Expression and Detection of Different Biotype-associated Cell-bound Haemagglutinins of *Vibrio cholerae* O1

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(Received 21 April 1988; revised 26 August 1988; accepted 30 September 1988)

The expression of two cell-bound haemagglutinins, one sensitive to L-fucose (FSHA) and the other to D-mannose (MSHA), on *Vibrio cholerae* O1 strains of both the classical and the El Tor biotypes was studied by (i) agglutination of chicken and human group O erythrocytes in the presence of L-fucose or D-mannose, (ii) binding of the bacteria to L-fucose- and D-mannose-coated agarose beads, and (iii) agglutination of the bacteria by 'biotype-specific' antisera. All of the 12 classical strains studied that were isolated before 1979 gave FSHA of human O erythrocytes whereas only 6 of 17 classical strains isolated during recent epidemics expressed FSHA; a few of the classical strains expressed MSHA in addition to FSHA. All the El Tor strains gave MSHA of chicken erythrocytes and one strain also expressed FSHA. Both the cell-bound HAs were optimally expressed during the exponential phase of growth; FSHA markedly decreased during the late exponential phase while the MSHA usually persisted into the stationary phase. The expression of FSHA and MSHA correlated very well with the direct binding of vibrios to fucose- and mannose-coated agarose beads, respectively. Antiserum 'specific' for classical vibrios agglutinated classical strains expressing FSHA and also the El Tor strain exhibiting FSHA. Similarly, the anti-El Tor serum agglutinated all El Tor strains and also classical strains expressing MSHA, suggesting that the 'biotype-specific' sera were specific for the biotype-associated cell-bound HAs.

INTRODUCTION

Cholera is caused by two different biotypes, classical and El Tor, of *Vibrio cholerae* O group 1, which are further divided into the serotypes Inaba and Ogawa. The two serotypes, which have both shared and non-crossreactive O antigenic determinants (Barua, 1974; Sakazaki *et al.*, 1970), can be differentiated by agglutination with serotype-specific antisera. The different biotypes, on the other hand, are distinguished by a number of non-immunological tests (Barua, 1974).

V. cholerae O1 bacteria agglutinate certain species of erythrocytes and this property correlates with adherence to brush border membranes (Jones & Freter, 1976). Adhesion to the intestinal epithelium and haemagglutination (HA) by the vibrios may be inhibited by different sugars (Bhattacharjee & Srivastava, 1978; Jones & Freter, 1976). There is some evidence that the cell-bound HAs expressed by *V. cholerae* are indeed responsible for the adherence of the bacteria to intestinal epithelium (Finn *et al.*, 1987; Guinée *et al.*, 1985; Jones *et al.*, 1976). However, a direct correlation between intestinal attachment and HA expression has not always been seen (Freter & Jones, 1976; Teppema *et al.*, 1987). At least three different types of cell-bound haemagglutinins (HAs) of *V. cholerae*, one being sensitive to D-mannose (MSHA), another to L-

Abbreviations: HA, haemagglutinin (or haemagglutination); FS, fucose-sensitive; MS, mannose-sensitive; FR, fucose-resistant; MR, mannose-resistant; HAI, haemagglutination inhibition; CI, classical; TSA, Trypticase Soy Agar; TSB, Trypticase Soy Broth; KRT, Krebs–Ringer solution.

fucose (FSHA), and the third being resistant to both sugars (MRFRHA) have been described (Bhattacharjee & Srivastava, 1978; Hanne & Finkelstein, 1982; Holmgren *et al.*, 1983); an MRFRHA was recently cloned (van Dongen & DeGraaf, 1986). In previous studies (Holmgren *et al.*, 1983) we have proposed that the FSHA is associated with the classical biotype and the MSHA with the El Tor biotype, whereas no biotype-association with the MRFRHA has been reported.

For many bacteria the HA property is associated with the possession of adhesive fimbriae on the bacterial surface (Gaastra & DeGraaf, 1982; Duguid & Old, 1980). Recently Taylor *et al.* (1987) convincingly demonstrated the existence of fimbriae on *V. cholerae*. These fimbriae are co-regulated with cholera toxin by the *toxR* gene. Studies in human volunteers have suggested that the fimbriae are important for the colonization of the small intestine by the vibrios (Herrington *et al.*, 1987). However, it is still unclear whether these fimbriae are associated with HA properties.

