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Cell Morphology of Dominant Bacteria in Tilapia Intestine*1

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Abstract

The cell morphology of four dominant bacterial species isolated from tilapia intestines was examined by using transmission and scanning electron microscopy. Cell lengths of *Bacteroides* sp. *ATS*, *Bacteroides* sp. *BTS*, *Aeromonas hydrophila* and *Plesiomonas shigelloides* were 4.0－6.0, 2.5－5.0, 1.5－2.0 and 2.0－2.5 μm, respectively. Some strains of *Aeromonas hydrophila* possessed numerous thin pili or fibrils as well as a single polar flagellum and concomitantly showed the hemagglutination to chicken blood. The cell elongation of *Bacteroides* sp. *ATS* was observed for the cells grown on EG agar under CO_2 gas phase but not under N_2+CO_2(9 : 1) gas phase. The addition of NaCl or $MgCl_2$(0.9% w/v) to EG agar repressed the cell elongation of *Bacteroides* sp, *ATS* even if the cells grew under CO_2 gas phase. The cells of *Bacteroides* sp. *BTS* were comparatively slender rod-shaped and did not change apparently in the cell form under the growth conditions tested in this study.

The digestive tract of animals is known to provide the favourable habitat for the normal microflora, which contributes to specific functions such as the host nutrition and defense systems. In general, it has been reported that the intestinal microflora of fish is relatively simpler in generic composition than that of terrestrial mammals. Many published studies[1,2] also have described that fish have a normal intestinal microflora with higher counts of facultative anaerobes and fewer anaerobes while the normal flora of mammals consist of a wide variety of obligate anaerobes outnumbering facultative forms. However, some omnivorous or herbivorous fishes such as tilapia, were found to harbor the characteristic obligate anaerobes predominating over facultative anaerobes in the intestine.[3,4] In the intestine of tilapia, only four species have been shown to be principally recovered as the dominant bacteria of normal microflora as reported in previous papers.[5,6] They were two different species of obligate anaerobes, *Bacteroides* spp. and two facultative anaerobes, *Aeromonas hydrophila* and *Plesiomonas shigelloides*.

The present study illustrated the cell morphology under various growth conditions of the representative strains of dominant bacterial species isolated from tilapia intestines by use of electron microscopies.

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Materials and Methods

Bacterial strains and culture conditions. The bacterial strains used in this study were isolated from the intestinal contents of tilapia (*Sarotherodon niloticus*) by use of three kinds of media. *Bacteroides* spp. *ATS* and *BTS* were grown on EG agar[7] or NBGT−1/3S agar[8] under anaerobic conditions (N_2 : CO_2= 9 : 1 or CO_2 gas phase) and *Aeromonas hydrophila* and *Plesiomonas shigelloides* were grown on Z−AII agar[6] aerobically. *Bacteroides* strains were also incubated in PYFGS liquid medium[7], which contained Trypticase peptone (BBL) 5.0 g, Proteose peptone №3 (Difco) 5.0 g, yeast extract (Difco) 10.0 g, Fildes solution 10.0ml, L-cysteine·HCl·H_2O 0.5 g, salts solution (consisted of $CaCl_2$ 0.2 g, $MgSO_4$ 0.2 g, K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, $NaHCO_3$ 10.0 g and NaCl 2.0 g per *l*) 40 m*l*, glucose 5.0 g and starch 3.0 g per *l* (pH 7.6), aerobically without shaking. In the experiment to examine the effect of salts on cell morphology, NaCl, $MgCl_2$ or $CaCl_2$ solution was added to EG agar in final concentration of 0.9% (w/v). Unless otherwise, all strains were incubated at 25 C for one day. Characterization and identification of test organisms were reported in previous papers.[4,9]

Negatively staining. The cells of facultative anaerobes grown in Z−AII broth or on Z−AII agar media were suspended in 1 % ammonium acetate and one drop of the suspension was put on a glass slide. A copper grid covered with collodion film was touched with the bacterial suspension to transfer bacterial cells on it. After one min, the excess water was blotted with a filter paper. The bacterial cells on copper grid were negatively stained with 1 % potassium phosphotungstate (PTA pH 6.8) for 30−40 s.

Scanning electron microscopy(SEM). The bacterial cells or intestinal tissues were adhered to a cover glass by using Bioden Meshcement (Oken Shoji). Specimens were fixed for 2 hr at 4 C in 0.1 M phosphate buffer containing 2.5 % glutaraldehyde (pH 6.8). After dehydration through acetone series solutions, they were transfered to isoamyl acetate for 30 min at room temperature and dried by a critical point dryer (Hitachi, HCP−2). The specimens were coated with gold about 20 nm in thickness by an ion coater (Eiko Engineering, IB−3) and observed by use of a scanning electron microscope (JEOL, JSM T−20).

