CURRENT MICROBIOLOGY Vol. 27 (1993), pp. 329–333

Current Microbiology

Potential Pathogenic Factors Produced by a Clinical Nontoxigenic *Vibrio cholerae* O1

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Abstract. A clinical isolate of nontoxigenic *Vibrio cholerae* O1 that caused intestinal fluid accumulation (FA) in adult mice produced proteolytic, hemolytic, and cytotoxic activities in in vitro assays. The linkage of these secreted factors to the FA activity was studied by transposon (Tn*phoA*) mutagenesis. Ten of the 12 Tn*phoA* insertion mutants that were defective for proteolytic activity produced FA, hemolytic and cytotoxic activities; the remaining two mutants lost these latter three activities. These results indicate that FA activity is independent of proteolytic activity but closely associated with cytotoxic and hemolytic activities. Our results with the adult mouse model and a nontoxigenic *V. cholerae* O1 are in general agreement with previous studies that demonstrated linkage of cytotoxin and hemolysin of toxigenic *V. cholerae* O1 and non-O1 with FA activity in rabbit ileal loops.

Vibrio cholerae serogroup O1 causes a severe diarrheal illness in humans by colonizing the small intestine and secreting cholera toxin (CT) [14]. However, attenuated *V. cholerae* O1 strains with a deletion of the genes encoding CT (*ctx*AB) caused a mild to moderate diarrhea in human volunteers [11]. This finding led to the hypothesis that *V. cholerae* O1 produces other factor(s) that stimulate secretory activity in intestinal epithelial cells [3]. Takeda et al. [24] detected heat-stable (ST) enterotoxin in a CT gene-positive strain of *V. cholerae* O1. Fasano et al. [3] reported the identification of a new toxin, ZOT (zonula occludens toxin), produced by both CT-producing and *ctx*A deletion mutants of *V. cholerae* O1 strain 395. Baudry et al. [1] cloned the *zot* gene, which is located immediately upstream of the *ctx* genes on the toxigenic *V. cholerae* chromosome, but the *zot* gene sequences could not be detected in a naturally occurring nontoxigenic (non-CT) *V. cholerae* O1 strain 1196-78. Taylor et al. [26] observed that an essential colonization factor for *V. cholerae* in animals, the cholera toxin co-regulated pilus (Tcp), is present in all toxigenic clinical isolates but absent in most environmental and non-CT isolates of *V. cholerae*.

Although lacking the *ctx* genes, some naturally occurring non-CT *V. cholerae* O1 strains have been associated with diarrheal diseases and other infections of humans [6, 9, 17]. The virulence properties of these strains are unknown, and their public health importance remains uncertain. We have recently observed that certain clinical and environmental isolates of non-CT *V. cholerae* O1 that did not hybridize CT, *Escherichia coli* heat-labile, heat-stable, and shiga-like toxins gene-probes produced positive intestinal fluid accumulation (FA) activity in adult mouse; the FA was comparable to that produced by a known toxigenic strain, 569B [18]. Some of these non-CT O1 mouse-enteropathogenic strains also produced proteolytic and hemolytic activities on plate assays (unpublished data) and cytotoxic activities on tissue culture cells.

The transposon, Tn*phoA*, mutagenesis has been applied to identify virulence determinants in *V. cholerae* and other intestinal pathogens [4, 8, 16, 19, 27]. Tn*phoA* creates fusions between target genes and the *E. coli* gene for alkaline phosphatase. Hybrid proteins encoded by such gene fusions exhibit little or no PhoA activity unless the target gene product is a secreted or membrane-spanning protein [13]. Because exported proteins represent the most frequent classes of proteins recognized to be involved in bacterial pathogenesis, the use of Tn*phoA* provides a strong enrichment for insertion mutations in virulence genes [25, 27]. In the present study, we generated Tn*phoA* insertion mutants of a naturally

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occurring non-CT *V. cholerae* O1 (strain 1029-84) that produced the highest FA activity [18] to determine the linkage of in vitro production of protease, hemolysin, and CHO-cell cytotoxin with the FA activity in the adult mouse intestine.

