CHARACTERIZATION AND DISTRIBUTION OF THE CELL-BOUND HAEMAGGLUTININS PRODUCED BY *VIBRIO CHOLERAE*

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INTRODUCTION

Vibrio cholerae agglutinates certain erythrocytes, and the haemagglutination has been suggested to mimick the interactions resulting in attachment of cholera vibrios to the intestine. A variety of haemagglutinins (HAs) have been identified on *V. cholerae*. Of special interests are cell-bound HAs which have evidently been found to play an adhesive role in cholera vibrios (Foo and Chaicumpa, 1981).

Even if an HA is required for the initiation of infection, it would not necessarily be associated with the toxin-producing property, and loss of toxin-producing capacity would not necessarily be correlated with loss of the HA activity or of the capacity to initiate asymptomatic infection. As yet, none of the previous, studies has examined the HA activities of antigenically rough mutants and of nonpathogenic strains of cholera vibrios. In addition, it has been suggested that HA-positive, non-flagellated mutants might be useful for studies on the nature of HAs since contamination by flagellar protein would be avoided. This study was carried out to determine the production of cell-bound HA and their distribution for pathogenic and non-pathogenic strains of *V. cholerae*, for its nonmotile mutants and for its rough mutants.

MATERIALS AND METHODS

Bacteria: The *V. cholerae* used are described in Table 1. Nonmotile mutants (de-

Table 1

Vibrio cholerae strains, characteristics, and origins.

Biotype	Serotype	Strain	Source
Classical	Inaba	CA 401	Calcutta, 1953
	Inaba	NIH 35A3	India, 1941
	Ogawa	CA 411	Calcutta, 1953
	Ogawa	NIH 41	India, 1941
	Ogawa	CA 414*	Calcutta, 1953
	Ogawa	VC 12 Rx1*	Dacca, 1964
El Tor	Inaba	8233	Manila, 1961
	Ogawa	HK 1	Hong Kong, 1961
		EW 6**	
		ME 7**	

All strains were obtained from the collection of R.A. Finkelstein (Hanne and Finkelstein, 1982).

* Antigenically rough strains.

** Nonpathogenic, water strains used in the investigation of live oral cholera vaccination (Mukerjee, 1963).

signated as M) were isolated following N-methyl-N'-nitro-N-nitroso-guanidine treatment (Adelberg *et al.*, 1965). Rough mutants, NIH35R and NIH41R, were isolated by incubating heated smooth cultures in salt-free tryptic soy broth (TSB; Difco) with 0.1% O antisera for 24 hours at 37°C. Cells taken from the broth were inoculated onto 0.5% soft tryptic soy agar (TSA; Difco) with 0.1% O antiserum, the conditions under which colonies of rough mutants were produced (Engelking, 1969).

HA production: The bacteria were grown in TSB for 12 hours at 37°C and adjusted to $2x10^5$ cells/ml. To inoculate solid medium, 1 ml of this suspension was spread over the surface of TSA plates and incubated for 12 hours at 37°C. To prepare broth cultures, 1 ml of the inoculum was inoculated in 40 ml of TSB and shaken 200 rpm for 12 hours in a 37°C water bath. Twenty-five ml of cell suspension was shaken for 1 hour at 37°C with an equal volume of 0.05 M. CAPS (Calbiochem) buffer. The bacteria were centrifuged at 5,860xg for 20 minutes. The supernate was filtered and the filtrate was dialyzed through four changes to distilled water. The preparation was then tested for HA activity.

Erythrocytes: Chicken erythrocytes were obtained from chickens which had been pretested to have erythrocytes giving positive HA reaction. Human group O erythrocytes were provided by volunteers. The erythrocytes were collected in sodium citrate and washed with 0.85% saline and maintained as a 10% packed cell volume in saline. For use, cells were diluted to 1.5% in KRT buffer (Jones *et al.*, 1976).

HA tests: HA preparation was studied for HA activity. Serial two-fold dilutions of the preparation was made in 0.025-ml volumes in microtiter plates (Dynatech). An equal volume of 1.5% erythrocytes was added to each well. The plates were incubated at 25°C for 30 minutes. The reciprocal of the highest dilution giving complete haemagglutination was defined as the HA titer.

Certain sugars including D-mannose, L-fucose, D-galactose, D-fructose, D-glucose, D-ribose, sucrose and D-xylose were tested for the ability to inhibit HA reaction. These sugars (25 mg/ml) were serially diluted in microtiter plates in a 0.025 ml of KRT buffer. Portions, 0.025 ml, of HA preparation, adjusted to contain 2 HA units, were added to each well and allowed to interact at 25°C for 15 minutes. Erythrocytes were then added and HA reaction was examined after 30 minutes. The inhibitory effect of each substance was determined by comparing the HA titers obtained in its presence and absence.