The present study was undertaken to characterize the FS and MS cell-bound HAs on a large number of *V. cholerae* O1 isolates with regard to biotype-association, expression during different culture conditions and reactivity with different species of erythrocytes. Another aim was to evaluate alternative methods for identifying the FSHA and MSHA, respectively.

METHODS

Bacterial strains. Twenty-nine classical and 19 El Tor *V. cholerae* O1 strains were used in this study. Their strain designations, biotypes, serotypes and sources are indicated in Table 1. All strains were kept at -70°C in broth containing 20% (v/v) glycerol.

Culture of bacteria. Colonies from a fresh blood agar plate inoculated with bacteria previously stored at -70°C were suspended in Trypticase Soy Broth (TSB) without glucose, and incubated without shaking at 37°C for 16 h. Then 1 ml of the culture was transferred into 40 ml TSB without glucose in a 250 ml flask and cultured with shaking (200 r.p.m.) at 37°C ; in some instances bacteria were also cultured at 27°C . In initial studies, samples from the cultures were collected every hour during the first 8 h and then at 10 (or 12), 16 and 20 h. Bacterial concentrations were determined according to optical density in a Corning 252 spectrophotometer using a 600 nm filter, and 1 ml samples of the cultures were centrifuged, washed and resuspended in modified Krebs-Ringer solution (KRT), pH 7.4 (Jones *et al.*, 1976).

Based on the results of the initial kinetic studies, when HA production for various *V. cholerae* strains was followed during 20 h, the bacteria were in subsequent cultures grown until they reached a concentration of $\sim 3\text{--}5 \times 10^9$ bacteria ml^{-1} ($\text{OD}_{600} \sim 1.0\text{--}1.5$), which was usually reached within 4–6 h and corresponded to the mid-exponential phase of the growth curve. The bacteria were then harvested by centrifugation at 8000 g at 4°C for 10 min and washed and resuspended in KRT buffer to give final concentrations of 10^{10} or 5×10^{10} bacteria ml^{-1} . In some instances the strains were grown in Syncase medium (Finkelstein *et al.*, 1966) or inoculated on blood agar or Tryptic Soy Agar (TSA) plates and incubated at 37°C for 16 h.

Purified antigens. Soluble HA was purified from the supernate of a stationary-phase culture of strain *V. cholerae* O17SR as previously described (Svennerholm *et al.*, 1983). In short, bacteria were cultured in TSB at 37°C for 18 h with shaking. The culture supernate was then subjected to precipitation with 50% saturated ammonium sulphate, gel filtration on a Sephacryl S-300 column and finally preparative agarose electrophoresis. Purified *V. cholerae* lipopolysaccharide (LPS) was prepared from strain 569B (Inaba) and strain 34 (Ogawa) by hot phenol/water extraction followed by repeated ultracentrifugation (Ørskov *et al.*, 1967). Purified cholera toxin was purchased from List Biological Laboratories.

Antisera. Antisera were prepared in New Zealand White rabbits weighing approximately 3 kg at the onset of immunization. Four to five injections were given subcutaneously with 2-week intervals; the initial two immunizations were given in Freund's complete adjuvant. The final bleeding was performed 1–2 weeks after the last immunization.

Antiserum 'specific' for El Tor vibrios (anti-El Tor) was produced by immunizing rabbits with 5×10^9 mid-exponential-phase, cell-bound HA-positive El Tor vibrios (strain X25049, Ogawa) per injection and subsequently absorbing the serum twice with washed and boiled bacteria of the homologous strain and thereafter twice with washed, live mid-exponential-phase classical vibrios (strain T19766, Ogawa). Antiserum 'specific' for classical vibrios (anti-CI) was prepared by immunizing with mid-exponential-phase cell-bound HA-positive classical vibrios (strain 1451, Ogawa) and subsequently absorbing the serum twice with washed and boiled mid-exponential-phase bacteria of the homologous strain and thereafter twice with washed, live mid-exponential-phase cell-bound HA-positive El Tor vibrios (strain T20567, Ogawa). The absorptions were performed by mixing

equal volumes of antiserum and bacterial suspension (final concentration 5×10^{10} organisms ml^{-1}) and incubating the mixtures at 37°C for 45 min with rotation. After centrifugation at $11\,000\text{ g}$ at 4°C for 10 min the supernates were heat-inactivated at 56°C for 30 min and stored in portions at -30°C until used.

