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Type 4 Pilus Biogenesis and Type II-Mediated Protein Secretion by *Vibrio cholerae* Occur Independently of the TonB-Facilitated Proton Motive Force

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In *Vibrio cholerae*, elaboration of toxin-coregulated pilus and protein secretion by the extracellular protein secretion apparatus occurred in the absence of both TonB systems. In contrast, the cognate putative ATPases were required for each process and could not substitute for each other.

Two systems that ultimately facilitate the extracellular localization of proteins by gram-negative bacteria are the type 4 pilus system and the type II protein secretion system. These two systems may be related since they share a number of protein homologs that are predicted, or have been demonstrated, to localize and function in a similar manner for the two systems (10, 19).

Type 4 pili are long, filamentous surface appendages expressed by numerous gram-negative bacteria (reviewed in reference 27). Type 4 pili are involved in a number of bacterial processes, including adherence to surfaces, cell-cell interaction, twitching motility, and biofilm formation (27, 31). Some type 4 pili also serve as receptors for bacteriophage. Biogenesis of the type 4 pilus fiber involves polymerization and secretion of the pilin subunit by a mechanism involving a set of proteins that likely compose an assembly-and-export apparatus. One such type 4 pilus is the toxin-coregulated pilus (TCP) expressed by *Vibrio cholerae*. TCP mediates bacterial colonization of the intestine by facilitating microcolony formation via pilus-mediated bacterial interactions and perhaps direct attachment to the intestinal brush border (7). TCP also serves as the receptor for the cholera toxin-encoding CTX phage (30). Biogenesis of TCP requires the activities of at least 11 accessory proteins, most of which are encoded by genes located in the *tcp* operon (9, 28).

The type II secretion pathway or main terminal branch of the general secretory pathway is involved in the secretion of a wide variety of protein monomers or complexes (reviewed in references 15 and 19). In *V. cholerae*, this type of protein export is accomplished by the extracellular protein secretion (EPS) system (22). This system, which facilitates the export of a variety of proteins, such as cholera toxin, chitinase, and lipase, requires the functions of at least 12 accessory proteins.

The type II secretion and type 4 pilus biogenesis systems have several features in common, suggesting that at least some aspects of the export mechanism are shared by the two (10, 19). Most strikingly, type II secretion requires the presence of proteins that are highly homologous to type 4 pilins. These proteins are generally termed type 4 pilin-like proteins and may form a pseudopilus that mediates export. In one instance, these proteins have been visualized to form a bona fide pilus structure that is potentially part of a type II export apparatus (24). In both systems, the prepilins or prepilin-like proteins are processed by a homologous type 4 prepilin peptidase. In addition, both systems have an outer membrane protein, known as the secretin, that is thought to multimerize and form an outer membrane channel through which proteins can pass during the extracellular translocation step. Both systems also have at least one protein that contains an ATP-binding domain and is thought to be an ATPase. Recent work by Sakai et al. has demonstrated the nucleoside triphosphatase activity of PilQ, an ortholog required for formation of the thin pilus in R64 (18). For several type II secretion and type 4 pilus biogenesis systems, the putative ATPase has been shown to be peripherally associated with the cytoplasmic side of the inner membrane via direct contact with transmembrane proteins that are conserved among both type II secretion and type 4 pilus biogenesis systems (10, 21). The results of studies on TCP, EPS, and other systems suggest that the presence of the putative ATPase is required for these systems to function (6, 21). Previous experiments have suggested that the cognate putative ATPase is required for these systems to function. For example, ExeA of *Aeromonas hydrophila* does not complement a *V. cholerae epsE* mutant (21). However, certain EpsE-ExeA protein chimeras were able to complement the *epsE* mutation, suggesting that protein domains can be defined that contribute to species specificity (21). However, it has not been clear whether or not the activity of the cognate ATPase is sufficient to drive protein secretion.

