

Selective Induction of Fimbriate *Vibrio cholerae* O1

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Abstract: Fimbriate *Vibrio cholerae* O1 (classical in biotype) were selectively induced in the presence of chitin and thioproline. Fimbriate vibrios detectable by cell agglutination with anti-E1 Tor fimbriae antiserum formed pellicle when cultured in liquid media under static conditions and were highly hydrophobic. Fimbriate vibrios agglutinated several kinds of red blood cells (RBCs, human type O, sheep, chicken, horse) and showed highest haemagglutinating (HA) activity for horse-RBCs, although parent non-fimbriate cells had no HA activity. Pellicle formation and HA activity of fimbriate cells were completely inhibited by 1% monosaccharides such as D-mannose, D-glucose, D-fructose, N-acetyl-D-glucosamine, but not by L-fucose. Numerous pores with a regular diameter were found on an outer membrane debris through which fimbriae may extend extracellularly.

Key words: *Vibrio cholerae* O1, Fimbriae, Cholera vaccine

INTRODUCTION

It is well known that natural infection with *Vibrio cholerae* give rise to long-lasting protective immunity against a second attack of cholera both from epidemiological studies of cholera in endemic areas (Mosley, 1969; Woodward, 1971; Glass *et al.*, 1982; Ehara *et al.*, 1985) and from experiments in human volunteers (Cash *et al.*, 1974; Levine *et al.*, 1981) and in rabbits (Lycke *et al.*, 1986). During infection, *V. cholerae* O1 seem to express protective antigens that stimulate immunity which extends across both biotype and serotype barriers. *V. cholerae* O1 organisms cause diarrhea by colonizing onto the epithelial cells of the small intestine. The cell-surface appendages (pili or fimbriae) play main roles in the colonization of enteropathogenic bacteria to the mucinous epithelial cells. Evidence for the presence of fim-

briae on vibrio species was shown by Tweedy *et al.* (1968), although the number of fimbriae demonstrated on each cell was small. We reported the evidence for the presence of fimbriae on vibrios adherent to the intestinal epithelial cells (Ehara *et al.*, 1986) and reported the purification of fimbriae of *V. cholerae* O1 (Ehara *et al.*, 1987).

Knowledge about the cell-associated haemagglutinin of *V. cholerae* O1 necessary for the infectious process is of central importance for an understanding of pathogenicity and immunity in cholera. Thus, it is essential to establish fimbriate strains of enteropathogenic *V. cholerae* O1 for better understandings of the haemagglutinating activity and the colonization mechanism of *V. cholerae* O1 and for the induction of protective immunity against the bacteria.

Here we report a method to induce fimbriate vibrios and a partial characterization of fimbriate vibrios.

MATERIALS AND METHODS

Chemicals

L-Thiazolidine-4-carboxylic acid (Thioprolin) was purchased from Wako Pure Chemicals Industries Ltd, Osaka, Japan. Ethyleneglycol bis (β -aminoethyl ether)-*N, N, N', N'*-tetraacetic acid (EGTA) was bought from Nakarai Chemicals Ltd, Kyoto, Japan. The other reagents used were of analytical grade.

Bacteria

Four enteropathogenic strains of *V. cholerae* O1, Bgd17, 86B3, 86B4, 86B31 were chosen from stock strains in our laboratory. All strains were classical in biotype, isolated in Bangladesh in 1982 and 1986.

Media

Vibrios were grown in an alkaline tryptone broth (AT broth, w/v, 1% Bactotryptone, 0.5% NaCl, 0.2% yeast extract, 0.1% NaHCO₃) or in TCG broth (1% Bactotryptone, 0.2% yeast extract, 0.5% NaCl, 0.1% NaHCO₃, 0.02% thioprolin, 0.1% mono-sodium L-glutamate, 1 mM EGTA) at 37°C overnight.

Selection and induction of fimbriate vibrios

Vibrio cells taken from a single colony of each strain were subcultured at 37°C twice with overnight intervals in Roux bottles containing 50 ml of AT broth in the presence of 0.5% chitin. After the first overnight subculture, the liquid medium containing non-adherent vibrios was removed by pipetting, leaving chitin behind. Then, chitins remaining in the bottles were washed thrice vigorously with saline to eliminate vibrios non-adherent to chitin. After the second subculture, 50 ml of TCG broth was added to the bottles containing vibrios adherent to chitin. These vibrios were further transformed by subculturing in TCG broth to form pellicle on the surface of the liquid medium. Vibrio cells which started pellicle forma-

tion were further grown in AT broth. The resultant pellicle of each strain was mixed with fresh AT broth in the presence of 30% glycerol and divided into 1 ml aliquotes for stocking at -80°C

A ligated rabbit ileal loop test

Ligated loops produced by the method of De and Chatterje (1953) were inoculated with 0.2 ml of overnight AT broth culture of fimbriate vibrio strains. The rabbit was sacrificed 8 hr post-injection for the observation of *V. cholerae* O1 colonized onto the surface of the small intestinal epithelial cells.