Haemagglutination tests. Haemagglutination of bacteria at an initial concentration of 10^{10} organisms ml^{-1} , and twofold dilutions thereof in KRT buffer (25 μl per well) was done in polyvinyl U-bottom microtitration plates (Dynatech Laboratories). To each well was added 25 μl of a 1% (v/v) erythrocyte suspension in KRT and the titre was recorded, after incubation at room temperature for 30–60 min, as the reciprocal of the highest dilution giving strong HA. All titrations were done in duplicate on the same day and in most instances the experiments were repeated on one or more separate occasions; the results given are the means of all separate titrations for each individual strain. The variation in the HA titre of each strain never exceeded a fourfold difference between the highest and lowest titre.

Slide agglutination was done by mixing 15 μl of bacteria (1×10^{10} organisms ml^{-1}) with 15 μl of a 3% (v/v) human or chicken erythrocyte suspension in KRT and then slowly rotating the slide for 3 min. HA was recorded as +++ (strong HA), ++ (medium HA) or + (weak HA). Haemagglutination inhibition (HAI) was similarly done by mixing the bacteria with a 0.1% (0.006 M) solution of D-mannose or L-fucose in KRT buffer for 30 s before the erythrocyte suspension was added (Holmgren *et al.*, 1983). All analyses were done in duplicate on the same day and in all instances repeated at least twice on separate days.

Binding to sugar-coated agarose beads. Classical and El Tor vibrios were tested for their ability to bind to fucose and mannose covalently attached to agarose beads (agarose-*p*-aminophenyl α -L-fucopyranoside and agarose-*p*-aminophenyl α -D-mannopyranoside) on a glass slide. Bacteria (10 μl of 1×10^{10} bacteria ml^{-1}) were mixed with 20 μl of the respective bead preparation diluted 1 in 4 in KRT buffer at room temperature. After rotating the slide gently for 1–2 min the binding of the bacteria to the different beads was determined under the phase-contrast microscope at a magnification of $400\times$ and recorded as +++, ++, or + according to the number of bacteria attached to individual beads. When binding was weak incubation was continued for up to 5 or 10 min. All experiments were repeated twice on separate days, and the results given are the means of the separate determinations. The variation in binding for each strain was low, with differences within one unit.

Agglutination with antisera. Fifteen microlitres of anti-El Tor serum, anti-CI serum and KRT buffer were each mixed with 15 μl of bacteria (5×10^{10} bacteria ml^{-1}) on a glass slide. After gently rotating the slide for 3 min, visible agglutination was recorded as + (strong agglutination), (+) (partial agglutination) or – (no visible agglutination). In cases of + reactions with antisera, threefold dilutions of the sera were tested; the agglutinating titre was determined as the dilution of antiserum giving a + reaction with 5×10^{10} bacteria ml^{-1} of the respective strain. Experiments were done in duplicate on the same day and in most instances also repeated on a new batch of bacteria on a separate day. Most results were fully reproducible; some varied with a threefold difference in agglutination titre.

ELISA methods. Anti-El Tor and anti-CI sera were evaluated for their reactivity with serotype-homologous *V. cholerae* LPS by ELISA (Holmgren & Svennerholm, 1973). Only sera that, in the concentrations used in the test, lacked significant levels of anti-LPS antibodies, i.e. titres not exceeding that of rabbit preimmune sera, were used. Lack of significant antibody titres in these sera against cholera toxin or soluble HA was also ascertained by ELISA methods (Holmgren & Svennerholm, 1973; Svennerholm *et al.*, 1983).

Physical and chemical treatment of bacteria. Suspensions of strains 1451 and Cairo 48 (classical), T19479 and X25049 (El Tor) were diluted tenfold in KRT buffer of different pH values to 2×10^9 bacteria ml^{-1} and incubated at pH values ranging from 2 to 10. After incubation at 4°C for 30 min the suspensions were neutralized and the HA titre determined. Bacteria (10^{10} organisms ml^{-1}) were also incubated at different temperatures (20, 37, 56 and 80°C) for 30 min or in 0.005 M-EDTA.

