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Genetics

# 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 Tn*phoA* 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 Tn*phoA*, 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*. Tn*phoA* is a derivative of Tn*5* 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 periplasmic, the application of Tn*phoA* 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 Tn*phoA* 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 Tn*phoA*. The use of pRT291 and other broad-host-range vectors for Tn*phoA* 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 Tn*phoA* were obtained in *V. cholerae* strains carrying pRT291 by superinfection with the incompatible plasmid pPH1JI and selection for resistance to kanamycin and gentamycin. *V. cholerae* colonies carrying inserts of Tn*phoA* 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 $NaDodSO_4$/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 $OD_{600}$ unit. Alkaline phosphatase (PhoA) activity was measured in permeabilized cells (9) and is expressed in enzyme units per $OD_{600}$ unit. Hemagglutination was performed by the method of Jones and

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

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Freter (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 L-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 Faris *et al.* (19), and the value reported is the molar concentration of $(NH_4)_2SO_4$ required to cause total agglutination of the bacterial culture.

**Preparation and Analysis of *V. cholerae* Pili.** Strain O395-*mot-39*, a nonflagellated mutant of O395-N1 (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 $MgCl_2$/4 mM $CaCl_2$. 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 NaDodSO$_4$/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 electroelution of the corresponding 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 $LD_{50}$ Assays.** The competition assay of Freter *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 $5 \times 10^4$ 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 $5 \times 10^4$ 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 intestinal 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 Tn*phoA* fusion strains. *In vivo* competitive indexes are the average of values obtained from 4–6 individual mouse experiments.

The $LD_{50}$ 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 inserts of Tn*phoA* 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 inserts of Tn*phoA* were blue on this medium indicating that these carried copies of Tn*phoA* 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 NaDodSO$_4$/PAGE. Seven mutants 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

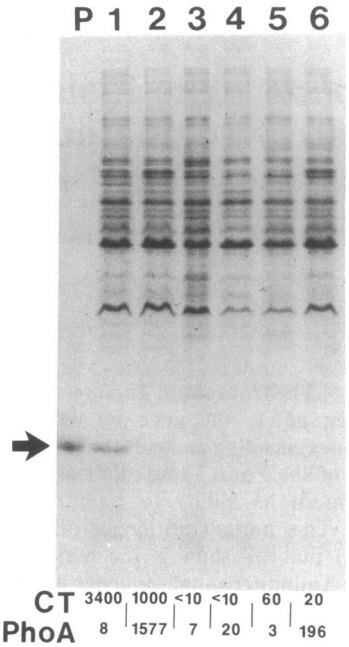


FIG. 1. Production of the 20.5-kDa pilus subunit, cholera toxin, and PhoA activity in strain O395 and Tn*phoA* mutant RT110.21. *V. cholerae* O395 (odd-numbered lanes) and Tn*phoA* insertion mutant 110.21 (even-numbered lanes) were grown in LB medium at a starting pH of 6.5 (lanes 1, 2, 5, and 6) or 8.0 (lanes 3 and 4) and at a temperature of 30°C (lanes 1–4) or 37°C (lanes 5 and 6). The total protein content of the bacterial cells was analyzed by NaDodSO$_4$/PAGE and the same cultures were also assayed for cholera toxin (CT; ng per $OD_{600}$ unit) and alkaline phosphatase (PhoA; units per $OD_{600}$ unit). Lane P contains a sample of partially purified *V. cholerae* O395 pili, which are composed largely of a single protein band migrating with an apparent molecular mass of 20.5 kDa (arrow).

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.J.M., unpublished data).

