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Single amino acid substitutions in the N-terminus of *Vibrio cholerae* TcpA affect colonization, autoagglutination, and serum resistance

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Summary

The toxin-coregulated pilus (TCP) of *Vibrio cholerae* O1 is required for successful infection of the host. TcpA, the structural subunit of TCP, belongs to the type IV family of pilins, which includes the PilE pilin of *Neisseria gonorrhoeae*. Recently, single amino acid changes in the N-terminus of PilE were found to abolish autoagglutination in gonococci. As type IV pilins demonstrate some similarities in function and amino acid sequence, site-directed mutagenesis and allelic exchange were used to create corresponding mutations in TcpA. All four mutant strains demonstrated autoagglutination defects, and all were highly defective for colonization in the infant mouse model. These results support the previously proposed correlation between autoagglutination and colonization. Finally, all four mutants are serum sensitive, indicating that TcpA plays a role in serum resistance, a phenotype previously attributed to TcpC. As the mutations have similar effects in *N. gonorrhoeae* and *V. cholerae*, our results support the idea that type IV pilins have similar functions in a variety of pathogenic bacteria.

Introduction

The Gram-negative bacterium *Vibrio cholerae* is the causative agent of cholera, a potentially fatal disease characterized by profuse watery diarrhoea. The pathogenesis of *V. cholerae* depends on the ability of the bacterium to produce cholera toxin, and to colonize the small intestine of the host. The secretory diarrhoea is caused by cholera toxin, an ADP-ribosylating toxin that constitutively activates adenylate cyclase (Kaper *et al.*, 1994), but colonization is absolutely essential for pathogenesis. Cholera toxin expression is regulated by ToxR, a transcriptional activator that co-ordinately regulates a number of genes in addition to cholera toxin (Ottemann and Mekalanos, 1994). A screen for additional ToxR-regulated genes identified the toxin-coregulated pilus (TCP), the most important colonization factor identified to date (Taylor *et al.*, 1987; Attridge *et al.*, 1993; Kaufman and Taylor, 1994). In the infant mouse model, *tcpA* mutations result in drastic reductions in colonization, and TCP is required for colonization in humans (Herrington *et al.*, 1988).

TCP is a polymer of subunits encoded by *tcpA*, one of at least 12 genes in the *tcp* gene cluster (Taylor *et al.*, 1987; Shaw and Taylor, 1990; Ogierman *et al.*, 1993; Brown and Taylor, 1995). TcpA belongs to the type IV family of pilins, which includes pilins from several pathogenic bacteria, including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and enteropathogenic *Escherichia coli* (EPEC). The type IV class of pilins is defined by amino acid sequence similarities and *N*-methylation of the N-terminal amino acid of the mature pilin polypeptide. Most type IV pilins fall into the group A classification, whose members share amino acid homology in several regions throughout the pilin. TcpA and the bundle-forming pilin of EPEC are group B pilins, and share homology with the group A pilins in their N-termini. The type IV pili in the pathogenic bacteria noted above are thought to be involved in adhesion to the host, suggesting that the structural similarities among the type IV pilins probably reflect certain functional similarities, including polymerization, pilus aggregation, cellular autoagglutination (see below), and possibly receptor recognition (Strom and Lory, 1993).

Another gene in the *tcp* operon, *tcpC*, encodes a lipoprotein that has been implicated in serum resistance (Parsot *et al.*, 1991). Although serum resistance was clearly associated with the *tcp* operon, this phenotype was not definitively linked to *tcpC*. This earlier work relied on Tn*phoA* insertions in *tcp* genes, whose polarity on genes downstream in the operon made it difficult to ascribe the

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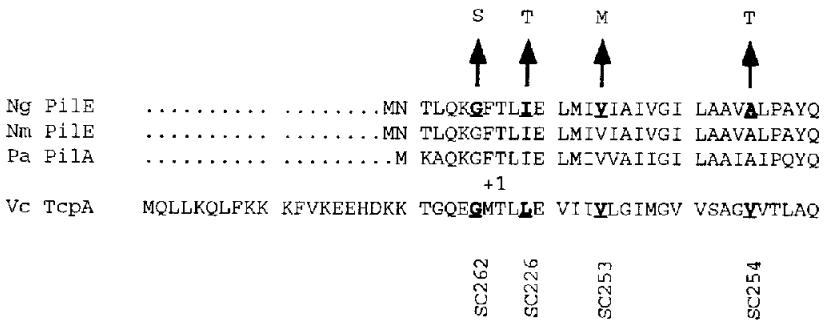

Fig. 1. Amino acid sequence comparison of N-termini of type IV pilins from *N. gonorrhoeae* (Ng), *N. meningitidis* (Nm), *P. aeruginosa* (Pa), and *V. cholerae* (Vc). Alignment was achieved using PILEUP (Genetics Computer Group, Inc., University of Wisconsin). Location of single amino acid substitutions in *N. gonorrhoeae* and *V. cholerae* are indicated by bold type, underlining, and arrows. The N-terminal amino acid of the mature pilin polypeptide is indicated by '+1'.

resulting mutant phenotypes to a single gene disruption. Several other genes in the *tcp* gene cluster are now known to be involved in TCP biogenesis (Kaufman *et al.*, 1991; Kaufman *et al.*, 1993; Iredell and Manning, 1994).

Autoagglutination is a phenotype tightly associated with type IV pilus expression in *N. gonorrhoeae* and *V. cholerae* (Swanson *et al.*, 1971; Taylor *et al.*, 1987). For example, certain single amino acid changes in the conserved N-terminus of gonococcal PilE abolish autoagglutination in that species (M. Koomey, unpublished). In some cases, the loss of autoagglutination reflected the failure of the altered subunit to be assembled, while pilus assembly was retained in other instances. Since there is no animal model for gonococcal infection, and because of the apparent conservation of structure and function among type IV pilins, we used site-directed mutagenesis to create analogous substitutions in TcpA in the *V. cholerae* classical Ogawa strain O395. Analysis of the resulting mutant strains demonstrated a correlation between autoagglutination and colonization, although the degree of autoagglutination did not correspond to the degree of colonization. The mutants are also serum-sensitive, indicating that it is TcpA rather than TcpC that mediates serum resistance.

