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Analysis of Expression of Toxin-Coregulated Pili in Classical and El Tor *Vibrio cholerae* O1 In Vitro and In Vivo

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The expression of toxin-coregulated pili (TCP) and their structural subunit TcpA was compared in 20 strains of *Vibrio cholerae* of the classical and El Tor biotypes. Bacteria were isolated from the intestines of rabbits with experimental cholera and compared with the same strains grown under optimal TCP expression conditions in vitro. Immunoblotting revealed that TcpA production was induced in both biotypes after vibrios entered the intestinal milieu; TcpA-negative inocula gave rise to TcpA-positive vibrios after multiplication in the gut. The levels of TcpA expressed during growth in the intestine were, for most strains, comparable to those attained under optimal growth conditions in vitro. Of 11 classical strains tested, 10 expressed TCP antigen on the bacterial surface at levels comparable to or exceeding those seen after growth in vitro as determined by an inhibition enzyme-linked immunosorbent assay. In contrast, only one of the nine El Tor strains studied produced detectable amounts of TCP surface antigen in vivo and no fimbriae or surface antigen reacting with anti-TCP serum was found on El Tor vibrios from human cholera stools. Distinct TCP fimbriae were observed by immunoelectron microscopy on classical-biotype vibrios grown either in rabbit intestines or in vitro but were not detected on El Tor vibrios. The results show that TCP is expressed on *V. cholerae* O1 of the classical biotype but not on *V. cholerae* O1 of the El Tor biotype in the intestines of rabbits with experimental cholera infection.

Cholera, which is the most severe of the diarrheal diseases, is caused by *Vibrio cholerae* O1 bacteria of either the classical or the El Tor biotype, each of which exists as serotype Inaba or Ogawa. After having been ingested together with contaminated water or food and having survived passage through the stomach, cholera vibrios must be able to colonize the small intestine and multiply there to cause disease. Various putative adhesins have been proposed to be involved in colonization (6, 8, 10, 34). One of these, a toxin-coregulated pilus (TCP) composed of repetitive 20.5-kDa subunits (TcpA), has been shown to be important for colonization of the human intestine by at least one classical *V. cholerae* strain (11, 34). Studies with mice have also shown that antibody preparations against TCP may provide protection against classical cholera (24, 25, 27, 28). The significance of TCP as a colonization factor for *V. cholerae* of the El Tor biotype remains to be defined. El Tor strains have been shown to possess DNA that reacts with *tcpA* probes derived from classical strains (24, 32), although the size of one of the digestion fragments tested was different from that of classical *tcpA* DNA (32). At least some El Tor strains have also been shown to express TcpA subunitlike proteins (27). A partial protective effect by high concentrations of anti-TCP antibodies against El Tor vibrios has been reported by Sun et al. (27, 28), whereas Sharma et al. found that an anti-TCP serum was protective against El Tor vibrios only if the El Tor strain was complemented with *tcp* genes from a classical strain (24). No experiments with human volunteers to assess the importance of TCP as a colonization factor in El Tor strains using El Tor *tcpA* mutants have been reported.

Production of cholera toxin (CT), TCP, and several other factors (not fully defined) of possible importance for virulence, as well as outer membrane proteins, is regulated by the *toxRS* genetic locus in conjunction with environmental signals (3, 4, 33). The regulation of putative virulence factors of *V. cholerae* O1 during cholera infection is poorly defined. We have previously shown that production of CT by classical *V. cholerae* strains is enhanced during growth in the intestines of cholera-infected rabbits, compared with expression under optimal conditions in vitro. At the same time, the amount of OmpU, which is a ToxR-regulated outer membrane protein, was lower in vibrios derived from diarrhea fluid than in vibrios grown in vitro (16, 17). El Tor strains, on the other hand, generally produced less CT in vivo than under optimal conditions in vitro (17). The aim of the present study was to determine, for a number of classical and El Tor *V. cholerae* strains, whether production of TCP and its structural subunit TcpA is enhanced during growth in the intestine.

MATERIALS AND METHODS

Bacterial strains. Eleven *V. cholerae* O1 strains of the classical biotype and nine of the El Tor biotype were used. The strains had been isolated in Bangladesh during 1986 and 1987 (1) and kindly provided by J. Clemens, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB), Dhaka, Bangladesh, or in various countries (India, Indonesia, Egypt, and Bangladesh) during 1947 to 1982, as described previously (13, 15). Strain JS1569 (23), a rifampin-resistant derivative of strain CVD 103 (strain 569B *ctxA*; J. Kaper, University of Maryland, Baltimore, Md.), was used as a TCP-positive reference (13). Strains were stored at −70°C in broth containing 20% (vol/vol) glycerol.

