569B and V663, and samples were loaded with equal amounts of protein for TcpA immunoblotting. The TcpA subunit was detected in membrane blebs harvested from V663 in proportions at least equal to its representation in whole cell fractions, as judged by immunoblotting and development with the NBT-BCIP colourimetric method (Fig. 3.7, overleaf). This experiment tells us that TcpA can be released with material from these cells that is not pelleted at 20,000xg for 30 minutes, and concords with IF experiments which suggests a periplasmic or outer membrane location. However, anomolous floatation in density gradients and the hydrophobic nature of the bundle-forming pilin meant that a weakly associated inner membrane or periplasmic pool fractionating non-specifically with the outer membrane could not be excluded by these data alone.

Fig. 3.7 TcpA is present in V663 outer membrane blebs

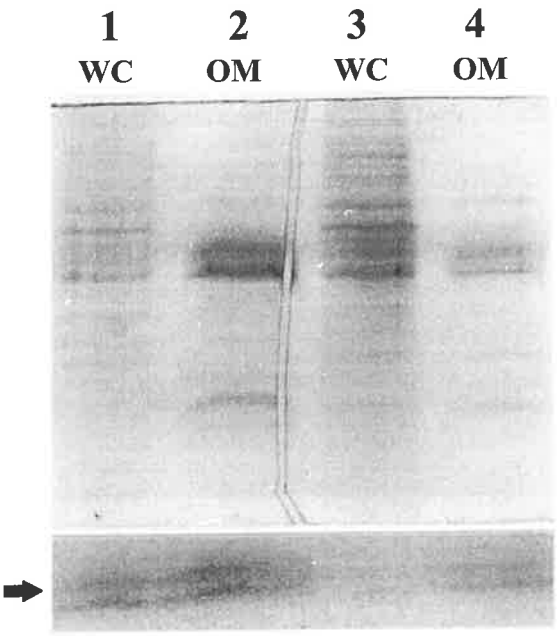


Fig. 3.7 LiAc/ LiCl/ EDTA-extracted membrane material contains TcpA in V663. Coomassie-stained SDS-PAGE, and the corresponding TcpA immunoblot (arrow), of 20,000xg pellets from 569B and V663 (whole cells, WC) and 100,000xg pellets (outer membrane blebs, OM) are shown.

3.3.5. TcpA localization in ultrathin cryosections of *rfb* mutants.

Much of the data implying that TcpA was present in the outer membrane of V663 is indirect, and floatation gradients (Fig. 3.5) had failed to clearly demonstrate the subcellular location of TcpA. Cells taken direct from culture and immediately plasmolysed in PBS/ sucrose, before ultrathin sectioning at -100°C, suffer minimal disruption to native structures and antigens, can be readily labelled with a monoclonal antibody, and are relatively free of fixation artefact (Geuze and Slot, 1980). V663 cells grown as described for TCP induction were therefore prepared, and 800μm sections incubated with the TcpA-specific monoclonal antibody, and gold-labelled (Fig. 3.8).

Fig. 3.8 Ultrathin cryosections of V663 with a monoclonal antibody to TcpA

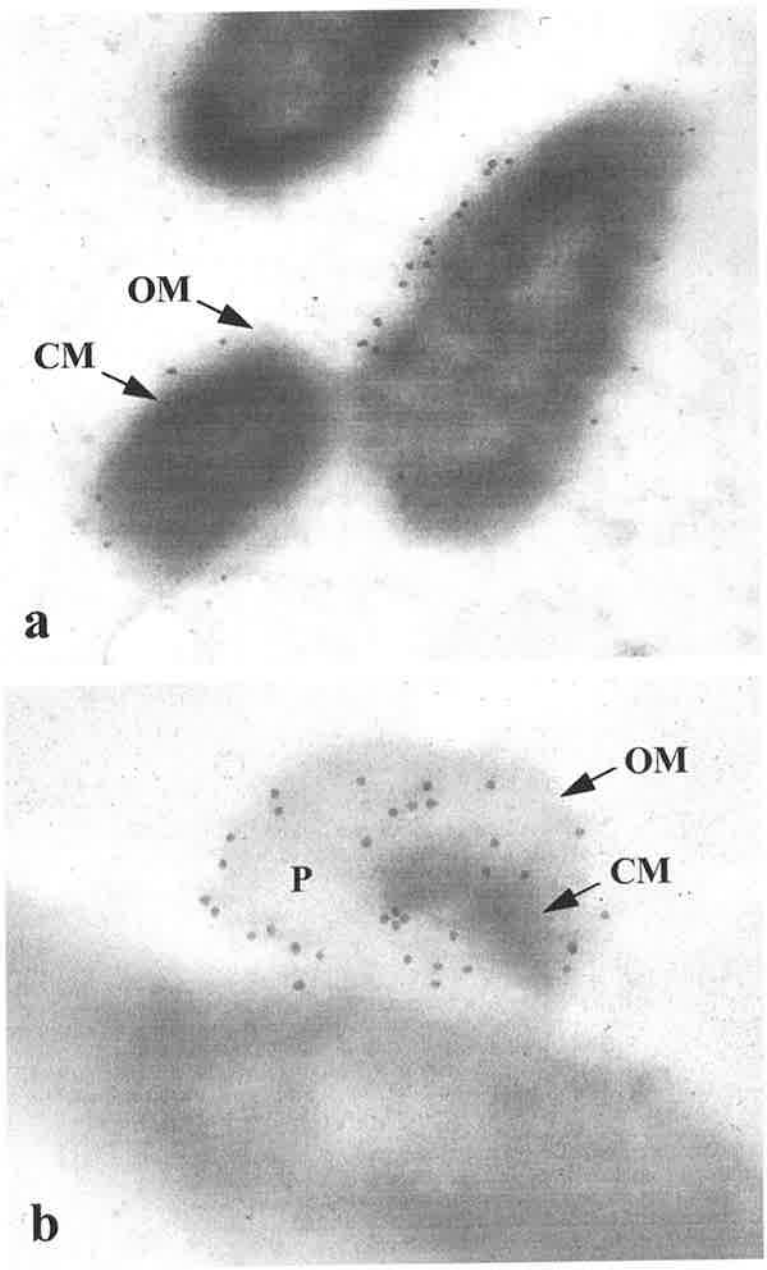


Fig. 3.8 Ultrathin cryosections of plasmolysed V663 reveal immunogold labelling of TcpA in both the cytoplasmic (CM) and outer membranes (OM), in panels a and b. A section apparently through the longtitudinal pole of a cell (panel b) shows gold particles in the periplasmic space (P). Bound Tc20.2 monoclonal antibodies were detected by goat-antimouse antibodies conjugated to 10nm gold particles.

3.4 Subunit disposition in the *rfb* mutant V663

3.4.1. TcpA subunit interactions within *wt* and *rfb* strains.

In view of the data demonstrating that TcpA could be found associated with the outer membrane in V663 but was not externally presented, it was of interest to determine the influence of this apparent export arrest on the physical characteristics and interactions of the TcpA subunit. Membrane fractions were treated with a 1.0M NaCl and a chaotropic/ denaturing agent (6.0M urea), and with detergents (2% Triton X-100 and 1% sarkosyl, both in PBS pH7.4 with no added $MgCl_2$ or EDTA) (Fig. 3.9).

Fig. 3.9 Salt and detergent extraction characteristics of TcpA in 569B and V663

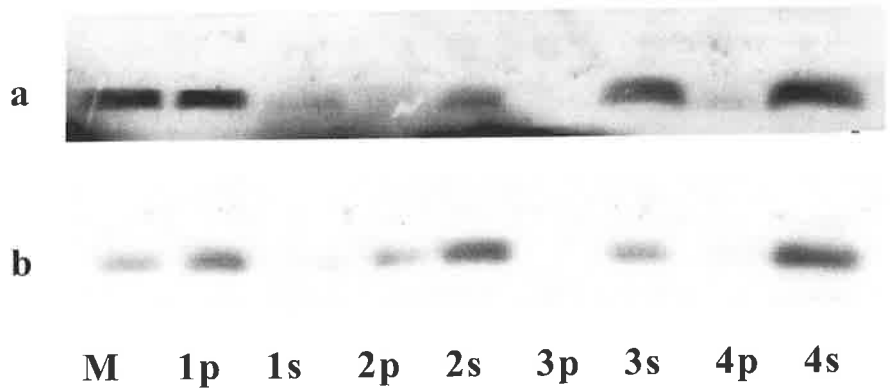


Fig. 3.9 TcpA in membrane extracts from 569B (panel a) and V663 (panel b). Paired samples of pellets (p) and supernatants (s) were derived after the various extractions (listed below) by centrifugation at 90,000xg for one hour. Pellets were resuspended in the original volume, equal to that of supernatant. Samples were subjected to SDS-PAGE, before immunoblotting with rabbit α-569B-165 polyclonal antiserum to TcpA (Sharma *et al.*, 1989). Lanes contain untreated membrane (M), or membrane pellet after treatment with NaCl 1.0M, 30mins on ice (1), Urea 6.0M, 30 mins 30°C (2), 2% Triton X-100, 30 mins 30°C (3), or 1% sodium lauryl sarcosinate (Sarkosyl), 30 mins 30°C (4).

TcpA could not be solubilised by the action of NaCl 1.0M alone in either case, but the capacity of high molarity urea to denature proteins and disaggregate complexes may be responsible for its ability to solubilise TcpA. The identical behaviour of TcpA in V663 membrane fractions, to that in TCP which was brought down with the same fraction in 569B, suggests that subunit-subunit interactions rather than peripheral membrane associations determine the behaviour of TcpA under these conditions.

3.4.2 Identical near-neighbour relationships of subunits in *wt* and *rfb* strains.

Membrane-permeable (Dithiobis succinimidyl propionate, DSP) and watersoluble (3,3'-diothiobis sulphonyl succinimidyl propionate, DTSSP) crosslinkers with reducible disulfide bonds were next used to explore near-neighbour relationships of TcpA subunits. Whole cells and gradient fractions from regions corresponding to inner and outer membranes were cross-linked in order to detect differences in multimerisation of TcpA. These agents span up to 11Å between crosslinkable residues with reactive amine groups (eg. lysyl and aspartyl), and gave reproducible and consistent results in a range of concentrations. An identical pattern was obtained in all specimens in which cross-links were not reduced by the addition of β-mercaptoethanol to sample buffer, in whole cell or density gradient fractions of V663 or 569B. TcpA migrated at the same molecular weight in reducing and non-reducing conditions in all samples, and there were no multimers evident in SDS-PAGE in the absence of cross-linker (Fig. 3.10).

Fig. 3.10 Chemical cross-linking of TcpA in 569B and V663

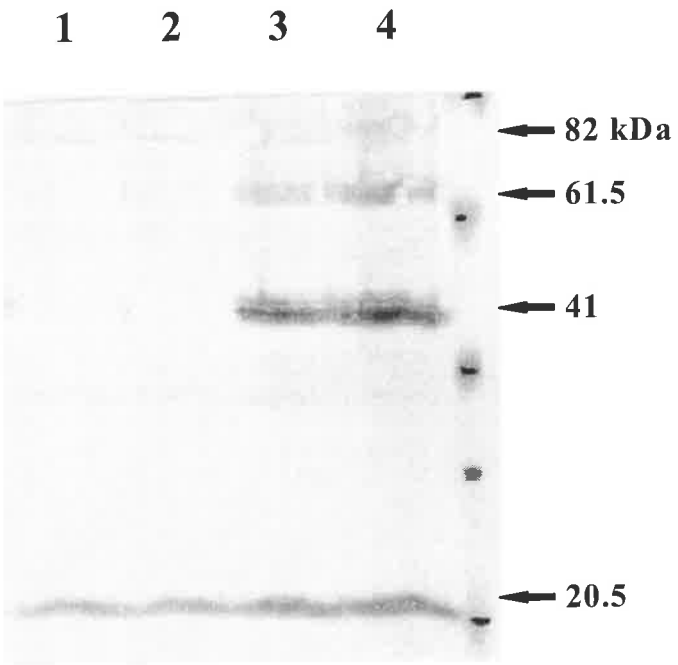


Fig. 3.10 Chemical crosslinking of the TcpA subunit in V663 is indistinguishable from that of TcpA subunit within *wt* TCP. DSP-treated whole cells of 569B (lanes 1 and 3) and V663 (lanes 2 and 4) were run in both reducing (lanes 1 and 2) and non-reducing (lanes 3 and 4) conditions. Molecular sizes are indicated in kDa.

3.4.3. Protease accessibility and haemagglutination characteristics in translocation - arrested subunits suggest a mature secondary structure.

Whole cells prepared as for IF, but without fixation, were exposed to proteinase K (10, 50, and 100 μg/ml) and trypsin (100 and 500 μg/ml) in an attempt to probe differences in protease accessibility in the presence and absence of EDTA. TcpA in both 569B and V663 was degraded no more readily than any of the major outer membrane proteins at high concentrations of proteinase K, consistent with the previously demonstrated

incomplete susceptibility of TcpA to proteases (Sun *et al.,* 1991), and no differently in the presence of EDTA pretreatment and washing, as for IF samples. Since identically prepared EDTA-pretreated cells can be labelled by monospecific antisera to TcpA in IF experiments, it is unlikely that accessibility to antigen explains this result. This suggests that translocation-arrested TcpA subunit in V663 is no more susceptible to these proteases under the conditions described than TcpA within the assembled pilus of the *wt* isogenic parent strain 569B.

Concentrated whole cell lysates of 569B and V663 were then tested for fucose-resistant haemagglutination of fresh murine (adult Balb/c.) red cells, by a slight modification of the method of Jones and Freter (1976) as described for TCP (Taylor *et al.,* 1987). While this method is relatively insensitive (requiring ca. 10^9 cfu/ml of *wt* 569B to cross-link 0.5% red cells in PBS or in the buffer described in 2 hours at room temperature), it discriminated clearly between TcpA-negative and TcpA-positive lysates. Assays were repeated on several occasions, and TcpA expression confirmed by immunoblot. Unmodified TcpA released from V663 by mechanical lysis alone is competent for fucose-resistant haemagglutination (Table 3.2). Significant reduction of titres by specific monoclonal antibody confirms that this is a TcpA-specific phenomenon, as previously demonstrated (Taylor *et al.,* 1987; Peek and Taylor, 1992). Incomplete blocking by this monoclonal may be explained by the large amounts of antigen present, since greater than 1:10 dilution gives poor labelling in IEM studies (data not shown; G. Jonson, pers. comm.).

Table 3.2 Fucose-resistant agglutination of murine red blood cells

	strain (growth conditions)			
	569B (AKI)	569B (NB)	V663 (AKI)	V663 (NB)
whole cells	**32, 64, 64 (53)**	**2, 4, 4 (3.3)**	**2, 4, 2 (2.7)**	**2, 2, 2 (2)**
lysates	**32, 64, 64 (53)**	**nd**	**32, 64, 32 (42.7)**	**nd**
`lysates + mab	**8, 32, 16 (18.7)**	**nd**	**8, 8, 16 (10.7)**	**nd**

Table 3.2. Agglutination titres for murine (Balb-c) red blood cells. Whole cells and lysates were adjusted to ca. 2 x 10^{11} cfu/ml in the starting material. Cultures were grown under optimal *tcp*-inducing conditions (AKI) or in non-inducing conditions (NB), as indicated.The results of three experiments are recorded as reciprocals of the highest dilution in which a mat of red cells, rather than a 'button' of non-crosslinked cells, was visible at the bottom of the wells. The arithmetic mean appears in brackets. Serial dilutions are twofold, and are recorded as the final dilution (first well is therefore a 1:2 dilution, and recorded as 2). The monoclonal Tc20.2 (mab) is used at a final dilution of 1:10. Cell numbers were estimated in a counting chamber before lysis. Total protein content was also calculated (BCA method) to confirm equivalence of final samples before use. The density of undiluted lysates and whole cells seemed to non-specifically prevent 'buttoning' of added red cells, and may explain the positive results at final dilutions of 1:2. 569B (and V663) produces some TcpA at 37°C (not shown).

3.5. Potential outer membrane elements in the Tcp system

3.5.1. Possible toxicity of the *tcpF* gene product in *Vibrio cholerae*

Early complementation studies of mutations in the *tcpT* gene (following chapters) involved attempts to introduce fragments containing the translationally linked region encoding *tcpT, E,* and *F* on multicopy vectors (Section 5.2). The *tcpF* gene was well tolerated in *E. coli* DH5α in high copy vectors (pPM4103 and pPM4107, detailed later in Fig. 5.1) under the control of the 'leaky' *lac* promoter (which is derepressed in *V. cholerae*). However, it could only be introduced into *V. cholerae* under the control of the tightly repressed arabinose-controlled pBAD promoter or the uninduced T7 promoter. Simple cutdowns which deleted half of the *tcpF* gene from the end of the *Bgl*II fragment permitted easy introduction of the otherwise 'toxic' *tcpF*-bearing plasmids.

Since *tcpF* was tolerated in the (uninduced) T7 orientation, it was decided to test the consequences of overexpression by introducing the T7 plasmid, pGP1-2, into a *V. cholerae* strain. Difficulties were experienced introducing the plasmid into 569B and Z17561, and because the *tcp* operon could be more reliably turned tightly 'off' under 'non-inducing' conditions, pGP1-2 was introduced into an attenuated El Tor strain, JBK70. This strain is derived from N16961, which is known to assemble TCP when grown in AKI medium with CO_2 (Voss and Attridge, 1993; Section 7.2.2), and has been employed in this manner previously (Kaufman *et al.,* 1993). JBK70[pGP1-2] carrying

plasmid pPM4201 (*tcpTEF*) was grown either in Difco nutrient broth at 30°C, or under the *tcp*-inducing conditions of AKI broth at 30°C 5% CO_2 (Voss and Attridge, 1993), before induction of T7 polymerase (Fig. 3.11).

Fig. 3.11 Pulse-labelling of TcpF in *Vibrio cholerae* JBK70

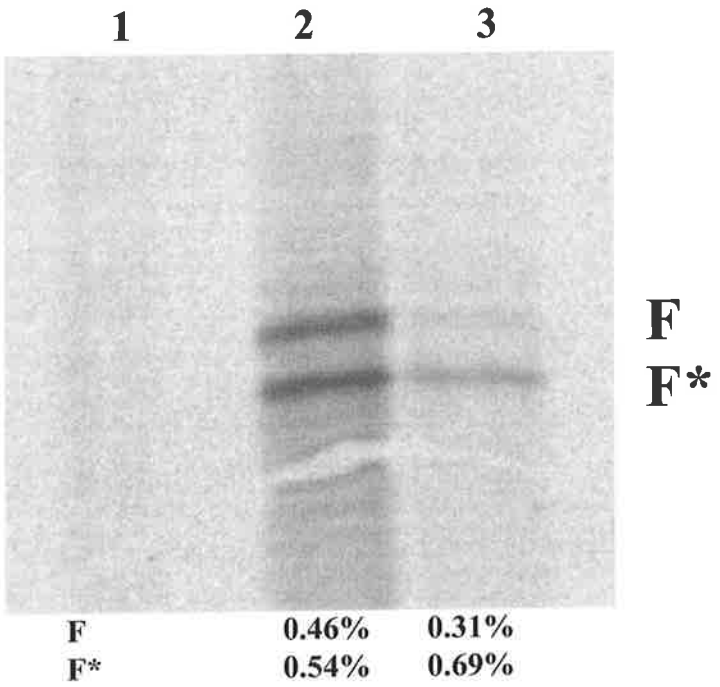


Fig. 3.11 In *V. cholerae* JBK70/pGP1-2[pPM4201], ^{35}S-methionine labelled TcpF is predominantly of the processed form at 20 minutes in the setting of prior *tcp* induction. Lane 1: vector control; lane 2: no prior *tcp* induction; lane 3: prior *tcp* induction. The numbers under each lane represent the proportion (of the total labelling for both bands in a given track) of the indicated band. The lower molecular weight species (F*) is proportionally greater in the presence of TCP induction.

Expression and labelling of TcpF-bearing plasmids in *V. cholerae* JBK70 was relatively poor (compared to that in *E. coli,* see Fig. 3.12, below), perhaps related to the toxicity of the *tcpF* gene product, but pulse-labelling for 20 mins with [^{35}S]-methionine at 37°C gave reasonable results (Fig. 3.11, above). A band at 38kDa, corresponding to the *tcpF*

product, is accompanied by a second band approximately 1.5 kDa smaller, consistent with the predicted cleavage of a type 1 peptidase presequence (Kaufman *et al.,* 1993; Ogierman *et al.,* 1993). Analysis of the apparent difference in TcpF/F* ratios was performed by density x area summation of individual bands of [^{35}S]-labelled protein detected by phosphor-imaging. While processing of the apparently typical signal peptide of TcpF proceeded in both conditions, the unprocessed form represents 69% of the total for the two bands in the setting of TCP induction (Fig. 3.11, lane 3), but only 54% in the absence of TCP induction (lane 2). While this may simply reflect better protein expression in non-induced conditions putting greater demand upon processing/ transport apparatus, it is conceivable that the processed form may have greater stability in the presence of other members of the *tcp* operon, as shown for outer membrane proteins in related systems (Jones *et al.,* 1994; Hardie *et al.,* 1996). It may even be that TcpF* is better inserted into the OM in the presence of other *tcp* products, and is thus more 'toxic' when overexpressed in that setting.

3.5.2 Instability of TcpF* when expressed at high level in *E. coli* DH5α

The long labelling time made effective cold chase of pulse-labelled TcpF difficult in *V. cholerae*, and the presence of kanamycin resistance in both the pGP1-2 plasmid and the Tn*5* insertion of V663 further complicated matters. *E. coli* DH5α is 'rough' also, and can assemble *V. cholerae* LPS in the same manner as V663 (Fig. 3.3). The processed form (TcpF*) can be seen to disappear in a pulse-chase experiment while unprocessed TcpF persists (Fig. 3.12), suggesting that TcpF* and TcpE (predicted to be an integral inner membrane protein) are unstable compared to unprocessed TcpF.

Fig. 3. 12 Pulse-labelling and cold chase of TcpF in *Escherichia coli*

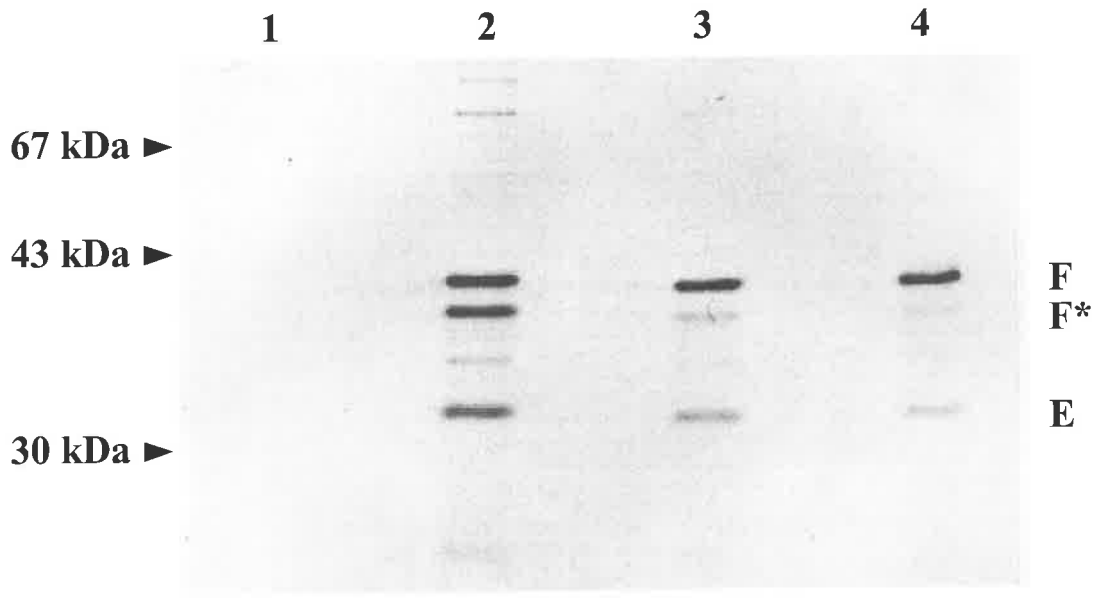


Fig. 3.12 The processed form of TcpF (F*) disappears in a pulse-chase of ^{35}S-methionine labelled TcpF in *E. coli* DH5α, while the unprocessed form (F) remains. Lanes 1-4 contains samples at t = 0, 2, 10, and 60 mins respectively after labelling (cold chase added at 1 minute). The putative integral cytoplasmic membrane protein TcpE can also be seen to diminish relative to TcpF with time.

3.6. Concluding remarks

Previously described *rfb* mutants of classical *V. cholerae* strain 569B that are unable to synthesize perosamine for LPS O-antigen production (Ward and Manning, 1989), appear to have a TcpA subunit translocation defect at the level of the outer membrane. The parent strain presents all detectable TcpA within the mature pilus, but there is identical and relatively uniform distribution of subunit in a floatation gradient whether or not it is exported as assembled pilin. Direct labelling of subunit in cryosections,

along with the immunofluorescence data and findings on simple physical fractionation, clearly shows TcpA to be accumulating in a stable configuration beyond the cytoplasmic membrane in the *rfb* mutant, V663. Although it is not surface presented, processed pilin can be identified by specific antisera in chemically extracted material and in ultrathin sections of the outer envelope. It remains a moot point as to whether periplasmic 'spillover' of subunit from the outer membrane is real, or whether it simply reflects the loose association of translocation arrested subunits. These would be expected to cofractionate with the membranes in any case, and the periplasmic 'pool' seen in ultrathin cryosections could simply be a consequence of loss into the (otherwise only potential) space created by plasmolysis for cryosectioning.

It has not been possible to determine the precise assembly state of TcpA subunits prior to their presentation in a complete external fibre from the data presented here, but a number of other lines of evidence suggest that simple conformationally directed self-association is the mechanism by which the fibre is formed. The requirement for periplasmic disulfide oxidoreductase activity for normal TCP function but not assembly (Peek and Taylor, 1992), the surface assembly of similarly dysfunctional pili lacking normal C-terminal disulphide loops in *P. aeruginosa* (Farinha *et al.,* 1994), and models derived from the recently solved crystal structure of the type-4 pilin of *N. gonorrhoeae* MS11 (Parge *et al.,* 1995) suggest that subunits are capable of packing into a multimer after processing at the cytoplasmic membrane. The haemagglutination and protease susceptibility data are consistent with normal folding of the globular head domain of TcpA in V663, and further imply that the TcpA subunit in V663 has achieved normal secondary structure in the periplasm. The apparent density of TcpA subunits in a

floatation gradient, their detergent solubility, salt elution characteristics, and ability to be chemically cross-linked do not differ between *wt* and *rfb* mutant by the methods used. The identical behaviour of TcpA, which we have shown is not detectable in the *wt* other than in the mature fibre by the methods used here, suggests that these are subunit-subunit rather than subunit-membrane interactions. The requirement for strong salt conditions to elute the subunit from membrane fractions is consistent with the predicted interaction centred on the +5 glutamate residue absolutely conserved in type-4 pilin and pilin subunit-like proteins (Hobbs and Mattick, 1993; Pugsley, 1993b), and probably critical for correct assembly in all of these (Pugsley, 1993b; Parge *et al.,* 1995).

There is no member of the GspD superfamily encoded within the *tcp* operon. While such a protein may be encoded elsewhere, there are other candidate outer membrane proteins, TcpC and TcpF, which may fulfill this role. TcpC is a lipoprotein, processed by a type II peptidase, but is of unknown function (Parsot *et al.,* 1991; Ogierman and Manning, 1992). TcpF is predicted to be an outer membrane porin-like protein, possessing a type I leader sequence and a number of possible transmembrane regions (Kaufman *et al.,* 1993; Ogierman *et al.,* 1993). Tn*phoA* insertions in both of these genes leads to a TCP negative phenotype in the classical strain O395 (Taylor *et al.,* 1988). Such a mutation in *tcpC* could lead to disruption of the trancriptionally linked downstream genes, but *tcpF* is immediately followed by a transcriptional termination sequence and a separate promoter for *toxT* and *tcpJ* (Higgins and DiRita, 1994). While neither TcpC nor TcpF have direct homologues in the type-4 pilin or general secretory pathways, they could conceivably contribute to a porin-like channel permissive for macromolecular passage, as proposed for filamentous phage (Russel, 1994a).

There are a number of examples to illustrate the importance of an intact LPS to the functioning of outer membrane structures. Surface-presentation of K99 (type 1) pili is reduced in *rfa* mutants of *E. coli*, and K99 is observed to bind LPS *in vitro* (Pilipcinec *et al.,* 1994). It has been observed that rough mutants of *Shigella flexneri* fail to correctly localize the polar surface protein IcsA (Sandlin *et al.,* 1995), and rough mutants of *E. coli* fail to normally assemble LamB and OmpF trimers (Sen and Nikaido, 1991; Laird *et al.,* 1994). An LPS-related effect may act indirectly by affecting a product or products of the *tcp* operon that contribute directly to the stable localization of TcpF within the OM, such as has been demonstrated in functionally related systems for DNA transfer (Jones *et al.,* 1994) and protein export (Hardie *et al.,* 1996). The profile of proteins detectable by Coomassie staining of SDS-polyacrylamide gels differs between the mutant and its isogenic parent (Figs. 3.4 and 3.5), but secretion of cholera toxin and DNase appears to be normal. One might speculate on the basis of the data presented that apparently greater instability of TcpF* in the rough *E. coli* strain DH5α owes to incorrect localization after processing, and that it may reflect the situation in V663. The nonspecific degradation of TcpF is an unlikely explanation, since the change in size of TcpF is consisted with the predicted Lep processing, is not associated with other smaller fragments, does not occur at the expense of unprocessed TcpF (which does not decline with time; see Fig. 3.12) and is identical in repeated experiments in both *E. coli* and in *V. cholerae* in separate laboratories (this work, and Kaufman *et al.,* 1991). This result might be better explained by failure of TcpF* to correctly localise after processing, when it becomes vulnerable to periplasmic and cytoplasmic membrane proteases. The data shown in Fig. 3.12 also suggest this fate for TcpE, predicted to be an integral inner

membrane protein in *V. cholerae* and likely to be part of a multiprotein complex *in vivo* (see Introduction). The *E. coli* host strain lacks O-antigen and the *lacZ* protein, unlike *V. cholerae* in which the vector *lac* promoter in pPM4201 is derepressed, and it is possible that incorporation of excessive amounts of the porin-like TcpF into the outer membrane explains the toxicity of *tcpF* in *V. cholerae*. The evidence for toxicity in *V. cholerae* but not in *E. coli* is arguably consistent with a failure to insert into the outer membrane in *E. coli*. The suggestion that processed TcpF may be more stable in the setting of *tcp* induction is also consistent with the data shown, but the evidence for this is most unsatisfactory in the absence of a similar pulse-chase experiment in *V. cholerae* (569B and V663) and trivial explanations may account for observed differences. Other protein such as TcpC have not been addressed, and may be equally valid candidates to explain the fault in V663. Despite all these reservations, TcpF remains a candidate protein which may simply be misassembled in the setting of abnormal outer membrane stoichometry in the O-antigen deficient V663 and DH5α strains.

An alternative explanation, which does not implicate other proteins, is a more direct effect of the loss of O-antigen to increase the hydrophobicity of LPS molecules, thus acting as a trap for hydrophobic TcpA subunits and preventing their assembly. A possible precedent is the inability of *N. gonorrhoeae* to correctly assemble the hydrophobic overlength L-pilus (Manning *et al.,* 1991). Resolution of these issues in TCP assembly await the definitive subcellular localization of TcpF and F*, and identification of the role/s, if any, of other proteins.

Chapter Four

TcpT is essential for translocation of TcpA subunit beyond the cytoplasmic membrane

4.1. Introduction

TcpT homologues are essential in a number of systems in which periplasmically pooled substrate is exported across the outer membrane (Pugsley, 1993; Turner *et al.*, 1993; Sandkvist *et al.*,1993). A closely related protein is also essential for DNA transfer into plants from a Gram-negative bacterium (Christie *et al.*, 1989), another for natural competence in a Gram-positive organism (Albano *et al.*, 1989), and others are to be found in a variety of macromolecular transporters across the bacterial membrane or membranes, including the assembly of type-4 pili (Stone *et al.*, 1996). The wide representation of type 4 pilin-like proteins in non-pilin transporters increases the importance of investigations into subunit translocation in pilin systems. Although the TcpT homologue within the type-4 pilin system of *P. aeruginosa* has been analysed by site-directed mutagenesis (Turner *et al.*, 1993), the resulting pilin translocation defect has not been described in detail. Chromosomal mutations in the *tcpT* gene are described in the following chapter, and their specificity demonstrated. The *tcp* region of Z17561 has been fully sequenced in this laboratory, and TCP expression is more tightly repressed in non-TCP-inducing conditions in Z17561 than in 569B. While most of the data pertaining to the *tcpT* mutations are therefore described for mutants in a Z17561

background, studies in the 569B background were performed in parallel so that data derived from isogenic *rfb* mutants could be reliably interpreted. In this chapter, the importance of *tcpT* is evaluated by the construction of specific mutants and analysis of their TcpA and TCP expression phenotypes.

4.2. *tcpT* is required to assemble TCP

4.2.1. Creation of chromosomal mutations in *tcpT*

Fig. 4.1 shows the *tcp* operon and the arrangement of *tcpT* within it, along with some of the most important restriction sites for mutagenesis strategies, for easy future reference The Walker A motif and the adjacent *Nde*I and *Hpa*II sites are shown in bold print. *TcpT* mutants were constructed in the two well-characterised Classical strains Z17561 and 569B, and an El Tor strain (H1). A 1.8kb *Cla*I fragment containing *tcpT* was cloned from a previously described plasmid, pPM2402 (Ogierman *et al.,* 1993), and the Tn*903*-derived GenBlock km^R cartridge (Oka *et al.*, 1981; Pharmacia, Uppsala, Sweden) inserted by blunt-end ligation into the unique *Nde*I site of *tcpT* (Fig. 4.1). This cartridge permits marker exchange mutagenesis and facilitates screening for clones of JRI1, JRI2, and JRI3, but V663 (the parent of JRI4) already harbours a kanamycin resistant transposon within the perosamine biosynthesis genes of the *rfb* operon (Section 3.2.1) .