Production and characterization of cell-associated haemagglutinin (HA)

Fimbriate strain of Bgd17 (kept at -80°C) was inoculated in a small test tube containing 4 ml of AT broth and cultured at 37°C overnight under a static condition. After mixing the pellicle formed by the fimbriate strain with 1 ml of fresh AT broth, a 25 μl of the mixture was inoculated in 6 test tubes containing 4 ml of AT broth, followed by incubation at 37°C under a static condition. Samples for testing were removed periodically. Vibrio cells were brought to sediment by centrifugation at $4,500\times g$ for 10 min at 4°C , and cells were resuspended with 1 ml of saline. These cell suspensions were used for monitoring bacterial growth turbidimetrically at 600 nm and for quantitation of HA. Effects of monosaccharides on HA inhibition were also examined. Techniques for quantitation of HA and HA inhibition with sugars were same as previously reported (Ehara *et al.*, 1987). Briefly, HA preparations were diluted in two fold series in U-bottomed microtitre plates in 25 μl of phosphate-buffered saline (10 mM PBS, pH 7.4), the plates were tapped to mix the interactants, and RBCs (human type O, sheep, horse) were allowed to settle at room temperature for 30 min. The titre was defined as reciprocal of the highest dilution in which HA was visible to naked eyes. To test whether the HA reaction was inhibitable by specific monosaccharides, the vibrio cell suspensions were serially diluted in microtitre plates in 25 μl of PBS. RBCs containing 1% (w/v) of a monosaccharide was added to each well and HA reactions were examined after 30 min. Sugars tested included D-mannose, α -methyl D-mannoside, L-fucose, D-glucose, methyl- α -D-glucoside, N-acetyl D-glucosamine, D-fructose, D-galactose.

Inhibition of pellicle formation by monosaccharides

Inhibition of pellicle formation of the fimbriate Bgd17 strain by 1% monosaccharides in AT broth was tested. The fimbriate Bgd17 strain (kept at -80°C) was inoculated in small test tubes containing 1% monosaccharides and cultured at 37°C overnight. Pellicle formation was judged macroscopically. Sugars tested were the same as those used for HA inhibition.

Electron microscopy

For negative staining, one drop of the sample was placed on a sheet of PARAFILM and a Formvar-coated copper grid was floated on the drop for 2 min. The excess liquid was removed with filter paper. The specimen was washed three times with deionized water each

time for 10 sec then stained with 1% uranyl acetate for 30 sec.

The excess stain was removed, using the tip of a filter paper. For shadowing, the negative stain was omitted and the air-dried grid was shadowed with platinum. For immunoelectron microscopy, labeling of bacteria with immunogold was carried out essentially as described by Faulk and Taylor (1971). Formvar-coated copper grids with air-dried fimbriate vibrios were reacted with anti-El Tor fimbriae antiserum (400 fold diluted with saline) for 15 min and washed 3 serial drops of deionized water and reacted for 15 min with a drop of 15 nm-colloidal gold labeled anti-rabbit IgG goat serum (E. Y. LABS, INC., SANMAEO, CA 94401, USA). The specimen was stained with 4% uranyl acetate for 30 sec and examined with a JEM 100CX electron microscope operated at 80 kV. For scanning electron microscopy (SEM), a part of the washed rabbit ileal loop was immediately fixed with 2% cold glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 (CB) and kept overnight at 4°C. The specimen was rinsed twice in CB and post-fixed with 1% osmium tetroxide in CB for 1 hr at 4°C. The sample was rinsed again in CB and dehydrated through a series of ethanol baths, dried at a critical point, and followed by coating with gold palladium. For the SEM observation of pellicle, a part of pellicle formed by the fimbriate Bgd17 strain cultured in AT broth at 37°C overnight was mixed with 200-fold diluted anti-El Tor fimbriae antiserum for immunofixation. The mixture was kept at 37°C for 30 min in a small test tube with shaking and washed thrice with saline by centrifugation, and then processed for sample preparation for SEM, omitting the post fixation by osmium tetroxide. These specimens were examined with a JSM 840A scanning electron microscope operated at 10 kV.

RESULTS

Selection of vibrios adhesive to chitin and induction of fimbriate vibrios

Vibrio cells are known to adhere to chitin (Amako *et al.*, 1987) and these adherent vibrios (Fig. 1) appeared to be more suitable candidates for further transformation to fimbriate form than non-adherent vibrios. In the absence of chitin, neither selection nor induction of fimbriate vibrios was successful in AT broth despite our efforts in subculture over several months. Even in the presence of chitin, fimbriate cells were not induced when subcultured in AT broth alone. The periods necessary for the formation of pellicle macroscopically visible required several days to several weeks depending on the strains when vibrios adherent to chitin were subcultured in TCG broth. Fimbriate vibrios were obtained from all of 4 non-fimbriate parent strains tested. Out of these 4 fimbriate strains, Bgd17 strain was used for further study on fimbriate vibrios.