In vitro growth conditions. Bacteria were grown at 30°C by the AKI-SW method (12) as described previously (13). All strains were grown on at least two different occasions, and the culture of each strain with the highest amount of TcpA was selected for further studies. AKI medium (1.5% Bacto Peptone, 0.4% yeast extract [Difco], 0.086M NaCl) was

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prepared freshly and adjusted to pH 7.2 to 7.4 by adding sterile $NaHCO_3$ (0.018 M, final concentration) immediately prior to use. Three colonies from a fresh culture on blood agar were mixed, and bacteria corresponding to one colony were inoculated into a tube with 10 ml of AKI medium. After standing still at 30°C for 3.5 to 4 h (optical density at 600 nm, 0.05 to 0.15), the whole culture was transferred to a 250-ml flask and incubation was continued at 30°C for 17 to 19 h with shaking (250 rpm); some strains were grown with shaking for only 2.5 h.

In vivo growth of *V. cholerae*. Bacteria were grown in rabbit small-bowel loops as previously described (29). Each loop (5 to 6 cm long) was inoculated with 1 ml of 10^6, 10^7, or 10^8 bacteria (TcpA negative) grown in Trypticase soy broth without glucose (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 3 to 4 h; before inoculation, bacteria had been washed and suspended in phosphate-buffered saline (PBS; pH 7.2). Each strain was inoculated in at least two loops in different rabbits, and bacteria from the loop in which the highest TcpA content was noted for each strain were selected and used in the study. In some cases, rabbits were infected with *V. cholerae* by using the removable intestinal tie-adult rabbit diarrhea (RITARD) (26) nonligated-intestine model as previously described (20). The fluids that had accumulated in the loops or in the entire small intestine (RITARD) after growth of the bacteria for 16 to 18 h, or in some instances for 7 h, were collected from the animals immediately after sacrifice, chilled on ice, and prepared as described below.

Preparations of bacteria grown in vitro and in vivo. Bacteria grown in vitro were centrifuged at 8,000 × *g* for 15 min at 4°C, washed twice, and adjusted to an optical density corresponding to 10^{10} cells ml^{-1} in PBS. In vivo-grown bacteria were prepared directly from intestinal fluids without subculturing. The fluids were initially centrifuged twice at 250 × *g* for 5 min at 4°C to remove large cells and debris and then centrifuged and subsequently washed in PBS as described for in vitro-grown vibrios. Bacteria prepared from one loop were suspended in 0.5 to 1.5 ml, and vibrios from an entire small intestine (RITARD) were suspended in ca. 3 ml of PBS. CFU/ml of fluid were determined, and possible contamination with other aerobic bacteria was analyzed by culturing the diarrheal fluid specimens on blood agar plates at 37°C overnight. Only fluids in which >98% of the aerobic bacteria were vibrios were used. In some instances, vibrios adhering to the intestinal epithelium were isolated by being scraped off the mucosa of infected intestinal loops after extensive washings in PBS. All bacterial preparations were stored frozen at −70°C.

Preparation of bacteria from human cholera stools. Liquid stool specimens were collected from cholera patients treated at the hospital of the ICDDRB in Dhaka, Bangladesh. The stools, kindly provided by M. M. Alam, ICDDRB, had been shown to be positive for *V. cholerae* O1 on the basis of dark-field microscopy followed by routine bacteriology tests. Immediately after collection, the specimens were transported chilled to Göteborg and bacteria were prepared as described above for in vivo-grown bacteria within 24 h. Only specimens in which >80% of the bacteria were *V. cholerae* O1 were used.

Antisera and monoclonal antibodies (MAbs) against TCP. Adult New Zealand White rabbits were immunized with bacteria of classical strain JS1569 grown by the AKI method at 30°C; a combination of subcutaneous and intravenous injections was used (13). Sera were absorbed twice with boiled and twice with live serotype-homologous, TCP-negative bacteria. The rabbit serum that contained the highest titer of TCP antibodies after absorption was used (13).

MAbs against TCP were produced by immunizing BALB/c mice repeatedly with crude TCP prepared from strain JS1569 as previously described (2, 13). One MAb, Tc 20:2 (57 μg of immunoglobulin G1 per ml of culture medium), that reacted with TCP-positive bacteria in an enzyme-linked immunosorbent assay (ELISA; titer, 1:13,000) and with TcpA in both classical and El Tor vibrios in immunoblotting (but not with lipopolysaccharide- or TCP-negative bacteria), was used in this study (13). In some experiments, another MAb, Tc 21:1, that has been shown to react only with classical TcpA was used (13).

Quantitation of CT. The amount of CT produced in vivo was determined by GM1-ELISA (30) by using a MAb against the B subunit of CT and a purified CT (List Biological Laboratories, Campbell, Pa.) as a reference. Intestinal fluids obtained after centrifugation at 8,000 × *g* for 15 min and 4°C were tested.

