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Fimbriae of *Vibrio cholerae* O1: Observation of Fimbriae on the Organisms Adherent to the Intestinal Epithelium and Development of a New Medium to Enhance Fimbriae Production

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Abstracts: The fimbriate phase of enteropathogenic strains of *Vibrio cholerae* O1 were observed under electron microscope during the colonization to the surface of rabbit small intestine. There was no difference in the shape of fimbriae between the two serotypes of El Tor Inaba and El Tor Ogawa. Pathogenic strains were confirmed to produce numerous fimbriae on a solid medium (TCG agar) with the same structure, namely 5 to 7 nm in width and flexible fibers as seen *in vivo*. Fimbriae of *V. cholerae* were easily removed from the cell wall at cell harvest, leaving holes behind. TCG medium which contains thioproline, sodium bicarbonate and EGTA was successful to produce fimbriae from *V. cholerae*, however the reason still remains to be elucidated.

Key words: *Vibrio cholerae*, Fimbriae, Colonization

INTRODUCTION

The adhesion of pathogenic *Vibrio cholerae* O1 to the human intestinal surface in the upper small intestinal tract has been reported to play a role in the pathogenesis of cholera (Freter, 1980 a, b; Jones, 1980).

However, despite the great interest in the pathogenesis of cholera in the past decade, very little is known about the initial steps of cholera infection. Strains of *V. cholerae* of the biotype El Tor produce a surface hemagglutinin "cholera rectin", which has

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been proposed as a candidate involved in mucosal adhesion factor recognizing specific receptor in the small intestine and promoting mucosal association (Finkelstein and Hanne, 1982). A very few investigations have revealed fimbriae of *V. cholerae* as adhesin candidates under electron microscopy (Faris *et al.*, 1982; Tweedy *et al.*, 1968). One of the primary problems facing investigators who want to prepare fimbriae from *V. cholerae* O1 is the difficulty of culturing the organism, especially in its fimbriate form.

This study was initiated to confirm whether *V. cholerae* O1 adherent to the epithelial surface of the small intestine are in fimbriate phase or not, and to develop a medium to facilitate fimbriae production. This paper presents the evidences for the presence of fimbriate *V. cholerae* during the colonization to the surface of the small intestine and for the reproducibility of fimbriae on the TCG agar.

MATERIALS AND METHODS

Chemicals: L-Thiazolidine-4-carboxylic acid (Thioproline) was purchased from Wako Pure Chemicals Industries, Ltd., Osaka, Japan. Ethylene glycol bis (β-aminoethyl ether) $-$N, N,N',N'$-$ tetraacetic acid (EGTA) was bought from Nakarai Chemicals, Ltd., Kyoto, Japan.

N-Acetyl cystein (Acetein) and casein (nach Hammarstem) were bought from Takeda Chemical Industries, Ltd. and Merck, respectively. The other drugs used were of analytical grade.

Selection of enteropathogenic strains of *V. cholerae* O1: Strains of *V. cholerae* O1 biotype El Tor, serotype Inaba and Ogawa were selected from the strains isolated in Kenya between 1983 to 1984 using casein agar plates composed of 1% casein, 1% agar and 1% pepton, pH 8.0 adjusted with 1 N NaOH. Strains with high proteinase and migration activity were injected in the rabbit ileal loop. Once strains showed positive in the tests, they were examined for their colonization to the epithelial surface under scanning electron microscopy (SEM). Finally, strains of 83K1 (El Tor, Inaba) and 83K23 (El Tor, Ogawa) were selected as the enteropathogenic candidates. Non-flagellar mutant strain of H218 (classical, Inaba) was used as a nonpathogenic control strain.

Rabbit ileal loop test: We followed the method by De and Chatterjee (1953). Results were read macroscopically 8 to 10 hr after the injection of 0.1 ml of overnight culture in heart infusion broth.

Recovery of *V. cholerae* O1 colonized to the epithelial surface: After removing the fluid accumulated in the ileal loop with a 10 ml syringe, the loop was cut at the both ligated ends and transfered into a Petri dish to open by a longitudinal incision. Then, the piece was washed by vigorous shaking with 100 ml of normal saline in a medium bottle. Washing was repeated 3 times changing the solution every time. The washed sample transfered into a Petri dish was scraped with a cover slip to remove the organisms colonized to the surface of the small intestine. The scraped material was collected into a small test tube with

a pipet and treated with 5% Acetein for 5 min at room temperature to solubilize glycocaryx of the epithelial cells. Then, the mixture was washed 3 times with normal saline by centrifugation at low speed and the final pellet was processed for the electron microscopic study.

Medium: We developed a medium to enhance fimbriae production from *V. cholerae* O1 and named it as TCG medium, composed of 1% Bactotriptone, 0.2% Yeast Extract, 0.5% NaCl, 0.3% $NaHCO_3$, 0.02% thioproline, 0.1% sodium L-glutamate, mono, 2% Agar and 1mM of EGTA.

