Plasmids and factors associated with virulence in environmental isolates of *Vibrio cholerae* non-O1 in Bangladesh

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Summary. Plasmid profiles and factors associated with toxigenicity in 51 strains of *Vibrio cholerae* non-O1 isolated from water samples collected in Bangladesh were analysed. Eleven (21.5%) strains were found to harbour at least one plasmid of 1.7–115 Mda; seven of these strains shared a 115-Mda plasmid. Six of 13 strains tested gave positive cytotoxic and enterotoxin responses. However, two non-cytotoxic strains were enterotoxigenic. Only three of the six cytotoxic and enterotoxin strains caused haemagglutination of human erythrocytes which indicated that toxin production and haemagglutinating activity were unrelated in these *V. cholerae* non-O1 strains. Conjugal transfer assays demonstrated that the 115-Mda plasmid harboured by some of the toxigenic *V. cholerae* non-O1 strains carried genes coding for antibiotic resistance and cytotoxin production but not for enterotoxin production. However, this plasmid was also carried by non-toxigenic strains. Some other strains carrying no plasmids or only small-mol.-wt plasmids, were found to be toxigenic. Therefore, toxin production is not plasmid-mediated in all *V. cholerae* non-O1 strains. Regardless of their pathogenic potential, *V. cholerae* non-O1 strains possessed the capacity to grow in conditions of iron limitation and, under these conditions, synthesis of at least two new outer-membrane proteins was induced.

Introduction

At present there is little doubt about the autochthonous aquatic nature of the non-O1 serovars of *Vibrio cholerae*. *V. cholerae* non-O1 strains are widely distributed in marine environments, especially bays, estuaries and other brackish waters, and in shellfish.^{1–4} Ecological studies^{1, 5} seem to indicate that *V. cholerae* non-O1 strains are not associated with sewage pollution, being more abundant in the natural environment where they are associated frequently with the micro- and macro-flora and fauna of fresh and coastal waters.^{6, 7}

The number of reports of diarrhoeal illness in different countries caused by *V. cholerae* non-O1 strains has increased over the past decade as a result of the recognition of their importance as pathogens for man^{8–10} and animals.^{3, 11, 12} Although it has been reported¹³ that *V. cholerae* non-O1 strains can produce more than one type of

cholera-like enterotoxin, the pathophysiological mechanism underlying *V. cholerae* non-O1 induced diarrhoea remains largely unknown because many strains do not produce cholera toxin (CT).^{1, 2, 12, 14} Furthermore, whether clinical or environmental isolates possess the higher enteropathogenic potential is controversial.^{14–16}

In the work reported here, several factors associated with the toxigenicity of environmental *V. cholerae* non-O1 isolates from waters in Bangladesh were studied and the drug resistance patterns and plasmid profiles of these strains were analysed.

Materials and methods

Bacterial strains and antimicrobial susceptibility tests

In this study, 51 *V. cholerae* non-O1 strains isolated from water samples collected at three different sites in Bangladesh—Dacca (D), Matlab (M) and Teknaf (TK)—were examined. Strains were cultured routinely at 25°C in Trypticase Soy Broth (TSB; Oxoid, CM129) supplemented with NaCl 1%, and maintained on Trypticase Soy Agar (TSA; Oxoid, CM131). Strains were identified by the procedures and criteria of West and Colwell.¹⁷

Resistance of the isolates to antimicrobial agents was determined by disk diffusion on Mueller-Hinton Agar (MHA; Oxoid, CM 337) with disks containing ($\mu\text{g}/\text{disk}$); ampicillin 10, chloramphenicol 30, gentamicin 10, kanamycin 30, tetracycline 30, streptomycin 10, trimethoprim-sulphamethoxazole 1.25/23.7 and sulphathiazole 1000.

The numbers and characteristics of the *Vibrio* isolates are shown in table I.

