INFECTION AND IMMUNITY, July 1991, p. 2508–2512
0019-9567/91/072508-05$02.00/0

Vol. 59, No. 7

Immunogenicity of *Vibrio cholerae* O1 Toxin-Coregulated Pili in Experimental and Clinical Cholera

ROBERT H. HALL,[1]† GENEVIEVE LOSONSKY,[1] ADRIANA P. D. SILVEIRA,[1] RONALD K. TAYLOR,[2] JOHN J. MEKALANOS,[3] NANCY D. WITHAM,[4] AND MYRON M. LEVINE[1]

Center for Vaccine Development, Division of Geographic Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201,[1] Department of Microbiology and Immunology, University of Tennessee, Memphis, Tennessee 38163,[2] Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115,[3] and Division of Tropical Medicine, U.S. Naval Medical Research Unit-2, Jakarta, Indonesia[4]

Received 12 December 1990/Accepted 15 April 1991

A functional *tcpA* gene, encoding the major subunit of toxin-coregulated pili (TCP), is necessary for *Vibrio cholerae* O1 Ogawa strain 395 to colonize the human intestine and confer protective immunity to virulent challenge. The immunogenicity of TCP and other antigens in experimental and naturally acquired cholera was determined. Seroconversion to cholera toxin (CT), whole cell preparations, and to Ogawa lipopolysaccharide but not to purified native TCP or to a TcpA mimiotope was found in volunteers. Local intestinal secretory immunoglobulin A from volunteers showed conversions to cholera toxin and lipopolysaccharide but not to TCP. Cholera patients in Indonesia showed a seroconversion rate to TCP of 3 of 6 and a seroconversion to a TcpA mimiotope of 1 of 6. Volunteer and patient sera showed similar vibriocidal seroconversions when assayed against either TCP-positive and TCP-negative *V. cholerae* O1 strains, suggesting that TCP do not contribute demonstrably to the vibriocidal antigen. We conclude that although seroconversion to TCP can occur in naturally acquired cholera, solid long-term protection can be engendered in the absence of a detectable anti-TCP immune response.

Results of experimental challenge studies in volunteers (3, 13, 19) and epidemiological studies in endemic areas (5) show that clinical cholera can be a highly immunizing infection. North American volunteers who ingested classical biotype *Vibrio cholerae* O1 and developed cholera diarrhea were all protected when rechallenged 1 to 2 months later with classical strains of either Ogawa or Inaba serotype (13, 19). Efforts to identify the key protective antibodies in anti-cholera immunity have focused on antitoxin, antibacterial, and vibriocidal antigens. Although cholera infection produces high antitoxin seroconversion rates, significant protection against cholera can be engendered without antitoxin immunity by toxin-negative cholera mutants (10, 16, 17) and by orally or parenterally administered killed *V. cholerae* O1 vaccines (2, 11). The titer of vibriocidal antibody in serum has been the immunological measurement that most closely correlates with protection, either natural or vaccine derived (15, 22, 23). Although most vibriocidal antibodies are directed against lipopolysaccharide (LPS) antigens (15), a small proportion of vibriocidal antibodies is directed against undefined protein antigens (1, 15, 24). The discovery of a toxin-coregulated pilus (TCP) colonization factor in *V. cholerae* O1 (31) with a key role in pathogenesis (8, 31) suggested that TCP may be such a vibriocidal antigen. To study the role of TCP in cholera immunity, we investigated the immunogenicity of TCP and other antigens in experimentally and naturally acquired cholera.

Preparation of TCP, LPS, and CT antigens. TCP were purified from *V. cholerae* 395 (classical biotype, Ogawa serotype) as described elsewhere (5a). Essentially, cultures were grown in colonization factor broth at 25°C with shaking, harvested, and sheared by passage through an 18-gauge needle. TCP were isolated by differential centrifugation and salt precipitation without denaturation in order to ensure that they possessed the native array of conformational epitopes. Transmission electron microscopy (6) verified that TCP preparations consisted of intact filaments. Contamination by LPS was detected by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), periodic acid oxidation, and silver staining (7). Ogawa LPS was isolated from *V. cholerae* strain 395 as described previously (32) and visualized by SDS-PAGE, oxidation, and silver staining. Protein contamination of TCP and LPS preparations was detected by SDS-PAGE (7). Cholera toxin (CT) was purchased from List Biologicals, Campbell, Calif.