Culture condition: Strains used were cultured at 37°C overnight or 30°C for 48 h on TCG agar plates.

Electron microscopy: For negative staining, strains of *V. cholerae* were resuspended in normal saline. One drop of this suspension was applied to a carbon coated Formvar grid and washed 3 times with distilled water for 10 sec each time, then stained with 1% uranyl acetate for 30 sec. The excess was removed by filter paper tip. Grids were examined in a JEM 100CX electron microscope operated at 60 kV.

For sample preparation for SEM, a part of the washed loop before scraping and a part of the mixture treated with Acetein were immediately placed in cold 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2 (CB) and kept overnight at 4°C. Specimens were rinsed twice in CB and postfixed for 1 hr with 1% osmium tetroxide in CB at 4°C. These samples were rinsed again in CB and dehydrated through a series of ethanol baths. The specimens were critical-point dried, coated with gold palladium, and examined by SEM.

For ruthenium red staining, ruthenium red was present in both fixative and wash solutions at a final concentration of 0.075% (Luft, 1971). A part of the washed loop before scraping was also processed for ruthenium red staining as follow. The specimens were fixed in 3% glutaraldehyde in CB for 1 h, washed, and postfixed for 1 h in buffered osmium tetroxide. Fixed specimens were dehydrated through graded ethanol solutions and embedded in Epon. Sections were cut with a Reichert Ultra Cut E and stained with uranyl acetate and lead salts. Specimens were examined with a JEM 100CX operated at 80 kV.

RESULTS

Characters of the strains used were summarized in Table 1. Motility, proteinase activity, colonization and fimbriae production were mutually related with the results in the ileal loop test.

Electron microscopy. Strongly adherent vibrio strains as shown in Fig. 1,2 possesed 5-7 nm flexible fimbriae as shown in Fig. 3-5. Strains of 83K1 and 83K23 cultured on TCG agar produced fimbriae as shown in Fig. 6, 7. There was no marked difference in the production of fimbriae between the 2 culture conditions (37°C, overnight and 30°C, 48 h). Numerous pores in the surface of the cultured cells were identified and the fimbriae released from the cell had a cap like structure at one end of what appeared to be a fimbrial

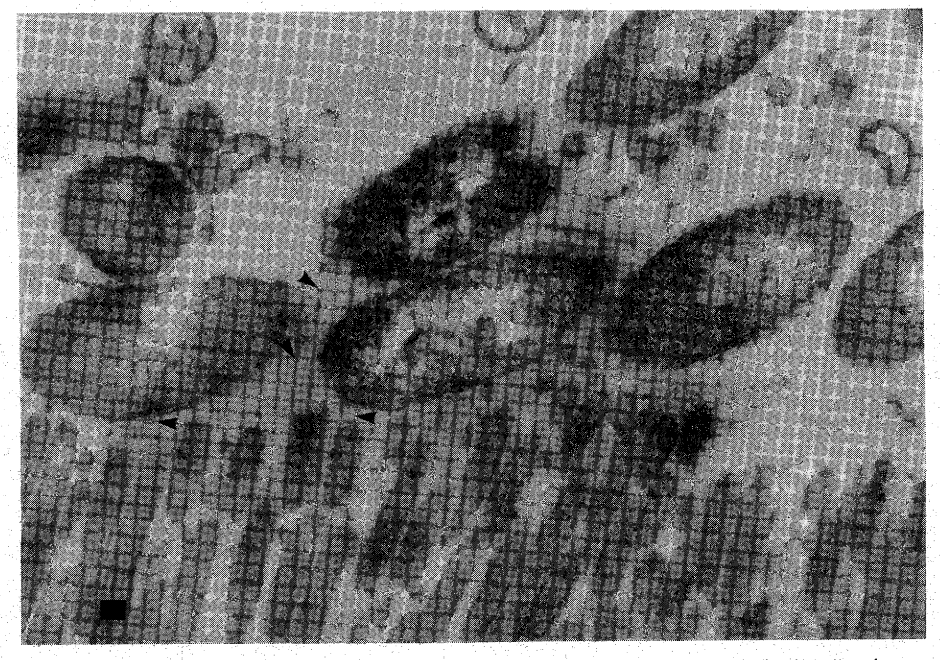


Fig. 1. Ruthenium red-stained preparation of strain of 83K23 showing fimbrially (arrows) mediated adhesion to the apex of brush border microvilli. Bar, 0.1 μm.

