

## Cell Surface Characteristics of Environmental and Clinical Isolates of *Vibrio cholerae* Non-O1

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**The cell surfaces of several toxigenic and nontoxigenic environmental and clinical isolates of *Vibrio cholerae* non-O1 have been examined. The environmental strains, irrespective of toxigenicity, are significantly more resistant to antibiotics and detergents than are *V. cholerae* O1 strains. The clinical isolates of non-O1 vibrios are as sensitive to a wide variety of chemicals as the O1 vibrios. The environmental non-O1 strains are also less susceptible to lysis when treated with protein denaturants or neutral and anionic detergents than are O1 vibrios and the clinical non-O1 strains. In contrast to O1 vibrios, the environmental non-O1 vibrios do not have exposed phospholipids in their outer membranes. These features of the cell surfaces of environmental non-O1 vibrios might have a role in the better survival of these organisms under environmental fluctuations.**

*Vibrio cholerae*, a noninvasive gram-negative bacterium and the etiological agent of cholera, a severe diarrheal disease, can cause acidosis and death. All epidemics recorded so far are caused by *V. cholerae* cells belonging to the serological group O1. Cells belonging to serovar non-O1 cause only sporadic infections. Non-O1 vibrios were previously designated nonagglutinating vibrios, since they failed to react with polyvalent O1 somatic antisera. The recent emergence of *V. cholerae* non-O1 as a significant cause of morbidity and mortality in Bangladesh (12) has created a major public health concern. In developed countries, gastroenteritis due to *V. cholerae* non-O1 predominates over the enteric infections caused by *V. cholerae* O1 (13). While the basis of pathogenicity of O1 vibrios has been examined in some detail, little is known about the pathophysiological mechanisms underlying non-O1 vibrio-induced diarrhea in spite of the fact that several virulence factors such as cholera toxin (CT) (35), Shiga-like toxin (23), heat-stable toxin (ST) (3), El Tor hemolysin (22), Kanagawa hemolysin (36), and other cell-associated hemagglutinins (4, 9) have been identified.

Other than serological differences, *V. cholerae* cells belonging to serovars O1 and non-O1 are biochemically and genetically indistinguishable (13). *V. cholerae* non-O1 strains are abundant in the environment and are widely distributed in bays, estuaries, and fresh water in India and Japan. These environmental non-O1 isolates are culturable under laboratory conditions (19, 33). Attempts to isolate culturable *V. cholerae* O1 from the environment of zones of endemicity like Calcutta, India, have not been successful. However, a large number of non-O1 vibrios were isolated during this study (19). It is generally believed that O1 vibrios do not survive under environmental fluctuations. It thus appears that cells belonging to serovars O1 and non-O1 adapt themselves differently to tolerate environmental fluctuations. In view of the fact that the cell surfaces of O1 vibrios are fragile (16, 17, 26, 27, 30, 31) and that the cell surface is the primary potential barrier between the cell and the environment, we wanted to examine whether the cell surfaces of non-O1 vibrios are relatively rigid and thus help these cells survive better in the environment.

*V. cholerae* O1 cells are highly susceptible to a wide range of chemicals, particularly hydrophobic compounds and neutral and anionic detergents (26). The reduced negative charges of lipopolysaccharide (LPS) molecules and exposed phospholipids in the outer membranes of O1 vibrios (26) might be responsible for the inability of these vibrios to survive under various environmental conditions. Furthermore, O1 vibrios are highly sensitive to protein denaturants, and it is possible to isolate the outer membrane directly from whole cells by treatment with urea (16). To examine whether the cell surface architecture of non-O1 vibrios can account for their better adaptability to various external conditions, this report describes experiments that analyze the outer membranes of several environmental and clinical *V. cholerae* non-O1 isolates. The results presented here show that the outer membranes of environmental non-O1 vibrios are much more resistant to hydrophobic compounds, detergents, and lysis in hypotonic medium than are cells belonging to serovar O1. Non-O1 cells do not have phospholipids in the outer leaflet of the outer membrane as has been reported for cells belonging to serovar O1 (26).

