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Iron-Binding Compounds and Related Outer Membrane Proteins in *Vibrio cholerae* Non-O1 Strains from Aquatic Environments

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A total of 156 strains of *Vibrio cholerae* non-O1 from aquatic origins were examined for the presence of iron uptake mechanisms and compared with O1 strains and other *Vibrio* species. All non-O1 strains were able to grow in iron-limiting conditions, with MICs of ethylenediaminedi (*O*-hydroxyphenylacetic acid) ranging from 20 μM to 2 mM. The production of siderophores was demonstrated by growth in chrome azurol S agar and cross-feeding assays. All strains produced phenolate-type compounds, as assessed by the chemical tests and by bioassays with *Salmonella typhimurium enb-7*. Some of the strains also promoted the growth of *S. typhimurium enb-1* (which can use only enterobactin as a siderophore) as well as some strains of *Vibrio anguillarum* deficient in the anguibactin-mediated system. The chromatographic analyses and absorption spectra of siderophores extracted from culture supernatants suggest that vibriobactin may be produced by the strains examined. Interestingly, some strains also produced hydroxamate-type compounds, as determined by chemical tests, and were able to promote the growth of an aerobactin-deficient strain of *Escherichia coli*. These results were confirmed by the absorption spectra and chromatographic analyses of the culture extracts. The synthesis of iron-regulated outer membrane proteins in representative strains was also examined. The molecular sizes of the main induced proteins ranged from 70 to 78 kilodaltons. These results indicate that several iron uptake mechanisms which could be involved in environmental survival and pathogenicity are present in environmental *V. cholerae* non-O1 strains.

Iron is an essential nutrient for the growth of virtually all bacteria. However, iron is highly insoluble in neutral or alkaline aerobic environments in which it forms biologically unavailable polymers (24). Microbial pathogens are further limited in iron acquisition, since the element is strongly bound to the high-affinity iron-binding proteins of animal hosts (8). To overcome this limitation, many bacteria possess complex systems to solubilize and transport iron into the cell; most of them consist of iron-binding ligands or siderophores and outer membrane proteins (OMPs) serving as specific receptors (4, 8, 24, 40). In addition, possession of a siderophore-mediated iron transport system is related to increased virulence of some bacterial pathogens (15, 23).

Vibrio cholerae non-O1 serotypes are autochthonous bacteria of aquatic environments (2, 14, 17, 18) that are presently recognized as potential pathogens. These species have been associated with choleralike diseases (5, 17) and other extraintestinal infections, not only in humans (6) but also in higher aquatic organisms (41). Although it has been demonstrated that some pathogenic *V. cholerae* non-O1 strains can produce choleralike toxins (17), most of them synthesize other biologically active factors (e.g., exohemagglutinins, hemolysins, proteases, and cytotoxins) that have been related to pathogenicity (7, 11).

Production of siderophores has been described for *V. cholerae* O1 (1, 26, 34), and a catechol siderophore named vibriobactin has been isolated and characterized from one strain of clinical origin (16). Moreover, Sigel and Payne (34) have described at least five OMPs that are derepressed by iron starvation in *V. cholerae* O1 strains. Likewise, it has been demonstrated that OMPs from strains grown in vivo closely resemble those found in cells grown in a low-iron culture medium (33).

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Although O1 and non-O1 strains share many biochemical and virulence characteristics, little is known about the relationship between the iron uptake systems in the two serotypes. In this work, we have studied the iron transport systems in a group of *V. cholerae* non-O1 strains isolated from environmental waters and compared them with several *V. cholerae* O1 strains and other strains of different *Vibrio* species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 156 strains of *V. cholerae* non-O1 were used in this study; 148 had been isolated from fresh water (Albufera Lagoon) and seawater (Mediterranean Sea) (2, 14), and 8 were from Bangladesh (kindly provided by A. E. Toranzo, University of Santiago, Santiago de Compostela, Spain) (Table 1). Other strains of O1 and non-O1 serotypes of *V. cholerae* from culture collections as well as other *Vibrio* species were included for comparative purposes (Table 1). All strains were routinely grown in CM9 medium (4) with rotary shaking (200 rpm). Strains were stored at −20°C in L broth containing 20% (wt/vol) glycerol.