Results

Design and construction of mutant tcpA *alleles*

Loss of autoagglutination is the standard phenotype scored to detect non-piliated variants in *N. gonorrhoeae* and *V. cholerae*. In the course of studies aimed at identifying novel gonococcal pilus assembly genes, an unanticipated class of spontaneous non-agglutinating mutants was found, in which pilus expression was quantitatively unchanged. Genetic analysis of these strains revealed that the mutations responsible for the loss of autoagglutination were single-base mutations that resulted in substitutions within the conserved hydrophobic N-terminus of the pilin subunit (Fig. 1; D. DeRyckere, P. Lauer, and M. Koomey, manuscript in preparation).

Site-directed mutagenesis was used to make corresponding amino acid changes in TcpA of *V. cholerae* O395. In two cases, the original amino acid in TcpA was not the same as the corresponding amino acid in gonococcal PilE (Leu_4 and Val_{20}, instead of Ile and Ala, respectively; numbering reflects amino acid sequence of mature TcpA pilin). A mutation changing the invariant glycine residue at the site of prepilin leader peptidase cleavage was also constructed. Based on prior studies in *N. gonorrhoeae* (Koomey *et al.*, 1991) and *P. aeruginosa* (Strom and Lory, 1991), this mutation should result in a TcpA protein that cannot be processed by TcpJ and, therefore, cannot be assembled. An alignment of the N-termini of several type IV pilins is shown in Fig. 1.

After verification of the sequences of the mutant *tcpA* alleles, pTZ18U-derived plasmids carrying these alleles were electroporated into BGD4, an O395 derivative with an internal non-polar deletion in *tcpA*. BGD4 does not assemble TCP, and is non-agglutinating and highly defective for colonization (M. Kaufman and R. K. Taylor, unpublished; Table 1). Western analysis of the resulting strains showed that all four mutant strains produced TcpA proteins with decreased electrophoretic mobility (data not shown). The G(−1)S TcpA showed the most marked decrease in mobility, presumably because the mutation prevented processing of the prepilin.

Restoration of wild-type phenotypes by allelic exchange with a wild-type tcpA *allele*

In order to study the phenotypes of our *tcpA* mutants, we required a method by which a wild-type copy of *tcpA* could restore wild-type phenotypes in a strain carrying a chromosomal *tcpA* mutation. We therefore used allelic exchange to place the wild-type *tcpA* allele on the chromosome of KP9.79, a non-agglutinating, colonization-defective O395 derivative that carries a Tn*phoA* insertion near the 5′ end of *tcpA* (Peterson and Mekalanos, 1988). Wild-type *tcpA* restored autoagglutination, serum resistance, and colonization to wild-type levels (Table 1 and Fig. 2).

Production of TcpA and TCP in strains derived from KP9.79

The wild-type and four mutant *tcpA* alleles were placed on

Table 1. Phenotypes of TcpA mutants.

Strain	Genotype	TcpC	Auto-agglutination	Pili	Competitive index (*in vivo*)[a]
O395	Wild type	+	+	+	~1[b]
KP9.79	*tcpA*::Tn*phoA*	−	−	−	<0.002[c]
SC224	TcpA$^+$	+	+	+	0.83
SC262	G(−1)S TcpA	+	−	−	NR[d]
SC226	L4T TcpA	+	−	−	NR[d]
SC253	V9 M TcpA	+	+[e]	+	1.7×10^{-4}
SC254	V20T TcpA	+	−	+	2.8×10^{-3}
BGD4	Δ*tcpA*	+	−	−	NR[d]

a. All competitive indices (C.I.) were calculated relative to CG842, except for KP9.79. KP9.79 C.I. is calculated relative to O395. Competitive index is defined as the output ratio of mutant to wild-type bacteria, divided by the input ratio of mutant to wild-type bacteria. For SC224, 262, 226, 253, and 254, the results are the averages of experiments from seven individual mice. No significant changes in ratio were observed in the *in vitro* competitions.
b, c. C.I. values for these strains were obtained from other sources (C. Gardel, personal communication; Peterson and Mekalanos, 1988). The C.I. for KP9.79 was calculated relative to O395.
d. NR = no mutant bacteria recovered from any mouse. In these cases, the C.I. is below the limit of detection (~1×10^{-4}).
e. Extent of agglutination was wild type, but aggregates were smaller.

the KP9.79 chromosome by allelic exchange, which was verified by Southern analysis (data not shown). Western analysis performed on whole cultures of wild-type and mutant strains demonstrated that the strains produced equivalent quantities of their respective TcpA proteins, and that the mutant TcpA proteins exhibited variations in electrophoretic mobility similar to those observed for proteins produced from sequenced mutant alleles (Fig. 3). Therefore, we conclude that these strains express the appropriate mutant TcpA proteins.

Surface TCP expression was examined by both electron microscopy and whole-cell enzyme-linked immunosorbent assay (ELISA). Electron microscopy showed that SC224, 253, and 254 produced bundled pili, while SC226 and SC262 produced no pili. With polyclonal α-TCP antibodies and gold-conjugated secondary antibody, we were able to immunolabel bundles of pili in SC224, 253 and 254. An immuno-electron micrograph of bundled TCP from SC254 is shown in Fig. 4, with similar results obtained for SC224 and 253. SC262 and 226 were unlabelled, as expected (data not shown). The results of whole-cell ELISA roughly paralleled those from electron microscopy (data not shown).

Autoagglutination, colonization, and serum-resistance phenotypes of strains derived from KP9.79

With strains grown under optimal conditions (30°C, in Luria–Bertani (LB) broth with a starting pH of 6.5), autoagglutination is easily scored visually. As expected, all four mutants exhibited changes in autoagglutination, with complete loss of autoagglutination in three mutants (Table 1). In SC253, although the extent of agglutination was wild type, the bacterial aggregates were smaller and required a few more minutes to settle out to the same extent as strains SC224 and O395.