Immunoblotting. Whole bacteria adjusted to 10^{10} vibrios ml^{-1} were boiled in sample buffer containing sodium dodecyl sulfate and 2-mercaptoethanol, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18) in 16% minigels with *N,N'*-dialyltartardiamide as a cross-linker (16), electrotransblotted to nitrocellulose paper, and developed essentially as previously described (16). Nitrocellulose sheets were incubated overnight with rabbit anti-TCP serum (dilution, 1:800) or an anti-TCP MAb (6 μg of immunoglobulin ml^{-1}) and then for 2 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G or anti-mouse immunoglobulin G as the secondary antibody (Jackson, West Grove, Pa.). H_2O_2 with 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, Calif.) was used as the substrate. Transblotted low-molecular-weight reference proteins (Bio-Rad) were stained for 5 min with amido black.

Inhibition ELISA for quantitation of TCP. Expression of TCP was determined by an inhibition ELISA method using TCP-positive vibrios (from strain JS1569) as the solid-phase antigen (13). The interpolated concentration of bacteria that caused inhibition of binding of specific anti-TCP antibody (rabbit anti-TCP serum or anti-TCP MAb) to the solid-phase-bound TCP by 50% was determined (13).

Electron microscopy (EM) and immunoelectron microscopy (IEM). Fresh *V. cholerae* bacteria grown either by the AKI method at 30°C (for 2.5 or 18 h) or in vivo (in rabbit intestines or in stools from cholera patients) and adjusted to ca. 5 × 10^{10} cells ml^{-1} in 1% bovine serum albumin (BSA)–PBS were applied to Formvar-coal-coated nickel grids for 2 to 4 min. After washing, the grids were negatively stained with 1% ammonium molybdate (pH 7). Vibrio-coated grids were also incubated on drops with anti-TCP serum or anti-TCP MAb Tc 20:2 (diluted 1:50 and 1:2 in BSA-PBS-Tween, respectively) for 15 min and, after washing, on drops of gold-protein A or gold–anti-mouse immunoglobulin G (Amersham International, Amersham, United Kingdom). Preimmune serum or a MAb against *Escherichia coli* heat-stable toxin was used as a negative control. After being washed in BSA-PBS-Tween and then H_2O, the grids were negatively stained with 1% ammonium molybdate (pH 7) for 35 to 60 s and examined in an electron microscope (JEOL Ltd., Tokyo, Japan).

RESULTS

Production of TCP in vivo and in vitro. Bacteria collected without subculturing from cholera-infected rabbit ileal loops

were used to evaluate the extent to which TCP is produced by classical and El Tor vibrios, respectively, during growth in the intestine. Bacteria grown in vitro under optimal TCP expression conditions for both biotypes, i.e., by the AKI-SW method at 30°C (13, 27), were used for comparison. The loops were inoculated with *V. cholerae* bacteria that were cultured in Trypticase soy broth without glucose at 37°C and were negative for TcpA. After growth for 16 to 18 h, bacteria had multiplied at least 300-fold in the intestines and all but one of the strains, X19128, caused significant fluid accumulation in the ligated loops (0.60 to 1.85 ml cm^{-1}). The concentration of bacteria in the intestinal fluid was approximately the same as that attained in the AKI cultures (4×10^9 to 7×10^9 cells ml^{-1}). Expression of TCP was determined by using an inhibition ELISA, and production of TcpA was evaluated by immunoblotting with a MAb or a polyclonal antiserum against TCP.

All of the 20 strains tested were shown to produce TcpA in vivo when anti-TCP serum was used in immunoblot analyses. Use of MAb Tc 20:2, on the other hand, developed TcpA in all classical but only four of nine in vivo-grown El Tor strains (data not shown). In vitro-grown El Tor strains more readily reacted with the MAb (seven of nine strains). In support of the surface expression of TCP antigen on most classical but practically no El Tor vibrios grown in vitro (13), 10 of 11 classical strains grown in vivo and only 1 of 9 El Tor strains were able to inhibit the binding of MAb Tc 20:2 to solid-phase-bound TCP-positive vibrios in an inhibition ELISA. Polyclonal anti-TCP serum was not better than MAb Tc 20:2 in detecting TCP in either of the two biotypes (data not shown).

The quantities of TcpA and TCP produced by different strains in vivo and in vitro were compared. In classical strains, the levels of TcpA were comparable after growth in vivo and in vitro, whereas there was a tendency toward higher levels of surface TCP in vivo as determined by inhibition ELISA (Fig. 1 and Table 1). For most El Tor strains, there was no marked difference in TcpA production in vivo and in vitro, as exemplified in Fig. 1, although there was a tendency toward lower TcpA production in vivo, and for some strains the difference was clear-cut (Table 1). With one exception, TCP antigen was not detected on the surface of El Tor vibrios (Table 1). This difference between classical and El Tor strains was seen even though the fluids from the infected loops contained high levels of CT for both biotypes.