Fig. 4.1 *tcpT* within the *tcp* operon

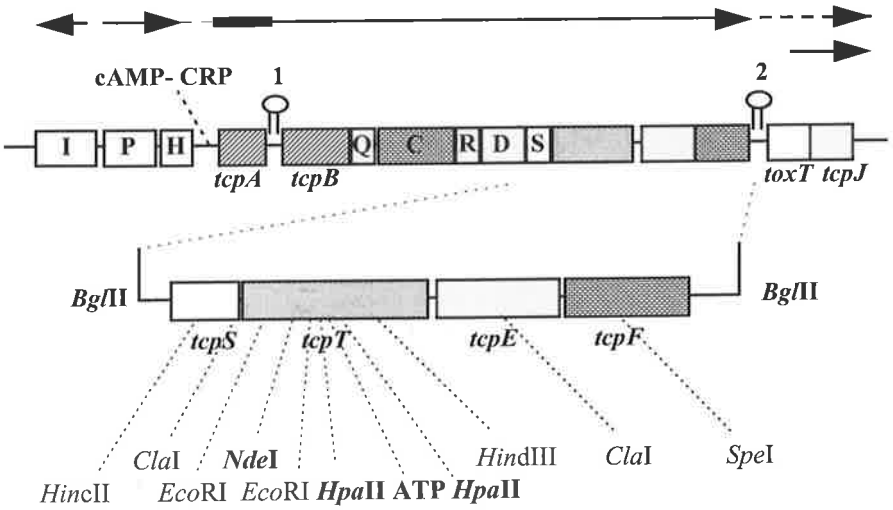


Fig. 4.1 Diagrammatic representation of the region encoding *tcpT*, showing important restriction sites. The locations of the Walker A motif (**ATP**), the unique *Nde*I site, and the two *Hpa*II sites in *tcpT* are shown in bold. The *tcp* operon is depicted above with direction of transcription (arrows), the cAMP-CRP site and attenuating sequences (1 and 2) indicated. Gene prefixes are *tcp(A-J)*, except *toxT*.

The Tn*903*-derived aminoglycoside 3'-phosphotransferase gene encoding kanamycin resistance (Oka *et al.*, 1981), here shown as the 1.1kb GenBlock™ cartridge (Pharmacia, Uppsala, Sweden).

GenBlock™ km^R cartridge:

(™ trademark; ^{R} resistance)

Appropriate transcriptional orientation of the cartridge within *tcpT* was confirmed by restriction analysis before subcloning into the pCACtus suicide vector (Clark *et al.*, in preparation) for homologous recombination into the chromosome (Section 7.1.12). Chromosomal DNA preparations of candidate strains were then digested with *Xba*I and probed with end-labelled *tcpT* to confirm allelic exchange (Fig. 4.2).

Fig. 4.2 Southern hybridisation of *tcpT*^{km} mutants with a *tcpT* probe.

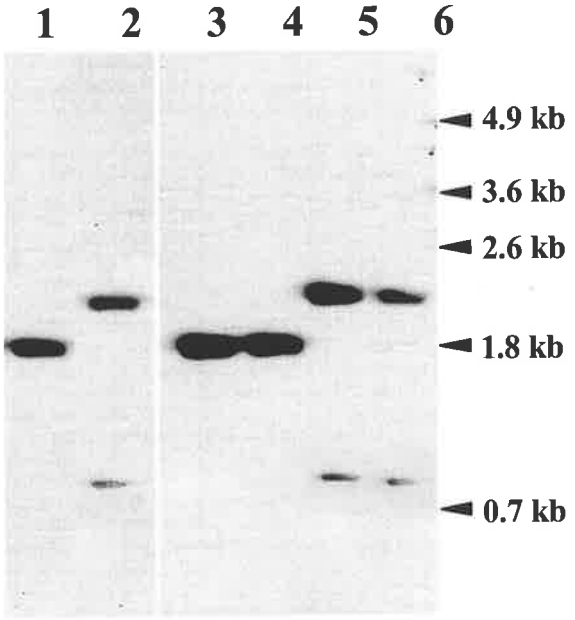


Fig. 4.2 A Southern blot probing *Cla*I restricted chromosomal DNA, using end-labelled *tcpT* (1.8 kb *Cla*I fragment from Z17561; see also Fig. 4.1). The blot shows replacement of the ca. 1.8 kb *Cla*I fragment in *wt* strains H1, Z17561 and 569B (lanes 1, 3 and 4), with two fragments of ca. 2.1 and 0.8 kb in the respective *tcpT*^{km} mutants (JRI1, JRI2, and JRI3 in lanes 2, 5, and 6, respectively). All tracks were loaded with 10 µg of DNA and electrophoresed in TAE/ agarose (0.8%), before Southern transfer and hybridisation (as described in Section 7.1.19).

4.2.2. Absence of TCP in *tcpT* mutants

All *tcpT* mutants were assessed for their ability to assemble TCP *in vitro* by transmission immune electron microscopy (IEM), using a polyclonal TcpA antiserum and immunogold labelling (Fig. 4.3, overleaf). These strains were repeatedly examined as negative controls in later complementation experiments. On every occasion, TCP bundles were absent in IEM preparations (see following, Table 4.1), and the coarse agglutination characteristic of high level TCP expression from the two Classical strains in broth culture (Taylor *et al.,* 1987) was lost.

Fig.4.3 Immune Electron Micrographs of Z17561 and JRI1 (Z17561*tcpT*^{km})

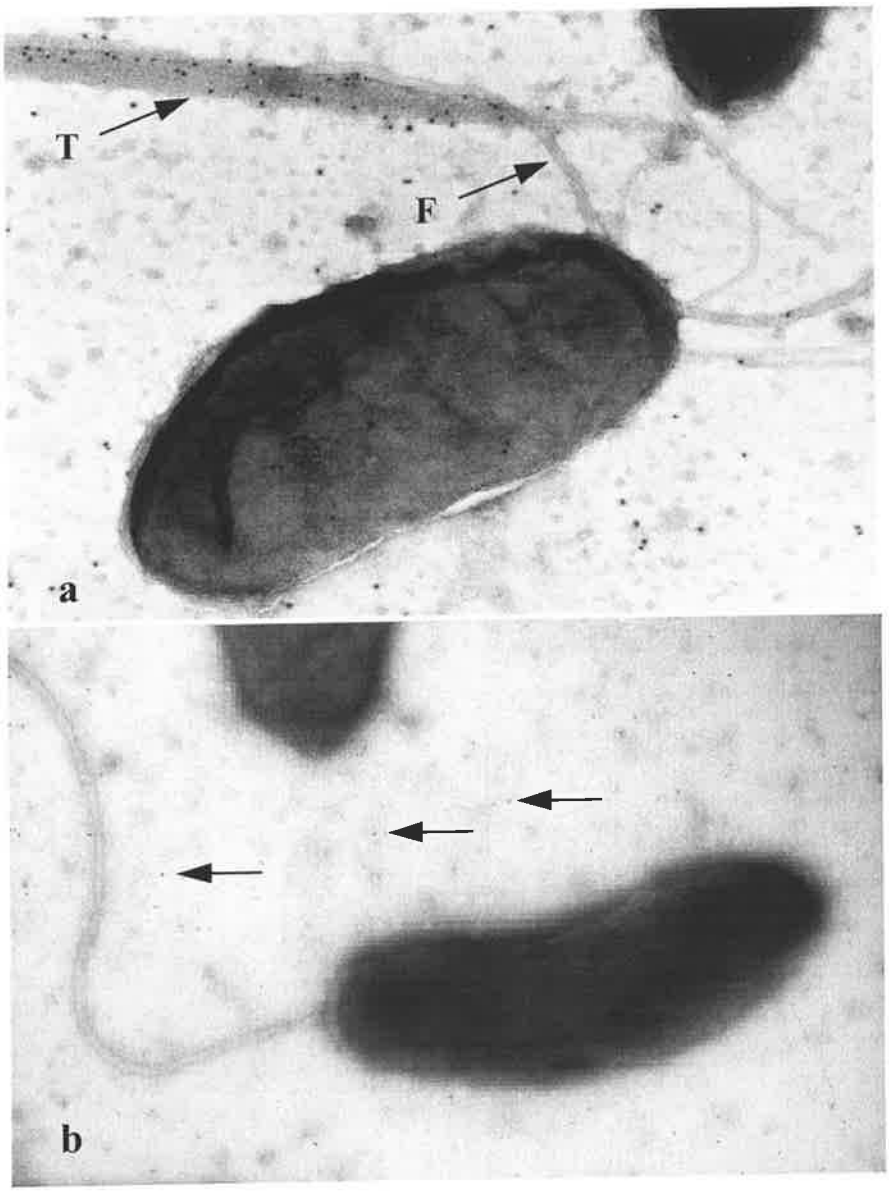


Fig. 4.3 A TCP bundle (T) is indicated in panel a, lying alongside the polar flagellum (F). Immunogold particles are clustered along the length of the bundle, but not on the cell surface. is No TCP bundles were found in association with JRI1 (Z17561*tcpT*^{km}) nor was it surface labelled by antiserum to TcpA (panel b). Some unbound gold-conjugated antibodies are indicated in panel b by horizontal arrows.

Table 4.1: Chromosomal *tcpT* mutants of *Vibrio cholerae* fail to export TcpA

Strain	Phenotype	IEM
Z17561	*wt* classical *V. cholerae* O1 Inaba	TCP
569B	*wt* classical *V. cholerae* O1 Inaba	TCP
H1	*wt* El Tor *V. cholerae* O1 Ogawa	TCP
JRI1	Z17561*tcpT*^{km} (km^{R} in *Nde*I)*	NIL
JRI2	569B*tcpT*^{km} (km^{R} in *Nde*I)	NIL
JRI3	H1*tcpT*^{km} (km^{R} in *Nde*I)	NIL
JRI5	Z17561*tcpT*^{20} (60bp deletion)	NIL
JRI6	569B*tcpT*^{20} (60bp deletion)	NIL

TCP: TCP bundles detected in IEM; NIL: no TCP identified by IEM. *km^{R}: Klenow-treated 1.1 kb kanamycin resistance cartridge (GenBlock^{TM}; Pharmacia, Uppsala, Sweden) cloned into the endfilled *Nde*I site (Fig. 4.1).

4.2.4. Confirmation of the specificity of the chromosomal *tcpT* mutation

TcpT is encoded from near the promoter-distal region of the transcriptionally linked *tcp* operon (Ogierman *et al.*, 1993). The phenotype resulting from the *tcpT*^{km} mutation might therefore be due to effects on downstream genes such as *tcpE* and *tcpF*, already identified as genes in which transposon insertions lead to a TCP-negative phenotype (Taylor *et al.*, 1988). Deletion of a 60 nucleotide region encoding the Walker A motif was therefore effected by primer overlap extension in a reverse circle polymerase chain reaction (RCPCR), utilising pPM4105, containing the 1.8 kb *tcpT*-encoding *Cla*I fragment subcloned into pBluescriptSK, as a template to create pPM4105 (Fig. 4.4).

Fig. 4.4 60 bp deletion of the *tcpT* region encoding the Walker A motif

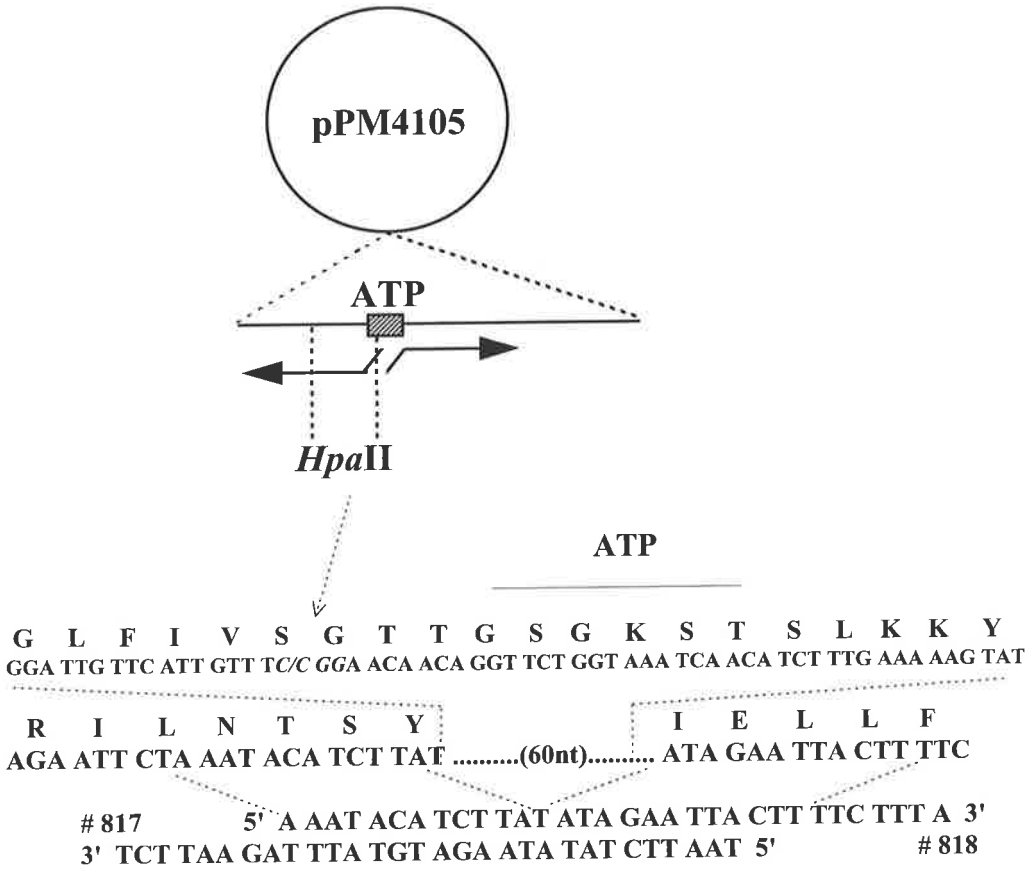


Fig. 4.4 RCPCR of pPM4105 (*tcpT Cla*I fragment) using oligonucleotides #817 and #818, to create pPM4114. The 3' regions of each oligonucleotide are mutually complementary, and span the 60 nt deletion (boxed). The 3' regions of initial template annealing for each oligonucleotide, as well as the 5' regions of complementarity between the two, have a predicted T_m of 50°C. The region encoding the Walker A motif (ATP) is deleted, thereby removing one of two *Hpa*II sites (*C/CGG*) normally present in the complete *tcpT* gene.

pPM4114 clones ($tcpT^{20}$ mutants of pPM4105) were confirmed by restriction analysis and automated sequencing, and introduced by allelic exchange into kanamycin resistant strains Z17561$tcpT^{km}$ (JRI1) and 569B$tcpT^{km}$ (JRI2) via the pCACtus suicide vector (Section 7.1.12). Isolates sensitive to kanamycin and chloramphenicol and resistant to sucrose at 30°C were presumed to have lost the chromosomal kanamycin resistance

cartridge, and the pCACtus-encoded *sacB* and chloramphenicol resistance genes. The chromosomal $tcpT^{20}$ mutation was confirmed by analysis of the altered restriction pattern of the direct *tcpT* amplification product, using oligonucleotides #721 and #1054 to amplify the entire chromosomal gene from isolated restreaked colonies of candidate strains. Loss of a 200 bp fragment from *Hpa*II digests of a PCR amplicon (using primers #721 and #1054) of the chromosomal copy of $tcpT^{20}$ confirms the deletion, as illustrated below for JRI5 (Fig 4.5). The $tcpT^{20}$ deletion mutants so created, JRI5 and JRI6, were completely TCP negative by IEM examination.

Fig. 4.5: Loss of the *Hpa*II site in the 60 bp chromosomal $tcpT^{20}$ deletion

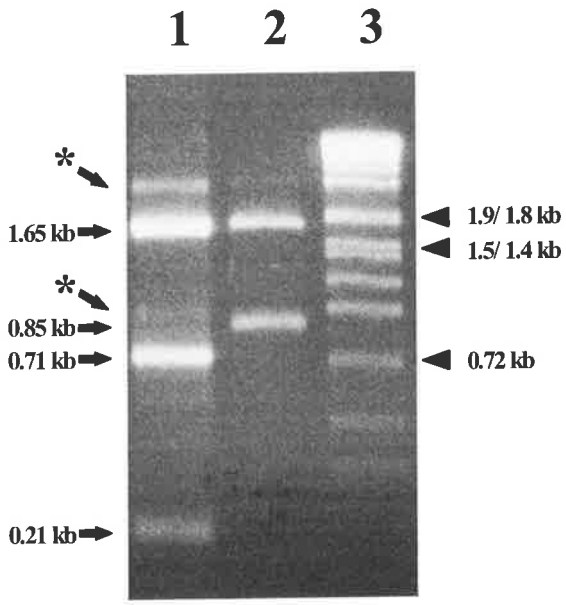


Figure 4.5 *Hpa*II restriction of PCR products (of #721 and #1054) of the *tcpT* gene from JRI5 shows the loss of the internal 200bp fragment (see also Fig. 4.4). In lane 1, a 2.6kb product of Z17561 (oligos #754 and #1047) is restricted to 1.6, 0.7, and 0.2 kb. In lane 2, the 2.54 kb product of JRI5 is restricted to 1.6 and 0.85 kb, with loss of the 60bp deletion and the *Hpa*II site within it. Uncut material (*) can be seen in each track. Lane 3 contains *Eco*RI digested SPP-1 as a molecular weight marker. The gel is 1.8% agarose, and DNA fragment sizes are given in kilobases.

A number of subclones of the *tcpT* gene and surrounding region were next constructed for use in initial complementation studies, and are detailed in Fig. 4.6 and Table 4.2 (to follow). Most are simple subclones, but a a large in-frame internal deletion of the 5'

region of *tcpT* (pPM4132) was created as follows: A *Hinc*II cutdown of pPM4104 removes a portion of *tcpS* and an *Eco*RI site from the vector MCS. A subsequent *Eco*RI cutdown in *tcpT* creates a 547 nt internal deletion which resumes in the correct open reading frame after end-filling with Klenow fragment of DNA polymerase I (see Fig. 4.6).

Thus,

```
41                              223
Glu     Phe                     Arg     Ile     Leu
G/AA    TTC ..................  AG/A    ATT     CTA
CTT     AA/G..................TCT     TAA/    GAT
```

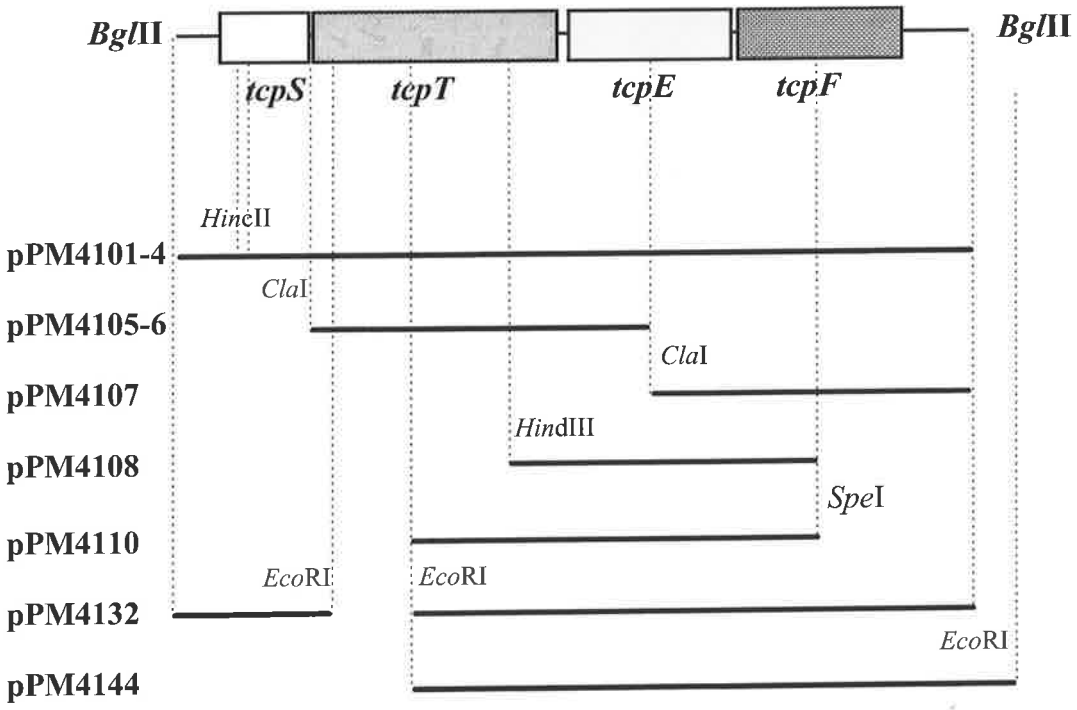
becomes

```
41              224
Glu     Leu     Ile     Leu
GAA     TTA     ATT     CTA
CTT     AAT     TAA     GAT
```

, after endfilling of the *Eco*RI (G/AATTC) sites.

The deleted Phe42 - Arg223 region contains 181 of the 503 residues in TcpT. The lost region therefore encodes most of the N-terminal of TcpT, and creates a fusion with an added Leu residue which preserves the generally hydrophobic nature of the amino acids immediately preceding the Walker A motif (which is itself centred on the Lys residue at position 242). Analysis of the derived secondary structure, according to the protocol of Chou and Fasman (1978) and performed in PROSIS ver. 6.0 (Hitachi Software), predicts minor disruption of the highly conserved region of α-helix which immediately precede such motifs (described in Section 1.12).

Fig. 4.6 Subclones of the *Bgl*II fragment



Exact location of important sites in the *Bgl*II fragment

(distance from the 3' end of the *Bgl*II fragment in nt)

***Bgl*II**	0; 4861*	***tcpT*:**	785 - 2296
***Nde*I**	1319*	**T^{20} deln:**	1471-1531
***Hinc*II**	643; 653	**Lys224:**	1507-1509
***Cla*I**	781**; 2565	***tcpE*:**	2329 - 3293
***Eco*RI**	905, 1452	***tcpF*:**	3303 - 4319
***Hpa*II**	1488; 1703		

*unique sites, i.e. not present in vector
**also present in km^R cartridge; cuts between RBS sequence and ATG codon of *tcpT*

Fig. 4.6. The *Bgl*II fragment encoding *tcpSTEF* is depicted in stylised form. Important restriction sites for subcloning are represented in the diagram (above) and their exact location also listed in tabular form. The plasmids so represented are further described in the table below (Table 4.2)

A combination of simple complementation experiments performed with these plasmids (Table 4.2) confirm a requirement for the *tcpT* gene for TCP biogenesis.

Table 4.2: Complementation by subclones of the *Bgl*II fragment

plasmid number	*tcp* genes	host vector	controlling promoter	TCP bundles in IEM JRI1 JRI2	JRI5 JRI6
nil	(see text)		*tcpA*	NIL	NIL
pPM4101[a]	*tcpSTEF*	pBluescript SK[b]	T7	TCP	TCP
pPM4102	*tcpSTEF*	pWSK29[c]	T7	TCP	TCP
pPM4103	*tcpSTEF*	pBluescript SK	*lacZ*[d]	TCP	TCP
pPM4104	*tcpSTEF*	pWSK29	*lacZ*	TCP	TCP
pPM4105[e]	*tcpT*	pBluescript SK	T7	nd	NIL
pPM4106	*tcpT*	pWSK29	T7	nd	nd
pPM4107	*tcpF*	pBluescript SK	T7	nd	nd
pPM4108	*tcpE*	pBluescript SK	T7	nd	nil
pPM4110	*tcpT*EF*	pET-17b[f]	T7	nd	nd
pPM4132	*tcpT**EF*	pBluescript SK	T7	NIL	NIL
pPM4135	*tcpT*	pTTQ181[g]	*tac*	NIL	TCP
pPM4144	*tcpEF****	pGem-*Zf*	*lacZ*	NIL	NIL

[a] pPM4105-7, pPM4108, and pPM4132 are derived from pPM4101-4 (as *Cla*I and *Hin*dIII-*Spe*I cutdowns, and an *Eco*RI endfill** respectively - see text), as is the *Nde*I-*Spe*I fragment in pPM4110.
[b] Stratagene, USA
[c] Wang and Kushner, 1991
[d] The *lacZ* promoter is provided without a functional repressor in all vectors except pTTQ181 (see d, below), resulting in significant derepression of the *lacZ* promoter, particularly in *V. cholerae*.
[e] pPM4105 and pPM4106 have lost the predicted *tcpT* RBS sequence in the *Cla*I cutdown.
[e] optimal T7 expression vector (Novagen, USA); see also Figs. 5.3 and 5.4
[e] pTTQ181 (Stark, 1987) is an expression vector into which *tcpT* is cloned in-frame for optimal expression, and contains the *lacI* repressor gene *in cis* (considered in detail in Section 5.3.1)
[c] optimal T7 expression vector (Novagen, USA); see also Figs. 5.3 and 5.4
[e] pTTQ181 (Stark, 1987) is an expression vector into which *tcpT* is cloned in-frame for optimal expression, and contains the *lacI* repressor gene *in cis* (considered in detail in Section 5.3.1)
**Nde*I-*Spe*I fragment (Fig. 4.1) cloned into an ATG site in optimal position for expression
***Eco*RI endfill in *tcpT* with ATP motif-encoding sequence in correct transcriptional frame (see text)
*** The *Eco*RI fragment extends from the second *tcpT Eco*RI site (1452 nt 3' from *Bgl*II).
[f] optimal T7 expression vector (Novagen, USA); see also Figs. 5.3 and 5.4
[g] pTTQ181 (Stark, 1987) is an expression vector into which *tcpT* is cloned in-frame for optimal expression, and contains the *lacI* repressor gene *in cis* (considered in detail in Section 5.3.1)
(TCP: bundles detected in IEM; NIL: no TCP bundles detected in IEM; nd: not tested)

4.2.6. The *tcpT* mutation is specific for TCP assembly

TcpT is predicted to be an ATPase, and any role in TCP assembly or export would be expected to be mirrored by TcpT homologues in other export systems (eg. Ctx, MSHA). A peptidase is shared between pilin and non-pilin systems in *P. aeruginosa* (Strom *et al.,* 1993) and the similarities between these systems and type-4 pilins such as MSHA and TCP are well known (discussed in detail in Section 1.12). There was no detectable effect of the $tcpT^{km}$ mutation however, with normal activity in mutant strains JRI1 and JRI2 of exported cholera toxin (as measured by Gm_1 -ELISA), and in DNAse or casein hydrolysis assays. Production of haemolysin, characteristic of the El Tor biotype (Alm *et al.,* 1990b), is unaffected in JRI3 compared to H1 as judged by zones of haemolysis on 5% sheep blood agar and haemolytic activity in the supernatant of a 4 hour culture. Chemotaxis was not specifically evaluated. Any effect on chemotaxis would be difficult to attribute to TcpT alone, since there may be direct and indirect links between various aspects of TCP biogenesis (see Section 1.12). The *tcpT* mutation does not affect the natural vigorous motility of the cholera organism however, as judged by hanging drop preparations, and by 0.3% agar overlays.

4.3. Cytoplasmic membrane arrest of the processed subunit

4..3.1 Absence of surface labelling of TcpA in IF studies

Whole cells of Z17561 (*wt*), JRI1 and JRI2 ($tcpT^{km}$), and JRI5 and JRI6 ($tcpT^{20}$) were prepared for IF experiments, in the manner already described for *rfb* mutants of 569B. Immunoreactive TcpA is clearly not surface presented in JRI2 (Fig.4.7), as already suggested by IEM studies, and identical results were obtained in JRI1, 5, and 6. EDTA pretreatment had no effect on these cells, unlike identically prepared V663 cells (Section 3.3.3). This does not exclude periplasmic exposure, however. These cells are not true sphaeroplasts and the outer membrane of the 569B *wt* parent strain is likely to be more resistant to EDTA-mediated disruption than that of the isogenic *rfb* mutant V663, as already evidenced perhaps by the diminished viability of V663 in late cultures (Section 3.2.2).

4.3.2 Preliminary physical fractionation suggests membrane localisation; processed TcpA is present in *tcpT* mutants in diminished amounts

Immunoblotting for TcpA in $tcpT^{km}$ and $tcpT^{20}$ mutants revealed all of the TcpA to be processed, located only in the membrane fraction, and present in lesser amounts than in the isogenic *wt* parent (Figs. 4.8 and 4.9). While the diminution of TcpA is more marked in the 569B background than the Z17561 background, the phenotypes are indistinguishable in all other respects.

Fig. 4.7 Immunofluorescent studies of 569B and JRI2

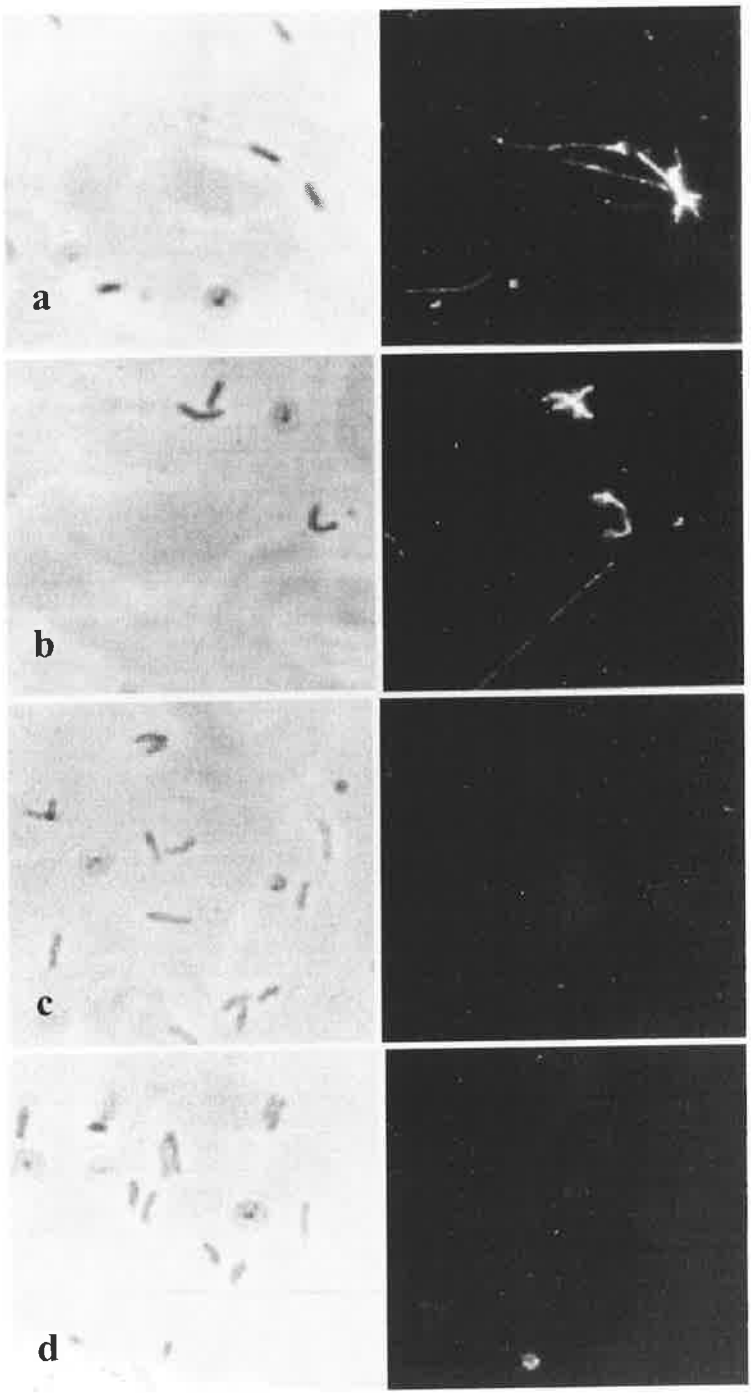


Figure 4.7 Phase contrast (left) and matching IF (right) images of 569B (panels a and b) and its *tcpT* mutant JRI2 (panels c and d). There was no apparent surface labelling of JRI2, nor any effect of EDTA pretreatment on 569B (panel b) or JRI2 (panel d). In these preparations, TCP fibres (panels a and b) appear to be the only form of TcpA detected. See also Fig. 3.6, p. 119

Fig. 4.8 Processed TcpA in the membrane fraction

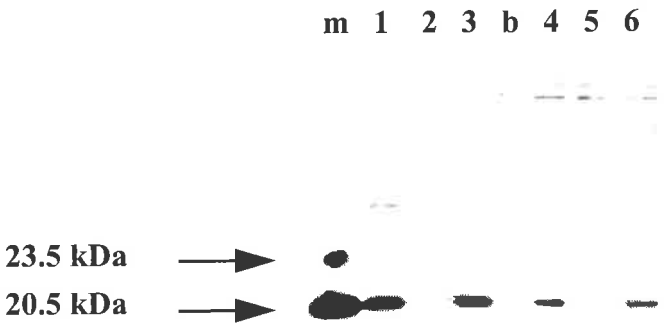


Fig. 4.8 TcpA immunoblot of Z17561 and JRI1 (Z17561*tcpT*^{km}), after being subjected to SDS-PAGE and Western transfer. Unprocessed (23.5 kDa) and processed (20.5 kDa) forms of TcpA as markers in the extreme left lane (m). Lane 1 contains Z17561 whole cells. Lanes 2 and 3 contain soluble (supernatant, periplasm and cytoplasm) and insoluble fractions (membranes and pilin bundles) after French press lysis and centrifugation at 90,000xg. Lane 3 (b) is left blank. Lanes 4-6 contain whole cells, soluble and membrane fractions respectively for JRI1. TcpA is detected by α569B-165 antiserum (1:2000) and secondary antibody (goat anti-rabbit conjugated to HRPO) is detected by the ECL method.