Preparation of synthetic putative TcpA peptide epitopes. The antigenicity prediction algorithm of Hopp and Woods (9) was used to select the regions of greatest hydrophilicity within the primary sequence of TcpA. These peptides have been reported to possess protective efficacy in the infant mouse model and have been proposed as components in an improved killed vaccine formulation (4, 26, 28). Only peptides which reacted with positive control rabbit anti-TCP antisera were used in the assay of volunteer and Indonesian sera.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was used to detect immunoglobulin G (IgG) and IgA antibodies in serum and jejunal fluids directed against CT, LPS, and whole vibrios (17, 20, 30). The coating condition for TCP antigen was 0.5 μg/ml in carbonate buffer (pH 9.6), determined from preliminary experiments using three standard positive antisera: anti-TCP (30a), anti-TcpA (20a), and anti-395 rabbit antiserum (6). Coating conditions for the other antigens were as described previously (17, 20, 30).

Volunteer serum and jejunal fluid. Published protocols

* Corresponding author.
† Present address: Virulence Assessment Branch, Division of Microbiology, Food and Drug Administration, HFF 236, 200 C Street SW, Washington, DC 20204.

TABLE 1. Serum and jejunal antibody responses[a] in volunteers after challenge with *V. cholerae* O1 classical Ogawa 395 challenge

Antigen	Serum responses[b]			Mucosal responses[b]	
	Response rate (%)	Peak GMT[c]		Response rate (%)	GMT[d] IgA
		IgG	IgA		
CT	15/15 (100)	1.19	—	3/9 (33)	8[e]
Ogawa LPS	6/15 (40)	400	95.5	2/9 (22)	7.4
WC	10/15 (66.7)	381.9	209.5	0/9	2
TCP	0/15	12.5	12.5	0/10	2
Peptide no. 7	0/13	25	33	—	—
Vibriocidal activity vs Ogawa 395	15/15 (100)	8127.55[f]	8127.55[f]	—	—

[a] The differences in response rates between TCP and CT, LPS, and WC were highly significant ($P < 0.001$ versus CT, $P < 0.016$ versus LPS, and $P < 0.001$ versus WC). —, not determined.

[b] Expressed as the number positive per number tested. A positive antibody response was considered to be a fourfold rise in titer from prechallenge to postchallenge antibody for all assays except for the IgG CT assay. A positive CT antibody response was a change in optical density units of 0.15 from pre- to postchallenge specimens.

[c] GMT for all assays except CT. Seroconversions include all IgG or IgA responses.

[d] GMT obtained from jejunal fluids drawn 7 days postinfection.

[e] CT result expressed in geometric mean absorbance units.

[f] Immunoglobulin class not discriminated in this assay.

were used to recruit, to screen medically, to obtain informed consent from, and to provide clinical supervision of volunteers (12, 13). The protocols were reviewed by the Human Volunteer Research Committee of the University of Maryland at Baltimore and the Clinical Research Subpanel of the National Institute of Allergy and Infectious Diseases. Challenge with virulent *V. cholerae* classical Ogawa strain 395 was carried out under quarantine as described elsewhere (12). Sera were collected from all volunteers before and after ingestion of pathogenic *V. cholerae* O1 strains. Jejunal fluid was obtained by tube from most volunteers on the day before challenge with *V. cholerae* 395 (prechallenge sample) and 7 days postchallenge. From two volunteers, a third specimen each was collected 28 days after challenge. Clinical and immunological observations of the immunizing infection and rechallenge of this cohort of volunteers have been previously described in detail (13, 17, 19). Sera and jejunal fluids obtained from volunteers before and after experimental cholera challenge allowed us to determine the relative immunogenicity of several *V. cholerae* macromolecules during a single, demonstrably protective immunizing infection.