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *V. cholerae* strains used in this study are described in Table 1. The O1 and non-O1 strains were obtained from the National Institute of Cholera and Enteric Diseases, Calcutta, India. Cells were grown in gyratory shakers at 37°C either in Casamino Acids-yeast extract broth (pH 7.4) or in nutrient broth containing 0.18 M NaCl, pH 8.0 (16). *Escherichia coli* C-600 was grown in nutrient broth at pH 7.4. Cell viability was assayed as CFU on nutrient agar.

**Toxin assay.** CT was assayed by the ligated-rabbit-ileal-loop assay (10) and by a monoclonal-antibody-based enzyme-linked immunosorbent assay (ELISA) (5), which can detect up to 1 ng of CT per 100  $\mu$ l of culture supernatant.

**Determination of MIC.** The MICs of various compounds for the test strains were determined by both broth dilution and agar dilution assays (31). For the broth dilution assay, about  $5 \times 10^4$  cells were inoculated into nutrient broth containing different concentrations of antibiotics and detergents and shaken for 16 h at 37°C. The lowest concentration of the compound at which there was no visible growth was

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TABLE 1. *V. cholerae* strains

Strain	Serovar	Source	Toxin production <sup>a</sup>	CT gene or non-O1 ST gene probe <sup>b</sup>
569B	O1	Clinical	+	CT+
EW6	O1	Environmental	-	CT+
VCE299	Non-O1	Environmental	-	CT-, ST-
VCE309	Non-O1	Environmental	-	CT-, ST-
VCE232	Non-O1	Environmental	+	CT+, ST-
VC9	Non-O1 (O9; Sakazaki)	Clinical	-	CT (ND), ST-
VC42	Non-O1 (O42; Sakazaki)	Clinical	-	CT (ND), ST-
NRT36	Non-O1 (O31; Smith)	Clinical	+	CT-, ST+

<sup>a</sup> Amount of toxin excreted in the culture supernatant was assayed either by rabbit ileal loop or plate ELISA for CT and in suckling mouse for ST. +, positive; -, negative.

<sup>b</sup> Presence of CT and/or ST gene was determined by hybridization with respective probes. ND, not determined.

considered the MIC. In the agar dilution assay, ca.  $10^3$  cells were spread on agar plates containing different concentrations of the test compound and incubated at 37°C for 18 h. The minimum concentration of the compound at which no CFU were observed was considered the MIC. The results of the two assays were comparable.

**Assay of secretory periplasmic proteins.** For the assay of secreted DNases, cells were inoculated onto DNase test agar (Difco) plates containing toluidine blue O (E. Merck, Darmstadt, Germany) and incubated at 37°C for 16 h (6). The diameters of the zones of clearance (also the changes in color from bluish to reddish) around the colonies were taken as the measure of the amount of DNases secreted. For the induction of alkaline phosphatase, a low-phosphate medium was used, and the enzyme was assayed with *p*-nitrophenyl phosphate as substrate (29). Cyclic phosphodiesterase was assayed by using bis-*p*-nitrophenyl phosphate as substrate (20).

**Lysis of whole cells.** Cells in the logarithmic phase of growth ( $2 \times 10^8$  to  $5 \times 10^8$  CFU/ml) were harvested by centrifugation ( $6,000 \times g$ , 10 min) and suspended in desired solutions. The extent of lysis was estimated by monitoring the decrease in  $A_{585}$  normalized with respect to the control cell sample suspended in 0.18 M NaCl.

**Membrane preparation.** The crude-cell envelope was isolated as described previously (11, 17). Fractionation into outer and inner membranes was carried out by treatment of the crude-cell envelope with 4 M urea for the analysis of LPS and phospholipids (16) and with 1% (wt/vol) Sarkosyl NL-97 (CIBA-Geigy) as described previously (17). Contamination in the outer membrane preparation due to inner membrane was always less than 8% as determined by cytochrome assay (25).