Thin sections and transmission electron microscopy(TEM). The preparation of stained thin sections for electron microscopy was carried out by the method of RYTER and KELLINBERGER[10] (RK). The cell pellets were resuspended in a mixture of the KELLENBERGER fixation solution containing OsO_4 1 % and 0.2 ml of Tripton broth. This was followed by incubation at room temperature for 16 h. After centrifugation, bacterial pellets were mixed with melted 2 % agar solution to be taken up into agar blocks. The bacterial cells in agar blocks were then washed in MICHAELIS veronal-acetate buffer, and postfixed with 0.5% uranyl acetate for 2 h. The cells were dehydrated in a graded acetone series and followed by embedding in epoxy resin according to the method of SPURR.[11] Thin sections were cut with a glass knife and put onto copper grids, after which they were stained for 20 min with saturated uranyl acetate and then with 0.1% alkaline lead citrate[12] for 5 min. A transmission electron microscope (Hitachi H−300 or JEOL JEM−100B) was used to examine and photograph the specimens.

Results and Discussion

I. Direct electron microscopic examination of tilapia intestine.

As shown in Fig. 1, scanning electron micrographs of the unwashed and washed intestinal surface of tilapia demonstrated the bacterial cells in the intestinal contents including the skeleton of diatoms and amorphous materials and also the bacteria embedded with mucus materials of intestinal surface. These latter bacteria were comparatively fewer and showed morphologically similar cells as compared with those in the intestine of various mammals. Recent studies[13,14] have shown the presence of attached bacteria on the surface layer of the digestive tract of various animals. These bacteria grow in glycocalyx-enclosed microcolonies adhered to the surface tissues of host animals. In the case of fish intestine, bacteria in the intestinal contents did not appear to associate with intestinal tissue so firmly as those in the intestine of mammals. These results are not inconsistent with the fact that the microflora in fish intestines has a tendency to vary more easily depending on the growth conditions of host animals when compared with those of mammalian intestines.

II. Cell morphology of facultative anaerobes.

A. Negatively staining. Electron micrographs of negatively stained preparations of facultative anerobes are shown in Fig. 2. Cell lengths of *Aeromonas hydrophila* and *Plesiomonas shigelloides* strains were 1.5−2.0 μm and 2.0−2.5 μm respectively and both cells had a single polar flagella of which wave length was 2.0−2.5 μm. The undulated structure of cell surface was well observed for the cells of *A. hydrophila* strains. Some strains of *A. hydrophila* also contained thin straight pili or fibrils structure and concomitantly showed hemagglutinating activities to chicken blood cells (data not shown). None of *Plesiomonas* strains tested in this experiment showed pili-like structure and hemagglutination.

Bacterial pili (fimbriae) are now known to occur widely among gram-negative bacteria and to have the functional role as attachment organelles for adherence to animal host cell surface, which have been associated with virulence, colonization or hemagglutination.[15] For example, Type 1 pili of enterobacteria are found to be responsible for adhesion properties, especially hemagglutiation. *Aeromonas hydrophila* strains isolated from tilapia intestines were found to contain hemagglutinating and non-hemagglutinating strains.

B. Thin sections. Electron micrographs of ultrathin section of *Aeromonas* and *Plesiomonas* strains are shown in Fig. 3. The thin sections of both bacteria showed a typical structure of gram-negative bacterial cells. The cells were enclosed by crenated outer membrane and underlying plasma membrane and contained a cytoplasmic region which consisted of electron-opaque ribosomes and fibrillar nucleoids. The cells of *Aeromonas* possessed round ends while *Plesiomonas* had relatively sharp cell ends. Electron micrographs of *Plesiomonas* demonstrated a fairly straight outer membrane and electron-dense appendages which were associated with cell surface. BAYER *et al*[16] observed that capsular polysaccharide of *E. coli* K29 collapsed during conventional fixation and dehydration process to small clumps of high electron density. They also reported that the collapse can be avoided by exposing the cells to anticapsular antibody. The electron dense appendages of *Plesiomonas* strains observed in

this experiment would suggest that they were artifact structure derived from a kind of exopolysaccharide or surface structure. On the other hand, *Aeromonas* showed occasionally mesosome like structure in the cytoplasmic region.

Ⅲ. Cell morphology of obligate anaerobes.