Fig. 4.9 Processed TcpA is present in diminished amounts

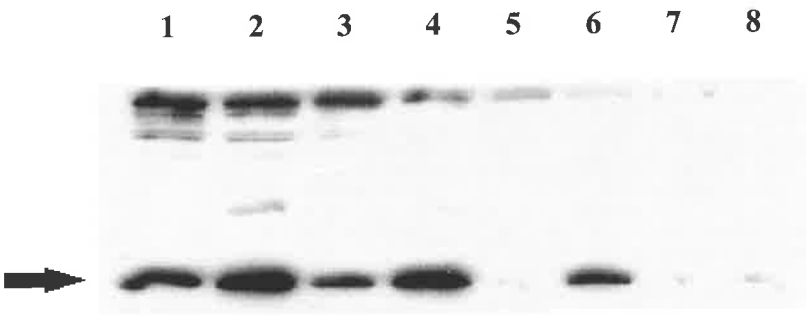


Fig. 4.9 TcpA immunoblot of serial two-fold dilutions of JRI1 (Z17561*tcpT*^{km}) and parent strain Z17561 after SDS-PAGE and transfer show that the amount of immunoreactive TcpA (arrow) is approximately halved by the *tcpT* mutation. Whole cells taken from a four hour culture were subjected to SDS-PAGE and immunoblotted with α569B-165 for detection by enhanced chemiluminescence as described (Methods). Lanes 1, 3, 5, 7 (JRI1) and 2, 4, 6, 8 (Z17561 parent) contained twofold dilutions of JRI1 and Z17561, respectively, from an initial load of ca. 10^9 cfu, to extinction for this exposure. TcpA is detected by the ECL method (as for Fig. 4.8).

4.3.3 Anomolous floatation of TcpA in sucrose density gradients

These data (above) show that the prepilin peptidase TcpJ is functional and that pilin subunits have at least associated with the cytoplasmic membrane. TcpA is found only in the membrane fraction of these strains by pelleting at 90,000xg after complete lysis (Fig. 4.8). Since this is also true of the TcpA within *rfb* strains and the assembled TCP of the *wt* parent, it was necessary to fractionate the subunit more accurately. TcpA subunits underloaded in sucrose flotation gradients are found throughout, with predominance in fractions corresponding to the location of the outer membrane proteins of *V. cholerae* as well as fractions of lower density (Fig. 4.10). The essentially identical nature of the distribution pattern in *wt* Z17561 and 569B strains, and in both *rfb* and *tcpT* mutants, suggests nonspecific association of pilin subunits with other cellular elements (Fig. 4.10, overleaf; see also Fig. 3.5). A possible explanation for the range of density distribution is variation in multimerisation, either *in vivo*, or as a result of degeneration of multimers during the gradient separation. This proved not to be the case in the *rfb* mutants, as judged by simple cross-linking experiments (section 3.4.2), and is also examined in these strains (Fig. 4.16). Variable casual association with other membrane elements is plausible. Entrapment in French pressure-cell generated vesicles of variable size seems a less likely explanation, since there is such consistency between *wt* and translocation mutants (whether *rfb* or *tcpT* mutants). While there is an impression that TcpA in the *tcpT* mutants have shifted to a higher density in floatation gradients (Fig 4.10), TcpA is still found in fractions clearly containing outer membrane proteins (tracks 2-5 in panels a-d, Fig. 4.10) and the results must be interpreted in the light of data to follow.

Figure 4.10 Sucrose floatation gradients: Z17561 and JRI1

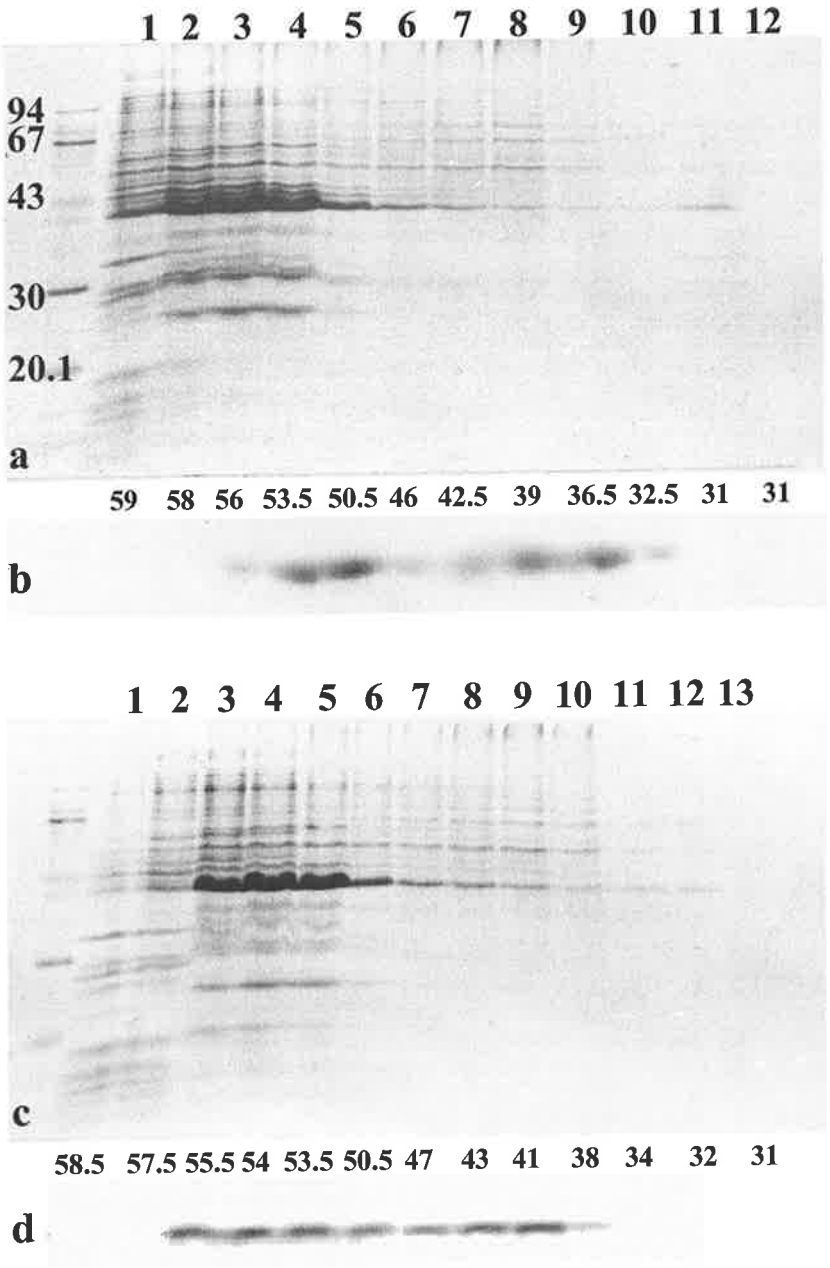


Fig. 4.10 SDS-PAGE and corresponding immunoblots of sucrose density gradient fractions from Z17561 and JRI1. PBS-washed whole cell lysates were underloaded into sucrose density gradients. Gradient fractions from Z17561 (panels a and b) and JRI1 (panels c and d) were loaded on SDS-PAGE gels and stained with Coomassie Brilliant Blue (panels a and c) or immunoblotted with α-569B-165 polyclonal antiserum to TcpA (panels b and d). Sucrose density is given under each lane (% w/v). Major outer membrane proteins, as previously indicated in Fig. 3.5, are prominent in high density fractions; left lanes contain size markers (given, in kDa).

4.3.4 EDTA washes do not release TcpA from the membrane of *tcpT* mutants

In order to test whether external pili or loosely associated subunit could be removed by washing in the presence of EDTA, *tcpT* mutants and their isogenic parents were repeatedly washed and vortexed in Tris-HCl (30mM, pH 8.0) EDTA (5mM) sucrose (30%). This concentration of EDTA is identical to that used in IF experiments, the use of which did not increase surface accessibility of immunoreactive TcpA. Assembled pili sheared from Z17561 cells were completely removed by repeated washings in this experiment, while $tcpT^{km}$ mutant cells retained TcpA in the sphaeroplast pellet. This does not appear to be a function of growth phase as it is equally true of 4 hr and 16 hr cultures (Fig. 4.11).

Fig. 4.11: Tris/ EDTA washes do not remove TcpA from *tcpT* mutants

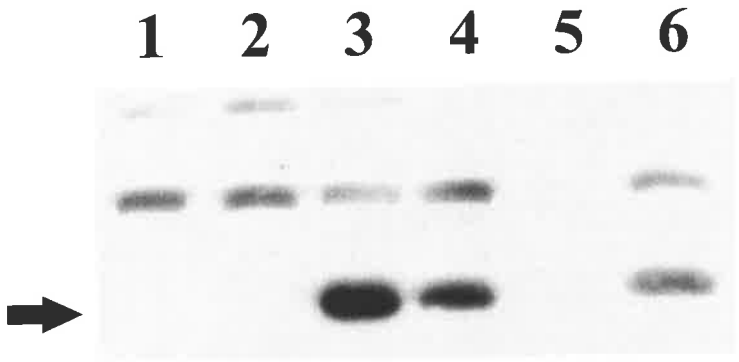


Figure 4.11 TcpA immunoblot of whole cells and EDTA-treated pellets. Cells were washed three times in Tris-HCl (30mM, pH 8.0) EDTA (5mM) sucrose (30%) were pelleted, resuspended in sample buffer, and subjected to SDS-PAGE followed by transfer to nitrocellulose membrane for immuno-blotting with polyclonal α569B-165 antisera. The reaction was detected by the ECL method. TcpA (arrow) is retained in $tcpT^{km}$, but not *wt*. Lanes 1,2: Z17561 4hrs, 16 hrs culture; 3,4: Z17561$tcpT^{km}$ 4 hrs, 16 hrs; 5: 569B 16hrs; 6: 569B$tcpT^{km}$ 16hrs.

4.3.5. LiAc/ LiCl-induced membrane blebs contain no TcpA in *tcpT* mutants

A membrane extraction procedure has been previously shown to be a highly effective method for harvesting small amounts of relatively pure outer membrane material, shed as membrane blebs during incubation in LiAc/ LiCl at 42°C (Pannekoek *et al.*, 1992; Section 7.2.7). This technique was applied to investigate subunit representation in the outer membrane compared with whole cells. Collected material constituted about 1% of total protein, as expected, and TcpA was well represented in material shed from *wt* Z17561, but not at all in material from the isogenic *tcpT* mutants (Fig. 4.12).

Fig. 4.12. LiAc/ LiCl-induced membrane blebs contain no TcpA in *tcpT* mutants

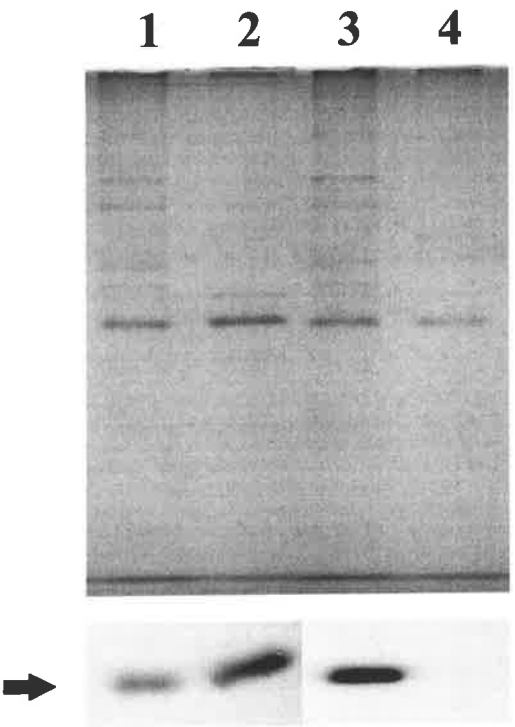


Figure 4.12 SDS-PAGE after staining with Commassie Brilliant Blue (top panel) is shown along with the corresponding immunoblot for TcpA (arrow). Whole cells incubated in LiAc/ LiCl at 42°C were pelleted at 20,000xg for 30 mins (lanes 1 and 3). Supernatant was pelleted at 100,000xg for I hour (lanes 2 and 4). Protein content was estimated by the BCA method, and equivalent amounts of each pellet run on SDS-PAGE for immuno-blotting with polyclonal α569B-165 antiserum to TcpA. Z17561 (Lanes 1 and 3) and Z17561*tcpT*^{km} (Lanes 3 and 4) are directly compared.

4.3.6 Ultrathin sectioning demonstrates membrane-associated TcpA

The data described above (Figs. 4.11 and 4.12) strongly suggest a firm association of TcpA with the cytoplasmic membrane fraction in *tcpT* mutants. No TcpA was previously detected in IEM or IF studies of whole cells (Figs. 4.2 and 4.7). It is also apparent that the TcpA in *tcpT* mutants is differently distributed from that in the *rfb* mutants (Fig. 4.8), but possible distortions of results (due to decreased amount of TcpA produced in *tcpT* mutants, increased fragility of *rfb* mutants, etc.) could not be absolutely dismissed. For this reason, and because of relatively inconclusive data from sucrose floatation gradients (Section 4.3.3), direct imaging methods were employed once more (this section), and isogenic double mutants created (Section 4.3.6).

Fresh cultures (4 hours) were washed and resuspended in PBS, before gluteraldehyde/ formaldehyde fixation. The impressive membrane and periplasmic labelling of TcpA seen in preparations of V663 (Fig. 3.8) was absent from the isogenic mutants JRI2 (569B*tcpT*^{km}) and JRI4 (V663*tcpT*^{km}; Section 4.3.6). In JRI1 (Z17561*tcpT*^{km}), as well as in JRI2 (569B*tcpT*^{km}), the small amount of detectable TcpA makes it impossible to clearly discriminate between inner and outer membrane locations (Fig. 4.13). Nevertheless, the location of labelling is consistent with the other lines of evidence already presented which point to the cytoplasmic membrane as the site of arrested subunit translocation.

Fig.4.13 TcpA in Ultrathin cryosections of JRI5

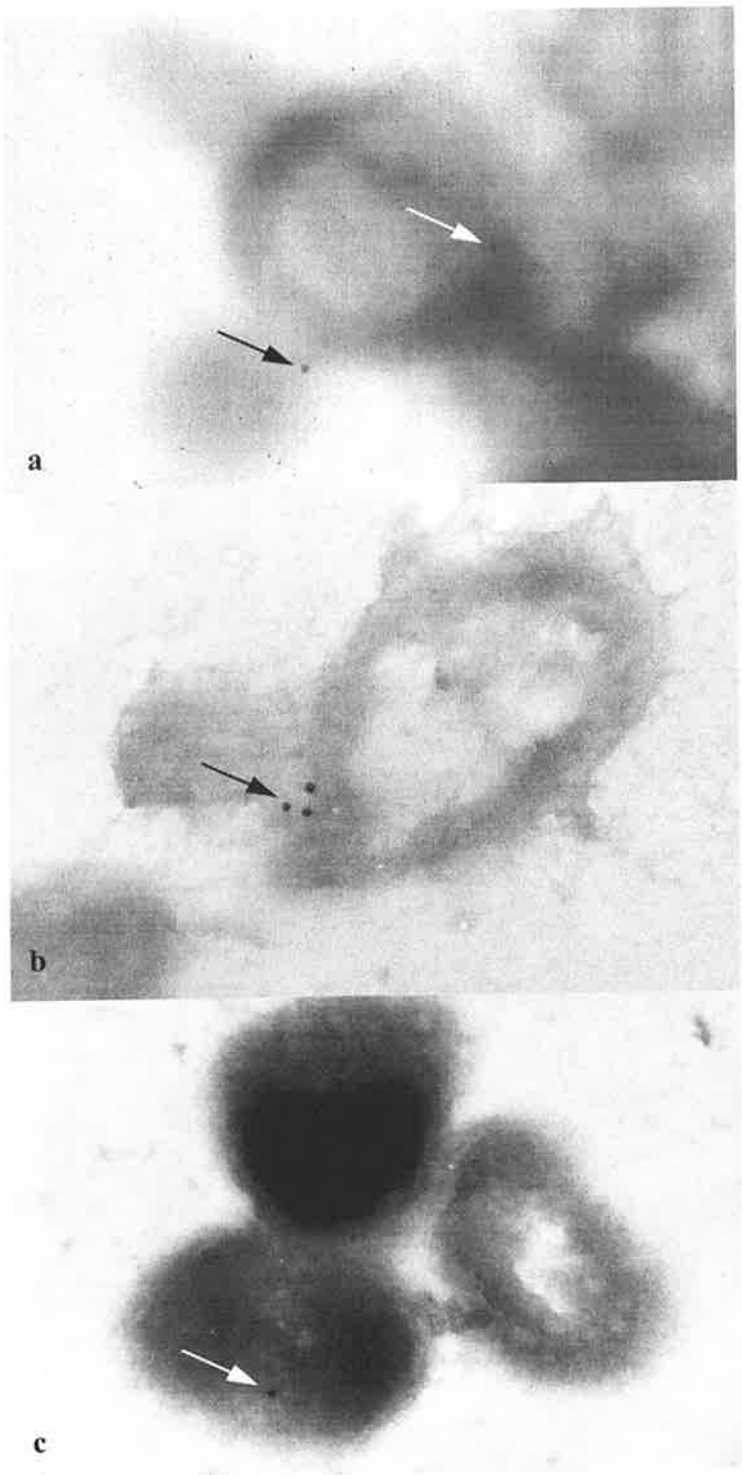


Fig. 4.13 Ultrathin cryo-sections of JRI5. Bound Tc20.2 monoclonal anti-bodies were detected by goat-anti-mouse antibodies conjugated to 10nm gold particles. TcpA (arrows) is detected in the membrane.

Panels a, b, and c show three different preparations of the same strain, and demonstrate membrane labelling. See also Fig. 3.8.

TcpA could not be detected in preparations of *wt* Z17561 other than in external TCP bundles. Since the immunogold labelling method is a relatively insensitive detection method, this does not exclude a membrane pool of subunit. It does imply that the *tcpT* mutants (and especially the *rfb* mutant) are trapping increased amounts of TcpA in the membrane fraction beyond that which is detectable in the native state. Diminution in the total amount of TcpA in *tcpT* strains also suggests that TcpA is either trapped in the inner membrane and subsequently degraded to some extent, or that the conformation/ associations of TcpA in the *tcpT* mutants is/ are less resistant to periplasmic degradation than in the *rfb* strains, in which the translocation failure is clearly at the level of the outer membrane (Section 3.3).

4.3.6 TcpA subunit in *rfb tcpT* double mutants

Ultrathin cryosectioning and physicochemical fractionation methods had shown that TcpA was arrested at the level of the OM in the *rfb* mutant strain V663. If the *tcpT* mutation did indeed lead to translocation arrest prior to the outer membrane, a *tcpT* mutation in V663 should behave the same as a *tcpT* mutation in the *wt* background. Isogenic double mutants (V663*tcpT*^{km}) were therefore constructed by allelic exchange in V663 (569B*rfb*) to allow direct comparison with JRI2 (569B*tcpT*^{km}) (Fig. 4.14).

Fig. 4.14 Isogenic *rfb,tcpT* double mutants have a *tcpT* phenotype

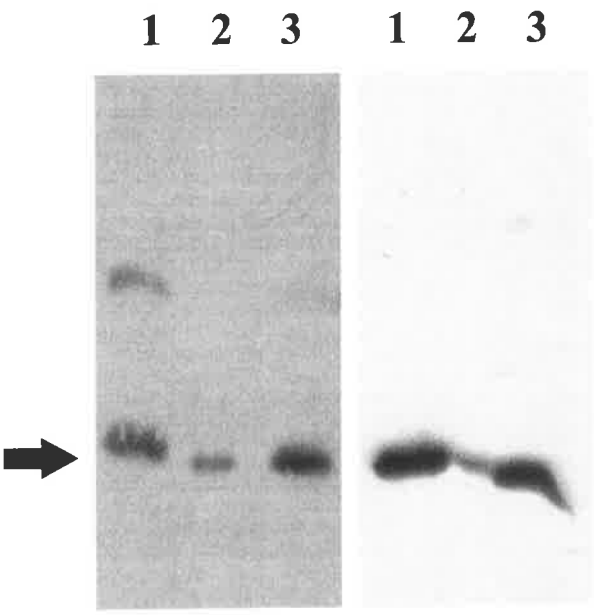


Fig. 4.14 TcpA immunoblot, after SDS-PAGE and Western transfer of equal amounts of 569B, JRI2, and JRI2[pPM4133] (Lanes 1, 2, and 3 left) and V663, JRI4, and JRI4[pPM4133] (Lanes 1, 2, and 3 right). pPM4133 is an IPTG-inducible tcpT-encoding construct (details in Section 5.3). The $tcpT^{km}$ defect is associated with an equivalent decrease in total amounts of TcpA (arrow) in both backgrounds, and this is fully restored in both backgrounds by complementation of the *tcpT* defect alone. TcpA is detected by α569B-165 polyclonal antiserum and detected by the ECL method, as previously described (Fig. 4.11).

The phenotype of the $tcpT^{km}$ mutation in V663 was also the same in IF and ultrathin cyosection preparations as the $tcpT^{km}$ mutation in 569B. Complementation of the *tcpT* defect in JRI3 (V663$tcpT^{km}$) was effected by electroporation of pPM4133 into JRI3, and this restored the typical V663 phenotype in IF preparations and in Western blot (Fig. 4.14). These results show that the effect of TcpT upon processed subunit occurs prior to the location of the translocation block in the *rfb* mutants. The next important issue is thus to determine whether the TcpA subunits in the *tcpT* mutant background differ in their physicochemical characteristics from those in TCP and in the *rfb* background.

4.4. TcpA configuration in *tcpT* mutants

4.4.1 Salt elution and detergent extraction of TcpA

The ease with which chaotropic, denaturing, and detergent solutions solubilise proteins from subcellular fractions often points to the nature of interactions of the target protein with the fraction from which it is extracted. TcpA was efficiently extracted from membrane fractions in both 2% Triton X-100 and 1% Sarkosyl (in the absence of $MgCl_2$ or EDTA; Fig. 4.15), consistent with the findings in *rfb* mutants (Section 3.4).

Figure 4.15 Salt and detergent extraction of TcpA from membrane fractions

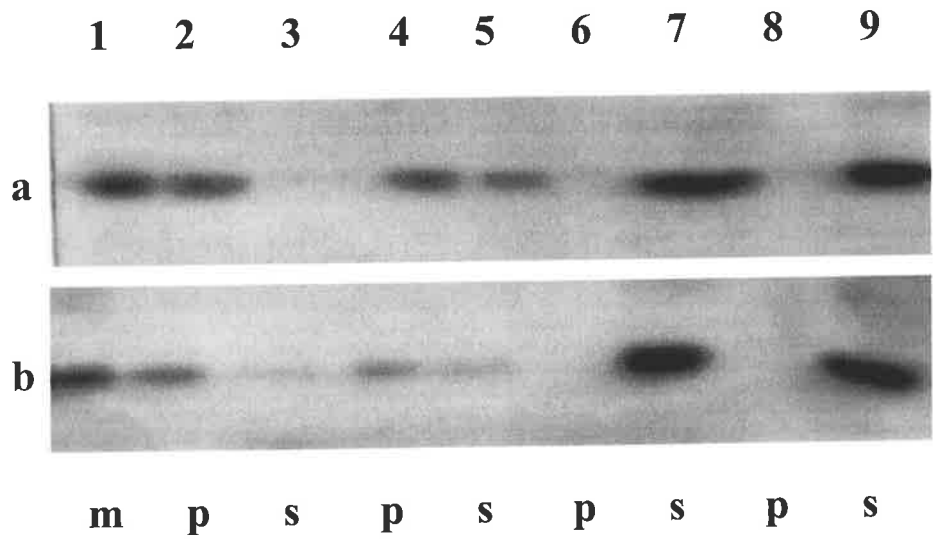


Figure 4.15 TcpA immunoblot of salt and detergent extractions of TcpA from membrane fractions of Z17561 (panel a) and JRI1 (panel b). Lane 1 contains untreated membrane (m) and all other lanes are paired. Pellet (p) and supernatant (s), respectively, were loaded in equal volumes as follows: lanes 2 and 3, NaCl 1.0M; 4 and 5, Urea 6.0M; 6 and 7, 2% Triton X-100; 8 and 9, 1% Sarkosyl. All samples were boiled in sample buffer before being subjected to SDS-PAGE for transfer and immunoblotting with α569B-165 polyclonal antiserum to TcpA.

Likewise, 6.0M Urea but not 1.0M NaCl was able to elute TcpA, again suggesting a requirement for protein denaturing activity to solubilise the pilin subunit from *wt* and isogenic *tcpT* strains. The behaviour of TcpA under these conditions suggests that the subunit is not casually membrane associated in *tcpT* mutants, and supports the previous assertions that subunit-subunit interactions determine behaviour in these extraction experiments.

4.4.2 Cross-linking experiments suggest early subunit-subunit association

As had been done with the *rfb* mutants (Section 3.4.2), membrane-permeable as well as watersoluble crosslinkers (Dithiobis succinimidyl propionate [DSP] and 3,3'-diothiobis sulphonyl succinimidyl propionate [DTSSP], respectively) were employed on whole cell and sucrose floatation gradient fractions. A pattern of ascending multimers was identical in all specimens in which cross-links were not reduced. This was true for 569B, V663, Z17561, and JRI5. TcpA migrated at the same molecular weight in reducing and non-reducing conditions in all untreated samples, and no multimers were evident in the absence of cross-linker (Fig. 4.16).

Fig. 4.16 Chemical crosslinking of TcpA subunits in *tcpT* strains

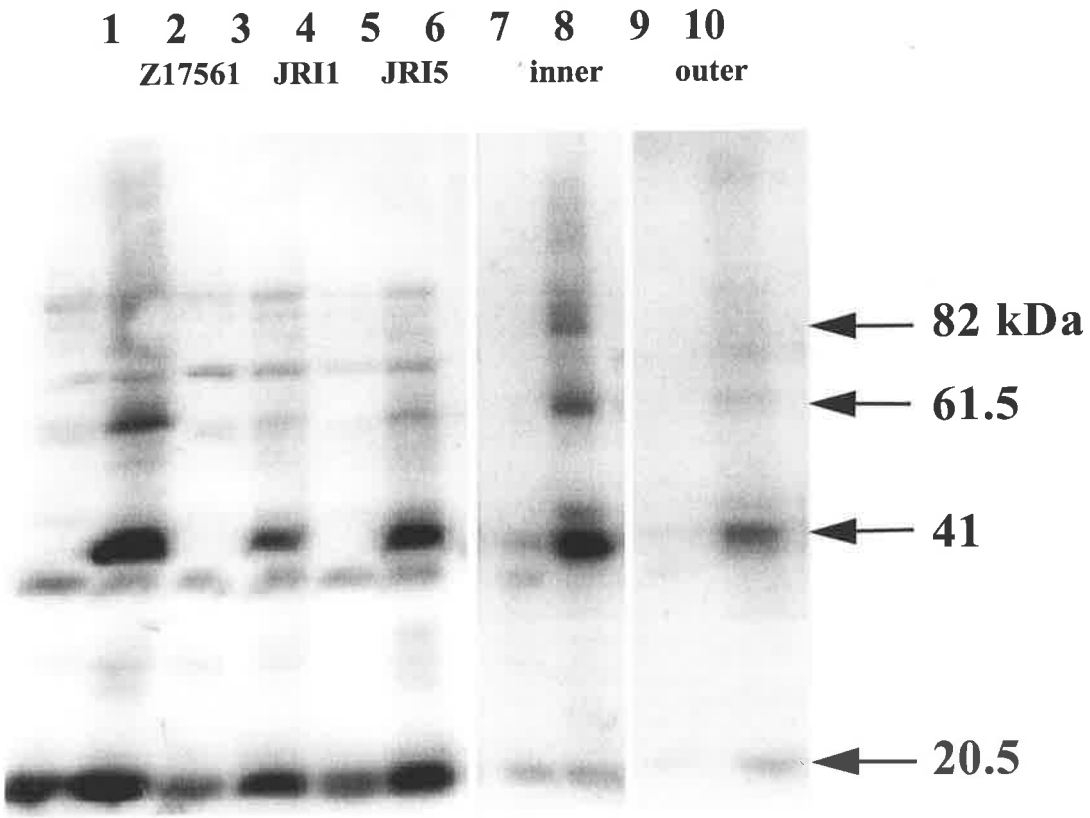


Figure 4.16 TcpA immunoblot of whole cells and sucrose floatation gradient fractions after SDS-PAGE and transfer. Crosslinking of whole cells (Lanes 1-6) and fractions from sucrose density gradients (7-10) do not differ significantly in *tcpT* mutants from *wt* parents. DSP cross-linked Z17561 whole cells are shown in reducing (lane 1) and non-reducing (lane 2) conditions, with JRI1 and JRI5 in the presence (lanes 3 and 5) and absence (lanes 4 and 6) of β-mercaptoethanol. DTSSP-crosslinked fractions 8 (inner) and 5 (outer) from the JRI5 sucrose density gradient (see Fig. 4. 9) are also shown in the presence (lanes 7 and 9) and absence (lanes 8 and 10) of β-mercaptoethanol (see Section 7.3.1 for details of reducing and non-reducing SDS-PAGE buffer conditions). TcpA was detected by α569B-165 antiserum and developed by the ECL method, as described (Fig. 4.11).

Fig. 4.16 Chemical crosslinking of TcpA subunits in *tcpT* strains

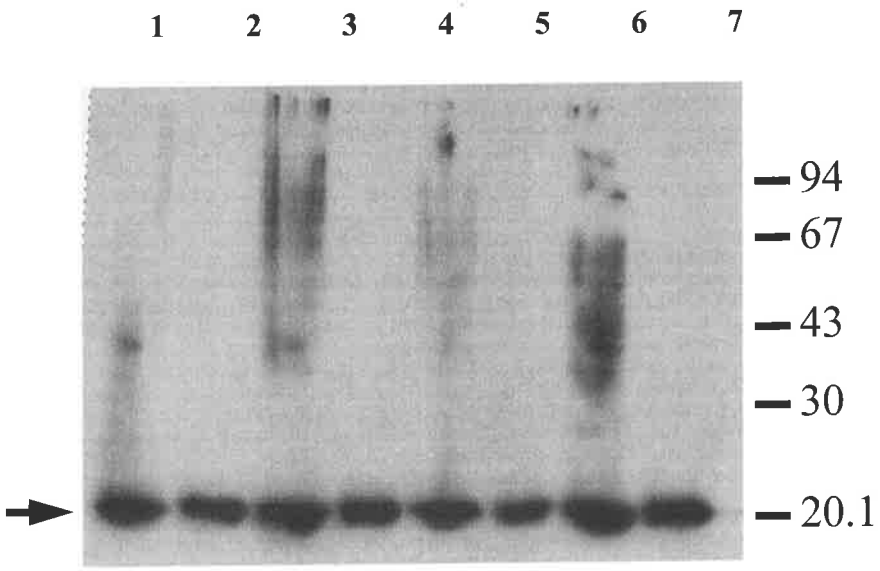


Fig. 4.16. An autoradiograph shows TcpA after crosslinking by exposure of whole cells to DSP. Visible smearing with discernible bands are seen in the tracks in which crosslinks have not been subsequently reduced in β-mercaptoetheanol prior to SDS-PAGE and transfer for immunoblot. TcpA is detected with Tc20.2, a murine monclonal antibody. Bound antibodies are detected by incubation with secondary antibody (goat-antimouse) and development of conjugated horseradish peroxidase activity by the ECL method, as described (Fig. 4.11). Lanes 1, 2: 569B (non-reduced; reduced); Lanes 3, 4: V663 (569B *rfb*) (non-reduced; reduced); Lans 5, 6: JRI2 (569B $tcpT^{km}$) (non-reduced; reduced); Lane 7, 8: JRI3 (V663 $tcpT^{km}$; i.e. 569b *rfb, tcpT*) (non-reduced; reduced).

4.4.3 Fucose-resistant haemagglutination by translocation-arrested TcpA subunits

Fucose-resistant haemagglutination of murine red blood cells by TCP (Taylor et al, 1987) is absent in *tcpG* mutants, despite assembly of macroscopically normal pilin bundles (Peek and Taylor, 1990). Deletions in the disulphide loop region of *P. aeruginosa* type-4 pilin also lead to an assembly-competent adhesion-defective phenotype, confirming that this region is essential for function but not assembly

(Farinha *et al.,* 1994). Z17561, JRI, 569B and V663 cells were grown in TCP inducing conditions, pelleted briefly and resuspended in Tris-HCl (30mM, pH 8.0) sucrose (20%) at ca. $2x10^{11}$ cfu/ml. Results are presented in Table 4.3

Table 4.3 Fucose-resistant haemagglutination in French press lysates

	Z17561 (AKI)	Z17561 (NB)	JRI1 (AKI)	JRI1 (NB)
unlysed whole cells	**32, 64, 32, 64 (48)**	**2, 4, 4 (3.3)**	**1, 2, 2, 2 (2)**	**2, 2, 2 (2)**
whole cell lysates	**32, 64, 64 (53)**	**nd**	**32, 64, 32 (42.7)**	**nd**
whole cell lysates + Tc20.2	**8, 32, 16 (18.7)**	**nd**	**8, 8, 16 (10.7)**	**nd**

Table 4.3 Wild-type (Z17561) and *tcpT* mutant (JRI1) strains were grown in TCP-inducing (AKI) conditions or in nutrient broth (NB, which results in no detectable TcpA by immunoblot) for haemagglutination. Taking as unity the undiluted lysates, the highest reciprocal of serial two-fold dilutions (represented as whole numbers) are given at which haemagglutination was clearly present in fresh murine (Balb/c.) red blood cells. The results of several individual haemagglutination experiments on identical samples are given, with the arithmetic mean presented in brackets underneath (note that Z17561 and JRI1 whole cells in AKI conditions were compared on one extra occasion). Bacterial cell count was estimated by haemocytometer, and adjusted to ca. 5 x 10^{11} cfu/ml. TCP induction was confirmed by visible agglutination of broth cultures and by TcpA immunoblot.

