APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1990, p. 1140–1147
0099-2240/90/041140-08$02.00/0

Vol. 56, No. 4

Surface and Virulence Properties of Environmental *Vibrio cholerae* Non-O1 from Albufera Lake (Valencia, Spain)

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Received 27 June 1989/Accepted 27 December 1989

A total of 140 environmental *Vibrio cholerae* non-O1 isolates, together with several culture collection strains from both environmental and clinical sources, were studied in relation to hemagglutination, surface hydrophobicity, and the enzymatic, hemolytic, cytotoxic, and enterotoxic activities of their extracellular products. A total of 78 and 62% of the strains produced hemagglutinins and exohemagglutinins, respectively. Four different hemagglutinating and two exohemagglutinating activities were found by using eight sugars in the inhibition assays. Cell-bound mannose-sensitive hemagglutination was detected mainly in chicken blood, whereas fucose-sensitive hemagglutination was recorded only in human blood. Cell-bound hemagglutinin resistant to all sugars tested was the only one related to surface hydrophobicity. The surface properties varied along the growth curves. The non-O1 strains displayed strong enzymatic and hemolytic activities, except for esculin hydrolysis. Of 26 non-O1 isolates selected for cytotoxin and enterotoxin production, 23 showed a wide spectrum of cytotoxic effects on cell lines of poikilothermic and homoiothermic species, but they were weakly enterotoxigenic in the infant mouse test. All extracellular products of cytotoxic strains were proteolytic, lipolytic, and hemolytic, and a high percentage produced hemagglutination of chicken blood. The cytotoxic factors in the non-O1 strains analyzed were not R plasmid mediated.

Vibrio cholerae non-O1 serotypes are autochthonous bacteria of aquatic environments (3, 10, 21, 30, 31, 43, 45) and have been associated with choleralike diseases (5, 34, 42) and other extraintestinal infections in humans (6). Recent reports have stressed their importance as pathogens for fish (23, 51).

It has been demonstrated that *V. cholerae* non-O1 strains can produce several toxins apart from cholera toxin (30, 33, 50). Hemagglutinins, hemolysins, proteases, and cytotoxins have been proposed as other virulence factors expressed by *Vibrio* species (8, 14, 19, 28). Several attempts have been made to relate these putative virulence factors to the pathogenicity of *Vibrio* species of environmental and clinical origins, but no clear correlation has been established (14, 29, 31, 44, 51).

In previous studies, we have shown that *V. cholerae* non-O1 is present as a free-living and zooplankton-associated organism in Albufera Lake, south of Valencia (Spain), and in coastal waters under its influence (3, 21). This region has been one of the areas most affected by cholera invasions in Spain during the last century, and some cases of confirmed cholera have been registered recently (Boletín Microbiológico Semanal 25, 34, 36, 38, 39, Ministerio de Sanidad y Consumo, Madrid, Spain, 1987). A high percentage of the *V. cholerae* non-O1 strains isolated during studies on Albufera Lake showed resistance to antibiotics and harbored an R plasmid of high molecular mass (around 74 megadaltons) responsible for their resistance phenotype (2). In the present work, we report on surface properties as well as exohemagglutinating, hemolytic, enzymatic, cytotoxic, and enterotoxic activities of the extracellular products (ECPs) of these non-O1 environmental isolates, compared with some O1 and non-O1 culture collection strains from clinical and environmental sources. Finally, the cytotoxic and enterotoxic properties of selected non-O1 strains harboring the 74-megadalton R plasmid (2) and their transconjugants were compared to elucidate the possible relationships between R plasmids and virulence factors in environmental *V. cholerae* strains.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. One hundred and forty *V. cholerae* non-O1 strains from samples of water and plankton (3, 21) and five *V. cholerae* O1 strains and one non-O1 strain from culture collections were used in hemagglutination (HA), hydrophobicity, enzymatic, and hemolytic assays (Table 1). Twenty-six *V. cholerae* non-O1 environmental strains were tested in cytotoxic and enterotoxic assays, as were seven transconjugants in *Escherichia coli* K-12 185 Nal^r obtained from cytotoxic *V. cholerae* non-O1 in a previous work (2). The following isolates of environmental *V. cholerae* were used as controls in toxicity assays: non-O1 strains from Bangladesh, M-17151 (Ent^+), D-17642 (Ent^+), TK17752 (Ent^-); O1 strains from Louisiana, LA 4808 (Ent^+); and an O1 strain from the Chesapeake Bay, V69 (Ent^+). All control strains were kindly supplied by R. R. Colwell, Department of Microbiology, University of Maryland, College Park. *E. coli* m452-C1 (LT^+ ST^+) and K-12 185 (LT^- ST^-) were also used as positive and negative controls, respectively. The strains were kept as permanent cultures in a lyophilized form. Working stocks were maintained on semisolid nutrient agar at room temperature with periodic subculturing.