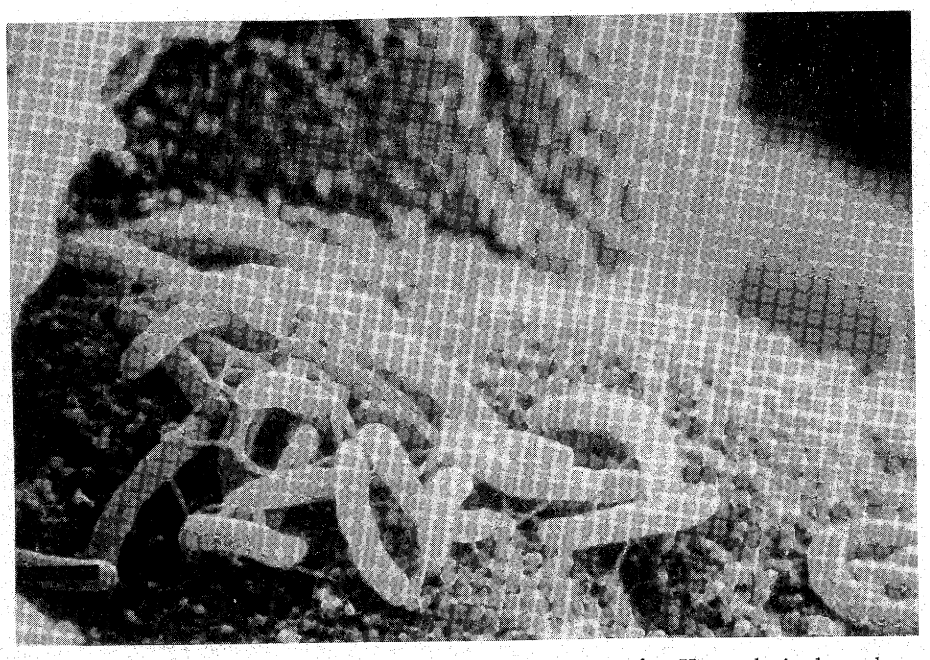


Fig. 2. Area of the mucous layer disruption showing strain of 83K23 colonized to the apex of microvilli. Bar, 0.5 μm.