Fucose-resistant haemagglutination titres of JRI1 cells lysed in a French pressure cell are compared with the levels of TcpA on immunoblot by serial two-fold dilutions to extinction (refer to Fig. 4.9), to demonstrate that the results are not explained simply by reduced levels of TcpA being present in JRI1 (Table 4.4, overleaf).

Table 4.4 TcpA-mediated haemagglutination (HA) in serial dilutions of lysates

a. French pressure cell-derived lysates

Lane	dilution	TcpA	HA
1 (wt)	1:1	detected	+
2 (JRI1)	1:1	detected	+
3 (wt)	1:2	reduced	-
4 (JRI1)	1:2	detected	+
5 (wt)	1:4	absent	-
6 (JRI1)	1:4	detected	+
7 (wt)	1:8	absent	-
8 (JRI1)	1:8	absent	+

b. Lysates derived by freeze-thawing and sonication

	Z17561 (AKI)	Z17561 (NB)	JRI5 (AKI)
membrane fraction	**32, 32 (32)**	**2,2 (2)**	**32, 16 (24)**
cytoplasmic fraction	**0**	**0**	**0**

Table 4.4

a. The HA result is indicated (+ or -) for serial dilutions of French pressure cell-derived whole cell lysates of Z17561 (wt) and JRI1. The Lane number refers to Fig 4.9, showing the same samples in a TcpA immunoblot, the result of which is indicated in the third column.

b. Lysates of Z17561 and JRI5 grown in TCP-inducing conditions (and confirmed by TcpA immunoblot) were prepared by successive cycles of freeze-thawing and sonication in PBS. These were tested twice and appear to mediate fucose-resistant haemagglutination in otherwise identical conditions to those for the samples described in Table 4.3. Bacterial cells were adjusted to approximately 3 x 10^{11} cfu/ml before lysis. Samples were serially diluted in two-fold dilutions.

The HA-negative phenotype of TcpA from *tcpT* mutants cannot be attributed to export failure alone, since non-surface presented TcpA in V663 lysates haemagglutinates relatively normally. A 10 to 20-fold decline in titre might be explained however by allowing that there is a roughly two-fold reduction in the amount of immunoreactive TcpA available (Figs. 4.9 and 4.14) and that most of the French press-derived vesicles would be inside-out. If TcpA is present on the periplasmic face of the cytoplasmic membrane, it might thus be internalised in the inside-out vesicles derived from cell lysis in the French pressure cell. Accordingly, lysates were prepared by freeze-thawing and sonication and were found to be competent for haemagglutination at levels comparable with TcpA from the parent strain (Table 4.4).

4.5 Concluding remarks

The *tcpT* gene product is here shown to be specifically and absolutely required for assembly of the Toxin-Coregulated Pilus (TCP). In the absence of its function, reduced amounts of processed pilin subunits are found in association with the cytoplasmic membrane. Transcriptional attenuation of genes downstream of the Tn*903*-derived kanamycin resistance cartridge has been previously demonstrated (Galan *et al.*, 1992), and the known requirement for sequence-identical *tcpE* and *tcpF* genes in TCP assembly by the classical *V. cholerae* strain O395 (Kaufman *et al.*, 1993; Ogierman *et al.*, 1993) necessitated the construction of discrete in-frame chromosomal deletions. Complementation studies confirmed the specificity of the *tcpT* mutation, and the essential nature of the Walker A ATP-binding motif. Mutations in *tcpT* are shown to

completely abrogate assembly of TCP in *V. cholerae,* and the TcpA subunit is shown by a number of different techniques to be localised to the cytoplasmic membrane. Subunit associations in these mutants are indistinguishable from those in normally assembled TCP, and the *tcpT* phenotype is shown to be the same in the background of isogenic *rfb* strains, in which transport of subunit across the outer membrane is defective (Section 4.3.6). The presence of normal fucose-resistant haemagglutination of murine red cells in the presence by TcpA also suggests that subunits have been normally folded by the periplasmic action of TcpG. The absence of this activity from French press lysates of JRI1 might be explained by the predominance of inside-out vesicles, and is consistent with a location on the periplasmic face of the inner membrane. Several attempts were also made to attack TcpA with trypsin and proteinase K, both in whole cells in the presence and absence of EDTA and in freeze-thaw/ sonicated lysates. Conditions in which TcpA was partially but not completely degraded were not achieved, but such an approach might still prove fruitful once appropriate conditions were defined. The evidence for N-terminal association of subunits (cross-linking, salt and detergent extraction) and for C-terminal folding (fucose-resistant haemagglutination) suggest that TcpA has been exposed to the action of the periplasmic disulphide oxidoreductase, TcpG, previously shown to be essential for the specific haemagglutinating activity of TcpA (Peek and Taylor, 1990). Thus, *tcpT* is required after the processing and N-terminal association of TcpA subunits in a stable fashion similar to their final configuration, but prior to that step in translocation which is blocked in isogenic *rfb* mutants (Section 3.3). Crosslinked trimers and tetramers are consistent with an organised subunit association rather than random or casual association, as suggested by the requirements for Urea treatment to solubilise subunits. Attempts to cross-link

overexpressed TcpA in an *E. coli* background were unsuccessful, resulting in (unprocessed) monomers, lysates of which were also incompetent in fucose-resistant haemagglutination experiments. The different host background and the requirement for high-level expression make this difficult to interpret, but would be consistent with the failure of TcpA to pass the cytoplasmic membrane.

Type-4 pilin biogenesis appears to be initiated by specific cleavage of pilin substrate by the unique peptidase in a Sec-independent fashion, and this same peptidase is capable of cleaving heterologous pilin subunits as well as non-pilin substrates in most systems (Section 1.11). The finding of subunit association after this stage is therefore to be expected, and one would further predict that the cross-linking exhibited by TcpA in the translocation mutants described here would be found in such heterologous pilin subunits as could be cleaved by the peptidase, including export-incompetent forms (such as large C-terminal fusions to proteins such as alkaline phosphatase) (Dupuy *et al.,* 1991). Substrate specificity is probably greater at or beyond the point which requires TcpT (see Sections 1.11, 1.12, and 3.8). Thus, the appropriate localisation or trans-cytoplasmic membrane passage of type-4 pilin-like proteins in other systems are not expected to be directly influenced by mutations in TcpT homologues, although secondary effects of misassembled or dysfunctional cytoplasmic membrane complexes may alter their stability. The requirement for the encoded ATP-binding site suggests therefore that energy from ATP hydrolysis is required for release of TcpA from the periplasmic face of the cytoplasmic membrane, but not engagement of the outer membrane translocation pore.

Chapter Five

Cytoplasmic Membrane-Association of TcpT *in vivo*

5.1. Introduction

It has been shown that TcpA is trapped at the level of the cytoplasmic membrane in *tcpT* mutants (Section 4.3). Since we also know that TcpT homologues are essential in outer membrane translocation systems, it is important to understand the exact localisation and relationships of the TcpT protein.

The unique type-4 prepilin peptidase, and cleavage specificity for the distinct leader peptide, are the defining features of the type-4 pilin system and related transport pathways (reviewed in Hobbs and Mattick, 1993), and may be shared between pilin and non-pilin pathways in some bacteria (Strom *et al.,* 1993). Indeed, the peptidase substrates universally found in non-pilin systems are sometimes termed "pseudopilins" because of the close homology to type-4 pilin sububits, especially in the N-terminal third of the protein (Bally *et al.,* 1992; Pugsley, 1993b). This N-terminal region in the processed substrate is thought to form the interacting structural backbone between subunits in pilin systems (Parge *et al.,* 1995), although attempts to demonstrate such an association between non-pilin ("pseudopilin") substrates of the peptidase in the general secretory pathway have not been successful (Pugsley and Possot, 1993).

The current assembly model for type 4 pili assumes Sec-independent translocation across the cytoplasmic membrane after processing, with N-terminal association of subunits at an early stage (Hobbs and Mattick, 1993; Parge *et al.,* 1995). The periplasmic action of a disulphide bond oxidoreductase is required to fold the externally presented highly conserved disulphide loop in which, or adjacent to which, are the major epitopes essential for function (Sun *et al.,* 1991; Lee *et al.,* 1994; Sheth *et al.,* 1994). Sec-independent processing of TcpA has been shown (Kaufman *et al.,* 1991), and the recently solved crystal structure of the *Neisseria gonorrhoeae* MS11 PilE subunit (Parge *et al.,* 1995) helps to explain the conserved structural features of type IV pili (Dalrymple and Mattick, 1987). A periplasmic disulphide bond oxidoreductase is required for normally functional TCP but not for assembly and export (Peek and Taylor, 1990), and deletions of the disulphide loop in *P. aeruginosa* type 4 pili affect adhesion but not assembly (Farinha *et al.,* 1994). Accumulation in the periplasm and outer membrane of export-arrested TcpA subunits capable of mediating the haemagglutination characteristic of assembled TCP are consistent with these data (Section 3.3), but there are very little data currently available which describe the fate of the type 4 pilin subunit between processing and final assembly as the external fibre.

EpsE is a TcpT homologue essential for cholera toxin export, and has been shown to stably associate with an integral inner membrane protein, EpsL. The specificity of this interaction is attributed to the N-terminus of EpsE, and the data suggest that TcpT particpates in an ATP hydrolysing cytoplasmic membrane complex which transduces energy for substrate export across the outer membrane (Sandkvist *et al.,* 1995). Homologues of EpsE and TcpT are required in a number of such outer membrane

transporters (Possot and Pugsley 1994, Sandkvist *et al.,* 1995, Christie *et al.,* 1989, Stephens *et al.,* 1995), which export from a periplasmic pool of substrate intermediate in a distinct two-step pathway (Hirst and Holmgren, 1987a,b; Poquet *et al.,* 1993).

Data from homologues in other systems strongly suggests that the hydrophilic TcpT protein is peripherally associated with the cytoplasmic membrane, possibly by complexing with integral membrane proteins encoded by the *tcp* operon. This chapter describes attempts to explore the normal context of TcpT *in vivo.* Expression of *tcpT* was performed *in vitro* to confirm the open reading frame, and issues relating to transcriptional attenuation are addressed. Protein purification and the raising of a polyclonal antiserum permitted precise subcellular localization and a better understanding of the association of the protein with other cellular elements. TcpT is a 57 kDa hydrophilic protein, and one member of a highly conserved superfamily of cytoplasmic membrane-associated ATP-binding proteins which is widely represented in type-4 pilin operons and related transport systems (Pugsley, 1989; Christie *et al.,* 1989; Turner *et al.,* 1993; Possot and Pugsley 1994; Sandkvist *et al.,* 1995). Expression of TcpT from a plasmid in the presence and absence of other members of the *tcp* operon is therefore described below, along with the effect of various N-terminal fusions of TcpT in complementation studies.

5.2. Attenuated transcription from the *tcpT* gene

5.2.1. TcpT is poorly expressed *in vitro* from the 4.8kb *Bgl*II fragment

TcpT is predicted to be a 57.5 kDa protein, encoded from near the promoter-distal end of the transcriptionally linked *tcp* operon (Ogierman *et al.,* 1993). The *Bgl*II fragment harbouring *tcpT, E,* and *F* was subcloned under the control of the T7 promoter to create pPM4101 and pPM4102. pPM4105 and pPM4107 represent *tcpT* and *tcpF* (without a ribosome binding site (RBS) sequence) respectively, both as *Cla*I cutdowns of pPM4103, and *tcpE* was cloned as a *Hin*dIII-*Spe*I cutdown of pPM4101 (to create pPM4108). These constructs have already been described in Fig.4.6 and Table 4.2. Fig. 5.1 shows autoradiographs of the [^{35}S]-methionine-labelled T7-polymerase expression products of pPM4101, pPM4102, pPM4105, and pPM4107 after electrophoresis on SDS-PAGE and drying for exposure to X-ray film.

tcpS encodes a protein of 152 residues including a typical signal prepeptide. The signal prepeptide is predicted to be susceptible in *E. coli* to cleavage by the Lep prepeptidase, resulting in a 17.4 kDa preprotein and a relatively hydrophilic 15.2 kDa mature form which may be periplasmic or exported (Ogierman *et al.,* 1993). Expression products of *tcpS* in *E. coli* have been shown to migrate at ca. 19 and 20 kDa on SDS-PAGE (Ogierman *et al.,* 1993), and there are bands (marked S1-4) in Fig. 5.1 migrating at between 20 and 15 kDa that could be attributed to TcpS and degradation products of TcpS and/ or other products of the *Bgl*II fragment in pPM4101 and pPM4102.

Fig. 5.1. T7-polymerase expression of *tcpT, E, and F* in *E. coli* DH5α

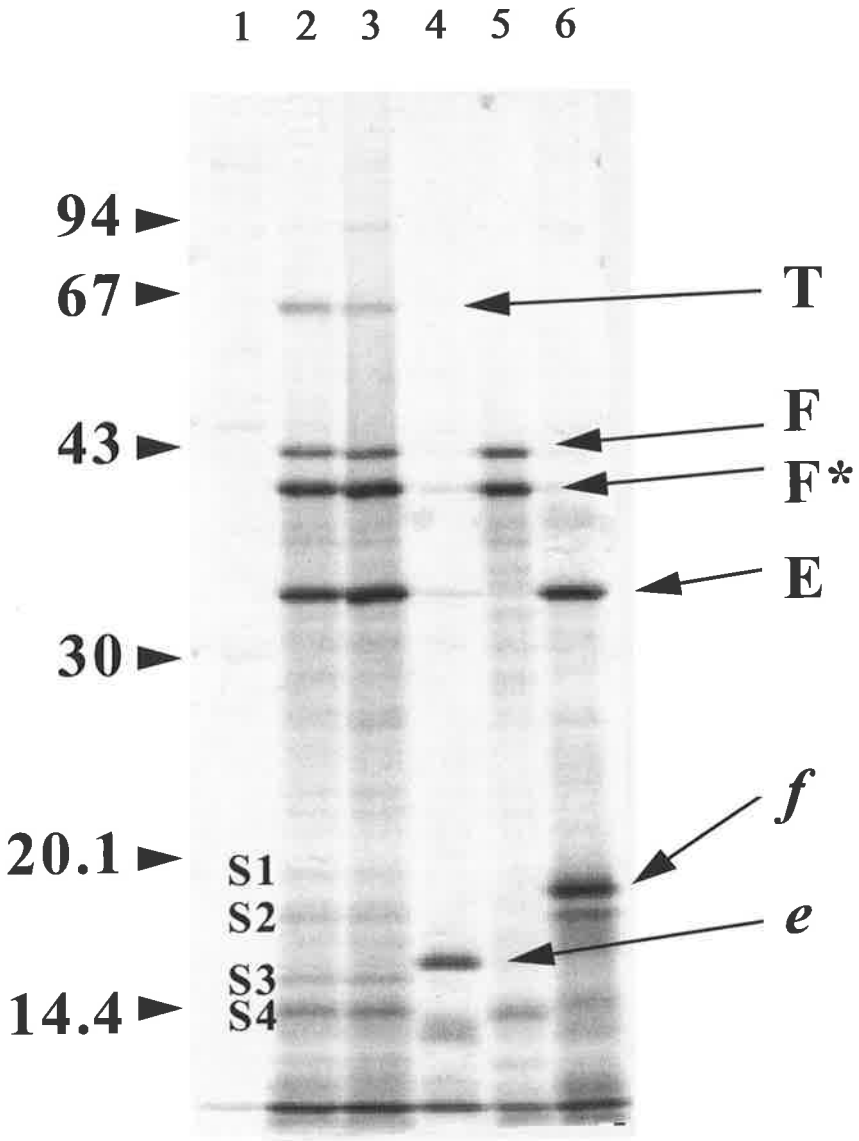


Fig. 5.1: Autoradiograph after drying of [^{35}S]-methionine labelled products electrophoresed on 12% acrylamide SDS-PAGE. Lane 1: (vector) pBluescript SK; Lane 2: pPM4101; Lane 3: pPM4102; Lane 4: pPM4105 encodes TcpT (**T**) with ca. 17 kDa of *tcpE* (***e***) from a *ClaI* cutdown of pPM4101 (thereby abolishing the *tcpT* RBS, and TcpT mRNA translation). Lane 5: pPM4107 encodes TcpF (**F**) and a lower molecular size form consistent with loss of the predicted signal prepeptide (**F***). These are also products of pPM4101 and pPM4103. Lane 6: pPM4108 (*Hin*dIII-*Spe*I cutdown of pPM4103, to give *tcpE* (**E**) and half of *tcpF* (***f***). S1, S2, S3, and S4 mark bands that appear to be unique to the *Bgl*II fragment (although S2 may be mirrored in lane 6), and may represent *tcpS* product/s (see text).

tcpE has been reported previously as a 38 kDa protein (Ogierman *et al.,* 1993; Kaufman *et al.,* 1993). The originally predicted RBS is unusual, and the spacing of 12 nt from the ATG would be rare indeed if it were a functional start codon (Fig. 5.2, below). T7 expression experiments clearly show TcpE to be approx. 31.5 kDa, which is consistent with a more typical RBS sequence that is found 171 nt (57 residues) further in the 3' direction (Figs. 5.2 and 5.3), 4 nt from an ATG codon.

When *tcpT* is expressed *in vitro* in the presence of [^{35}S]-methionine from the T7 promoter using the native RBS sequences (Fig. 5.1, above), the amount of radiolabelled product is markedly less than that from the two downstream genes *tcpE* and *tcpF*. Although a palindromic sequence is apparent in the precoding region of *tcpT* (nt #-22 to -34), there are no obvious potential stem-loop structures that might directly sequester the RBS (Fig 5.2a), nor does a search of the region from the *Bgl*II cleavage site to the ATG codon of *tcpT* using DNAsis (Hitachi Software) reveal any significant stem-loop structures that might otherwise diminish the expression of *tcpT*. The small AT-rich palindromic structure in the late coding region of *tcpS* (Fig. 5.2a) would not be expected to greatly attenuate transcription of the *tcpT* gene (Ogierman *et al.,* 1993), and the two transcriptionally linked genes downstream of *tcpT* are evidently not affected.

A search done by reference to the codon usage of *V. cholerae* (Stroeher, 1992) and of *E. coli* (Anderson and Kurland, 1990) discloses no apparent requrement for rare tRNA species in the N-terminal region removed from TcpT (to form TcpT*, the product of pPM4110). In particular, while more than half of the Arg residues within the deleted

region were encoded from CGT or CGC codons, only one each of the rare CGG and AGG codons were found (at positions #46 and #111, respectively). No other codons calling for rare tRNA species in *E. coli* (Anderson and Kurland, 1990) were identified in the deleted region.

In order to explore whether protein instability or reduced translation of *tcpT* mRNA (due to a poor or sequestered RBS, or due to a requirement for rare tRNA species) was at issue, a number of different constructs were made (Fig. 5.2). The 2.1 kb *Nde*I-*Spe*I fragment of pPM4101, encoding all of TcpE and a truncated form of TcpT, was subcloned into the T7-driven pET-17b expression vector (Novagen, USA) to create pPM4110. pPM4105 (the 1.8kb *Cla*I fragment cloned into pBluescript SK, under conmtrol of the T7 promoter) was also subjected to oligonucleotide-directed mutagenesis in order to introduce an optimal RBS in front of *tcpT*, thereby creating pPM4142 which served as a useful control in T7 expression experiments (see Table 5.1 and following text; also Figs. 5.1 and 5.2 - 5.4).

Fig. 5.2 The precoding region and RBS sequence of *tcpT*

a. The tcpT precoding region from -61 nt to the ATG codon

1 Gly Trp Lys Phe Ile Glu Asp Gln Asn Lys Ile Gln Phe Phe
2 GGT TGG AAA TTC ATT GAA GAC CAA AAT AAA ATT CAA TTT TTC
3 -61 -34

1 Lys Gly Asn Lys Val Ile Asp Val Asn * (TcpS)
2 AAG GGC AAT **AAG GTA** ATC GAT GTC AAT TGA
3 -19 -10 -7 Met (TcpT)

b. Comparative RBS sequences

tcpT	TAAGGTAAT*CGATG	(*Cla*I)
SD	**AAGGAGGT** (5-8nt†) ATG	
pPM4110	AG**AAGGAGA**TATACA*TATG	(*Nde*I)
#1047(5'-3')	TCG*GTACC**AGGAGGA**TAAT*CGATGTCAATTGATA	(*Kpn*I, *Cla*I)
pPM4142	**GGAGGA**TAAT*CGATG	
tcpE	T**AAGGA**TAGGATG	
prev. *tcpE*Φ	**GAGGATGAA**AATATAATATATG	
tcpF	T**GAGGA**TATATG	
epsE	C**GAGGAGA**AAAAATAATG	

Fig. 5.2 The precoding region and RBS sequence of *tcpT* .

a. The precoding region of *tcpT*. 1: Predicted amino acid sequence of TcpS in standard 3-letter code. 2: DNA sequence, with the RBS sequence (nt #-7 to -10) in bold and a palindromic sequence (nt #-22 to -34) underlined. 3: Nt number with respect to ATG of *tcpT* and predicted amino acid sequence in standard 3-letter code.

b. Sequences of constructs encoding genes are given up to the start codons, and the entire sequence of oligo #1047, isogenic to *tcpT* from 5' of the ATG codon) is given. SD: Shine-Dalgarno optimal RBS sequence (Shine and Dalgarno, 1975); †optimal spacing between RBS and ATG: 7 nt. Potential RBS sequences are in bold type and restriction endonuclease sites are underlined (*cleavage point; enzyme stated after sequence). ΦThis sequence (prev. *tcpE*) is the previously predicted *tcpE* (Ogierman *et al.*, 1993; Kaufman *et al.*, 1993) and is not associated with a detectable expression product (see Fig. 5.1). *epsE* encodes the TcpT homologue responsible for Ctx export (Section 1.12; Sandkvist *et al.*, 1993).

5.2.2 Codon usage or ribosome binding site ?

The *tcpT* RBS sequence itself is not strikingly unusual on first inspection, and is comparable to the Shine-Dalgarno RBS sequence and RBS sequences from relevant genes (Fig. 5.2b). The unique *Nde*I site of *tcpT* permits cloning into a start codon present within the unique *Nde*I site (CA/**TATG**) of pET-17b, itself placed optimally with respect to an RBS sequence and the -10 and -35 sequences, without disruption to the open reading frame of the newly added *Nde*I-*Spe*I fragment downstream. pPM4110 therefore differs from pPM4101 and pPM4102 essentially in that the first third of the *tcpT* gene is missing and the remainder is positioned downstream from the (vector-encoded) optimal RBS. The transcriptional linkage of *tcpT* and *tcpE* (Section 1.12.6) means that labelling of TcpE serves as an internal control in expression experiments, and attests to message stability.

Expression of the radiolabelled products of pPM4110, pPM4101, and pPM4102 is shown in Fig. 5.3 (overleaf). It can be seen that the 38 kDa product (TcpT*) is present in equal amounts to TcpE in pPM4110 despite losing 6 of the 11 methionine residues available for ^{35}S-labelling in the N-terminal truncation, while TcpT expressed from the large *Bgl*II fragment within pPM4101 or pPM4102 is barely detectable. This implies either that the full length TcpT is unstable when expressed in *E. coli*, that there is a requirement for rare tRNA species encoded in the first third of the gene (deleted in pPM4110), or that diminished transcription of *tcpT* occurs from an inefficient or sequestered native RBS in the *Bgl*II fragment (pPM4101/ pPM4102).

Fig. 5.3 Expression of truncated TcpT from an optimal RBS in *E. coli* DH5α

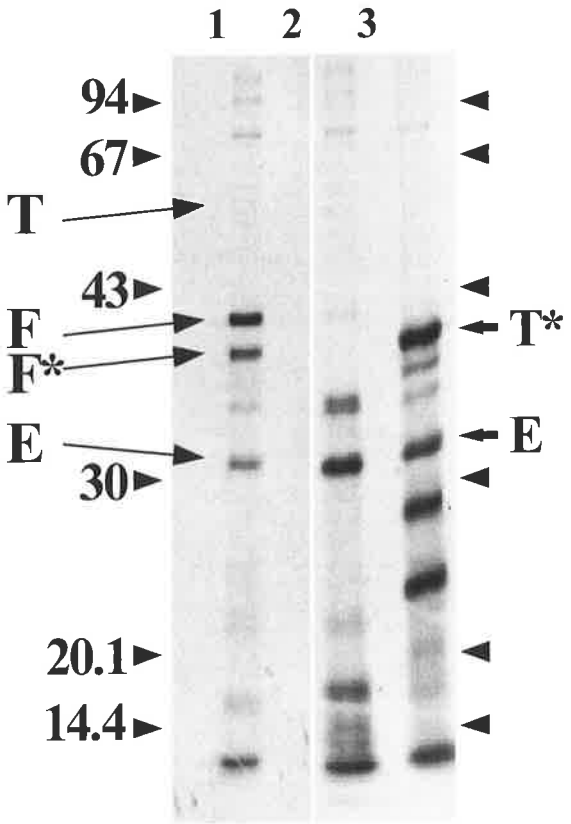


Fig. 5.3 Autoradiograph of T7-expressed [^{35}S]-methionine labelled proteins after SDS-PAGE and drying. Expression of a truncated form of TcpT from an optimal RBS in pPM4110 (**T*** is missing the N-terminal one third of TcpT). TcpE (**E**) is encoded by both pPM4101 and pPM4108 (Lanes 1 and 2; see also Fig. 5.1) for comparison with T7-expressed [^{35}S]-methionine labelled products of the *Nde*I-*Spe*I fragment (pPM4110), encoding the truncated TcpT (T*) and TcpE (E). Molecular size markers are shown (solid triangles at both margins), and actual values indicated along the left hand border in kDa.

5.2.3 Substitution of the RBS sequence facilitates expression of TcpT *in vitro*

The markedly increased expression of the truncated TcpT* product from pPM4110 therefore suggests that either the *tcpT* RBS sequence is indeed suboptimal, or that the TcpT N-terminal region (which is absent from TcpT*) is responsible for instability in *E. coli*. An optimal RBS sequence was therefore introduced by oligonucleotide-directed mutagenesis in front of *tcpT*, by subcloning *tcpT* as the product of PCR primer #1047 (Fig. 5.2) and the M13 (forward) primer (using pPM4105 as template) into pBluescript SK. This results in pPM4142, which differs from pPM4105 in the incorporation of an

RBS sequence (lost in pPM4105 in the cleavage of the *Cla*I site) and in the alteration of the potential loop region (nt #-22 to -34) by introduction of the *Kpn*I site (refer back to Fig. 5.2), and differs from pPM4110 in harbouring only the complete *TcpT* gene.

To eliminate possible variations in TcpT stability (since the protein is predicted to participate in a cytoplasmic membrane complex) or the availability of rare tRNA species in *E. coli*, pPM4142 was also expressed in a *V. cholerae* background. In order to do this, the T7 RNA polymerase-encoding plasmid pGP1-2 (Tabor and Richardson, 1985) was first electroporated into *V. cholerae* JBK70 to create JRI7, and pPM4105 and pPM4110 introduced separately thereafter. Cultures were grown in Tcp-inducing conditions prior to T7 polymerase induction (refer to Sections 7.2.2 and 7.2.5).

It can be seen in Fig. 5.4 (overleaf) that TcpT is readily expressed from pPM4142 in *V. cholerae* and *E. coli,* and that expression in the *V. cholerae* background is no greater than in *E. coli*. It is therefore unlikely that diminished labelling is due to requirements for tRNA species that are rare in *E. coli*, consistent with the predictions made above (Section 5.2.2).

Fig.5.4 Radiolabelling of TcpT expressed from pPM4142 in *V. cholerae* and *E. coli*

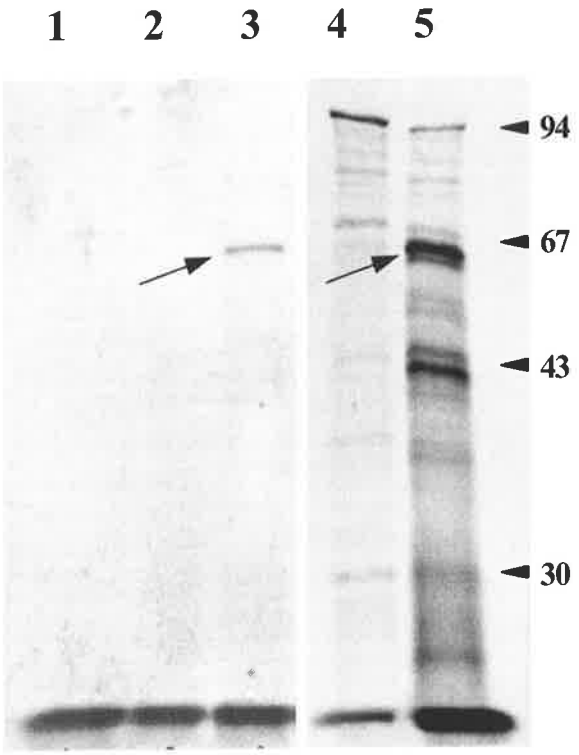


Fig. 5.4 Autoradiograph after drying of [^{35}S]-methionine labelled products electrophoresed on SDS-PAGE. TcpT (arrows) is expressed from pPM4142 in *V. cholerae* JRI7 (JBK70[pGP1-2]) (lane 3) and *E. coli* DH5α (lane 5). Lanes 1 and 4 contain vector only, in *V. cholerae* and *E. coli* respectively. Lane 2 contains pPM4143 (opposite orientation to pPM4142). Molecular weight is given along the right hand margin in kDa.

5.3. TcpT can be purified from the cytoplasm of *V. cholerae* when maximally expressed *in vitro*

5.3.1 Maximal expression of TcpT as fusion proteins

In order to harvest large amounts of TcpT for protein purification and for the raising of antisera, *tcpT* (from pPM4101) was cloned into the expression vectors listed in Table 5.2. The *tcpT*^{20} mutation was also subcloned from pPM4114 (see Fig. 4.4) as an *Nde*I-*Spe*I fragment into pPM4133 and pPM4136 to create pPM4134 and pPM4137, respectively (Table 5.2). These vectors all contain a powerful *tac* promoter optimally

spaced from the vector RBS, the *lacI* gene *in cis*, and an MCS to facilitate cloning in the correct reading frame for the RBS. Several clones were picked from each cloning experiment and tested by restriction enzyme analysis and fusion junction sequencing.

Table 5.2 TcpT in specific expression vectors

plasmid number	gene product	host vector	controlling promoter
pPM4133	$TcpT^{F}$	pFLAG-MAC (IBI)	*lacZ*[1]
pPM4134[2]	$TcpT^{F20}$	pFLAG-MAC (IBI)	*lacZ*
pPM4135[3]	$TcpT^{C}$	pFLAG-MAC (IBI)	*lacZ*
pPM4136	$TcpT^{Q}$	pTTQ181 (Stark, 1987)	*lacZ*
pPM4137	$TcpT^{Q20}$	pTTQ181 (Stark, 1987)	*lacZ*

[1] The pFLAG-MAC, pFLAG-CTC, and pTTQ181 all additionally provide the *lacI* gene *in cis*
[2] pPM4134 and pPM4137 are identical to pPM4133 and pPM4136 respectively, except for deletion of the 60 nt encoding the Walkjer A box of *tcpT*
[3] pPM4135 is isogenic with pPM4133 except for the region encoding the FLAG ($AspTyrLysAsp^{4}Lys$) octapeptide in $TcpT^{F}$, absent from $TcpT^{C}$ - details in Section 5.5.2

pPM4133 and pPM4134 encode the N-terminal FLAG octapeptide which is fused in-frame, along with a small section of the MCS, with the N-terminus of TcpT. The pFLAG-MAC vector system (International Biotechnologies Inc., USA) offers a specific monoclonal antibody (M2) to the N-terminal (FLAG) epitope, and a corresponding affinity column containing the M2 antibody coupled to agarose beads. The pFLAG-CTC vector is identical to pFLAG-MAC except for the absence of the N-terminal (FLAG) octrapeptide. The protein product ($TcpT^{F}$) can be found in inclusion bodies as well as in membrane and cytoplasmic fractions, when maximally expressed from

pPM4133 in *E. coli* (Fig. 5.5) and *V. cholerae* (Fig. 5.6), and TCP induction does not appear to affect the distribution of maximally expressed $TcpT^F$ from pPM4133 in *V. cholerae*.

Fig. 5.5 Maximal expression of $TcpT^F$ in *E. coli* DH5α

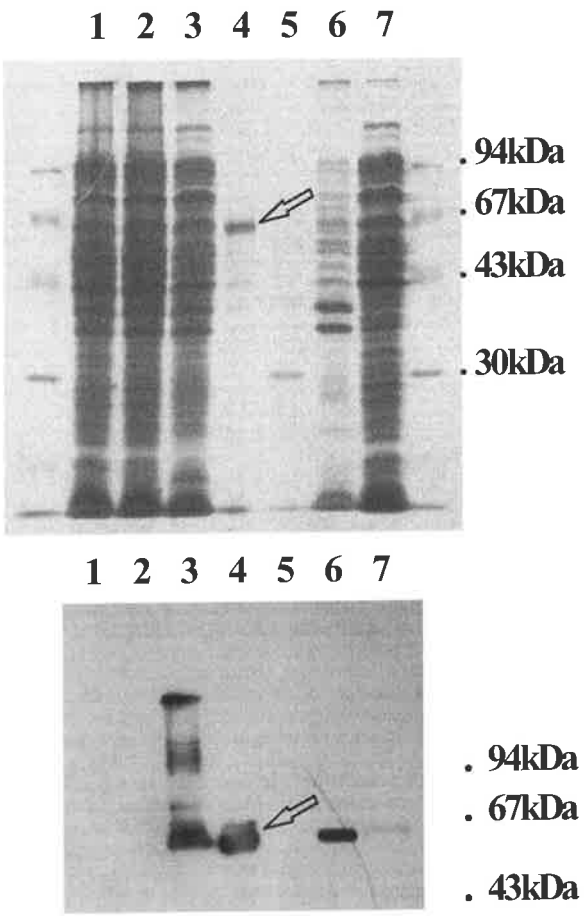


Fig. 5.5 Cellular fractions after IPTG-induced derepression of pPM4133 are subjected to SDS-PAGE and Coomassie Brilliant Blue staining (upper panel), and to transfer and immunoblot with the M2 monoclonal antibody to the N-terminal FLAG epitope in $TcpT^F$ (lower panel). Lanes 1 and 2: vector alone; lane 3: pPM4133; lane 4: inclusion body fraction; lane 5: periplasmic fraction; lane 6: membrane fraction; lane 7: cytoplasmic fraction. Fractions were derived as described in Section 7.2.6 (Method 1), and loaded according to equal representation (according to volume of original whole cell fraction). Molecular sizes are given along the right side of both panels, in kiloDaltons. The location of the band of expected size (57.5 kDa) is indicated in both panels (arrow). Slowly migrating components as well as apparent degradation products are also seen (lower panel, lanes 3 and 4).