All strains were routinely cultured with agitation (200 rpm) in peptone water, nutrient broth, and tryptose soy broth (TSB) at 37°C for 24 h. For estimation of growth curves, samples were removed periodically and the optical density at 600 nm was determined in a Beckman DU-7 spectrophotometer. Bacterial cultures grown without agitation were used for electron microscopic observations.

TABLE 1. Origin, serology, and Heiberg biogroup of *V. cholerae* strains studied

Serology	No. of strains	Origin	Heiberg group	Serotype
Non-O1	12	Fresh water	I	
Non-O1	8	Seawater	I	
Non-O1	3	Sea plankton	I	
Non-O1	54	Fresh water	II	
Non-O1	36	Seawater	II	
Non-O1	12	Lake plankton	II	
Non-O1	6	Sea plankton	II	
Non-O1	1	Seawater	IV	
Non-O1	2	Fresh water	V	
Non-O1	5	Seawater	V	
Non-O1	1	Sea plankton	VII	
Non-O1	1	ATCC 11195	II	
O1	1	ATCC 25870		Inaba
O1	1	NCTC 7270		Hikojima
O1	1	NCTC 7254		Inaba
O1	1	NCTC 8021		Ogawa
O1	1	NCTC 3661		Inaba

HA, inhibition of HA, and surface hydrophobicity. The ability of *V. cholerae* to adhere to host cells was tested by agglutination of chicken and human erythrocytes and by the surface hydrophobicity test. Strains were harvested in the early logarithmic and stationary phases of growth. Cells were washed in phosphate-buffered saline (PBS, pH 7.4), resuspended in the same buffer, and adjusted to a concentration of 10^9 cells per ml. Human group O blood collected by venipuncture and chicken blood from a slaughterhouse were mixed immediately with 0.5% (wt/vol) sodium citrate and stored for no more than 2 days at 4°C. Assays for HA and inhibition of HA were done with a 3% suspension of erythrocytes in PBS as previously described (44). Reactions were considered negative if no agglutination occurred within 10 min, and the degree of HA was scored from + to +++ depending on intensity and time. Inhibition of HA was performed by mixing the bacterial suspensions with 0.5 to 2% (wt/vol) solutions in PBS of D-mannose, D-mannitol, D-fructose, D-glucose, D-galactose, D-ribose, L-arabinose, and L-fucose (Sigma Chemical Co., St. Louis, Mo.). A negative control of blood plus sugar in PBS was used.

The relative cell surface hydrophobicity was evaluated by the salt aggregation test (SAT) conducted by the method of Faris et al. (17, 18). Serial dilutions of ammonium sulfate solutions (0.1 to 4.0 M) in PBS were prepared. The SAT value was defined as the lowest molarity which caused a visible white bacterial clumping.

Electron microscopy observations and cell envelope preparations. Selected strains from the hemagglutinating groups were grown in nutrient broth to an optical density at 600 nm of 0.4, and washed cells in PBS were used for microscopic observations and cell envelope preparations. Strains were negatively stained with 1% phosphotungstate (pH 7.2) on carbon-coated Formvar grids and examined for pili or fimbriae with a JEOL 100 S electron microscope.

For envelope preparations, these selected strains were sonicated in a Labsonic 2000 (Braun) ultrasound disintegrator and suspensions were centrifuged at 8,000 × *g* for 10 min to remove cell debris. A second centrifugation at 48,000 × *g* for 1 h was performed for membrane isolation. The pellets were suspended in 1 ml of PBS and used for HA assays.

Exohemagglutinins. Strains were incubated in TSB for 24 h, and samples were removed periodically. Supernatants were obtained by centrifugation (16,000 × *g* for 20 min) at 4°C. Assays were conducted by a slide test (20) by mixing 20 μl of supernatant with 20 μl of the blood suspension in Krebs Ringer solution as modified by Jones et al. (28). For inhibition assays, erythrocytes were directly suspended in Krebs Ringer solution containing the same sugars at the same concentrations used in the cell-bound HA assays.

Detection of extracellular enzymes and hemolytic activity. Proteolytic (caseinase, gelatinase, and elastase), lipolytic (lipase and phospholipase), amylolytic, and DNase activities and esculin hydrolysis were evaluated by a plate assay method by the procedures of West and Colwell (47) and Wakabayashi et al. (46). Elastase activity was determined as described by Hsu et al. (26). Hemolysin production was detected on CDC anaerobe blood agar (32) containing 5% (vol/vol) defibrinated human or chicken blood cells at 37°C both aerobically and anaerobically. The ratio between halo and colony diameters was considered as a quantitative estimate of enzymatic activity (26). The correlation matrix between the different enzymatic and hemolytic activities was obtained with the stepwise regression program of BMDP statistical software (15).

