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Immunology

ToxR regulates the production of lipoproteins and the expression of serum resistance in *Vibrio cholerae*

(complement/mucosal immunity/toxin coregulated pili/Tn*phoA*)

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ABSTRACT **The genes encoding three lipoproteins of *Vibrio cholerae* were identified by a combination of DNA sequence analysis and [^{3}H]palmitate labeling of hybrid proteins encoded by Tn*phoA* gene fusions. The expression of these three lipoproteins, TagA, AcfD, and TcpC, was controlled by ToxR, the cholera toxin transcriptional activator. The involvement of other bacterial lipoproteins in conferring resistance to the bactericidal effects of complement prompted us to examine this possibility in *V. cholerae*. Remarkably, mutations in *toxR* and *tcp* genes (including *tcpC*), involved in the biogenesis of the toxin coregulated pili, rendered *V. cholerae* about 10^4–10^6 times more sensitive to the vibriocidal activity of antibody and complement. Since *V. cholerae* is a noninvasive organism and *toxR* and *tcp* mutants are highly defective in intestinal colonization in animals and humans, these results raise the possibility that resistance to a gut-associated, "complement-like" bactericidal activity may be a major virulence determinant of *V. cholerae* and other enterobacterial species.**

The most important virulence properties of *Vibrio cholerae*, the causative agent of the diarrheal disease Asiatic cholera, are cholera toxin production and the ability to adhere to and colonize the small intestine of the host (1, 2). Cholera toxin is encoded by the *ctxAB* operon (3), and essential colonization properties of *V. cholerae* have been associated with the production of fimbriae encoded by the *tcp* locus (4, 5). The production of toxin coregulated pili (TCP) and cholera toxin are both modulated by the same *in vitro* growth conditions (4, 6) and are under the control of the transcriptional activator encoded by the *toxR* gene (4, 6–9).

The ToxR regulon, defined as the set of genes whose expression is under the direct or indirect control of ToxR, comprises, in addition to the *ctx* and *tcp* genes, the genes encoding the outer membrane proteins OmpU and OmpT, the *acf* genes specifying an accessory colonization factor, and a group of genes, the *tag* genes (ToxR activated genes), which have not yet been associated with a virulence property in immunologically naive infant mice (10). Most of these genes have been characterized through a Tn*phoA* mutagenesis (11) of the *V. cholerae* chromosome and the subsequent screening for the active alkaline phosphatase hybrid proteins whose expression was modulated by the environmental growth conditions known to regulate toxin production (10).

To understand the structure and regulation of expression of the ToxR-regulated genes, we have cloned the Tn*phoA* fusions to two of these genes (*tagA* and *acfD*; ref. 10) and determined the nucleotide sequences at the junction with Tn*phoA* (C.P. and J.J.M., unpublished results). Sequence analysis of the open reading frames corresponding to the N-terminal parts of TagA and AcfD revealed, at the end of potential signal sequences, the presence of the motif Leu-Xaa-Gly-Cys, which suggests that the *tagA* and *acfD* genes could encode lipoproteins. Mature lipoproteins have an N-terminal cysteine residue that is modified by the addition of two fatty acyl groups through ester linkage involving a glyceride residue and by the addition of a third fatty acyl group via an amide linkage (12). Some bacterial outer membrane lipoproteins have previously been associated with the property of serum resistance (13–15). Although the mechanism is not yet understood, it is thought that resistance to the bactericidal effects of complement involves preventing the deposition of the membrane attack complex (composed primarily of polymerized complement component C9) into the bacterial outer membrane (13, 16, 17).

We report here that *V. cholerae* produces three ToxR-regulated lipoproteins (TagA, AcfD, and TcpC). Mutations that affect the expression or proper outer membrane localization of TcpC cause both a pronounced sensitivity to the bactericidal effects of anti-vibrio antibody and complement and a severe defect in intestinal colonization.