The fluids were stored lyophilized and prior to ELISA were reconstituted to a secretory IgA concentration of 20 mg/100 ml of buffer. Sera and jejunal fluids were diluted by sequential twofold steps and assayed as previously described. End-point absorbance values were determined by using 30 negative control sera and 15 jejunal fluids obtained from subjects participating in previous noncholera studies at the Center for Vaccine Development. In all titrations, fourfold or greater rises in antibody titer from prechallenge to postchallenge specimens were considered significant.

Patient sera. Acute and convalescent (+14 days) sera were obtained from six patients diagnosed with clinical cholera in Indonesia. In all cases, the *V. cholerae* strain isolated was of O1 serogroup, El Tor biotype, and Ogawa serotype. Acute and convalescent sera were assayed for vibriocidal activity, and by ELISA against CT, whole inactivated bacterial cells (WC), TCP, and Ogawa LPS.

Immune responses of North Americans. Sera and jejunal fluids from volunteers challenged with *V. cholerae* 395 were assayed against CT, WC, Ogawa LPS, and TCP in parallel. Peptides corresponding to epitopes of TcpA were also assayed for immunological recognition in volunteer sera. The data in Table 1 show the response rate and peak geometric mean titer (GMT) for IgG and IgA. The anti-CT seroconversion rate was 100%, the anti-WC rate was 66.7%, and anti-LPS rate was 40%. In contrast, none of the 15 volunteers demonstrated rises in serum antibody to purified native TCP. The difference in immunogenicity between TCP and WC and between TCP and CT was highly significant ($P = 0.0002$ versus WC; $P = 0.00000001$ versus CT; $P = 0.016$ versus LPS). Regrettably, the time points of jejunal fluid collection postchallenge from most volunteers (day 7) were different from the time points used previously in studies on enterotoxigenic *Escherichia coli* fimbriae (18) and probably too early to detect optimally a local secretory IgA response (14). Nevertheless, although no response to inactivated vibrio WC or to TCP was detected, the anti-CT secretory IgA response rate was 33%, and the anti-LPS response rate was 22% (Table 1). No immune response to any peptides tested was detected, with the exception of the conformational epitope peptide no. 7 (Table 2). The sera and jejunal fluids studied were samples from persons with proven, 100% solid protective immunity, as evidenced by subsequent rechallenge of these volunteers 2 months later with virulent *V. cholerae* strain 395 (the homologous strain) or strain 569B (classical Inaba).

We determined whether an anti-TCP immune response followed an immunizing infection of experimental cholera. None of the North American volunteers tested showed an immune response to TCP. This was in contrast to rises in vibriocidal titer and rises in levels of antibody to Ogawa LPS, whole Ogawa 395 organisms, and toxin detected with the same sera (Table 1). Our data indicate that, in stark contrast to experimental enterotoxigenic *E. coli* infection (14, 18), a single-dose, experimental infection of North American volunteers with *V. cholerae* O1 strain 395 induces no detectable serological IgG or IgA response to the fimbrial colonization factor.

It is unclear why TCP are poor immunogens during experimental cholera infection. We assume that TCP are expressed in vivo because strains unable to express TCP do not colonize (8, 31), and furthermore, the disease state and the antitoxin immune response show that the B subunit of CT, a protein whose expression is intimately linked to that of TCP (31), is expressed.