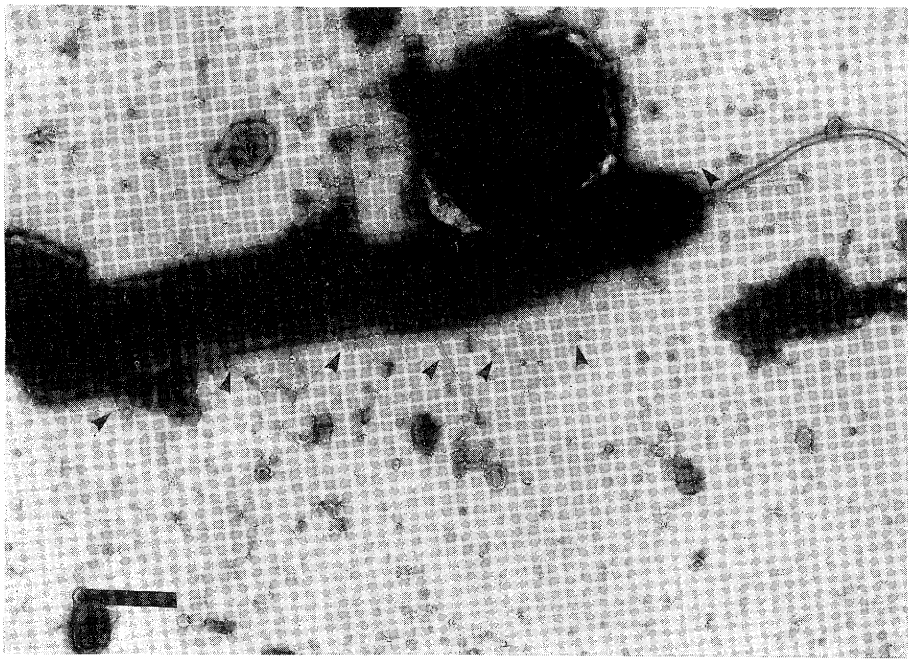


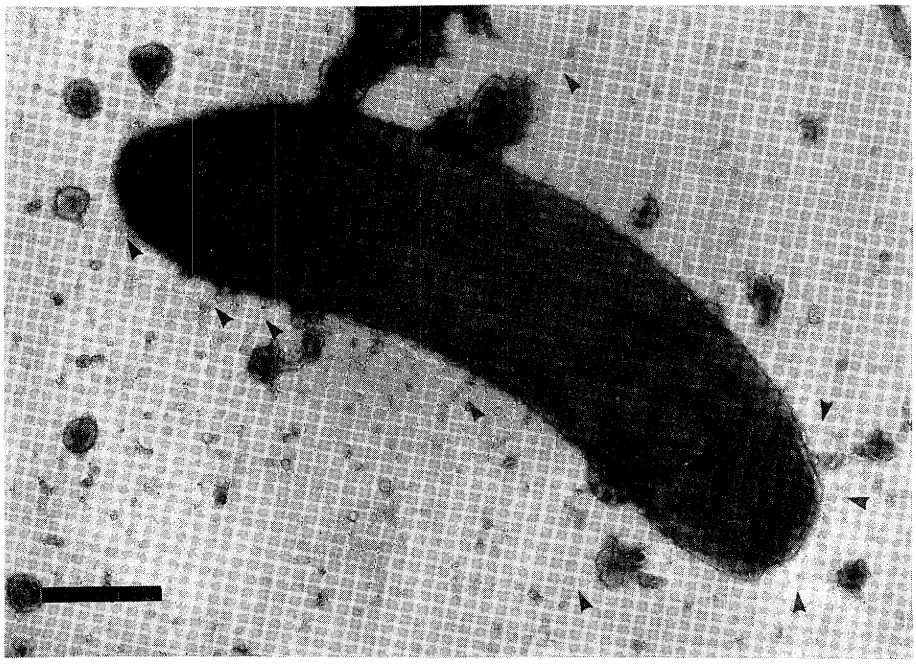
Fig. 3. Scraped specimen of 83K1 showing fimbriae (arrow heads). Bar, 0.5 μm.

Table 1. Characterization of the strains used.

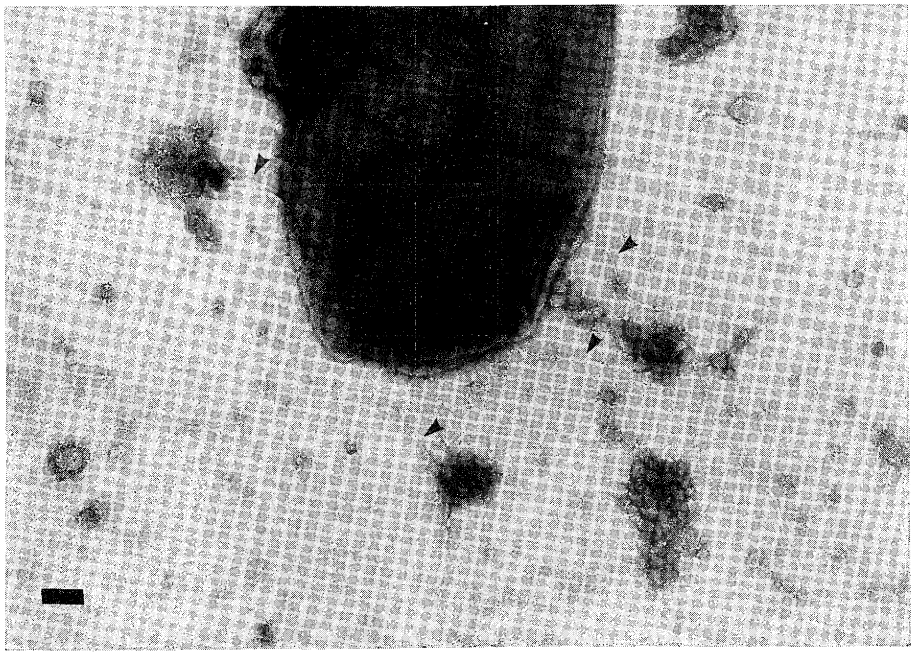
Strain	Characteristics				
	Motility	Proteinase	Ileal loop test	Colonization	Fimbriae
83K1 (El Tor, Inaba)	+	++	+	+	+
83K23 (El Tor, Ogawa)	+	++	+	+	+
H218 (Classical, Inaba)	±	±	−	−	−

Proteinase activity was determined on the casein plate after overnight culture by measuring the diameter of the translucent area. Undigested casein was fixed with 15% trichloroacetic acid. Colonization and the presence of fimbriae were examined under SEM and negative staining EM respectively.

stub (Fig. 8). Strain of H218 used as a non pathogenic control never produced fimbriae on TCG agar in both culture conditions (Fig. 9).



(a)



(b)

Fig. 4. (a) Scraped specimen of 83K23 showing flexible fimbriae (arrow heads). Bar, 0.5 μm.
(b) Higher magnification showing long fimbriae (arrow heads). Bar, 0.1 μm.