**Analytical methods.** Protein was measured by the method of Markwell et al. (18) with bovine serum albumin as standard. LPS was isolated from crude-cell envelope by 45% (wt/vol) aqueous phenol at 68°C (26, 27). Heptose amounts were estimated by the cysteine-sulfuric acid method (34). Phospholipids were extracted from membranes by chloroform-methanol (2:1, vol/vol) and quantitated by assaying for lipid phosphate (2). The phospholipids were free of LPS contamination, as no 3-hydroxytetradecanoic acid could be detected on analysis of the fatty acids by gas-liquid chromatography (27). Phospholipids were analyzed by thin-layer chromatography (TLC) on silica gel G (Merck) plates with chloroform-methanol-acetic acid (65:25:10). The chromatogram was observed during a brief exposure to iodine vapor and developed in ninhydrin spray reagent (0.2% ninhydrin in acetone). For quantitative analysis, the spots containing the

phospholipids were scraped into a tube and eluted by chloroform-methanol, and their phosphate contents were determined (17).

**Isolation of peptidoglycans and analysis of synthesis.** Estimation of amounts of peptidoglycan was done by using cells in the logarithmic phase of growth labeled with 2.5  $\mu$ Ci of [<sup>14</sup>C]acetate per ml (specific activity, 56.7 mCi/mmol). After 2 h of growth, cells were harvested ( $6,000 \times g$ , 10 min), suspended in 4% sodium dodecyl sulfate (SDS) (pH 6.0), and incubated at 100°C for 2 h. The cells were then brought to room temperature and centrifuged at  $120,000 \times g$  for 2 h at 15°C, and the pellet containing the peptidoglycan sacculus was washed with distilled water to remove SDS and the radioactivity assayed.

**Cyanogen bromide (CNBr)-activated dextran coupling.** Preparation of CNBr-activated dextran T-10 (molecular mass, 10 kDa) and its coupling to whole cells were done by the method of Kamio and Nikaido (14) as described previously (26). Cells were labeled by growing them in medium containing [<sup>3</sup>H]glycerol (2  $\mu$ Ci/ml; specific activity, 1 Ci/mmol; Amersham Inc., Buckinghamshire, United Kingdom) for 2 h, harvested, suspended in fresh prewarmed nutrient broth containing 0.05% glycerol, and shaken for 1 h. The cells were then harvested and suspended in 50 mM Na-borate buffer (pH 8.5). The suspension was divided into two parts, 0.1 M ethanolamine (pH 8.5) was added to the control tube at 0 min, and the coupling reaction with freshly prepared CNBr-dextran complex was carried out for 1 h at 25°C. The reaction was terminated with excess ethanolamine (pH 7.4). Phospholipids were isolated from both control and experimental cells and analyzed by TLC as described above. In some experiments, control and experimental cells were directly solubilized in SDS (2%, wt/vol; 2 min; 100°C), and the SDS lysate was directly applied to TLC plates and analyzed in the same way as lipid samples.

**SDS-PAGE.** The outer membrane proteins were analyzed by SDS-15% polyacrylamide gel electrophoresis (PAGE) as described earlier (28).

## RESULTS

Several representative toxigenic and nontoxigenic strains of environmental and clinical isolates of non-O1 vibrios were selected for this study. Except for strain VCE232, all the non-O1 vibrios examined (environmental and clinical) lack the CT gene (Table 1). Strain VCE232, although it carries the CT gene, secretes at least 10 times less CT in the medium than the hypervirulent strain 569B belonging to serovar O1. Strain NRT36 carries the ST gene (24). Strain EW6, although

TABLE 2. MICs of various antibiotics and detergents for *V. cholerae*

Inhibitory substance	MIC ( $\mu\text{g/ml}^a$ )							
	Serovar O1		Serovar non-O1					
	Clinical (569B)	Environmental (EW6)	Clinical			Environmental		
VC9			VC42	NRT36	VCE299	VCE309	VCE232	
<b>Antibiotics<sup>b</sup></b>								
Penicillin G	2	2	2	2	20	20	20	20
Ampicillin	2	2	2	2	20	20	20	20
Novobiocin	2	2	2	2	20	10	10	10
Rifampin	0.1	5	2	2	5	5	5	2
Chloramphenicol	5	1	2	2	1	1	1	1
Nalidixic acid	1	1	2	2	10	5	5	2
Streptomycin	2	10	10	10	>50	10	10	10
Polymyxin B	0.2	2	20	20	20	20	20	20
<b>Detergents</b>								
SDS	0.01	0.05	0.02	0.02	2	2	2	2
Sodium deoxycholate	0.05	0.2	0.1	0.1	2.5	2.5	2.5	2.5
Triton X-100	0.02	0.05	1.0	1.0	>4	>4	>4	>4
CTAB	20	20	10	10	50	20	20	20

<sup>a</sup> MICs for SDS, sodium deoxycholate, and Triton X-100 are expressed as percentages.