Thin sections of *Bacteroides* spp. demonstrated a typical outer membrane structure of gram-negative bacteria. The cytoplasm was enclosed with double layers of membrane structure as shown in Fig. 4. Cell envelope of *Bacteroides* sp. *ATS* had a fairly wide periplasmic space between outer membrane and cytoplasmic membrane. On the other hand, outer membrane of *Bacteroides* sp. *BTS* contacted closely with cytoplasmic membrane and a electron transparent space was sometimes difficult to be recognized. Outer membrane of *Bacteroides ATS* was observed to be well undulated whereas that of *Bacteroides BTS* was comparatively thick and straight. Although the surface structure of cell was modified artifactly during the preparation procedure, it suggested the difference in chemical composition or physical state between both bacteria.

Under N_2+CO_2(9 : 1) gas phase on EG agar, *Bacteroides ATS* showed a large round cell form (4.0—6.0 μm) but under CO_2 gas phase on EG agar, the cell form of *Bacteroides ATS* became much longer (8.0—15.0 μm) as demonstrated in Fig. 5. However, even if *Bacteroides ATS* was grown under CO_2 gas phase, the addition of NaCl or $MgCl_2$(0.9%) to EG agar repressed the elongation of cells (Fig. 6). On the contrary, the addition of $CaCl_2$ to EG agar (0.9%) led the cells to be much longer than those grown on EG agar under CO_2 gas phase. As compared with *Bacteroides ATS*, *Bacteroides BTS* showed thin and longer cells when they were grown on EG agar under N_2+CO_2 gas phase. Under CO_2 gas phase, *Bacteroides BTS* could grow very slightly on EG agar but not showed the cell elongation. These results indicate that the cell form of *Bacteroides ATS* varies depending on gas phase during incubation and also influenced with the presence of Na, Mg or Ca salts in relatively high concentrations.

In *Bacteroides ATS* strains, it was observed that the nucleoids were localized in half part of cytoplasmic region when grown in PYFGS broth, while they were dispersed in the cells grown on EG agar (Fig. 4 a and b). Hobot *et al*[18] reported that under RK conditions of OsO_4 fixation the shape of nucleoid changed depending upon the external salt concentration in the medium. In the case of *Bacteroides ATS* isolated from fish intestines it is quetionable that the change of nucleoid shape occures depending on the salt concentration of the medium. Although PYFGS broth contained higher salt concentration than EG agar, the addition of salt to EG agar did not necessarily lead to the nucleoid localization. Many investigators[17] that bacterial cells could not initiate septum formation and therefore grew as long multinucleate filamentous forms under the sublethal conditions such as the addition of antibiotics. The cell elongation was scarcely observed in direct microscopic examination of the intestinal contents of tilapia. Consequently a relatively round-shaped rod which was observed under N_2+CO_2(9 : 1) gas phase seemed to be normal cell form for *Bacteroides ATS*. On the other hand, each species of *Bacteroides* was easily discernible from the cell form when they were incubated on EG agar under CO_2 gas phase, since *Bacteroides ATS* showed the cell elongation.