The role of the ATPase in the pilus and protein secretion systems is not yet understood. The putative ATPase might provide energy for polymerization of pili or pilus-like structures that function to extrude proteins through the outer membrane. Alternatively, it might provide energy to the outer membrane that is necessary to translocate the exoproteins or pilins across the membrane or to modulate a gating mechanism for the outer membrane channel. There is no direct source of energy at the outer membrane to facilitate active processes that occur at that location. The outer membrane cannot main-

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tain an electrochemical gradient, and the periplasm lacks an ATP pool. The only known mechanism for energy transfer to the outer membrane is via the TonB system, which transduces the proton motive force (PMF) generated by the electrochemical gradient across the cytoplasmic membrane to the outer membrane in the form of mechanical energy (reviewed in reference 14). The amino-terminal portion of TonB is anchored in the cytoplasmic membrane, and the protein is stabilized by ExbB and ExbD (4, 11). The high percentage of proline residues in the central domain of the TonB protein is proposed to allow it to adopt an extended constrained conformation, which would allow it to span the periplasmic space and directly contact the outer membrane receptors. Most outer membrane proteins that interact with TonB possess a consensus heptapeptide sequence, termed the TonB box, near the amino terminus. The TonB box is believed to be required for interaction of the receptor with the carboxy terminus of TonB (3). However, there are exceptions to this rule. For example ViuA, the *V. cholerae* vibriobactin receptor, is TonB dependent but does not have an obvious TonB box. The outer membrane proteins of the type 4 pilus and type II secretion systems lack a TonB box, but the similarities between filamentous phage morphogenesis and these systems have prompted investigators to postulate that either TonB-mediated PMF or PMF transduction via another mechanism can fulfill the energy requirement in these systems.

Previous experiments have attempted to resolve the energy requirements for the process of type II protein secretion. These experiments utilized the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to dissipate PMF and arsenate to deplete ATP levels (8, 13). An inherent problem associated with using chemical inhibitors to study type 4 pilus biogenesis systems is that it is difficult to uncouple the outer membrane translocation from the cytoplasmic translocation steps of the pathways since the cytoplasmic translocation step requires the Sec machinery, which utilizes both ATP hydrolysis and PMF (16, 26). These potential complications prompted us to further investigate the role of PMF in these systems.

We investigated the role of TonB-facilitated PMF in type 4 pilus biogenesis and type II secretion in *V. cholerae* by using mutants defective for TonB. The *V. cholerae* genome encodes two sets of TonB, ExbB, and ExbD proteins (25). The two TonB proteins of *V. cholerae* share relatively low homology and differ in the length of the proline-rich central domain. Interestingly, mutations that result in strains that are differentially lacking in the TonB proteins have demonstrated that there are differences in their functions under various osmotic conditions (25). This finding is consistent with the proposition that the proline domain is critical for allowing the carboxy terminus of TonB to contact a receptor in the outer membrane. In *V. cholerae*, the TonB1 protein that contains a longer proline-rich domain has the potential to interact with proteins in the outer membrane under conditions of high osmolarity, whereas the shorter TonB2 interacts with certain outer membrane proteins only when the outer membrane is physically closer to the cytoplasmic membrane under low osmotic conditions. In the present study, we took advantage of the recently isolated mutations that disrupt the TonB energy transduction system in *V. cholerae* to study the role of TonB-facilitated PMF in EPS.

TCP biogenesis by mutants lacking TonB. To determine whether TonB-facilitated PMF is required for type 4 pilus biogenesis, various mutants deficient for TonB production were tested for production of TCP. Mutant derivatives of CA401S, AMV524, and DOV221 contain polar transposon insertions in *tonB1* and *exbB2*, the first genes in the *tonB1* and *tonB2* loci, respectively (25). Mutant DOV300 contains polar insertions in both *exbB1* and *exbB2*. AMV524 has been demonstrated to be defective for schizokinen utilization and for heme uptake under high-osmolarity conditions, whereas DOV221 was defective for enterobactin utilization, rendering a specific function to each of the two TonB systems. DOV300 is defective for growth on all of these iron sources (25). Prior to examination for TCP production, the mutants were tested for their iron transport phenotypes and verified to be defective for transport of the respective siderophores.