Serological responses of Indonesian cholera patients. Acute

TABLE 2. Immune response to peptides derived from the TcpA sequence[a]

Peptide no.	Sequence	Residue no.	Indonesian sera (n = 6)	
			IgA	IgG
1	GKISSDEAKNPFIG	77–90	0	0
3	AQRAIDSQNMTKAAQSLNS	24–42	0	0
4	ADLGDFENSAAAAETGVGVIKSIA	145–168	0	0
5	AETGVGVIKSIAPASKNLDLTNITHV	157–182	0	0
6	LDLTNITHVEKLCKGTAPFGVAFGNS	174–199	0	0
7	DGLTQAQCKTLITHVEKLCKGTAPFG	113–125[b] 181–193[b]	0	1 (16.7%)

[a] The description, derivation, and protective efficacies of these peptides are discussed elsewhere (28). No immune response was detected with IgG or IgA in sera from volunteers (n = 12).
[b] Mimiotope derived from cysteine loop identified by Sun et al. (28).

and convalescent (+14 days) sera were obtained from six patients diagnosed with naturally acquired clinical cholera in an endemic area of Indonesia. In all cases, the *V. cholerae* strain isolated was of O1 serogroup, El Tor biotype, and Ogawa serotype. The serological responses to CT, Ogawa LPS, TCP, and vibriocidal activity were determined and are presented in Table 3. Acute and convalescent sera were assayed by ELISA against CT, TCP, and Ogawa LPS. Of the paired acute and convalescent sera, samples from three of six patients exhibited a detectable albeit meager rise in anti-TCP titer from acute to convalescent phase (Table 3). The significance of these positive responses is difficult to interpret. Questions exist concerning the patients' infectious disease histories, the *V. cholerae* dose received, the pathogenesis and antigenic makeup of the strains ingested, and the times between infection, incubation, phlebotomy, and treatment. It is possible that multiple or high subsequent exposures to *V. cholerae* O1 in endemic areas may eventually induce an immune response to TCP which could conceivably be protective. However, if individuals in an endemic area received several exposures to cholera, one might expect elevated serum vibriocidal activity in the acute serum samples. These imponderables serve to emphasize the value of controlled clinical trials of well-characterized strains.

The indicator *V. cholerae* 395 derivatives 395N1 (*ctxA* mutant) (21) and TCP2 (*ctxA tcpA* mutant) (8, 21) were used in the vibriocidal assays after growth in CFB media (6) to induce expression of toxin-coregulated proteins (which include TcpA) (31). The vibriocidal activity of volunteer sera showed a 100% pre- to postchallenge conversion and a peak GMT of 8,128 (Table 1).

TABLE 3. Immune responses of Indonesian sera to cholera antigens

Activity	Seroconversion rate[a] (%)	GMT[b]	
		Acute	Convalescent
Vibriocidal			
Inaba	6/6 (100)	40	1,613
Ogawa	6/6 (100)	28	5,120
Anti-CT (IgG)	6/6 (100)	0.56	1.64
Anti-TCP			
IgG	1/6 (16.7)	6	7
IgA	3/6 (50)	16	25
Anti-peptide 7			
IgG	1/6 (16.7)	45	45
IgA	0/6	56	40

[a] Expressed as the number positive per total number tested.
[b] Acute samples were obtained on admission to hospital. Convalescent samples were obtained 14 days later.

The vibriocidal GMT of the acute sera to the homologous biotype (Ogawa) was 28, seroconverting to a GMT of 5,120 on convalescence. Seroconversion to vibriocidal activity against both indicator strains was found for six of six (100%) Indonesian patients (Table 3), indicating that although TcpA can be expressed, this protein constitutes a negligible component of the vibriocidal antigen array. With a heterologous biotype (Inaba) strain as the indicator organism, an acute GMT of 40 seroconverted to 1,613 on convalescence. All six Indonesian patients had serum vibriocidal activity during the acute phase of infection which was comparable to that of immunologically naive North American volunteers. The same three pairs showed the anti-TCP seroconversion and the greatest vibriocidal seroconversion. Possibly these individuals ingested the greatest dose of *V. cholerae*.