Toxic activities of ECPs in in vivo and in vitro assays. Filtered supernatants obtained from bacterial cultures grown in TSB were assayed for toxicity in vivo by the infant mouse test (IMT) with 2- to 3-day-old BALB/c mice (7). One drop of 2% (wt/vol in PBS) Evans blue dye per milliliter of sample was added, and each sample was assayed, at least, in a group of three mice. After 4 h of incubation, the mice of each group were killed and their intestines were weighed together. The IMT ratio (intestinal weight/remaining body weight) was determined, and fluid accumulation (FA) ratios above 0.09 were recorded as positive.

The cell-free supernatants were further assayed for cytotoxicity with six cell lines: CHSE-214 (chinook salmon embryo), FHM (fathead minnow peduncle), L929 (mouse lung fibroblast), BGM and Vero (African green monkey kidney), and HeLa (human cervix epithelioid carcinoma) cells. Cell lines of poikilothermic and homoiothermic species were cultured and maintained in Eagle minimum essential medium containing Earle salts and 10% (vol/vol) fetal calf serum. For cytotoxic assays, cells were grown as monolayers in 24-well culture plates (Costar, Cambridge, Mass.) with Leibovitz medium (L-15; Flow Laboratories, Inc., McLean, Va.). Plates were incubated at 18°C (fish cell lines) or 37°C (homoiothermic cells). The assays were done basically as described by Toranzo et al. (44) and Alonso et al. (1). Wells showing totally or partially destroyed monolayers within 2 days were scored as positive cytotoxic responses.

Heat treatment. Thermolability of hemagglutinins, surface hydrophobicity, and exotoxins was determined by heating samples to 80°C for 30 min.

RESULTS

Hemagglutinating and hydrophobic properties of *V. cholerae* non-O1. We found that the hemagglutinating activity of our strains was affected by both medium and growth phase, being stronger when the strains were grown in nutrient broth or TSB than in peptone water and harvested in the early logarithmic phase of growth (between 4 and 6 h of incubation). In Table 2, the strains are grouped on the basis of their hemagglutinating responses under the above-mentioned optimal conditions. Of 141 environmental non-O1 strains assayed, 109 (78%) possessed agglutinating capacity. Of these, 88 (81%) expressed adhesins for both types of erythrocytes, and 21 (19%) agglutinated only one type. On the other hand,

TABLE 2. Hemagglutinating, exohemagglutinating, hydrophobic, and cytotoxic groups of the *V. cholerae* strains

Group and no. of strains	Cell-bound HA[a]		Hydrophobicity test (SAT)[b] (M)	Soluble hemagglutinin[c]		No. of strains selected	Cytotoxic activity[d]
	Human	Chicken		Human	Chicken		
Group 1							
19	MSHA+++	MSHA+++	0.2–1.0		RHA	6	+
1	MSHA+++	MSHA+++	0.4				
1	MSHA+++	MSHA+++	0.8		SHA		
23	−	MSHA+++	0.3–1.5		RHA	2	+
1	−	MSHA+	1.0			1	+
Group 2							
7[e]	RHA++	RHA++	0.1–0.4		RHA	1	+
1	RHA++	RHA++	0.3		SHA		
8	RHA++	MSHA++	0.1–0.4		RHA	1	+
1	RHA+	MSHA+	2				
1	RHA++	MSHA+	0.4	RHA	RHA		
2	RHA++	−	0.1–0.4		RHA		
6	RHA+++	MSHA+++	0.1–0.3			1	+
6	RHA++	MSHA++	0.2–0.4				
4	RHA+	MSHA+	0.8–1.0			1	+
Group 3							
20[f]	FSHA++	MSHA++	0.3–2.0		RHA	4	+
7	FSHA++	RHA++	0.6–0.8		RHA		
4	FSHA+++	MSHA++	0.6–0.8			1	+
Group 4 (2 strains)	GSHA+++	MSHA+++	0.5–1.0	RHA	RHA		
Group 5							
12	−	−	>1.5		RHA	6	+
20	−	−	1.0–1.5			3	−

[a] Results were obtained from cultures in TSB at 37°C for 4 to 6 h of incubation. +++, Very fast and strong; ++, strong; +, moderate; −, no activity.
[b] Results were obtained from cultures in TSB at 37°C for 4 to 6 h. SAT is expressed as the lowest molarity of ammonium sulfate that caused a visible white clumping.
[c] Soluble hemagglutinin production was detected by agglutination of human or chicken erythrocytes. Supernatants were obtained from TSB cultures after 18 h of incubation at 37°C. SHA, Exohemagglutination sensitive to sugars.
[d] Supernatants of selected strains were obtained from TSB cultures after 18 h of incubation at 37°C. Toxic effects were recorded after 12 h at 37°C or 24 h at 18°C for homoiothermic and poikilothermic cell lines, respectively. Cytotoxicity was positive (+) when at least one of the six cell lines used gave this result.
[e] Group in which the culture collection non-O1 strain ATCC 11195 is located.
[f] Group in which the culture collection O1 strains NCTC 3661, NCTC 8021, and ATCC 25870 are located.

three of the five O1 clinical strains analyzed showed hemagglutinating activity on human and chicken blood.