The amounts of TcpA-TCP observed in bacteria derived from the intestines of rabbits infected in the nonligated RITARD model were similar to those found in the corresponding strains collected from ligated intestinal loops (data not shown). Bacteria adhering to the intestinal mucosa of loops infected with *V. cholerae* of either biotype for 7 or 17 h did not express higher quantities of TcpA or TCP than did vibrios collected from the luminal fluid. Luminal vibrios did not express more TcpA when harvested after 7 h than when harvested after 18 h. In vivo-grown vibrios of both biotypes were also tested for reactivity with MAb Tc 21:1, which has previously been found to be specific for classical TcpA (13). The result was similar to that obtained with in vitro-grown vibrios: only classical strains produced TcpA containing this epitope.

Differential expression of TCP on the bacterial surface of classical and El Tor strains. Two classical strains, JS1569 (which is derived from 569B) and X28214, which produced high and medium levels of TCP, respectively, and three El Tor strains, Phil 6973, N16961, and E7946, which were all TcpA positive by immunoblotting, were analyzed with EM and IEM for expression of TCP fimbriae after growth in vivo and in vitro. Strain JS1569, grown by the AKI method, produced a large number of TCP fimbriae that reacted strongly with the polyclonal antiserum (Fig. 2A) and did not react with the corresponding preimmune serum (Fig. 2B). TCP was also detected by IEM on in vitro-grown X28214 bacteria. When the TCP-positive bacteria were probed with MAb Tc 20:2, large numbers of gold particles were seen in short rows in the vicinity of the fimbriae, suggesting shedding of TCP epitopes; no such labeling was seen when a MAb against an unrelated antigen, *E. coli* heat-stable toxin, was used. No TCP fimbriae were detected by EM or IEM on AKI-grown El Tor bacteria when either anti-TCP serum or two antisera from rabbits immunized with TcpA-containing El Tor vibrios were used (data not shown).

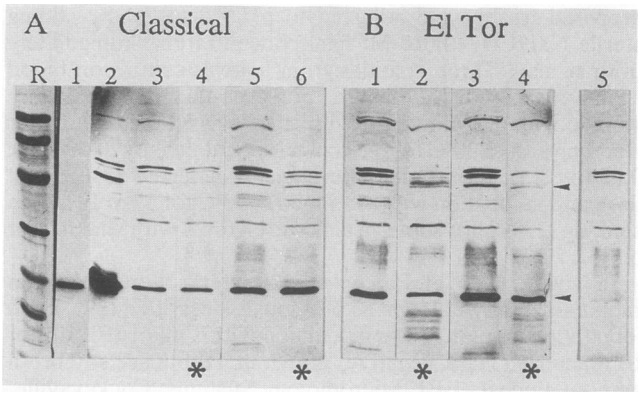


FIG. 1. Immunoblot analysis showing TcpA production in vitro and in vivo by classical and El Tor *V. cholerae*. Lane R, low-molecular-weight references (from the top): 95,000, 67,000, 45,000, 31,000, 21,500, and 14,400. (A) Classical strains: lanes 1 and 2, JS1569; lanes 3 and 4, VM69716; lanes 5 and 6, T19766. (B) El Tor strains: lanes 1 and 2, C5; lanes 3 and 4, X25049. Lanes without an asterisk contained vibrios grown by the AKI method at 30°C, and lanes with an asterisk contained bacteria harvested from rabbit ileal loops. Lane 5 in panel B contained X25049 bacteria grown in CFB at 27°C and was a negative control. Comparable amounts of bacteria were applied for each strain. TcpA was detected by using polyclonal anti-TCP serum, except for lane 1, where anti-TCP MAb Tc 20:2 was used. Arrowheads indicate the positions of TcpA (lower) and an ~50-kDa protein (upper) that is coexpressed with TcpA (13).

Classical vibrios (strain X28214) prepared from rabbit ileal loops 17 h after inoculation were found to react with both anti-TCP serum and MAb Tc 20:2 when examined by IEM. Distinct fimbriae in typical bundles were strongly labeled by polyclonal anti-TCP serum (Fig. 2C), whereas MAb Tc 20:2 bound to the cells or to material that appeared to be shed from TCP fimbriae (data not shown). No gold labeling was observed when preimmune serum or anti-*E. coli* heat-stable toxin MAb was used. To further exclude the possibility of nonspecific staining by MAb Tc 20:2 or the gold conjugate used, strain X28214, with a deleted *toxR* gene (and TcpA negative), was tested in IEM after growth in rabbit intestines; these bacteria showed no reaction with either anti-TCP serum or MAb Tc 20:2 (data not shown). In no instance were TCP fimbriae detected by EM on the El Tor strains grown in vivo, and only few gold particles were found on these bacteria when they were examined by IEM by using either of the anti-TCP preparations (data not shown).