Materials and Methods

Bacterial strain and chemical reagents. The study strain 1029-84, a *V. cholerae* serogroup O1 and biotype E1 Tor, was isolated from a patient with watery diarrhea and did not produce CT in the Y-1 adrenal cell assay and the ganglioside GM1-ELISA [18]. The strain was stored in 30% glycerol at −70°C and grown in Luria broth (LB) or on LB agar [21]. Antibiotics (Sigma Chemical Co., St. Louis, Missouri) used were: ampicillin, 100 μg/ml; gentamicin, 30 μg/ml; kanamycin, 300 μg/ml; streptomycin, 100 μg/ml; and tetracycline, 15 μg/ml. The chromogenic substrates (Sigma Chemical Co.) 5-bromo-4-chloro-3-indolyl-phosphate (XP) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (used to detect alkaline phosphatase activities of the mutants and beta-galactosidase activity of the subclone carrying Tn*phoA*-probe, respectively) were incorporated into the agar at a final concentration of 100 μg/ml. Glucose supplements were at 0.2% final concentration [27]. For in vitro DNA manipulations, restriction endonucleases (Boehringer Mannheim Biochemicals, Indianapolis, Indiana), T_4 DNA ligase, and *E. coli* DH5-α cells (GIBCO BRL) were used.

Construction and isolation of Tn*phoA* insertion mutants. Plasmid pRT291 carrying Tn*phoA* (Tc^r and Km^r) in *E. coli* SM10 [27] was conjugated into a streptomycin-resistant derivative of the non-CT *V. cholerae* O1 strain 1029-84 by cross-streaking on LB agar and incubating at 30°C for 16–24 h. The mating mix was then spread after dilution onto agar containing streptomycin, tetracycline, and kanamycin. Transconjugant colonies were subsequently mated with *E. coli* MM294 carrying the gentamycin-resistant plasmid pPH1JI, which is incompatible with pRK290 [2]. The mating mix was plated on LB agar containing gentamycin, kanamycin, and streptomycin, thereby simultaneously selecting for pPH1JI and retention of Tn*phoA* within the chromosome. All of the resulting colonies were Tc^s and carried Tn*phoA* transposed from the pRT291 vector. The colonies that carried active *phoA* gene fusions appeared blue on LB agar containing XP.

Isolation of nonproteolytic, nonhemolytic, and noncytotoxic mutants. All of the blue colonies were purified and initially screened for their proteolytic activity on dialyzed brain–heart infusion–milk medium as described by Sokol et al. [22]. Hemolytic activities of the mutants were detected on Trypticase soy agar plates containing 5% sheep red blood cells (Carr-Scarborough Microbiologicals Inc., Decatur, Georgia) after 24 and 48 h of incubation at 30°C. Proteolytic and hemolytic activities were determined by measuring the zone of clearing around each colony (distance from the edge of the colony to the edge of clearing) and were recorded as strong (+4: >2.5 mm), moderate (+3: 2.0–2.5 mm; +2: 1.5–1.9 mm), weak (+1: 1.0–1.5 mm), questionable (±: <1.0 mm), and none (−). Cytotoxic activities of the mutants were determined by the Chinese hamster ovary (CHO)-cell assay as described earlier [18].

Quantitation of alkaline phosphatase (PhoA) activity. Single colonies of the non-CT *V. cholerae* wild-type and nonproteolytic mutant strains were grown in LB containing 0.2% glucose at 30°C with aeration. Alkaline phosphatase (PhoA) activity was measured from the rate of hydrolysis of *p*-nitrophenyl phosphate by permeabilized cells [15]. The cells were washed in saline and resuspended (10^8–10^9 cells) in 1 ml of 1 M Tris hydrochloride (pH 8), and the optical density (*D*) was measured at 600 nm on a spectrophotometer. To 1 ml of the cell suspension one drop of 0.1% SDS and 2 drops of chloroform were added. The suspension was incubated at 37°C for 5 min prior to the addition of 100 μl of 0.4% *p*-nitrophenyl phosphate [19]. The assay tubes were incubated at 37°C for 30 min or until a yellow color was observed, and the *D* was measured at 420 nm and 550 nm. The PhoA activity of all nonproteolytic mutant strains and the control wild-type strain was based on at least five sets of observations spanning an 18-month period. The activity was expressed in enzyme units per D_{600} unit as described previously [15].