MATERIAL AND METHODS

Bacterial Strains and Growth Media. Derivatives of *V. cholerae* O395 Sm were maintained at −70°C in LB medium (18) containing 25% (vol/vol) glycerol or on LB plates. O395-N1 (4) and TCP2 (5) have deletions within *ctxA* and a deletion insertion in *tcpA*, respectively. JJM43 is a *toxR* deletion derivative of O395-N1 (4) and O395-12 is a *toxR* insertion derivative of O395 Sm (4). Construction of strains carrying Tn*phoA* insertions in *ctxA* (KP2.36), *tagA* (KP8.56), *tagB* (KP5.51), *tagC* (KP2.16), *tagD* (KP8.74), *acfA* (KP9.62), *acfB* (KP3.51), *acfC* (KP3.44), *acfD* (KP8.11), *tcpA* (KP9.79), *tcpB* (KP8.97), *tcpC* (KP8.87 and KP11.30), *tcpD* (KP9.53), and *tcpE* (KP2.21) has been described (10).

***In Vivo* Labeling with [^{3}H]Palmitate.** *V. cholerae* cells were grown at 30°C in either LB or M9 medium (19) and labeled with [^{3}H]palmitate (25 μCi per nmol per ml) for 3 hr (about four generations) or for 16 hr; similar results were obtained in both cases.

Preparation of Outer Membrane Protein-Enriched Fractions. Outer membrane proteins were separated from inner membrane and cytoplasmic proteins by fractionation in Triton X-100 as described by Hantke (20). The Triton X-100-soluble material was precipitated by addition of 5 volumes of cold acetone.

SDS/PAGE and Immunoblotting. Electrophoresis in 8% polyacrylamide gels in the presence of SDS was performed as described by Laemmli (21). After electrophoresis, proteins were stained with Coomassie brilliant blue or transferred by

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Abbreviation: TCP, toxin coregulated pilus (pili).

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electrophoresis to nitrocellulose sheets (22). The preparation and use of the anti-PhoA antiserum has been described (10).

Serum Sensitivity Assay. Bacterial strains were grown under ToxR-inducing conditions in LB broth at 30°C for 19 hr as described (6), collected by centrifugation, and resuspended in phosphate-buffered saline at a concentration of ≈5 × 10^7 bacteria per ml. Guinea pig complement (Difco) was added to a final concentration of 22%, and the reaction mixture was split into two tubes; rabbit antiserum against whole *V. cholerae* O395 cells was added (1/1500th volume) to one tube, and then both tubes were incubated at 37°C for 1 hr. The reaction was stopped by diluting 10-fold in cold LB, and the number of viable bacteria was determined by plating dilutions on LB plates. The percent survival in the complete reaction mixture relative to the control reaction mixture, lacking only the rabbit anti-vibrio serum, was calculated. Results are reported as relative sensitivity (i.e., the fold decrease in survival of mutant strains relative to appropriate parental strains O395 or O395-N1).

RESULTS

Hybrid PhoA Lipoproteins in *V. cholerae* Tn*phoA* Mutants. We have recently determined (C.P. and J.J.M., unpublished results) the nucleotide sequences of the 5′ part of some ToxR-regulated genes previously identified through Tn*phoA* mutagenesis (10). Examination of the amino acid sequences deduced for the N-terminal part of the *tagA* and *acfD* gene products revealed a stretch of hydrophobic and nonpolar residues that is likely to represent the signal sequences involved in the periplasmic or membrane localization of these proteins; such a localization is assumed since both the TagA–PhoA and the AcfD–PhoA hybrid proteins are endowed with alkaline phosphatase activity (11). The motif Leu-Xaa-Gly-Cys, which is present at the processing site of lipoproteins (12), was detected at the end of these putative signal sequences (Fig. 1), suggesting that the *tagA* and *acfD* genes could encode lipoproteins.