Four distinct patterns of inhibition by sugars could be detected among the *Vibrio* strains: mannose-sensitive HA (MSHA), fucose-sensitive HA (FSHA), galactose-sensitive HA (GSHA), and HA resistant to all sugars at all concentrations tested (RHA). In our work, MSHA of chicken blood was the dominant pattern and was expressed by all O1 hemagglutinating strains and 97 non-O1 isolates (89%). MSHA was also inhibited to a different extent by fructose and glucose (0.5 to 1%, wt/vol) and, depending on the strain, by D-mannitol and D-ribose (1 to 2%, wt/vol) as well. FSHA and RHA clearly dominated in human erythrocytes. The former was only observed in human blood and was expressed, together with MSHA of chicken blood, by the 3 hemagglutinating O1 strains (NCTC 3661, NCTC 8021, and ATCC 25870) and 28 environmental non-O1 isolates (25.7%). *V. cholerae* non-O1 ATCC 11195 produced RHA of both types of erythrocytes. This kind of hemagglutinating activity was only found in non-O1 strains and was expressed by 43 (39%) of our environmental isolates. Finally, a new type of HA, GSHA of human erythrocytes, was detected in two *V. cholerae* non-O1 strains from plankton samples (Table 2).

Hydrophobicity was assessed by the SAT. Table 2 shows the SAT values corresponding to the different hemagglutinating groups. They ranged from 0.1 M (hydrophobic) to more than 1.5 M (nonhydrophobic). Of the non-O1 strains, 50% were hydrophobic (0.1 to 1.0 M) and 23.6% were nonhydrophobic (>1.5 M). Like HA, surface hydrophobicity was influenced by the composition of the growth medium and the growth phase and was lost by heating at 80°C for 30 min. Hydrophobicity was lowest when cells were grown in peptone water and recovered in the stationary phase of growth (SAT > 2 M). No relationship between HA pattern and hydrophobicity values was observed.

We randomly selected one *V. cholerae* non-O1 strain representative of each of the different HA groups for electron microscopic observation and membrane isolation. The membranes retained HA and hydrophobic properties, and no specialized structures like pili or fimbriae were observed on cell surfaces.

Production of exohemagglutinins, extracellular enzymes, and hemolysins. All culture collection O1 strains and approximately 62% of our environmental non-O1 strains produced exohemagglutinins when cells were cultured in TSB. The most common type was active against chicken blood and was resistant to all sugars at all concentrations tested (RHA). Three strains out of the total produced RHA on human erythrocytes, and only one strain showed soluble HA sensitive to glucose, mannose, and fructose. In contrast to cell-bound HA, the exohemagglutinating activity of the supernatants was only just detectable in the early logarithmic phase and reached its maximum in the stationary phase of growth. The exohemagglutinating properties were lost by

heat treatment (80°C, 30 min), suggesting that these thermolabile factors could be protein in nature.

All *V. cholerae* non-O1 environmental strains tested produced amylase and DNase. A large number of environmental isolates also produced gelatinase (97%), lipase (87%), phospholipase (76%), and elastase (62%). Only a small percentage of strains (25%) were positive for esculin hydrolysis. Only two of the O1 strains analyzed (NCTC 3661 and ATCC 25870) hydrolyzed casein or esculin, whereas the culture collection non-O1 strain gave a strong positive response on all substrata. The relative activity (zone ratio) varied in the different reactions. The highest activity was observed for elastin and gelatin, and the lowest was observed for esculin hydrolysis. Correlation analysis with quantitative data revealed a positive significant correlation ($P < 0.01$) between lipolysis and phospholipolysis ($r = 0.63$) and among gelatinase, caseinase, and elastase ($r = 0.33$ to 0.55).

Nearly all strains (97%) displayed hemolytic activity for human (92%) and chicken (97%) blood, which were significantly correlated ($r = 0.69$, $P < 0.01$), and more than 50% of environmental strains produced strong hemolysis (zone ratio, >3.0). A correlation matrix revealed that the hemolytic activities measured were also significantly correlated ($P < 0.01$) with lipolysis ($r = 0.45$ to 0.56) and phospholipolysis ($r = 0.4$ to 0.50). No relationship between hemagglutinating and hemolytic activities was observed.