Electron microscopic studies on fimbriate vibrios

Fimbriate Bgd17 vibrios cultured in AT broth at 37°C overnight were examined under EM together with those inoculated in a ligated rabbit ileal loop. An EM specimen taken from AT broth overnight culture was shown in Fig. 2a in which one vibrio cell and a membrane debris were present together with several fimbriae and numerous pores. Each pore has a

regular diameter corresponding to the width of the fimbriae. Two filaments were shown to connect themselves with pores. Fimbriate Bgd17 vibrios produce morphologically different two types of fimbriae, namely, hydrophobic fimbriae (Fig. 3a–d) which cause auto-agglutination and hydrophilic fimbriae (Fig. 3e). Fimbriate vibrios with high hydrophobicity were observed in the specimens taken from pellicle and fimbriate cells with low hydrophobicity were seen in the specimens taken from AT broth culture after removing the pellicle. A scanning electron micrograph of a pellicle formed by fimbriate Bgd17 strain revealed numerous filamentous structures (strands) between vibrios and vibrios (Fig. 3f). Fimbriate Bgd17 vibrios showing a localized colonization onto epithelial cells of a rabbit small intestine (Fig. 3g) exhibited also filamentous surface appendages between vibrios and vibrios, and between vibrios and epithelial cells similar to those as shown in the pellicle (Fig. 3f). Fimbriae of the Bgd17 strain (a classical strain) were immunodecorated by the anti-El Tor fimbriae antiserum (Fig. 3h). When reacted with the antiserum, the fimbriae were shown as several bundles on the cell surface.

Production of cell-associated haemagglutinin

Production of cell-associated haemagglutinin by fimbriate Bgd17 strain and the sensitivity of the haemagglutinin to various RBCs were summarized in Fig. 4. This strain showed the highest HA titre at the late logarithmic phase in parallel with the cell-growth curve. The cell-associated haemagglutinin of this strain was most sensitive to horse-RBCs.

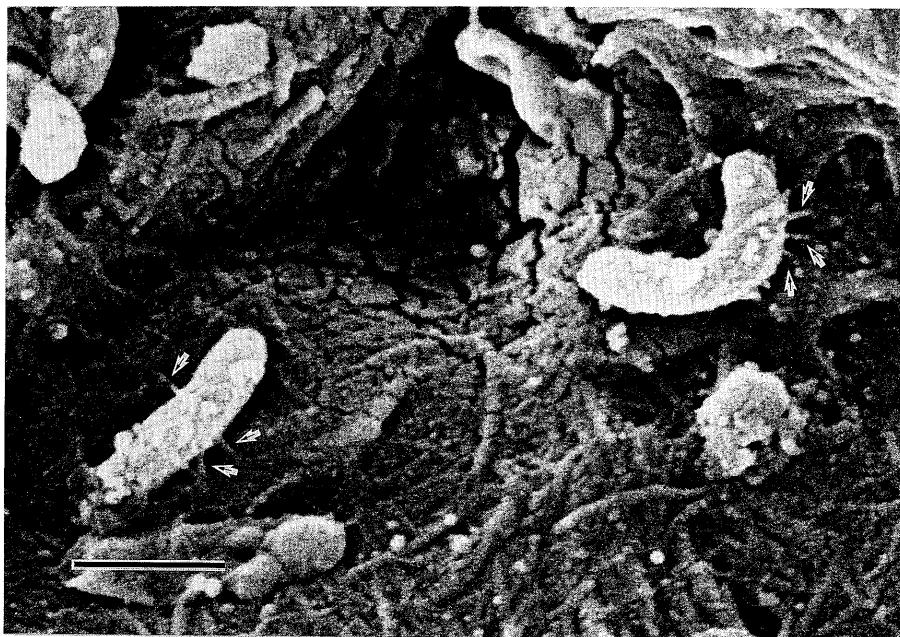
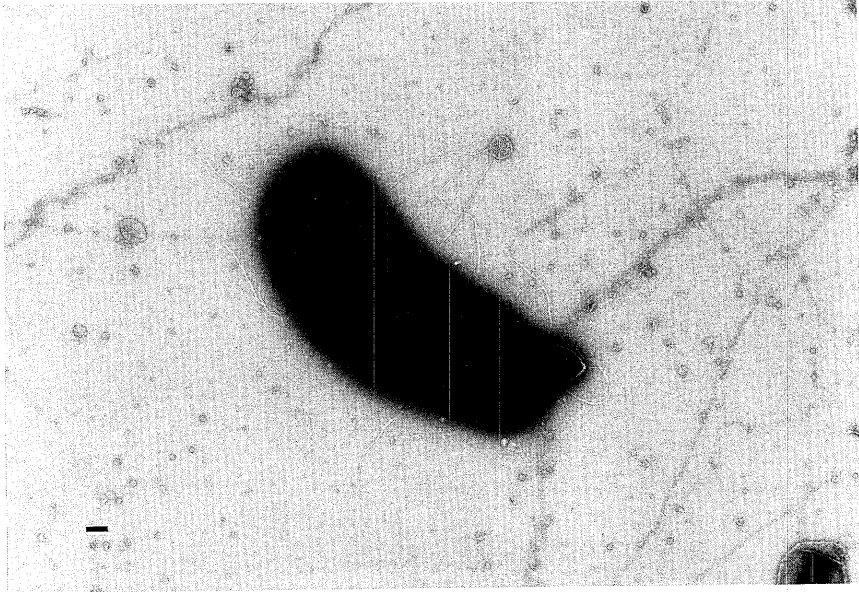