To characterize the lipoproteins synthesized in *V. cholerae*, we performed *in vivo* labeling experiments with [^{3}H]palmitate as described by Ichihara *et al.* (24). The region of the gel encompassing the proteins with a molecular mass of 38 kDa or less was heavily labeled and was not accessible to the analysis (see, for example, the bottom part of the gels in Fig. 4*B*); this was probably the result of the incorporation

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TraT                    MKMKKLMMVALMSSTLA L S G C AMS..
                                ----------
Iss                     MQDNKMKKMLFSAALAML I T G C ANN..
                                ----------
Bor                     MKKMLLATALALL I T G C ANN..
                                ----------

                                          *
Consensus sequence                  L A G C
of lipoprotein                        S A
processing sites                      V

TagA                    MVVRYSLLMKVSFAILIF L V G C NEN..
                                ---------
AcfD                    MKIAIVSLIVLGF L I G C KHE..
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FIG. 1. Comparison of the signal sequences and potential lipoprotein processing sites of serum-resistance proteins TraT, Iss, and Bor with those of TagA and AcfD. The N-terminal sequences of *Escherichia coli* TraT (13, 14), Iss (18), and Bor (23) precursors have been aligned above the consensus sequence of the bacterial lipoprotein processing site (12) and the N-terminal sequences of TagA and AcfD deduced from the nucleotide sequences upstream of Tn*phoA* fusions carried by *V. cholerae* strains KP8.56 and KP8.11 (C.P. and J.J.M., unpublished results). The hydrophobic and nonpolar residues are underlined, and the cysteine residue at which the processing takes place is indicated by a star.

of radioactive palmitate or one of its metabolites into the lipopolysaccharide. This difficulty in identifying the ToxR-regulated genes encoding lipoproteins was overcome by taking advantage of the collection of Tn*phoA* mutants; we reasoned that the insertion of Tn*phoA* into a gene encoding a lipoprotein would give rise to a hybrid protein with a molecular mass of at least 48 kDa (corresponding to the molecular mass of the PhoA portion of the hybrid) that should still be labeled with [^{3}H]palmitate.

The pattern of proteins labeled with radioactive palmitate in wild-type *V. cholerae* O395 Sm, in a *toxR* mutant (JJM43), and in strains carrying Tn*phoA* insertions in *tagA*, *tagB*, *tagC*, and *tagD* as well as in *acfA*, *acfB*, *acfC*, and *acfD* is shown in Fig. 2. Two proteins of estimated molecular masses of 62 kDa and 53 kDa were detected in O395 Sm, and additional proteins of molecular masses of 58 kDa and 55 kDa were detected in the extracts of the strains carrying Tn*phoA* insertions in *tagA* and *acfD*, respectively. As these additional proteins were not present in the extracts of the other strains that also carried Tn*phoA* insertions, we can conclude that they are not encoded by the Tn*phoA* transposon. The relative intensity of the 58-kDa protein detected in KP8.56 (*tagA:phoA*) was enhanced in the extract of O395 Sm harboring the pVC8 plasmid into which was cloned the same *tagA:phoA* fusion (see Fig. 2, lane 7); this plasmid expresses 10 times more alkaline phosphatase activity than the chromosomally encoded fusion (C.P. and J.J.M., unpublished results). Thus, the 58-kDa lipoprotein is the TagA–PhoA hybrid protein encoded by KP8.56. Because no lipoproteins are detected in O395 Sm that are subsequently replaced by the 58-kDa or 62-kDa lipoproteins present in the *tagA:phoA* or *acfD:phoA* fusion strains, the wild-type TagA and AcfD proteins probably each have a molecular mass <38 kDa and are masked by the bulk of radioactivity due to the lipopolysaccharide.

The 53-kDa lipoprotein detected in the wild-type O395 Sm strain was still present in the *tagA:phoA* and *acfD:phoA* mutants, indicating that this lipoprotein was not encoded by either *tagA* or *acfD*. On the other hand, this 53-kDa protein was not present in the *toxR* mutant (see Fig. 2, lane 1), suggesting that the expression of the gene encoding this lipoprotein was also under the control of ToxR. This led us to investigate the lipoproteins synthesized in strains carrying Tn*phoA* insertions in the *tcp* genes, as these genes are known to be ToxR-regulated (4, 10, 25). As shown in Fig. 3, the