To determine whether TCP fimbriae are expressed on the surface of El Tor bacteria in an earlier growth phase, expression of TcpA and TCP by El Tor strains N16961 and

TABLE 1. Production of TcpA and TCP by *V. cholerae* O1 grown[a] in vivo and in vitro

Strain (serotype)[b]	In vivo		In vitro	
	TcpA[c]	TCP[d]	TcpA	TCP
Classical				
395 (O)	+++	23	+++	5
569B (I)	++++	30	++++	30
Cairo 48 (I)	++	4	++	2
Cairo 50 (O)	(+)	<1	−	<1
C21 (O)	+++	8	+++	10
T19766 (O)	+++	3	++(+)	<1
X28214 (I)	+++	6	+++	2
X23332 (I)	+++	2	+++	1
VM11647 (O)	+++	12	+++	4
VM47760 (O)	++	3	++	<1
VM69716 (O)	++	1	++	<1
El Tor				
Phil6973 (I)	+	<1	+++	1
N16961 (I)	+	<1	+++	1
E7946 (O)	+	<1	++	<1
C5 (O)	++	<1	+++	<1
T19479 (I)	+	<1	+	<1
X19128 (O)	(+)	<1	(+)	<1
X24761 (I)	++	<1	+++	<1
X25049 (O)	++	<1	+++	<1
VM12260 (O)	+++	1	+++	<1

[a] Bacteria were prepared from rabbit ligated ileal loops or grown at 30°C by the AKI method, as described in the text, and adjusted to 10^{10} bacteria ml^{-1}.

[b] O, Ogawa; I, Inaba.

[c] TcpA was detected in immunoblot analyses by using polyclonal anti-TCP serum and graded subjectively as follows: −, negative; (+), very weak; +, weak; ++, intermediate; +++, strong; ++++, very strong.

[d] TCP was detected by an inhibition ELISA using MAb Tc 20:2. Inhibitory titers are expressed as the reciprocal values of bacterial dilutions that caused 50% inhibition of binding of the MAb to solid-phase-bound, TCP-positive vibrios.

X25049 was compared with that by classical strain X28214 after growth by the AKI method with shaking for 2.5 h (instead of for 17 to 19 h). Although all three strains produced CT and TcpA in large amounts, only the classical strain was TCP positive in an inhibition ELISA and expressed typical TCP fimbriae detected by EM and IEM.

Bacteria from cholera stools. Bacteria prepared from five cholera stool samples, four with El Tor Ogawa and one with El Tor Inaba vibrios, were examined by immunoblotting and IEM for expression of TcpA and TCP fimbriae, respectively. Only one of the stools contained TcpA-positive vibrios as determined by use of polyclonal anti-TCP serum. Aggregated TCP-like fimbriae were found on some cells from the TcpA-positive specimen (Fig. 3A), but these fimbriae were not stained to any significant extent by polyclonal anti-TCP serum (Fig. 3B).

DISCUSSION

In previous studies, we have found that although TcpA, the structural subunit of TCP, is produced by *V. cholerae* O1 bacteria of both the classical and El Tor biotypes, only classical strains seem to express significant amounts of assembled TCP antigen-fimbriae on the bacterial surface. We have now investigated the extent to which TCP and TcpA are expressed by *V. cholerae* O1 in the intestine during cholera infection and, specifically, whether the in vivo growth conditions would enhance TCP expression on the surface of El Tor vibrios.

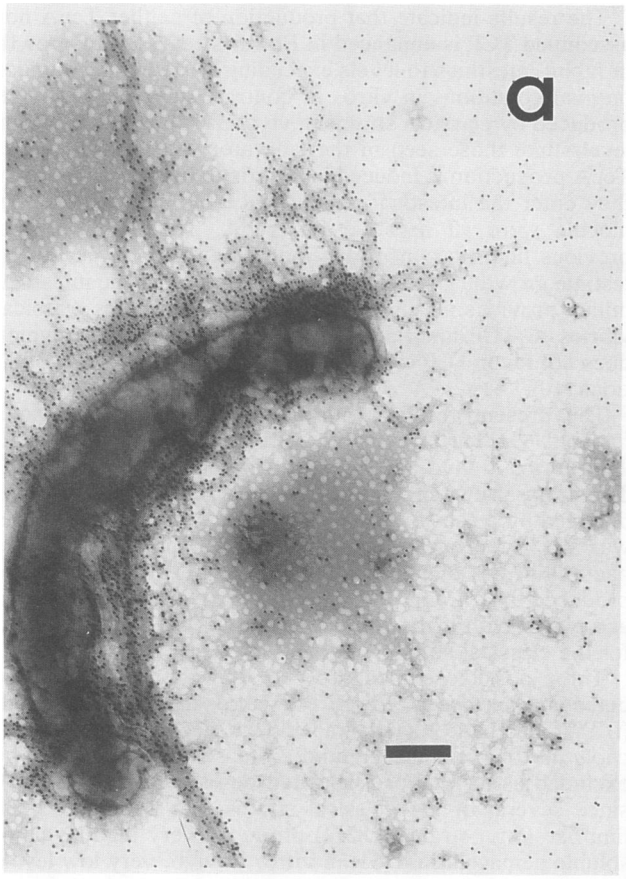
The results indicate that production of neither TcpA nor assembled TCP is enhanced in El Tor strains during growth in rabbit intestines to levels exceeding those obtained under optimal conditions in vitro. In contrast, the levels of TCP produced by classical strains in vivo may reach even higher levels than those seen in the laboratory. It is evident that TcpA production is induced in vibrios of both biotypes after they enter the intestinal milieu, since the bacteria used as inocula were all negative for TcpA. This confirms our previous findings concerning induction of TcpA during intestinal growth (17). We have shown here that the intestinal milieu provides an environment which stimulates classical vibrios to TCP fimbria production at a temperature which does not favor TCP expression under in vitro growth conditions (10).