Cytotoxicity assays

Supernates obtained by centrifugation (10 000g for 5 min) of bacteria cultured in TSB for 18 h at 25°C were filtered (0.2 μm ; Millipore membrane) and tested for cytotoxicity on Y-1 mouse adrenal cells and Chinese Hamster Ovary (CHO) cells. Assays were performed as described elsewhere.¹ Cytotoxic activity was indicated by rounding, shrinking, granule formation and vacuolisation of the cells. In these assays, the filtered culture supernate of *V. cholerae* LA 4808 (cholera enterotoxin positive; CT⁺) was included as a positive control.

Production of enterotoxins

Enterotoxin activity of cytotoxic and non-cytotoxic strains of *V. cholerae* non-O1 was measured by the suckling mouse assay following essentially the procedures of Nishibuchi and Seidler.¹⁸ Filtered supernates (100 μl) of bacterial cultures were administered to 2–4-day-old mice intra-gastrically. After incubation for 3 h at 30°C, the animals were killed and the ratio of intestinal weight to remaining body weight (IW/BW) was measured. Ratios greater than 0.08 were regarded as indicating enterotoxin production. Filtered culture supernates of *V. cholerae* LA 4808 (CT⁺) and *Escherichia coli* K12 185 ST-LT⁻ (thermostable and thermolabile enterotoxin negative) were used as controls.

Culture filtrates of some enterotoxin-producing strains were heated at 100°C for 30 min and the IW/BW ratios induced by the heated and unheated filtrates were determined.

Haemagglutination assays and haemolytic activity

Human blood group O was collected by venepuncture and placed into Alsever's solution. For haemagglutination assays, blood cells were washed three times in phosphate-buffered saline (PBS), pH 7.4, and a 3% v/v final suspension was prepared in this solution. Bacterial strains were grown at 25°C on TSA and on blood agar prepared from TSA with citrated sheep blood 5%. After 24 and 48 h, suspensions of the bacteria were prepared in PBS and adjusted to an optical density (OD) to yield c. 10^9 cells/ml. Slide agglutination tests were performed essentially as described previously.¹⁹ Strains were considered to be non-haemagglutinating if agglutination did not occur within 10 min. The Minimum Haemagglutinating Dose (MHD) was defined as the smallest number of bacterial cells/ml that gave visible haemagglutination

Table I. Drug-resistance markers and plasmid profiles of *V. cholerae* non-O1 strains isolated from water samples in Bangladesh

Strain no.	Presence (and number) of plasmids	Size of plasmids (Mda)*	Resistance patterns
M-17151	+(1)	115	Am K S St Te
M-17153	+(1)	115	Am K S St Te
M-17214	—	—	St
M-17221	—	—	St
M-17225	—	—	Am S St
TK-17065	+(3)	115; 20; 1.7	Am S St Te
TK-17068	+(1)	115	Am
TK-17081	—	—	Am St
TK-17085	—	—	Am
TK-17121	+(1)	4.6	Am S St
TK-17599	+(3)	115; 28; 4.8	Am St Te
TK-17600	—	—	Am K S St
TK-17604	—	—	St
TK-17610	—	—	Am St
TK-17747	—	—	Am S
TK-17750	—	—	Am S
TK-17751	—	—	Am St Te
TK-17752	+(1)	115	Am St
TK-17755	—	—	Am St
TK-17841	+(1)	18	Am St
D-17372	—	—	St
D-17382	—	—	S St
D-17387	—	—	St
D-17401	—	—	Am St
D-17409	—	—	...
D-17410	—	—	...
D-17417	—	—	St
D-17419	—	—	...
D-17421	—	—	...
D-17424	—	—	St
D-17432	—	—	St
D-17435	—	—	S St
D-17447	—	—	St
D-17450	—	—	...
D-17451	+(2)	115; 3.4	Am S St Te
D-17467	—	—	St
D-17492	—	—	St
D-17642	+(1)	2.5	St
D-17833	—	—	...
D-17892	—	—	St
D-17897	+(2)	3.4; 2	Am St
D-17912	—	—	St
D-17914	—	—	St
D-17917	—	—	St
D-18009	—	—	...
D-18045	—	—	...
D-18046	—	—	...
D-18047	—	—	...
D-18048	—	—	...
D-18050	—	—	...
D-18076	—	—	St

D, Dacca; M, Matlab; TK, Teknaf.

