Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin

(transcription activation /secretion/fimbriae/virulence/toxR gene)

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ABSTRACT The transposon TnphoA was used to generate fusions between phoA, the gene for alkaline phosphatase (PhoA), and genes encoding proteins that are secreted by Vibrio cholerae. One of the PhoA+ mutants isolated showed a dramatic reduction in its ability to colonize the intestines of suckling mice. This mutant no longer produced a 20.5-kDa protein (TcpA) that we show is the major subunit of a V. cholerae pilus. Amino-terminal sequence analysis of the TcpA pilus subunit showed that it shares amino acid homology with the pilins produced by several other pathogenic bacteria. The TcpA pilus was coordinately expressed with cholera toxin under various culture conditions, and this effect appeared to be dependent on the transcriptional activator encoded by the toxR gene. We conclude that the toxR gene plays a central role in the transcriptional regulation of multiple virulence genes of V. cholerae.

Vibrio cholerae is a bacterial species that can cause a diarrheal disease in humans by colonizing the small intestine and secreting a protein toxin (1). While the action of cholera toxin is well understood, the equally important property of colonization has resisted detailed biochemical and genetic analysis. Motility, chemotaxis, and hemagglutinin/protease production have been shown to be properties that enhance colonization of V. cholerae (2, 3), but to date the molecular components actually involved in adherence of V. cholerae to the intestinal mucosa have eluded identification.

For other bacterial pathogens, colonization of mucosal surfaces frequently depends on the production of filamentous surface appendages, called fimbriae or pili, that mediate the adherence of bacteria to specific receptors on host tissues (2, 4–7). However, production of pili by V. cholerae has been demonstrated by only a few laboratories and never been shown to correlate with colonization properties of V. cholerae (2).

Exported proteins, such as the individual subunits making up pilus filaments, utilize hydrophobic amino-terminal signal sequences to accomplish their transport across the bacterial cytoplasmic membrane (4–8). We have used this property of secreted bacterial proteins and TnphoA, the vector recently described by Manoil and Beckwith (9, 10), to provide a strong enrichment for mutations that affect the colonization properties of V. cholerae. TnphoA is a derivative of Tn5 that can be used to create fusions between target genes and phoA, the gene for Escherichia coli alkaline phosphatase. Such gene fusions encode hybrid proteins composed of a carboxyl-terminal portion of alkaline phosphatase (PhoA) fused in-frame to an amino-terminal portion of a target gene product. The most critical property of these hybrid proteins is that they exhibit little or no PhoA activity unless the target gene encodes a secreted or membrane-spanning protein (9, 10).

Because virtually all bacterial proteins implicated as virulence factors are extracellular, surface-associated, or peri-plasmic, the application of TnphoA should provide a strong enrichment for insertion mutations that affect pathogenic properties of bacteria. Using this approach, we have identified the gene for a pilus colonization factor that is coordinately regulated with cholera toxin in V. cholerae.

MATERIALS AND METHODS

Bacterial Strains and Genetic Methods. V. cholerae strains used in this study were maintained at −70°C in LB medium (11) containing 25% (vol/vol) glycerol. Random insertions of TnphoA into the chromosome of V. cholerae were accomplished through the use of pRT291, a derivative of the broad-host-range P-group plasmid pRK290 that carries a copy of TnphoA. The use of pRT291 and other broad-host-range vectors for TnphoA will be described in detail in a separate report (R.K.T., C. Manoil, and J.J.M., unpublished data). In brief, chromosomal inserts of TnphoA were obtained in V. cholerae strains carrying pRT291 by superinfection with the incompatible plasmid pPH11 and selection for resistance to kanamycin and gentamycin. V. cholerae colonies carrying inserts of TnphoA were screened for the PhoA* phenotype on LB agar containing 0.2% glucose and 20 μg of 5-bromo-4-chloro-3-indolyl phosphate (X-P) per ml at 30°C. The toxR insertion mutation toxR55 was introduced into various strains by integration of plasmid pVM55 into the chromosomal copy of toxR (12). The toxR43 deletion mutation was introduced into O395-N1 (13) by recombination with plasmid pVM41 (12). The correct introduction of toxR55 and toxR43 mutations was confirmed by Southern blot (14) analysis with appropriate gene probes for toxR. Complementation of toxR55 mutations was performed by introduction of toxR* plasmid pVM53-D (15) into the toxR55 mutants O395-55 and CA401-55.