In vivo assessment of virulence of the mutant strains. The enteropathogenicity of the nonproteolytic mutant strains was examined in the sealed-adult-mouse model of Richardson et al. [20], as modified earlier [18]. A group of six Swiss Webster mice weighing ca. 16–20 g were challenged orally with the live cells of each strain (ca. 2×10^{10} CFU/ml) suspended in 0.5 ml of sterile 4% $NaHCO_3$ in heart infusion broth. At 5 h post-inoculation the animals were killed with CO_2, and the FA ratios were determined. FA ratios of 100 and above were considered positive [18]. FA values produced by the mutant strains were statistically compared with the FA value produced by the wild-type strain by use of Student's independent *t*-test.

Mapping of Tn*phoA* insertions to chromosomal fragments by Southern blot analysis. The restriction enzymes *Xba*I and *Eco*RV do not cut within the Tn*phoA* vector [27]. Chromosomal DNA from nonproteolytic, nonhemolytic, and noncytotoxic mutants was isolated by the method of Silhavy et al. [21]. The DNA (10 μg) was digested with *Eco*RV, electrophoresed through a 1% agarose gel, denatured, blotted onto nylon (Schleicher and Schuell, Inc., Keene, New Hampshire), and hybridized as described by Southern [23]. We obtained the probe DNA for Tn*phoA* by subcloning a 3.4-kb *Hin*dIII fragment from the plasmid pRT291 into PUC18. The probe fragment was isolated from a low-melting agarose gel, purified with Gene-clean II (BIO 101, La Jolla, California), and labeled with $(\alpha\text{-}^{32}P)^{dCTP}$ (New England Nuclear Corp., Boston, Massachusetts) by nick translation (Boehringer Mannheim Biochemicals).

Results

Two hundred of approximately 2000 *V. cholerae* colonies carrying inserts of Tn*phoA* appeared as blue colonies on LB agar containing selective antibiotics and the chromogenic substance, XP. Of the 200 Tn*phoA* fusion mutants, 12 did not produce any proteolytic activity (Table 1), and two (strains MM346 and MM347) of the 12 did not produce any hemolytic or cytotoxic activities. The remaining 188 retained all three biological activities similar to those of the wild-type strain 1029-84.

The 12 nonproteolytic mutants produced greater alkaline phosphatase activity than the wild-type strain (Table 1). The two nonproteolytic mutants

Table 1. Alkaline phosphatase, proteolytic, hemolytic, cytotoxic, and FA activities of the wild-type (1029-84) and Tn*phoA* mutant strains[a]

Strain	Alkaline phosphatase (Units)	Proteolytic activity[b]	Hemolytic activity[c]	CHO titer[d]	FA values[e]
1029-84	5	+3	+2	400	149.9
MM301	50	–	+2	128	115.2
MM303	115	–	+2	32	116.5
MM304	197	–	+2	128	113.4
MM307	353	–	+2	128	112.8
MM309	370	–	+2	128	116.1
MM311	268	–	+2	128	114.5
MM314	259	–	+2	32	115.7
MM317	338	–	+2	128	116.6
MM321	386	–	+2	128	113.3
MM346	634	–	–	–	87.1
MM347	772	–	–	–	84.2
MM870	39	–	+2	400	146.5

[a] The results are the mean values of at least three tests for each activity.
[b,c] Relative zone of clearing around colonies (distance from the edge of the colony to the edge of clearing); +3, 2.0–2.5 mm; +2, 1.5–1.9 mm; +1, 1.0–1.5 mm; and –, no clearing.
[d] The titers are expressed as the reciprocal of the highest dilution that exhibits cytotoxicity in 50% or more of the cells.
[e] FA values represent mean fluid accumulation ratios in six mice for each strain.