In vivo colonization was assayed by competition, in which the mutant strain was mixed at approximately three-fold excess with CG842, and passaged through infant mice. No significant difference in the ratio of mutant to wild type was observed in *in vitro* competitions in LB broth culture, indicating that the mutant strains suffer no pronounced growth defects in laboratory media. All four TcpA substitution mutants were highly defective for colonization *in vivo*, although not to the same degree (Table 1). From these results, we conclude that although autoagglutination defects accompany defects in colonization, the correlation between degree of autoagglutination and degree of colonization is poor; SC253 agglutinates almost as well as wild type, but colonizes extremely poorly, while the non-agglutinating strain SC254 colonizes slightly better than SC253.

Another phenotype associated with the *tcp* operon is resistance to the bacteriocidal effects of antibody and complement. This phenotype previously correlated with production of an outer membrane lipoprotein called TcpC, since all previous mutations in the *tcp* and ToxR operons that caused serum sensitivity also reduced expression of TcpC (Parsot *et al.*, 1991). Our TcpA substitution mutant strains produced wild-type amounts of outer membrane-associated TcpC (Fig. 5), and thus presumably are also producing normal amounts of the other *tcp* operon gene products. Since the five strains generated from KP9.79 by allelic exchange are non-polar on TcpC, they permitted us to investigate the possibility of TcpA involvement in serum resistance (Fig. 2).

Although the degree of serum sensitivity reported for KP9.79 (Parsot *et al.*, 1991) was not apparent in our experiments, KP9.79 was nonetheless consistently more serum sensitive than CG842, the TcpA$^+$ control strain.

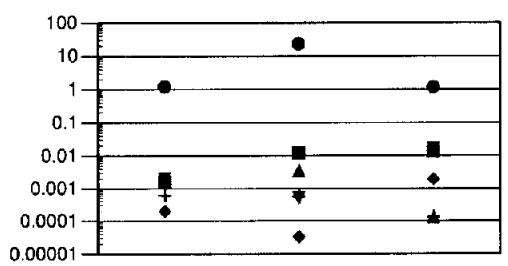

Fig. 2. Serum resistance competitions of KP9.79-derived strains versus CG842. Each vertical group of data points shows the results of a single set of experiments. Serum resistance is expressed as the ratio of a strain to CG842 in the presence of antiserum, divided by the ratio of the strain to CG842 in the absence of antiserum. Symbols: KP9.79 (■), SC224 (●), SC262 (▲), SC226 (◆), SC253 (+), SC254 (▼).

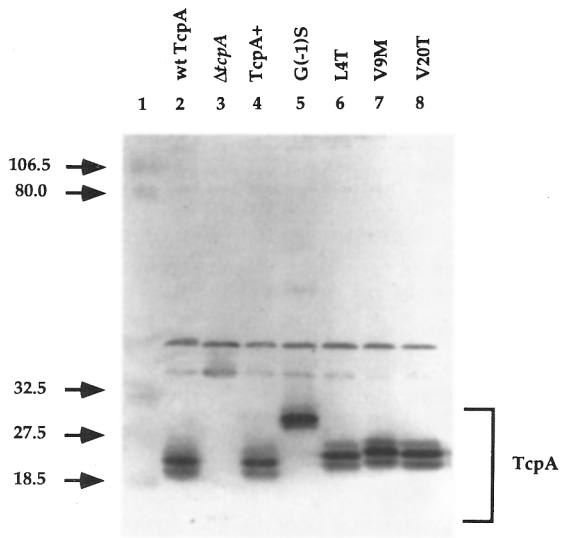

Fig. 3. Western immunoblot analysis of TcpA expression. Samples from whole cell lysates were adjusted for culture density, separated by 12.5% SDS–PAGE, transferred to nitrocellulose, and probed with polyclonal rabbit α-TCP antiserum. Lane 1, Bio-Rad low range molecular weight markers; lane 2, CG842; lane 3, BGD4; lane 4, SC224; lane 5, SC262; lane 6, SC226; lane 7, SC253; lane 8, SC254. Arrows to the left indicate positions and molecular masses (in kDa) of protein standards.

SC262, 226, 253, and 254 were also consistently more sensitive to killing by antibody and complement, although there was some experimental variation in the relative level of sensitivity. In contrast, SC224, the TcpA$^+$ recombinant of KP9.79, displayed a level of sensitivity comparable to TcpA$^+$ strain CG842. In additional experiments, BGD4 was also highly serum sensitive (data not shown). These results suggest that TcpA or TCP pili play a direct role in serum resistance.

Discussion

In previous studies, autoagglutination, colonization, and serum resistance were all associated with TCP, but it was unclear whether these phenotypes were separable, and which of the *tcp* operon gene products were responsible for these phenotypes (Taylor *et al.*, 1987; Parsot *et al.*, 1991). These problems can be attributed primarily to the polarity exerted on the remainder of the *tcp* operon by the Tn*phoA* insertion mutations used in those studies. In contrast, the TcpA mutations investigated here were single amino acid substitutions, whose construction was patterned on pilin mutations known to abolish autoagglutination in *N. gonorrhoeae*. We reasoned that if the TcpA