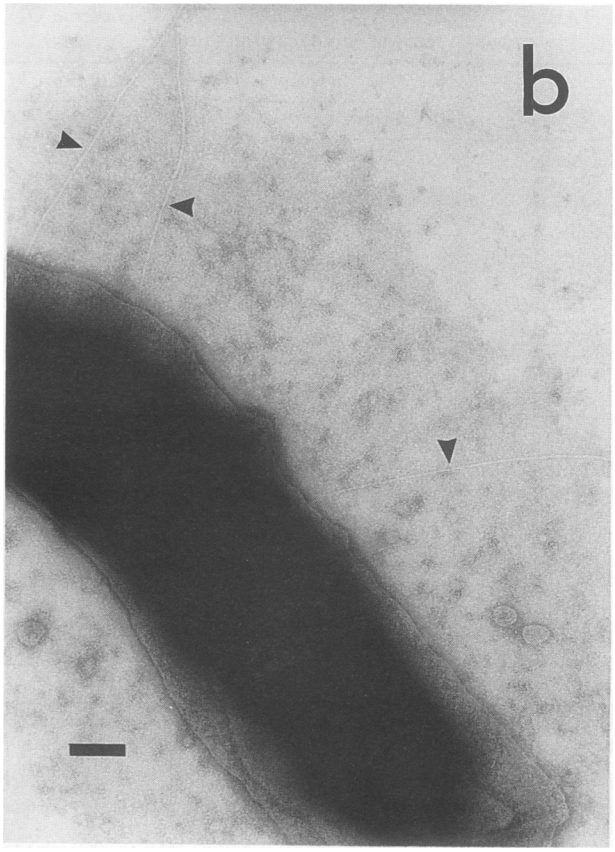
The present results confirm and extend our previous findings (13) that TCP fimbriae are poorly expressed on El Tor vibrios in vivo as well as in vitro. Only one of nine El Tor strains grown in vivo had TCP antigen on the surface as detected by a sensitive and specific inhibition ELISA. This was supported by the absence of TCP fimbriae on El Tor bacteria in EM and IEM. Although high levels of CT were produced by strains of both biotypes in vivo, this was associated with high-level expression of TCP-TcpA in vivo for the classical strains only.

Three possible explanations for the poor expression of detectable surface TCP in El Tor vibrios may be considered. (i) TCP may have been degraded by proteolytic enzymes. *V. cholerae* soluble hemagglutinin-protease (7) can probably be excluded as an El Tor-associated degrading factor (5, 17), since several of the classical strains that expressed TCP fimbriae (e.g., strain X28214) produced very high levels of soluble hemagglutinin (31) in vitro. In vivo, very low levels of soluble hemagglutinin were produced by both biotypes; ≤23 ng ml^{-1} in intestinal fluids. Other proteases may be responsible for such degradation of TCP, but none have been identified. We also examined vibrios grown by the AKI-SW technique only to the exponential growth phase, partly to try to avoid possible degradation of TCP by proteases. Although strains of both biotypes produced large amounts of TcpA, only the classical strain was positive for TCP in an inhibition ELISA and expressed TCP fimbriae detected in EM and IEM.

(ii) The regulatory system that controls expression of TcpA and TCP in El Tor strains may differ from that described for classical strains (4). ToxR expression is reduced at body temperature compared with that at 30°C. This may partly explain the observed down regulation of OmpU and, in El Tor strains, the tendency of reduced levels of TcpA in vivo, since both proteins are positively regulated by ToxR (22, 33, 34). However, none of the classical strains tested produced less TcpA during growth in vivo compared with growth at 30°C under conditions that promote ToxR expression in vitro. Because classical and El Tor strains (also the strains scored only as weakly positive in Table 1) produced similar, high levels of CT in vivo, suggesting good expression of ToxR in both biotypes under in vivo conditions, other regulatory proteins in addition to ToxR (and ToxT) may be involved in TcpA expression in El Tor strains (4, 21). In addition, El Tor strains may lack proteins-factors needed for assembly and/or membrane translocation of TcpA (33).