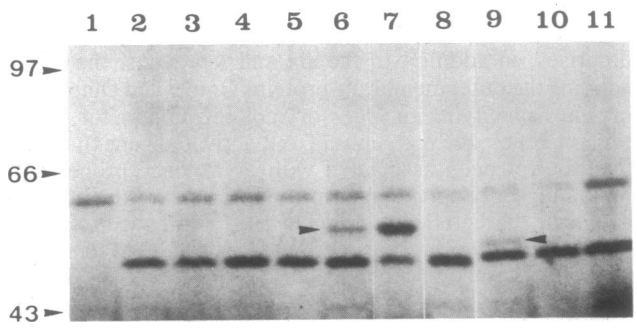

FIG. 2. Hybrid PhoA lipoproteins in *V. cholerae* strains carrying Tn*phoA* insertions in the *tag* and *acf* genes. *V. cholerae* cells grown in M9 medium containing glycerol (0.4% wt/vol) and Casamino acids (0.4% wt/vol) were labeled with [^{3}H]palmitate for four generations. Crude extracts were analyzed by SDS/PAGE, and the gel was processed for fluorography. Lane 1, JJM43 (*toxR*); lane 2, O395 Sm (wild type); lane 3, KP2.16 (*tagC:phoA*); lane 4, KP5.51 (*tagB:phoA*); lane 5, KP8.74 (*tagD:phoA*); lane 6, KP8.56 (*tagA:phoA*); lane 7, O395 Sm with pVC8 (*tagA:phoA*); lane 8, KP9.62 (*acfA:phoA*); lane 9, KP8.11 (*acfD:phoA*); lane 10, KP3.44 (*acfC:phoA*); lane 11, KP3.51 (*acfB:phoA*). Arrowheads by the numbers indicate the positions and molecular masses (in kDa) of protein standards. Other arrowheads indicate the positions of new labeled lipoproteins.

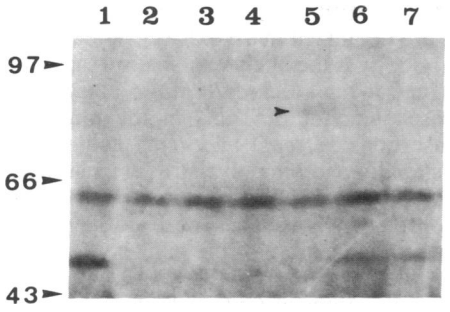

FIG. 3. Hybrid PhoA lipoproteins in *V. cholerae* strains carrying Tn*phoA* insertions in the *tcp* genes. Crude extracts of *V. cholerae* cells grown in M9 medium containing glycerol (0.4% wt/vol) and Casamino acids (0.4% wt/vol) and labeled with [^{3}H]palmitate for 16 hr were analyzed by SDS/PAGE, and the gel was processed for fluorography. Lane 1, O395 Sm (wild type); lane 2, JJM43 (*toxR*); lane 3, KP9.79 (*tcpA:phoA*); lane 4, KP8.79 (*tcpB:phoA*); lane 5, KP8.87 (*tcpC:phoA*); lane 6, KP9.53 (*tcpD:phoA*); lane 7, KP2.21 (*tcpE:phoA*). Arrowheads by the numbers indicate the positions and molecular masses (in kDa) of protein standards. The remaining arrowhead indicates the position of a new labeled lipoprotein in the *tcpC:phoA* mutant.

53-kDa protein was not present in the extracts of the strains with Tn*phoA* insertions in *tcpA*, *tcpB*, and *tcpC*, and the relative amount of this protein was lower in the *tcpD* and *tcpE* mutants than in the wild-type strain. A new labeled lipoprotein estimated to be 85 kDa was detected in the extract of the strain carrying the *tcpC:phoA* fusion, indicating that the 53-kDa protein was the *tcpC* gene product. This result was confirmed by examining another *tcpC:phoA* mutant (see lane 5 in Fig. 4).