Iron-limiting conditions were routinely obtained by adding the iron chelator α-dipyridyl (20 μM) or ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) (20 μM) to the CM9 medium. The low-iron medium MM9 (32) was also used, with 0.2% succinic acid as the carbon source. In the latter case, all solutions were deferrated by extraction (three times) with an equal volume of 8-hydroxyquinoline (3% [wt/vol] in chloroform) and then washed three times with chloroform to eliminate residual 8-hydroxyquinoline. The medium was buffered with 50 mM Tris hydrochloride (pH 6.8).

MICs of EDDA were determined in CM9 medium, as previously described (4).

TABLE 1. Growth under iron-limiting conditions and siderophore production of *Vibrio* spp.

Organism and origin	No. of strains	MIC (mM) of EDDA	Result of:		
			CAS agar assay[a]	Arnow test[b] (A_{550})	Csáky test[b] (A_{520})
V. cholerae					
O1					
Clinical	5	0.10	+	+ (0.038)	−
Clinical (NCTC 3661)	1	0.30	+	+ (0.115)	−
Estuarine water[c]	1	0.20	+	+/− (0.018)	−
Non-O1					
River (ATCC 11195)	1	2.00	+	+ (0.042)	−
Lake water[d]	3	1.00	++	+ (0.027)	−
	4	0.40	+++	+/− (0.005)	−
	1	0.20	+++	ND	ND
	1	0.20	++	+/− (0.015)	−
	3	0.20	−	−	−
	7	0.07	+	+ (0.052)	−
	3	0.07	−	−	−
	1	0.02	+	+ (0.040)	−
Seawater[e]	3	0.40	+++	+/− (0.019)	+ (0.029)
	1	0.20	−	−	−
	1	0.02	+	+ (0.044)	−
Estuarine water[f]	8	0.15	+	+ (0.039)	−
Seawater, plankton[e]	5	2.00	++	+/− (0.020)	−
	4	0.70	+	+/− (0.012)	−
	5	0.40	++	+ (0.049)	−
	12	0.40	+	+ (0.052)	−
	12	0.20	+	+ (0.049)	−
	7	0.07	+	+/− (0.017)	−
	3	0.07	−	− (0.003)	−
Lake water, plankton[d]	23	1.00	+	+ (0.061)	−
	6	0.40	+++	+ (0.056)	−
	4	0.40	+++	+/− (0.011)	+ (0.068)
	10	0.40	+	+/− (0.013)	−
	20	0.20	+	+ (0.032)	−
V. anguillarum					
775, diseased fish	1	0.04	+	+ (0.040)	−
H775-3, mutant from 775	1	0.01	−	−	−
RV22, diseased fish	1	0.80	++	+ (0.063)	−
Unspecified, fish farm water	2	0.50	+	+ (0.107)	−
V. parahaemolyticus, mussel[e]	2	0.80	++	+ (0.052)	+ (0.013)
V. fluvialis, mussel[e]	2	0.80	++	+ (0.023)	+ (0.031)
V. mediterranei, seawater[e]	2	0.10	+	− (0.009)	−

[a] Ratio of orange halo to colony diameter in CAS agar plates. −, <1.3; +, 1.3 to 2.0; ++, 2.0 to 3.0; +++, >3.0.
[b] +, Positive; −, negative; +/−, equivocal; ND, not determined.
[c] Strain from Chesapeake Bay (provided by A. E. Toranzo, University of Santiago, Spain).
[d] Strains from Albufera Lagoon (14).
[e] Strains from Mediterranean Sea (14).
[f] Strains from Bangladesh (provided by A. E. Toranzo).

Bioassays. Siderophore activity was determined by cross-feeding assays, which tested the ability of each strain to promote the growth of other strains subjected to iron starvation (1, 4). Several *Vibrio* species were used as indicator strains in these assays (Table 2). Furthermore, the following mutants of *Vibrio anguillarum* deficient in the anguibactin-mediated iron uptake system were used to detect anguibactinlike siderophores: H775-3 (cured from plasmid pJM1), 775::Tn*1-5* (receptor$^+$, anguibactin$^-$), and 775::Tn*1-6* (receptor$^-$, anguibactin$^-$). The wild-type strain 775 was used as a positive control (all strains were kindly provided by J. H. Crosa, Oregon Health Sciences University, Portland). *Salmonella typhimurium enb-1* and *enb-7* mutants, deficient for enterobactin biosynthesis (29), and the positive control *S. typhimurium* LT2 were used for enterobactin assays. *Escherichia coli* LG1522, deficient for aerobactin biosynthesis, and the positive control *E. coli* LG1315 were used for aerobactin detection (39) (Table 3).