Exotoxic activity of ECPs. We investigated the toxicity of ECPs in 26 randomly selected *V. cholerae* non-O1 isolates and the culture collection non-O1 strain. Two O1 enterotoxic strains (LA 4808, V69) and two non-O1 enterotoxic strains (M-17151, D-17642) were used as positive controls. Of the 26 strains selected, 23 showed some cytotoxic effect on both poikilothermic and homoiothermic cell lines (Table 3). In general, positive effects were observed within 6 h, and some ECPs caused total destruction of the monolayer in less than 2 h. The enterotoxic controls were cytotoxic on all cell lines used, whereas the nonenterotoxic control strain (*V. cholerae* non-O1 TK17752) had no cytotoxic effect. Cytotoxic activities were manifested by pyknotic nuclei, vacuolization, rounding, shrinking, dendritic elongation, and finally cell detachment (Fig. 1). Heat treatment (80°C, 30 min) of our positive ECPs resulted in a loss of cytotoxicity.

Supernatant fluids of 18 strains of *V. cholerae* non-O1 tested for cytotoxicity were used in enterotoxigenic assays by the IMT. The FA ratio ranged between 0.061 and 0.080 (Table 3). The non-O1 strain, ATCC 11195, gave a strong positive response (FA ratio = 0.115). In addition, the cytotoxic titers on Vero cells of non-O1 isolates were lower (1/4 to 1/128) than the titers exhibited by the enterotoxic ATCC 11195 strain (1/4,096).

Plasmid content and cytotoxic activity. In a previous study, we reported the presence of R plasmids in our environmental isolates (2). To investigate whether these R plasmids of high molecular mass (around 74 megadaltons) were related to cytotoxic activity, we assayed seven transconjugants in *E. coli* K-12 185 Nal^r for cytotoxicity, using the parental and recipient strains as positive and negative controls, respectively. These R plasmids coded for Am^r Ax^r Sn^r (T1, T5, T33, and T78), for Am^r Ax^r (T153 and T155), and for Am^r Ax^r Fo^r Km^r Sn^r (T255). No cytotoxic activity was exhibited by any of the transconjugants, suggesting that the genes for cytotoxic production and/or regulation of cytotoxic production are not located in these R plasmids.

DISCUSSION

Adhesion to the intestinal mucosa represents the first step in the infectivity of bacterial pathogens such as *V. cholerae* (9, 19, 28). This process is mediated by nonspecific (mainly hydrophobic) and specific (binding of the bacterial adhesin with its receptor on the epithelial cell) interactions (16, 29). Agglutination of erythrocytes and the SAT are the most useful assays to test the attachment ability of potential pathogens (4, 18, 28). Strains and biotypes of *V. cholerae* O1 produce several hemagglutinins (9, 24, 28) whose importance in the adherence process in vivo has already been suggested (49). However, at present there is insufficient information on this subject concerning *V. cholerae* non-O1 isolates, in which the ability to attach to different substrata (chitin, detritus . . .) is also important for their survival in aquatic environments (3, 13, 27).

We found that *V. cholerae* non-O1 displayed a broad range of hemagglutinating activities and that no marked differences could be noted between environmental non-O1 and clinical O1 strains. The most frequent pattern of HA found among our environmental non-O1 isolates was sensitivity to D-mannose, which has been described as the predominant cell-bound HA in *V. cholerae* O1 Eltor biotype (24). On the other hand, FSHA, previously ascribed to the O1 serotype-classical biotype, was found in the O1 strains NCTC 3661, NCTC 8021, and ATCC 25870 (biotypes Eltor, cholera, and cholera, respectively), and in 28 environmental non-O1 isolates. Therefore, no apparent relationships between type of HA and serotype-biotype could be established. Similar results have been reported by Booth and Finkelstein (9) in both O1 and non-O1 strains. Hemagglutinating activity resistant to all sugars was only found in non-O1 strains. This pattern of HA has been described in marine *Vibrio* species (36) and in some mutants of *V. cholera* O1 that developed a resistant hemagglutinin in the stationary phase of growth (24). In this sense, we also observed that some strains exhibited different HA depending on the growth phase (data not shown), although the main response was a weaker HA or absence of HA in the stationary phase. Interestingly, a new type of HA, GSHA on human erythrocytes, previously described in environmental strains of *Aeromonas hydrophila* (4), was detected in a few of our strains of planktonic origin.