Fig. 1. A scanning electron microscopic picture showing vibrio cells adherent to chitin with the aid of fimbriae as indicated by arrows. Bar indicates 1 μ m.]

a.



b.

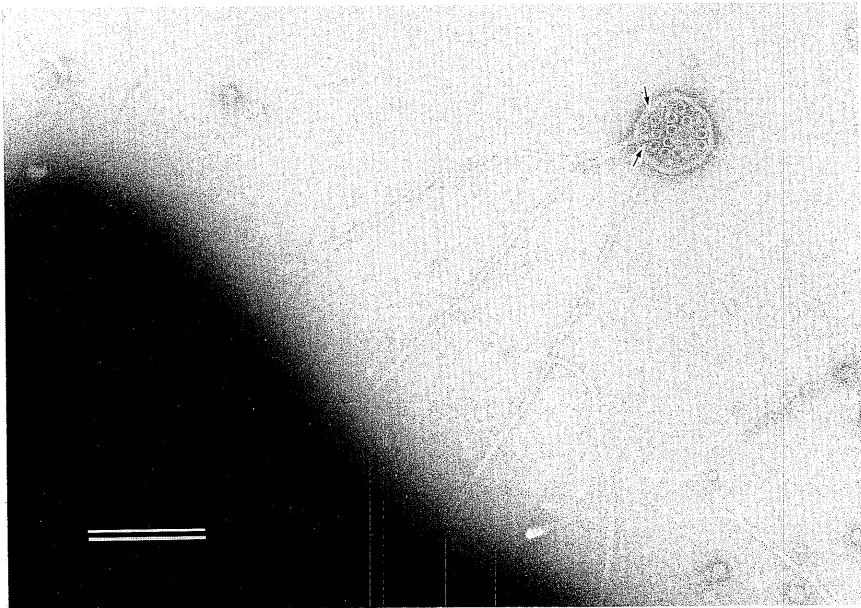


Fig. 2 a. A picture of a membrane debris with many pores (or rings) connected with fimbriae as indicated by arrows.
b. enlarged. Bars indicate 100 nm.

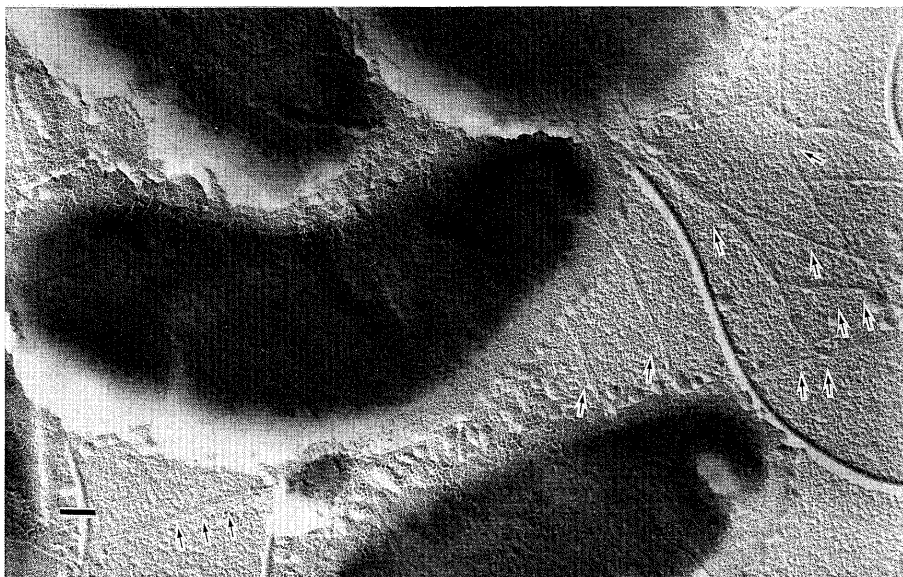


Fig. 3. a)



Fig. 3. b)

Fig. 3. Electron micrographs of the fimbriate Bgd 17 strain.

a) shadowed, b) – e) negatively stained with 1% uranyl acetate. Arrows indicate fimbriae. Bars, 100 nm. Note that the surface of *Vibrio* cells are covered with hydrophobic fimbriae (Figs. 3b and c). f) A scanning electron micrograph of pellicle showing groups of vibrios with numerous strands (fimbriae) between vibrios and vibrios. The specimen was treated with anti-E1 Tor fimbriae antiserum for immuno-fixation. g) Colonization of the fimbriate Bgd 17 strain on the mucous epithelial cells of a ligated rabbit ileal loop. Note the strands between vibrios and vibrios, and between vibrios and epithelial cells. h) An immuno-electron micrograph of the fimbriate Bgd17 strain reacted with anti-E1 Tor fimbriae antiserum. Bar indicates 100 nm. Note the heavily decorated fimbriae forming several bundles. Fimbriae of *V. cholerae* O1 are highly antigenic and cross-reactive independent of biotype and serotype.