Each indicator strain was inoculated into soft CM9 agar plates supplemented with appropriate concentrations of EDDA, and the strains to be assayed were seeded on top of the agar (4).

Siderophore detection. Production of siderophores was investigated by using the universal assay of Schwyn and Neilands (32) in solid and liquid media, with suitable *Vibrio* strains as positive and negative controls (Table 1). Siderophore determination on chrome azurol S (CAS) agar plates was performed as previously described (4). The ratio of orange halo diameter to colony diameter was considered an estimative quantification of the siderophore levels produced.

TABLE 2. Cross-feeding assays of the *V. cholerae* strains tested with different *Vibrio* indicator strains

Assayed strain (no. tested)	Growth[a] after 18 h with indicator strain:					
	V. cholerae non-O1 V137	*V. cholerae* O1 ATCC 25870	*V. anguillarum* 775	*V. parahaemolyticus*	*V. fluvialis*	*V. mediterranei*
V. cholerae						
Non-O1 (29)	++	++	+	+	+	+
Non-O1 (2)	+	+	+	+	+	+
O1 (6)	+	++	+	+	+	+[b]
V. anguillarum						
775 (1)	+	+	++	+	+	+
Unspecified (2)	++	+	++	++	++	+
V. parahaemolyticus (2)	++	+	++	++	++	+
V. fluvialis (2)	++	+	++	++	+	+
V. mediterranei (2)	+	+	+	+	+	++

[a] + and ++, Growth zone diameter of ≤12 or >12 mm, respectively.
[b] Result obtained after 48 h of growth.

For siderophore determination in liquid media, supernatants from MM9 cultures were mixed with CAS solution (32), and the A_{630} of the mixture was measured. The ratio of the A_{630} of iron-deficient and iron-enriched cell-free supernatants was considered a relative quantification of the iron chelators produced. The data obtained from detection of siderophores in agar and liquid media by the CAS method were subjected to a correlation analysis with the P2R program of BMDP (12).

Phenolates were detected in supernatants by the colorimetric test of Arnow (3). A standard curve was determined by using increasing concentrations of 2,3-dihydroxybenzoic acid (2,3-DHBA) and the corresponding A_{550}. Presence of hydroxamic acids was determined by the method of Csáky (10).

Extraction of siderophores. Cells were grown in deferrated MM9 for 24 h, reinoculated into flasks containing 100 ml of fresh medium, and incubated until the pH had risen to 7.5 to 8.0 (36 to 48 h). Siderophore production was indicated by the observance of a blue fluorescence under UV illumination and confirmed by the Arnow and Csáky tests. Supernatants were obtained by centrifugation at 16,000 × *g* for 20 min followed by filtration through 0.22-μm-pore-size membranes.

Catechols were extracted by using a minor modification of the method of Griffiths et al. (16). Briefly, supernatants were adjusted to a pH of 6.0 and extracted three times with an equal volume of ethyl acetate. The organic fractions were pooled and washed three times with 0.1 M sodium citrate (pH 5.5) and three times with water. The extracts were dried in a rotary evaporator at 30°C and then redissolved in 1 ml of ethanol.

Hydroxamates were extracted with phenol-chloroform (1:1 [vol/vol]) and ethyl ether by the method of Simpson and Oliver (36). Extracts were dissolved in 1 ml of deionized water and sterilized by filtration.

Biological activities of extracts were tested by adding 100 μl to 3 ml of deferrated MM9 broth inoculated with appropriate indicator strains. Growth stimulation of indicator strains was measured by A_{600}.

Paper chromatography and absorbance spectra. Supernatants and freeze-dried concentrates (50-fold), as well as the extracts obtained as described above, were chromatographed through no. 1 (3MM) filter paper (Whatman, Inc., Clifton, N.J.). For catechol-type siderophores, the methanol extracts were chromatographed for 2 h with 5% ammonium formate plus 0.5% formic acid as a solvent. For hydroxamic compounds, chromatography was carried out with ammonia (25%)-ethanol-water (1:16:3, vol/vol/vol) and butanol-acetic acid-water (60:15:25, vol/vol/vol) mixtures as solvents. Papers were air dried, and spots of iron-binding compounds were visualized by illumination with UV light and by spraying the paper with CAS solution (32) or with 0.4% $FeCl_3 \cdot 6H_2O$ in 0.04 M HCl.