Fig. 5.6 Maximal expression of TcpT^F in *V. cholerae* Z17561

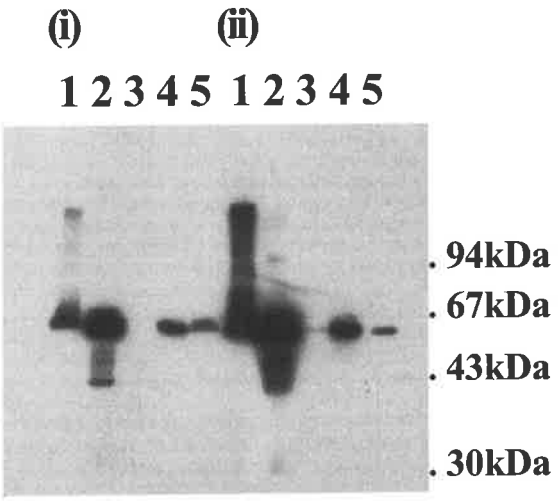


Fig. 5.6 TcpT^F expression from pPM4133 in *V. cholerae* in nutrient broth at 37°C (i), and in AKI (TCP-inducing) conditions (ii), is here shown after transfer and immunoblot of subcellular fractionations, which were treated as in Fig. 5.5. Loading was performed according to representative volumes, as follows: lane 1: whole cells; lane 2: inclusion bodies; lane 3: periplasm; lane 4: membrane fraction; lane 5: cytoplasm.

Figures 5.5 and 5.6, taken together, suggest that TcpT^F can be found in the membrane fraction of *E. coli* and *V. cholerae* in the absence of TCP induction. Refractile "inclusion bodies" could be seen under phase contrast microscopy after high level expression in this manner, consistent with the formation of membrane-bound insoluble aggregates. The high level of expression complicates the interpretation of membrane localisation, but there is an apparently increased representation of TcpT^F in the membrane compared with the cytoplasmic fraction in TCP-induced *V. cholerae*, which prompted further examination of the influence of other *tcp*-encoded proteins on the subcellular localisation of TcpT and will be discussed later in this chapter. Slower migrating and faster migrating forms on SDS-PAGE are visible in Figs. 5.5 and 5.6.

5.3.2. Maximally expressed TcpT can be recovered from the cytoplasm

TcpT is predicted to be highly hydrophilic (Ogierman *et al.*, 1993), and affinity-purification of the $TcpT^F$ protein was achieved from the cytoplasm of both DH5α[pPM4133] and JRI1[pPM4133] by employing an affinity column with the M2 monoclonal antibody (directed against the FLAG epitope of $TcpT^F$) coupled to agarose beads. Elution was performed with 0.1M glycine/ HCl (pH 3.0)/ PBS into equilibrating Tris buffer, according to the manufacturer's instructions (Fig. 5.7, overleaf).

Immunoblot of maximally expressed $TcpT^F$ in the cytoplasmic fraction of DH5α (in lane 1 of Fig. 5.7) again shows a slower migrating species on SDS-PAGE, as also seen above (Figs 5.5 and 5.6). Note that slowly migrating species of $TcpT^F$ (Fig. 5.8: panel b, lane 1) are less prominent in successive eluates (Fig. 5.7; panel c). There is relatively poor initial elution in Lane 7, presumably as a result of dilution of the eluting buffer in the TBS-washed column. This suggests that these slowly migrating $TcpT^F$ species consist of large aggregates that are either washed out early, or broken down by acid exposure (pH3.0), to the 57.5 kDa $TcpT^F$ monomer. No degradation products were observed in the eluates. Eluates were collected directly into a neutralising buffer (Tris 1.0M pH8.0) and stored at -20°C in the presence of a protease inhibitor (PMSF ImM) (see Section 7.4.4 for methodological details).

Fig. 5.7 Affinity purification of TcpT^F from the cytoplasm.

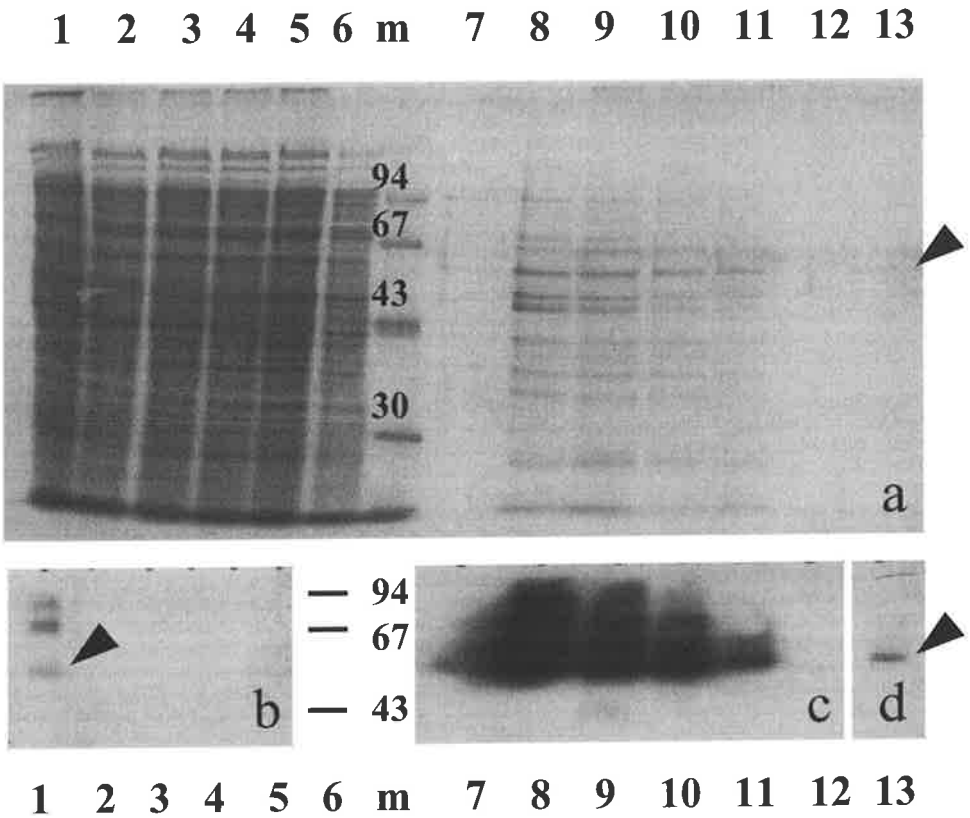


Fig. 5.7 Binding and subsequent elution of maximally expressed TcpT^F from the cytoplasmic fraction of DH5α[pPM4133]. Panel a shows the cytoplasmic fraction after each passage, after SDS-PAGE and staining with Coomassie Brilliant Blue. Lane 1 contains the original sample; lanes 2-6 illustrate successive passages through the column; lanes 7-12 show successive 1 ml eluates after addition of glycine 0.1M/ HCl pH 3.0. After collection and two further passages, a relatively pure (and dilute) fraction is obtained, which remains completely soluble at pH 7.4 (Panel d; arrowhead). Panel b-d show the corresponding M2 immunoblots after transfer, developed with the ECL method. Molcular sizes (in kDa) are shown (m), and indicated by bars adjacent to the right of panel b (lane 6).

5.3.3 Purified protein could not be shown to bind [γ-^{32}P] ATP *in vitro*

A number of attempts were made to demonstrate ATP-binding activity by the TcpT protein. Binding of ATP to affinity-purified TcpT^{F} could not be demonstrated by incubation (in PBS in the presence or absence of 5mM $MgCl_2$) with γ-[^{32}P] ATP according to the method which had been successful for EpsE (Sandkvist *et al.,* 1995). *V. cholerae* were therefore grown in *tcp*-inducing conditions prior to expression of TcpT^{F} and TcpT^{F20} (from pPM4133 and pPM4134, respectively), in order to provide a native background to test for such activity. TCP induction was confirmed by the presence of visible clumping and by immunoblot for TcpA using polyclonal α569B-165 antiserum, and TcpT^{F} and TcpT^{F20} expression was confirmed by immunoblot using M2 monoclonal antiserum. Membrane samples were lysed in PBS by freeze-thawing and sonification.

Cross-linking of γ-[^{32}P] ATP to the ATP-binding site by successive oxidation and reduction (Scherer *et al.,*1990) was then attempted, in the presence of appropriate positive (*N. gonorrhoeae* P1 protein) and negative (TcpT^{F20}) controls. In view of the presence of a Walker B motif (typical of the Mg^{2+}-dependent ATPases) in TcpT (Section 1.12.4). $MgCl_2$ and EDTA were used in different samples. Despite clear evidence for activity *in vivo* of overproduced proteins and the TCP negative phenotype of the *tcpT* mutant strain, γ-[^{32}P] ATP activity could not be detected at an appropriate molecular weight (Fig. 5.8, overleaf).

Fig. 5.8 Failure to demonstrate ATP-binding *in vitro*

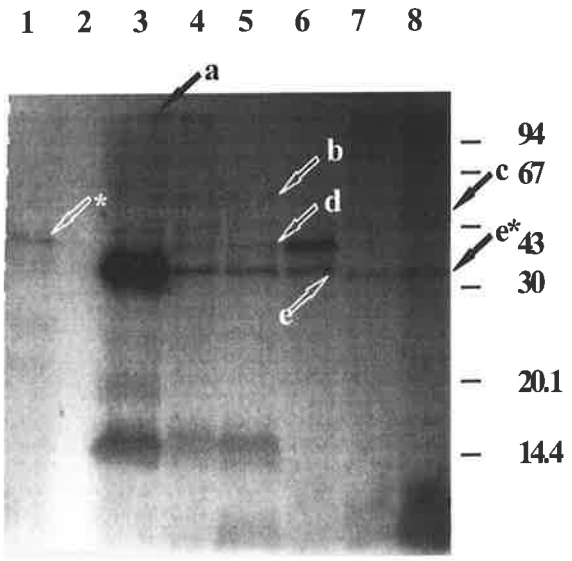


Fig. 5.8 Autoradiograph of SDS-PAGE to demonstrate binding of γ-[^{32}P] ATP. A purified extract (0.2 μg) of the *Neisseria gonorrhoeae* P1 protein was kindly provided (Prof. T. F. Meyer, Max-Planck Institut, Tuebingen) as a positive control sample in lane 1 (*). Loading is as follows: Lane 3: *E. coli* DH5α[pPM4133] (TcpT^{F}) in 5mM EDTA; 4: as for 3, with 5mM $MgCl_2$; 5: *E. coli* DH5α[pPM4134] (TcpT^{F20}) in 5mM EDTA; 6: as for 5, in 5mM $MgCl_2$; 7: Z17561[pPM4133] with 5mMEDTA; 8, as for 7, in 5mM $MgCl_2$. Molecular weights are given in kDa along the right margin.

E. coli samples produced faint high molecular weight bands (Fig. 5.8, a and b) that were present equally in pPM4134, from which the putative binding site has been deleted. A number of other bands of activity were identifed (c, d, e, and e*), but there was no band that appeared to be unique to TcpT^{F}. Is should be pointed out that ATP-binding activity of the EpsE protein (since TCP-inducing conditions are also Ctx secretion-inducing conditions) is likely to be present in lane 8 (with $MgCl_2$) but not lane 7 (with EDTA), and that the band marked as c may be consistent with this.

The Walker A motif and highly conserved residues within it have been shown to be essential for the function of TcpT homologues in pilin and non-pilin systems. The TcpT protein from which the Walker A motif-containing regions has been deleted produces a stable product similar to TcpT, and is found in the same fraction as TcpT *in vivo* (see

below, Fig. 5.9). It therefore seems that deletion of this region from TcpT does not affect its stability nor its subcellular fractionation by these measures, but completely abolishes its function. Inability to demonstrate ATP-binding suggests that the site is not available in these assays, or that the ATP-bound form is so unstable under the conditions described that the amount of this form is not readily detected.

5.3.4 Raising of antisera to TcpT

The monoclonal antibody to the N-terminal FLAG octapeptide of $TcpT^F$ has several useful applications, but antisera to the native protein is necessary in order to detect TcpT in the natural state in *V. cholerae*. Accordingly, maximally expressed $TcpT^F$ was harvested from *E. coli* to raise a polyclonal antiserum. The total protein concentration in the $TcpT^F$ inclusion body fraction depicted in Fig. 5.5 was estimated at 2 mg/ml by the BCA method (Pierce Chemicals, USA). 200µl (0.4 mg) of this was electrophoresed, excised as a band from SDS-PAGE, and prepared for immunisation of a rabbit in 200µg-equivalent doses after unambiguous identification by M2 immunoblot. Doses were divided and administered by deep subcutaneous injection at four different sites on each occasion.

After a course of six such immunisations at 2 week intervals, a test bleed (10ml) revealed that the antiserum recognised $TcpT^F$ protein in an immunoblot experiment at a dilution of 1:2000. The antiserum obtained was adsorbed against appropriate whole cell and membrane fractions to improve its specificity. This required the preparation of whole cells of *E. coli* DH5α (3 adsorptions) and *V. cholerae* JRI1 (3 adsorptions), as

well as membrane fractions of *V. cholerae* JRI1 (16 adsorptions) (Section 7.4.2). The resulting antisera is shown employed in immunoblots against various TcpT-containing preparations in Figs 5.9 and 5.10.

Fig. 5.9 Antisera raised against the TcpT^F protein recognises bands of different molecular sizes in *V. cholerae* Z17561 and JRI5

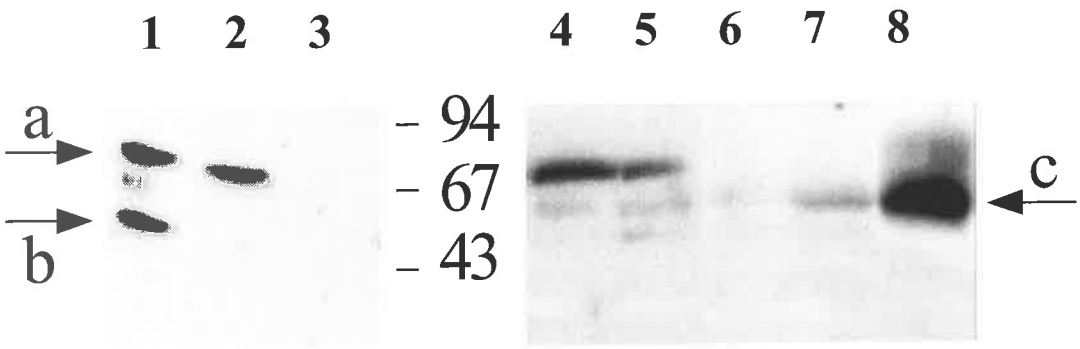


Fig. 5.9 Immunoblot with polyclonal antiserum to TcpT after SDS-PAGE, Western transfer, and development by the ECL method. The antiserum raised against TcpT^F-enriched *E. coli* inclusion bodies recognises bands of different apparent molecular sizes (a and b) in Z17561 (lanes 1 and 4) and JRI5 (lanes 2 and 5). A band at the expected size (57.5 kDa) is recognised in the lane containing E1315[pPM4133] whole cells (lane 7: without induction, lane 8: +1mM IPTG), is faintly visible in most tracks, including E1315 alone (lane 6). Lane 3 contains JRI1.

Maximally expressed TcpT^F in *E. coli* (recognised by the M2 monoclonal antibody at the expected weight in Figs. 5.6 and 5.7) is detected as a prominent band (Fig. 5.9, c) by the polyclonal antiserum. A reactive band migrating at the position consistent with the predicted size of 57.5 kDa was largely removed from JRI1 (Z17561*tcpT*^{km}) by adsorpton of antisera against whole cell and membrane fractions, as described.

Since the antiserum was raised by excision of *E. coli*-derived inclusion bodies of $TcpT^F$ from an acrylamide gel, *E. coli* fractions remain slightly reactive after the adsorptions described. However, there are also bands of higher and lower apparent molecular sizes than this in the immunoblot of TCP+ve Z17561 and JRI5 using the polyclonal antiserum (lanes 1,2,4, and 5 in Fig. 5.9, overleaf). These bands (a and b) are absent from the *tcpT*^{km} strain, JRI1 (lane 3), but slowly migrating bands in SDS-PAGE are detected in the htrack containing *E. coli* DH5α[pPM4133], after maximal expression of $TcpT^F$ (c, in lane 8). Note that a band consistent with the size predicted for TcpT is also detected by the M2 monoclonal antibody (Fig. 5.7, Lane 1), and therefore cannot be dismissed as an artefact attributible to the cross-reactivity of the polyclonal antiserum. The apparently lower molecular size species (indicated as b) is detected only in TcpT-expressing strains, and may represent a breakdown product of TcpT. Consistent with this, apparent degradation products of $TcpT^F$, as well as slowly migrating forms, are detected in M2 monoclonal immunoblots after $TcpT^F$ expression in both *E. coli* and *V. cholerae* (Figs. 5.6 and 5.7). A side-by-side comparison of immunoblots using polyclonal and monoclonal antisera is shown below, with $TcpT^F$ produced at low levels (grown in the absence of IPTG) (Fig. 5.10).

Fig. 5.10 confirms that $TcpT^F$ produced at low level in a *V. cholerae* background migrates at other than the predicted molecular size, consistent with the data already shown. It is interesting that the $TcpT^{F20}$ protein is most conspicuous at the predicted molecular size (band c, at 57.5 kDa, in lane 2 of both blots), as is $TcpT^F$ when expressed in *E. coli* (Fig. 5.9).

Fig. 5.10 Comparison of monoclonal and polyclonal antisera

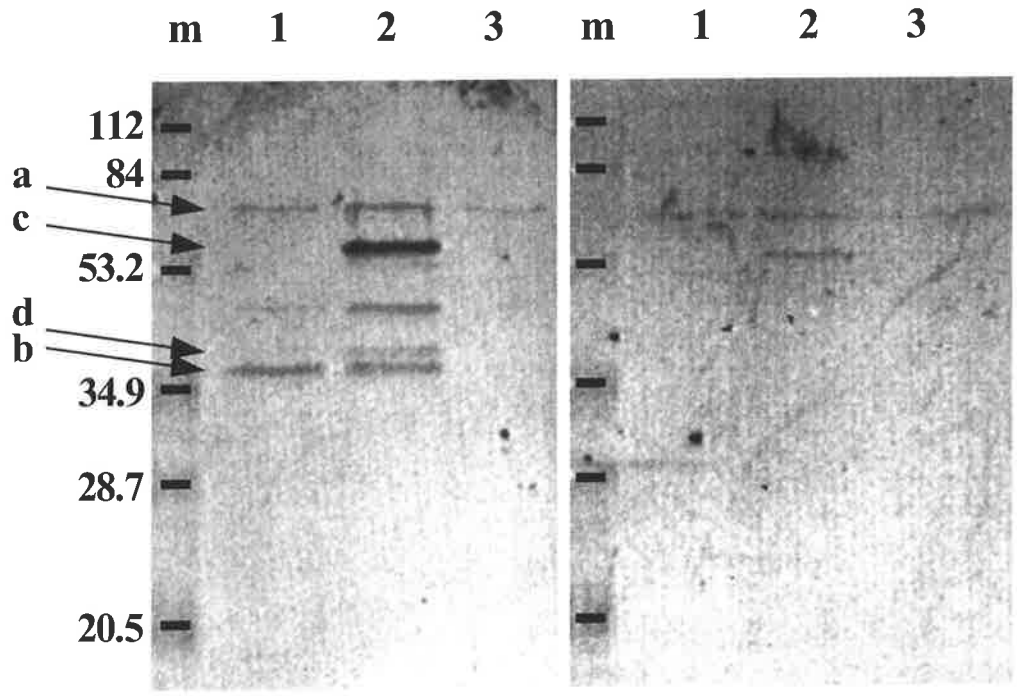


Fig. 5.10 Immunoblots of whole cell fractions after SDS-PAGE and transfer, using polyclonal αTcpT antiserum (1:2000) (left panel) and monoclonal M2 antiserum (right panel). After incubation in secondary antisera, blots were developed using NBT-BCIP (Section 7.4.4) .All strains were TCP-expressing (as judged by typical clumping in AKI broth, and +ve immunoblot for TcpA), and were grown in the absence of IPTG. Loading was in equal amounts, and was as follows: Lane 1: Z17561[pPM4133]; Lane 2: Z17561[pPM4134]; Lane 3: Z17561[pPM4133]. Molecular sizes (m) are indicated in kDa along the left hand border. Letters are referred to in text (below).

Band a is detected by both polyclonal (left panel) and monoclonal (right panel) antisera in all tracks. Secondary bands (b, d, and e) are again detected by the polyclonal antiserum but are not seen in the other panel, although the band at position b appears to be common to both monoclonal and polyclonal blots previously shown. Indeed, bands a, b, and d are equivalent in Lanes 1 and 2, and one is tempted to speculate that $TcpT^{F20}$ is less prone to degradation than $TcpT^{F}$, perhaps relating to its position or associations

in the cell. The complementary data from the monoclonal and polyclonal immunoblots confirm that the band (a) at approx. 70 kDa is $TcpT^{F}$, and that $TcpT^{F20}$ is present at the predicted size.

5.4. Cytoplasmic membrane-association of TcpT *in vivo*

5.4.1. TcpT is exclusively found in the cytoplasmic membrane *in vivo*

In order to determine whether TcpT was found in the cytoplasm or membrane *in vivo,* Z17561 was grown under *tcp*-inducing conditions, washed and resuspended in PBS. The finding of the hydrophilic TcpT as a soluble protein in overexpression systems (above) was not surprising, but TcpT and $TcpT^{20}$ were found exclusively in the membrane fraction when expressed from the native promoter after conventional TCP induction in Z17561 and JRI5, respectively (discussed in further detail in Section 5.5). In order to examine the natural state of TcpT, PBS-washed whole cells lysed in a French press were underloaded and floated up through a sucrose density gradient for 48 hours. The antiserum to TcpT raised as described (Section 5.3.4) recognised the characteristic bands (see Fig. 5.6) floating above the major outer membrane proteins (Figure 5.11).

Fig. 5.11 TcpT floats into a position above the major outer membrane proteins

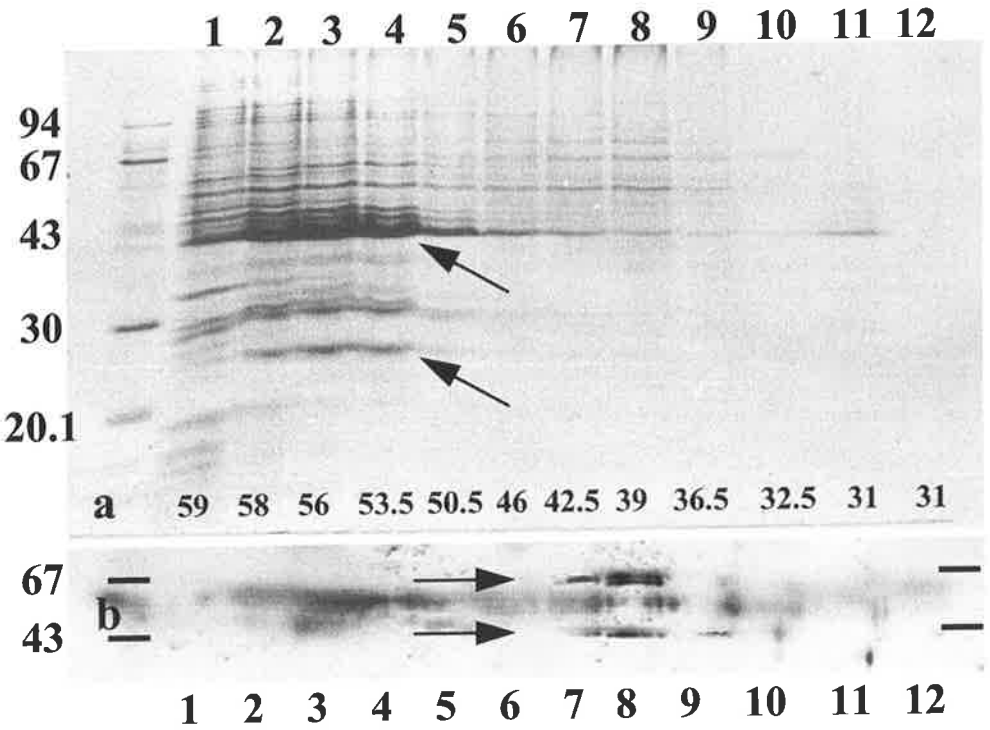


Fig 5.11 Coomassie Brilliant Blue-stained SDS-PAGE gel (upper panel) and corresponding TcpT immunoblot (lower panel) of sucrose density gradient fractions of TCP-induced Z17561. Immunoblot for TcpT is performed with polyclonal αTcpT antiserum, and detected by the ECL method. The SDS-PAGE gel has been shown as Fig. 4.10 (with TcpA immunoblot), and is presented again for ease of comparison. Location of molecular size markers (horizontal bars, left border in panel a; two horizontal bars at each border in panel b) are shown, in kDa. Specific bands attributable to TcpT are indicated by horizontal arrows, and correspond to those previously seen in Figs 5.5, 5.6, 5.9, and 5.10. Oblique arrows point to outer membrane proteins of *V. cholerae* (Sengupta *et al.,* 1992; see also Figs 3.5 and 4.10). The sucrose concentrations (% w/v) for each fraction are given at the bottom of panel b. Gels were loaded in equal volumes for each fraction.

5.4.2. TcpT is efficiently extracted from the membrane fraction by detergents

In an attempt to further define the nature of membrane association/s of TcpT, whole membrane fractions of TCP-induced Z17561 wereincubated in 1.0M NaCl (on ice) and 6.0M Urea (at 30°C) under identical conditions to those previously described for TcpA

subunits (Section 4.15). TcpT was completely solubilised from the membrane fraction by 1% Sarkosyl, and 6.0M Urea but not 1.0M NaCl appeared to partially elute TcpT in these experiments (Fig. 5.12).

Fig. 5.12 Salt and detergent extraction of TcpT

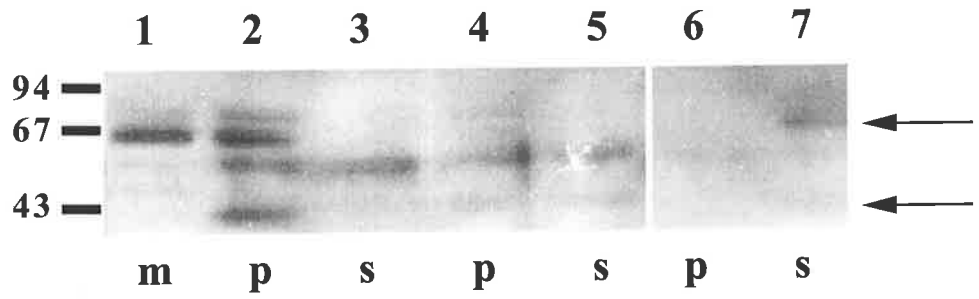


Figure 5.12 Immunoblot of salt and detergent extractions of TcpT (position of major bands marked by horizontal arrows) from membrane fractions of Z17561. Lane 1 contains untreated membrane (m) and all other lanes are paired with pellet (p) and supernatant (s) loaded, in equal volumes: Lane 2: pellet (p) following 1.0M NaCl; Lane 3: NaCl-soluble (s) extract; Lane 4: (p) following 6.0M Urea; Lane 5: Urea (s) extract; Lane 6: (p) following 1% Sarkosyl; Lane 7: Sarkosyl (s) extract. Molecular sizes are shown along the left margin (kDa).

The complete extraction of TcpT by detergent is consistent with an inner membrane location, as Sarkosyl in the absence of $MgCl_2$ will extract inner membrane proteins almost exclusively in *E. coli*, and the apparent requirement for the denaturing effect of concentrated Urea to significantly solubilise TcpT adds to the impression that TcpT participates in a protein complex..

5.5. Does TcpT participate in a membrane complex ?

5.5.1. Co-expression of TcpS, T, E, and F in complementation studies

The complementation (albeit incomplete) of JRI1, JRI2, JRI5 etc. by pPM4101 (*tcpSTEF*) in the absence of a source of T7 polymerase has already been described (Table 4.2). TcpT is clearly required, since internal in-frame deletions in *tcpT* render it ineffective. This must be produced at very low levels however, as TcpT (and TcpS) is poorly expressed from pPM4101 *in vitro* even in the presence of T7 polymerase (Section 5.2.1; Fig. 5.1). The essential requirement for downstream genes *tcpE* and/ or *tcpF* for TCP assembly (as reported by Taylor *et al.,* 1988) is supported by the capacity of several *tcpT*-containing constructs to effectively complement JRI5 and JRI6 ($tcpT^{20}$) but not JRI1 or JRI2 ($tcpT^{kan}$) (Section 4.2).

JRI1 and JRI2 contain a kanamycin resistance gene as a large insertional mutation in *tcpT* with a TAA (stop) codon at the end of the cartridge (Fig. 4.2). The only differences in complementation requirements between JRI1/ JRI2 and JRI5/ JRI6 should therefore pertain either to transcriptional attenuation of *tcpE* and *tcpF* in JRI1 and JRI2, or to an interference effect by the chromosomal $tcpT^{20}$ mutant gene product in JRI5 and JRI6. Attempts to demonstrate any interference by a $tcpT^{20}$ product maximally expressed *in trans* from the fully derepressed *tac* promoter of either pPM4137 (encoding $TcpT^{Q20}$) or pPM4134 (encoding $TcpT^{F20}$) were negative, however. Since it is possible that $TcpT^{20}$ may enter into a complex directly after translation, IPTG-induced derepression of the *tac* promoter was performed both from the outset (with 1mM IPTG present in the

medium before innoculation) and after TCP production was established (when early agglutination became visible in broths) Expression of these proteins in the *wt* Z17561 background had no detection influence on levels of TCP expression, and plasmid retention in both instances was confirmed at greater than 95% by comparison of serial dilutions onto selective media (ampicillin 100μg/ml) and plain nutient agar.

Constructs in which *tcpSTEF* are under the control of the derepressed *lac* promoter appear to be tolerated in *V. cholerae* only if *tcpF* is partially deleted (pPM4139 and pPM4145), and the possible toxicity of TcpF has already been discussed (Section 3.5.1). Interestingly, both pPM4139 and pPM4140 (*tcpSTE,* under the control of the *lac* promoter and the uninduced T7 promoter, respectively)[1] are as effective as pPM4101 or pPM4102 (*tcpSTEF*) in complementing JRI1, implying that attenuation of *tcpF* (and therefore probably *tcpE*) is incomplete[2] (Table 5.2, overleaf). Further, pPM4139 and pPM4140 also appear to be more effective than pPM4135 (encoding $TcpT^{C}$), pPM4136 (encoding $TcpT^{Q}$), or pPM4143 (encoding TcpT) in complementing JRI5 and JRI6 (Table 5.2). This is despite the fact that TcpT alone is clearly required and sufficient to complement the defect in the $tcpT^{20}$ strains, and that interference from $TcpT^{20}$ cannot be demonstrated. Since one would predict TcpS to be completely unaffected by either of the downstream mutations, and TcpF appears not to explain the difference between complementation requirements, these data suggest TcpE to be the critical element.

[1] Plasmids pPM4101, pPM4140 and pPM4143 are in pBluescript backgrounds and therefore have no functional *lacI* gene, unlike plasmids pPM4133-4137. The *lac* promoter is derepressed in *V. cholerae* unless *lacI* is also provided, and thus IPTG derepression is not required in the absence of *lacI* on the plasmid. T7 polymerase must be supplied separately by the pGP1-2 plasmid (eg. JRI7) for normal expression from the T7 promoter to proceed in pPM4101, pPM4140, etc.

[2] Otherwise, one must propose that the highly conserved *tcpF* gene is redundant or inessential and that the *tcpF* defect in the classical strain O395 (Taylor *et al.*, 1988), sequence identical at amino acid level to Z17561 and 569B throughout the *tcp* operon (Taylor, R.K.; pers. comm.), is somehow unique.