<sup>b</sup> See reference 26.

it belongs to serovar O1 and biotype El Tor, was isolated from the water of a river at Calcutta (8). This environmental O1 strain, unlike other O1 strains, is culturable and has been maintained for a long time. Although strain EW6 carries the CT gene (7), it did not cause fluid accumulation in the ligated rabbit ileal loop and secreted no CT in the medium according to results of ELISA.

**Susceptibility toward various compounds.** Susceptibilities of strains belonging to non-O1 serovars toward antibiotics and detergents were compared with those of hypotoxigenic strain 569B and nontoxigenic strain EW6, both belonging to serovar O1. Non-O1 environmental strains are 5 to 20 times more resistant than the toxigenic O1 strain 569B toward different antibiotics tested (Table 2) except chloramphenicol, for which the non-O1 strains were about fivefold more sensitive. The sensitivity of the ST-producing clinical non-O1 strain NRT36 toward these antibiotics was similar to that of the non-O1 environmental strains. On the other hand, the sensitivities of the non-O1 clinical isolates VC9 and VC42 were similar to those of O1 strains with the exception of polymyxin B (Table 2). These strains did not secrete CT, as revealed by both rabbit-ileal-loop assay and plate ELISA. Except for strains VC9 and VC42, all non-O1 strains examined in this study were more than 200-fold more resistant to neutral and anionic detergents than were cells belonging to serovar O1. However, both O1 and non-O1 strains examined were equally resistant to the cationic detergent cetyltrimethyl ammonium bromide (CTAB) (Table 2) and were sensitive to EDTA.

**Secretion of periplasmic proteins.** One of the typical features of *V. cholerae* cells belonging to serovar O1 is secretion of periplasmic proteins, including CT, into the medium. The leakiness of these cells has been attributed to an unstable outer membrane (16, 26, 31). All the non-O1 vibrios examined in this study also secreted as many periplasmic proteins such as DNase (Fig. 1), cyclic phosphodiesterase, and alkaline phosphatase as did the O1 vibrios. This was unexpected because the environmental non-O1 strains were similar to enteropathogenic *E. coli* strains (26) with respect to sensitivity towards various antibiotics and because *E. coli*

cells do not secrete periplasmic proteins. These observations prompted us to examine the cell surface architecture of the non-O1 vibrios.

**Lysis of *V. cholerae* cells.** The structure of the murein network plays a significant role in determining the stabilities of cell surfaces in bacteria. Several studies have indicated the fragile nature of the murein networks of *V. cholerae* O1 cells (17, 31). These cells lyse rapidly in hypotonic media (17) and in the presence of chelating agents like Tris and EDTA (1). To investigate whether the cells belonging to serovar non-O1 are equally susceptible to lysis, cells in the logarithmic phase of growth were suspended in distilled water, protein denaturants, or detergents, and the extent of lysis was compared with that in cells belonging to serovar O1. Strains of *V. cholerae* belonging to O1 and non-O1

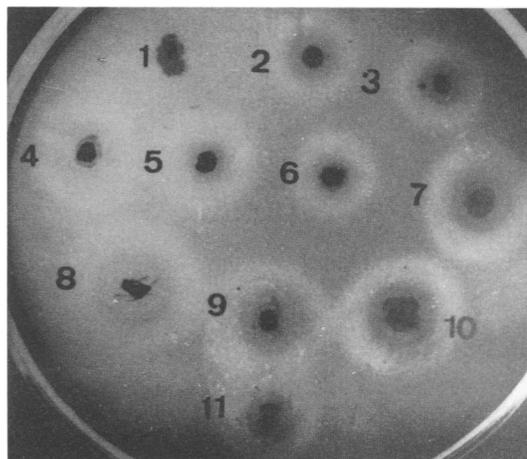


FIG. 1. Secretion of periplasmic DNase into the medium from *V. cholerae* O1 and non-O1 strains. Spots: 1, *E. coli* C-600; 2 through 11, *V. cholerae*; 2, 569B; 3, VCE299; 4, VCE232; 5 and 6, VCE309; 7 and 8, VC9; 9, NRT36; 10, VC42; 11, EW6.