Localization of the Lipoproteins. To further characterize the lipoproteins detected in the previous experiments, we performed a Western blot analysis using anti-PhoA antibodies and attempted to localize the PhoA hybrid proteins in either the Triton X-100-soluble or -insoluble fractions; this latter fraction was enriched for outer membrane proteins (23). As shown in Fig. 4, the new molecular species labeled with [^{3}H]palmitate (Fig. 4*B*) in the Tn*phoA* mutant extracts had the same electrophoretic mobilities as the hybrid proteins recognized by the anti-PhoA antibodies (Fig. 4*C*). This result confirmed that these new lipoproteins actually corresponded to PhoA fusion proteins. The 53-kDa protein that we identified as the *tcpC* gene product was visible on the Coomassie-stained gel in both the whole-cell extract (Fig. 4*A Top*, lanes 1, 3, and 4) and the Triton X-100-insoluble fraction (Fig. 4*A Bottom*, lanes 1, 3, and 4), and was absent in the Triton X-100-soluble fraction (Fig. 4*A Middle*, lanes 1, 3, and 4). This partitioning of TcpC was confirmed by examination of the [^{3}H]palmitate labeled proteins (Fig. 4*B*). These results indicated that TcpC was one of the major outer membrane proteins of *V. cholerae*. Whereas TcpC was present almost only in the Triton X-100-insoluble fraction, this was not the case for the TcpC–PhoA hybrid protein (72 kDa), detected by either the [^{3}H]palmitate labeling (Fig. 4*B Middle*, lane 5) or the anti-PhoA antibodies (Fig. 4*C Middle*, lane 5). This might be due to the instability or improper localization of the PhoA hybrid protein. Similarly, the relative amounts of the TagA–PhoA and AcfD–PhoA chimers were not enriched in the Triton X-100-insoluble fraction. Inasmuch as the wild-type proteins were not detected by Coomassie staining or by [^{3}H]palmitate labeling, the exact localization of the TagA and AcfD proteins could not be determined by these methods.

The Expression of Serum Resistance Is ToxR-Regulated. The expression of some bacterial lipoproteins has been associated with resistance to the bactericidal effects of complement (13–15). That three ToxR-regulated genes encoded lipoproteins prompted us to test wild-type, *toxR*, *tagA*, *acfD*,