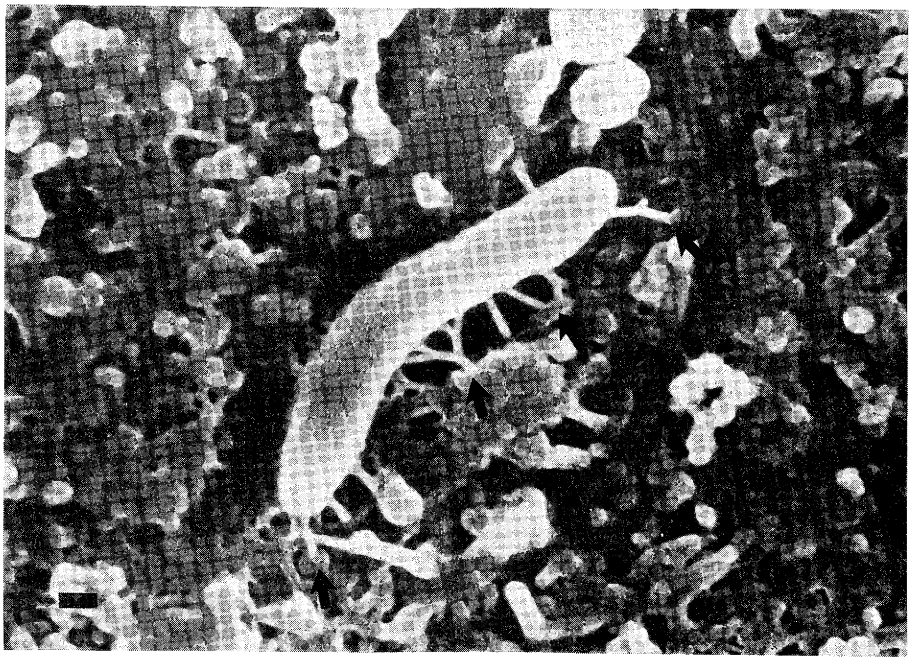


Fig. 5. Scraped specimen of 83K23 prepared for SEM showing bundles of fimbriae (arrows). Bar, 0.2 μm.

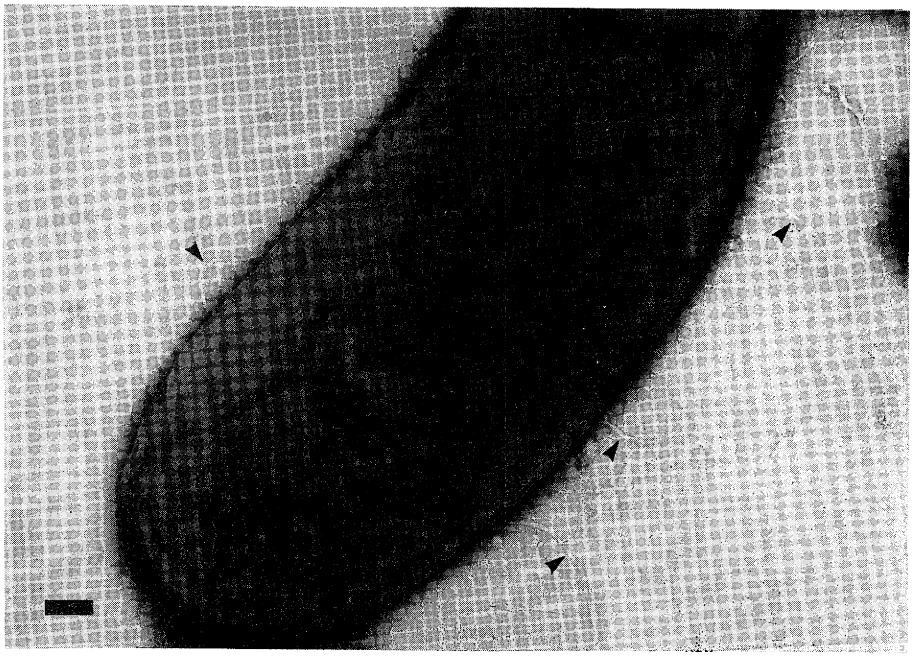
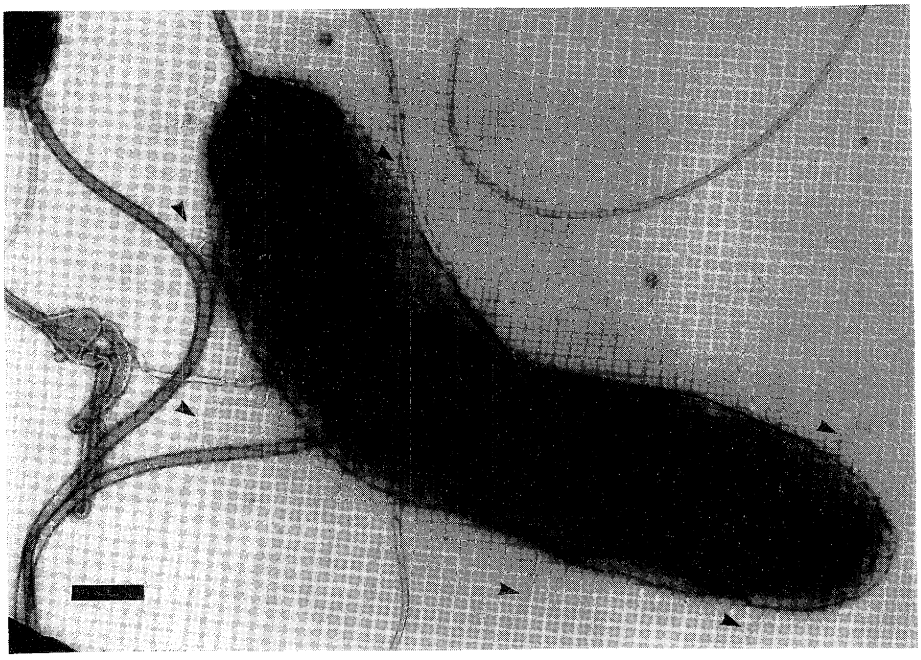