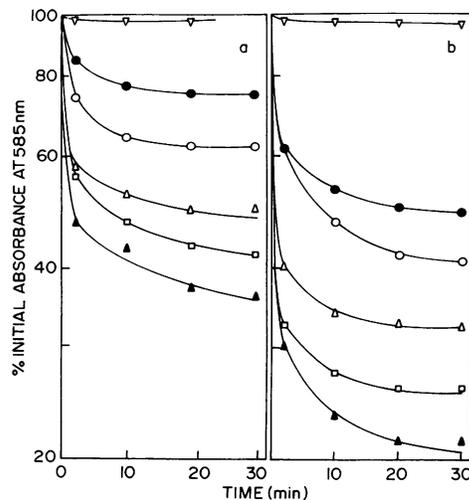


FIG. 2. Lysis of *V. cholerae* cells. Cells in the logarithmic phase of growth were harvested, suspended in distilled water (a) or 1 M urea (b), and kept at room temperature (25°C). Lysis was monitored by the fall in  $A_{585}$ . Symbols:  $\nabla$ , *E. coli* C-600;  $\bullet$ , VCE309, VCE299, and VCE232;  $\circ$ , 569B;  $\triangle$ , VC9;  $\square$ , EW6;  $\blacktriangle$ , NRT36.

serovars were much more sensitive to lysis upon suspension in distilled water, urea (Fig. 2), and detergents (Fig. 3) than was strain C-600 of *E. coli*. The environmental non-O1 strains, irrespective of the presence or absence of the CT gene, were resistant to lysis relative to both the O1 strains and the clinical isolates of non-O1 vibrios. The lytic patterns of environmental strains VCE299, VCE232, and VCE309 were identical (Fig. 2 and 3). The sensitivities to lysis of these strains when they were subjected to a cationic detergent like CTAB was similar to those of O1 strains and clinical isolates of non-O1 vibrios (Fig. 3c). The ST-producing clinical strain NRT36 and the O1 strains were sensitive to most of the agents examined. When these experiments were repeated with cells in the stationary phase of growth, the extent of lysis in all cases was less than that for cells in the

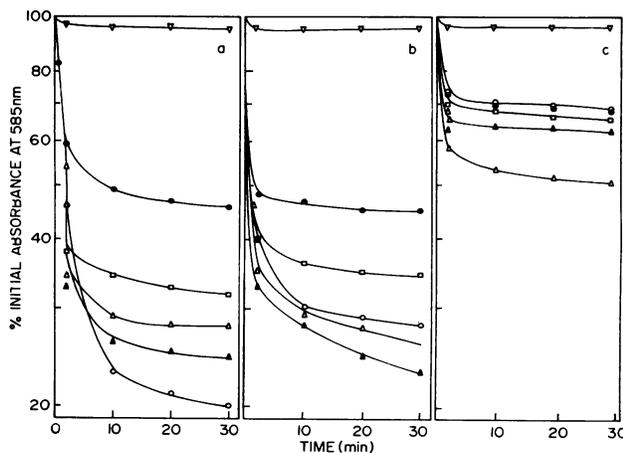


FIG. 3. Lysis of *V. cholerae* cells. Cells in the logarithmic phase of growth were harvested and suspended in 0.01% SDS (a), 0.01% Triton X-100 (b), or 0.01% CTAB (c). Lysis was monitored by the fall in  $A_{585}$ . Symbols:  $\nabla$ , *E. coli* C-600;  $\bullet$ , VCE309, VCE299, and VCE232;  $\circ$ , 569B;  $\triangle$ , VC9;  $\square$ , EW6;  $\blacktriangle$ , NRT36.