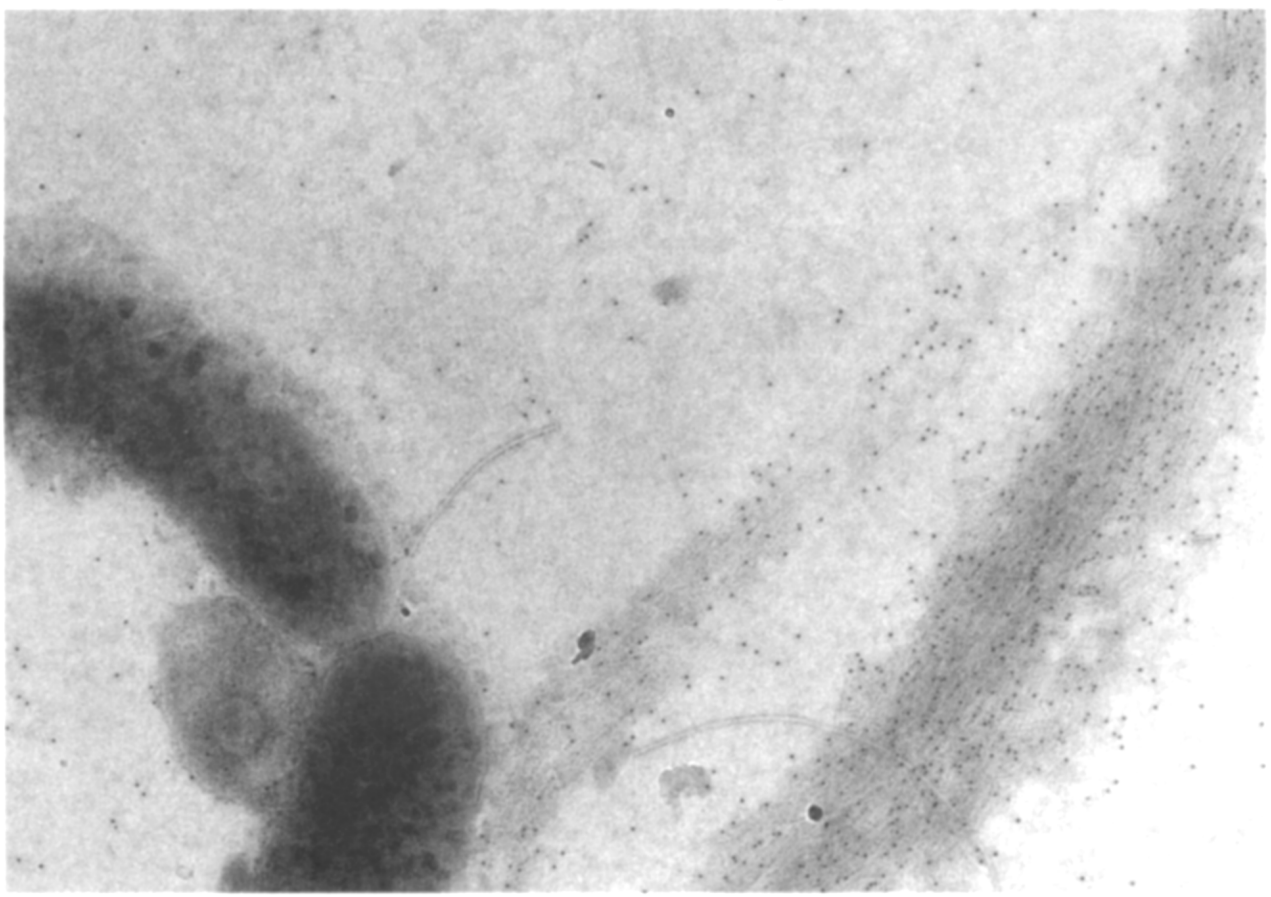

Fig. 4. Immuno-electron micrograph of SC254. Immunogold labelling was carried out using polyclonal α-TCP antiserum and gold-conjugated secondary antibody.

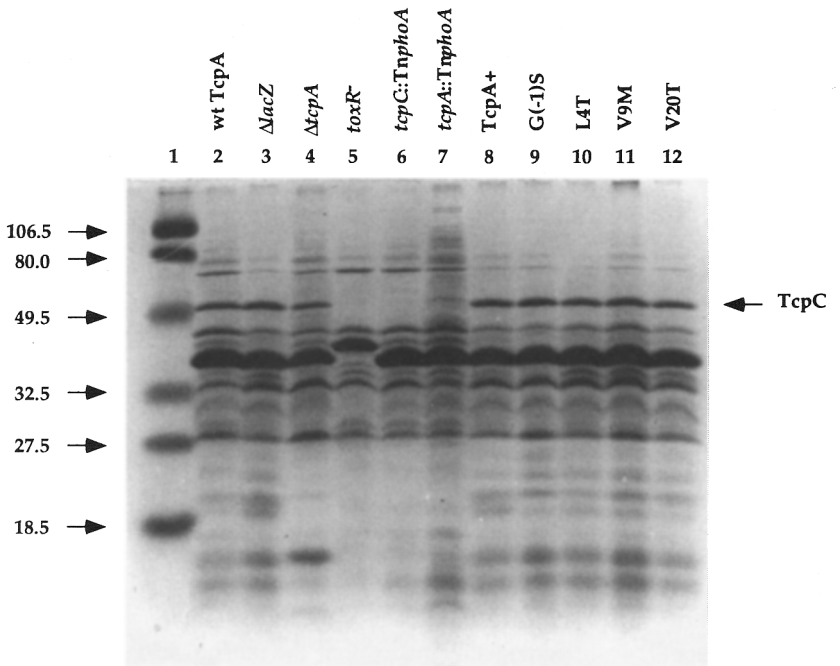

Fig. 5. Coomassie brilliant blue-stained 12.5% SDS–polyacrylamide gel of outer membrane protein-enriched fractions. Lanes: 1, Bio-Rad low range molecular weight markers; 2, O395; 3, CG842; 4, BGD4; 5, JJM43 (*toxR*⁻); 6, KP8.87 (*tcpC*::Tn*phoA*); 7, KP9.79 (*tcpA*::Tn*phoA*); 8, SC224; 9, SC262; 10, SC226; 11, SC253; 12, SC254. Arrows to the left indicate positions and molecular masses of protein standards (in kDa). The arrow to the right indicates the position of TcpC.

mutations affected autoagglutination and were non-polar, they might allow us to separate the various TCP phenotypes and assess the role of TcpA alone in these phenotypes.

The phenotypes of SC262 and 226 can be attributed simply to a lack of TCP, and this lack is probably due to inability to export or assemble the mutant TcpA proteins into TCP. The G(−1)S mutation in SC262 appears to interfere with leader peptide cleavage, and this protein is probably not exported. The L4T TcpA in SC226 undergoes leader peptide cleavage, but may be impaired for export or assembly. SC253 and 254, however, display similar defects (see below) despite producing surface TCP. Since the mutations in SC226, 253, and 254 all decrease the hydrophobicity of the TcpA N-terminus, one possibility is that these amino acid changes interfere with subunit–subunit interactions in such a way as to alter the export, assembly, or properties of TcpA.