The mutants were initially analyzed for TCP production by the in vitro autoagglutination test. Wild-type strains grown overnight under TCP-expressing conditions (Luria-Bertani broth, pH 6.5, 30°C) form aggregates that clump and settle to the bottom of the tube. AMV524, DOV221, and DOV300 exhibited autoagglutination, similar to CA401S, the isogenic wild-type strain. To visualize any morphological changes in the pilus, the strains were viewed by transmission electron microscopy. Wild-type pili were clearly observed in all of the mutants (Fig. 1). Furthermore, the CTXΦ transduction frequencies of the mutants were similar to that of the wild type (Table 1). These results indicate that TonB-mediated PMF does not play a role in TCP biogenesis.

Secretion of proteins by the EPS pathway in mutants deficient for TonB. Cholera toxin and protease are secreted by the type II secretion mechanism, representing the main terminal branch of the general secretory pathway of *V. cholerae* (15, 22). As noted above, the similarities between type II secretion and type 4 pilus biogenesis prompted us to investigate the effect of loss of TonB on toxin secretion. The toxin levels in the culture supernatant of the mutants were observed to be similar to that of the wild type (Table 1). The mutants were further tested for protease secretion by plating on 2% milk agar plates. Protease secretion was also found to be unaffected in these mutants (Fig. 2). Taken together, these data indicate that type II secretion is unaffected by loss of TonB function, indicating that it is also likely to be independent of TonB-facilitated PMF.

Effect of a *tcpT* deletion on TCP biogenesis and toxin secretion. To determine if ATP is the prime source of energy for TCP biogenesis, an O395 *tcpT* deletion strain, RT4376, was tested for production of TCP. TCP production was assessed by the in vitro autoagglutination test and the CTX-KnΦ phage transduction assay. The mutant did not agglutinate and could not be transduced by the phage (Table 1). These results are consistent with previous studies in which *tcpT* mutants containing a deletion of the Walker box region were found to be defective for TCP biogenesis (6). To determine if TcpT activity was specific for TCP production or if other systems were affected in a *tcpT* mutant, toxin secretion by the EPS system was examined and found to be unaffected by the deletion of *tcpT* (Table 1). These results suggest specificity for the putative ATPase function with respect to the cognate secretion system.

Effect of an *epsE* insertion mutation on TCP biogenesis. It has been previously demonstrated that EpsE is required for