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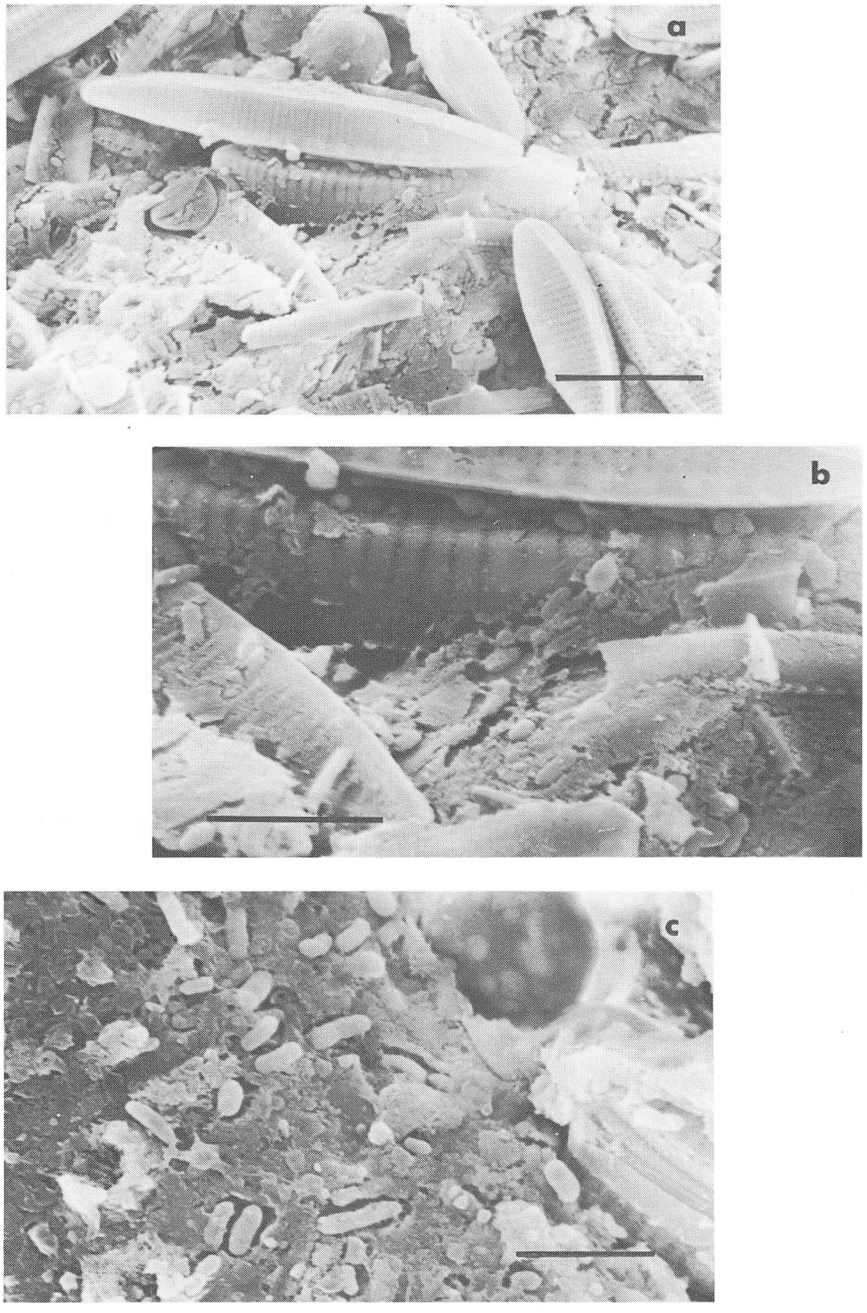

Fig. 1. SEM of critical point dried preparations of tilapia intestine.
a : Non-washed intestinal surface and contents of tilapia. Bar = 20 μm.
b : Non-washed intestinal surface and contents of tilapia. Bar = 10 μm.
c : Washed intestinal surface of tilapia. Bar = 10 μm.