RESULTS

Table 2 represents the HA production by *V. cholerae*. When grown on agar, El Tor vibrios, their nonmotile mutants (except HK1M-6) and nonpathogenic El Tor vibrios were HA-positive, whereas classical vibrios were either HA-positive or negative. In addition, all of the rough mutants of classical vibrios examined were HA-positive. Identical results were obtained when vibrios were grown in broth. However, some change in HA production was observed. El Tor 8233 produced HA only in moderate titers and its mutant, 8233M-7, failed to produce HA. El Tor HK1 and its 3 mutants produced high titers of haemolysin which masked the HA. Classical NIH41 and its mutants produced only poor HA titers.

The HAs of El Tor and HA-positive classical vibrios were equally active on both human group 0 and chicken erythrocytes. The El-Tor HA was inhibited by D-mannose at a concentration as low as 3 μg/ml. It was also sensitive to D-fructose, D-glucose and sucrose. By contrast, the classical HA was not

Table 2

Production of cell-bound HAs by *Vibrio cholerae* and nonmotile mutants, and antigenically rough mutants and nonpathogenic *V. cholerae*.

Classical	Strain		HA titer	
			Agar	Broth
Classical	CA401:	Parent	0	0
		M-1,M-4,M-5,M-6	0	0
	CA411:	Parent	0	0
	NIH35A3:	Parent	10	0
		M-1,M-2,M-3,M-4,M-5	0	0
		M-6,M-7	10	0
	NIH41:	Parent	40	10
		M-1,M-2,M-3	40	20
		M-4	40	10
		M-5	10	20
		M-6	20	10
Classical, rough mutants	CA414		40	0
	NIH35R		40	10
	NIH41R		40	10
	VC12Rx1		40	10
El Tor	8233:	Parent	80-160	80
		M-6	40-320	40
		M-7	40-80	0
	HK1:	Parent	80	*
		M-1	80	*
		M-4	160	*
		M-6	0	0
El Tor, nonpathogenic	EW6		80	20
	ME7		80	10

* Haemolysin.
Cultures were incubated 12 hours at 37'C, except for cultures of El-Tor 8233 and its mutants which were incubated for 16 hours.
HA activity was examined with either of human group 0 or chicken erythrocytes.

sensitive to D-mannose but was sensitive to L-fucose.

Kinetic studies of HA production by El Tor HK1 and classical NIH41 and CA401 were performed in shaking and stationary cultures at 28°C and 37°C. Bacteria were grown in dialyzed TSB (DTSB), prepared by dialyzing 10xTSB in 10 volumes of distilled water overnight, and monitored at 2-hour intervals. Each sample was centrifuged at

5,860xg for 20 minutes and cells were resuspended in 0.85% saline to the original volume. Whole cultures, supernatant culture fluids, and washed cell suspensions of each culture-age sample were then examined for HA activities.

HA production of El Tor HK1 was temperature-dependent whereas HA production by classical NIH41 and CA401 was determined by shaking conditions. El Tor HK1 and classical NIH41 produced HAs during all phases of growth. A similar result was obtained in whole cultures of both strains. Classical CA401 expressed an HA in a different manner (Fig. 1 and 2). It produced HA

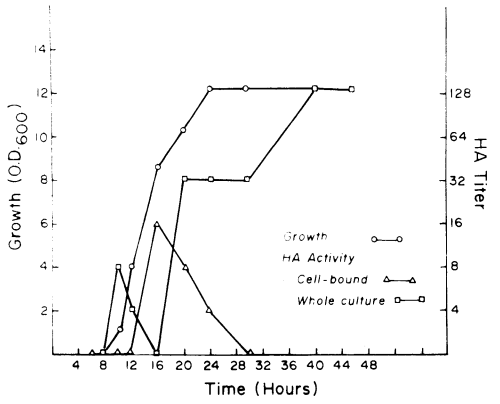

Fig. 1—HA production by shaking cultures at 28°C of *V. cholerae* strain CA401 (Inaba serotype, classical biotype).