The absorbance spectra of supernatants and extracts were determined in a DU-7 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

To determine the biological activities of the different spots, iron-reactive or fluorescent spots from paper chromatograms were cut and placed on the surface of CM9-EDDA agar plates inoculated with indicator strains.

Analysis of OMPs. Cells were grown in both CM9 plus 10 μM $FeCl_3$ and in CM9 supplemented with EDDA in a concentration equivalent to one-fifth of the corresponding MIC for each strain. Total outer membrane proteins (OMPs) were prepared as previously described (4). Outer membrane fractions were obtained by the method of Filip et al. (13) by differential solubilization of the bacterial cell envelopes with

TABLE 3. Cross-feeding assays of the *V. cholerae* and *Vibrio* spp. strains tested with various indicator strains

Assayed strain (no. tested)	Growth[a] after 18 h with indicator strain:				
	V. anguillarum[b]		*S. typhimurium*		*E. coli* LG1522
	R^+S^-	R^-S^-	*enb-1*	*enb-7*	
V. cholerae					
Non-O1 (22)	(+)	(+)	(+)	++	−
Non-O1 (7)	(+)	(+)	(+)	++	+
Non-O1 (2)	−	−	−	+	−
O1 (11)	(+)	(+)	(+)	+	−
V. anguillarum 775 (1)	++	−	−	+	−
V. anguillarum (2)	++	++	++	++	−
V. parahaemolyticus (2)	+	+	+	++	−
V. fluvialis (2)	+	+	++	++	−
V. mediterranei (2)	−	−	−	+	−

[a] + and ++, Growth zone diameter of ≤12 or >12 mm, respectively (values in parentheses were obtained after 48 h of growth).
[b] Phenotype of anguibactin-mediated system. R, Receptor; S, siderophore.

1.0% sodium lauroyl sarcosinate. Total proteins and OMPs were examined in sodium dodecyl sulfate-polyacrylamide gels. Gels and buffers were prepared as described by Laemmli (19). The concentrations of acrylamide were 6% in the stacking gel and 12.5% in the separation gel. Electrophoresis was carried out at a constant current of 15 mA through the stacking gel and 20 mA through the separating gel. Densitometry of gels, stained with Coomassie brilliant blue R-250, was performed with a Laser Ultroscan 2202 densitometer (LKB Instruments, Inc., Rockville, Md.). The molecular weights of the protein bands were determined by using a robust, modified hyperbola model (28) adapted for the PAR program of BMDP (31).

RESULTS

Growth under iron-limiting conditions and siderophore detection. To evaluate the ability of *V. cholerae* to grow in low-iron environments, the MICs of EDDA were determined (Table 1). All strains were able to grow in the presence of 10 μM EDDA. MICs ranged from 20 μM to 2 mM; the modal value was in the range of 0.2 to 0.4 μM (56.8% of strains), and 21.6% of strains gave values between 1.0 and 2.0 mM. There was no appreciable correlation between the MICs and the serotypes or origins of the strains. MICs for other *Vibrio* species showed similar variations (0.1 to 0.8 mM).

The method of Schwyn and Neilands was used to relate the growth in iron-deficient medium to the production of siderophores. In the CAS agar test, a ratio between halo and colony diameter of less than 1.3 was considered a negative result. This value was established by testing the two negative controls of *V. anguillarum* deficient for anguibactin production. A total of 93.5% of *V. cholerae* O1 and non-O1 strains gave positive results (Table 1). Of these, 41.3% showed a halo-to-colony ratio of around 1.5, and six non-O1 environmental strains reached a ratio greater than 5.2 (Table 1). Interestingly, the lowest positive values (around 1.35) were obtained with the O1 strains of clinical origin. Other *Vibrio* species also showed positive, intermediate values (1.5 to 3.0).

The siderophore detection assay using the cell-free supernatants of deferrated MM9 medium confirmed the results obtained in CAS agar plates. In general, only the strongest siderophore-producing strains (those producing the largest orange halos) were able to change the color of the CAS agar from blue to orange. A significative correlation between the data obtained by the two methods was achieved ($r = 0.82$, $P < 0.01$).