TABLE 3. Composition of outer membranes of representative strains of O1 and non-O1 vibrios<sup>a</sup>

Bacterial strain	Amt ( $\mu$ g)/mg of outer membrane			Ratio <sup>b</sup>	Amt ( $\mu$ g)/mg of OMP	
	Protein	LPS	Phospholipid		LPS	Phospholipid
569B <sup>c</sup>	618	223	157	3.9:1.4:1.0	360	256
VCE309	600	234	166	3.6:1.4:1.0	390	276
NRT36	700	184	116	6.0:1.6:1.0	262	156

<sup>a</sup> Each value is the mean of at least three sets of independent experiments. OMP, outer membrane protein.

<sup>b</sup> Ratio of protein/LPS/phospholipid.

<sup>c</sup> Data were taken from reference 26.

logarithmic phase of growth. A comparison of the lytic behavior of environmental non-O1 vibrios with that of other strains examined suggests that the murein network may be less fragile in the environmental strains. To investigate this possibility further, representative O1 and non-O1 clinical and environmental strains were labeled with [<sup>14</sup>C]acetate, and the amount of acetate label in the peptidoglycan sacculus isolated from these cells was estimated. The percentages of acetate label of the whole cells recovered in the peptidoglycan fractions were 5.4, 9.7, and 24.3 in 569B, NRT36, and VCE309 cells, respectively. Thus, there is apparently about fivefold more peptidoglycan in the environmental non-O1 strains than in O1 vibrios. Clinical non-O1 vibrios have about twofold more peptidoglycan than O1 toxigenic strains. These observations indicate that the environmental non-O1 strains have relatively rigid murein networks.

**Cell surface constituents.** The amounts of protein, LPS, and phospholipid in the outer membranes of some representative strains of O1 and non-O1 serovars were estimated (Table 3). Except for the ST-producing clinical strain NRT36, amounts of protein, LPS, and phospholipid in the outer membranes of cells belonging to serovar O1 and non-O1 were almost identical. Strain NRT36 has more protein and less phospholipid in the outer membrane than other strains.

The outer membrane proteins of the non-O1 vibrios were analyzed by SDS-PAGE, and the gels were scanned densitometrically (Fig. 4). The outer membrane protein profiles of clinical isolates of non-O1 vibrios (Fig. 4c and d) and an environmental isolate carrying the CT gene (Fig. 4f) are similar and are different from those of toxigenic or nontoxigenic strains of O1 serovar (Fig. 4a and b). The nontoxigenic environmental non-O1 strains have a 33- and a 30-kDa major outer membrane protein besides the 38- and 36-kDa proteins common to all non-O1 vibrios (Fig. 4e). Two proteins of sizes 23 and 27 kDa are present in all strains irrespective of serovar. It has recently been shown that the 23-kDa protein is responsible for maintaining the comma shape of this organism and represents an OmpA-like protein of *V. cholerae* (31).

More than 60% of the phospholipids in the outer membrane of non-O1 vibrios were phosphatidyl ethanolamine (PE). The other 40% consisted of phosphatidyl glycerol, diphosphatidyl glycerol, and lyso-PE (Table 4). These values are identical to those for O1 vibrios (26). Thus, in spite of the apparent similarity in the macromolecular contents of the outer membranes of O1 and non-O1 vibrios, non-O1 vibrios were much more resistant to various agents. The high sensitivity of O1 vibrios to hydrophobic compounds has been attributed to the presence of exposed phospholipids in the outer membranes of these cells (26). The relatively more

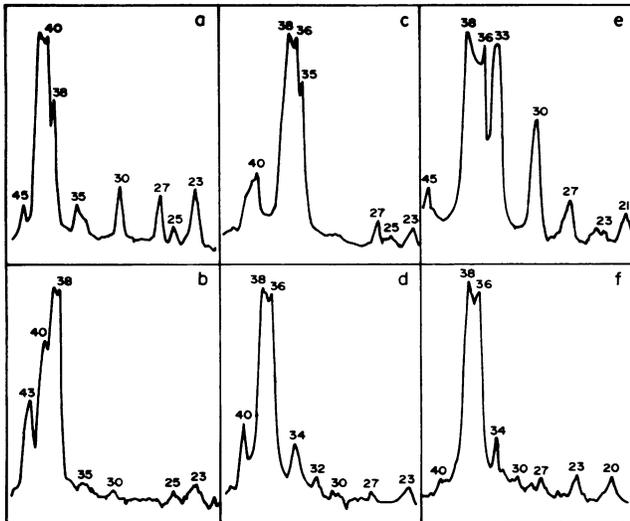


FIG. 4. Densitometric scans of SDS-PAGE of outer membrane proteins of *V. cholerae* O1 and non-O1 strains. Numbers indicate molecular masses of proteins in kilodaltons. (a) 569B; (b) EW6; (c) VC9; (d) VC42; (e) VCE309; (f) VCE232.