Fig. 3. c)

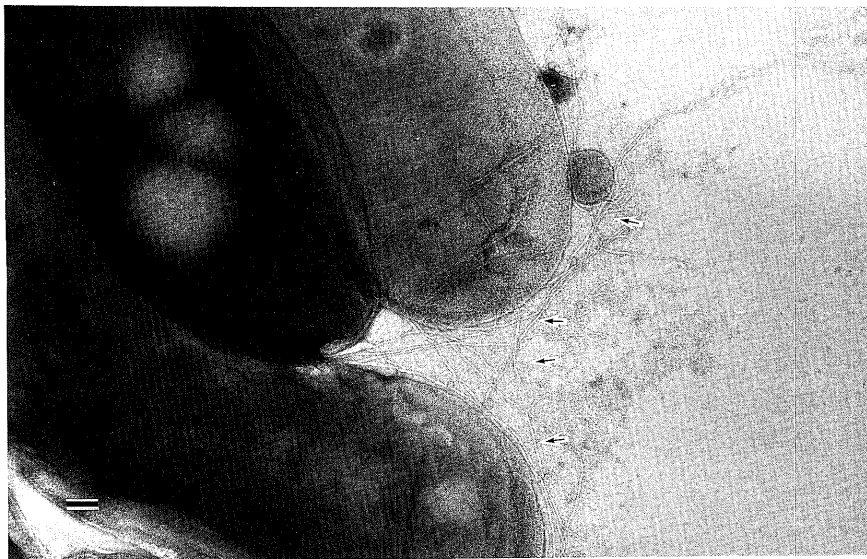


Fig. 3. d)

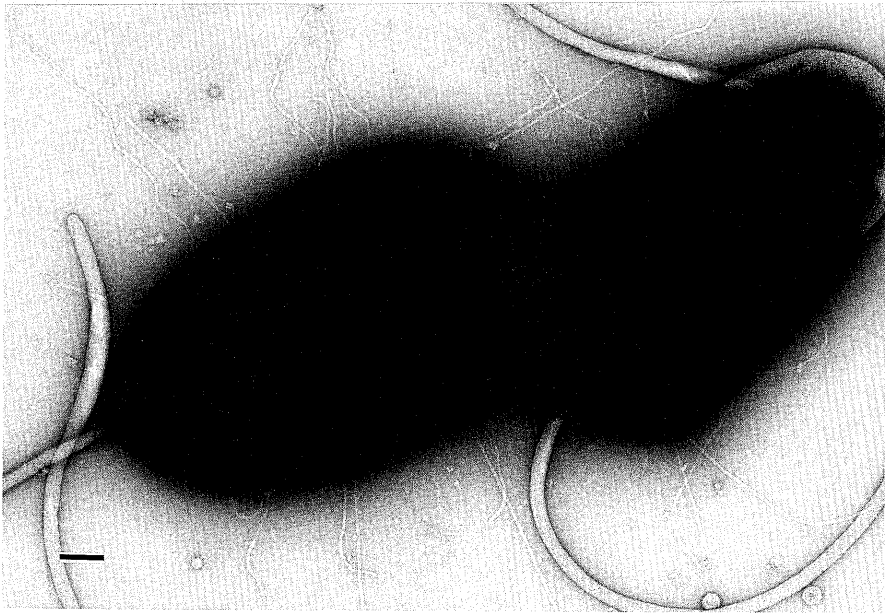


Fig. 3. e)