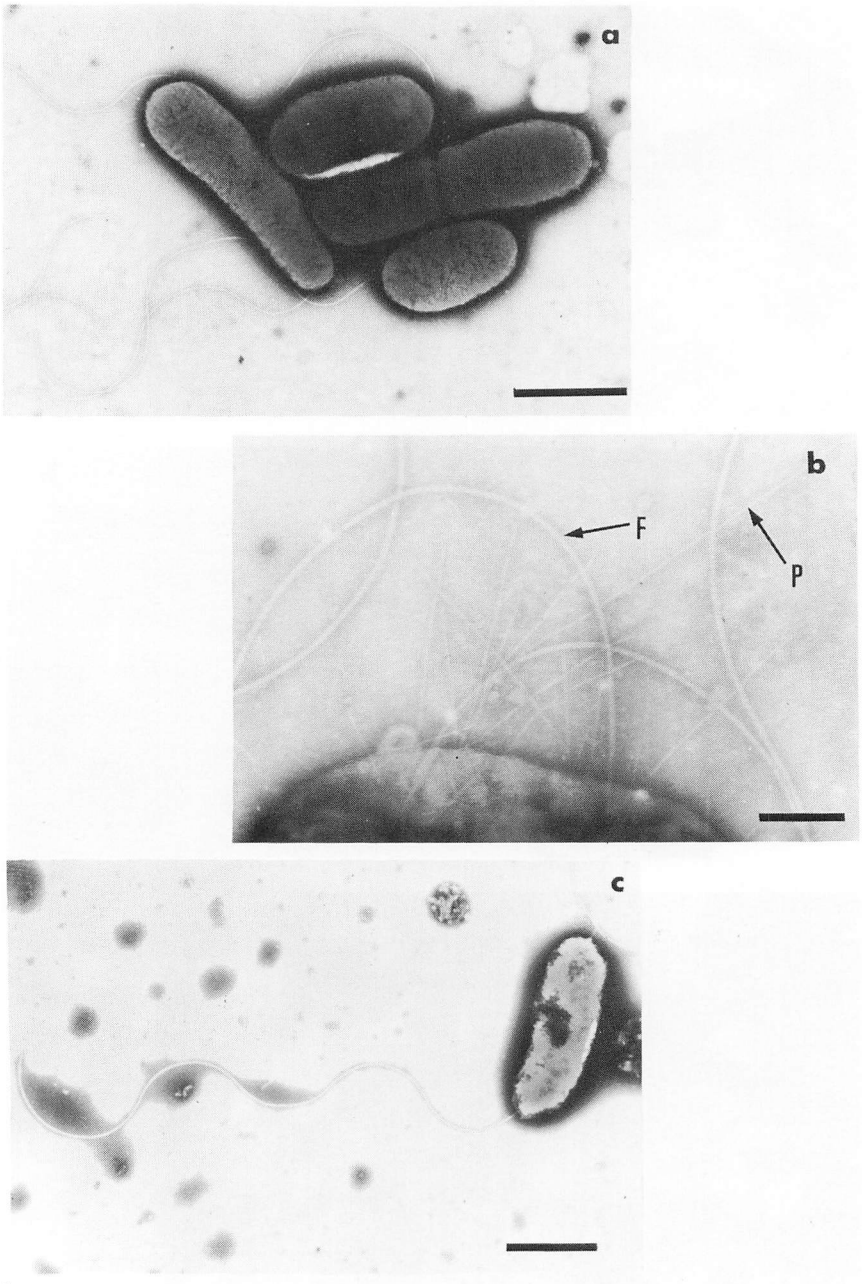

Fig. 2. TEM of the cells negatively stained with 1 % PTA.
a : *Aeromonas hydrohpila* NIF-166 grown in Z-AII broth. Bar = 1.0 μm.
b : *A. hydrohpila* NIF-166 grown in Z-AII broth. Bar = 0.1 μm.
F ; flagellum, P ; pili.
c : *Plesiomonas shigelloides* NIA-113 grown in Z-AII broth. Bar = 1.0 μm.