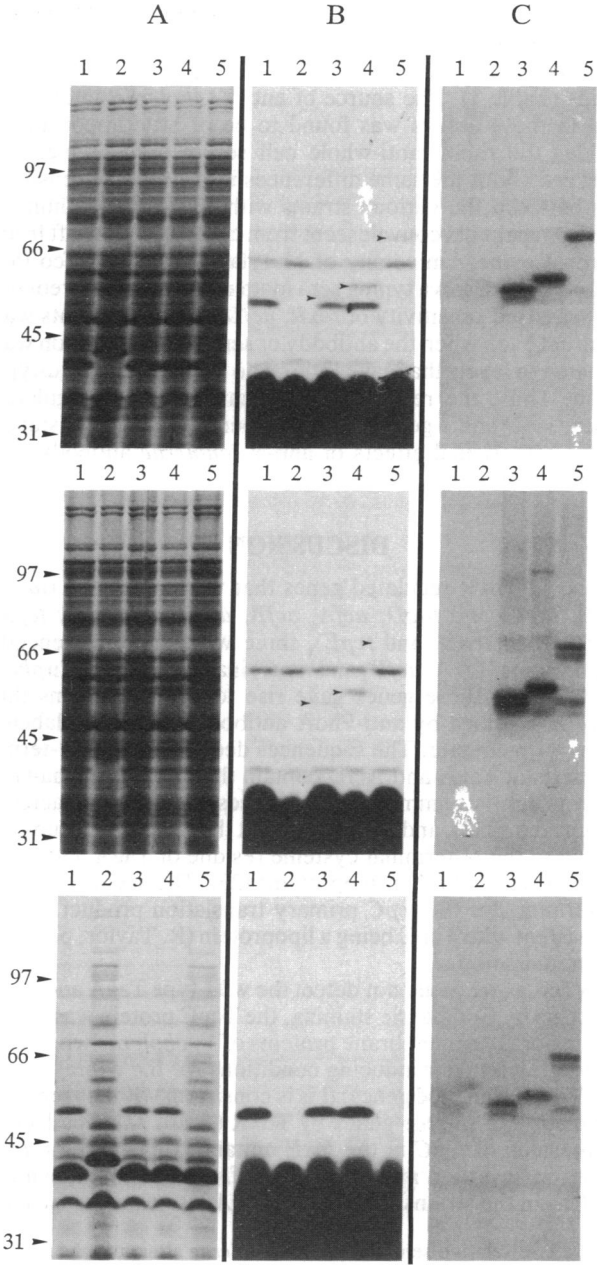

FIG. 4. [^{3}H]Palmitate labeling and immunoblot analysis of the TagA–PhoA, AcfD–PhoA, and TcpC–PhoA fusion proteins. *V. cholerae* cells were grown in LB medium and labeled with [^{3}H]palmitate for 16 hr. Crude extracts (*A–C Top*), Triton X-100-soluble fractions (*A–C Middle*), and Triton X-100-insoluble fractions (*A–C Bottom*) were analyzed by SDS/PAGE and either stained with Coomassie brilliant blue (*A*) and processed for fluorography (*B*) or transferred to nitrocellulose and probed with anti-PhoA antibodies (*C*). Lane 1, O395 Sm (wild type); lane 2, JJM43 (*toxR*); lane 3, KP8.11 (*acfD:phoA*); lane 4, KP8.56 (*tagA:phoA*); lane 5, KP11.30 (*tcpC:phoA*). Arrowheads by the numbers indicate the positions and molecular masses (in kDa) of protein standards. Other arrowheads indicate the positions of new labeled proteins in the Tn*phoA* mutants.

and *tcp* mutant strains for resistance to guinea pig serum in the presence or absence of anti-vibrio cell antibodies. In preliminary experiments, we found that 22% guinea pig serum showed no vibriocidal activity against any of the strains tested but, in contrast, all strains were sensitive to the bactericidal effects of 22% guinea pig serum when mixed with as little as a 1:1500 dilution of rabbit anti-*V. cholerae* O395 sera. Most important, certain strains were much more sensitive to the vibriocidal activity than others; the *acfD* mutant

was about 10 times and two *toxR* mutants and *tcpA*, *tcpB*, *tcpC*, *tcpD*, and *tcpE* mutants were about 10^4–10^6 times more sensitive than the wild-type, O395-N1, *tagA*, or *ctxA* mutant strains (Table 1). The source of anti-*V. cholerae* antibodies used in these assays was found to be of little importance. Besides the rabbit anti-whole cell serum used above, we observed about the same differences in the relative sensitivities between the various strains with either pooled human sera from patients convalescent from cholera (a kind gift from Myron Levine, University of Maryland) or with Difco (lot 2431-47) anti-Ogawa typing sera (data not shown). Moreover, the increased sensitivity of *toxR*, *acfD*, and *tcp* mutants was apparent even when the antibody or serum concentration was adjusted to levels that gave little or no killing of the wild-type strain. Thus, the results suggest that two ToxR-regulated genes, *acfD* and a gene in the *tcp* operon, encode resistance to the bactericidal effects of anti-*V. cholerae* antibody and complement.

DISCUSSION

Of the 13 ToxR-regulated genes that we have tested (*tagA*, *tagB*, *tagC*, and *tagD*; *acfA*, *acfB*, *acfC*, and *acfD*; *tcpA*, *tcpB*, *tcpC*, *tcpD*, and *tcpE*), three were shown to encode lipoproteins (*tagA*, *acfD*, and *tcpC*) inasmuch as the Tn*phoA* insertions in these genes gave rise to hybrid proteins that were recognized by anti-PhoA antibodies and were labeled with [^{3}H]palmitate. The sequences deduced for the N-terminal parts of TagA and AcfD contain the motif Leu-Xaa-Gly-Cys, which is common to the processing sites of bacterial lipoproteins (12), and we assume that the fatty acyl groups are linked to the N-terminal cysteine residue of TagA and AcfD as in the case of other lipoproteins (25). The sequence of the N terminus of the *tcpC* primary translation product is also consistent with TcpC being a lipoprotein (R. Taylor, personal communication).