RESULTS

Expression of cell-bound HAs in different media

The optimal culture conditions for expression of the cell-bound HAs on classical and El Tor *V. cholerae* O1 were evaluated. Six of the strains representing both biotypes and serotypes were cultured in parallel in different liquid and solid media and their ability to agglutinate different erythrocytes was determined. Human group O and chicken erythrocytes were used because previous studies have shown that these red blood cells give optimal agglutination by classical and El Tor *V. cholerae*, respectively (Holmgren *et al.*, 1983). The titre of cell-bound HA on classical *V. cholerae* was usually 2–4-fold higher after growth in TSB without glucose than after growth in Syncase, and 8–16-fold higher than that of bacteria grown on TSA or blood agar. The cell-bound HA titre of El Tor vibrios was similar after growth in TSB without glucose and in

Syncase but was 8-fold higher than after growth on solid media. TSB without glucose was therefore chosen as the culture medium for subsequent studies.

Specificity of cell-bound HAs for different species of erythrocytes

The specificity of the two biotypes of *V. cholerae* in agglutinating chicken and human O erythrocytes was evaluated. A number of classical and El Tor strains were titrated in parallel against the two types of erythrocytes. Most (10 of 13) of the classical strains that agglutinated human O erythrocytes also reacted strongly with chicken erythrocytes although titres against human O erythrocytes were ≥ 4 -fold higher for 8 of the 13 HA-positive strains. All the El Tor strains agglutinated chicken as well as human O erythrocytes; the HA titre was usually somewhat higher against the chicken cells but a ≥ 4 -fold difference was seen only in 4 of the 14 strains.

Sugar specificity of cell-bound HAs

Strains were tested for HA with human O and chicken erythrocytes and for HA inhibition with mannose and fucose, respectively. All classical strains isolated before 1979 (except 569B) strongly agglutinated human O erythrocytes and this HA could in all instances be completely or partly inhibited by 0.1% fucose (Table 1a). Classical strains isolated in Bangladesh after 1978 only rarely (6 of 17) agglutinated human O erythrocytes; however, some of the HA-negative strains that were tested after passages on meat extract agar, pH 8.5, agglutinated human O cells in an FS manner (not shown). The sugar inhibition pattern differed somewhat when chicken erythrocytes were used; e.g. strains 569B and Cairo 50 were not inhibited by 0.1% fucose but were inhibited by 0.1% mannose, demonstrating the expression of both FSHA and MSHA by these strains (Table 1a). All the El Tor strains tested agglutinated chicken erythrocytes (Table 1b). This HA could in all instances be completely or partly inhibited by 0.1% mannose whereas 0.1% fucose with one exception had no effect; mannose was often less effective in inhibiting HA by El Tor vibrios when human erythrocytes were used (Table 1b).

Identification of cell-bound HAs by binding to sugar-coated beads and agglutination with antisera

The possibility of using a method based on binding of bacteria to agarose beads with covalently coupled mannose or fucose for screening of the two HAs was evaluated by testing 15 classical and 13 El Tor strains in parallel for HAI with the two sugars and for binding to sugar-coated agarose beads (Table 2). All classical strains which exhibited ++ or +++ HA that could be inhibited by fucose, bound to fucose-coated agarose beads. With the exception of strain Cairo 50, which also expressed MSHA, classical strains failed to bind to mannose-coated beads. All the El Tor strains which expressed MSHA, bound strongly to mannose-coated agarose beads and only one strain that also repeatedly exhibited FSHA (Phil 6973) also bound significantly to fucose-coated beads (Table 2).

The different strains were also tested, in parallel with HAI and binding to sugar-coated beads, for agglutination with the 'biotype-specific' anti-CI and anti-El Tor *V. cholerae* sera, respectively. All but one (569B) of the classical strains were agglutinated by the anti-CI serum. In some instances the anti-El Tor serum agglutinated the classical strains but usually in lower titre (Table 2). One strain, Cairo 50, that repeatedly expressed MSHA and bound strongly to mannose-coated beads, was strongly agglutinated by the anti-El Tor serum.

The El Tor strains were all agglutinated by the anti-El Tor serum, which had a high titre against most strains, whereas the anti-CI serum was relatively ineffective (Table 2). Prior incubation of El Tor T19479 with 1% (v/v) mannose repeatedly inhibited agglutination at least fourfold with anti-El Tor antibodies, and preincubation of classical Cairo 48 with fucose partly inhibited agglutination with anti-CI antibodies (Table 3).