Table 5.2 Complementation efficiency

plasmid	*tcp* genes	comment	controlling promoter	TCP production* JRI1 JRI2	JRI5 JRI6
pPM4101	*tcpSTEF*	native context[1]	uninduced T7	(+)	+
pPM4145	*tcpSTE*	*Spe*I cutdown pPM4101	uninduced T7	+	+
pPM4139	*tcpSTE*	pBlue-Cml KS[2]	*lacZ* (no *lacI*)[4]	++	++
pPM4140	*tcpSTE*	opp.orientation to pPM4139	uninduced T7	+	+
pPM4142	*tcpT*	optimal *tcpT* RBS[3]	uninduced T7	-	(+)
pPM4143	*tcpT*	opp.orientation to pPM4142	*lacZ* (no *lacI*)	-	+
pPM4133	$tcpT^{F}$	*tcpT* in pFLAG-MAC	*lacZ* (repressed)[4]	++	+++
			lacZ (IPTG)	++	+++
pPM4134	$tcpT^{F20}$	$tcpT^{20}$ in pFLAG-MAC	*lacZ* (repressed)	-	-
pPM4135	$tcpT^{C}$	*tcpT* in pFLAG-CTC	*lacZ* (repressed)	-	(+)/+
			lacZ (IPTG)	(+)	+
pPM4136	$tcpT^{Q}$	*tcpT* in pTTQ181	*lacZ* (repressed)	-	+
			lacZ (IPTG)	(+)	+
pPM4137	$tcpT^{Q20}$	$tcpT^{20}$ in pTTQ181	*lacZ* (repressed)	-	-

				Z17561
nil				+++
pPM4134	$tcpT^{F20}$	$tcpT^{20}$ in pFLAG-MAC	*lacZ* (IPTG)	+++
pPM4135	$tcpT^{C}$	*tcpT* in pFLAG-CTC	*lacZ* (IPTG)	+++
pPM4136	$tcpT^{Q}$	*tcpT* in pTTQ181	*lacZ* (IPTG)	+++
pPM4137	$tcpT^{Q20}$	$tcpT^{20}$ in pTTQ181	*lacZ* (IPTG)	+++

Table 5.1 The complementing efficiency of various constructs are compared. JRI1 and JRI2 ($tcpT^{km}$ mutants) were tested in parallel and gave identical results in complementation studies, as did JRI5 and JRI6 ($tcpT^{20}$ strains). The lower panel shows the (lack of) effect of expression of $TcpT^{F20}$ and $TcpT^{Q20}$ *in trans* in the *wt* (Z17561) background.

KEY TO TABLE

[1] *Bgl*II fragment (Fig. 4.6) with native RBS sequence for *tcpT*

[2] *Spe*I fragment pPM4103 (bisects *tcpF*) recloned into pBlue-Cml KS *Spe*I site

[3] introduced by oligonucleotide-directed mutagenesis of RBS (Fig. 5.2). pPM4143 is isogenic, with the *tcpT*-containing fragment in the opposite orientation in the MCS

[4] *lacI* present in pPM4133-4137. IPTG derepression had no effect on complementation phenotype

* The scoring of TCP production is described in detail in Section 7.2.3

5.5.2. A small fusion alters the N-terminal isoelectric point and hydrophobicity of TcpT and enhances complementation of the *tcpT* mutation *in trans*.

Despite uniformly high plasmid retention in complementation experiments and close similarities in expression systems, there are striking differences apparent in the complementing efficiency of different constructs (Table 5.2, Fig. 5.13). In contrast to the partial complementation of the defects in JRI5 and JRI6 by provision of TcpT, $TcpT^{C}$, or $TcpT^{Q}$, impressive complementation of the TCP assembly defect is achieved with low levels of $TcpT^{F}$ (without IPTG derepression of the *tac* promoter in pPM4133) in chromosomal *tcpT* mutants of both genotype (Table 5.2; Fig.5.13).

Since the *tcpT*-containing 1.8kb *Cla*I fragment is identical in each strain, derived by separate subclonings, the most likely explanation for variation is the difference in the acquisition of the small N-terminal fusions acquired during the process of cloning into the MCS of each vector. While in a similar genetic background to pPM4136, and differing from pPM4135 only in encoding the octapeptide, the product of pPM4133 ($TcpT^{F}$) differs greatly in its ability to suppress both forms of *tcpT* mutation in both 569B and Z17561 backgrounds, and the $tcpT^{km}$ mutation in V663 (Fig. 5.13). The small fusion unique to $TcpT^{F}$ substantially alters overall charge and hydrophobicity in the N-terminal region of the protein. The relative amount of TcpT produced from each construct along with a TcpA immunoblot and schematic representation of the TCP phenotype is illustrated in Fig. 5.13.

Fig. 5.13 Efficient complementation by TcpT^F at low expression levels

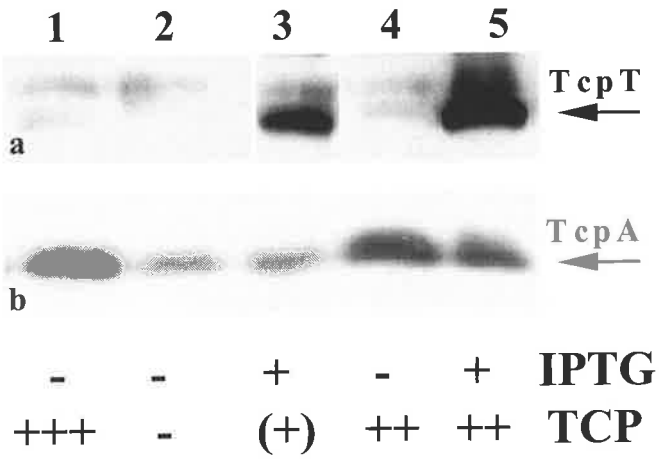


Fig. 5.13 TcpT and TcpA immunoblots after SDS-PAGE and transfer. Lanes 1-5 contain Z17561, JRI1, JRI1[pPM4135] with IPTG added, JRI1[pPM4133} without IPTG, and JRI1[pPM4133] wih IPTG, respectively. Where indicated (+ or -), IPTG was added to broths (to a final concentration of 1mM) at time of innoculation to ensure derepression of *tac* promoters. TCP production is scored as for Table 5.2. TcpT and TcpA were detected by αTcpT and α569B-165 antisera as indicated.Bound secondary antibodies were detected by the ECL method. Panel a was relatively underexposed (for tracks 1 and 2) so as to effectively compare lanes 3, 4, and 5. All tracks were equally loaded by volume.

This shows that differences in efficiency of complementation between pPM4133 (encoding a product bearing the DYKDDDDK N-terminal epitope) and isogenic pPM4135 (lacking the DYKDDDK fusion) cannot be explained by differences in the amount of TcpT protein produced. Changes in the mean hydrophobicity indices and isoelectric points of TcpT^C (lacking only the Flag octapeptide) and TcpT^Q, with a similar sized neutral N-terminal fusion, are presented in Table 5.3. TcpT^C differs from TcpT^F only in the presence of the FLAG octapeptide and thus provides for a very direct comparison.

Table 5.3 Effects of different N-terminal extensions on TcpT

	fusion sequence (P_i)	N-terminal region		entire protein	
		P_i	HI	P_i	HI
TcpT^F (pPM4133)	M**DYKDDDDK**VKLLENSA (3.97)	5.1	-0.43	6.26	-0.27
TcpT^C (pPM4135)	MVKLLENSA (6.79)	7.15	-0.21	6.85	-0.23
TcpT^Q (pPM4136)	MTMITNSSSVPGPPSRSTVS (10.90)	8.52	-0.25	7.15	-0.24
TcpT	-	7.15	-0.27	6.85	-0.24

Table 5.3 Amino-terminal fusion extensions to TcpT. N-terminal extensions are shown using the conventional single-letter amino acid code, up to the residue immediately before the +1 Met residue of native TcpT for the (P_i in brackets), with the calculated P_i and mean hydrophobicity indices (HI) for the entire protein and for the N-terminal region (first 100 residues). Calculations are based on the algorithm of Kyte (1989) in PROSIS ver. 6.0 (Hitachi Software, 1990). The FLAG epitope is shown in bold type

The amino-terminal region has already been identified as particularly important in the function of closely related transport system proteins PulE (Possot and Pugsley, 1994) and EpsE (Sandkvist *et al.,* 1995). It can be seen that the hydrophobicity and isoelectric point of the first 100 residues of the N-terminal region are dramatically altered by the presence of the positively charged octapeptide.

5.5.3 The influence of TcpE co-expression on the membrane localisation of TcpT

It is clear that alterations in the N-terminal region, as well as co-expression of other Tcp proteins, has dramatic effects upon the results of complementation studies. Taken together, a reasonable explanation for the data presented thus far is that TcpT associates stably in a cytoplasmic membrane complex *in vivo*, and that the presence of protein

such as TcpE may participate directly or indirectly. Previous experiments have shown that TcpT is a cytoplasmic membrane protein *in vivo*, and yet, consistent with its predicted hydrophilic nature, can be found in the cytoplasm when present in excess. In oredr to test whether subcellular localisation of TcpT in *E. coli* is also affected by the presence of co-expressed TcpE (or TcpS) or by the N-terminal fusion, cells were grown for TCP induction (Z17561) or for low-level expression of $TcpT^F$ (*E. coli* DH5α [pPM4133] in the absence of IPTG), washed in PBS pH7.4, and lysed in a French pressure cell. Lysates were centrifuged for 60 mins at 90 000 xg to separate pellet from supernatant; these were subjected to SDS-PAGE and immunoblot for TcpT, and the results are shown in Fig. 5.14.

Fig. 5.14 Influence of TcpE and the FLAG fusion on TcpT localisation in *E. coli*

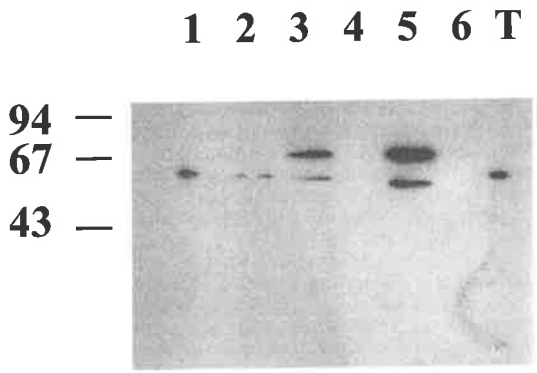


Fig. 5.14 Immunoblot for TcpT of pellets and supernatants after lysis and high-speed centrifugation. TcpT expressed alone as $TcpT^C$ from pPM4135 in lanes 1 (membrane) and 2 (soluble in PBS pH 7.4), as$TcpT^F$ from pPM4133 (membrane and soluble fractions in lanes 3 and 4, respectively) and expressed with TcpE (and TcpS) from pPM4139, as membrane and soluble fractions in lanes 5 and 6, respectively) in *E. coli* DH5α. The right hand lane (T) contains semi-purified TcpT from the affinity column (see Fig. 5.9) as a marker.

When expressed in the presence of TcpE (whether from the *tcp* operon in *V. cholerae*, or from pPM4139 in *E. coli*), TcpT is detected wholly in the insoluble fraction and predominantly as the higher molecular weight species (Figs. 5.9-12). When expressed alone from pPM4135 in *E. coli*, $TcpT^{C}$ is detected in both soluble and insoluble fractions and predominantly in the lower molecular weight form. $TcpT^{F}$ expressed from pPM4133 appears to be distributed in the same way that native TcpT is distributed when expressed in the *wt* organism or from pPM4139 in *E. coli*. A requirement for TcpE co-expression to efficiently complement *tcpT* mutations in *V. cholerae* would be consistent with this, in that there is an apparent effect on both the localisation and the apparent molecular size of TcpT. The observation that $TcpT^{F}$ degradation *in vitro* appears to be from the C-terminal end (see Fig. 5.5) further suggests that the effect of TcpE and the FLAG fusion may be similar, acting perhaps to promote or stabilise the membrane association/s of TcpT. The fact that $TcpT^{F}$ (but not $TcpT^{C}$) is preferentially located in the *E. coli* membrane fraction, independent of the presence of TcpE, implies that the strongly charged octapeptide serves a nonspecific role in membrane localisation and/ or stabilisation, and that it substitutes for the role performed by TcpE to this end. While it is conceivable that TcpT could interact with another protein in *E. coli* DH5α, such an interaction clearly does not prevent the alteration in apparent molecular weight, nor loss into the soluble fraction, of $TcpT^{C}$. The finding of TcpT in the *E. coli* soluble fraction cannot simply be dismissed as an artefact of *in vitro* expression, since pPM4133 and pPM4135 are isogenic, the apparent molecular sizes also differ (suggesting degradation), the apparent amounts of $TcpT^{C}$ are comparable to or less than those generated from pPM4139 (TcpT co-expressed with TcpE), and because the complementation data are both complementary and consistent. The inactive but stable

$TcpT^{20}$ does not interfere *in trans* (as $TcpT^{F20}$ or $TcpT^{Q20}$), and is found only in the insoluble fraction of French press-lysed or sonication/ freeze-thaw-lysed cells of *V. cholerae* JRI5 (not shown), suggesting that localisation and stability of TcpT is independent of any role in ATP binding by the Walker A motif.

5.5.4 Crosstalk and co-existing homologues

The presence of at least two related export systems in *V. cholerae,* for cholera toxin (Sandkvist *et al.*, 1991) and for the MSHA type 4 pilin (Jonson *et al.*, 1994), make for the possibility of crosstalk between systems. EpsE is the TcpT homologue in the cholera export system and, since the regulation of the *tcp* operon is co-ordinately linked to that of cholera toxin, the most significant. While *tcpT* mutants are normal with respect to cholera toxin export, they are absolutely TCP negative. The relatedness of TcpT and EpsE is underscored by the ability of overexpressed EpsE to partially complement $tcpT^{20}$ mutants, restoring occasional TCP bundles in IEM preparations (not shown). *EpsE* null mutants were constructed in *V. cholerae* El Tor strain phil 6973, in collaboration with Dr. G. Jonson, and MSHA and TCP production were found to be unaffected (as measured by mannose-sensitive haemagglutination in appropriate culture conditions for MSHA, and IEM for TCP). This was done by introduction of the Tn*903*-derived kanamycin cartridge (Fig. 4.2) into an unique site in *epsE,* which was amplified by PCR using oligomers designed for this purpose from the published sequence (Sandkvist *et al.,* 1993), and introduced into the pCACtus vector (Clark *et al.,* in press) for chromosomal recombination. The introduction of this mutation into Z17561 was not

completed at time of writing, and the effect of an *mshE* mutation on TCP production in *V. cholerae* is unknown.

5.6 Concluding remarks

The *tcpT* gene product has been shown in the previous Chapter to be specifically and absolutely required for assembly of the Toxin-Coregulated Pilus (TCP) of *V. cholerae*. Complementation studies and construction of discrete in-frame chromosomal deletions demonstrate the specificity of the *tcpT* mutation, and the essential nature of the Walker A ATP-binding motif. Mutations in *tcpT* are shown to completely abrogate assembly of the Toxin-Coregulated Pilus in *V. cholerae,* and reduced amounts of the TcpA subunit are localised to the cytoplasmic membrane. Subunit associations in these translocation mutants are indistinguishable from those in TCP, and the *tcpT* phenotype is shown to be the same in the background of isogenic strains in which a second mutation prevents transport of subunit across the outer membrane (Chapter Three).

The evidence for N-terminal association of subunits (cross-linking, salt and detergent extraction) and for C-terminal folding (fucose-resistant haemagglutination) suggest that TcpA has been exposed to the action of the periplasmic disulphide oxidoreductase, TcpG (DsbA), previously shown to be essential for the specific haemagglutinating activity of TcpA (Peek and Taylor, 1992). The loss of this activity in French press lysates might be explained by the predominance of inside-out vesicles (Dean *et al.*, 1989; 1992), restoration in sonicated freeze-thaw lysates being consistent with a

location on the periplasmic face of the inner membrane. Thus, *tcpT* is required after processing and after N-terminal association of TcpA subunits in a stable fashion similar to their final configuration has occurred, but prior to the outer membrane translocation block in isogenic *rfb* mutants. The only model for type-4 pilin assembly consistent with the recently defined fibre and subunit crystal structure is that of sequential incorporation of monomers (Parge *et al.,* 1995), rather than the dimeric assembly model arising from previous observations of subunit characteristics in laboratory conditions (Paranchych *et al.,* 1979; Parge *et al.,* 1990). Crosslinked trimers and tetramers are consistent with an organised subunit association rather than random or casual association (as also suggested by the requirements for urea extraction to solubilise subunits), and are not explained by simple subunit pairing *in vitro* to minimise surface hydrophobicity, as has been previously described (Parge *et al.,* 1990).

Type-4 pilin biogenesis appears to be initiated by specific cleavage of the pilin substrate by the unique peptidase, TcpJ, in a Sec-independent fashion, and this same peptidase is capable of cleaving heterologous pilin subunits as well as related non-pilin substrates in some systems (reviewed in Hobbs and Mattick, 1993; Pugsley, 1993a). Substrate specificity may be greater than this at or beyond the TcpT-dependent stage. Thus, appropriate localisation/ trans-cytoplasmic membrane passage of type-4 pilin-like proteins in other systems might not be expected to be directly influenced by mutations in TcpT homologues, although secondary effects of misassembled or dysfunctional cytoplasmic membrane complexes may alter their stability. Production of a stable mutant TcpT protein by $tcpT^{20}$ mutants is demonstrated as part of investigations into the location and membrane interactions of the TcpT protein, and appears to behave as *wt*

TcpT protein in localization to the membrane. Thus, ATP hydrolysis may be required for release of TcpA from the periplasmic face of the cytoplasmic membrane, but not for engagement of the outer membrane translocation complex.

TcpT is clearly stable as a hydrophilic protein, and can be purified from the cytoplasmic fraction of *E. coli* DH5α cells in which it has been overexpressed as a fusion protein. The $TcpT^F$ protein is fully functional and is found in the membrane fraction, but the cytoplasmic localisation may well be a 'spillover' effect due to saturation of suitable membrane sites. The independence of this phenomenon from influence by co-expression of other Tcp proteins in Z17561 supports this, but it must be pointed out that these latter experiments were performed in the presence of native TcpT produced in an intact *wt* organism. Thus, one might expect little in the way of 'free' sites. The finding of large amounts of membrane-associated material suggests either that 'inclusion bodies' of overexpressed insoluble protein aggregates are present in membrane-bound vesicles generated by the French pressure cell, and thus non-specifically fractionating with the native membrane material, or that there is a relatively non-specific association of TcpT with the membranes of both *V. cholerae* and *E. coli*. It seems unlikely that native TcpT is naturally present in the cytoplasm at all, since it is almost certainly co-expressed as part of an operon, along with TcpE and the export substrate TcpA, and is only detected floating above the outer membrane fraction in a sucrose density gradient. Conformational change of TcpT related to binding and/or hydrolysis of ATP in the cytoplasmic membrane is a more satisfying hypothesis, and is consistent with existing models of the analogous (not homologous) SecA protein (Lill *et al.*, 1989; Kim *et al.*, 1994; Economou and Wickner 1994, Rajapandi and Oliver, 1996).

The requirement for co-expression of TcpE apparent in complementation studies might be simply explained by a requirement for TcpE in the translocase, and the transcriptional polarity of the *tcpT*^{km} mutation. This is consistent with previous data from Taylor *et al.* (1988), attesting to the importance of *tcpE*, and with the position of *tcpE* immediately downstream from *tcpT* in the operon. The relief of this requirement by a small highly charged fusion at the N-terminus of TcpT^{F} is more interesting, and reminiscent of data from related systems which point to the significance of this amino-terminal region in interacting with the membrane and/or membrane anchor protein/s (Possot and Pugsley, 1994; Sandkvist *et al.*, 1995). While it is conceivable that TcpT is interacting with another protein in *E. coli* DH5α, such an interaction does not prevent the alteration in apparent molecular size nor loss into the soluble fraction. The observation that TcpT^{F} degradation products remain immunoreactive to the monoclonal antibody after maximal expression *in vitro* (implying that the N-terminal end is intact) suggests that the effect on TcpT localisation of the FLAG octapeptide fusion and of co-expression of TcpE may be similar, acting perhaps to anchor or stabilize TcpT in the membrane. The fact that the TcpT^{F} protein is apparently stable in the *E. coli* membrane fraction, independent of the presence of TcpE, implies that the strongly charged octapeptide serves a nonspecific role in membrane localisation and/ or stabilisation, and that it may substitute for the role performed by TcpE to this end. TcpT is clearly able to localise to the membrane even in the absence of any members of the *tcp* operon. The finding of TcpT in the *E. coli* soluble fraction cannot simply be dismissed as an artefact of *in vitro* overexpression, since pPM4133 and pPM4135 are isogenic, the apparent molecular weights also differ (suggesting degradation of TcpT^{C}), the apparent amounts of TcpT^{C} are comparable or less than those generated from pPM4139 (TcpT co-

expressed with TcpE) or from the chromosome in the *wt*, and the complementation data cannot be readily explained as artefactual in the same manner. The inactive but stable $TcpT^{20}$ does not interfere *in trans*, and is found only in the membrane fraction in *V. cholerae* JRI5, suggesting that localisation and stability of TcpT is independent of any role in ATP binding by the Walker A motif. The inactive but stable $TcpT^{20}$ does not interfere *in trans* when expressed as $TcpT^{Q20}$ or $TcpT^{F20}$, and chromosomally encoded $TcpT^{20}$ is found only in the membrane fraction in *V. cholerae* JRI5, suggesting that localisation and stability of TcpT is independent of any role in ATP binding by the Walker A motif.

TcpT and TcpE therefore appear to participate in the same translocation machine. These data imply that TcpT is able to associate with the membrane independent of other members of the Tcp system, but that this is enhanced by co-expression of TcpE. Thus, TcpE appears to stabilise and/ or promote the membrane association of TcpT in both *V. cholerae* and *E. coli* backgrounds and is necessary for the efficient function of TcpT in *V. cholerae*.

Chapter Six -

General Discussion

Most of the experimental work has been put in context by the opening discussions, and the results reviewed in detail at the end of each experimental Chapter. It remains to summarize the important points and identify future directions for research. This final summation will therefore deal with major issues arising during the course of the thesis and conclude with some simple models to incorporate the new data.

The Toxin-Coregulated Pilus of *V. cholerae* is important because it serves as a useful model of the export and assembly of a type-4 pilin. The type-4 pili are sufficiently similar to the important main terminal branch of the General Secretory Pathway (the presence of the type-4 pilin and the peptidase, as well as the cytoplasmic membrane ATPase; reviewed in Pugsley, 1993a), and the Tcp system is sufficiently different (in the absence of homologues of GspF and GspD) that the study of this system is especially instructive. TCP is also the only adhesin of proven pathogenic significance for the important cholera organism (Attridge *et al.,* 1994; Attridge *et al.,* 1996; discussed in Section 1.2.3). The *tcpA* gene is therefore subjected to scrutiny in the initial part of this work.

Data collected during the course of the thesis work suggests that TcpA and TCP are proteolytically degraded *in vitro,* and that this may be mediated by the soluble haemagglutinin/ protease (SHA). This has some interesting implications for the model

of pathogenesis (discussed in Section 1.2.3), being consistent with a 'detachase' role for SHA and supportive of a previously proposed model (see Finkelstein *et al.,* 1992; Sections 1.2 Supplement [pp18-21] and 1.2.3; Fig. 1.2).

TcpA and TCP: implications of data presented in the opening chapter

TcpA has here been shown by genetic methods to be biotype-specific and highly conserved in all of 18 virulent isolates examined (Section 2.7), consistent with data from this and other laboratories (Voss and Attridge, 1993; Keasler and Hall, 1993; Ogierman *et al.,* 1996; pers. commun., R.K. Taylor).

The biotype specificity is also likely to be a useful marker for diagnostic and epidemiological purposes, although such a prediction would need to be validated by much more extensive surveys than that attempted herein. It has been demonstrated in IEM studies by a colleague in this laboratory that El Tor and classical TCP can be co-expressed in *V. cholerae* (Voss, 1995), and it can be confidently predicted from the work presented (Chapters 3 and 4) that these would prove to be heteromers rather than two distinct populations of pili. Indeed, TcpA (of either biotype) may prove to be an ideal marker for the "pathogenic" or potentially pathogenic environmental Vibrios, since TCP expression is required for infection by lysogenic phage carrying the CTX virulence cassette (Waldor and Mekalanos, 1996; see also Section 2.8).

TcpB and pseudopilins in type-4 operons

The type-4 pilin structure of TcpA is shown (Section 2.7) to be rigidly conserved in the N-terminal region, which is thought to be so vital for pilus assembly (Parge *et al.,* 1995) and which is so highly conserved amongst these pilins (Dalrymple and Mattick, 1987). The folding of the C-terminal region of TcpA may well occur immediately after processing, and prior to entry into the OM (Section 4.3.6). A finding of normal C-terminal folding of heterologous type-4 pilin subunits in *V. cholerae* would tend to confirm that the initial assembly process is truly very simple, and that structures such as the 'pseudopilins' (in the General Secretory Pathway) are probably only involved in outer membrane translocation. In view of the absence of typical 'pseudopili' and of a PilQ (GspD) homologue in *tcp,* and the essential nature of the GspGHIJ proteins in outer membrane translocases, this is not an unreasonable model to challenge experimentally. A suitable approach would be introduction of an inducible plasmid containing a type-4 pilin gene (e.g. the *P. aeruginosa* PAK *pilA* gene) into *V. cholerae*, and use of an appropriate antiserum. One might predict from the data presented in this work that such heterologous type-4 pili expressed in *V. cholerae* would be assembled at the level of the cytoplasmic membrane and perhaps the C-terminal region folded normally in the periplasm. However, unlike the El Tor TcpA subunit, one would predict that a different pilin such a PAK PilA may well fail to pass the outer membrane, since it is likely that this depends on interaction with the outer membrane translocase, and there is no GspD homologue in Tcp (see Introduction, and discussions of Chapters 3 and 4).

Since the specificity of the peptidase presumably allows for at least two substrates (ie. TcpA and TcpB in the case of TcpJ), and often more in other systems (eg. *P. aeruginosa*; see Nunn and Lory, 1991), I would argue that it is both highly likely and appropriate that subsequent steps (at or prior to outer membrane entry) would have the greater specificity.

It is thus probable that the initial assembly of pilin (prior to the action of the GspE protein, TcpT in this case) is specifically exclusive of the non-pilin components (the 'pseudopilins'). The notion of a dynamic (even rotating?) scaffold which winds in substrate or pilin subunits to the outer membrane (and, in other systems, winds in DNA?) is appealing, perhaps. However, were these pseudopili to act as a scaffold for the passage of pilin subunits to an outer membrane assembly point, one would expect that CM-associated TcpA (in *tcpT* mutants) would only be found as monomers, or the dimers observed of other type-4 pilin subunits in hydrophobic environments *in vitro* (Parge *et al.,* 1990), rather than the trimers and higher order multimers evident in crosslinking studies (Section 4.4.2).

The intriguing question of the role of the "pseudopilin" class of prepilin peptidase substrates remains open at this point, but the single *tcpB* gene should lend itself readily to molecular approaches. A mutation in *tcpB* could be readily engineered in a 'suicide' vector suitable for homologous recombination into the genome. A small 'in frame' deletion is necessary because of the probable monocistronic nature of mRNA encoding TcpB. The study itself is important because TcpB is significantly different to the pseudopilins in pilin and related non-pilin operons (Bally *et al.,* 1992; Lindeberg and

Collmer, 1992; Howard *et al.,* 1993), and is the only such protein in the *tcp* operon (see Section 1.12.3 for discussion). Previous data from transposon insertions are uninterpretable because of the likelihood of transcriptional attenuation of downstream genes (Taylor *et al.,* 1988). Essential components of the analysis would necessarily include the localisation of TcpB (which may require immunofluorescent and ultrathin sectioning studies, since chemical and physical fractionation methods may well be misleading: see Chapters 3 and 4), and the localisation of TcpA using methods such as those employed in Chapters 3 and 4.

The assembly and export of the TCP is a two-step procedure

The TcpA structural analysis and translocation phenotypes of the *tcpT* and *rfb* mutants described in this thesis allows development of a model suitable for future experimental testing, outlined below.

1. The type-4 pilin subunit is subject to specific cleavage by TcpJ on the inner face of the cytoplasmic membrane.

This is an accepted model (Hobbs and Mattick, 1993; Pugsley, 1993a) based on previous work (eg. Kaufman *et al.,* 1991), as discussed in Section 1.12. Specificity at this point may be limited, in that TcpJ has been shown to be essential for TCP biogenesis, but processing of heterologous type-4 pili and pseudopili by TcpJ homologues has been demonstrated in a number of systems (reviewed in: Hobbs and Mattick, 1993; Pugsley, 1993a). While TcpJ processing of TcpA has been shown to be

intact in the presence of Sodium azide (Kaufman *et al.,* 1991), this tells us nothing of the actual relationship between initial subunit processing and subsequent insertion into the membrane.

2. Localisation within the membrane and initial subunit interassociations occur prior to and independent from passage across the outer membrane.

Analysis of isogenic *tcpT, rfb,* and *tcpT, rfb* double mutants shows that TcpA is associating at least in trimeric form prior to passage through the outer membrane. Unless TcpA is fundamentally different to the MS11 subunit in the critical N-terminal region (which is clearly not the case: see Sections 1.12 and 2.8), the finding of trimers strongly implies that N-terminal interassociation occurs at the level of the cytoplasmic membrane and presumably therefore immediately follows processing. This is apparently also associated with normal or near-normal folding of the C-terminal region of the protein, since both cytoplasmic membrane and outer membrane-arrested subunits in cell lysates appear to haemagglutinate normally (Sections 4.4.3 and 5.4), further implying that exposure to the essential periplasmic disulphide oxidoreductase (TcpG) has occurred (see Peek and Taylor, 1992, Farinha *et al.,* 1994, and discussions in Sections 1.9.2, 1.11, and 1.12). The reduced haemagglutinating activity in French press-derived lysates compared to freeze-thaw lysates of *tcpT* strains might be explained by subunit localisation to the cytoplasmic membrane with orientation to the periplasmic space. Failure to gain more useful data from protease accessibility studies means that a clearer statement about the 'maturity' of subunit interassociations in *tcpT* mutants cannot be

3. Failure of translocation at the outer membrane in rfb mutants leads to accumulation of TcpA in the membrane and periplasm prior to surface presentation.

Transposon insertions in the putative perosamine biosynthesis genes of the *rfb* operon of classical strain 569B lead to reduced viability and autoagglutination of cells (Chapter 3). Motility and secretion of other elements (eg Ctx) is unaffected, but TCP assembly is defective (Fig. 6.1).

Figure 6.1 TcpA fails to traverse the outer membrane of *rfb* mutants

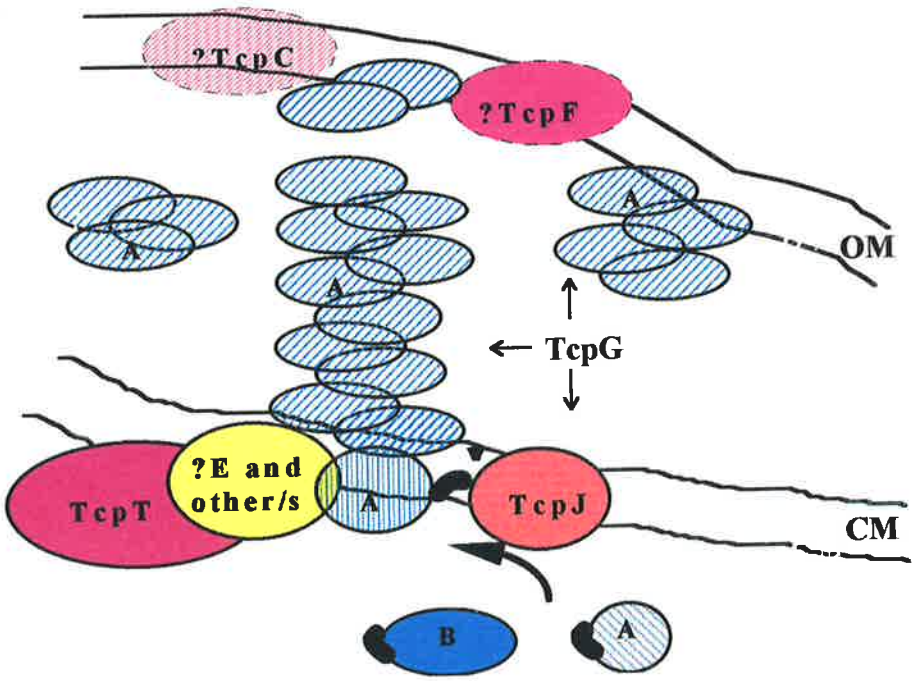


Fig. 6.1 TcpA pilin subunits fail to pass beyond the periplasmic face of the OM in *rfb* mutants after processing, and subsequent folding of the C-terminal in a TcpG-dependent fashion.

Isogenic in-frame mutations in the perosamine biosynthesis genes were not constructed, but complementation of the *rfb* mutation *in trans* restores TCP assembly *in vitro*. The absence of a gene encoding a GspD homologue in the *tcp* operon means that there is little guide to candidate proteins, but the function of TcpC is unknown and the porin-like TcpF may well be unstable or disassembled in an abnormal outer envelope (Section 3.5; Fig. 6.1).

4. TcpT is required for transport beyond the cytoplasmic membrane

The apparent localisation and accumulation of TcpA in *rfb* mutants suggests that TcpA is in a more stable configuration or site than in isogenic *tcpT* strains. Simple physicochemical distinctions could not be made, and protease accessibility studies were not successfully completed, but if TcpA is 'trapped' at the level of the cytoplasmic membrane, one might well predict that either the system would 'jam' or that accumulating subunits would be catabolised in the inner membrane. This latter appears to be the case, since TcpA subunits are clearly diminished in amount in such mutants (Fig. 6.2, overleaf). It is also likely that stable complexing occurs with other elements associated with translocation at or beyond the action of TcpT, but prior to passage of the outer membrane. A 'chaperone'-like role for as yet unidentified protein/s is not excluded by any existing data, although it seems that subunits are folded to at least some extent before the action of TcpT, and traditional chaperone and chaperone-like paradigms (Sections 1.5, 1.7.5, 1.10.4, and 1.11.2) seem unlikely.