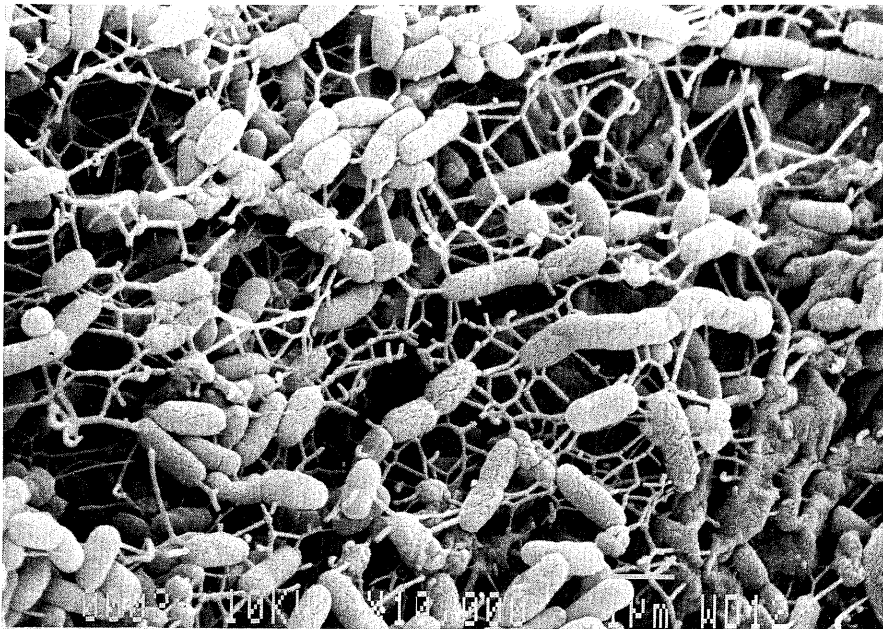


Fig. 3. f)

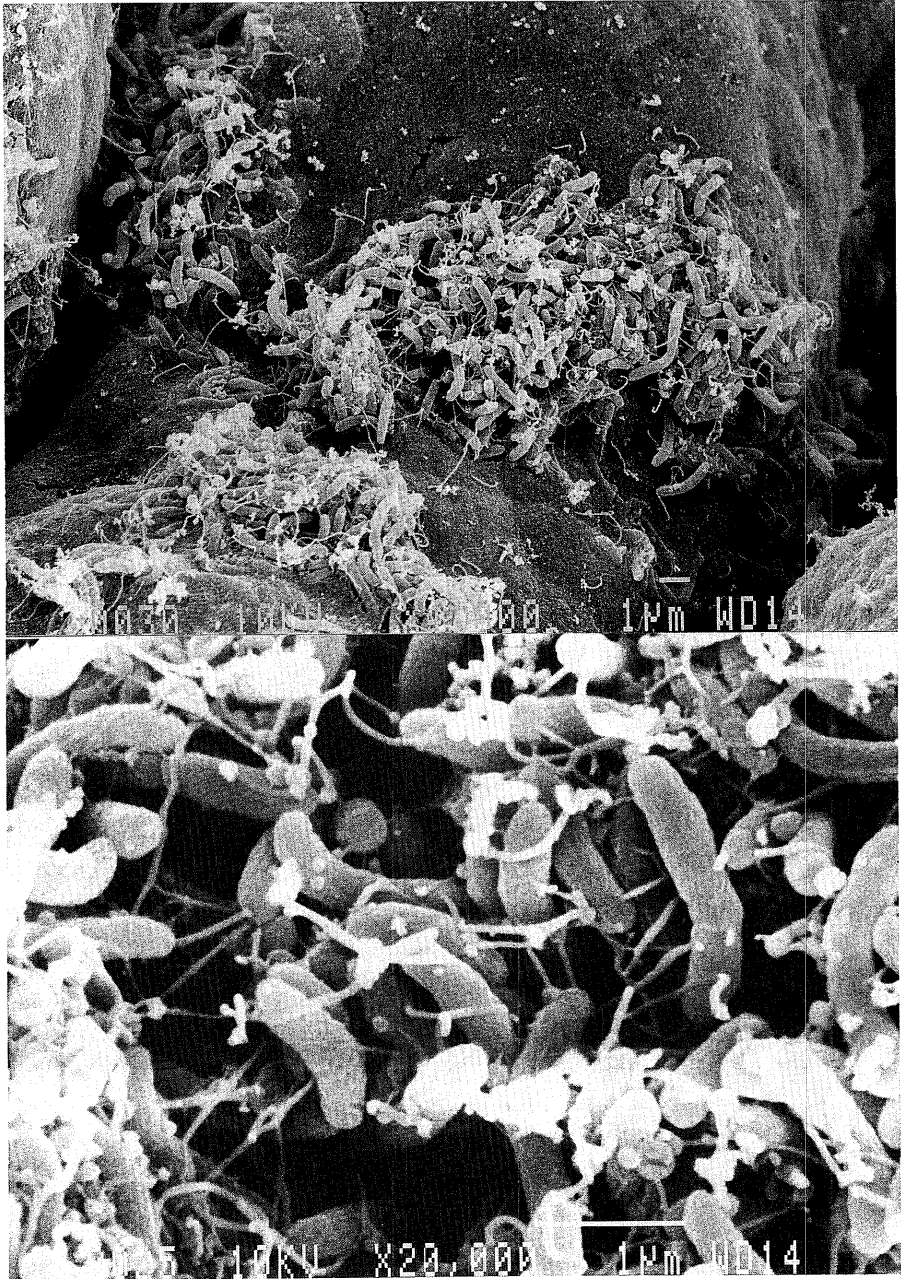


Fig. 3. g)