(strains MM346 and MM347) that showed highest alkaline phosphatase activity lost hemolytic, cytotoxic, and FA activity (<100). Nine other nonproteolytic mutants retained hemolytic activity but had reduced cytotoxic and FA activity; and one (MM870) retained hemolytic, cytotoxic, and FA activity comparable to that of the wild-type strain (Table 1). All the mutants retained these characteristics and did not revert back to the wild-type phenotype during the entire course of the study. The ranges of FA activities in mice were 119–192 (SD = 23) for wild-type strain, 96–128 (SD = 10) for mutants producing FA ratios of 112–116, and 82–92 (SD = 6) for nonenteropathogenic mutants. The difference between the FA ratios produced by the enteropathogenic wild-type strain and nonenteropathogenic mutants were highly significant ($P < 0.001$). Also, the difference between the FA ratios produced by the wild-type strain and the mutants that lost partial cytotoxic and FA activities was significant ($P < 0.05$).

Southern blot analysis with a probe from Tn*phoA* showed that the inserts in the 12 mutants fell into two classes based on the size of the restriction fragment harboring Tn*phoA* (approximately 10 or 20 kb; Figs. 1 and 2). A single Tn*phoA* chromosomal insertion was present in each of the nonproteolytic

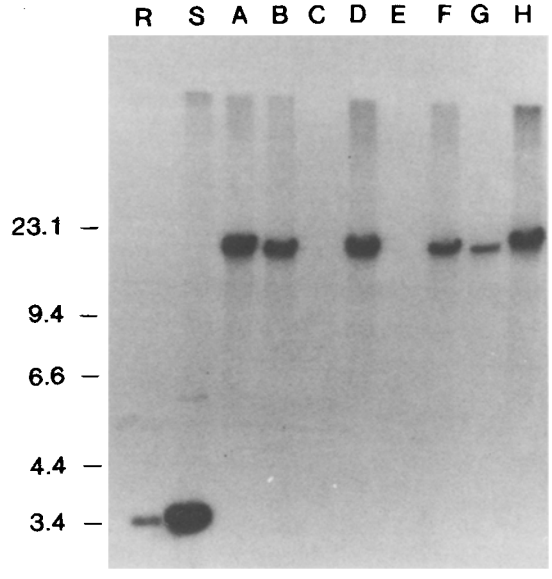

Fig. 1. Southern blot of chromosomal DNA demonstrating insertion of Tn*phoA* in groups of nonproteolytic mutant strains with similar FA activity. DNA was digested with *Eco*RV and probed with a ^{32}P-labeled internal fragment of Tn*phoA*. Lanes: A, MM301; B, MM303; C, 1029-84 (wild-type parent strain); D, MM311; E, SM10 (*E. coli*); F, MM314; G, MM317; H, MM321; R, pRT291 (plasmid carrying Tn*phoA*) digested with *Hin*dIII and *Eco*RI; S, pMM001 (plasmid carrying a fragment of Tn*phoA*) digested with *Hin*dIII. The numbers to the left of the gel indicate the sizes (in kilobases) of the bands.