Figure 6.2 TcpA fails to reach the OM translocation apparatus in *tcpT* mutants

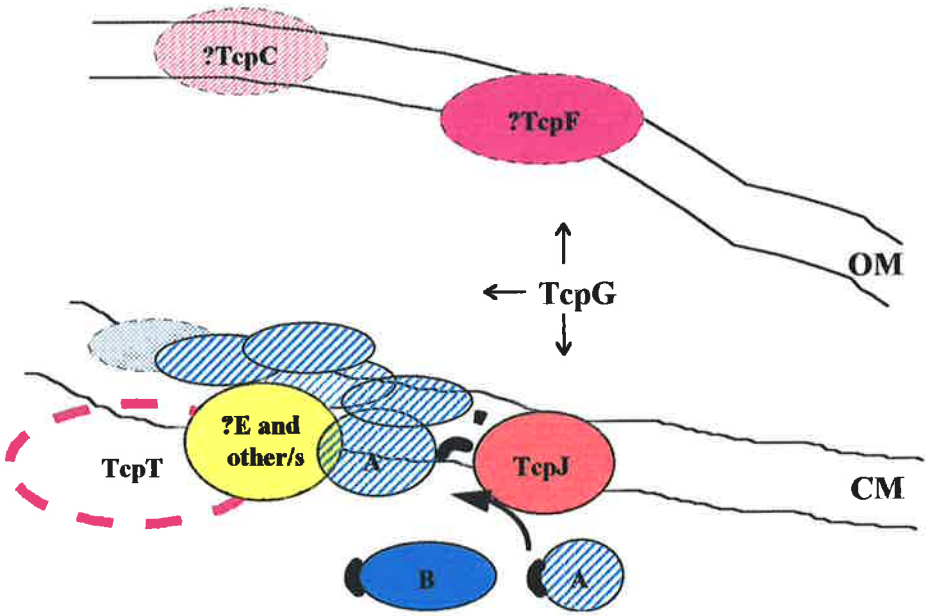


Fig. 6.2 The translocation defect in *tcpT* mutants. TcpA is processed and is exposed to the periplasmic disulphide bond oxidoreductase, TcpG, but does not leave the CM-associated stage of translocation and is probably degraded in the CM. Whether TcpB shares such a fate in *tcpT* mutants is unknown but would be interesting to test experimentally. This phenotype is independent of any defect in the OM translocation apparatus, with restoration of the ATP-binding (and/ or hydrolysing) activity of TcpT appears allowing normal levels of TcpA to reach the outer membrane.

5. The cytoplasmic membrane-associated ATPase is part of a complex in the inner membrane, and requires a highly conserved ATP-binding motif

The high degree of conservation at amino acid level in the GspE subclass of cytoplasmic membrane-associated ATPases is reflected in TcpT, and this protein is clearly essential for TCP biogenesis. While predicted to be hydophilic, and able to be purified from the cytoplasm when overexpressed *in vitro*, TcpT fractionates to the cytoplasmic membrane *in vivo* and in the presence of another integral inner membrane

protein, TcpE. When expressed in the absence of this latter protein, TcpT has different electrophoretic mobility and can be found in the cytoplasmic fraction. Interestingly, alterations in the P_i of the extreme N-terminal region of TcpT has the capacity to partially relieve the apparent requirement for co-expression of TcpE, influencing both the subcellular fractionation of TcpT and the efficient complementation of chromosomal *tcpT* mutations. This is consistent with data from other workers pointing to protein-protein interactions in the inner membrane involving the N-terminal region. Neither ATP-binding nor ATPase activity could be demonstrated for TcpT, but deletion of 60 nt encoding the highly conserved Walker A motif from the chromosomal copy of *tcpT* alters neither the subcellular fractionation nor apparent electrophoretic mobility of the resultant $TcpT^{20}$ protein.

The failure of overexpression of the $TcpT^{F20}$ or $TcpT^{Q20}$ proteins *in trans* to interfere with TCP biogenesis is difficult to explain. It may be that complex formation occurs immediately upon emerging from the ribosome in this operon, as discrete in-frame mutations in *tcpA* have also proven difficult to complement *in trans* (Attridge *et al.*, 1993). It may also be that membrane association of $TcpT^{20}$ occurs independently of any associations for which *wt* TcpT competes, and that the absence of these 20 residues abolishes complex formation and participation in the translocase. In any case, it is clear that while small amounts of TcpT can complement the chromosomal mutation, $TcpT^{F}$ can be found in insoluble cell fractions of *E. coli* and *V. cholerae* in the absence of TcpE or any other products of the *tcp* operon, and that $TcpT^{20}$ does not participate in the translocation of TcpA.

TCP and TcpA quantification: a methodologic problem

An important issue is the objective assessment of the complementation efficacy of the various constructs tested (Chapters 4 and 5). Section 7.2.3 deals with the methodology of TCP scoring in detail, but quantification of TCP production was in essence performed by the pooling of a number of measures which appeared to cross-correlate and give reproducible results. These were based on three fundamental observations which have already been described: (i) TcpA is reduced in amount in *tcpT* mutants, and is restored with complete or near-complete complementation (Sections 4.3.2 and 4.3.6); (ii) complementation restores TCP bundles and these can be quantified as proportion of the number of cells present in IEM grids; and (iii) TCP causes agglutination of broth culture which is roughly proportional in degree to the amount of TCP present (as judged by IEM or IF, for example), and results in visible clumping of cells and clearing of the broth medium.

Antigen-based methods (eg. ELISA) would not distinguish TcpA from TCP, but might be very useful if a reliable method of harvesting pili without damaging cells could be developed. Quantification of undifferentiated protein in the medium is plagued by the export of other substances (Ctx, proteases, etc.) and measures of hydrophobicity are similarly indirect and proved to be very insensitive. An adhesion assay has the greatest potential to separate unexported TcpA from TCP with some level of sensitivity and specificity, but the target for the TCP adhesin has not yet been identified and no *in vitro* adhesion models presently exist. Animal models (competition experiments, infective dose quantitations etc.) are generally insensitive and complementation studies in animal

models are made problematic by expression control and plasmid retention issues, even if all the mutants strains were made *recA* to rule out homologous recombination (after the single and double mutations were introduced).

Closing remarks

Conserved themes are seen in membrane translocation systems, and some of these are described in the introduction to this thesis. Energy from ATP is likely to be harnessed into work by TcpT in a cytoplasmic membrane protein complex, by transduction of phosphate bond-derived energy into conformational change. The initial interassociation of subunits after cleavage and methylation does not appear to require this energy, but these subunits are probably not released from the cytoplasmic membrane without it. An active transmembrane gradient is predicted to be necessary for passage of the inner membrane, but this was not tested in the course of this work.

Once assembled subunits reach the inner face of the outer membrane, they are unable to pass through in the absence of a normal 'smooth' OM, and this may well relate to failure to assemble a translocation channel involving proteins such as TcpF. The accumulated subunits are apparently associated in a 'mature' form, but are not seen as stunted CM-based pili in cryosections. Since TCP are usually seen as randomly broken semi-rigid pili *in vivo* and *in vitro*, one might predict that similar mutations in other organisms might actually lead to such observations. Indeed, it may be that mutants in the gene encoding the GspD homologue (*omc*) in *Neisseria gonorrhoeae* MS11 behave in this manner, since small stunted projections sheared off from pilin-expressing *omc*

mutants (encoding the PilQ/ Gsp(Pul)D homologue) are covered by OM and appear to contain mostly the MS11 PilE main structural subunit (T. Rudel and T.F.Meyer, pers. comm.).

While the concept of retractable pili is suggested by the "twitching motility" characteristic of type-4 pilin-expressing organisms (Whitchurch *et al.,* 1991; Hobbs and Mattick, 1993) and homologies in DNA uptake systems (Dubnau, 1991), and is neatly consistent with the observations that phage infectivity requires such pili to be expressed (Waldor and Mekalanos, 1996), no retractile pili or twitching motility has been demonstrated for *V. cholerae*. Indeed, the variation in length of externalised TCP that is observed *in vitro* as well as *in vivo* suggests that there is no 'anchoring' or 'capping' occurring, and that the brittle pili clump in skeins that entrap daughter cells in the ideal milieu. As the local concentration of secreted proteases such as SHA/ HAP rise (coincident with increased density of cells, depletion of nutrients, and increasing effect of cholera toxin), TCP dispersal would facilitate dispersal of organisms to new sites or the external environment. This hypothesis is supported by data described in the Introduction (pp. 18-21), is consistent with previous suggestions (Finkelstein *et al.,* 1992), and warrants more definitive investigation.

The apparent requirement for co-expression of TcpE in complementation might be most simply explained by a requirement for TcpE in the translocase, and the transcriptional polarity of the $tcpT^{km}$ mutation. Indeed, the relationship of *tcpE* to *tcpT* within the operon makes this finding essentially unremarkable. The relief of this requirement by a

small charged fusion at the N-terminus of $TcpT^F$ is more interesting, and reminiscent of data from PulE (Possot and Pugsley, 1994) and EpsE (Sandkvist *et al.,* 1995) which point to the significance of this region of the GspE class of proteins. While an improvement in efficiency of a native protein is not inconceivable (see Rusch *et al.,* 1994; Izard and Kendall, 1994; Section 1.6), the suggestion is that TcpT and TcpE participate in the same translocation machine. TcpT is wholly present in the *E. coli* insoluble fraction when co-expressed with TcpE, but both $TcpT^F$ and $TcpT^C$ can be found in this fraction of *E. coli* in the absence of TcpE. The data suggest that TcpT is able to associate with the membrane independent of other members of the Tcp system, but that this is enhanced by co-expression of TcpE. Thus, TcpE may stabilise and/ or promote the membrane association of TcpT in both *V. cholerae* and *E. coli* backgrounds and be necessary for the efficient function of TcpT. Nevertheless, a direct physical association remains unproven. The ability to radiolabel individual proteins in a *V. cholerae* background, and to purify TcpT, should facilitate the direct examination of associations within the Tcp translocase in floatation gradients and cross-linking experiments. The influence of other proteins on the stability and elution characteristics of membrane-associated TcpT is also a matter for examination in the future.

Chapter Seven

Materials and methods

7.1 DNA sources and techniques

7.1.1 Bacterial strains and plasmids

The *V. cholerae* strains used are listed with their sources in Table 3.3, except for *V. cholerae* El Tor Phil6973 (G. Jonson), which was used to demonstrate *wt* MSHA expression (see Chapter 1). Strains of the El Tor biotype were distinguished from the classical biotype by resistance to polymyxin B (50 units/ml) and sensitivity to biotype specific typing phages.

The plasmid cloning vectors which were used in this study are listed in Table 2.3. Most work was done in the specified *V. cholerae* strains or in the background of *E. coli* DH5a [F'*supE*44 D*lacU*169 (Φ80*lacZ*DM15) *hsdR*17 *recA*1 *endA*1 *deoR gyrA*96 *thi*-1 *relA*1], supplied by J. Pohlner of the Max Planck Institut für Biologie, Tubingen, Germany.

Table 7.1 Vectors and expression systems

vector/ phagemid	resistance	reference/ source
pBluescript SK II	ampicillin	Stratagene
pBluescript KScml	chloramphenicol	Stratagene
pWSK29	ampicillin	Wang and Kushner, 1991
pCACtus	chloramphenicol	Clark *et al.*, in preparation
pET-17b	ampicillin	Novagen
pTTQ181	ampicillin	Stark, 1987
pFMAC	ampicillin	IBI
pCTC	ampicillin	IBI
pGP1-2	kanamycin	Tabor and Richardson, 1985

7.1.2 Plasmid DNA extraction procedures

Method 1:

Triton X-100 cleared 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 50 mM Tris-HCl, pH 8.0. Lysozyme (50 ml, 10 mg/ml freshly prepared in H_2O) and 50ml of 0.25M EDTA, pH 8.0 were added to cells in Eppendorf tubes and left to stand on ice for 15 min. 0.5 ml TET buffer (50mM Tris-HCl, 66mM EDTA, pH 8.0, 0.4% Triton X-100) was added followed by a brief mixing by inversion of the tubes. The chromosomal DNA was then pelleted by centrifugation (20 min, 4^{o}C, Eppendorf). 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 weight of propan-2-ol and allowed to stand at -70^{o}C for 30 min. The precipitate was collected (10 min, Eppendorf), washed once with 1 ml 70% (v/v) ethanol, dried *in vacuo* and resuspended in 50 ml 1X TE buffer.

Method 2:

Large scale plasmid purification was performed by the three step alkaline 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 24 ml of solution 1 (50mM glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA). Freshly prepared lysozyme (4 ml of 20 mg/ml in solution 1) was mixed with the cell suspension and incubated at room temperature for 10 min. Addition of 55 ml of solution 2 (0.2M NaOH, 1% (w/v) SDS), followed by a 5 min incubation on ice resulted in total lysis of the cells. After the addition of 28 ml solution 3 (60 ml 5M potassium acetate, pH 4.8, to which was added 11.5 ml glacial acetic acid and 28.5 ml of H_2O) and incubation on ice for 15 min, protein, chromosomal DNA and high molecular weight RNA were removed by centrifugation (8,000 rpm, 20 min, 4°C, GSA, Sorvall). The supernatant was then extracted with an equal volume of a TE saturated phenol, chloroform, isoamyl alcohol mixture (25:24:1). Plasmid DNA from the aqueous phase was precipitated with 0.6 weight of 100% (v/v) propan-2-ol at room temperature for 10 min and collected by centrifugation (10,000 rpm at 4^{o}C, 35 min, GSA, Sorvall). After washing in 70% (v/v) ethanol, the pellet was dried *in vacuo* and resuspended in 4.8ml TE. Plasmid DNA was purified from contaminating protein, chromosomal DNA and RNA by centrifugation on a two step CsCl ethidium bromide gradient according to Garger *et al.* (1983). The DNA band was removed by side puncture of the tube with a 19 gauge needle attached to a 1 ml syringe. The ethidium bromide was extracted using isoamyl alcohol. CsCl was then removed by dialysis overnight against three changes of 5 litres 1x TE at 4^{o}C. DNA was stored at 4^{o}C.

Method 3:

Small scale plasmid purification was performed by a four-step alkaline lysis method according to Morelle (1989). Overnight bacterial cultures (1.5 ml) were transferred to a microfuge tube, harvested by centrifugation (45 sec, Eppendorf), and resuspended in 0.1 ml of solution 1 (50mM glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA, 4mg/ml lysozyme). The addition, after 5 minutes at room temperature, of 0.4 ml of solution 2 (0.2M NaOH, 1% (w/v) SDS) followed by a 10 min incubation on ice resulted in cell lysis. After the addition of 0.3ml of solution 3 (7.5M Ammonium acetate, pH 7.8 without adjustment), and a 10 min incubation on ice, protein, chromosomal DNA and high molecular weight RNA were collected by centrifugation (5 minutes, 15000rpm in a Heraeus bench microfuge). The supernatant was transferred to a fresh tube containing 0.6 volumes isopropanol, and held at room temperature for 15 minutes before centrifugation for 15 minutes as above, washing with 70% (v/v) ethanol and drying *in vacuo*. The pellet was resuspended in 30-50 µl of 1x TE.

7.1.3 DNA purification and quantitation

Purification of DNA after gel excision in agarose is described below. The Wizard (Strategene, USA) and GeneClean (IBI) silicon-binding systems were also occasionally used for DNA fragments and plasmids for sequencing or clonings, as per the manufacturers instructions. The concentration of DNA in solutions was determined by measurement of absorption at 260 nm, assuming an A_{260} of 1.0 to equal 50 mg DNA/ml (Miller, 1972).

7.1.4 Restriction endonuclease digestion of DNA

Most cleavage reactions were done using the restriction enzyme buffer SPK (10x: 200 mM Tris-HCl pH 7.5, 50mM $MgCl_2$, 5mM dithiothreitol, 1mM EDTA, 500mM KCl and 50% glycerol). The remaining restriction digests were carried out using EB buffer (10mM Tris-HCl, pH7.5, 6mM $MgCl_2$, 1mM DTT) as a basis, with either the addition of NaCl or KCl as described by the manufacturers. 0.1-0.5 µg of DNA or purified restriction fragments were incubated with 2 units of each restriction enzyme in a final volume of 20 µl, at 37°C, for 1-2 hr. 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 µg/ml RNase A) was added.

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 calculated sizes of the SPP1 *Eco*RI standard fragments used differ from those published (Ratcliff *et al.*, 1979) and were calculated with the program DNAFRAG (Rood and Gawthorne, 1984) using bacteriophage lambda and plasmid pBR322 as standards. The sizes (kilobases, kb) used were: 8.37; 7.2; 6.05; 4.9; 3.55; 2.68; 1.73; 1.61; 1.29; 1.19; .99; .86; .63; .48; .38; (Franzon and Manning, 1986).

7.1.5 Analytical and preparative separation of restriction fragments

Electrophoresis of digested DNA was carried out at room temperature on horizontal, 0.6%, 0.8% or 1% (w/v) agarose gels (Seakem HGT), 13 cm long, 13 cm wide and 0.7 cm thick. Gels were run at 100V for 4-5 hr in either 1x TBE buffer (67mM Tris base, 22mM boric acid and 2mM EDTA, final pH 8.8), or 1x 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 either Polaroid 667 positive film or 665 negative film.
For preparative gels Sea Plaque (Seakem) low-gelling-temperature agarose at a concentration of 0.6% (w/v) was used for separation of restriction fragments, which were recovered by the following methods:

Method 1:

DNA bands were excised and the agarose melted at 65^{o}C. Five volumes of 20mM Tris-HCl, 1mM EDTA, pH 8.0 buffer were added and the agarose extracted with phenol:water (1:1) and then phenol:chloroform (1:1). Residual phenol was removed with chloroform and the DNA precipitated with two volumes of ethanol and one tenth volume of 3M sodium acetate, pH 5.0. DNA was collected by centrifugation (15 min, Eppendorf), washed once with 70% (v/v) ethanol and dried *in vacuo* before being resuspended in 1x TE buffer.

Method 2:

After electrophoresis the required DNA bands were excised and then placed inside dialysis tubing. This was then positioned in an electrophoretic tank filled with 0.5x TAE buffer. The DNA was electrophoresed from the gel fragment into the buffer contained in the dialysis tubing. The DNA was then extracted with an equal volume of TE saturated phenol and precipitated with two volumes of ethanol and one tenth volume of 3M sodium acetate, pH 5.0.

7.1.6 Dephosphorylation of DNA using alkaline phosphatase

Restriction enzyme digested DNA was treated with alkaline phosphatase by the following method. 0.1-0.5 μg of digested plasmid DNA was incubated with 1 unit of alkaline phosphatase (Calf intestinal: CIP), for 30 min at 37^{o}C. The reaction was terminated by the addition of EDTA, pH 8.0 to a final concentration of 3mM followed by heating at 65^{o}C for 10 min. The reaction mix was then extracted twice with hot (56^{o}C) TE saturated phenol and twice with diethyl ether. DNA was precipitated overnight at -20^{o}C with two volumes of ethanol and 1/10 volume of 3M sodium acetate pH 8.0. The precipitate was collected by centrifugation (15 min, Eppendorf), washed once with 1 ml 70% (v/v) ethanol, dried *in vacuo* and dissolved in 1x TE buffer.

7.1.7 End-filling with Klenow fragment

Protruding ends created by cleavage with restriction endonucleases were filled in using the Klenow fragment of *E. coli* DNA polymerase I. Typically, 1μg of digested DNA or purified DNA fragments, 2μl of 10 x nick-translation buffer (Maniatis *et al.*, 1982), 1μl of each dNTP (2mM) and 1 unit of Klenow fragment were mixed in a final volume of 20ml and incubated for 30min. Samples were phenol/chloroform extracted twice in a total volume of 100μl and precipitated in 2 volumes of 100% ethanol and 1/10 volume of 3M sodium acetate for 30min in a dry ice/ethanol bath. Pellets were dried *in vacuo* and resuspended in a total volume of 20ml of 1 x TE.

7.1.8 *In vitro* cloning

DNA to be subcloned (200 ng) was digested by appropriate restriction endonucleases. This was combined with 20 ng of similarly cleaved vector DNA, then ligated with 2 units of T4 DNA ligase in a volume of 50 μl in a final buffer concentration of 20mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 10mM dithiothreitol (DTT), 0.6mM ATP for 16 hr at 4°C, or on the bench for 2 hours at 25 °C. The ligated DNA was then used directly for transformation of *E. coli* strains. Digests and endonucleases for ligation performed at 25°C for 2 hours were either purified or heat-killed (where the endonuclease was heat-sensitive) beforehand.

7.1.9 Transformation procedure

Transformation was performed essentially according to the method described by Brown *et al.* (1979). *E. coli* K-12 strains were made competent for transformation with plasmid DNA as follows: an overnight shaken culture (in NB) was diluted 1:20 into NB and incubated with shaking until the culture reached an A_{650} OD of 0.6 (4x 10^8 cells/ml). The cells were chilled on ice for 20 min, pelleted at 4°C in a bench centrifuge, resuspended in half volume of cold 100mM $MgCl_2$, centrifuged again and resuspended in a tenth volume of cold 100 mM $CaCl_2$. The cells were allowed to stand for 15 min on ice before addition of DNA. Competent cells (0.2ml) were then mixed with DNA (volume made to 100ml with 1x TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and left on ice for a further 15 min. The cell/DNA mixture was heated at 42°C for 2 min and then 3ml NB was added followed by incubation with shaking at 37°C for 1-2 hr. The culture was then plated onto selection plates directly or concentrated by centrifugation and plated. Cells with sterile buffer were included as a control.

7.1.10 Electroporation of *V.cholerae*

Electroporation of plasmid DNA was performed essentially as described by Stoebner and Payne (1988). Cells were grown in LB to an A_{650} of 0.5. They were then washed in sucrose electroporation buffer (272mM sucrose, 7mM sodium phosphate buffer, pH 7.4, 1mM $MgCl_2$) and resuspended in ice-cold sucrose buffer at 1/10 the original volume. 5µg plasmid DNA was added to 100 ml of cell suspension, placed in a cuvette and left on ice for 30 min. Electroporation conditions were 2000 V at 25 mF capacitance and 200 ohms. The cells were returned to ice for 30 min, then diluted in LB (3ml) and incubated at 37°C for 45 min, and plated out on appropriate media.

7.1.11 Bacterial conjugation

Overnight broth cultures grown in NB or LB were diluted 1:20 and grown to early exponential phase with slow agitation. 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 200 ml of broth and spread onto a cellulose acetate membrane filter (0.45 mm, type HA, Millipore Corp.) on a NA plate. This plate was incubated for 6-16 hr at 37°C. The cells were then resuspended in 10 ml NB and samples plated onto selective agar and incubated overnight at 37°C.

7.1.12 Homologous recombination using pCACtus

The pCACtus (Clark *et al.,* in preparation) multiple cloning site was used to receive genetic material suitable for chromosomal exchange. pCACtus - based contructs were introduced by electroporation into the target *V. cholerae* host strain and incubated at 30°C on chloramphenicol (25 µg/mL) Luria agar to select for electroporants. Cml- resistant colonies were picked and amplified overnight at 30°C shaking at 200 oscillations per minute in broth culture in the presence of chloramphenicol and subsequently plated onto Luria agar at 42°C overnight to select for chromosomal integrants. Where an additional antibiotic selection was available, this was utilised. At this temperature, the pCACtus vector is unable to replicate and only cells in which chromosomal integration had occurred (of the chloramphenicol resistance gene on the vector) were able to grow. Colonies were picked and amplified at 37°C in Luria broth before plating in serial dilutions (to 10^{-6}) onto Luria agar at 30°C in the presence of sucrose (5g/100mL) and chloramphenicol (25mcg/mL) and absence of NaCl, to ensure that organisms in which the *sacB* gene was retained (in other words, where the pCACtus vector remained, either chromosomally integrated or extrachromosomally) did not grow because of the toxicity of this gene product in the presence of sucrose. Organisms that grew had either lost the whole contruct originally integrated or had lost the extraneous vector DNA by a second crossover event. Approximately 50% of colonies are expected to be mutants, and this was selected for by antibiotic resistance marker where marker exchange was performed (eg.

introduction of a kanamycin resistance cartridge). Mutants were evaluated by Southern hybridisation, and by PCR and RFLP.

7.1.13 Preparation of oligodeoxynucleotides

Oligodeoxynucleotides were synthesized using reagents purchased from Applied Biosystems or Ajax Chemicals, on an Applied Biosystems 381A DNA synthesizer in the trityl-off mode and butanol-extracted prior to use. Annealing temperatures were estimated by allowance of 2°C per base (A and T) or 4°C per base (G and C) up to a temperature of 60°C.

7.1.14 Polymerase Chain Reaction Protocols

The procedure used essentially as described by Delidow (1993). PCR amplification was performed in a 50 µl reaction volume containing PCR buffer (1.5mM $MgCl_2$, 10mM Tris pH8.4, 50mM KCl), 1.5U of Taq polymerase (Cetus), 20pM of primer and 100ng of *V. cholerae* chromosomal DNA. The dNTPs were used at a concentration of 2mM. The thermocycler (Perkin Elmer Cetus) was programmed to incubate samples at 95°C for 5 min and then to carry out 25 cycles consisting of 95°C for 30 sec., 62°C for 30 sec., 72°C for 1 min followed by a final extension at 72°C for 5 min. 5µl of this reaction product was analysed on a 0.8% agarose gel, the remaining product was purified and used for cloning. Annealing temperatures (T_m) of oligonucleotides of 20 nt or less were estimated by allowing 4°C (G and C), or 2°C (A and T) per base. Reduced stringency reactions (see Chapter 2) were performed with lower annealing temperatures without alteration of salt concentrations in the reaction mixtures.

Recombination PCR and reverse circle PCR were essentially performed as per Jones and Howard (1991). Oligonucleotides were designed to include altered sequences as specified in the experimental sections of this thesis, whether this was to incorporate new restriction sites for cloning or as markers, areas of overlap extension for a deletion, or simple substitutions for site-directed mutageneses. Care was taken to ensure that complementary 5' tails of mutagenic primers in overlap extension reactions had annealing temperatures in the regions of complementarity close (within 2°C) to those 3' regions of primer annealing to the original template. This was set at around 50°C for convenience, in most cases. Reactions with large regions of template-primer disparity requiring reduced annealing temperatures (eg. introduction of a Shine-Dalgarno site) were performed as staged reactions, typically with 5 cycles at low T_M (10°C below initial template-primer annealing T_M) followed by 18-20 cycles at the T_M of the modified strands, so as to reduce background. All primers were designed with terminal free energies of the 3' pentamer greater than -9 (most around -6) kCal/mol, as calculated from estimates tabulated for each nucleotide pairing, for optimal efficiency.

7.1.15 Sequencing using dye-labelled primers

Sequencing reactions were carried out on 1μg of double stranded plasmid DNA using the protocol provided by Applied Biosystems. In dye-labelled primer sequencing the DNA was split into four tubes containing 160ng (A and C) and 320ng (G and T) of DNA, respectively. To each tube the following reagents were added: Dye primer 0.4pmol, 5x cycle sequencing buffer (400mM Tris-HCl, pH8.9, 100mM $(NH_4)_2SO_4$ pH9.0, 25mM $MgCl_2$), d/dNTP mixes (Applied Biosystems) and Taq polymerase 0.5 units.

Reagent	**A**	**C**	**G**	**T**
Dye-primer	1μl	1μl	2μl	2μl
d/dNTP mix	1μl	1μl	2μl	2μl
5x Cycle buffer	1μl	1μl	2μl	2μl
DNA template	1μl	1μl	2μl	2μl
Diluted Taq	1μl	1μl	2μl	2μl
Total Vol.	5μl	5μl	10μl	10μl

Each reaction was overlayed with 20μl of light mineral oil and centrifuged briefly.

Samples underwent 15 cycles (95°C 30sec; 55°C 30sec; 70°C for 60sec), followed by 15 cycles (95°C 30sec; 70°C 60sec; 15 cycles total), and were then held at 4°C. Reactions were combined in 80μl of 95% (v/v) ethanol with 3μl of 3M sodium acetate and precipitated on ice. DNA was pelleted at 13,000 rpm for 15 min (Hereaus bench microfuge). Samples were dried *in vacuo* and stored at -20°C.

7.1.16 Sequencing with dye-labelled terminators

Plasmid DNA was purified prior to dye terminator sequencing with kits supplied by Boehringer-Mannheim. 0.5 ml thin walled tubes (Gene Amp, Perkin Elmer) containing 1-2 μg of template DNA and 3.2 pmol primer, made up to a final volume of 20 μl with 9.5 μl of "Go" pre-mix (Boehringer Mannheim) and sterile water, were overlaid with mineral oil (Nujol, Perkin Elmer) and subjected to 25 cycles (96°C 30 sec; 50°C 15 sec; 60°C 4 min) before three extractions with phenol:chloroform:water (70:20:10 μl, added to reaction mix which was first made up to 100 μl with water). DNA was precipitated at -20°C overnight by addition of 100% ethanol (300 μl) and 3M Na Acetate (10 μl) to the 100 μl extracted aqueous phase, and was then collected and washed in the usual manner.

7.1.17 Analysis of DNA sequences

The dried pellets for sequencing were stored at -20°C until required, when they were resuspended in 4.5 µl loading buffer (83% deionised formamide, 8.3mM EDTA pH8.0), heated (95°C for 2min), and run on a 6% polyacrylamide-8M urea gel in an Applied Biosystems 373A DNA sequencer. Raw sequencing data from the 373A automated sequencer were analysed using the Applied Biosystems Seq Ed program version 6.0. Sequencing data were analysed using the LKB DNA and protein analysis programs, DNASIS and PROSIS (Hitachi Software).

7.1.18 Preparation of DNA probes

DNA fragment and plasmid probes were labelled with digoxigenin-11-dUTP (Boehringer-Mannheim) according to the manufacturers protocol, using random-labelling or end-labelling (oligonucleotides and small fragments). Random-primed labelling was performed with heat denatured (95°C 10 mins) DNA (10 ng - 3 µg) chilled on ice (3 mins) prior to addition of 2µl hexanucleotide mix and dNTP labelling mix (Boehringer-Mannheim) and 2 U Klenow fragment DNA polymerase 1. This was made up to 20 µl with sterile distilled water and held at 37°C for 20 minutes. The reaction was stopped with 2 µl of 0.2M EDTA pH8.0, and DNA precipitated with ethanol and LiCl (75 µl ethanol 100%; 25 µl LiCl 4.0M) on ice before washing, vacuum drying, and resuspension in 20 µl of TE (10mM Tris HCl, 1mM EDTA pH8.0).

For end-labelling, ca. 200ng of oligo in a final volume of 25 ml was mixed with tailing buffer (10x buffer: 1.4M potassium cacodylate, 300mM Tris pH7.2, 1mM DTT), 2.5 µl dig-11-dUTP, 1 µl terminal transferase and I µl 400mM $CoCl_2$, and incubated at 37°C for 60 minutes. All probes were held at -20°C before use.

7.1.19 Preparation of *V. cholerae* genomic DNA

Genomic DNA from *V. cholerae* 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 then 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. TE buffer (0.75ml) 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 vortexed, transferred to a 50 ml Ehrlenmeyer flask and incubated at 56°C for 60 min. This was followed by three extractions with TE-saturated phenol and two extractions with diethyl-ether. The genomic DNA was precipitated with four volumes of 100% ethanol and resuspended in 1ml of TE.

7.1.20 Southern transfer and hybridisation

Bi-directional transfers of DNA from agarose gels to nitrocellulose paper (Schleicher and Schuell) were performed as described by Southern (1975) and modified by Maniatis *et al.* (1982). Prior to hybridization with digoxigenin-labelled probe, filters were incubated for 4 hr at 44°C in a pre-hybridization solution containing 50% (v/v) formamide, 50mM sodium phosphate buffer, pH 6.4, 5xSSC 7.0), 5x Denhardt's reagent and 83mg/ml single stranded herring sperm DNA (Sigma) (Maniatis *et al.*, 1989). Pre-hybridization fluid was discarded and replaced with fresh hybridization buffer (as for pre-hybridization solution, with the exclusion of herring sperm DNA). Denatured probe was added and hybridization allowed to occur for 16-24 hr at 44°C, or 25°C for conditions of reduced stringency.

Filters were washed twice with shaking at 37°C for 30 min in 2xSSC, containing 0.1% (w/v) SDS. This was followed by two further washes in 0.1xSSC plus 0.1% (w/v) SDS at 65°C, or at 25°C for conditions of reduced stringency. After drying in the air (15min, room temperature), the filters were developed using an enhanced chemiluminescence kit (Boehringer-Mannheim) according to the manufacturer's instructions, after detection of probe with anti-digoxigenin Fab fragments conjugated to horseradish peroxidase.

7.2. Bacterial growth conditions and protein expression

7.2.1 Growth media and viability assays

The following nutrient media were used for bacterial cultivation. Nutrient broth (NB) (Difco), prepared at double strength (16 g/l) with added sodium chloride (NaCl) (5 g/l) or Luria broth (LB), were the general growth medium for both *V. cholerae* and *E. coli* K-12 strains. Luria broth (LB) is composed of bacto-tryptone (10 g/l) (Difco), bacto-yeast (5 g/l) (Difco) and NaCl (5 g/l). Minimal medium (M13 minimal media) was prepared as described by Miller (1972) and supplemented prior to use with $MgSO_4$, glucose and thiamine-HCl to concentrations of 0.2 mg/ml, 0.5%(w/v) and 50mg/ml, respectively. Terrific broth was prepared as described by Maniatis *et al.* (1989). AKI medium (0.3% $NaHCO_3$, 0.5% NaCl, 1.5% Bactopeptone (Difco), 0.4% yeast extract (Difco) as described by Iwanaga and Yamamoto (1985) was used as the growth medium for *tcp* induction. NA is nutrient agar composed of Lab-Lemco powder (Oxoid) (10 g/l), peptone (Oxoid) (10 g/l), NaCl (5 g/l) and Agar (Media Makers) (15 g/l). Soft agar contains equal volumes of NB and NA. Incubations were at 37°C unless otherwise specified. Liquid cultures, were normally grown in 20 ml McCartney bottles.

Antibiotics were added to broth and solid media at the following final concentrations: ampicillin (Ap), 25 mg/ml; chloramphenicol (Cm), 25 mg/ml; kanamycin (Km), 25 mg/ml; rifampicin (Rif), 200 mg/ml; gentamycin (Gm), 40 mg/ml; tetracycline (Tc), 12 mg/ml for *E. coli* and 4 mg/ml for *V. cholerae* strains.