Whereas we could not detect the wild-type TagA and AcfD proteins by Coomassie staining, the TcpC protein was one of the major outer membrane proteins of *V. cholerae* when cells were grown under inducing conditions for the expression of the ToxR-regulated genes; this is consistent with the reported regulation of the *tcp* genes by ToxR (4, 10, 22). The lack of expression of *tcpC* in the *toxR* mutant JJM43 and the *tcpA* deletion/insertion mutant TCP2 (ref. 5; data not shown), as well as in the strains carrying Tn*phoA* insertions in *tcpA* and *tcpB*, constitutes strong evidence that the *tcpA*, *tcpB*, and *tcpC* genes are organized in a ToxR-regulated operon. Transcriptional polarity is the likely explanation for the decreased amount of TcpC in *tcpA* and *tcpB* Tn*phoA* insertion mutants. The decreased amount of TcpC in the mutants carrying Tn*phoA* insertions in *tcpD* and *tcpE* could be due to a decreased stability of a *tcp* polycistronic messenger RNA, if these genes are transcribed in the same operon as *tcpA*, *tcpB*, and *tcpC*, or to an increased rate of degradation of the TcpC protein when the assembly of the pilus is not completed. In this regard, we have been unable to detect TcpC in outer membrane preparations of *tcpD* or *tcpE* mutants, suggesting that the small amount of TcpC detected by [^{3}H]palmitate labeling in these mutants is not localized correctly (data not shown). In the case of the P pilus of *E. coli*, accessory proteins have been shown to act as "chaperons" in the proper localization of the pilus subunits to the outer membrane (26), and perhaps the *tcpD* and *tcpE* gene products perform a similar function for TcpA and TcpC in TCP biogenesis.

Table 1. Relative sensitivity of *V. cholerae* strains to the vibriocidal activity of anti-vibrio whole-cell antibodies and complement

Strain	Genotype	Relative sensitivity*
O395 Sm	Wild type	1.0
KP2.36	*ctxA*::Tn*phoA*	1.8
KP8.56	*tagA*::Tn*phoA*	1.4
KP2.37	*acfD*::Tn*phoA*	9.6
KP9.79	*tcpA*::Tn*phoA*	4.0×10^5
KP8.97	*tcpB*::Tn*phoA*	4.0×10^6
KP8.87	*tcpC*::Tn*phoA*	3.0×10^5
KP9.53	*tcpD*::Tn*phoA*	1.0×10^6
KP2.21	*tcpE*::Tn*phoA*	4.6×10^6
O395-12	*toxR*::pJM703.12	7.3×10^4
O395-N1	Δ*ctxA*	1.0
JJM43	Δ*ctxA*, Δ*toxR*	2.7×10^4
TCP2	Δ*ctxA*, Δ*tcpA*	6.3×10^5

*Relative sensitivity is the fold decrease in survival of a mutant strain compared to its parental strain. All Tn*phoA* mutants and strain O395-12 were normalized to O395 Sm. Strains JJM43 and TCP2 were normalized to O395-N1. Under the conditions of the assay, strains O395 Sm and O395-N1 showed about 1% survival in the presence of anti-vibrio antibodies as compared to in their absence.

The production of bacterial lipoproteins (13–15) has been associated with resistance to the bactericidal effects of complement. This consideration led us to examine whether there existed a relationship between production of the lipoproteins TagA, AcfD, and TcpC and resistance to the bactericidal effects of antibody and complement. Remarkably, *V. cholerae toxR* and *tcpA*, *tcpB*, *tcpC*, *tcpD*, and *tcpE* mutants were all found to be about 10^4–10^6 times more sensitive to the bactericidal effect of anti-whole cell antibody and guinea pig complement than isogenic strains carrying a Tn*phoA* insertion in *ctxA* or *tagA*. Mutants defective in AcfD showed only a 10-fold increase in sensitivity, suggesting that the majority of the resistance phenotype was encoded by the *tcp* gene cluster. Given that resistance to complement killing in Gram-negative organisms frequently involves the production of either proven (13–15) or putative (18, 23) outer membrane lipoproteins, we consider TcpC to be the best candidate for mediating serum resistance encoded by the *tcp* gene cluster. This is simply because TcpC is the most abundant outer membrane lipoprotein encoded by the *tcp* locus and that all *tcp* mutations tested caused both hypersensitivity to complement killing and also poor expression and/or localization of TcpC in the outer membrane of *V. cholerae*. This deleterious effect of *tcpA*, *tcpB*, *tcpD*, and *tcpE* mutations on both the expression and outer membrane localization of TcpC has made it difficult to prove TcpC is solely responsible for serum resistance. The proof will require construction of a mutated *tcpC* gene that encodes no serum resistance but whose gene product is correctly localized in the outer membrane of *V. cholerae*. Alternatively, evidence that TcpC encodes serum resistance might be obtained by engineering the expression of this protein in a heterologous serum-sensitive organism like *E. coli* K12. However, this approach too might require additional *tcp* chaperon gene products to obtain correct outer membrane localization of TcpC.