Effect of physical and chemical treatment of HAs

The effect of incubation at different pH values and temperatures or in EDTA on the cell-bound HA activity was evaluated by comparing HA titres for four different vibrio strains before and after various treatments (Table 4). Cell-bound HA activity of classical as well as El Tor

Table 1. *Haemagglutination by classical and El Tor V. cholerae O1 strains of different species of erythrocytes and inhibition of haemagglutination with monosaccharides*

Strain†	Serotype‡	Year of isolation	Haemagglutination*					
			Human O erythrocytes			Chicken erythrocytes		
			Inhibitor:			Inhibitor:		
			KRT	Mannose	Fucose	KRT	Mannose	Fucose
(a) Classical strains								
1 ^a	I	<1970	+++	+++	—	+	+	—
34 ^a	O	<1970	+++	+++	—	+	+	—
35 ^a	I	<1970	+++	+++	—	++	++	—
48 ^a	I	1958	+++	+++	—	+	+	—
84 ^a	I	<1970	+++	+++	—	++	+	—
86 ^a	I	1941	+++	+++	(+)	++	++	—
569B ^a	I	<1970	+	(+)	—	(+)	—	(+)
1449 ^a	I	1962	+++	+++	+	++	++	—
1451 ^a	O	1962	+++	+++	—	++	++	—
57 rough ^a	O	<1970	+++	+++	—	+	++	—
Cairo 48 ^b	I	1949	+++	+++	—	+++	+++	—
Cairo 50 ^b	O	1949	+++	+	—	+++	—	+++
V63P ^{—c}	I	1979	+++	+++	—	++	+	—
T19766 ^c	O	1979	+++	+++	—	+++	+++	—
T19767 ^c	O	1979	—	—	—	—	—	—
T19765 ^d	O	1979	—	—	—	—	—	—
T19768 ^d	O	1979	—	—	—	—	—	—
U10198 ^d	O	1980	—	—	—	—	—	—
X20988 ^c	I	1982	++	+	—	+	—	—
X20838 ^c	I	1982	+++	++	—	—	NT	—
X29057 ^c	I	1982	—	—	—	—	—	—
X20752 ^c	I	1982	—	—	—	—	—	—
X27495 ^c	I	1982	—	—	—	—	—	—
X20492 ^c	I	1982	+	+	—	—	—	—
X19073 ^d	I	1982	—	—	—	—	—	—
X19203 ^d	I	1982	—	—	—	—	—	—
X23332 ^d	I	1982	—	—	—	—	—	—
X24782 ^d	I	1982	—	—	—	—	—	—
X28214 ^d	I	1982	+	+	—	—	—	—
(b) El Tor strains								
1796 ^a	O	<1973	++	—	++	+++	—	+++
1824 ^a	O	<1973	+++	+	++	+++	—	+++
1836 ^a	I	<1973	++	—	++	+++	—	+++
1843 ^a	I	<1973	++	—	++	+++	—	+++
1850 ^a	I	<1973	++	—	++	+++	—	+++
Phil 6973 ^e	I	<1973	+++	++	+	+++	+	++
O17SR ^f	O	?	++	—	++	+++	—	+++
N16961 ^g	I	?	+	(+)	+	+++	—	+++
E7946 ^g	O	?	+	—	+	+++	—	+++
T19218 ^c	I	1979	++	—	++	+++	—	+++
T19479 ^c	I	1979	+	—	+	+++	—	+++
T20567 ^c	O	1979	++	—	++	+++	—	+++
T20859 ^c	O	1979	+	(+)	+	+++	—	+++
X28378 ^c	I	1982	++	+	++	+++	(+)	+++
X20581 ^d	I	1982	+++	+++	+++	+++	(+)	+++
X24761 ^d	I	1982	+++	++	+++	+++	(+)	+++
X25049 ^d	O	1982	++	+	++	+++	(+)	+++
X19215 ^d	I	1982	+++	++	+++	+++	(+)	+++
X19128 ^d	O	1982	+	+	+	+	—	+

* The results are based on two to four determinations on different days. See Methods for details. NT, Not tested.