Am, ampicillin; K, kanamycin; S, streptomycin; St, sulphathiazole; Te, tetracycline.

..., Sensitive strains.

* Mol. wt in Mda, determined by agarose gel electrophoresis.

in 10 min and the haemagglutination power of a culture was calculated as 10^{11} /MHD.²⁰ Inhibition of haemagglutination was performed in the same way as the agglutination test but with bacterial suspensions containing 10 MHD in PBS supplemented with D-mannose (Sigma) 1% w/v.

Haemolysin production by the *V. cholerae* non-O1 strains was detected by eye on TSA plates supplemented with defibrinated sheep blood 5% after incubation for 24 and 48 h at 25°C.

Detection of plasmids by agarose gel electrophoresis

Plasmid DNA was isolated from small volumes (3–4 ml) of bacterial cultures, following basically the method of Kado and Liu²¹ with several modifications.²² DNA samples (25 µl) were electrophoresed through agarose (type I, Sigma) 0.7% in Tris-acetate buffer (40 mM Tris, 2 mM Na₂-EDTA, adjusted to pH 7.9 with glacial acetic acid) at 150 V, for 4 h in a horizontal apparatus. The gels were stained in ethidium bromide 0.5 µg/ml of water. Plasmids used as markers were: TP 116 (143.7 Mda), R40a (96 Mda), R1 (62 Mda), R27 (112 Mda), and plasmids from *E. coli* V517 1.4–35.8 Mda.

Curing experiments

To assess the possible relationship between plasmid content and phenotypic traits, *V. cholerae* non-O1 strains were treated with several curing agents: mitomycin C (Sigma), ethidium bromide (Sigma), and sodium dodecyl sulphate (SDS; Sigma). Cells were grown for 24 h at 25°C in broth containing mitomycin C 0.5, 0.05 and 0.005 µg/ml, or ethidium bromide 0.4, 0.04 and 0.004 mg/ml. The SDS concentrations used were 1% and 0.1% w/v. After these treatments, cultures were plated on TSA and the colonies were replica-plated on to the same media with and without the appropriate antibiotics. Only those colonies were picked from the antibiotic-free master plate whose replicas failed to grow on media containing antibiotics, and their plasmid content was determined. In addition, colonies of toxigenic strains were selected at random from the master plates and their cytotoxic activity determined.

Conjugal-transfer assays

Four multiresistant *V. cholerae* non-O1 strains, each carrying a 115-Mda plasmid, were used as potential donors in mating experiments with *E. coli* K12 185 Na⁺lac⁺ ST⁻ LT⁻ (nalidixic acid resistant, lactose positive) as the recipient strain. Matings were performed in TSB at 25°C as previously described.²³ At 4 and 18 h after the start the experiment, each mating mixture was plated on BTB-lactose agar—Nutrient Agar (Difco) containing bromothymol blue 0.0045% and lactose 1%—supplemented with nalidixic acid 100 µg/ml and selected antibiotics (kanamycin, streptomycin and tetracycline) 25 µg/ml. The frequency of transfer was calculated as the number of transconjugants/initial number of donors/ml.

For the strains showing donor ability to *E. coli*, these conjugal studies were extended with the nalidixic acid-resistant derivatives obtained from *Aeromonas hydrophila* 67-P-24, *V. anguillarum* PB-15 (fish pathogenic strains) and *V. harveyi* KN-96 as recipient strains.

Transconjugants isolated from each selective plate were checked for drug resistance and the presence of plasmids. The *E. coli* transconjugants were also assayed for cytotoxic (with Y-1 cells) and enterotoxigenic activities.