Biochemical Analyses. Except where otherwise noted V. cholerae wild-type and mutant strains were grown in LB broth (pH 6.5) at 30°C for 18 hr with aeration. Cells were collected by centrifugation, lysed in sample buffer with a reducing agent, and analyzed by NaDodSO4/12.5% polyacrylamide slab gel electrophoresis (PAGE) as described (16). The same cultures were assayed for cholera toxin by GM1 ELISA (17), and the amount produced is expressed in nanograms of toxin antigen per OD600 unit. Alkaline phosphatase (PhoA) activity was measured in permeabilized cells (9) and is expressed in enzyme units per OD600 unit. Hemagglutination was performed by the method of Jones and

Abbreviation: X-P, 5-bromo-4-chloro-3-indolyl phosphate.

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Frerer (18) with bacterial cells grown as described above. Erythrocytes from CD-1 mice were used and the reaction was performed at room temperature in KRT buffer (18) containing 1-fucose at 2.5 mg/ml. The hemagglutination titer is the reciprocal of the highest dilution of the culture that still produced detectable agglutination of the CD-1 erythrocytes after 2 hr of incubation. Hydrophobicity of bacterial cells was determined by the method of París et al. (19), and the value reported is the molar concentration of (NH₄)₂SO₄ required to cause total agglutination of the bacterial culture.

Preparation and Analysis of V. cholerae Pili. Strain O395-mont, a nonflagellated mutant of O395-N (13) was used to prepare purified pilus filaments and was grown in LB broth (pH 6.5). A volume of 400 ml of broth per 2-liter flask was used and the culture was incubated at 150 revolutions per min for 16 hr at 30°C. The cells were collected by centrifugation and resuspended in 12.5 mM Tris-HCl, pH 7.0/25 mM NaCl/4 mM MgCl₂/4 mM CaCl₂. After the bacterial cells were sheared by passage through a 21-gauge needle, the pili were purified by several rounds of differential centrifugation to remove cells and soluble proteins. The isolation of the pili was monitored by following the purification of the 20.5-kDa pilus subunit by NaDodSO₄/PAGE. Purified pili were observed by electron microscopy after preparations were stained with 2% ammonium molybdate. The 20.5-kDa pilus subunit was further purified by electrophoresis of the corre- sponding protein band after PAGE. The amino-terminal amino acid sequence of this protein was determined using an Applied Biosystems model 470A automated sequencer.

Colonization and LD₅₀ Assays. The competition assay of Frerer et al. (20) was performed essentially as described. The competitive index is defined as the change in the ratio of two strains after growth together under experimental conditions. The in vitro condition was growth at 37°C for 18 hr in LB broth at a starting density of 5 × 10⁴ colony-forming units (cfu) per ml. The in vivo condition was intraintestinal growth in 3- to 5-day-old suckling CD-1 mice inoculated orally with 5 × 10⁴ cfu and killed 24 hr later. For these experiments the input ratio was ∼1.0 in both types of competitions. Viable cell counts were obtained by plating dilutions of broth or intesti- nal homogenates on LB agar containing streptomycin (Sm; 100 µg/ml) and X-P (20 µg/ml). The ratio of the two strains was determined either by replica plating to media containing differentially selective antibiotics or by scoring the PhoA+ blue colony phenotype of TnphoA fusion strains. In vitro competitive indexes are the average of values obtained from 4–6 individual mouse experiments.

The LD₅₀ values were determined by oral challenge of 3- to 5-day-old CD-1 mice with various doses of viable bacteria grown in LB broth at 30°C and suspended in saline solution. Four or more mice were used per dose of bacteria, and the results were analyzed after 36 hr as described (21).

RESULTS

Isolation of Colonization-Deficient Mutants of V. cholerae. A pool of several thousand V. cholerae colonies carrying insertions of TnphoA was screened for mutations that encoded PhoA+ fusion proteins by growth on LB agar containing 0.2% glucose and 20 µg of X-P per ml. About 1% of the colonies carrying insertions of TnphoA were blue on this medium indicating that these carried copies of TnphoA fused to genes encoding secreted or membrane proteins that are expressed in this medium.

Forty of these blue colonies were purified and analyzed for their total protein profile by NaDodSO₄/PAGE. Seven mu- tants showed detectable differences in their protein profile that involved the loss of one or more bands. One type of mutant, represented by strain RT110.21, had lost a single protein of 20.5 kDa (Fig. 1, lanes 1 and 2). Another type of mutant, represented by RT102.2, has lost a 25-kDa protein (data not shown). Subcloning of a V. cholerae chromosomal DNA fragment carrying the fusion to the 25-kDa protein has demonstrated that this fusion lies within the same stretch of DNA that encodes a highly immunogenic, 25-kDa outer membrane protein called OmpV, previously characterized by Stevenson et al. (22). DNA sequence analysis of the subclone has shown that the fusion joint occurred at codon 64 of the ompV gene and that OmpV is synthesized with a typical hydrophobic amino-terminal signal sequence (R.K.T. and J.M., unpublished data).