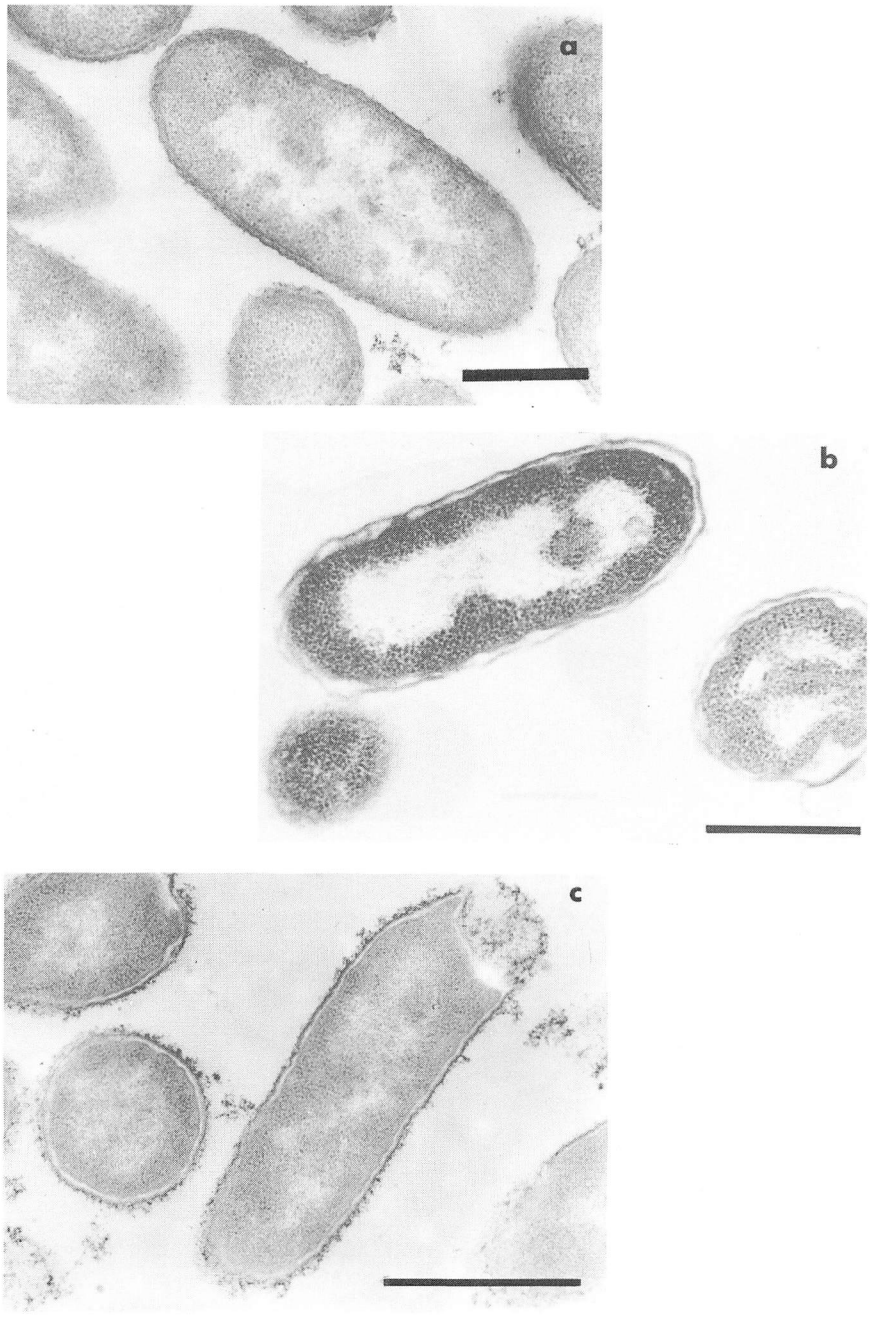

Fig. 3. TEM of thin sections of facultative anaerobes.
a：*A. hydrohpila* NIF-172 grown on Z-AII agar. Bar = 0.5 μm.
b：*A. hydrohpila* NIF-101 grown in Z-AII broth. Bar = 0.5 μm.
c：*P. shigelloides* NIA-105 grown on Z-AII agar. Bar = 1.0 μm.