Growth under iron-limiting conditions and analysis of cell-envelope proteins

V. cholerae non-O1 strains were cultured in M9 minimal medium (composition in g/l: Na₂HPO₄, 6; KH₂PO₄, 3; NaCl, 5; NH₄Cl, 1; CaCl₂, 0.0015; MgSO₄, 0.012; glucose, 5; pH 7.2) supplemented with Casamino acids (Difco) 0.2% w/v. The iron chelator ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDA) (Sigma) was added to produce a concentration of 10 µM to achieve the iron-limitation conditions.

Total and outer-membrane proteins were prepared as previously described.^{24, 25} The outer membranes were obtained by treatment of the cell envelopes with Sarkosyl 1.5% w/v in 10 mM Tris-HCl (pH 8.0) at room temperature for 20 min to dissolve the inner membranes. Pellets from total cell envelopes or outer membranes were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on separating gels of acrylamide 12.5% and stacking gels of 3%. Induction of new proteins under iron limitation was revealed by comparisons between the protein patterns of the strains grown in M9 minimal medium containing either 10 µM EDDA or 10 µM FeCl₃.

Results

Cytotoxic and enterotoxigenic activities

The cell-free culture supernates of 13 strains of *V. cholerae* non-O1 were tested for cytotoxic activity on Y-1 and CHO cells. Four strains showed a cytotoxic response similar to *V. cholerae* LA 4808 in both cell lines employed and two strains exhibited activity only on Y-1 cells (table II). Although culture supernates of some strains (D-17451, D-17447 and D-17642) produced morphological changes in CHO or Y-1 cells within 6 or 8 h, the extracts of other positive strains (M-17151, M-17153 and TK-17068) required 20 h to display the same degree of activity.

The enterotoxigenic activity of the supernates, as determined by the suckling mouse assay, revealed that all the cytotoxic *V. cholerae* non-O1 strains also produced enterotoxins. However, two of the non-cytotoxic strains (TK-17121 and TK-17599) were also enterotoxigenic.

The enterotoxins produced by the *V. cholerae*

Table II. Cytotoxic, enterotoxic and haemagglutinating activities of *V. cholerae* non-O1 and other strains

Strain no.	Cytotoxic response*		Enterotoxin activity in suckling mouse assay (IW/BW)†	Haemagglutination power‡
	Y-1	CHO		
M-17151	+	+	+(0.095)	2.5 × 10 ³ (R)
M-17153	+	+	+(0.090)	2.5 × 10 ³ (R)
TK-17068	+	—	+(0.084)	—
TK-17065	—	—	—(0.070)	—
TK-17081	—	ND	—(0.070)	—
TK-17121	—	—	+(0.091)	—
TK-17599	—	—	+(0.084)	—
TK-17599-B§	—	—	+(0.088)	—
TK-17752	—	—	—(0.071)	—
TK-17841	—	ND	ND	ND
D-17447	+	ND	+(0.083)	—
D-17451	+	+	+(0.10)	—
D-17642	+	+	+(0.087)	6.6 × 10 ⁴ (R)
Controls				
<i>V. cholerae</i> LA4808	+	+	+(0.11)	8.3 × 10 ⁴ (R)
<i>E. coli</i> K12 185	—	—	—(0.060)	ND

ND, Not determined.

* Responses of cell lines to culture supernates were recorded after treatment for 6–20 h; +, a morphological response exhibited by >75% of the cells; —, no morphological response.

† Fluid accumulation ratio (intestinal weight remaining/body weight). Results are expressed as mean values of three different experiments for each strain.

‡ Results were obtained from cultures grown on TSA at 25°C for 48 h. (R), indicates haemagglutination resistant to inhibition by D-mannose; —, non-haemagglutinating.