Hydrophobic properties of the bacterial surface have been related to hemagglutinating activity in *E. coli* and *V. cholerae* (17, 18, 22). In the majority of our strains, the relative surface hydrophobicity was comparable to that reported by others for *V. cholerae* and for strains of *E. coli* that are negative for colonization factor antigens I and II (17, 18, 22). As we found for HA, no differences between clinical and environmental strains and serotypes O1 and non-O1 could be established. Surface hydrophobicity was also influenced by growth medium and growth phase and was thermolabile, suggesting that both activities could be coexpressed in *V. cholerae* strains. Finally, the evidence obtained after electron microscopic observation suggests that adhesins could be distributed in a layer over the cell surface.

Pathogenic *V. cholerae* O1 strains secrete a hemagglutinin-protease that has been proposed previously to be cholera lectin (19) and that has the ability to activate the cholera enterotoxin (8). However, the information on exohemagglutinin production by non-O1 strains from environmental origin is very scarce. We found that approximately 62% of our *V. cholerae* non-O1 strains were exohemagglutinating. In contrast to cell-bound HA, all O1 strains produced exohemagglutinin in the stationary phase of growth, which is in

TABLE 3. Characteristics of selected environmental *V. cholerae* non-O1 strains

Strain	HA of erythrocytes from[a]:		Hydrophobicity test (SAT) (M)	Exohemagglutination[b]	Enzymatic activity[c]				Hemolysis of erythrocytes[d]		Cytotoxic response on[e]:						Enterotoxigenicity (IMT)[f]
											Fish cell line		Homoiotherms				
	Human	Chicken			Cas	Elas	Lip	Phos	Human	Chicken	CHSE	FHM	L929	Vero	HeLa	BGM	
V1	MSHA+++	MSHA+++	1.0	+	+	+	+	−	−	+	+	+	+	+	+	−	0.071
V5	RHA+	MSHA+	1.0	−	+	+	+	+	+	+	−	−	−	+	+	−	0.076
V18	FSHA+++	MSHA++	0.8	−	+	−	+	+	+	+	+	+	+	+	+	+	0.069
V33	FSHA++	MSHA++	1.5	+	+	+	+	+	−	+	−	+	+	+	+	+	0.070
V35	−	MSHA+	1.0	−	+	+	+	−	−	+	−	−	−	+	+	−	0.069
V78	−	MSHA+++	1.5	+	+	−	+	+	+	+	−	+	+	−	−	+	0.079
V94	−	MSHA+++	0.3	+	+	+	+	+	+	+	+	+	+	+	+	+	0.064
V110	FSHA++	MSHA++	0.5	+	−	−	−	+	+	+	−	−	+	+	+	−	0.079
V114	−	−	2.0	+	+	−	+	+	+	+	+	+	+	+	+	+	0.077
V116	FSHA++	MSHA++	1.0	+	−	+	+	+	+	+	+	+	+	+	+	+	0.065
V137	−	−	2.0	+	+	+	+	−	−	+	+	+	+	ND[g]	ND	−	ND
V148	FSHA++	MSHA++	1.0	+	+	−	−	+	+	+	+	+	+	−	−	+	0.080
V155	−	−	1.8	+	+	+	+	+	+	+	+	+	+	+	+	−	0.076
V161	−	−	1.5	+	−	+	+	+	+	+	+	+	+	ND	ND	+	ND
V166	−	−	1.5	+	+	−	−	+	+	+	+	+	+	ND	ND	+	ND
V167	−	−	1.5	+	+	−	+	+	+	+	+	+	+	−	−	+	0.061
V210	−	−	2.0	−	+	−	−	−	−	−	−	−	−	ND	ND	−	ND
V242	MSHA+++	MSHA+++	0.4	+	+	+	+	+	+	+	−	+	+	+	+	+	0.074
V244	MSHA+++	MSHA+++	0.2	+	+	−	+	+	+	+	+	+	+	−	−	+	0.073
V252	MSHA+++	MSHA+++	0.3	+	+	+	+	+	+	+	+	+	+	+	+	+	0.067
V255	MSHA+++	MSHA+++	0.4	+	+	+	+	+	+	+	+	+	+	+	+	+	0.072
V268	RHA++	MSHA++	0.3	+	+	+	+	+	+	+	+	+	+	ND	ND	+	ND
V73P	MSHA+++	MSHA+++	0.2	+	−	+	−	−	−	+	−	+	+	ND	ND	+	ND
V82P	RHA++	MSHA++	0.2	−	+	+	+	+	+	+	+	+	+	−	−	+	0.072
V140P	−	−	1.8	−	−	−	−	−	−	−	−	−	−	ND	ND	−	ND
V142P	−	−	>2.0	−	−	−	−	−	−	+	−	−	−	ND	ND	−	ND
ATCC 11195	RHA++	RHA++	0.4	+	+	+	+	+	+	+	+	+	+	+	+	+	0.112