Mutants of strain O395 lacking the 20.5-kDa and 25-kDa proteins, together with control strains, were tested for colonization defects by a competition assay (20) 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 Tn*phoA* 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 nontoxinogenic deletion mutant constructed by recombinant DNA methods (13), did not show a colonization defect in the competition assay relative to its toxinogenic 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 $LD_{50}$ for mutant strain RT110.21 is more than 5 orders of magnitude greater than the $LD_{50}$ 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 Pilin.** Cell-fractionation methods showed that the 20.5-kDa protein was

Table 1. Characterization of wild-type and mutant strains

| Strain | Relevant genotype | Competing strain | Competitive index *In vitro* | Competitive index *In vivo* | $LD_{50}$, no. of bacteria |
|---|---|---|---|---|---|
| O395 Sm | Wild type | — | — | — | $4 \times 10^3$ |
| RT110.21 | *tcpA*::Tn*phoA* | O395 Sm | 1.4 | 0.002 | $8 \times 10^8$ |
| RT102.2 | *ompV*::Tn*phoA* | O395 Sm | 0.15 | 1.1 | ND |
| RT102.5 | ? ::Tn*phoA* | O395 Sm | 0.6 | 0.9 | ND |
| O395-NT | *ctxAB211* | O395 Sm | 2.0 | 1.9 | $>6 \times 10^8$ |
| O395-55 | *toxR55* | O395 Sm | 2.3 | <0.002 | ND |
| CA401-55 | *toxR55* | CA401 Sm | 1.2 | <0.002 | $2 \times 10^7$ |
| JJM43 | *toxR43 ctxA23* | O395-NT | 5.2 | 0.02 | $>9 \times 10^8$ |
| RT110.21 | tcpA::Tn*phoA* | JJM43 | 0.5 | 0.13 | ND |

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 Tn*phoA*-induced mutants that had lost the 20.5-kDa protein (data not shown).

The 20.5-kDa protein was further purified by electroelution after $NaDodSO_4$/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 Tn*phoA* 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).

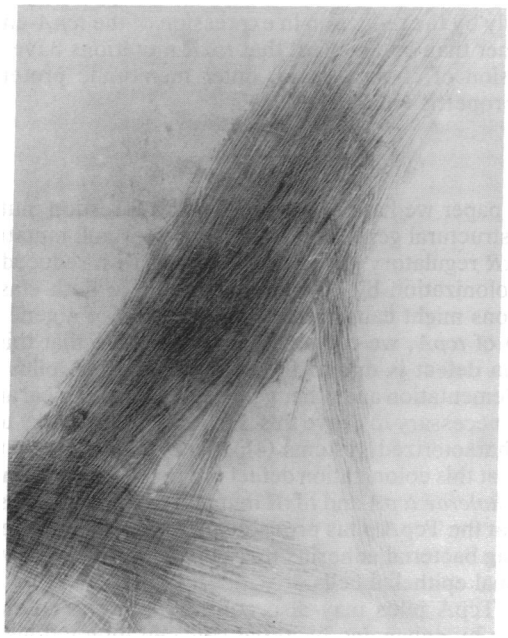

FIG. 2. Electron microscopic appearance of pili purified from *V. cholerae* O395-N1 *mot-39*. (×58,000.)

**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 pili to hemagglutinate erythrocytes of CD-1 mice. The nature of the host receptors recognized by the *V. cholerae* pilus is unknown, but the agglutination of CD-1 erythrocytes was detected in the presence of L-fucose, a sugar that inhibits most of the hemagglutination mediated by *V. cholerae* O395 (2, 18). This property was lost in mutants, such as RT110.21, that carry an insertion mutation in the pilin structural gene (i.e., the hemagglutination titer was reduced from 8 to 0 by this Tn*phoA* mutation). Thus, *V. cholerae* produces multiple hemagglutinins (2, 3), and one of these is apparently associated with the pilus identified by our Tn*phoA* 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 Tn*phoA* 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 Pilus 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 Tn*phoA* fusion strain RT110.21 in response to temperature and pH of the medium. In other experiments, we found that the osmolarity 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 *t*oxin-coregulated *p*ilus.