† Strains were obtained from: *a*, Dr H. Smith, Vibrio Reference Laboratory, Philadelphia, PA, USA, and isolated in Dhaka, Bangladesh or Calcutta, India; *b*, National Bacteriological Laboratory, Stockholm, Sweden; *c*, Dr I. Huq, ICDDR, B, Dhaka, Bangladesh; *d*, Dr S. Sanyal, ICDDR, B, Dhaka, Bangladesh; *e*, Statens Seruminstitut, Copenhagen, Denmark; *f*, Dr W. Chaicumpa, Bangkok, Thailand; *g*, Dr M. Levine, Baltimore, USA.

‡ I, Inaba; O, Ogawa.

Table 2. Comparison of haemagglutination (HA) and HA inhibition (HAI) with binding to sugar-coated agarose beads and with agglutination by 'biotype-specific' antisera for demonstration of cell-bound HAs on classical and El Tor *V. cholerae* O1

Strain	HA	HAI*	Binding to beads coated with:		Agglutinating titre of:	
			Fucose	Mannose	Anti-Cl	Anti-El Tor
<i>Classical</i>						
34	++	FSMR	+++	—	12	3
35	+++	FSMR	++	—	48	<6
569B	+	FSMS	(+)	—	<6	<3
1451	+++	FSMR	+++	—	†	†
Cairo 48	+++	FSMR	++	—	58	3
Cairo 50	+++	FS(MS)	+	+++	20	90
T19766	+++	FSMR	+++	—	†	†
X20988	++	FS(MS)	++	—	58	10
X20838	+++	FS(MS)	++	—	20	4
X20492	+	FSMR	—	—	12	12
X29057	(+)		—	—	6	<6
X28214	+	FSMR	+	—	15	3
U10198	—		++‡	—	6	<3
X19073	—		++‡	—	6	<3
X23332	—		++‡	—	6	<3
<i>El Tor</i>						
1824	+++	FRMS	—	++	6	180
1836	+++	FRMS	—	+++	6	30
Phil 6973	+++	(FS)(MS)	++	+++	12	60
O17SR	+++	FRMS	—	++	†	†
N16961	+++	FRMS	—	+++	<6	10
E7946	+++	FRMS	—	+++	<6	20
T19479	+++	FRMS	(+)	+++	<6	90
T20567	+++	FRMS	—	+++	<6	90
T20859	+	FRMS	—	+++	<6	30
X24761	+++	FRMS	—	+++	<6	10
X25049	+++	FRMS	(+)	+++	6	90
X19215	+++	FRMS	—	+++	<6	180
X28378	+++	FRMS	—	+++	<6	90

* Classical vibrios were agglutinated with human O erythrocytes and El Tor vibrios with chicken erythrocytes. Brackets indicate partial inhibition.

† Spontaneous agglutination.

‡ Positive after incubation with beads for 10 min.

vibrios was destroyed or reduced by incubation at pH values below 4, whereas HA titres were similar throughout the pH range 4–10. The classical-associated HA was not, or was only slightly, affected by heating to 56 °C but was destroyed at 80 °C; this HA did not agglutinate erythrocytes in the presence of EDTA, suggesting that Ca²⁺ is necessary for the binding of these bacteria to the erythrocytes (Table 4). The El Tor-associated HA was destroyed by incubation at 56 °C. EDTA had no effect on the HA titre of the El Tor vibrios.

Kinetics of expression of cell-bound HAs

The kinetics of the expression of cell-bound HAs during culture in TSB without glucose was studied.

For classical vibrios, as exemplified by strain 1451 in Fig. 1, the HA titre was optimal within 4 h, i.e. before maximal bacterial concentration was reached. The HA titre then decreased during the late exponential phase and was usually below the detection limit after 10–12 h incubation. Similarly, strain 1451 bound strongly to fucose-coated beads only during the early exponential phase; it did not react with mannose-coated beads during any phase of growth (Fig. 1a). Agglutination by 'biotype-specific' antisera could not be assessed during the initial 8 h of

Table 3. *Inhibition by different monosaccharides of V. cholerae* O1 agglutination with 'biotype-specific' antisera

Inhibitor	Serum . . .	Agglutinating titre*	
		El Tor (T19479)	Classical (Cairo 48)
		Anti-El Tor	Anti-CI
KRT (control)		48	12
1% L-fucose		48	<6
1% D-mannose		12	9

* The mean agglutinating titres of 10 µl of bacteria after preincubation with 10 µl of inhibitor for 30 s. Results from two different batches of bacteria varied within one twofold titre unit.