All four mutant strains produced wild-type quantities of their respective TcpA protein, and were non-polar on TcpC and, presumably, the remainder of the *tcp* operon. All four mutants showed autoagglutination defects, with three of the four demonstrating complete loss of autoagglutination. Upon electron microscopic examination, two of the non-agglutinating strains (SC226 and 262) were found to produce no surface TCP, while both agglutinating SC253 and non-agglutinating SC254 produced pili. The non-agglutinating phenotype of SC254 cannot be explained by poor surface localization of TCP, since whole-cell ELISA demonstrated that SC253, SC254, and O395 produced similar quantities of surface TCP. However, production of surface TCP does correlate with colonization, since SC253 and 254 were the only mutants able to persist in the mouse intestine, albeit at drastically reduced levels relative to wild-type strains.

Autoagglutination has been proposed as an indicator of ability to form microcolonies on the intestinal epithelium (Taylor *et al.*, 1987), and indeed, all of our mutants showed both autoagglutination and colonization defects. However, a strain with essentially wild-type agglutination (SC253) was no better a colonizer than a non-agglutinating strain (SC254). This demonstrates that although the slight change in autoagglutination observed in SC253 may reflect a serious *in vivo* defect, there is no correlation between extent of *in vitro* autoagglutination and colonization ability.

Our results show that TcpA also affects serum resistance, a phenotype previously attributed to the outer membrane protein TcpC (Parsot *et al.*, 1991). Although it is unclear how TcpA or TCP might mediate serum resistance, a potential explanation is that TCP-dependent agglutination sterically decreases the accessibility of complement to bacterial cells, resulting in increased survival of bacteria 'buried' deep within bacterial clumps. However, if agglutination were the primary determinant of serum resistance, the agglutinating mutant SC253 would be expected to be more serum resistant than the other mutant strains, a prediction not supported by our data.

Complement resistance in bacteria and parasites is achieved through a variety of mechanisms, including

blockage of complement activation, deposition, or activity, or shedding of molecules that destroy or deplete complement (Joiner, 1988). Most studies of serum resistance have implicated lipopolysaccharide and outer membrane proteins as serum-resistance determinants (Moll *et al.*, 1980; Joiner, 1988). The role of piliation in serum resistance has been investigated in *N. gonorrhoeae*, but with conflicting results (McCutchan *et al.*, 1976; Taha, 1993). If type IV pili are general targets for complement, it may be that TCP increases serum resistance directly by activating and depleting complement at a distance from the cell membrane, or by otherwise interfering with complement deposition on the bacterial membrane. Alternatively, TcpA may influence serum resistance indirectly by affecting the activity of a serum-resistance determinant. For example, if in addition to TcpA, TCP contains a very small proportion of a serum-resistance determinant, TcpA mutations might result in incorrect assembly of TCP and thereby prevent proper localization of the serum-resistance determinant. Incorrect assembly of TCP might also cause changes in the bacterial surface that render it more susceptible to complement attack (e.g. changes in lipopolysaccharide structure or density). Further analyses will be performed to address these possibilities.

There is evidence that in El Tor strains, *tcpA* mutations do not decrease serum resistance, even though TCP pili are required for colonization (Attridge *et al.*, 1993). However, serum-resistance assays developed for classical strains may not be applicable to El Tor strains. TCP expression in El Tor strains is regulated differently than in classical strains, and El Tor TcpA differs from classical TcpA genetically and antigenically, with amino acid similarity reduced to 68% between biotypes in the C-terminal third of TcpA (Sun *et al.*, 1990; Jonson *et al.*, 1991; Voss and Attridge, 1993; Iredell and Manning, 1994; Rhine and Taylor, 1994). A more intriguing idea is that El Tor strains are intrinsically more serum resistant than classical strains, and thus the link in classical strains between TCP and serum resistance has been broken in El Tor strains. If so, the separation of serum resistance from TCP might represent a step in the evolution of *V. cholerae* as a pathogen, since El Tor strains have displaced classical strains as the predominant etiologic agents of cholera. Interestingly, in the newly emergent pathogenic O139 strains of *V. cholerae*, a correlation between serum resistance and colonization has also been observed. In this case, serum resistance is dependent upon expression of capsular polysaccharide (Waldor *et al.*, 1994).

It has been proposed that serum resistance might reflect a comparable resistance to a gut-associated, 'complement-like' bacteriocidal activity (Parsot *et al.*, 1991). Although serum resistance correlates with colonization, it is still probable that the colonization defects of TCP mutants are caused by a decrease in adhesion rather than a decrease in resistance to gut-associated bacteriocidal activities. The involvement of type IV pili in adhesion has been documented by a number of investigators (Strom and Lory, 1993), and studies of the type IV pilins in *N. gonorrhoeae* and *N. meningitidis* have determined that changes in the pilin primary amino acid sequence lead to changes in ability to adhere to cultured cell lines and fixed tissue (Virji *et al.*, 1993; Jonsson *et al.*, 1994). No host receptor for TCP has been identified, but the theory that TCP functions as an adhesin is supported by the finding that polyclonal α-TCP antibodies confer passive protection in the infant mouse model (Sun *et al.*, 1990). It will therefore be of interest to determine whether our mutant strains possess different abilities to adhere to host tissue, although unfortunately no *in vitro* experimental system to date detects differences between TCP^+ and TCP^- strains on the basis of adherence.

The mutations described in this work cause autoagglutination defects in both *N. gonorrhoeae* and *V. cholerae*, supporting the theory that the amino acid homologies among PilE, TcpA, and perhaps other type IV pilins, reflect certain structural and functional similarities. That colonization is concomitantly decreased argues that autoagglutination is an indicator of colonization ability, although it is not a quantitative one since the extent of autoagglutination did not correlate with extent of colonization. Since the mutants also showed decreased serum resistance, the results of this study are consistent with previous findings in that the TCP-associated phenotypes of autoagglutination, colonization, and serum resistance were not clearly separable. Future studies will focus on further characterizing the mechanisms of these phenotypes and the relationships among them.