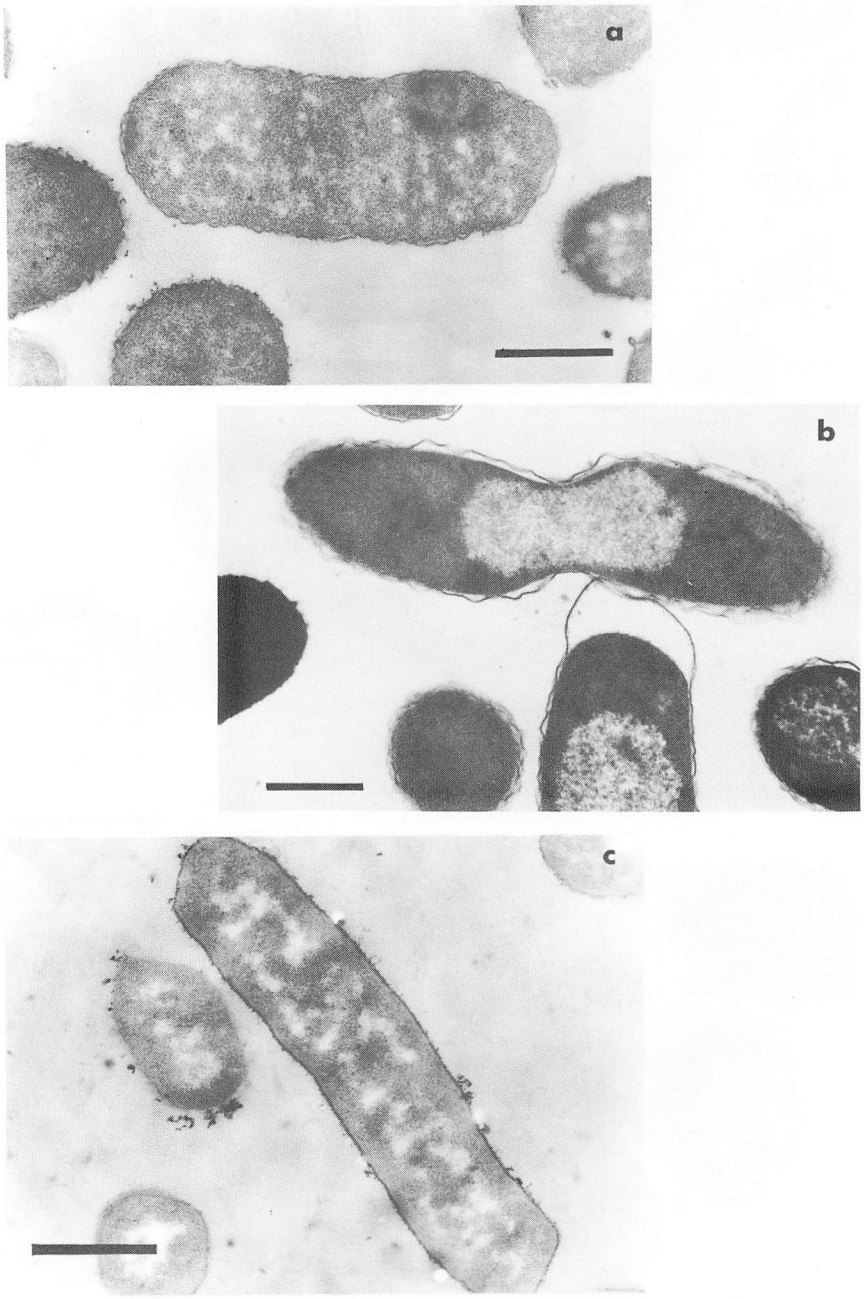

Fig. 4. TEM of thin sections of obligate anaerobes.
a : *Bacteroides sp. ATS* NOX-C201 grown on EG agar under N_2+CO_2 (9 : 1).
Bar = 1.0 μm.
b : *B. ATS* NIV-207 grown in PYFGS broth. Bar = 1.0 μm.
c : *B. BTS* NOM-219 grown on EG agar under N_2+CO_2(9 : 1). Bar = 1.0 μm.