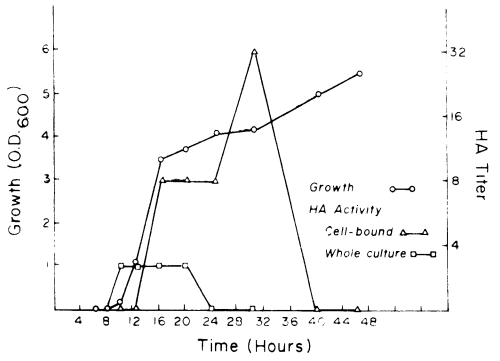

Fig. 2—HA production by stationary cultures incubated at 28°C of *V. cholerae* strain CA401 (Inaba serotype, classical biotype).

transiently in early-log cultures shaking at both 28° and 37°C; this HA was fucose-sensitive. Whole cultures of CA401 produced weak HA very early during log-phase growth. This HA disappeared transiently, and it was detected again only in shaking cultures at the mid- to late-log phase of growth. The late-appearing HA remained active as long as it was monitored. Of interest was the repeated observation that the weak HA disappeared before the maximal stationary phase. Although the HA activity of whole culture was negative, cells from these cultures still produced HA in moderate titers. In addition, samples from whole cultures taken just before the disappearance of HA were often found to give anomalous patterns of haemagglutination, i.e., their first three dilutions did not give complete HA reactions. As the cultures were diluted further, the reactivity declined in the usual manner. The HA reaction of these cells was somewhat atypical, but if the cells were washed once and tested, a normal reaction was obtained,

DISCUSSION

HA production by *V. cholerae* in this study is related to biotype. El Tor vibrios, most of their nonmotile mutants and nonpathogenic El Tor strains produced a mannose-sensitive HA constitutively under all growth conditions. Some classical vibrios, their nonmotile mutants and rough mutants of classical strains produced a fucose-sensitive HA continually. Other classical vibrios produced neither HA nor a fucose-sensitive HA transiently. The HAs of both biotypes were equally active on human and chicken erythrocytes, in agreement with a previous report by Hanne and Finkelstein (1982).

Of interest is the transient expression of HA by classical strain CA401. Cells taken from the HA-negative culture of this strain and washed were capable of producing HA.

This finding, together with the repeated observation of anomalous patterns of HA activity for whole cultures tested just before the disappearance of HA activity, suggests the existence of an HA inhibitor in the culture supernatant fluid. A continually expressed and a transiently expressed cell-bound HAs have previously been described in classical vibrios (Jones *et al.*, 1976; Hanne and Finkelstein, 1982). It is possible that most classical vibrios which continually produce HA fail to produce an HA inhibitor after its production. Bhattacharjee and Srivastava (1978) found that all their classical vibrios tested showed good adhesion to the intestine even though some were HA-positive ane others negative. Their HA-negative cultures possibly contained an HA inhibitor, thus giving apparently no HA activities during the time of testing. Studies on the nature of such an inhibitor may be useful in solving the unexplained variations in kinetics of HA production and in the degree of adhesion of cholera vibrios to the intestinal epithelium.

Antiflagellar (Yancey *et al.*, 1979), antisomatic (Chitnis *et al.*, 1982) and antitoxic (Svennerholm, 1976) antibodies have been found to protect against experimental cholera. The identification of these antibodies however, does not exclude the possible role of additional antibodies directed against other bacterial surface molecules. Foo and Chaicumpa (1981) have reported the protective role of cell-bound-HA antibodies in experimental cholera, thus implying a major role of this antigen as a bacterial adhesin. The HA production by non-flagellated mutants, by the rough mutants and by nonpathogenic vibrios in this study was similar to that of the wild-type strains. These results indicate that the lack of either the flagellar or somatic antigen and of the capacity to produce enterotoxin is not necessarily associated with the lack of cell-bound HA or the vibrio adhesin.

The results obtained in this study may be of some use in the selection of HA-positive strains for future studies. A mutant of an HA-negative El Tor strain would be valuable for absorption of antiserum or for comparison for pathogenicity with its parent. The finding that nonpathogenic and apparently nontoxigenic El Tor vibrios have strong cell-bound HA is interesting. These strains proliferate in the gut of suckling rabbits without inducing disease, and they can be used as live oral vaccine strains to prevent ileal loop distension by virulent vibrios in adult rabbits (Mukerjee, 1963). The means by which they confer immunity, therefore, is of interest.

SUMMARY

The two biotypes of *Vibrio cholerae* were found to produce two distinct cell-bound haemagglutinins (HAs). El Tor vibrios, most of their nonmotile mutants and nonpathogenic El Tor strains produced a mannose-sensitive cell-bound HA constitutively under all growth conditions examined. Some classical vibrios, their nonmotile mutants and antigenically rough mutants of classical strains produced a fucose-sensitive cell-bound HA continually. Other classical vibrios produced neither cell-bound HA nor a fucose-sensitive cell-bound HA transiently.

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