Experimental procedures

Bacterial strains, plasmids, media, and growth conditions

Bacterial strains and plasmids used in this study are shown in Table 2. *E. coli* and *V. cholerae* strains were grown in LB broth, pH 6.5, at 37°C and 30°C, respectively. For long-term storage, all strains were frozen at −70°C in LB containing 20% glycerol (v/v). For short-term storage, strains were maintained at room temperature on LB plates containing appropriate antibiotics.

Nucleic acid manipulations

All nucleic acid manipulations were accomplished according to standard molecular biology techniques (Ausubel *et al.*, 1991).

Site-directed mutagenesis

Site-directed mutagenesis of pSC18.1 was carried out

Table 2. Strains and plasmids used in this study.

Strain/Plasmid	Description	Source/Reference
Strain		
V. cholerae		
O395 Sm	classical O1 Ogawa strain; Sm^R	Taylor *et al.* (1987)
BGD4	O395 Δ*tcpA4*	M. R. Kaufman and R. K. Taylor (unpublished)
CG842	O395 Δ*lacZ*	C. Gardel (in preparation)
JJM43	O395 Δ*toxR43*	Taylor *et al.* (1987)
KP8.87	O395 *tcpC*::Tn*phoA*	Peterson and Mekalanos (1988); Parsot *et al.* (1991)
KP9.79	O395 *tcpA*::Tn*phoA*	Peterson and Mekalanos (1988); Parsot *et al.* (1991)
SC224	$TcpA^+$ recombinant of KP9.79	This work
SC226	L4T TcpA	This work
SC253	V9M TcpA	This work
SC254	V20T TcpA	This work
SC262	G(−1)S TcpA	This work
E. coli		
BW19851	RP-4-Tc::Mu-1kan::Tn*7* integrant/*creB510 hsdR17endA1 zbf-5 uidA* (Δ*Mlu1*)::*pir recA1 thi* (λ*pir* host for ori R6K plasmids)	Metcalf *et al.* (1994)
SM10λpir	*thi thr leu tonA lacY supE recA*::RP-4-Tc::Mu (λ*pir*)	Taylor *et al.* (1987)
Plasmid		
pGP704	Suicide vector; Ap^R	Miller and Mekalanos (1988)
pMD12	Suicide vector; Ap^R	M. Dziejman (personal communication)
pSC18.1	Wild-type *tcpA* allele in pTZ18U	This work
pSC20.1	*tcpA* G(−1)S allele in pTZ18U	This work
pSC21.5	*tcpA* L4T allele in pTZ18U	This work
pSC22.6	*tcpA* V9M allele in pTZ18U	This work
pSC23.1	*tcpA* V20T allele in pTZ18U	This work
pSC24.2	Wild-type *tcpA* allele in pMD12	This work
pTZ18U	Vector for site-directed mutagenesis	Bio-Rad Laboratories

according to the protocol supplied with the Muta-Gene® Phagemid In Vitro Mutagenesis Kit, Version 2 (Bio-Rad Laboratories, Inc.). Putative mutant clones were sequenced using an Applied Biosystems Model 373A DNA Sequencing System and the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.), according to the manufacturer's directions. The sequence of the entire *tcpA* gene was verified for mutant alleles selected for further analysis.

Oligonucleotides for mutagenesis and sequencing were obtained from Oligos, Etc., Inc. The following oligonucleotides were used to create the four TcpA mutations: oSC4 (5′-TGTCATAGACTCTTGACC-3′), oSC5 (5′-CACTTCGTTAAT-TGTCAT-3′), oSC6 (5′-GCCTAGCATGATGATCAC-3′), and oSC7 (5′-AGTAACAGTCCCCGCCGA-3′). The respective nucleotide changes are: GGT → TCT for the G(−1)S mutation, CTC → ACC for the L4T mutation, GTT → ATG for the V9M mutation, and GTT → ACT for the V20T mutation.

Allelic exchange

The wild-type and mutant *tcpA* alleles were cloned into suicide vector pGP704 (Miller and Mekalanos, 1988) or pMD12 (M. Dziejman, personal communication), and mated into *V. cholerae* strain KP9.79. Transconjugants were screened by Western analysis for production of appropriately sized TcpA. For each construction, an appropriate transconjugant strain was grown through several passages at 37°C in the absence of selection for the integrated plasmid, and then plated on L-agar containing 5-bromo-4-chloro-3-indolyl-phosphate (40 μg ml^{-1}) and 0.2% glucose to screen for loss of Tn*phoA*. White colonies were picked and retested for absence of *phoA* activity, as well as kanamycin (Km) and ampicillin (Ap) sensitivity. Pho^- Ap^S Km^S isolates were retained for further analysis.

SDS–PAGE and Western analysis

Proteins from whole cell lysates were separated electrophoretically in 12.5% polyacrylamide gels in the presence of SDS as previously described (Laemmli, 1970). For Western analysis, proteins were transferred from gels to BA85 nitrocellulose (Schleicher and Schuell) at 4°C in Towbin buffer (25 mM Tris; 192 mM glycine; 20% methanol; 1% SDS; pH 8.3) using a Transphor unit (Hoefer Scientific) in accordance with the manufacturer's recommendations. The α-TCP antiserum was polyclonal rabbit antiserum raised against purified TCP (gift of K. Peterson).

Preparation of outer membrane protein-enriched fractions

Outer membrane proteins were prepared by fractionation in Triton X-100 as previously described (Hantke, 1981).

Electron microscopy and immuno-electron microscopy

Strains were grown as described below for autoagglutination. For electron microscopy, samples were prepared by floating carbon film on a drop of bacterial culture for 30 s, transferring the film to 2% ammonium molybdate (w/v in water), pH 6.5, for 1 min, and collecting the film on a copper grid. For immunogold labelling, cultures were vortexed for 1 s prior to processing according to a method adapted from Voss and Attridge (1993). Each IEM sample was prepared by floating a carbon Type-A grid (300 mesh copper; Ted Pella, Inc.) for 5 min on a 50 μl drop of bacterial culture spotted on Parafilm®, with

the Formvar surface facing the drop. All subsequent steps were also performed on drops on Parafilm®. The grid was tranferred to a 50 µl drop of 3% bovine serum albumin (BSA)-phosphate-buffered saline (PBS) (w/v) for 5 min, and then to a 25 µl drop of α-TCP antiserum (diluted 1:20 in 3% BSA-PBS) for 15 min, followed by two 5-min washes on 50 µl drops of 0.15 M NaCl. The grid was then transferred to a 25 µl drop of goat α-rabbit IgG gold conjugate for 10 min (10 nm gold particle, diluted 1:50 in deionized, distilled water; Ted Pella, Inc.). After two 5-min washes on 50 µl drops of deionized, distilled water, the grids were stained for 30 s by floating on 0.5% phosphotungstic acid (w/v in water), pH 6.5. All samples were examined with a Hitachi H-7100 electron microscope at 75 kV.