Fig. 6 (a)



(b)

(c)

Fig. 6. Strain of 83K23 cultured on TCG agar at 37°C overnight showing several long fimbriae which are not straight. Bar, (a) 0.1 μm, (b) 0.2 μm, and (C) 0.1 μm.

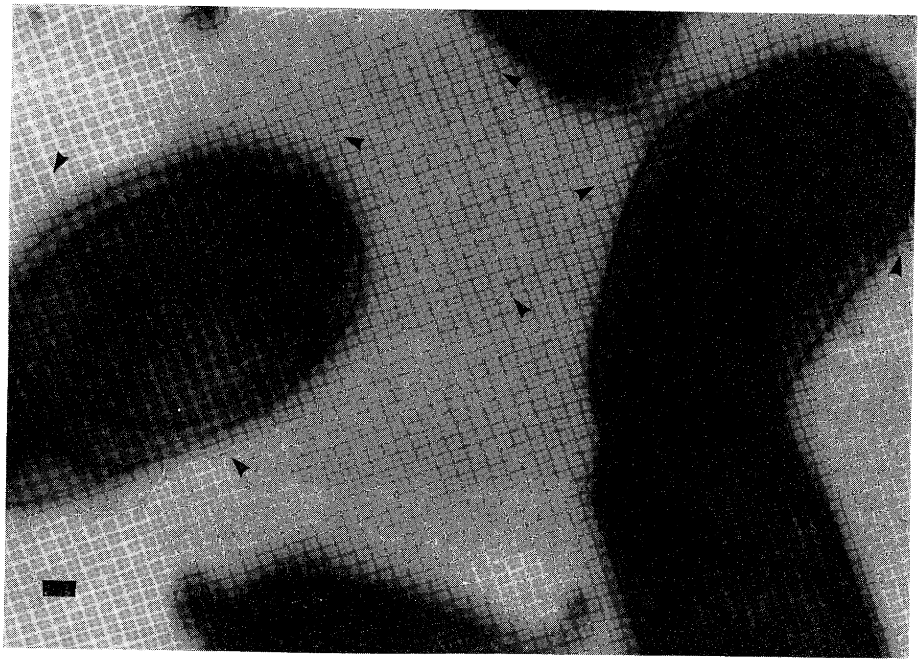


Fig. 7. Strain of 83K1 cultured on TCG agar at 37°C overnight. Note the same type of fimbriae as seen in the strain of 83K23. Bar, 0.1 μm.