The cell-free supernatants were also examined by the Arnow and Csáky tests for the presence of catechols and hydroxamates. All strains which were positive in the CAS agar test also gave a positive result in the Arnow test, indicating the presence of phenolates (Table 1). Moreover, the 50-fold-concentrated supernatants became purple when $FeCl_3$ solution was added. The siderophore concentrations in supernatants ranged from 5 to 30 μM, as estimated from the standard 2,3-DHBA curve.

Only seven environmental *V. cholerae* non-O1 strains gave positive results in the Csáky test, and these corresponded to the strains producing the largest halos in the CAS agar plates. Their concentrated supernatants became yellowish brown when $FeCl_3$ was added. *Vibrio parahaemolyticus* and *Vibrio fluvialis* strains also gave positive reactions in the Arnow and Csáky tests.

Bioassays. The secretion and utilization of biologically active iron uptake compounds were tested for the selected strains shown in Tables 2 and 3. All strains were able to stimulate the growth of the homologous iron-starved strains and also to stimulate the growth of other strains belonging to different species (Table 2).

TABLE 4. R_fs in paper chromatography for iron-reactive compounds of supernatants from MM9 medium cultures

Strain	R_f [a]
V. cholerae	
Non-O1	
ATCC 11195	0.11, 0.62
1	0.15, 0.64
5	0.12, 0.56
137	0.13, 0.58
114	0.15, 0.63
167	0.15, 0.56
O1	
NCTC 3661	0.13, 0.60
Lou 4808	0.15, 0.66
V. anguillarum	
775	0.24, 0.40
RV22	0.18, 0.67
S. typhimurium LT2	0.30, 0.67

[a] R_fs are for paper chromatography with 5% (wt/vol) ammonium formate–0.5% (vol/vol) formic acid as the solvent.

In order to determine the biological activity of the siderophores produced by the *V. cholerae* strains, cross-feeding assays were performed with different mutants of *V. anguillarum*, *S. typhimurium*, and *E. coli* deficient in the anguibactin-, enterobactin-, and aerobactin-mediated systems, respectively.

The *V. cholerae* strains were separated into three groups according to their responses in these bioassays (Table 3). The majority of the non-O1 strains and all O1 strains were able to stimulate strongly the growth of the *S. typhimurium enb-7* mutant and, to a minor extent, the growth of the *S. typhimurium enb-1* mutant and both anguibactin-deficient mutants. Moreover, seven non-O1 strains were also able to promote the growth of *E. coli* LG1522, a mutant deficient in the biosynthesis of aerobactin. These strains were the same strains which gave positive results in the Arnow and Csáky tests. The third group of strains (two strains), although positive in the CAS assay, were negative in both chemical tests and stimulated the growth of only the *S. typhimurium enb-7* mutant. The same results were obtained with the two strains belonging to *Vibrio mediterranei*. Finally, *V. fluvialis*, *V. parahaemolyticus*, and *V. anguillarum* strains were able to stimulate, to different degrees, the growth of all iron-deficient mutants except *E. coli* LG1522 (Table 3).

Paper chromatography, absorbance spectra, and activity of extracts. Since the chemical and biological tests indicated that *V. cholerae* non-O1 strains could produce phenolate and hydroxamate iron chelators, cell-free supernatants of selected strains and their 50-fold concentrates were analyzed by paper chromatography. Table 4 shows the R_fs corresponding to the spots showing a blue fluorescence under UV light. These spots were confirmed as iron-reactive compounds by spraying with CAS or $FeCl_3$ solution.

V. cholerae supernatants showed two spots when an ammonium formate-formic acid mixture was used as the solvent. One spot, with an R_f of about 0.14, did not have a counterpart in the supernatants of *S. typhimurium* LT-2 or *V. anguillarum* 775. This spot, when placed on the surface of the EDDA-supplemented agar plates used for the bioassays,

promoted the growth of *V. cholerae* O1 and non-O1 strains and also of the *S. typhimurium enb-7* mutant. The second spot, with an R_f of around 0.6, was similar to that exhibited by pure 2,3-DHBA and was able to stimulate the growth of the *S. typhimurium enb-7* mutant and, to a lesser extent, of *V. cholerae* O1 and non-O1 strains. Similar spots were observed in the 50-fold concentrates. However, none of these iron-reactive spots displayed biological activity in the bioassays, suggesting that the freeze-drying process could alter the structure of the siderophores.