Autoagglutination assay

Single colonies growing on L-agar plates were resuspended in 1 ml LB, and 10 µl of this suspension were then inoculated into 5 ml LB containing appropriate antibiotics. Cultures were then grown for 17–18 h on a roller shaker at 30°C. Extent of agglutination was scored by visual examination.

Infant mouse competition

Infant mouse competitions were carried out as previously described (Freter *et al.*, 1981; Taylor *et al.*, 1987), with minor modifications. Strains were mixed at threefold excess with CG842, washed once in 0.15 M NaCl, diluted 1:1000 in 0.15 M NaCl, and mixed with blue food colouring. This inoculum was plated at appropriate dilutions to determine the exact ratio of input strains. An *in vitro* competition was carried out by subculturing 50 ml of the inoculum into 5 ml of LB, incubating the culture at 37°C on a roller for 5–6 h, and then plating at appropriate dilutions. The Lac^- strain CG842 was used as the reference strain because it is easily distinguishable from the Lac^+ test strains by plating on medium containing X-gal.

For the *in vivo* competition, no less than four 4- to 5-day-old CD1 mice were selected from mixed litters, inoculated orally with 50 ml of the mixed culture, and incubated at 30°C for 21–24 h. The small intestine was then removed, homogenized in 5 ml of LB, and plated at appropriate dilutions to determine the output ratio of competing strains.

Serum resistance competition

This assay was carried out as described by Parsot *et al.* (1991), with minor modifications. Strains were grown as described above for the autoagglutination assay. Cultures were then vortexed vigorously for 1 min. Cells were pelleted in a microfuge and resuspended in the same volume of PBS. Equal volumes of CG842 and a test strain were then mixed at 1:100 dilution in PBS ($\sim 5 \times 10^7$ cells ml^{-1}). Reconstituted lyophilized guinea pig complement (Accurate Chemical and Scientific Corporation) was added to a final concentration of 20%. This mixture was divided into 0.5 ml aliquots to which were added either no antibody, or anti-Ogawa typing serum at a final dilution of 1:80 000. All samples were incubated at 37°C in microfuge tubes on a roller shaker for 1 h, and then diluted 1:10 into ice-cold LB. Appropriate dilutions of cells were plated on solid media. Serum resistance is expressed as the ratio of a strain to CG842 in the presence of antiserum, divided by the ratio of the strain to CG842 in the absence of antiserum, and results were arranged in a scatter plot using DeltaGraph® Pro3 (DeltaPoint, Inc.). The anti-Ogawa antiserum was a polyclonal rabbit antiserum raised against O395.

Whole-cell ELISA

Strains were grown as described above for the autoagglutination assay, then vortexed vigorously for 1 min. In order to correct for differences in agglutination, 0.5 ml of each culture was microfuged at 325 × *g* for 2 min to remove larger aggregates, and 400 µl of the resulting supernatant were removed to new tubes. The OD_{600} of these samples was determined, and equivalent cell numbers were pelleted in a microfuge. These cells were resuspended in 200 µl PBS, and dispensed in twofold dilution series in a 96-well Linbro®/Titertek® EIA flat bottom microtitration plate (Flow Laboratories, Inc.), with 100 µl per well. After incubation at 37°C for 1 h, the wells were washed twice with PBS, blocked for 1 h at 37°C with 5 mg ml^{-1} BSA in PBS (200 µl per well), and washed twice with PBS. Polyclonal rabbit α-TCP antiserum was added at 1:1000 dilution in PBS (100 µl per well), and incubated for 1 h at 37°C, followed by two PBS washes. Next, 100 µl of goat α-rabbit IgG alkaline phosphatase conjugate (Boehringer Mannheim) was added to each well at a 1:1000 dilution in PBS, and incubated for 1 h at 37°C. After two PBS washes, the wells were rinsed once with 0.1 M Tris, pH 8.0. Enzymatic detection was performed by adding to each well 100 µl of 2 mg ml^{-1} *p*-nitrophenyl phosphate in 0.1 M Tris, pH 8.0, and incubating the plate at 37°C for approximately 10 min. A null control well was treated identically, except that PBS alone was used in the initial binding step. The absorbance at 420 nm was read using a Series 700 microplate reader (Cambridge Technology, Inc.). The microplate reader zero value was set to an untreated well containing the enzymatic substrate solution, and all A_{420} values were corrected for background activity by subtracting the A_{420} value of the null control well.

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References

Attridge, S.R., Voss, E., and Manning, P.A. (1993) The role of toxin-coregulated pili in the pathogenesis of *Vibrio cholerae* O1 El Tor. *Micro Pathog* **15:** 421–431.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.E., Seidman, J.G., Smith, J.A., and Struhl, K. (1991) *Current*

Protocols in Molecular Biology. New York: Greene Publishing Associates.

Brown, R.C., and Taylor, R.K. (1995) Organization of *tcp, acf*, and *toxT* genes within a ToxT-dependent operon. *Mol Microbiol* **16:** 425–439.

Freter, R., O'Brien, P.C.M., and Macsai, M.M.S. (1981) Role of chemotaxis in the association of motile bacteria with intestinal mucose in vivo studies. *Infect Immun* **34:** 234–240.

Hantke, K. (1981) Regulation of ferric ion transport in *Escherichia coli* K12: isolation of a constitutive mutant. *Mol Gen Genet* **182:** 288–292.