Responses to synthetic TcpA epitopes. Analysis of the *tcpA* gene sequence (4, 26) with the antigenicity algorithm of Hopp and Woods (9) revealed that TcpA possesses potential epitopes which compare favorably on the basis of hydrophobicity with colonization factor antigen I and coli surface antigen 3 of enterotoxigenic *E. coli*. Taylor et al. (30b) recently described a discontinuous epitope which was immunogenic and protective for mice. The seroconversion to peptide no. 7 mimicking this epitope (mimiotope) in one Indonesian sera pair holds out the hope that immunization with peptides may engender protective immunity (Table 2). Recent evidence suggests that in the infant mouse cholera model, passive immunization with anti-TcpA antibodies results in significant protection from infection (26, 27, 29, 30b).

TCP are immunogenic in rabbits when either purified pili or piliated vibrios are administered by intravenous or subcutaneous injection, and no difficulty in raising antisera reagents in our laboratories has been encountered (6). However, such immunization protocols are markedly different from natural infection. TCP is not the only virulence factor expressed by *V. cholerae* O1 which does not stimulate an immune response: the enzymatic A subunit of CT is also only minimally immunogenic.

Killed vaccines lacking TCP can confer significant protection. A recent field trial in Bangladesh of an oral vaccine consisting of three doses of inactivated *V. cholerae* O1 grown in conditions repressing TCP expression nevertheless conferred approximately 50% protection for at least 3 years (11). We can conclude that even if anti-TCP immunity is generated in natural infection by whatever means, TcpA is not the vibriocidal (and hence probably not the protective)

TABLE 4. Vibriocidal activity of sera from volunteers to *V. cholerae* 395N1 and to *tcpA* mutant strain TCP2[a]

Volunteer	395N1					TCP2				
	Pre	+10	+21	+28	+31	Pre	+10	+21	+28	+31
2002										
9	<20	10,240			2,560	<20	20,480			2,560
11	<20	10,240			NS	<20	10,240			NS
12	<20	640			320	<20	1,280			640
14	<20	1,280			<20	<20	1,280			<20
15	<20	<20			<20	<20	<20			<20
16	<20	5,120			2,560	20	10,240			10,240
2003										
4	<20	10,240	2,560	640		<20	5,120	2,560	1,280	
5	<20	640	160	160		<20	640	320	320	
6	<20	2,560	1,280	640		<20	2,560	1,280	640	
7	<20	1,280	320	20		<20	1,280	160	20	
11	<20	2,560	1,280	160		<20	2,560	1,280	160	
13	<20	10,240	2,560	1,280		<20	10,240	5,120	2,560	
18	80	640	<20	<20		80	1,280	<20	<20	
19	<20	2,560	2,560	640		<20	1,280	5,120	2,560	
21	<20	2,560	640	640		<20	1,280	640	1,280	

[a] Sera were drawn prechallenge (pre) and 10, 21, 28, and 31 days after challenge (+10, +2, +28, and +31, respectively). NS, no serum.

antigen in natural infection. It has been suggested that maximization of TCP expression by bacteria before they are inactivated may further enhance the protective efficacy conferred by such an oral vaccine (8, 27, 31). Despite the poor immune response to TCP in the course of experimental cholera, this does not preclude TCP or other coregulated structural proteins from being important, even critical, components of vaccines. The fact that TCP expression by live, orally administered, attenuated, nonenterotoxigenic *V. cholerae* O1 vaccine strains increases the vibriocidal antibody response (8) (presumably through some colonization mechanism) makes it important to ensure that TCP are present in live or killed orally administered vaccines.

We thank Mardi Reymann for conducting the ELISAs.

This study was funded by research contract N01 AI 62528 and research grants AI 25096 and AI 19716 from the National Institutes of Health.