resistant phenotype of environmental non-O1 vibrios towards hydrophobic compounds (Table 2) might be due to the absence of phospholipids in the outer leaflet of the outer membrane. To investigate this possibility, several topological probes were used. CNBr-activated dextran is one such probe useful for detecting PE molecules on the outer leaflet of the outer membrane (14). This nonpermeating probe was used to detect whether phospholipids are exposed in the outer membranes of non-O1 vibrios.

[2-<sup>3</sup>H]glycerol-labeled cells were treated with CNBr-activated dextran for 1 h at 25°C. Following the reaction,

TABLE 4. Distribution of [2-<sup>3</sup>H]glycerol-labeled phospholipids in O1 and non-O1 vibrios after treatment with CNBr-activated dextran<sup>a</sup>

Vibrio strain	Source of whole-cell lipids	% Radioactivity recovered from <sup>b</sup> :					
		Origin	Lyso-PE	PE	PG	DPG	Solvent front
<b>O1</b>							
569B <sup>c</sup>	Control	3	9	65	16	3	2
	Experimental	3	14	54	20	6	3
EW6	Control	1	9	66	15	7	2
	Experimental	1	13	59	19	6	2
<b>Non-O1</b>							
VCE309	Control	2	10	67	14	5	2
	Experimental	2	11	63	17	6	2
VCE232	Control	1	8	69	13	8	1
	Experimental	1	8	70	14	6	1
VC9	Control	2	10	64	13	8	2
	Experimental	2	15	54	21	6	3
NRT36	Control	1	10	66	15	7	1
	Experimental	1	11	67	13	7	1

<sup>a</sup> Treatment of [2-<sup>3</sup>H]glycerol-labeled cells (2.5 to 4 mg [dry weight],  $2 \times 10^6$  to  $3 \times 10^6$  cpm) with CNBr-activated dextran and subsequent fractionation of control and experimental cells were as described in Materials and Methods. The total amount of radioactivity in each sample was about  $2 \times 10^4$  cpm, and recovery was about 80%.

<sup>b</sup> PG, phosphatidyl glycerol; DPG, diphosphatidyl glycerol.

<sup>c</sup> See reference 26.

phospholipids were isolated from whole cells and from outer and inner membranes of treated and untreated cells and analyzed by TLC. With whole cells, the only significant difference in the amount of <sup>3</sup>H label recovered in the different phospholipids was a reduction in PE-associated radioactivity in the CNBr-dextran-treated cells of O1 vibrios (Table 4). Since CNBr-activated dextran is a nonpermeating probe, the PE-associated loss of radioactivity is not due to the phospholipid from the inner membrane. This was further confirmed by analyzing the outer and inner membranes from CNBr-dextran-treated and untreated cells. The loss of PE-associated radioactivity was strictly restricted to phospholipids isolated from the outer membranes of CNBr-dextran-treated cells. Surprisingly, the amounts of radioactivity recovered at the origin of the chromatogram were identical for treated and untreated cells (Table 4). It was expected that the loss in PE-associated radioactivity in the treated cells would be associated with dextran and that the radioactivity would remain at the origin. It is possible that because of the hydrophilic nature of PE-dextran complex, the radioactivity was not extracted by the chloroform-methanol used to isolate phospholipids. To recover the PE bound to activated dextran, [2-<sup>3</sup>H]glycerol-labeled cells were lysed with SDS (2%, wt/vol; 2 min; 100°C) and directly spotted onto TLC plates. Under these conditions, PE-associated radioactivity bound to dextran was recovered from the origin of the chromatogram. After extensive hydrolysis with HCl (4 M, 100°C, 4 h) and analysis by TLC, the radioactive material recovered at the origin was identified as PE complexed with activated dextran. Only background-level radioactivity was recovered from the origins of chromatograms of untreated cells. When these experiments were repeated with cells belonging to serovar non-O1, no reduction in PE-associated radioactivity was observed following treatment with CNBr-activated dextran. Since reduction in PE-associated radioactivity is a measure of the amount of phospholipids in the outer leaflet of the outer membrane, these observations indicate that phospholipids are not exposed in the outer membranes of non-O1 vibrios. However, phospholipids are exposed in the outer membranes of clinical nontoxicogenic isolates of non-O1 vibrios (Table 4). These results are in agreement with the observed susceptibilities of these organisms to hydrophobic compounds and detergents.