The expression of the cholera toxin operon (*ctx*) is known to be controlled at the transcriptional 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 569B 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

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V. cholerae      1      X  Thr Leu Leu Glu Val Ile Ile Val Leu Gly Ile 12
                                  *       *   *               *   *
N. gonorrhoeae   1  Me-Phe Thr Leu Ile Glu Leu Met Ile Val Ile Ala Ile 12
                                  *       *   *               *   *
P. aeruginosa    1  Me-Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile 12

V. cholerae     13 Met Gly Val Val Ser Ala Gly Val Val Thr Leu Ala Gln 25
                    *   *   *   *       *   *   *       *
N. gonorrhoeae  13 Val Gly Ile Leu Ala Ala Val Ala Leu Pro Ala Tyr Gln 25
                    *   *   *   *       *   *   *
P. aeruginosa   13 Ile Gly Ile Leu Ala Ala Ile Ala Ile Pro Gln Tyr Gln 25
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FIG. 3. Amino-terminal sequence of the *V. cholerae* pilus subunit. Amino acid residues homologous to the amino-terminal sequence of mature pilus subunits of *Neisseria gonorrhoeae* (5) and *Pseudomonas aeruginosa* (6) are underlined, and conserved hydrophobic residues are marked with an asterisk. The amino-terminal sequences of the pilus subunits produced by *Moraxella bovis* and *Bacteroides* sp. are not shown but are also highly homologous in this region (7). The first residue of the *V. cholerae* pilus was not identified but may be *N*-methylphenylalanine, by analogy to all the other pilus subunits in this group (5–7).

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*–Tn*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 coordinate production 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).

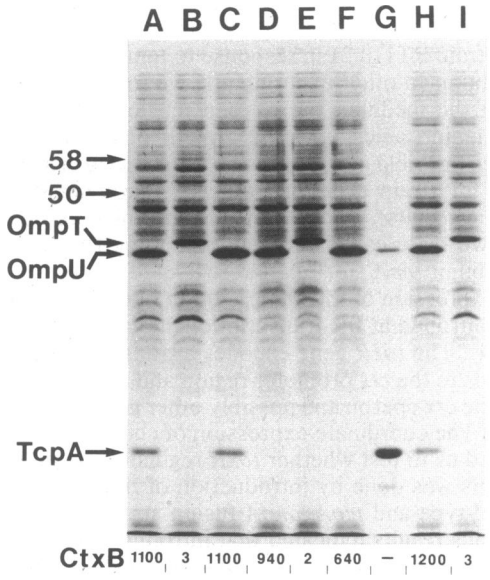


FIG. 4. Coordinate regulation of cholera toxin, TcpA, and other proteins by the *toxR* gene of *V. cholerae*. All strains were grown in LB broth (pH 6.5) and were then analyzed for their total protein content by $NaDodSO_4$/PAGE and amount of cholera toxin B subunit (CtxB). Lanes: A, O395; B, O395-55; C, O395-55(pVM53-D); D, CA401; E, CA401-55; F, CA401-55(pVM53-D); G, partially purified TcpA pili; H, O395-N1; I, JJM43. The positions of the outer membrane proteins OmpT and OmpU are indicated as well as the position of two unidentified *toxR*-regulated proteins (58 and 50 kDa).

To test the hypothesis that *toxR* regulates production of the *tcpA* pili 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* pili even in strains, such as CA401, that do not express the pili 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-base-pair deletion mutation of the *toxR* structural gene (the *toxR43* allele carried by strain JJM43) has the same phenotype as the *toxR55* mutation in terms of the protein profile of cells analyzed by $NaDodSO_4$/PAGE and the colonization defect seen in animals (Table 1). Competition experiments between JJM43 and RT110.21 show that JJM43 is the better colonizer (Table 1), suggesting that the small amount of *tcpA*-encoded pilus that is still made by strain JJM43 in the absence of a functional *toxR* gene is better than no pili 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 pili 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 level in broth culture. This clumping of piliated 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 precluded 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 mucosa 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 pilus 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 pilus 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 pilus.

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

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