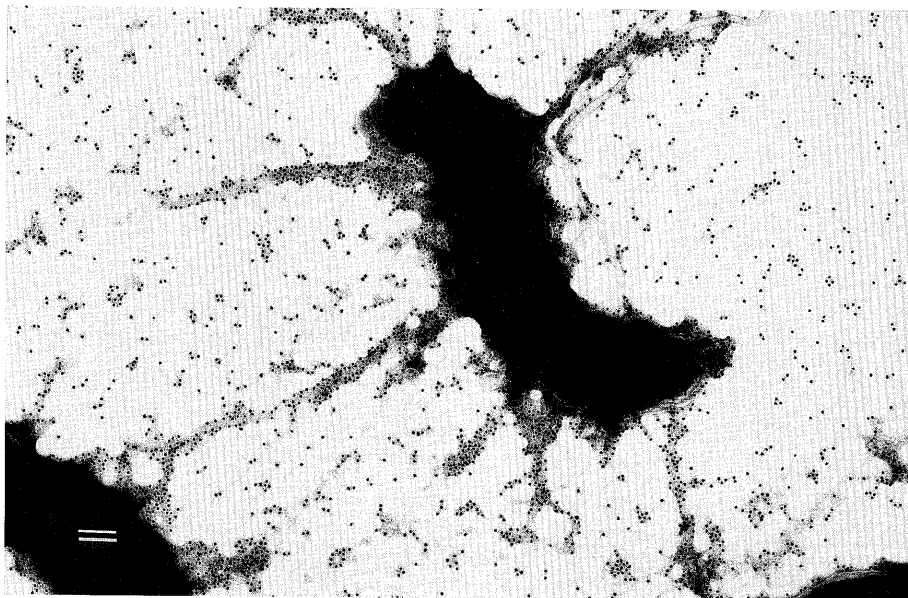


Fig. 3. h)

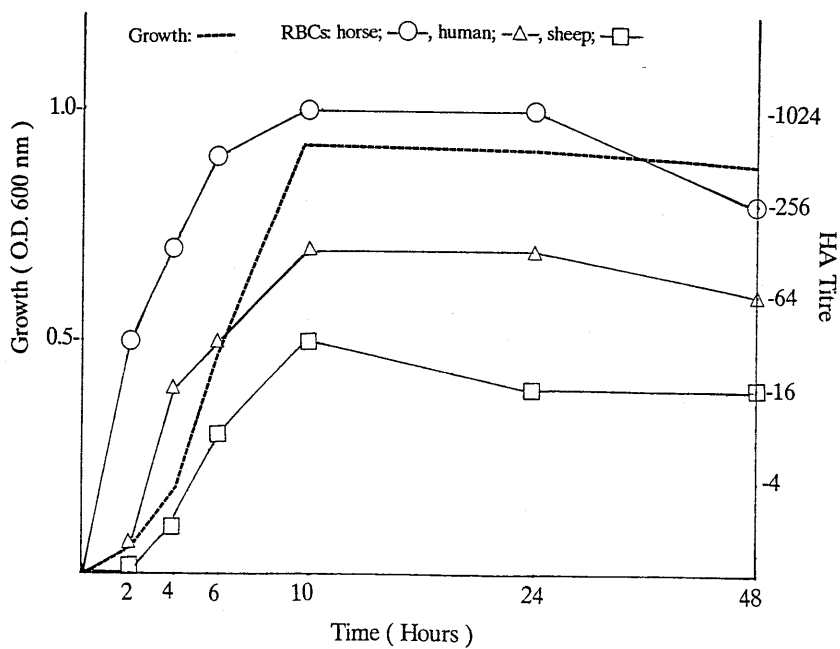


Fig. 4. HA production by the fimbriate *V. cholerae* O1 strain Bgd 17 (classical biotype, Inaba serotype). Note that the HA titre was highest at the end of logarithmic phase.

Inhibition of cell-associated haemagglutinin and pellicle formation by specific monosaccharides

Inhibition patterns of cell-associated haemagglutinin and pellicle formation by monosaccharides were shown in Table 1. Note the finding that the inhibition pattern of pellicle formation by monosaccharides is correlated with that of cell-associated haemagglutinin.

Table 1. Sensitivities of pellicle formation and cell-associated HA activity of fimbriate Bgd 17 strain to monosaccharides. Note the correlation in the sensitivity to monosaccharides between pellicle formation and HA activity

	Pellicle formation	Cell-associated HA activity
AT broth/2% horse-RBCs in the presence of 1% —	+	1,024
D-mannose	—	0
α -methyl D-mannoside	—	0
L-fucose	+	1,024
D-glucose	—	0
methyl- α -D-glucoside	—	0
N-acetyl D-glucosamine	—	0
D-fructose	—	0
D-galactose	+	1,024