Mutants of strain O395 lacking the 20.5-kDa and 25-kDa proteins, together with control strains, were tested for colonialization defects by a coinfection assay (22) involving coinfection of the mutant with the parental strain into 3- to 5-day-old CD-1 mice. Table 1 shows that an O395 strain carrying a randomly selected TnphoA fusion (RT102.5), as well as the mutant derivative that had lost the 25-kDa OmpV protein (RT102.2), competed effectively with the parental strain and therefore retain all important colonization properties. Similarly, strain O395-NT, a nontoxicogen deletion mutant constructed by recombinant DNA methods (13), did not show a colonization defect in the competition assay relative to its toxigenic parent strain O395. In contrast, the mutant strain RT110.21, which had lost the 20.5-kDa protein, showed a marked decrease in its ability to compete with the parental strain in vivo but not in vitro. Table 1 also shows that the LD₅₀ for mutant strain RT110.21 is more than 5 orders of magnitude greater than the LD₅₀ for the wild-type strain, suggesting that the 20.5-kDa protein is an important virulence factor.

Identification of the 20.5-kDa Protein as a Plin. Cell- fractionation methods showed that the 20.5-kDa protein was

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Table 1. Characterization of wild-type and mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Competitive index</th>
<th>LD90, no. of bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>O395 Sm</td>
<td>Wild type</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>RT110.21</td>
<td>tcpA::TnphoA</td>
<td>O395 Sm</td>
<td>1.4</td>
</tr>
<tr>
<td>RT102.2</td>
<td>ompV::TnphoA</td>
<td>O395 Sm</td>
<td>0.15</td>
</tr>
<tr>
<td>RT102.5</td>
<td>? ::TnphoA</td>
<td>O395 Sm</td>
<td>0.6</td>
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<td>2.0</td>
</tr>
<tr>
<td>O395-55</td>
<td>toxR55</td>
<td>O395 Sm</td>
<td>2.3</td>
</tr>
<tr>
<td>CA401-55</td>
<td>toxR55</td>
<td>CA401 Sm</td>
<td>1.2</td>
</tr>
<tr>
<td>JJM43</td>
<td>toxR43 ctxA23</td>
<td>O395-NT</td>
<td>5.2</td>
</tr>
<tr>
<td>RT110.21</td>
<td>tcpA::TnphoA</td>
<td>JJM43</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The in vivo competitive indexes obtained for strains RT110.21, O395-55, CA401-55, and JJM43 were all significantly different (P < 0.002) from the competitive indexes obtained for strains RT102.2, RT102.5, and O395-NT, which were not significantly different from each other (P > 0.2). ND, not determined.

associated with macromolecular structures that were located on the bacterial cell surface. A nonflagellated mutant of O395 was used to partially purify these structures from sheared cells by several rounds of differential sedimentation. Examination of these preparations in the electron microscope (Fig. 2) showed long, laterally associated fimbria or pili (7 nm in diameter). Individual pilus filaments could be seen on the surface of cells of strain O395 but were not seen on cells of TnphoA-induced mutants that had lost the 20.5-kDa protein (data not shown).

The 20.5-kDa protein was further purified by electroelution after NaDodSO4/PAGE and subjected to amino-terminal amino acid sequence analysis. The sequence data obtained support the identification of the 20.5-kDa protein as the major subunit (or pilin) of the V. cholerae pilus, because its amino-terminal sequence is highly homologous to the amino-terminal sequence of pilus subunits produced by several widely different pathogenic organisms (Fig. 3). This sequence is highly hydrophobic and may represent part or all of a secretory signal sequence. Consistent with this conclusion, subcloning of the TnphoA fusion from strain RT110.21 and DNA sequencing have shown that the PhoA gene is fused to the pilin-coding sequence 92 codons downstream from the sequence encoding this hydrophobic stretch of amino acids (unpublished data).

Properties of V. cholerae Cells Expressing Pili. Like other fimbriae and pili (4–7), the V. cholerae pili apparently bind to receptors on the surface of host cells, as indicated by the ability of O395 cells expressing pilus to hemagglutinate erythrocytes of CD-1 mice. The nature of the host receptors recognized by the V. cholerae pili is unknown, but the hemagglutination of CD-1 erythrocytes was detected in the presence of L-fucose, a sugar that inhibits most of the hemaggulination mediated by V. cholerae O395 (2, 18). This property was lost in mutants, such as RT110.21, that carry an insertion mutation in the pilus structural gene (i.e., the hemagglutination titer was reduced from 8 to 0 by this TnphoA mutation). Thus, V. cholerae produces multiple hemagglutinins (2, 3), and one of these is apparently associated with the pili identified by our TnphoA insertion.