§ Plasmid-less strain derived from the parental *V. cholerae* non-O1 strain TK-17599.

non-O1 strains differed in their stability to heat. Whereas the enterotoxin from strain D-17451 was heat-labile, those produced by strains M-17151 and M-17153 were heat stable.

Haemagglutinating and haemolytic activities

Only three strains (M-17151, M-17153, and D-17642) together with *V. cholerae* LA 4808 displayed strong agglutination of human erythrocytes that was resistant to inhibition by D-mannose. These strains were also cytotoxic and enterotoxic. However, the other toxigenic strains were unable to haemagglutinate human O erythrocytes.

To detect possible differences in adhesin expression under in-vitro conditions, the haemagglutination powers of the strains grown on TSA and blood agar were compared and found to be the same. Transient haemagglutination by the negative strains was not observed after growth on either of these media.

Analysis of the haemolytic activity of these estuarine vibrios revealed that only strains with

cytotoxic activity produced haemolysis of sheep erythrocytes (data not shown).

Plasmid content of V. cholerae non-O1 strains

Analysis of the plasmid content of the *V. cholerae* non-O1 strains revealed that 11 (21.5%) strains carried one or more plasmids of 1.7–115 Mda (table I). Seven strains carried the 115-Mda high-mol.-wt plasmid and three strains carried only small (< 5 Mda) mol.-wt plasmids. Only in the *V. cholerae* non-O1 strain TK-17599 was a 28-Mda plasmid band detected.

Curing experiments

To determine relationships, if any, between plasmid carriage and antibiotic resistance or cytotoxic properties or both, curing experiments were performed with four of the 11 plasmid-bearing strains—D-17642, M-17151 and D-17451, which had exhibited cytotoxic activity on Y-1 and CHO cells, and strain TK-17599 which had not. Tetra-

cycline-sensitive derivatives were isolated only from *V. cholerae* non-O1 strain TK-17599 after growth in media containing either ethidium bromide or mitomycin. The loss of tetracycline resistance was accompanied by the loss of the small-mol.-wt plasmid (4.8 Mda) in all the susceptible derivatives but their enterotoxigenic abilities were not modified.

Because neither drug-sensitive nor plasmid-less derivatives of the other *Vibrio* strains were isolated, it was not determined whether drug resistance or cytotoxic activity is plasmid-mediated in these strains.

Mating assays

In an attempt to characterise the 115-Mda plasmid present in a group of multiresistant *V. cholerae* non-O1 strains, four of these strains were mated with *E. coli* K12 185 Na^r. The results (table III) show that only the toxigenic strains M-17151 and M-17153 transferred drug resistance genes coding for streptomycin, kanamycin and sulphathiazole and did so with frequencies of *c.* 10⁻⁴. Furthermore, both strains transmitted the 115-Mda plasmid to the environmental *Aeromonas* and *Vibrio* strains used as recipients, with similar or higher frequencies than obtained with *E. coli*.

Interestingly, culture filtrates of the transconjugant *E. coli* strains displayed cytotoxic activity on

Y-1 cells similar to that given by those of the respective donor strains. However, culture filtrates of these transconjugants did not exhibit enterotoxin activity, which indicated that the enterotoxin production was not plasmid mediated in these *V. cholerae* non-O1 strains.

By analysis of the plasmid content of transconjugants we demonstrated a correlation between the specific 115-Mda plasmid and the antibiotic resistances and cytotoxin production transferred. The fact that other non-toxigenic strains with different drug resistance patterns also carried the 115-Mda plasmid, indicated that the plasmid coded for different phenotypic traits in these *V. cholerae* non-O1 strains.