The results presented here may have important implications in the understanding of cholera pathogenesis and the immunobiology of gut and other mucosal surfaces. It is striking that the serum sensitivity of *tcp* and *toxR* mutants is only the second phenotype, besides the loss of TCP pili, that strongly correlates with their poor intestinal colonization in immunologically naive animals (4, 10) and human volunteers (5). *V. cholerae* is known to be a noninvasive organism that colonizes the extracellular mucosal surface and is only very rarely isolated from blood (1). Therefore, the differences seen here in the relative serum sensitivity of *toxR* and *tcp* mutants may be considered of little importance in cholera pathobiology or immunity. However, there is the possibility that the bactericidal activity of antibody and complement artificially mimics another type of bactericidal process that occurs in the

gut of immune or even immunologically naive animals and humans. In support of this possibility, we have recently found that, compared to its wild-type parental strain, *tcpA*$^-$ mutant KP9.79 is over 100-fold more sensitive to a bactericidal process occurring in the gastrointestinal tract of mice during the first 7 hr of infection (J.J.M. and E.T., unpublished results). Because neither shedding nor net growth of either strain is apparent in this short-term infection, we believe that these results demonstrate the existence of a bactericidal activity in the gut of these animals to which *tcp*$^-$ mutants are significantly more sensitive than *tcp*$^+$ strains.

What is the nature of this gut-associated bactericidal activity and could it be related to the complement sensitivity we observe here? Previously, bactericidal activity has been associated with the gastrointestinal epithelium of nonimmune adult rabbits (27), suckling mice (28, 29), and adult mice (30, 31). In the latter case, the lymphocytes thought to be responsible have been called antibody-dependent cytotoxic cells because their bactericidal activity was greatly enhanced by secretory IgA (31). It is therefore of possible significance to this study that perforin, a cytolytic molecule produced by cytotoxic lymphocytes, is highly homologous to complement component C9, the terminal component of the membrane attack complex of complement (32). Furthermore, several different classes of membrane-disrupting, bactericidal peptides, including the cryptins (33), cecropins (34), and defensins (35), have been postulated to have a protective role in the intestinal mucosa. Thus, resistance to the membrane attack complex of complement may be reflective of resistance to a bactericidal process occurring at the mucosal surface involving cytotoxic cells, perforin, antibacterial peptides, or perhaps even complement and natural (or induced) antibodies.

If such a "complement-like" bactericidal activity is operative in the intestinal mucosa, then ToxR-regulated serum resistance properties could play an essential role in intestinal colonization and thus formally explain the reduced colonization observed with *toxR* and *tcp* mutants (4, 5, 10). Clearly, this possibility does not preclude additional roles for TCP fimbriae in adherence, autogglutination, or other important colonization properties of *V. cholerae* (4, 36). Significantly, Kapperud *et al.* (37) have reported that mutants of *Yersinia entercolitica* that lack the YOP1 fimbrial protein are also serum-sensitive and highly defective in intestinal colonization. Unlike *V. cholerae*, *Y. enterocolitica* is an invasive bacterial pathogen that readily penetrates the intestinal epithelium and is probably exposed to complement in the lamina propria (37). If, however, the colonization defect of YOP1 mutants is related to the colonization defect of *V. cholerae tcp* mutants, then the postulated complement-like bactericidal activity may actually be present on the luminal side of the epithelium. We can further speculate that evasion of this complement-like mucosal bactericidal activity may be a previously unrecognized factor contributing to the colonization properties of a variety of bacterial pathogens, thus explaining the dissemination of serum-resistance genes on plasmids (13–18) and phages (23) of even noninvasive bacterial isolates.

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