[a] Results were obtained from cultures in TSB at 37°C for 4 to 6 h of incubation. +++, Very fast and strong; ++, strong; +, moderate; −, no activity.
[b] Soluble hemagglutinin production was detected by macroscopic agglutination of chicken erythrocytes. Supernatants were obtained from TSB cultures after 18 h of incubation at 37°C.
[c] Extracellular enzymatic activities were evaluated by plate assay. Cas, Caseinase; Elas, elastase; Lip, lipase; Phos, phospholipase.
[d] Hemolysis on CDC plates incubated anaerobically at 37°C for 24 h.
[e] Supernatants were obtained from TSB cultures after 18 h of incubation at 37°C. Toxic effects were recorded after 12 h at 37°C or 24 h at 18°C for homoiothermic and poikilothermic cell lines, respectively.
[f] IMT (7). Data express the FA ratio. Ratios of *E. coli* controls were as follows: m452-C1 (LT^+ ST^+), 0.157; K-12 185 (LT^- ST^-), 0.066.
[g] ND, Not determined.

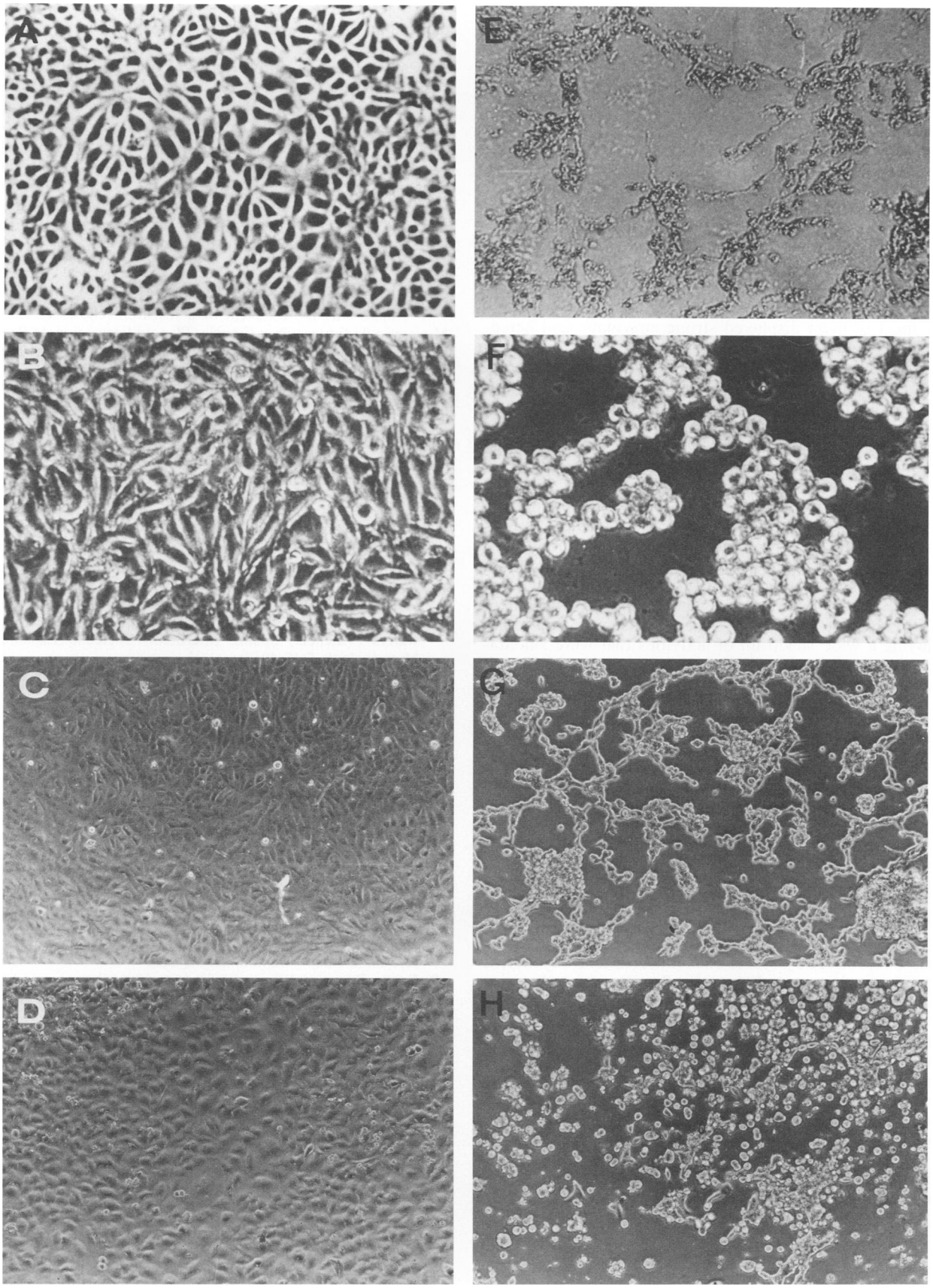

FIG. 1. Cytotoxic effects displayed by the ECPs of selected *V. cholerae* non-O1 strains isolated from Albufera Lake. (A, B, C, D) Control cells (CHSE-214, L929, Vero, and HeLa, respectively) inoculated with sterile broth; (E, F, G, H) cytotoxic effects on the respective cell lines.