Induction of outer-membrane proteins in conditions of iron limitation

We compared the ability of selected strains, with and without toxic activities (table II) to grow in the presence or absence of iron (as FeCl₃). Growth of all strains tested was found to be independent of the presence of iron in the medium. Analysis of total cell envelopes, as well as outer membranes, showed that an intense major outer-membrane protein of 25 Kda was common to most of the strains and that, among the enterotoxin-forming strains tested, three different patterns of outer-membrane proteins were induced in conditions of

Table III. Conjugal transfer assays of drug-resistance factors from selected *V. cholerae* non-O1 strains to *E. coli* K12 and different environmental *Aeromonas* and *Vibrio* strains

Donor strain	Drug resistance pattern*	Plasmid content (Mda)	Selective resistance markers	Transfer frequency‡ to recipient strain				Resistance transferred	Plasmid (Mda) transferred
				<i>E. coli</i> K12 185 Na ^r	<i>A. hydrophila</i> 67-P-24 Na ^r	<i>V. anguillarum</i> PB-15 S ^r Na ^r	<i>V. harveyi</i> KN-96 S ^r Na ^r		
<i>Toxigenic strains</i>									
M-17151	Am K S St Te	115	K	1.5×10^{-4}	2.0×10^{-2}	2.0×10^{-3}	1.4×10^{-4}	K S St†	115
			S	8.0×10^{-5}	4.5×10^{-3}	NA	NA	K S St†	115
			Te	...	...	...	...		
M-17153	Am K S St Te	115	K	2.5×10^{-4}	4.5×10^{-2}	4.0×10^{-3}	2.2×10^{-4}	K S St†	115
			S	1.3×10^{-4}	5.0×10^{-3}	NA	NA	K S St†	115
			Te	...	...	...	...		
<i>Non-cytotoxic strains</i>									
TK-17065	Am St Te	115; 20; 1.7	Te	...	NT	NT	NT		
TK-17599	Am St Te	115; 28; 4.8	Te	...	NT	NT	NT		

NA, Not applicable.

..., No transconjugants recovered.

NT, Not tested.

* See footnote to table I.

† Transfer frequencies expressed as the number of transconjugants/initial donor cell/ml.

† The transconjugants in *E. coli* showed also cytotoxic activity on Y-1 cells.

iron limitation (see figure). Enterotoxigenic and cytotoxigenic strains M-17153, M-17151 and D-17642 showed induction of three proteins of 76, 74 and 72 Kda (figure; lanes B₂, C₂ and G₂) whereas in strain D-17451, outer-membrane proteins of 72, 70 and 65 Kda were induced (figure; lane D₂). For strains TK-17599 and TK-17121, with only enterotoxic activity, the mol. wts of the induced proteins were 76 and 70 Kda (figure; lanes E₂, H₂). Differences in membrane proteins between parental and plasmid-cured derivative isolates of *V. cholerae* non-O1 TK-17599 were not detected (figure; lanes E, F).

All of the outer-membrane proteins induced in *V. cholerae* non-O1 strains were different in mol. wt from the OM2 protein (86 Kda) mediated by the virulence plasmid pJM1 and from the chromosome-mediated OM3 protein (79 Kda) present in *V. anguillarum* strain 775²⁴ (figure; lane A₂).

Discussion

Serology is not a definitive indicator of pathogenicity because *V. cholerae* O1 strains have been isolated that do not possess the genetic potential for

Figure. SDS-page of total cell envelopes of selected *V. cholerae* non-O1 strains with and without induction of outer-membrane proteins under iron limitation conditions: A, *V. anguillarum* 775; B, *V. cholerae* non-O1 M-17153; C, M-17151; D, D-17451; E, TK-17599, parental strain; F, tetracycline-sensitive derivative of TK-17599; G, D-17642; H, TK-17121; 1 indicates cells grown in medium containing 10 μ M FeCl₃; 2 indicates cells grown under conditions of iron limitation. Some of the induced outer-membrane proteins are indicated by bars. The position of OM2 and OM3 proteins of the *V. anguillarum* 775 strain is shown as reference. Numbers indicate mol. wt in Kda.