The expression of some pilus types has been correlated with increased hydrophobicity and bacterial autoagglutination (19, 23). Similarly, the hydrophobicity of V. cholerae is greatly increased due to the expression of these pili, and the vibrios autoagglutinate when expressing pili in broth culture. The TnphoA insertion mutant RT110.21 is not hydrophobic, and it does not autoagglutinate in broth (e.g., when grown under optimal conditions for pilus expression, O395 had a hydrophobicity value of 0.03, whereas the hydrophobicity value for RT110.21 was 2.0).

Coordinate Regulation of Pili and Toxin Expression. The V. cholerae pili identified here have not been characterized previously, perhaps because they are not produced under the most common conditions used for growing V. cholerae and for production of pili by other organisms. The culture conditions that lead to optimal production of this pilus (amino acid-containing broth with a pH of 6.5 and a salt concentration of about 60 mM, moderate aeration, and incubation at 25–30°C) are identical to those which lead to optimal production of cholera toxin by strain O395. Fig. 1 shows the coordinate expression of the 20.5-kDa pilus subunit, cholera toxin, and PhoA produced by strain O395 and the TnphoA fusion strain RT110.21 in response to temperature and pH of the medium. In other experiments, we found that the osmolality of the medium, the oxygen tension, the presence of certain amino acids, and the growth phase of the culture influence the yield of the pili and cholera toxin in a coordinate fashion for strain O395 and other V. cholerae strains (unpublished results). Because of this striking coordinate regulation, we have called the gene encoding this V. cholerae pilus subunit tcpA, for toxin-coregulated pilus.

The expression of the cholera toxin operon (ctx) is known to be controlled at the transcriotional level by a gene called toxR (24). The toxR gene encodes a transmembrane protein that binds to the ctx promoter region and activates transcription of the ctx operon and possibly other genes of V. cholerae (12, 15). The coordinate expression of cholera toxin with pili prompted us to test whether toxR regulates the pilin gene as well. This was done by introduction of toxR null mutations into wild-type and tcpA–phoA fusion strains.

Previous results indicated that introduction of the toxR55 null mutation in V. cholerae strain 596B reduced the production of both cholera toxin and a 38-kDa outer membrane protein called OmpU (12). As shown in Fig. 4 (lane B), O395-55, a toxR55 null mutant of strain O395 (lane A), shows not only reduced production of cholera toxin B subunit (CtxB) and the OmpU protein but also of the pilin protein (TcpA) and an unidentified 50-kDa protein. O395-55 shows increased production relative to O395 of the 40-kDa outer membrane protein OmpT and an unidentified 58-kDa protein. The protein expression pattern of O395-55 was returned to a
wild-type pattern (Fig. 4, lane C) by complementation of this toxR55 mutant with plasmid pVM53-D, which carries an active copy of the cloned toxR gene (15). Therefore, pilin protein expression appears to be also dependent on functional toxR gene. This was further confirmed by introduction of toxR55 null mutations into TcpA-PhoA fusion strain RT110.21 and measuring PhoA expression of cells grown under optimal conditions for toxin and pilus expression. While strain RT110.21 produced 1349 units, the toxR55 derivative of this strain (RT110.21-55) produced only 144 units of PhoA activity. Similar results were obtained for two other tcpA-PhoA fusions isolated in strain O395, thus supporting the conclusion that coordination of cholera toxin and the tcpA-encoded pilus in strain O395 is mediated by the transcriptional activator encoded by toxR.

Another strain of V. cholerae CA401 did not produce enough of the tcpA-encoded pilin or the unidentified 50-kDa protein to allow detection of the effect of a toxR null mutation on the production of these two proteins. However, the toxR55 mutant of CA401 (strain CA401-55) does show the same overall response in production of cholera toxin, OmpU, OmpT, and the 58-kDa proteins as the toxR55 mutant O395-55 (Fig. 4, lanes A–F).

To test the hypothesis that toxR regulates production of the tcpA pilin produced by CA401 in vivo (i.e., in the intestine), we analyzed the colonization properties of the toxR55 mutants of CA401-55 and O395-55 in infant mice (Table 1). In the competition assay for intestinal colonization, CA401-55 and O395-55 show a severe defect in colonization relative to their respective parental strains. Although we cannot rule out that some effect of the toxR null mutation other than its effect on tcpA expression is responsible for this colonization defect, these data do suggest that toxR regulates the in vivo expression of tcpA pilin even in strains, such as CA401, that do not express the pilin in large amounts in laboratory media.