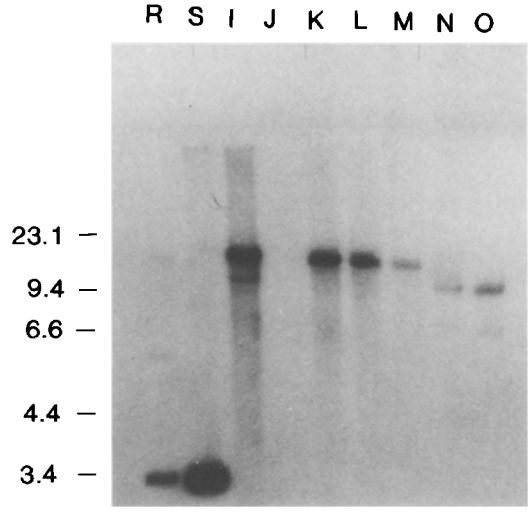

Fig. 2. Southern blot of chromosomal DNA demonstrating insertion of Tn*phoA* in groups of nonproteolytic mutant strains with different FA activities. DNA was digested with *Eco*RV and probed with a ^{32}P-labeled internal fragment of Tn*phoA*. Lanes: I, MM309; J, 1029-84 (wild-type parent strain); K, MM870; L, MM307; M, MM304; N, MM346; O, MM347; R, pRT291 (plasmid carrying Tn*phoA*) digested with *Hin*dIII and *Eco*RI; S, pMM001 (plasmid carrying a fragment of Tn*phoA*) digested with *Hin*dIII. The numbers to the left of the gel indicate the sizes (in kilobases) of the bands.

mutants except one (strain MM309), which appeared to have two inserts (Fig. 2, lane I). The Tn*phoA* insert in strains MM346 and MM347, which lacked proteolytic, hemolytic, cytotoxic, and FA activities, appeared to be in the same chromosomal location (Fig. 2, lanes N and O).

Discussion

Tn*phoA* mutagenesis of non-CT *V. cholerae* O1 produced protease-deficient mutants that were positive for phoA activity. The transposon was inserted, most probably, into a gene encoding a secreted or membrane protein [13]. Mutants that lost proteolytic activity alone retained FA activity; thus, FA activity appears independent of protease expression.

The two mutants that simultaneously lost hemolytic and cytotoxic activities lost FA activity completely. Nine other mutants that retained hemolytic activity but lost partial cytotoxic activity retained some FA activity. FA activity comparable to the wild type was observed only with a single mutant that produced hemolytic and cytotoxic activities equivalent to those of the wild type. Probably, some levels of cytotoxic and hemolytic activities are required for FA activity. The transposon insertion in the two FA-negative mutants that lacked proteolytic, hemolytic, and cytotoxic activities may have affected regulatory genes or genes that encode products necessary for transport or assembly of these secreted factors.

The possible role of extracellular hemolysins and cytotoxins in disease pathogenesis by different *Vibrio* spp. has been described [5, 7, 10, 28, 29]. CT-producing *V. cholerae* O1 and non-O1 strains are known to produce cytotoxin which stimulates FA in rabbit ileal loops [12]. Our results with Tn*phoA* mutants of a clinical non-CT *V. cholerae* O1 and a mouse model support previous observations that the cytotoxic and hemolytic factors are linked with enteropathogenicity [7, 12]. Production of cytotoxin and hemolysin may play a role in the pathogenesis of infections caused by non-CT *V. cholerae* O1.

Literature Cited

1. Baudry B, Fasano A, Ketley J, Kaper JB (1992) Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. Infect Immun 60:428–434
2. Beringer JE, Beynon JL, Buchanan-Wollaston AV, Johnston AWB (1978) Transfer of the drug resistance transposon Tn5 to *Rhizobium*. Nature 276:633–634
3. Fasano A, Baudry B, Pumplin DW, Wasserman SS, Tall BD, Ketley JM, Kaper JB (1991) *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proc Natl Acad Sci USA 88:5242–5246
4. Goldberg MB, DiRita VJ, Calderwood SB (1990). Identification of an iron-regulated virulence determinant in *Vibrio cholerae*, using Tn*phoA* mutagenesis. Infect Immun 58:55–60
5. Gray LD, Kreger AS (1987) Mouse skin damage caused by cytolysin from *Vibrio vulnificus* and by *Vibrio vulnificus* infection. J Infect Dis 155:236–241
6. Honda SI, Shimoirisa K, Adachi A, Saito K, Asano N, Taniguchi T, Honda T, Miwatani T (1988) Clinical isolates of *Vibrio cholerae* O1 not producing cholera toxin. Lancet ii:1486
7. Ichinose Y, Yamamoto K, Nakasone N, Tanabe MJ, Takeda T, Miwatani T, Iwanaga M (1987) Enterotoxicity for E1 Tor like hemolysin of non-01 *Vibrio cholerae*. Infect Immun 55:1090–1093
8. Jerse AE, Yu J, Tall BD, Kaper JB (1990) A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Natl Acad Sci USA 87:7839–7843
9. Johnston JM, McFarland LM, Bradford HB, Caraway CT (1983) Isolation of nontoxigenic *Vibrio cholerae* O1 from a human would infection. J. Clin Microbiol 17:918–920
10. Kreger AS (1984) Cytolytic activity and virulence of *Vibrio damsela*. Infect Immun 44:326–331
11. Levine MM, Kaper JB, Herrington D, Losonsky G, Morris JG, Clements ML, Black RE, Tall B, Hall R (1988) Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect Immun 56:161–167
12. Madden JM, McCardell BA, Shah DB (1984) Cytotoxin production by members of genus *Vibrio*. Lancet i:1217–1218
13. Manoil C. Beckwith J (1985) Tn*phoA*: A transposon probe for protein export signals. Proc Natl Acad Sci USA 82:8129–8133