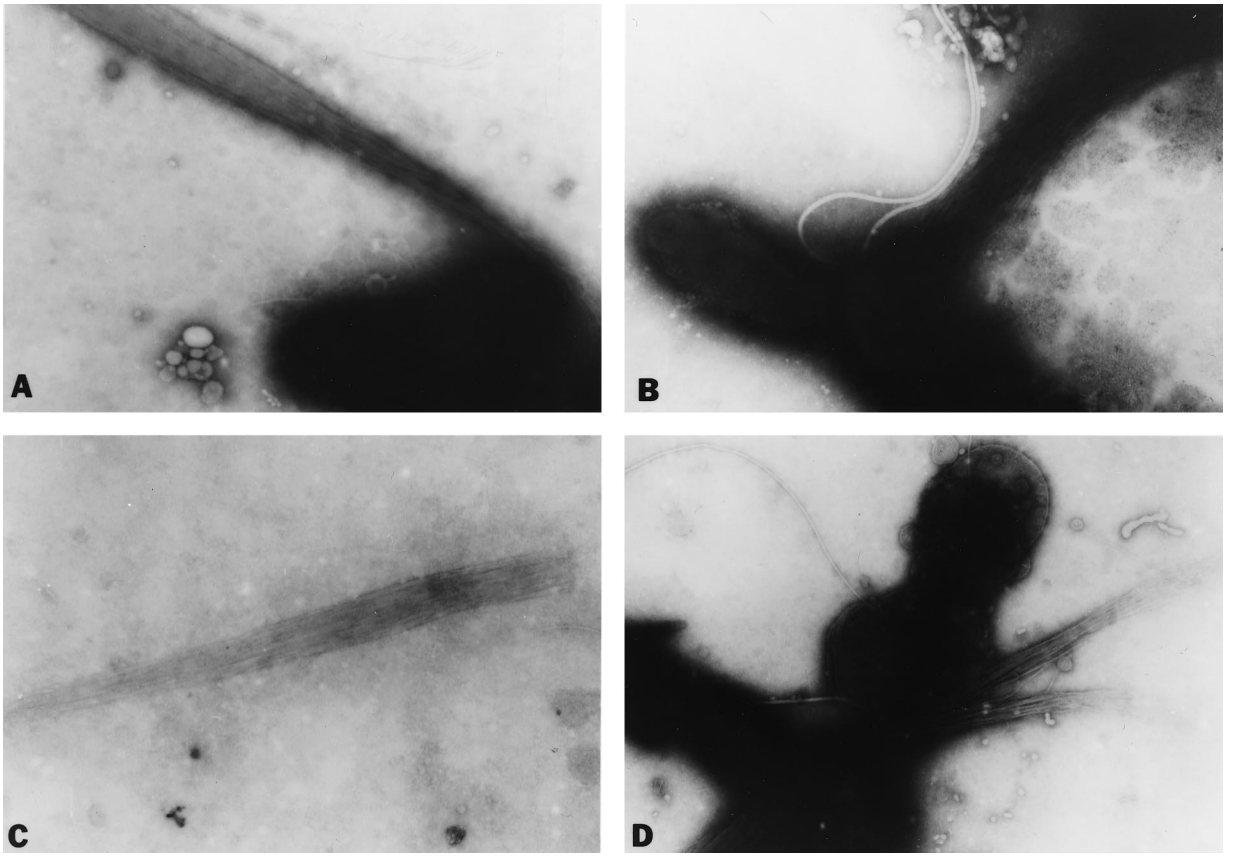

FIG. 1. Transmission electron micrographs of TCP produced by *V. cholerae* wild-type and TonB-deficient strains CA401 (A), AMV524 (B), DOV221 (C), and DOV300 (D). The strains were grown under TCP-expressing conditions, adsorbed onto Formvar-coated copper grids, negatively stained with 0.1% phosphotungstic acid (pH 6.8), and viewed by transmission electron microscopy.

secretion of proteins by the EPS pathway (21, 23). In order to determine if EpsE is specific to the EPS pathway, analogous to the specificity of TcpT with respect to TCP, an *epsE*::*kan* derivative of O395 (21) was examined for TCP production. Despite a growth defect, the strain elaborated TCP in a normal manner (Table 1). Taken together, these findings further support the specificity of each cognate ATPase for a specific secretion system.

General considerations. In this study, we have provided evidence that the type 4 pilus biogenesis and type II secretion systems in *V. cholerae* do not require TonB in the energy transduction process. These results, along with the fact that the cognate Walker box-containing proteins are required for each system, lead us to propose that ATP is likely to be the sole source of energy for these systems and that TonB-facilitated PMF is not required. These results suggest the possible exis-

TABLE 1. TCP- and EPS-related phenotypes in wild-type and TonB- and ATPase-deficient *V. cholerae* strains

Strain	Relevant characteristics	Agglutination[f]	TCP morphology	CTX-Gm/KnΦ transduction efficiency[a]	Secreted cholera toxin production[b] (%)
CA401S	O1 classical biotype; Sm^r	+++	WT	1	100
AMV524	CA401S; $TonB1^-$	+++	WT	0.6	>95
DOV221	CA401S; $TonB2^-$	+++	WT	1.4	>95
DOV300	CA401S; $TonB1^-$ $TonB2^-$	+++	WT	0.9	>95
O395 Sm	O1 classical biotype; Sm^r	+++	WT	1	100
RT4376	O395; Sm^r $TcpT^-$	−	ND[c]	<0.002[d]	>95
O395 *epsE*	O395; Sm^r $EpsE^-$	+++	WT	1.1	<10[e]

[a] The CTX-GmΦ and CTX-KnΦ transduction assays were carried out as previously described (30). Transduction frequency was calculated based on the number of Gm^r or Kn^r transductants per input CFU and is represented as efficiency relative to that of the wild type.
[b] Toxin secretion was quantitated by using the GM_1 enzyme-linked immunosorbent assay as previously described (2).
[c] ND, not determined.
[d] No Kn^r bacteria were recovered, so the actual transduction frequency was below the limit of detection.
[e] As previously reported (21).
[f] +++, maximal agglutination; −, no agglutination.