Herrington, D.A., Hall, R.H., Losonsky, G., Mekalanos, J.J., Taylor, R.K., and Levine, M.M. (1988) Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J Exp Med* **168:** 1487–1492.

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

Joiner, K.A. (1988) Complement evasion by bacteria and parasites. *Annu Rev Microbiol* **42:** 201–230.

Jonson, G., Holmgren, J., and Svennerholm, A.M. (1991) Epitope differences in toxin-coregulated pili produced by classical and El Tor *Vibrio cholerae* O1. *Microb Pathogen* **11:** 179–88.

Jonsson, A.-B., Ilver, D., Falk, P., Pepose, J., and Normark, S. (1994) Sequence changes in the pilus subunit lead to tropism variation of *Neisseria gonorrhoeae* to human tissue. *Mol Microbiol* **13:** 403–416.

Kaper, J.B., Fasano, A., and Trucksis, M. (1994) Toxins of *Vibrio cholerae*. In *Vibrio cholerae and Cholera: Molecular to Global Perspectives*. Wachsmuth, I.K., Blake, P.A., and Olsvik, Ø. (eds). Washington, D.C.: ASM Press, pp. 145–176.

Kaufman, M.R., and Taylor, R.K. (1994) The toxin-coregulated pilus: biogenesis and function. In *Vibrio cholerae and Cholera: Molecular to Global Perspectives*. Wachsmuth, I.K., Blake, P.A., and Olsvik, Ø. (eds). Washington, D.C.: ASM Press, pp. 187–202.

Kaufman, M.R., Seyer, J.M., and Taylor, R.K. (1991) Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by gram-negative bacteria. *Genes Dev* **5:** 1834–1846.

Kaufman, M.R., Shaw, C.E., Jones, I.D., and Taylor, R.K. (1993) Biogenesis and regulation of the *Vibrio cholerae* toxin-coregulated pilus: analogies to other virulence factor secretory systems. *Gene* **126:** 43–49.

Koomey, M., Bergstrom, S., Blake, M., and Swanson, J. (1991) Pilin expression and processing in pilus mutants of *Neisseria gonorrhoeae*: critical role of Gly-1 in assembly. *Mol Microbiol* **5:** 279–287.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

McCutchan, J.A., Levine, S., and Braude, A.I. (1976) Influence of colony type on susceptibility of gonococci to killing by human serum. *J Immun* **116:** 1652–1655.

Metcalf, W.W., Jiang, W., and Wanner, B.L. (1994) Use of the *rep* technique for allele replacement to construct new *Escherichia coli* hosts for maintenance of R6K-gamma origin plasmids at different copy numbers. *Gene* **138:** 1–7.

Miller, V.L., and Mekalanos, J.J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170:** 2575–2583.

Moll, A., Manning, P.A., and Timmis, K.N. (1980) Plasmid-determined resistance to serum bactericidal activity: a major outer membrane protein, the *traT* gene product, is responsible for plasmid-specified serum resistance in *Escherichia coli*. *Infect Immun* **28:** 359–367.

Ogierman, M.A., Zabihi, S., Mourtzios, L., and Manning, P.A. (1993) Genetic organization and sequence of the promoter-distal region of the *tcp* gene cluster of *Vibrio cholerae*. *Gene* **126:** 51–60.

Ottemann, K.M., and Mekalanos, J.J. (1994) Regulation of cholera toxin expression. In *Vibrio cholerae and Cholera: Molecular to Global Perspectives*. Wachsmuth, I.K., Blake, P.A., and Olsvik, Ø. (eds). Washington, D.C.: ASM Press, pp. 177–185.

Parsot, C., Taxman, E., and Mekalanos, J.J. (1991) ToxR regulates the production of lipoproteins and the expression of serum resistance in *Vibrio cholerae*. *Proc Natl Acad Sci USA* **88:** 1641–1645.

Peterson, K.M., and Mekalanos, J.J. (1988) Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. *Infect Immun* **56:** 2822–2829.

Rhine, J.A., and Taylor, R.K. (1994) TcpA pilin sequences and colonization requirements for O1 and O139 *Vibrio cholerae*. *Mol Microbiol* **13:** 1013–1020.

Shaw, C.E., and Taylor, R.K. (1990) *Vibrio cholerae* O395 *tcpA* pilin gene sequence and comparison of predicted protein structural features to those of type 4 pilins. *Infect Immun* **58:** 3042–3049.

Strom, M.S., and Lory, S. (1991) Amino acid substitutions in pilin of Pseudomonas aeruginosa. Effect on leader peptide cleavage, amino-terminal methylation, and pilus assembly. *J Biol Chem* **266:** 1656–1664.

Strom, M.S., and Lory, S. (1993) Structure-function and biogenesis of the type IV pili. *Annu Rev Microbiol* **47:** 565–596.

Sun, D.X., Mekalanos, J.J., and Taylor, R.K. (1990) Antibodies directed against the toxin-coregulated pilus isolated from *Vibrio cholerae* provide protection in the infant mouse experimental cholera model. *J Inf Dis* **161:** 1231–1236.

Swanson, J., Kraus, S.J., and Gotschlich, E.C. (1971) Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. *J Exp Med* **134:** 886–906.

Taha, M.-K. (1993) Increased sensitivity of gonococcal *pilA* mutants to bactericidal activity of normal human serum. *Infect Immun* **61:** 4662–4668.

Taylor, R.K., Miller, V.L., Furlong, D.B., and Mekalanos, J.J. (1987) Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci USA* **84:** 2833–2837.

Virji, M., Saunders, J.R., Sims, G., Makepeace, K., Maskell, D., and Ferguson, D.J.P. (1993) Pilus-facilitated adherence

of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. *Mol Microbiol* **10:** 1013–1028.

Voss, E., and Attridge, S.R. (1993) *In vitro* production of toxin-coregulated pili by *Vibrio cholerae* El Tor. *Microb Pathogen* **15:** 255–268.

Waldor, M.K., Colwell, R., and Mekalanos, J.J. (1994) The *Vibrio cholerae* 0139 serogroup antigen includes O-antigen capsule and lipopolysaccharide virulence determinants. *Proc Natl Acad Sci USA* **91:** 11388–11392.