The supernatants of only two of six of the selected strains of *V. cholerae* non-O1 (strains V114 and V167) migrated in the two solvent systems used for detection of hydroxamates. R_fs were 0.16 for the butanol-acetic acid-water solvent and 0.3 for the ammonia-ethanol-water solvent. These two strains were also positive in the Csáky test, and their R_fs were similar to those observed for extracts corresponding to the aerobactin producer *E. coli* LG1315. The chromatographic spots obtained by using cell-free supernatants and their 50-fold concentrates were able to stimulate the growth of the aerobactin-deficient mutant *E. coli* LG1522, giving the same response as the positive control *E. coli* LG1315.

For two strains, V137 (which had been shown to be a strong producer of phenolic compounds) and V167 (which was also a hydroxamate producer), the siderophores were extracted from supernatants. Paper chromatography of these extracts revealed two iron-reactive spots in the phenolate extract and one iron-reactive compound for the hydroxamate extract. Their R_fs were quite similar to those observed for the crude supernatants when ammonium formate-formic acid was used as the solvent. The spot from the phenolate extract with an R_f of 0.14 greatly stimulated the growth of all *V. cholerae* and other *Vibrio* strains, the *S. typhimurium enb-7* mutant, and the mutant strains of *V. anguillarum* deficient in the anguibactin-mediated system. The spot for the hydroxamate extraction enhanced the growth of only the aerobactin-deficient mutant.

The absorption spectra obtained after extraction revealed that the phenolic compounds absorbed mainly in the UV range, with a narrow peak at 257 nm and a smaller and broader peak between 320 and 325 nm. The spectra obtained for hydroxamate extracts also showed absorption in the UV range, with a main peak at 262 nm.

Induction of OMPs. OMPs of 12 strains of *V. cholerae* non-O1 (including ATCC 11195) and 1 O1 strain (*V. cholerae* NCTC 3661) grown in iron-rich and iron-deficient media were examined. A comparison of OMP patterns of the non-O1 strains (Fig. 1) shows that a variety of iron-regulated proteins are produced. Variations in at least four OMPs of high molecular mass (78,76, 74, and 70 kilodaltons) are produced as a result of iron starvation in *V. cholerae* O1 and non-O1 strains. Of these proteins, only the 74-kilodalton band was derepressed in all OMP patterns. This protein also showed great variability depending on the concentration of EDDA added to the medium to achieve the iron-limiting conditions, which may indicate its regulation by iron availability (data not shown).

Apparently, hydroxamate-producing strains displayed OMP profiles similar to those of the phenolate producers (data not shown).

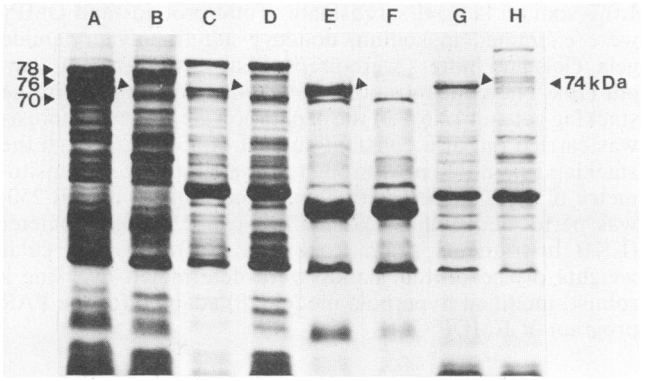

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OMPs of *V. cholerae* non-O1 strains. Lanes A and B, V35; lanes C and D, V18; lanes E and F, V161; lanes G and H, V24. OMPs were from cells cultured in CM9 plus either 20 μM EDDA (Lanes A, C, E, and G) or 10 μM $FeCl_3$ (lanes B, D, F, and H). Each lane was loaded with 60 to 80 μg of protein. The common derepressed protein (74 kilodaltons [kDa]) is marked in each lane.

DISCUSSION

Vibrio species, like other aerobic and facultatively anaerobic organisms, synthesize a number of iron-chelating substances which are apparently necessary for iron uptake from the microbial surroundings (1). In this study, we have focused on the possible iron transport mechanisms present in environmental strains of *V. cholerae* non-O1 in comparison with *V. cholerae* O1 strains and other *Vibrio* species.