Viable counts of cultures were derived by comparing estimates of broth cultures in a Neubauer chamber, after vigorous vortexing to separate clumped cells, with serial dilutions onto non-selective media and overnight aerobic incubation at 37°C.

7.2.2 *Tcp*-inducing and non-inducing growth conditions:

Induction of the *tcp* operon was essentially by the method refined by Voss (Voss, 1995) from that of Jonson *et al.* (1991a), and consisted of 2 hours standing at 30°C followed by 2 or 14-16 hours shaking at 200 rpm (Orbital shaking water bath, Paton Industries). An overnight culture from a single colony, shaken aerobically at 30°C in nutrient broth, was inoculated 1:50 into AKI medium supplemented with 1:50 of $NaHCO_3$ (7.5 mg/ml) in a conical flask of 20 x the volume of the final culture, after gassing out with 5% CO_2. Both 569B and Z17561 (classical *wt*) strains agglutinated heavily and were positive for cholera toxin and TcpA, under these conditions

Non-*tcp*-inducing conditions used routinely were those of aerobic incubation in nutrient broth at 37°C. TcpA was not detectable by immunoblot of H1, Z17561, or their derivatives under these conditions. Strain 569B and its derivatives produced sufficient amounts of TcpA under these conditions to be detectable at ca. 10^{10} cfu per track by the sensitive ECL method, using α569B-165 as the primary antobody.

7.2.3 Scoring of TCP production

In the absence of a defined adhesion specificity, the only measure available for functional activity of TCP and TcpA are the infant mouse cholera model and the fucose-resistant haemagglutination (FRHA) of mouse red blood cells. While competition and virulence behaviour in the IMCM is a useful and direct measure, it is impractical in complementation studies because of issues of plasmid stability, chromosomal integration, uncontrolled expression, etc. The FRHA characteristic of TcpA (Taylor *et al.*, 1987) was found to require high concentrations of TcpA and to depend on presentation (Chapter 5). Hydrophobicity as measured by ammonium sulphate precipitation (Taylor *et al.*, 1987) was also found to be a relatively insensitive measure, unable to discriminate clearly between various levels of incomplete complementation. Accordingly, a simple composite measure was used:

1. **TcpA levels on immunoblot** were simply scored as 'normal', 'reduced', or 'absent'. In certain circumstances, serial dilutions were used to refine this measure (Chapter 5).

2. **IEM fields**: scored on the **average number of bundles per cell** in a 4 hour culture:

1.sparse: < 1 bundle per 10 cells

2. few: < 1 bundle per 2-5cells, or only ocasional fibres detected

2.moderate: > 1 bundle per 2 - 5 cells

3.normal: copious bundle formation with large tangled fibre bundles

3. **agglutination** (micro):

1.nil under phase contrast

2.visible globular coalescence under PC on gentle agitation

3.normal levels: almost clearing of culture peripheries with a central mass

4. **macroscopic agglutination**:

1.nil

2.fine

3.coarse

4.*wt* levels

Assessments were made with reference to the isogenic parent positive control and a number of 'standards' or the various positive scores allowed, grown simultaneously by the 4-6 hour AKI method (above) so as to minimise inter-experiment variation. Immunoblot, IEM and macroscopic agglutination were used for routine scoring. Microscopic (phase contrast) asessment was done only to score levels of incomplete complementation.

The scales were found to be internally consistent, and compared as follows:

score	TcpA blot (polyclonal)	IEM score	phase contrast	agglutination
-	absent/ reduced	nil detected	nil	nil
(+)	reduced	sparse (<1:10)	1	1
+	reduced	few (<1:2-5)	1	1
++	normal	moderate (>1:2)	2	2
+++	normal	normal (copious)	3	3-4

Great care was taken to ensure uniform inoculation of cultures. Culture growth with increased OD_{550} over 0.6 tends to obscure lesser degrees of aggltination in shaking cultures. Excessive initial inoculation of cultures (OD_{550} > 0.05) and prolonged growth (> 6 hours) of strains bearing ampicillin-resistant plasmids was noted to lead to some plasmid dropout, while chloramphenicol-based vectors tended to be more

stable in late cultures. Plasmid stability at 4 hours was greater than 95% for all constructs tested, and all complementation measurements were done at this point unless otherwise specified.

The inherent subjectivity of such a measure was countered only by demonstration of important results to disinterested observers and by the composite nature of the measure. For this reason, the scale is simple and only clear and reproducible differences were reported.

7.2.4 Maintenance of strains

For long term storage, all strains were maintained as lyophilized cultures, stored *in vacuo* in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth. Half the contents were then transferred to a 10 ml bottle of NB and incubated with shaking overnight at the appropriate temperature. The other half was streaked onto two nutrient agar plates and incubated overnight at the appropriate growth temperature, with the appropriate antibiotic selection. Short-term storage of strains in routine use was as a suspension of freshly grown bacteria in glycerol (32%v/v) and peptone (0.6%w/v) at -70^{o}C. Fresh cultures from glycerols were prepared by streaking a loopful of the glycerol suspension onto a nutrient agar plate followed by incubation overnight just prior to use.

Bacterial strains were prepared for long-term storage by suspension of several loopfulls in a small volume of sterile skimmed milk (5g%). Approximately 0.2 ml aliquots of this thick bacterial suspension were dispensed into sterile 0.25x4 inch freeze drying ampoules and the end of each ampoule was plugged with cotton wool. The samples were then lyophilized in a freeze drier. The ampoules were evacuated to a partial pressure of 30 microns, sealed without releasing the vacuum, and stored at 4^{o}C.

7.2.4 Derepression of *lac* and *tac* promotors

An overnight culture was diluted 1:10, with appropriate antibiotic, and incubated with shaking for 90 mins at 37^{o}C, or until the OD_{600} reached approx. 0.8 (late lagarithmic phase). The culture was divided, IPTG was added to $^{1}/_{2}$ of the culture at a final concentration of 1mM, and cells were incubated for a further 3 hrs at 37^{o}C. 1 ml sample of culture was placed in a microcentrifuge and cells were collected by centrifugation and resuspended in 50 ml of 1x SDS smaple buffer. 10 µl of sample was boiled and loaded onto SDS-PAGE gels for analysis.

7.2.5 T7 RNA Polymerase Expression System

The plasmid pGP1-2 carries the T7 RNA polymerase under the control of the lambda P_L promoter. This plasmid was transformed into *E. coli* or *V.cholerae* strains containing a plasmid with the specific gene of interest under control of the T7 RNA polymerase promoter. A 10 ml LB broth with ampicillin and kanamycin was inoculated with a single colony and shaken at 30°C overnight. The culture was subcultured 1:10 and incubated with constant shaking at 30°C. When an A_{590} O.D. of 0.6 was reached, the cells were incubated at 42°C for 20 minutes to induce the pGP1-2 P_L promoter by the inactivation of cI_{ts}, allowing expression from the λpL promoter. Rifampicin was added to a final concentration of 200 μg/ml to inactivate the *E. coli* RNA polymerase and incubation was continued at 42°C for a further 20 minutes. The culture was then left for at least 2 hr shaking at 37°C. 1 ml of culture was transferred to microfuge tubes, spun to pellet the cells and resuspended in 100 ml of 1x SDS sample buffer. 10 ml of sample was boiled for 2 min and loaded onto SDS-PAGE gels for analysis. Gels were subsequently stained with Coomassie G250. *V.cholerae* strain JBK70 harbouring pGP1-2 and the plasmid of interest was grown in *tcp*-inducing conditions (Section 7.2.2) for certain experiments, prior to T7 induction.

7.2.6 Cell Fractionation

Method 1

The cell fractionation procedure was a modification of that described by Osborn *et al.* (1972a). Cells were grown in BHI to mid-exponential phase at 37°C (50 ml, OD_{650} of 0.6). Cells were pelleted in a Sorvall SS-34 rotor, (10,000 rpm, 10 min, 4°C) and resuspended in 1 ml of 20%(w/v) sucrose, 30mM Tris-HCl pH 8.1, transferred to SM-24 tubes and chilled on ice. Cells were converted to sphaeroplasts with 0.1 ml of 1 mg/ml lysozyme in 0.1M EDTA pH 7.3 for 30 min on ice. Cells were centrifuged as above and the supernatant collected (periplasmic fraction). The cell pellet was frozen in an ethanol dry ice bath for 30 min, thawed and dispersed vigorously in 3 ml 3mM EDTA, pH 7.3. Cells were lysed with a Branson Ultrasonifier (45% cycle, intermittent), by successive freeze-thawing, or by serial passage through a French pressure cell at 40-50 psi release pressure, until visibly lysed. Unlysed cells and large cell debris were removed by low speed centrifugation (5,000 rpm, 5 min, 4°C). The supernatant containing the membranes and the cytoplasm was centrifuged at 35,000 rpm in a 50Ti rotor for 60 min at 4°C in a Beckman L8-80 ultracentrifuge. The supernatant (cytoplasmic fraction) was collected and the membrane pellet was resuspended in 25% sucrose, 10mM Tris-HCl pH 7.8, 1mM EDTA.

Method 2

The harvesting of outer membrane blebs using LiAc/ LiCl was as described previously for *Neisseria gonorrhoeae* (Pannekoek *et al.,* 1992). This technique relies on the induction of blebbing of the outer membrane into the medium in the presence of LiCl/ LiAc/ EDTA at 42°C. Lysed cells and large membrane fragments are pelleted (at 20,000xg) before collection of outer membrane blebs from the supernatant at 100,000xg.

Method 3

Discontinuous sucrose density floatation gradients were created in 10 ml Beckman Ultra-Clear polyallomer tubes with 1ml layers each of 60, 56, 52, 48, 44, 40, 36, 32, 28 and 24% w/w sucrose/ PBS, and specimens (membrane fractions or whole cell lysates) were underloaded in 60% sucrose/ PBS. Centrifugation was performed in a Beckman SW41Ti swing-out rotor for 48 hours at 220,000xg. Aliquots of approximately 700µl were drawn from the bottom of the tube through a large bore needle and immediately frozen at -20°C for further analysis.

7.3 Electrophoretic methods

7.3.1 SDS-Polyacrylamide Gel Electrophoresis of Proteins

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on either 11-20% polyacrylamide gradients (for proteins) or straight 20% polyacrylamide gels (for lipopolysaccharides) using a modification of the procedure of Lugtenberg *et al.* (1975) as described previously by Achtman *et al.* (1978). Samples were heated at 100°C for 3 min in SDS sample buffer (.25mM Tris-HCl pH 6.8, 2%(w/v) SDS, 10%(v/v) glycerol, 5%(v/v) b-mercaptoethanol, 15(w/v) bromophenol blue) prior to loading. Gels were generally electrophoresed at 100 V for 5 hr (11-20% gradient gels) or 10mA constant current for 16 hr (20% PAGE gels). Proteins were stained with gentle agitation overnight at room temperature in 0.06% (w/v) Coomassie Brilliant Blue G250 (dissolved in 5% (v/v) perchloric acid). Destaining was accomplished with several changes of 5% (v/v) acetic acid, with gentle agitation for 24 hr. Size markers (Pharmacia) were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and a-Lactalbumin (14.4 kDa).

7.3.2 SDS-PAGE for Lipopolysaccharide (LPS)

Whole cell lysates were prepared by the method of Hitchcock and Brown (1983). Cells were grown overnight in NB and 1.5 ml was spun down in an Eppendorf centrifuge for 5 min. The pellets were solubilized in 50 µl of lysing buffer containing 2%(w/v) SDS, 4%(v/v) β-mercaptoethanol, 10%(v/v) glycerol, 1M Tris-HCl pH 6.8, and 0.1%(w/v) bromophenol blue. Lysates were heated at 100°C for 10

min. 2.5 mg of Proteinase K solubilized in 10 ml of lysing buffer was added to each sample and incubated at 60°C for 2-4 hr. Samples were stored at -20°C.

7.3.3 LPS silver staining

Silver staining of LPS in polyacrylamide gels was performed after the method described by Tsai and Frasch (1982), as follows:

i) fixation overnight in 40%(v/v) ethanol, 10%(v/v) acetic acid;
ii) oxidation for 5 min with 0.7%(w/v) periodic acid in 40%(v/v) ethanol, 10%(v/v) acetic acid;
iii) 4 washes with water at 30 min each;
iv) staining for 10 min, in a solution containing 28 ml 0.1N NaOH, 2 ml concentrated NH_4OH and 5 ml 20% (w/v) $AgNO_3$ in a total volume of 150 ml;
v) developing in a solution of 50 mg citric acid and 0.5 ml formaldehyde in 1 litre. The citric acid was dissolved in water and heated to 37°C, and formaldehyde added just before use.

This method is extremely intolerant of protein or ion contamination of any form. Distilled, deionized water which had been passed through a series of Millipore filters and had a conductivity of not more than 18 mega ohms/cm was used to rinse all glassware and in the preparation of solutions.

7.3.4 Western Transfer and Protein Blotting

The procedure used was a minor modification of that described by Towbin *et al.* (1979). Samples were subjected to SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell) at 200 mA for 2 hr in a Trans-Blot Cell (Biorad). The transfer buffer used was 25mM Tris-HCl pH 8.3, 192mM glycine and 5%(v/v) methanol. For LPS transfer the buffer contained 20%(v/v) methanol and the transfer time was 1hr at 500mA. After transfer, the nitrocellulose sheet was incubated for 30 min in 5%(w/v) skim milk powder in TTBS (0.05%(v/v) Tween 20, 20mM Tris-HCl, 0.9%(w/v) NaCl) to block non-specific protein binding sites. The primary antiserum was diluted 1/100 in TTBS, 0.02%(w/v) skim milk powder and incubated with gentle agitation at room temperature for 2-16 hr. The nonspecific antibody was removed by washing the nitrocellulose sheet three times for 10 min in TTBS with shaking. Bound antibody was detected using an anti-antibody coupled with horseradish peroxidase and peroxidase substrate. This was accomplished by incubating the filter for 2-16 hr (gentle agitation) with goat anti-rabbit IgG coupled with horseradish peroxidase (Nordic Immunology) at a dilution of 1/5,000 in TTBS. The filter was then washed four times (5 min intervals) with TTBS, followed by two 5 min washes in TBS (20mM Tris-HCl, 0.9%(w/v) NaCl). The antigen-antibody complexes were then visualized using peroxidase substrate (9.9mg 4-chloro-1-napthol dissolved in 3.3 ml -20°C methanol added to 16.5 ml TBS containing 15 ml hydrogen peroxide) which was added and allowed to incubate for 10-15 min with shaking, as described

by Hawkes *et al.* (1982), or by the enhanced chemiluminescence kit (Boehringer-Mannheim) as per manufacturers instructions). Alkaline phosphatase-labelled goat anti-rabbit antibody was detected by the addition of nitroblue-toluidine (NBT) and bromochloroindolyl phosphate (BCIP) as described (Maniatis *et al.,* 1989).

7.4 Antisera and other labelling methods

7.4.1 Animals

Fresh blood was obtained by retroorbital bleeding of ether-anaesthetised adult balb/c. mice for haemagglutination assays. The polyclonal antiserum against TcpT was raised in an adult New Zealand white rabbit, obtained from the Central Animal House of the University of Adelaide. The rabbit was immunized without adjuvant by subcutaneous injection of gel homogenates on days 1, 10, 24, and 38, and terminally bled by cardiac puncture under anesthesia 10 days after the last immunization. The band of interest was excised from $TcpT^F$-enriched inclusion bodies, and the excised acrylamide bands was destained over two days, then rinsed in PBS overnight with three changes of wash. After administration, the antiserum was adsorbed against whole cells of *E. coli* DH5α (3 adsorptions) and *V. cholerae* JRI1 (3 adsorptions), as well as membrane fractions of *V. cholerae* JRI1 (16 absorptions) to improve its specificity. Whole cells were prepared by growth in nutrient broth (*E. coli*) and in TCP-inducing conditions (*V. cholerae*)(see above, Section 7.2.2) to ca. 10^{10} cfu/ml, killed by the addition of Na azide 0.1%, subsequent centrifugation at 7000 xg for 10 mins and resuspension in antiserum at 20:1. Each adsorption was held for 4-6 hours with gentle agitation (100 opm) on a rotary table in a water bath at 37°C, repelleted and poured off, and then resusupended in fresh (killed) cells in the same concentration for holding at 4°C overnight. Adsorptions against membrane fractions were conducted in the same manner, except that centrifugations were performed at 90,000 xg for 60 mins and adsorptions of antiserum were against PBS-washed azide-treated membrane fractions, which were added to a total final concentration of ca. 4 mg/ml.

7.4.2 Antibodies

Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Kirkegaard and Perry Laboratories, Inc. Anti-digoxigenin-POD (Fab fragments) was obtained from Boehringer Mannheim. Protein A-gold (10nm) and goat anti-mouse antisera coupled to 10nm gold particles were obtained from Amersham. Antisera (goat anti-mouse and goat anti-rabbit) conjugated to alkaline-phosphatase, or the fluorochrome Texas Red, were obtained from Amersham or Pierce Chemicals.

Table 7.2 Primary antisera

	target	working dilution	source/ reference
a569B-165 polyclonal (rabbit)	(native and) denatured TcpA (classical >> El Tor)	1:1500-2000 (immunoblot); 1:50 (IEM)	Sharma *et al.*, 1989; Voss, 1995
Tc20.2 monoclonal (mouse IgG_1)	native and denatured TcpA (classical >> El Tor)	1:100 (immunoblot) 1:10 (IEM)	G. Jonson/ J. Holmgren, Univ. Göteborg, Sweden
17:10 monoclonal (mouse IgG)	native and denatured MshA	1:100 (immunoblot) 1:10 (IEM)	G. Jonson/ J. Holmgren, Univ. Göteborg, Sweden
αHAP polyclonal (rabbit)	SHA/ protease	1:100 (inhibition)	Svennerholm *et al.*, 1983
αTcpT polyclonal (rabbit)	TcpT	1:2000 (immunoblot)	this work (Section 5.3.4)
αM2 monoclonal (mouse IgG)	FLAG octapeptide (DYKDDDDK)	2-5 mg/ml (immunoblot)	International Biosystems Inc.,USA

7.4.3 Autoradiography and densitometry with [^{35}S]-methionine labelling

SDS-PAGE gels were dried on Whatman 3MM chromatography paper at 60°C for 2 hr on a Bio-Rad gel drier. [^{35}S]-methionine autoradiography was performed at room temperature for 1-7 days without intensifying screens using Kodak XR-100 film. For autoradiography with [^{32}P] labelled DNA, the gels were exposed to film for 6-72 hr at -70°C, using intensifying screens. Pulse-chase experiments were conducted by cold chase of label with excess L-methionine and/ or the addition of Luria broth 1.0 volumes before transfer back to 30 or 37°C, as appropriate for the conditions specified. Aliquots were taken at t = 0, and at intervals indicated for each experiment, immediately transferred to ice, and centrifuged at 4°C for 30 seconds before addition of sample buffer (Lugtenberg *et al.*, 1975) and storage at -20°C.

After exposure of the [^{35}S]-labelled protein to a BAS-IIIS imaging plate (Fuji Photo Co., Ltd.), densitometry was performed in a Fuji BAS-1000 phosphorimager and analysed with the AIS software package (Version 2.0, Rev. 1.5, 1995; Imaging Research, Ontario, Canada) by density x area summation for individual bands.

7.4.4 Western immunoblots - ECL and NBT-BCIP

SDS-PAGE was performed in 15% polyacrylamide under constant voltage conditions (180V), otherwise according to Lugtenberg *et al.* (25). Western transfer and immunoblotting was performed in the usual manner (58) with either anti-569B-165 (αTcpA) serum at 1:2000 or the Tc20.2 mab (αTcpA) at 1:100 final dilution. Antisera to TcpT were used at 1:2000. Detection of immunoblots was performed by using either alkaline phosphatase-labelled goat anti-rabbit antibody, with addition of nitroblue-toluidine (NBT) and bromo-chloro-indolyl phosphate (BCIP) as previously described (Sambrook *et al.*, 1989), or by using horseradish peroxidase-labelled goat anti-rabbit antibody and the enhanced chemiluminescence (ECL) detection kit according to the manufacturers instructions (Boehringer-Mannheim).

7.4.5 ATP binding assay

In situ periodate oxidation was performed according to a published method (Peter *et al* 1992). 10^7 cfu/ ml of PBS-washed cells are permeabilized on ice (5 mins) by 50μg/ ml L-α-lysophosphatidylcholine (Sigma) in Hepes buffer (20mm HEPES pH 7.8; 2.5mM $MgCl_2$; 10mM NaCl; 140mM KCl). Cells are washed in the same buffer and incubated with 1μM [γ-^{32}P] ATP (Amersham, 100 Ci/mmol) at 37°C. Oxidation of nucleotides is effected in 1mM $NaIO_4$ (Sigma; 37°C, 1 min) and reduction in 20mM $NaCNBH_3$ (Sigma; 37°C, 1 min). 20mM $NaBH_4$ (Sigma) is added to stop the reaction , and samples are transferred to ice and pelleted at 7000xg 3 mins. Samples are resuspended in loading buffer and subjected to SDS-PAGE. The gel is dried for autoradiography with intensification (as described), and films developed after 48 hours at -70°C, or by exposure to Fuji-BAS 1000S Phosphorimaging plates (Fuji Film Co., Ltd.).

7.5 Protein manipulation techniques

7.5.1 Salt elution and detergent extractions

Membrane fractions derived by centrifugation at 90,000xg, after washing and removal of unlysed cells as described above, were resuspended in PBS containing either 1.0M NaCl, 6.0M Urea, 2% Triton X-100, or 1% sarkosyl (no $MgCl_2$). NaCl elution was performed on ice, while urea and detergent treatments were performed at 30°C, all for 45 mins. Pellets obtained at 90,000xg (1 hour) were compared with equal volumes of supernatant run on SDS-PAGE and immunoblotted for TcpA.

7.5.2 Chemical cross-linking of proteins

In a slight modification of a published method (Scherer *et al.*, 1990), cells from 569B or V663 cultures were washed and resuspended in 50µl of buffer [20mM Hepes (pH 7.8); 140mM Kcl; 10mM NaCl; 2.5mM $MgCl_2$] at ca. 5 x 10^7 cfu/ml, and mixed with 2µl or 4 µl of freshly prepared solution of 5mM dithiobis (succinimidyl propionate) (DSP) in dimethyl sulphoxide (34). Sucrose density gradient fractions corresponding to the location of major outer membrane proteins and to the location of the cytoplasmic membrane protein TcpT were taken for each strain. These were mixed with 2µl of 5mM 3,3'-dithiobis (sulphonyl succinimidyl propionate) (DTSSP) in the buffer as above in equivalent amounts of protein. Crosslinking was performed on ice for 30 mins, and quenched with L-lysine (10mM final concentration). DSP and DTSSP were prepared fresh on each occasion in dimethyl sulphoxide and sterile water, respectively. Treated specimens were subsequently run on SDS-PAGE in the presence or absence of reducing conditions (boiling for 3 mins with or without β-mercaptoethanol, respectively).

7.5.3 Protease accessibility

These experiments were performed according to a published procedure (Klauser *et al.*, 1990b), with minor modifications. Whole cells grown for *tcp* induction were resuspended in PBS/ 30% sucrose with or without 5mM EDTA and held for 30 minutes on ice. Trypsin (at 100 and 500 µg/ml) or proteinase K (at 10, 50, and 100 µg/ml) were added after washing of cells in PBS/ sucrose, and samples were kept on ice for a further 30 minutes before pelleting and resuspension in sample buffer. Samples were stored at -20°C before electrophoresis as described above.

7.5.4 Affinity purification of protein

This was performed according to the manufacturers instructions (International Biotechnologies Inc., USA): agarose beads coupled to the M2 monoclonal antibody are packedin a 1.0 ml column with teflon filter and washed in Tris-buffered saline (TBS pH7.4), followed by 3 x washes with glycine 1.0M Hcl pH3.0 (to remoev non-specifically bound protein) and finally washed x 4 in TBS pH7.4. Sample (cytoplasmic fraction) is passed through the column several times to remove target antigen (the column has approximately 5 µg carrying capacity). After TBS washing, the bound antigen is eluted in 0.1M glycine/ TBS/ HCl pH3.0 into a neutralising buffer (Tris 1.0M pH8.0). Most antigen is eluted in 5 ml, and column is washed and stored in TBS/ Na azide (0.05%) pH7.4. All steps are performed on ice. Samples are stored at -20°C.

7.6 Imaging methods

7.6.1 Immuno-electron microscopy (IEM)

Immuno-gold labelling adapted from the method of Levine *et al.* (1984). Colloidin-coated nickel grids (200 mesh, Graticules) were treated with poly-L-lysine (0.1 mg/ml) and placed face down on 40µl of a washed bacterial suspension (10^{10}cfu/ml). Grids were then successively transferred onto 25 µl drops spotted onto Parafilm M laboratory film (American National Can) as follows:

3% BSA/ PBS, 3 mins; antiserum as appropriate (see Table 7.1), 15 mins; two saline washes; Protein-A-gold (Amersham: 1:80 in distilled water, 10nm gold particles), 10 mins; two distilled water washes; uranyl acetate 1.0% for 10-60 seconds. Grids were examined in a Phillips EM300, CM100, or CM200 transmission electron microscope, usually at 80 kV accelerating voltage

7.6.2 Ultrathin Cryosectioning

The procedures used were modified from a published method (Geuze and Slot, 1980). Cells were washed and resuspended in 30% sucrose/ PBS and held for 10 mins in a 1.5 ml Eppendorf microfuge tube, before pelleting and resuspending in 6.7M (ca.70%) sucrose 1% agarose/ PBS, which was kept at 50°C prior to use. The cells were cooled at 4°C before being cut from the agarose and kept in 2.3M (ca. 70% w/v) sucrose/ PBS (for 2 hours) before sectioning. Sections were cut at -100°C in a Reichert Ultracut S cryomicrotome, and the specimens mounted directly on Ni grids (200 mesh, Graticules) for examination in a Philips CM100 transmission electron microscope. 10nm gold particles conjugated to Protein A or goat-antimouse antisera (Amersham) were used to detect bound primary antibodies.

7.6.3 Immunofluorescent (IF) techniques

The technique is a minor modification of a published method (Klauser *et al.,* 1990b). Round glass cover slips were boiled for 1 minute in 0.1M HCl before storage in 95% ethanol prior to air-drying for use. Poly-L-lysine (100µl 0.1mg/ml) was pipetted onto cover slips placed in a 24-well flat-bottomed tissue culture tray (Costar) andincubated at room temperature for 5 minutes before being washed with PBS. Cells were prepared in 30% sucrose in phosphate-buffered saline (PBS) with or without 3mM EDTA at approximately 5 x 10^7 cfu/ml and held on ice for 10 mins. 100 µl of the cell suspension was pipetted gently over the cover slip and 400 µl of sterile PBS added at 25°C before centrifugation at 500 rpm for 10 mins. The wells were aspirated dry and 300 µl of 2% paraformaldehyde/ 0.1% gluteraldehyde added for a further 10 mins. After 3 gentle washes in PBS, 1% (w/v) foetal calf serum (FCS) in PBS was added in a 10 mins blocking step before replacement with the primary antibody in 10% FCS in PBS. After 90 mins

covered with a moistened paper towel, a further 3 PBS washes were followed by the addition of secondary antibody in the same manner for 30 mins. After a further 3 washes as above, with the last held for 5 mins, cover slips were gently aspirated dry and mounted upside down on a clean glass microscope slide with a 25µl drop of 1% glycerine/ PBS before sealing with acrylic varnish. All procedures after fixation were performed at room temperature (20 - 25°C).

7.7 Functional Assays

7.7.1 *V. cholerae* motility assay

Motility was tested by swarming of the bacteria in soft agar and is based on a modification by S. Attridge (PhD Thesis, University of Adelaide, 1979) of the sloppy agar overlay method devised by Stocker (1949). A fresh culture of a test organism was diluted and plated onto NA such that 100-200 colonies would develop per plate. Following overnight incubation, each plate was overlaid with 5ml of 0.3% soft agar, allowed to set at room temperature and incubated at 37°C for 2-3hr. Colonies which comprise motile bacteria develop a halo as the organisms swim in the soft agar overlay.

7.7.2 Haemolysin activity

Haemolysin activity was measured by a slight modification of that used by Alm *et al* (1990b). Stains were grown to an OD_{600} of 1.0. 0.5 ml of culture supernatant after centrifugation was admixed with an equal volume of 0.5% sheep red bloodcells (in PBS) and incubated at 37°C. 200 µl aliquots collected every 30 minutes for 4 hours were assayed for Haemoglobin release at A_{414} after clearing by centrifugation.

7.7.3 Cholera toxin activity

Supernatant was collected from cultures grown for *tcp/ctx* induction and estimated by a minor modification of the procedure described by Holmgren (1973). Microtitre trays (96 well, Costar or Nunc) were coated with 2 µg/ml monosialoganglioside-GM_1 (Sigma) diluted in PBS. Following overnight incubation at 4°C, trays were washed 4 times in PBS/ 0.05% Tween20, and BSA20% w/v-PBS/ Tween (0.05% Tween 20, 0.0125M triethanolamine, 0.14M NaCl) was used to block for 1 hour.

Replicate serial dilutions of a 4 µg/ml cholera toxin preparation (Sigma) and appropriate dilutions of culture supernatants were incubated for 2 hours at 37°C. Trays were washed 5 times with PBS-Tween and then incubated with antiserum (IgG fraction of polyclonal anti-CTX 1:70,000). After 4 further washes, trays were incubated overnight at 4°C with sheep anti-rabbit IgG conjugate with alkaline

phosphatase (1:1000 in enzyme diluent: 0.14M NaCl, 0.25mM triethanolamine, 0.002% (w/v) BSA, 0.5mM $MgCl_2$, and 1.25μM $ZnCl_2$). 5 further washes with PBS-Tween preceded the addition of substrate (1 mg/ml pNitrophenylphosphate, di-sodium (Sigma) in 10.5% diethanolamine, 1mM $MgCl_2$, pH 9.8) at 37°C for 90 minutes. OD_{405} was measured using a Titertek Multiscan ELISA tray reader, and the cholera toxin concentration in each sample estimated from a standard curve.

7.7.4 Protease and DNAse activity

Protease activity was measured in a qualitative fashion only by growth on casein plates and observation of areas of hydrolysis as clearing around the agar. Dnase activity was measured by growth on DNA-containing plates and comparison of zones of DNA hydrolysis visualised by the addition of HCl 1.0M.

7.7.5 Fucose-resistant haemagglutination

Cells grown under *tcp*-inducing conditions were pelleted, washed, and resuspended directly in PBS at ca. $2x10^{11}$ cfu/ml before lysis in a French press or by successive cycles of freeze-thawing and sonication. Fresh murine red cells, obtained by retro-orbital bleeding of anaesthetised adult balb/c. mice, were washed and resuspended at 1:200 dilution (packed cell volume) in a modified KRT buffer solution (0.13M NaCl, 5mM KCl, 1.3 mM Mg SO_4, 2.7 mM$CaCl_2$, 10mM Tris HCl pH7.4, 10mM ADA pH7.0) with 1% w/v L-fucose for use in the assay. Lysates were stored at -20°C and were loaded in equal amounts of total protein, as estimated by the BCA method. Serial two-fold dilutions in the same fucose-containing buffer were performed by multichannel pipette in a 96-well round-bottomed microtitre tray (Costar). 40 μl of lysate or cell suspension were serially diluted and mixed with 40 μl of erythrocyte suspension, and results read after 2 hours standing at room temperature.

7.8. Reagents and suppliers

7.8.1 Chemicals and reagents

Phenol, polyethylene glycol-6000 (PEG), sodium dodecyl sulphate (SDS) and sucrose were obtained from BDH Chemicals. Tris was Trisma base from Boehringer Mannheim. Caesium chloride (Cabot) was technical grade. Ethylene-diamine-tetra-acetic-acid, disodium salt (EDTA) was Analar analytical grade from Ajax Chemicals. Antibiotics were purchased from Sigma (ampicillin, kanamycin sulphate, rifampicin, gentamycin), and Calbiochem (tetracycline, chloramphenicol). All other anti-microbial agents (dyes, detergents and antibiotics) were purchased from Sigma Chemical Co., BDH Chemicals Ltd., Glaxo, or Calbiochem. Electrophoresis grade acrylamide and ammonium persulphate were obtained from Bio-Rad, and ultra pure N,N'-methylene bis-acrylamide and urea from BRL. Sodium lauryl sarcosinate (Sarkosyl) was obtained from from Geigy, and Tween20 from Sigma.

The four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and their corresponding dideoxy-ribonucleotide triphosphate homologues (ddATP, ddCTP, ddGTP and ddTTP), were obtained from Boehringer-Mannheim. Adenosine-5'-triphosphate, sodium salt (ATP), herring sperm DNA and dithiothreitol (DTT) were obtained from Sigma. X-gal (5-Bromo-4-chloro-3-indolyl-b-D-galacto-pyranoside) and IPTG (isopropyl-b-D-thiogalacto-pyranoside) were purchased from Boehringer-Mannheim. [γ-^{32}P] ATP, at a specific activity of 1,700 Ci/mMole, was obtained from BRESATEC (Adelaide). [^{35}S]-methionine (1,270 Ci/mmole) was purchased from Amersham. Digoxigenin (DIG) DNA labeling and detection kits were purchased from Boehringer-Mannheim.

7.8.2 Enzymes

Pronase and proteinaseK were from Boehringer-Mannheim, and lysozyme and trypsin from Sigma. 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: New England Biolabs (T4 DNA ligase), Amersham (T4 DNA polymerase, T4 DNA ligase) and Boehringer-Mannheim (DNA polymeraseI, Klenow fragment of DNA polymeraseI, and molecular biology grade alkaline phosphatase). Taq polymerase (Ampli Taq)was purchased from Perkin Elmer Cetus Corp. Sequencing kits using either dye-labelled primer or dye-labelled terminators were purchased from Applied Biosystems.

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