Table 4. *Cell-bound HA titre of V. cholerae* O1 after treatment at different temperatures and pH values, or in EDTA

Treatment	Erythrocytes . . .	Haemagglutination of indicated erythrocytes*			
		Classical		El Tor	
		Human O 1451	Cairo 48	Chicken T19479	X25049
Control: pH 7.4, 4 °C		128	128	64	64
Temperature: 20 °C		128	128	64	64
37 °C		128	128	64	64
56 °C		128	32	1	<1
80 °C		1	<1	<1	<1
pH: ≤3		8	<8	8	32
4		64	128	64	64
6		128	128	64	64
8		128	128	64	64
9		128	128	64	64
10		128	128	64	64
EDTA (0.005 M)		<1	<1	64	64

* The HA titres for 10¹⁰ bacteria ml⁻¹ are shown. The experiments were done in duplicate on the same day. Treatments were repeated with different batches of bacteria. The results given are the means of four titrations. Most results were fully reproducible, but some varied within one titre unit.

the growth curve due to strong autoagglutination of the bacteria during the exponential phase. Samples of bacteria collected after 10 and 16 h were not agglutinated by either the anti-CI or the anti-El Tor serum.

For the El Tor vibrios studied there was a parallel increase in cell-bound HA activity and bacterial concentration during the exponential phase of growth. During the late stationary phase the cell-bound HA activity decreased markedly (Fig. 1 b, c) and was usually below the level of detection after 20 h. The El Tor strain Phil 6973, which expressed both MSHA and FSHA in the exponential phase, bound strongly to both mannose-coated and fucose-coated beads in the early exponential phase and retained the mannose-binding ability during the stationary phase. The kinetics of the binding of the El Tor strain T19479 to mannose-coated beads mimicked that of the HA with chicken erythrocytes (Fig. 1 c). Agglutination of the vibrios by the anti-El Tor and anti-CI sera followed the mannose- and fucose-binding ability, respectively, during the initial 12 h. Bacteria collected thereafter were unspecifically agglutinated by both antisera at low level, although no HA was detected. Neither FSHA nor MSHA could be detected in the supernates of the bacterial cultures during any stage of the growth curve, whereas soluble HA (Svennerholm *et al.*, 1983) was produced during the late exponential and stationary phases.

Fig. 1. Kinetics of bacterial growth (□), expression of cell-bound HA activity (▼), and binding to fucose- (F) and mannose-coated (M) agarose beads, for different *V. cholerae* strains. Bacteria were cultured in TSB without glucose at 37 °C with shaking (200 r.p.m.). Growth was determined spectrophotometrically. The HA titres were determined after washing the bacteria once and resuspending them in KRT buffer to the original sample volume. HA of strain 1451 was tested using human O erythrocytes and HA of strains Phil 6973 and T19479 using chicken erythrocytes. The binding of bacteria to sugar-coated beads was determined after adjusting to 10^{10} organisms ml^{-1} ; arrows indicate time of sampling. HA titres given are the mean of two independent titrations of each specimen on the same day. HA titres were fully reproducible or varied within one titre unit, and binding results were always fully reproducible.

Fig. 2. Kinetics of bacterial growth (solid lines) and of cell-bound HA production (broken lines) by (a) classical Cairo 48 and (b) El Tor T19479 vibrios after culture at 37 °C (●) and 27 °C (○) with shaking in TSB without glucose. The HA titre of classical vibrios was determined using human O erythrocytes and that of El Tor vibrios using chicken erythrocytes. The results are the mean of HA titrations in duplicate. The experiment was repeated once and the results were in most instances fully reproducible; some titres varied within one titre unit.

The kinetics of the expression of cell-bound HA at different temperatures was also evaluated. The relatively high HA titres persisted longer after culturing at 27 °C than at 37 °C both for the classical strain Cairo 48 and the El Tor strain T19479 tested (Fig. 2). Similar results were observed for the additional two strains tested (not shown). The ratio between the HA and OD₆₀₀ value for bacteria grown at 27 °C and 37 °C did not differ during the exponential phase but was considerably higher after culture at 27 °C in the stationary phase.