(iii) Detection of El Tor TCP may be hampered because of lack of appropriate antiserum. Owing to difficulties in producing anti-El Tor TCP antibodies, despite repeated immunizations using different TcpA-containing El Tor strains,





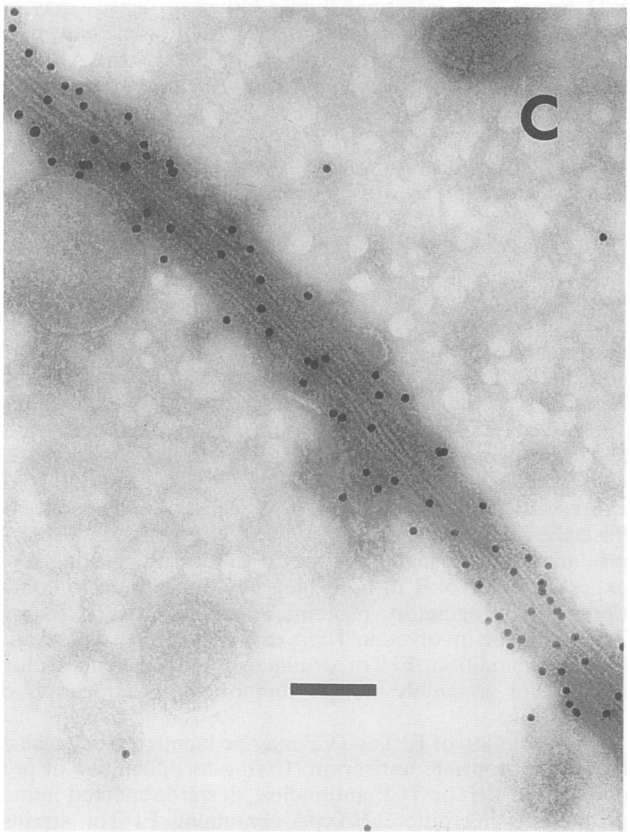


FIG. 2. Electron micrographs showing TCP fimbriae expressed by classical *V. cholerae* O1 and immunogold labeled by using anti-TCP serum and gold-protein A after growth in vitro by the AKI method at 30°C or in vivo in a rabbit ileal loop. Panels: A, strain JS1569 grown in vitro and probed with anti-TCP serum; B, JS1569 probed with preimmune serum (arrowheads indicate pili); C, TCP bundle from strain X28214 grown in vivo and probed with anti-TCP serum. Bars: A, 200 nm; B, 100 nm; C, 100 nm.

only expression of biotype-cross-reactive TCP epitopes could be examined. The lack of an immune response against homologous El Tor TcpA supports the idea that TCP is poorly expressed on the surface of El Tor bacteria. The lower degree of protection by antibodies to classical TCP against El Tor vibrios than against classical vibrios observed by Sun et al. (27, 28) was suggested to be due to differences in the pilin sequence or in TCP expression by the two biotypes (28). Although we have found epitope differences between TcpA in classical and El Tor vibrios (13), our results support mainly the latter hypothesis by showing poor expression of surface TCP on El Tor vibrios. The absence of TCP fimbriae also in EM preparations of El Tor vibrios containing large amounts of TcpA suggests that the inability to detect El Tor TCP is not due to the use of biotype-heterologous antibodies.

The lack of detectable surface TCP on most El Tor vibrios despite substantial TcpA production is a reverse analogy to the essential absence of El Tor-associated mannose-sensitive hemagglutinin pili on classical strains, although the mannose-sensitive hemagglutinin pilin protein is found intracellularly in bacteria of both biotypes (14). These data may reflect differences in accessory proteins in the two biotypes. In addition, the differences in TcpA epitopes per se (with at