All strains of *V. cholerae* non-O1 were able to grow in the presence of 10 μM EDDA, a concentration sufficient to induce iron-limiting conditions (21), and MICs for these strains were as high as 2.0 mM. All strains also gave positive results in the Schwyn and Neilands test (32), which is based on the competitive exchange of iron(III) between the CAS dye and potential chelators, such as siderophores. This finding indicates that the *V. cholerae* non-O1 strains may produce some kind of iron chelators. This hypothesis was also confirmed by the results obtained in the bioassays, since all strains were able to relieve the iron starvation of other strains grown under iron-limiting conditions.

In the chemical tests utilized, all strains produced phenolate compounds and in addition some also excreted hydroxamates. The latter strains showed the strongest reactions in the CAS assay. Simultaneous production of phenolate and hydroxamate siderophores has been reported for *Salmonella* spp. (4, 30), *Klebsiella pneumoniae* (23), *Shigella sonnei* (27), *E. coli* (39), and *Vibrio vulnificus* (40). However, production of hydroxamate compounds has so far not been described in *V. cholerae*. Some of these hydroxamates have been identified in members of the family *Enterobacteriaceae* as aerobactin, and their presence has been related to increased pathogenicity (9).

Siderophores from the *V. cholerae* strains were extracted from the culture medium by using organic solvents and subjected to paper chromatography. The majority of extracts from phenolate-producing strains showed two iron-reactive spots, one of which showed an R_f (0.6) very similar to that of the 2,3-DHBA used as a control. This compound is known to be a precursor for most of the catechol-based siderophores, such as enterobactin or vibriobactin (16, 25). The R_f of the second spot (0.14) was quite similar to that shown by extracts from vibriobactin-producing strains of *V. cholerae* O1 and very distinct from those of the spots shown by *V. anguillarum* 775 or *S. typhimurium* LT-2. Similarly, the absorbance spectra for these extracts were also similar to the spectrum described for purified vibriobactin (16). Thus, it is

likely that these *V. cholerae* strains may produce the siderophore vibriobactin, although more evidence is needed.

Extracts from the hydroxamate-producing strains showed a spot with an R_f very similar to that exhibited by *E. coli* LG1315, the aerobactin-producing control. In addition, the absorbance spectrum was also similar to that reported for aerobactin (38). This suggests the production of a siderophore closely related to aerobactin.

The biological activity of the siderophores produced by the *V. cholerae* strains was tested by cross-feeding assays with different mutants deficient in the enterobactin-, aerobactin-, and anguibactin-mediated systems (Table 3). As expected, all strains promoted the growth of the *S. typhimurium enb-7* mutant, since this strain can grow in iron-limiting conditions if enterobactin or 2,3-DHBA is made available externally. Most strains of *V. cholerae* non-O1 weakly stimulated the growth of the *S. typhimurium enb-1* mutant, which can grow only in the presence of enterobactin. However, strains of *V. anguillarum* and *V. fluvialis* strongly enhanced the growth of this enterobactin-requiring mutant. This fact has been reported previously for *V. anguillarum* (20) but not for other *Vibrio* species. Most of the strains also promoted the growth of the *V. anguillarum* mutants, regardless of the presence or absence of the anguibactin receptor. This could indicate some nonspecific growth stimulation.

The analysis of the OMPs from strains of O1 and non-O1 serotypes of *V. cholerae* showed the induction of a great variety of proteins under iron-limiting conditions. The function of these proteins is unknown, although some of them are likely to be involved in the iron uptake process, considering the role of other similar proteins found in *V. cholerae* O1 strains (33, 34).

Results reported here indicate that environmental *V. cholerae* non-O1 strains possess a strong iron uptake system or systems. This fact was previously reported by Sigel and Payne (34), who pointed out that aquatic environments are likely to be limited in soluble iron. Thus, possession of an efficient iron transport system would be very advantageous for survival and growth in these environments.

The involvement of these systems in pathogenicity needs further study, although Sigel et al. (35) found that the production of diarrhea in suckling mice by *V. cholerae* non-O1 is not affected by mutations in vibriobactin synthesis or transport. However, the pathogenic process in cholera and noncholera diseases requires the assembly of several virulence factors which have not been characterized sufficiently. Several authors suggest that the extremely low concentration of free iron in host tissues may be the environmental signal that triggers the coordinated expression of all virulence determinants, including siderophores and their receptors (22, 37).

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