REFERENCES

1. **Attridge, S. R., and D. Rowley.** 1983. Prophylactic significance of the nonlipopolysaccharide antigens of *Vibrio cholerae*. J. Infect. Dis. **148**:931–939.
2. **Black, R. E., M. M. Levine, M. L. Clements, C. R. Young, A.-M. Svennerholm, and J. Holmgren.** 1987. Protective efficacy in humans of killed whole-vibrio oral cholera vaccine with and without the B subunit of cholera toxin. Infect. Immun. **55**:1116–1120.
3. **Cash, R. A., S. I. Music, J. P. Libonati, J. P. Craig, N. F. Pierce, and R. B. Hornick.** 1974. Responses of man to infection with *Vibrio cholerae*. II. Protection from illness afforded by previous disease and vaccine. J. Infect. Dis. **130**:325–333.
4. **Faast, R., M. A. Ogierman, U. H. Stroeher, and P. A. Manning.** 1989. Nucleotide sequence of the structural gene, tcpA, for a major pilin subunit of *Vibrio cholerae*. Gene **85**:227–231.
5. **Glass, R. I., A.-M. Svennerholm, R. N. Khan, S. Huda, M. I. Huq, and J. Holmgren.** 1985. Seroepidemiological studies of El Tor cholera in Bangladesh: association of serum antibody levels with protection. J. Infect. Dis. **151**:236–242.

5a. **Hall, R. H., et al.** Submitted for publication.

6. **Hall, R. H., P. A. Vial, J. B. Kaper, J. J. Mekalanos, and M. M. Levine.** 1988. Morphological studies on fimbriae expressed by *Vibrio cholerae* O1. Microb. Pathog. **4**:257–265.
7. **Hames, B. D.** 1981. An introduction to polyacrylamide gel electrophoresis, p. 1–91. *In* B. D. Hames and D. Rickwood (ed.), Gel electrophoresis of proteins. IRL Press, Oxford.
8. **Herrington, D. A., R. H. Hall, G. A. Losonsky, J. J. Mekalanos, R. K. Taylor, and M. M. Levine.** 1988. Toxin, toxin co-regulated pili and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med. **168**:1487–1492.
9. **Hopp, T. P., and K. R. Woods.** 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA **78**:3824–3828.
10. **Kaper, J. B., H. Lockman, M. M. Baldini, and M. M. Levine.** 1984. Recombinant nontoxigenic *Vibrio cholerae* strains as attenuated cholera vaccine candidates. Nature (London) **308**: 655–658.
11. **Levine, M. M.** 1990. Vaccines against enteric infections. Lancet **335**:958–961.
12. **Levine, M. M., R. E. Black, M. L. Clements, C. Lanata, S. Sears, T. Honda, C. R. Young, and R. A. Finkelstein.** 1984. Evaluation in humans of attenuated *Vibrio cholerae* El Tor Ogawa strain Texas Star-SR as a live oral vaccine. Infect. Immun. **43**:515–522.
13. **Levine, M. M., R. E. Black, M. L. Clements, D. R. Nalin, L. Cisneros, and R. A. Finkelstein.** 1981. Volunteer studies in development of vaccines against cholera and enterotoxigenic *Escherichia coli*: a review, p. 443–459. *In* T. Holme, J. Holmgren, M. Merson, and R. Mollby (ed.), Acute enteric infections in children: new prospects for treatment and prevention. Elsevier/North Holland Publishing Co., Amsterdam.
14. **Levine, M. M., R. E. Black, M. L. Clements, C. R. Young, C. P. Cheney, P. Schad, and E. Boedeker.** 1984. Prevention of enterotoxigenic *Escherichia coli* diarrheal infection in man by vaccines that stimulate anti-adhesion (anti-pili) immunity, p. 223–244. *In* E. Boedeker (ed.), Attachment of organisms to the gut mucosa, volume II. CRC Press, Boca Raton, Fla.
15. **Levine, M. M., J. B. Kaper, R. E. Black, and M. L. Clements.** 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. **47**:510–550.
16. **Levine, M. M., J. B. Kaper, D. Herrington, J. Ketley, G. Losonsky, C. O. Tacket, B. Tall, and S. Cryz.** 1988. Safety, immunogenicity and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet **ii**:467–470.
17. **Levine, M. M., J. B. Kaper, D. Herrington, G. Losonsky, J. G. Morris, M. L. Clements, R. E. Black, B. Tall, and R. Hall.** 1988. Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect. Immun. **56**:161–167.
18. **Levine, M. M., J. G. Morris, G. Losonsky, E. Boedeker, and B. Rowe.** 1986. Fimbriae (pili) as vaccines, p. 143–145. *In* D. Lark (ed.), Protein-carbohydrate interactions in biological systems.