DISCUSSION

By using both human O and chicken erythrocytes in an HAI system with L-fucose and D-mannose as inhibitors, it was possible to detect co-expressed FSHA and MSHA on vibrios in the mid-exponential phase of growth. However, since HAI is sometimes difficult to read, direct binding of the vibrios to a defined receptor or receptor analogue may be preferable or be used as a complementary method. We used a method based on binding of vibrios to agarose beads with covalently bound L-fucose or D-mannose. We showed that binding of *V. cholerae* to these beads is closely related to HA and HAI with specific sugars. The advantages of using the beads include lack of need for fresh biological reagents and a higher specificity for the sugar to be studied. For quantitative studies, however (and for detection of HAs not sensitive to mannose or fucose), HA titration in microtitration plates is superior since it is more sensitive and permits determination of end-point titres.

By absorbing antisera against *V. cholerae* O1 with biotype-heterologous vibrios of the homologous serotype, sera that agglutinated biotype-homologous vibrios to a considerably higher extent than biotype-heterologous vibrios were obtained, suggesting the presence of biotype-specific antigens. The possibility of inhibiting at all the biotype-homologous serum agglutination of El Tor vibrios by addition of mannose, and that of classical vibrios by fucose, suggests that the sera were at least partly directed against the respective cell-bound HA.

In contrast to the MSHA that is expressed through to the stationary phase, the FSHA activity disappeared quite abruptly in the late exponential phase. Possible explanations for this difference may be that FSHA is expressed only on an early population of bacteria (phase variation), as observed for *Escherichia coli* fimbriae (Nowicki *et al.*, 1986). An alternative explanation is that FSHA is more sensitive to one or more substances, possibly proteolytic enzymes, formed during *in vitro* culture. Although we did not identify any FSHA activity in any of the culture supernates tested, either directly or in HAI studies, we cannot exclude the possibility that the outer-membrane structure responsible for binding to erythrocytes is shed from the bacteria with simultaneous loss of HA activity. Alternatively, the FSHA may be masked by a surface structure expressed during the late exponential phase.

An interesting observation during the kinetic experiments was that strain 1451 was strongly autoagglutinating in KRT buffer as long as FSHA was expressed on the bacterial surface. Several other *V. cholerae* strains, e.g. Phil 6973, O17SR, 1449 and T19766, autoagglutinated in the early exponential phase. A possible explanation for this agglutination is the presence of hydrophobic adhesins or fimbriae on the vibrio surface as described for the classical *V. cholerae* strain 395 (Taylor *et al.*, 1987). A high degree of hydrophobicity during exponential growth has been described for vibrios of both biotypes (Kabir & Ali, 1983). We have observed that *V. cholerae* often have a tendency to autoaggregate after being kept for long periods in deep agar. This may suggest a change in the surface structure, e.g. length of polysaccharide chains, resulting in a better exposure of the FSHA. This phenomenon may explain why all the old classical strains isolated before 1973, which had been subcultured in deep agar before this study was initiated, exhibited greater FSHA than the fresh isolates.

The possibility that either the MS or the FS cell-bound HA may correspond to or be associated with the recently described *toxR*-gene-regulated pili (Taylor *et al.*, 1987) is less likely. In contrast to observations for the pili, we did not find a clear correlation between the expression of the two HAs and the levels of cholera toxin (not shown). Furthermore, the pilated *V. cholerae* strain used by Taylor *et al.* (1987) bound to fucose-beads but not to mannose-beads after culturing under conditions favouring production of pili, suggesting that MSHA does not involve the fimbrial structure. One way to determine whether FSHA is related to the pili would be to find out whether pili-negative mutants (Taylor *et al.*, 1987) still bind to fucose-beads.

This study shows that *V. cholerae* FS and MSHAs are mainly biotype-associated but can be expressed and detected on both biotypes by HAI, by binding to sugar-coated agarose beads or by antibodies. The expression of FSHA is growth-phase dependent and difficult to demonstrate on many strains.

Although studies in different laboratories suggest that expression of FSHA or MSHA could promote bacterial adhesion *in vitro*, it remains to be seen whether any of these HAs are operating

as adhesins during infection. Since both the FSHA and the MSHA are immunogens, passive protection studies in experimental animals may help to determine whether antibodies to these structures play a role in protection against cholera.

This study was supported by grants from the Swedish Medical Research Council (grant 16X-3382) and the Faculty of Medicine, University of Göteborg, Göteborg.

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