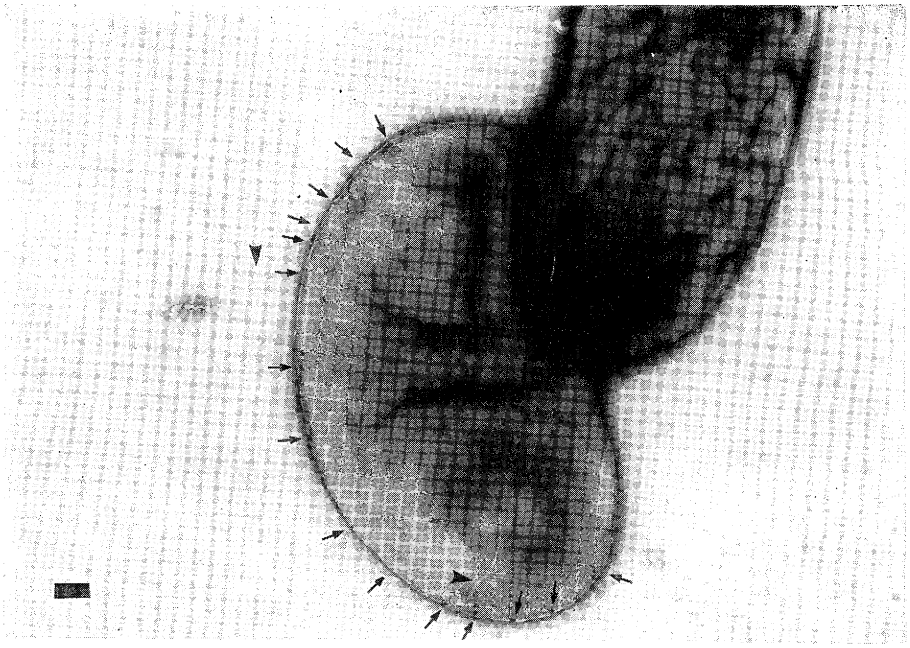
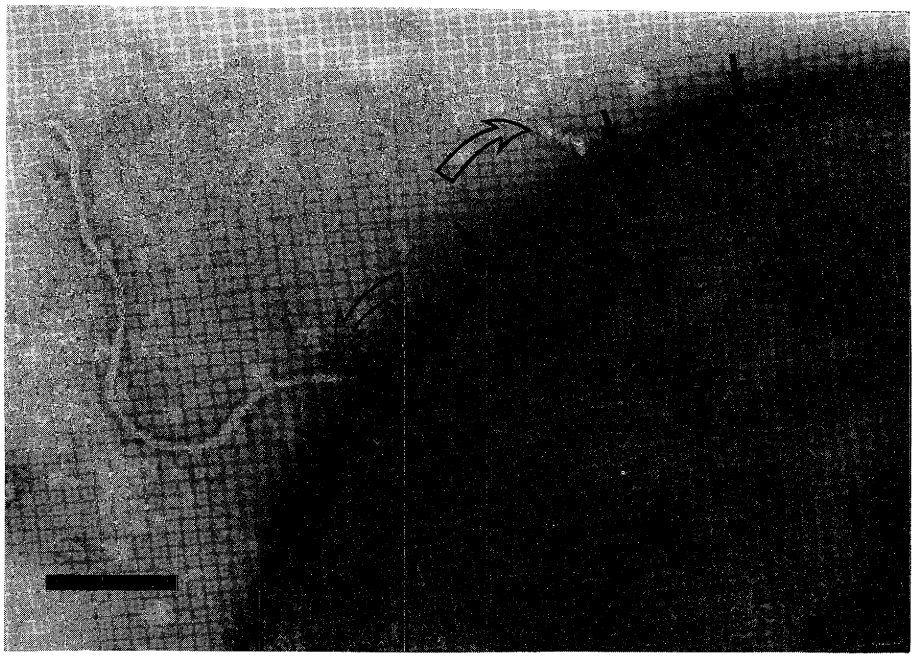


Fig. 8 (a)



(b)

Fig. 8 (a) Strain of 83K23 cultured on TCG agar showing numerous peritrichous holes (arrows) and fimbriae (arrow heads). Bar, 0.1 μm.
(b) Higher magnification showing fimbriae and base plates of fimbriae (white arrows). Bar, 0.1 μm.

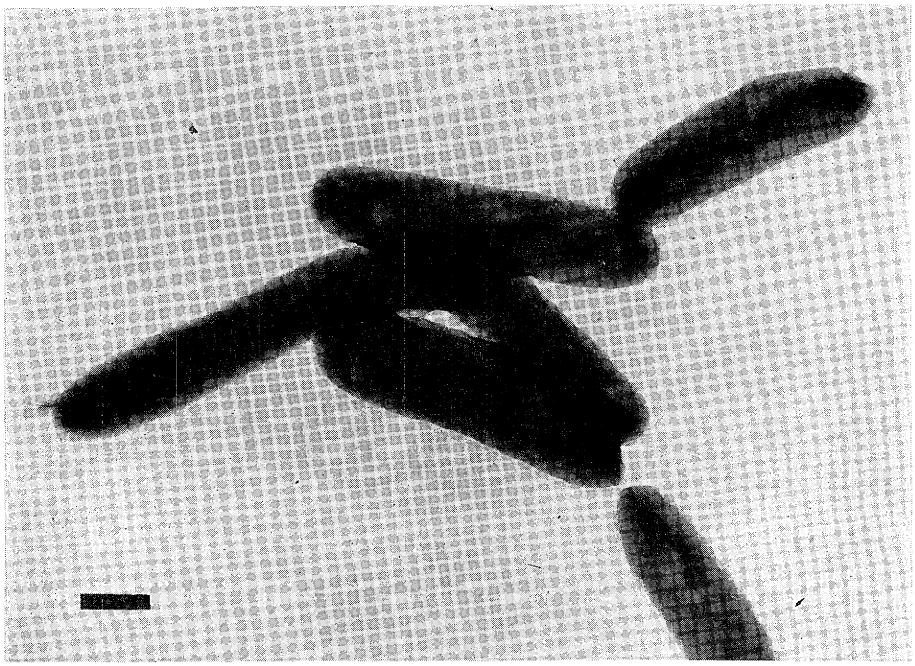


Fig. 9. Non-enteropathogenic strain of H218 cultured on TCG agar showing smooth surface without fimbriae. Bar, 0.5 μm.