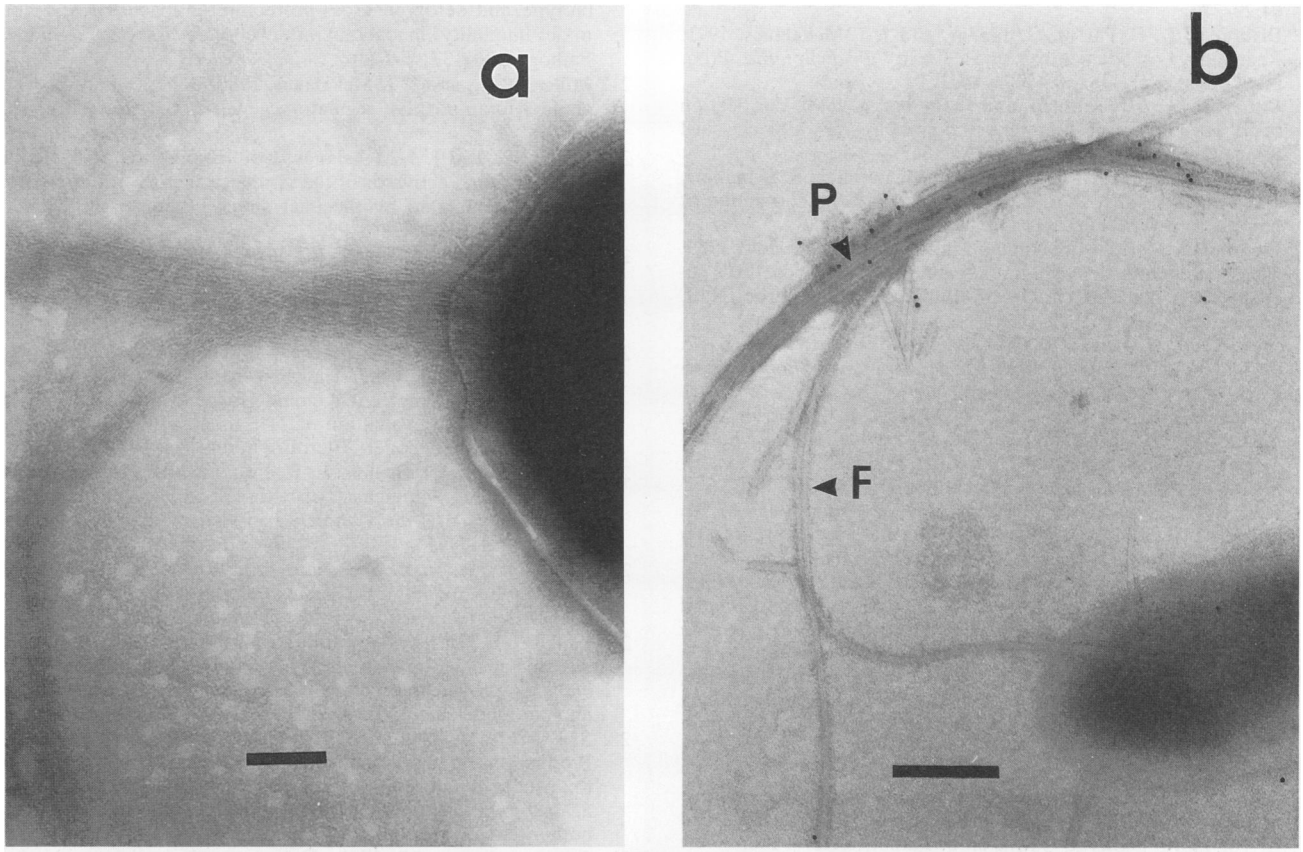


FIG. 3. Electron micrographs showing TCP-like bundles expressed by bacteria isolated from human cholera stool containing El Tor vibrios after negative staining (A) and after incubation with anti-TCP serum and probing with gold-protein A (B). A pilus bundle (P) and flagella (F) are indicated. Bars: A, 100 nm; B, 200 nm.

least one epitope in the C-terminal portion of classical TcpA lacking in El Tor TcpA [13]) may reflect structural differences in the TcpA pilin protein. It is possible that an altered C-terminal part of the pilin protein is a reason for the apparent inability of most of El Tor TcpA to form fimbriae.

Studies with human volunteers have shown that a TCP-positive classical-biotype strain colonized considerably better than a TCP-negative isogenic mutant (11). At the same time, several reports have described poor immune responses against TCP both in serum and in the intestine after experimental and clinical cholera, as well as in rabbits after RITARD infection (9, 12a, 19). This suggests that TCP fimbriae are expressed in small quantities or for a limited period during infection.

The finding that TCP fimbriae were detected on classical *V. cholerae* bacteria grown in rabbit intestines supports the notion that the rabbit model is useful for studying expression of *V. cholerae* surface antigens in vivo. However, it is noteworthy that TcpA was found in only one of five human cholera (El Tor) stool samples examined and also that some bacteria in this specimen had fimbriae which morphologically resembled TCP although they were not stained by anti-TCP serum. However, even though the stool specimens were transported chilled, the 24-h period from collection to preparation of the vibrios for analyses could have had an effect on bacterial surface antigens. Further studies of fresh vibrios from human cholera stool or jejunal aspirates are therefore needed to determine conclusively the expression of TCP on vibrios isolated from human intestines.

In conclusion, this study shows that production of TcpA is induced in both biotypes during experimental infection in rabbit intestines, while TCP surface antigen and TCP fimbriae are expressed mainly or exclusively on vibrios of the classical biotype.

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