production of cholera toxin and, in contrast, some non-O1 *Vibrio* strains produce diarrhoea mediated by cholera toxin (CT) or CT-like toxins.^{1, 5, 26} The toxicity assays on *V. cholerae* non-O1 strains isolated from water in Bangladesh revealed a high percentage of toxigenic strains (table II). This finding differs from that of Twedt *et al.*,² in which none of the *V. cholerae* non-O1 strains tested was toxigenic. The fact that we have found non-cytotoxic strains with enterotoxin activity indicates that cytotoxicity tests alone do not permit detection of all the potentially pathogenic *V. cholerae* non-O1 strains in a sample. We have reported similar findings for environmental *V. anguillarum* and *A. hydrophila* strains.^{19, 27, 28}

Only three of the enterotoxic and cytotoxic strains of *V. cholerae* non-O1 agglutinated human erythrocytes. This agglutination was found to be resistant to inhibition by D-mannose (table II) suggesting that this carbohydrate, or an analogue of it, is part of a receptor for bacterial adhesins in these strains. This type of haemagglutination is different from the D-mannose-sensitive haemagglutination reported in *V. cholerae* by Jones and Freter.²⁹ The fact that other toxigenic strains were not haemagglutinating suggests that there is no correlation between toxin production and haemagglutinating activity in these *V. cholerae* non-O1 strains.

Although all cytotoxic *Vibrio* strains displayed haemolytic activity, it is not possible to state what role the haemolysins play in disease because, as previously reported,¹⁹ both pathogenic and non-pathogenic marine vibrios are able to produce them.

The analysis of plasmids in the *Vibrio* isolates revealed that strains containing either no plasmid or several different plasmids (table I) showed similar cytotoxic, enterotoxic and haemagglutinating activities (table II), indicating that these properties are not necessarily plasmid-mediated in all the strains. The transfer studies conducted to characterise the 115-Mda plasmid common to a group of toxigenic and non-toxigenic, multiresistant, *V. cholerae* non-O1 strains, allowed us to demonstrate that, in the toxigenic strains tested, this plasmid carried genes coding for kanamycin, streptomycin and sulphathiazole resistance and cytotoxin production but not enterotoxin production. However, the 115-Mda

plasmid was associated with distinct phenotypic traits, because it was also carried by strains lacking cytotoxic or enterotoxic activities, or both, and with different antibiotic resistance patterns.

The availability of host iron has been proposed as a contributory factor in infections caused by diverse bacterial pathogens. The growth of pathogenic strains in low-iron medium can induce the synthesis of new outer-membrane proteins, some of which constitute a surface receptor for the siderophores.^{25, 30-32} In this study, some *V. cholerae* non-O1 strains were able to grow under iron-limiting conditions and the synthesis of at least two new outer-membrane proteins was induced thereby (figure). However, the fact that the non-toxigenic vibrios examined also showed the same properties indicates that the iron-sequestering system is not the only factor playing a role in the toxigenicity of these *V. cholerae* non-O1 strains.

Analysis of total cell-envelope proteins revealed the presence of a major outer-membrane protein of 25 Kda common to the majority of the *Vibrio* strains examined (figure). Interestingly, this protein was also detected in all the *V. cholerae* as well as in the *V. cholerae* non-O1 strains studied by Manning *et al.*³³ The outer-membrane-protein analysis also provided a useful tool to determine relatedness between strains. Thus, strains with comparable cytotoxic, enterotoxic, haemagglutinating and haemolytic activities (M-17151, M-17153 and D-17642) yielded the same iron-limitation induced outer-membrane-protein patterns (figure).

From the results reported here, we conclude that, as with other vibrios,^{25, 27, 31, 34} factors associated with virulence in *V. cholerae* non-O1 strains can be determined by chromosomal- or plasmid-borne genes, depending on the strain. Furthermore, the detection in some *V. cholerae* non-O1 strains of cytotoxic activity and drug resistance coded by a plasmid transferable to enteric bacteria as well as to environmental *Aeromonas* and *Vibrio* strains, is a matter of concern for public health.

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