## DISCUSSION

To examine why environmental non-O1 vibrios can survive under various environmental conditions while O1 vibrios cannot be recovered from the environment in culturable form, studies to investigate the role of the cell surface in survival were undertaken. The results presented in this report show that environmental non-O1 vibrios are more resistant to various agents than are cells belonging to serovar O1. It has been observed that unlike cells belonging to serovar O1, non-O1 cells have no exposed phospholipids in their outer membranes. However, clinical nontoxicogenic non-O1 isolates have exposed phospholipids in their outer membranes. It is not known whether these cells can survive in the environment in culturable form. A preliminary analysis also suggests that environmental non-O1 vibrios possess more peptidoglycan, which might give these cells more rigidity. Minor changes in the outer membrane protein profiles of cells belonging to O1 and non-O1 serovars suggest some differences in organization of the cell surfaces of O1 and non-O1 vibrios. These observations indicate that the difference in the cell surface architecture of cells belonging

to O1 and non-O1 serovars might play a significant role in the better survival of non-O1 vibrios.

The O1 vibrios behave more like deep rough, *traT*, or *envA* mutants of *E. coli* and *Salmonella typhimurium* (21, 32). On the other hand, environmental non-O1 vibrios might possess an asymmetric organization of the outer membrane like that in *E. coli* or *S. typhimurium* wild-type cells. Interestingly, the ST-producing clinical non-O1 strain examined in this study does not have phospholipids exposed in the outer membrane like the environmental non-O1 cells, but at the same time, it lyses extensively in hypotonic solutions, as do cells belonging to serovar O1 (Fig. 2). A rough estimate shows that this ST-producing clinical non-O1 strain has about 2.5-fold less peptidoglycan than the environmental non-O1 strains and about 2-fold more than the O1 vibrios. The lytic behavior of all O1 and non-O1 strains examined in this study clearly shows that even the environmental non-O1 vibrios are osmotically much more fragile than other gram-negative enteric pathogens (Fig. 2 and 3). Lysis of a bacterial cell results from damage to the peptidoglycan layer. This damage could be due to the action of enzymes which hydrolyze linkages in cell wall peptidoglycan, the presence of an inherently fragile cell-supporting structure, or an osmotic effect. The nearly instantaneous lysis of *V. cholerae* organisms belonging to serovars O1 and non-O1 when suspended in distilled water, urea, or detergents argues strongly against an enzymatic mechanism. On the other hand, the fact that the lysis is faster for cells in the logarithmic phase of growth than for those in stationary phase suggests that autolytic enzymes may be involved. In *E. coli*, the peptidoglycan network is thin but "more than a monolayer" (15). The peptidoglycan content of *V. cholerae* 569B is about 0.5% of the cellular dry weight, a percentage much lower than that in other gram-negative bacteria (17). The environmental non-O1 strains have about fivefold more peptidoglycan, and hence in these cells, the peptidoglycan network is probably more than a monolayer. The clinical non-O1 strains, on the other hand, have only twofold more peptidoglycan and might not give enough rigidity to the cells to protect cellular lysis. Thus, relatively less lysis of environmental non-O1 cells compared with their clinical counterparts or with cells belonging to serovar O1 might reflect a difference in the organization in the outer membrane, the only potential barrier between the cells and its environment, which in turn might allow better survival of the environmental non-O1 vibrios.

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