The toxR null mutations used in the above experiments represent a specific type of insertion mutation (the toxR55 allele). Fig. 4 (lanes H and I) also shows that an internal 25-bp deletion mutation of the toxR structural gene (the toxR43 allele carried by strain JMM43) has the same phenotype as the toxR55 mutation in terms of the protein profile of cells analyzed by NaDodSO4/PAGE and the colonization defect seen in animals (Table 1). Competition experiments between JMM43 and RT110.21 show that JMM43 is the better colonizer (Table 1), suggesting that the small amount of tcpA-encoded pilus that is still made by strain JMM43 in the absence of a functional toxR gene is better than no pilus at all (the RT110.21 case). Thus, the colonization defect of toxR mutants of V. cholerae is probably caused primarily by the reduction in expression of the tcpA-encoded pilus rather than by an effect that toxR mutations have on the expression of cholera toxin, outer membrane proteins, or other properties of V. cholerae.

**DISCUSSION**

In this paper we have shown that either insertion mutations in the structural gene for the TcpA pilin or null mutations in the toxR regulatory gene are associated with reduced intestinal colonization by V. cholerae. Because both classes of mutations might cause reduced expression of a gene downstream of tcpA, we cannot directly conclude that this colonization defect is due to the loss of the TcpA pilus alone. Complementation and other genetic and biochemical analysis will be necessary to prove this. However, by analogy to other well-characterized systems (4), it seems reasonable to propose that this colonization defect is due to reduced adherence of V. cholerae tcpA and toxR mutants to the mucosal surface and that the TcpA pilus probably mediates this adherence by carrying bacterial adhesins that bind to surface receptors on intestinal epithelial cells.

The TcpA pilus may also contribute to the colonization process by enhancing bacterial cell–cell interactions on the mucosal surface. Expression of the TcpA pilus results in a dramatic increase in the surface hydrophobicity of V. chol-
erae. The lateral association of pilus filaments into bundles (Fig. 2) and the hydrophobic properties of the TcpA pili may together cause the autoagglutination of V. cholerae cells when pili are expressed at high levels in broth culture. This clumping of pilated cells in culture may reflect similar processes occurring on the mucosal surface. Potential support for this hypothesis comes from the work of Nelson et al. (25), who noted the rapid appearance of microcolonies and adherent plaques of vibrios on intestinal villi at a rate that paralleled multiplication as a mechanism for their formation. In the same study, thick strands of a fibrous material were observed occasionally connecting vibrios to each other and the mucosal surface (25). Similar thick strands of extracellular material have been identified as bundles of pilus filaments in the case of the gonococcus (26), which is of particular interest given that autoagglutination of gonococci is also associated with pilus expression (23).

Consistent with an important role in cholera pathogenesis, the expression of the tcpA-encoded pili is coordinately regulated with cholera toxin both at the physiological level (by nutritional and physical growth parameters) and at the genetic level (by the toxR gene product). This coordinate regulation of the ctxAB operon and the tcpA gene probably occurs at the transcriptional level via the activation of the ctx and tcp promoters by the toxR gene product (15, 24). ToxR apparently also regulates production of OmpU and OmpT, two major outer membrane proteins of V. cholerae (12), as well as several other V. cholerae proteins. Some of these other proteins, like OmpT and the unidentified 58-kDa protein, are expressed well only in the absence of a functional toxR gene, suggesting that the toxR gene product might also act as a repressor for some genes of V. cholerae. Thus, ToxR may be a global regulatory protein capable of producing coordinate and opposite shifts in the expression of two distinct families of genes. Whereas one family may contain genes involved in virulence, the other family might include primarily genes important to the survival of V. cholerae in nonhuman reservoirs such as mollusks, crustaceans, copepods, or various water ecosystems (27).

The TcpA pili may represent an important new immunogen for incorporation into improved dead-whole-cell cholera vaccines or heterologous carrier vaccines currently under development. Previously tested dead-cell vaccines for cholera have been shown to be only partially protective in humans, but these vaccines were probably prepared under conditions that resulted in poor expression or destruction of the TcpA pili.

The unique genetic properties of TnphoA greatly facilitated the identification and characterization of the tcpA-encoded pili. These results have demonstrated the value of TnphoA in the genetic analysis of bacterial virulence and its potential application in the field of vaccine development.

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