Academic Press, Inc., London.
19. **Levine, M. M., D. R. Nalin, J. P. Craig, D. Hoover, E. J. Bergquist, D. Waterman, H. P. Holley, R. B. Hornick, N. F. Pierce, and J. P. Libonati.** 1979. Immunity of cholera in man: relative role of antibacterial versus antitoxic immunity. Trans. R. Soc. Trop. Med. Hyg. **73**:3–9.
20. **Levine, M. M., C. R. Young, R. E. Black, Y. Takeda, and R. A. Finkelstein.** 1985. Enzyme-linked immunosorbent assay to measure antibodies to purified heat-labile enterotoxins from human and porcine strains of *Escherichia coli* and to cholera toxin: application in serodiagnosis and seroepidemiology. J. Clin. Microbiol. **21**:174–179.
20a. **Mekalanos, J. J.** Unpublished data.
21. **Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306**:551–557.
22. **Moseley, W. H.** 1969. The role of immunity in cholera. A review of epidemiological and serological studies. Tex. Rep. Biol. Med. **27**(Suppl. 1):227–244.
23. **Moseley, W. H., W. M. McCormack, A. Ahmed, A. K. M. E. Chowdray, and R. K. Barui.** 1969. Report of the 1966-67 cholera vaccine trial in rural East Pakistan. 2. Results of the serological surveys in the study population—the relationship of case rate to antibody titre and an estimate of the apparent infection rate with *Vibrio cholerae*. Bull. W. H. O. **40**:187–197.
24. **Sears, S. D., K. Richardson, C. Young, C. D. Parker, and M. M. Levine.** 1984. Evaluation of the human immune response to outer membrane proteins of *Vibrio cholerae*. Infect. Immun. **44**:439–444.
25. **Sharma, D. P., C. Thomas, R. H. Hall, M. M. Levine, and S. R. Attridge.** 1989. Significance of toxin-coregulated pili as protective antigens of *Vibrio cholerae* in the infant mouse model. Vaccine **7**:451–456.
26. **Shaw, C. E., and R. K. Taylor.** 1990. *Vibrio cholerae* O395 *tcpA* pilin gene sequence and comparison of predicted protein structural features to those of type 4 pilins. Infect. Immun. **58**:3042–3049.
27. **Sun, D., J. J. Mekalanos, and R. K. Taylor.** 1990. Antibodies directed against the toxin-coregulated pilus of *Vibrio cholerae* provide protection in the infant mouse experimental cholera model. J. Infect. Dis. **161**:1231–1236.
28. **Sun, D., J. M. Seyer, I. Kovari, R. A. Sumrada, and R. K. Taylor.** 1991. Localization of protective epitopes within the pilin subunit of the *Vibrio cholerae* toxin-coregulated pilus. Infect. Immun. **59**:114–118.
29. **Sun, D., D. M. Tillman, T. N. Marion, and R. K. Taylor.** 1990. Production and characterization of monoclonal antibodies to the toxin coregulated pilus (TCP) of *Vibrio cholerae* that protect against experimental cholera in infant mice. Serodiag. Immunother. Infect. Dis. **4**:73–81.
30. **Tacket, C. O., B. Forrest, R. Morona, S. R. Attridge, J. LaBrooy, B. D. Tall, M. Reymann, D. Rowley, and M. M. Levine.** 1990. Safety, immunogenicity, and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* Ty21a. Infect. Immun. **58**:1620–1627.
30a. **Taylor, R. K.** Unpublished data.
30b. **Taylor, R. K., et al.** Submitted for publication.
31. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.
32. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure, p. 83–91. *In* R. L. Whistler and M. L. Wolfram (ed.), Methods in carbohydrate chemistry, volume 5. Academic Press, Inc., New York.