agreement with the results obtained by Hanne and Finkelstein (24) and Honda et al. (25). No correlation between soluble hemagglutinin and cell-bound hemagglutinin seems to exist in these *V. cholerae* non-O1 strains.

Proteasic and hemolytic activities have been related to virulence in *Vibrio* spp. and *Aeromonas* spp. by several authors (14, 26, 38, 40) and, as has been already suggested, could be of relevance in those illnesses different from cholera that are caused by non-O1 *V. cholerae* (6). In addition, it is known that *V. cholerae* non-O1 strains of clinical origin produce a hemolysin (48), but little information is available on hemolysin production in environmental strains. In accordance with Simpson et al. (40), who studied O1 and non-O1 *V. cholerae* strains from environmental and clinical sources, we found no differences between O1 and non-O1 strains since most of them displayed strong enzymatic and hemolytic activities. The hydrolytic profile of our *V. cholerae* non-O1 isolates indicates a high potential for substratum utilization. Some of these activities were significantly correlated, suggesting that some overlapping of reactions occurs.

Although our *V. cholerae* non-O1 strains were producers of cell-bound and soluble hemagglutinins, as well as hemolysins against different types of erythrocytes, the precise role of these factors in vivo is still obscure (14). Bacterial pathogenesis of cholera and noncholera infections requires a coordinated assembly of such virulence factors, as well as toxin production. It is now widely accepted that non-O1 *V. cholerae*, in most instances, does not produce cholera toxin but does possess other mechanisms, possibly including cytotoxins, which make it pathogenic for humans and fish (6, 23, 33). Most of our *V. cholerae* strains were cytotoxic, displaying a wide spectrum of activities that were simultaneously evaluated in homoiothermic and poikilothermic cell systems. The morphological changes induced were similar to the ones shown by the positive non-O1 and O1 control strains. The fact that the heat treatment (80°C, 30 min) of our positive ECPs resulted in a loss of cytotoxicity suggests that proteolytic enzymes are involved in the production of cytotoxic effects. In fact, our environmental vibrios have been revealed as active enzyme producers, which could explain their marked cytotoxicity. One commonly reported feature of the strains from aquatic origin is their lower enteropathogenic potential compared with clinical isolates (41, 42, 45). Even between O1 strains from clinical and environmental sources, some differences have been described (45). Moreover, the relationships between cytotoxicity and enterotoxicity in non-O1 strains are controversial because these strains can produce cytotoxic elements distinct from cholera toxin (33, 35). In our study, nearly all environmental strains tested for cytotoxicity yielded positive results, whereas none was enterotoxigenic when stringent criteria were applied to the IMT (positive test, FA ratio, >0.09). If the criteria are relaxed to a lower value (FA ratio between 0.07 and 0.08), as used by others (37, 38), some of our strains could be considered borderline enterotoxin producers. This type of situation has been reported by Cumberbatch et al. (12) and Pathak et al. (37) in clinical and environmental strains of *A. hydrophila*.

The frequent involvement of accessory genetic elements, such as plasmids, bacteriophages, and transposons that code for virulence factors, is well known. It has been described in gram-negative bacteria that enterotoxins and siderophores may be codified in plasmids (11, 39) and that some of them are conjugative (39). Since none of our transconjugants showed cytotoxic activity, we conclude that the R plasmids carried by our non-O1 isolates are not implicated in the dissemination of the virulence factors studied in the environment.

Despite the low enterotoxic capability exhibited by *V. cholerae* non-O1 strains from surface waters near Valencia (Spain), the high numbers of *V. cholerae* present during the warm season constitute a public health hazard, since most of them are producers of other putative virulence factors. Moreover, the importance of such factors in pathogenic processes other than cholera infections remains to be investigated. Further studies are required to ascertain the exact nature of the thermolabile ECPs that cause cytotoxicity but not appreciable enterotoxicity in these strains.

ACKNOWLEDGMENTS

C. Amaro was the recipient of a research fellowship from the Ministerio de Educación y Ciencia, Plan de Formación de Personal Investigador. This study was partially supported by the Comisión Asesora de Investigación Científica y Técnica (PR83-3166).

We also thank Isabel Bandin for her helpful technical assistance and C. A. Dawson for critical reading of the manuscript.

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