14. Mekalanos JJ (1985) Cholera toxin: genetic analysis, regulation, and role in pathogenesis. Curr Top Microbiol Immunol 118:97–118
15. Michaelis S, Inouye H, Oliver D, Beckwith J (1983). Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. J Bacteriol 154:366–374
16. Miller I, Maskell D, Hormaeche C, Johnson K, Pickard D, Dougan G (1989) Isolation of orally attenuated *Salmonella typhimurium* following Tn*phoA* mutagenesis. Infect Immun 52:2758–2763
17. Morris JG, Picardi JL, Lieb S, Lee JV, Roberts A, Hood M, Gunn RA, Blake PA (1984) Isolation of nontoxigenic *Vibrio cholerae* O group 1 from a patient with severe gastrointestinal disease. J Clin Microbiol 19:296–297
18. Moyenuddin M, Wachsmuth K, Richardson SH, Cook WL (1992) Enteropathogenicity of nontoxigenic *Vibrio cholerae* O1 for adult mice. Microb Pathogen 12:451–458
19. Peterson KM, Mekalanos JJ (1988) Characterization the *Vibrio cholerae* toxR regulon: identification of novel genes involved in intestinal colonization. Infect Immun 56:2822–2829
20. Richardson SH, Giles JC, Kruger KS (1984) Sealed adult mice: new model for enterotoxin evaluation. Infect Immun 43:482–486
21. Silhavy TJ, Berman ML, Enquist LW (1984) Experiments with gene fusions. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory, pp. 137–139
22. Sokol PA, Ohman DE, Iglewski BH (1979) A more sensitive plate assay for detection of protease production by *Pseudomonas aeruginosa*. J Clin Microbiol 9:538–540
23. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517
24. Takeda T, Peina Y, Ogawa A, Dohi S, Abe H, Nair GB, Pal SC (1991) Detection of heat-stable enterotoxin in a cholera toxin gene-positive strain of *Vibrio cholerae* O1. FEMS Microbiol Lett 80:23–28
25. Taylor RK, Miller VL, Furlong DB, Mekalanos JJ (1987a) Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc Natl Acad Sci USA 84:2833–2837
26. Taylor RK, Spears P, Mekalanos JJ (1987b) Applications of the Tcp pilus in cholera vaccine development. In: Abstr., 23rd Joint Conference on Cholera. The U.S.-Japan Cooperative Medical Science Program, Williamsburg, Virginia.
27. Taylor RK, Manoil C, Mekalanos JJ (1989) Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J Bacteriol 171:1870–1878
28. Wall VW, Kreger AS, Richardson SH (1984) Production and partial characterization of a *Vibrio fluvialis* cytotoxin. Infect Immun 46:773–777
29. Yamamoto K, Honda T, Takeda Y, Miwatani T (1983) Production and increased vascular permeability in rabbits by purified thermostable direct hemolysin from *Vibrio parahaemolyticus*. J Infect Dis 148:1129