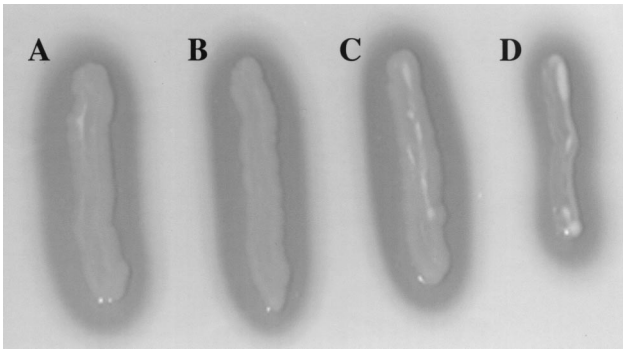

FIG. 2. Milk agar plate assay for protease secretion by *V. cholerae* wild-type and TonB-deficient strains. Streaks: A, CA401S (wild type); B, AMV524 (TonB1$^-$); C, DOV221 (TonB2$^-$); D, DOV300 (TonB1$^-$ TonB2$^-$). The strains were grown at 37°C for 24 h.

tence of a mechanism by which to transfer energy, derived from ATP hydrolysis at the cytoplasmic membrane, to the outer membrane in order to facilitate the final step of translocation. The energy transduction apparatus could be in the form of a protein bridge between the cytoplasmic and outer membranes. There are two likely mechanisms by which this could occur. One mechanism is that the energy is directly transferred via the type 4 pilins or pilin-like proteins of these systems. Another possibility is that some of the proteins of unknown function with a predicted periplasmic location or domains in the type 4 pilus or type II secretion system are part of the apparatus. Indeed, such a role has been proposed for components of several type II secretion systems (1, 5, 29). The inner membrane proteins could form a basal secretion apparatus, and ATP hydrolysis could change the conformation of the inner membrane and/or periplasmic proteins, leading to the formation of a bridge between the membranes. A similar mechanism for energy transduction has been proposed for the aerolysin secretion system of *A. hydrophila.* Energy derived from hydrolysis of ATP by the cytoplasmic membrane ATPase, ExeA, is hypothesized to be transduced to the outer membrane by TonB-like protein ExeB (5). Interestingly, *V. cholerae* has homologs in EpsA and EpsB but their role has not been determined. The transduced energy in the various systems might play a role in the opening and closing of the outer membrane channel and/or in exoprotein translocation across the outer membrane. It is also possible that these putative ATPases play a role in substrate selection (17) or provide energy for the assembly of the secreton or the pilus biogenesis apparatus. However, preliminary data (N. Bose and R. K. Taylor, unpublished) suggest that, overall, the TCP biogenesis apparatus is not disrupted in a *tcpT* mutant. An alternative hypothesis is that there are no energy requirements at the outer membrane for these secretion systems and that the proteins are forced through the secretin channel as they are extruded away from the cytoplasmic membrane. However, it is difficult to envision how gating of the secretin pore would be regulated if this mechanism were utilized.

It should be noted that even though we have obtained conclusive evidence that rules out a role for TonB in type 4 pilus biogenesis and EPS-mediated protein secretion by *V. cholerae*, pH and proton ionophore experiments suggest a role for PMF in toxin secretion (20). Toxin secretion was affected under conditions of low pH, as well as in the presence of CCCP. Similarly, studies utilizing arsenate and CCCP have also suggested a role for PMF in the pullulanase type II secretion system (13). If all type II secretion systems derive energy by the same mechanism, then our findings can be reconciled if there is a way, other than via TonB, for PMF to be transduced to the outer membrane. In such cases, components such as PulC might transduce PMF rather than energy obtained from ATP hydrolysis (12). There is no obvious homology between TonB and any of the components of the type 4 pilus and type II secretion systems, so any such mechanism of PMF-derived energy transfer would have to differ significantly from that utilized by TonB. Overall, results from the present study bring forth the possibility that the cognate putative ATPase provides the sole source of energy for type 4 pilus and type II secretion systems.

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