DISCUSSION

Two important and interrelated findings have emerged from this study: the observation of fimbriate *V. cholerae* O1 during the colonization to the surface of the upper small intestine and the development of a appropriate medium (TCG agar) for the production of fimbriae from *V. cholerae* O1.

The development of cholera vaccine has been remaining as one of the most important themes for cholera researchers. After the report by Tweedy *et al.* (1968), no one has reported the evidence for the presence of fimbriae on *V. cholerae* O1 except Faris *et al.* (1982).

This is the first report on the evidence for the presence of fimbriate *V. cholerae* O1 during the colonization of the organism to the surface of the small intestine. According to our experience in the rabbit ileal loop test, enteropathogenic strains of *V. cholerae* O1 exhibit positive even if they were cultured in normal saline, heart infusion broth or in any other medium in which they can grow. This fact means that *in vitro* culture conditions do not affect *V. cholerae* on their production of the colonization factor and cholera toxin, once they had the chance to interact with the epithelial surface.

Then, what mechanism can change *V. cholerae* from non-fimbriate phase to fimbriate phase? We thought that this change might be referred to the differentiation of *V. cholerae* as malignant cells differentiate into benign cells in the eukaryotes.

Differentiated cells like nerve cells in the central nervous system contain tubulin in the cytoplasm relatively much more than undifferentiated cells. However, no one has reported the presence of tubulin in *V. cholerae*, our hypothesis that even bacteria have tubulin in the cytoplasm led us to develop a new medium to differentiate *V. cholerae*. Thioproline was reported as an inducer of reverse transformation and induced dose-dependent cyclic nucleotides elevation from cerebral tumor cells (Brugarolas and Gosalvez, 1980; Caciacli *et al.*, 1980).

This amino acid was also reported to stimulate germ tube formation in *Candida albicans* (Hagihara *et al.*, 1985). Sodium glutamate, mono is a tubulin stabilizer (Arakawa and Timasheff, 1984). One milli molar of EGTA inhibits proteinase secreted by *V. cholerae* as an extracellular protein and chelates calcium ion in the medium. One per cent Bactotriptone compensates for a very small amount of metal ions necessary for CT production.

TCG medium developed on the above reasons was successful to produce fimbriae from *V. cholerae*, however the reasons remain to be elucidated.

A cap like structure observed under EM seems to represent the base plate of the fimbriae in the cytoplasm. Numerous holes identified in the surface of the cell appear to be the channels through which fimbriae emerge, since their diameter was the same as that of the fimbrial strand, and their frequency and peritrichous distribution corresponded to the degree and pattern of cell fimbriation. This observation suggests that the vast ma-

jority of the fimbriae are already detached at cell harvest and that enteropathogenic strains of *V. cholerae* produce several hundreds of fimbriae per cell. This apparent ease with which *V. cholerae* fimbriae are shed from the cell is a significant feature of this organism.

Whole cell vaccines which do not include the fimbriate cells nor the supernatant (i. e., fimbrial) fraction may have reduced efficacy, since the fimbriae are considered to be important protective antigens against cholera infection. Our findings presented in this paper could provide the definitive answer for the controversial question of whether *V. cholerae* O1 produces fimbriae which participate in the attachment of the vibrios to the small bowel. We suppose that this paper will encourage cholera researchers to develop parenteral cholera vaccine by using the fimbriae.

Purification and characterization of fimbriae are in rapid progress.

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コレラ菌の線毛: 腸管上皮に定着したコレラ菌における線毛の観察と線毛産生を増強する新しい培地の開発

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岩永正明（琉球大学医学部細菌学教室）

病原性のコレラ菌は小腸上皮へ定着するということは，下痢発症の初期に生じる現象として一般的に認められている．しかしながら，定着因子は未だコレラ菌に関しては定かなものは確認されていない．我々は定着因子を確認するために以下の実験を行った．病原株 83K23 (El Tor，小川型)，83K1 (El Tor，稲葉型) を教室保存株から兎ループテストにより選択した．対照として無鞭毛・非病原株 H218 を用いた．ループテストで明らかな液体貯溜を認めた各株を貯溜液吸引後，切除，切開したループを生食で 100ml 用びんに入れ，3 度洗浄後滅菌カバーグラスで小腸上皮よりかきとり，5％アセテイン処理して，電顕下で観察し，線毛 (pili or fimbriae) の出ていることを確認した．コレラ菌の線毛は幅 5〜7nm のしなやかな線維で ETEC の pili とは異なり，*Bacteroides* のそれに類似していた．我々が試作したチオプロリン，EGTA，重そうを含む TCG 培地上でも全く同様の pili が観察され，小川型でも稲葉型でも全く形態上の変化は認められなかった．なお対照株 H218 には線毛を認めなかった．何故今回我々が試作した培地で pili 産生が成功したのか，今後，pili の精製と共に，その理由を明らかにしていきたい．