DISCUSSION

We were surprised to see the pellicle formed by fimbriate vibrios, when we were getting tired of subculturing vibrio strains in TCG medium. Anyhow, four enteropathogenic, classical strains of *V. cholerae* O1 used were induced to the fimbriate phase. The reason why vibrio strains adhesive to chitin were transformed to fimbriate cells by subculturing in TCG broth is still remained to be clarified. The key substances in TCG broth seemed to be EGTA and thioproline. Both are chelating agents, *i. e.*, EGTA for calcium ion and thioproline for ferric ion. Furthermore, thioproline is shown to elevate the cytoplasmic level of cyclic nucleotides, cAMP, cGMP in eukaryotic cells (Caciaci *et al.*, 1980). The effect of thioproline on cell differentiation, *i. e.*, from malignant cells to benign cells (reverse transformation) (Brugarolas and Gosalvez, 1980) was applied to induce fimbriate vibrios using non-fimbriate vibrios. Although the cytoplasmic levels of cyclic nucleotides in vibrio cells were not titrated during the subculturing term, the switching mechanism of fimbriation of *V. cholerae* O1 may depend on the level of cAMP. If so, the cAMP-binding protein may play an important role in a gene regulation of an ON-OFF switch of fimbriation. Further studies are also necessary to understand the role of chelating ferric ion in fimbriation of vibrio cells.

EM pictures of fimbriate vibrio strains were not shown previously. This paper is the first report on fimbriate *V. cholerae* O1. EM pictures of fimbriate vibrios revealed several

interesting findings. Even membrane debris and ghost cells are the good sources for the anatomical surface-information of fimbriate cells. Regular rings found on a membrane debris, together with the immunodecoration of a fimbriate cell are the direct evidence showing that fimbriae of *V. cholerae* O1 are produced extracellularly as several bundles from various parts of the cell. The hydrophobic nature of fimbriate cells is derived from the fimbriae itself covering the cell surface. Faris *et al.* (1982), reported the correlation between high surface hydrophobicity and haemagglutinating activity of *V. cholerae* O1. After that report, there is no other paper concerning the cell-surface hydrophobicity of *V. cholerae* O1 except one paper by Honda *et al.* (1988) on non-O1 *V. cholerae*.

Parent cells of strain Bgd17 (non-fimbriate) have no HA activity and form no pellicle, however its fimbriate cells show a high HA activity and pellicle formation sensitive to D-mannose, D-glucose, D-fructose, N-acetylglucosamine. These natures may be applied for the treatment of clinical cholera. In fact, the oral rehydration salts (ORS) recommended by World Health Organization contains 2% glucose and has been shown effective for an early intervention of cholera. Possibly, the efficacy of glucose may be derived from two reasons. One is the well-known Na-glucose co-transport to compensate the electrolyte imbalance and the other is the inhibition of colonization of *V. cholerae* O1 on the surface of small intestinal epithelial cells. Therefore, the administration of ORS to a cholera patient is "the earlier, the better".

Based on the observation through EM, biochemical natures of vibrio cells, it may be possible to classify the phase of *V. cholerae* O1 into 3 phases according to the function of flagella and fimbriae. The first one is a non-fimbriate phase (or flagellate phase). Vibrio cells in this phase are motile and possess only a few number of fimbriae less than ten per cell. The HA activity of vibrio cells in this phase is nil or low and shows unstable sensitivities to monosaccharides, particularly, to D-mannose. The second is a transitional phase. Vibrio cells in this phase are motile and possess several tens of fimbriae. The HA activity of vibrios in this phase is intermediate and shows a weak sensitivity to D-mannose. These two phases of vibrios have hydrophilic cell surfaces. Fimbriae of *V. cholerae* O1 are easily digested or modified by the protease purified from *V. cholerae* O1, strain K23 (manuscript in preparation). These modified fimbriae show a hydrophilic appearance under EM. The third is a fimbriate phase (or non-flagellate phase). Vibrio cells in this phase are non-motile, aggregated by the presence of hydrophobic fimbrial adhesins forming pellicle. Accordingly, the HA activity of vibrio cells in this phase is high and shows clear sensitivities to various monosaccharides. As most of the previous works on the colonization factor and HA activity of *V. cholerae* O1 were performed using non-fimbriate cells without attention to the phase variation, their results were not so fruitful (Iwanaga *et al.*, 1987). Sciortino and Finkelstein (1983) showed that some surface proteins of *V. cholerae* are expressed during growth in infant rabbits but not during *in vitro* growth. Jonson *et al.* (1989) reported the similar result using adult rabbits. Further, Richardson *et al.* (1989), suggested that studies of protective immunity and *V. cholerae* O1 pathogenesis should include examination of both *in vitro*- and *in vivo* (rabbit ligated ileal loop)-grown *V. cholerae* O1 cellular antigens. However, those vibrios grown *in vivo* (in accumulated intestinal fluid) are possibly in non-fimbriate phase, because most of fim-

briate cells colonize on the mucous epithelial cells. Therefore, the essential antigens for analyzing the colonization factor of *V. cholerae* grown *in vivo* should be collected by scraping the epithelial surface after vigorous washing. Now it is the time for us to analyze the protective antigen which evokes a long-term immunity in humans, since we could establish several fimbriate vibrio strains. In the accompanying paper, we present the direct evidence showing that fimbriae of *V. cholerae* O1 function as fimbrial adhesins as those of *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa* and *Bacteroides nodosus*.

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