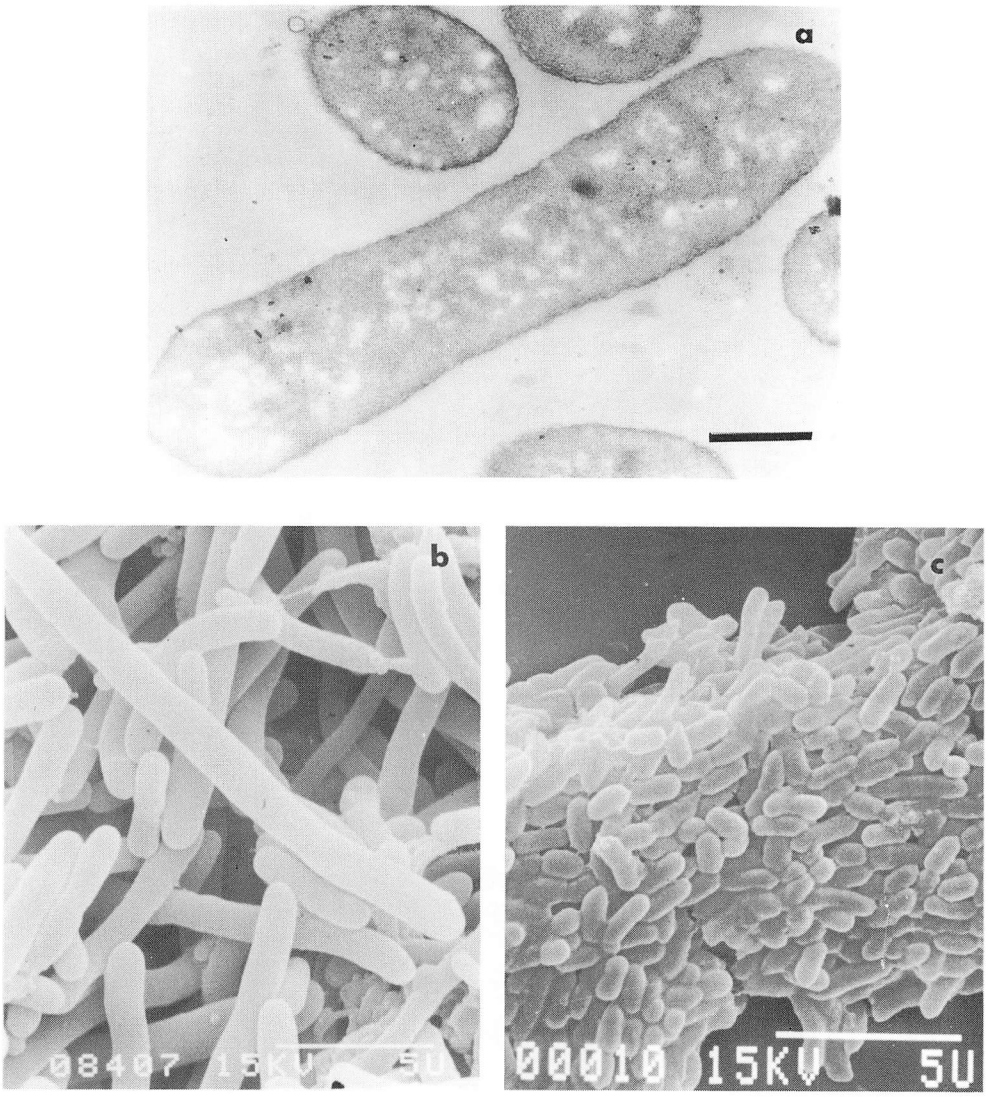

Fig. 5. TEM and SEM of obligate anaerobes.
a : TEM of *B. ATS* NOX-C201 grown on EG agar under CO_2. Bar = 1.0 μm.
b : SEM of *B. ATS* NOX-204 grown on EG agar under CO_2. Bar = 5.0 μm.
c : SEM of *B. BTS* NIV-233 grown on EG agar under N_2+CO_2(9 : 1). Bar = 5.0 μm.

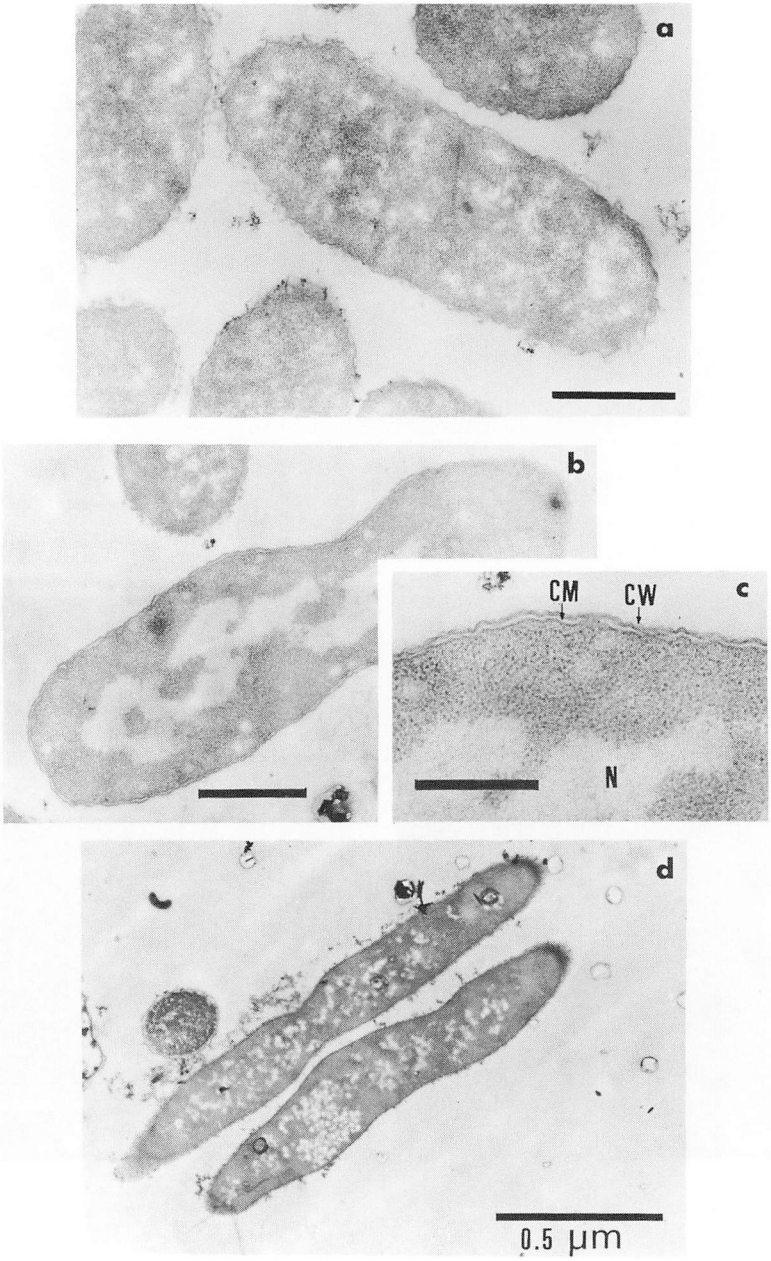

Fig. 6. TEM of thin sections of obligate anaerobes.
a : *B. ATS* NOX-C201 grown on EG+NaCl(0.9%) agar under CO_2. Bar=1.0 μm.
b : *B. ATS* NOX-C201 grown on EG+$MgCl_2$(0.9%) agar under CO_2. Bar=1.0 μm.
c : Same as b. Bar=0.5 μm. CM ; cytoplasmic membrane, CW ; cell wall, N ; nucleoid.
d : *B. ATS* NOX-C201 grown on EG+$CaCl_2$(0.9%) agar under CO_2. Bar=0.5 μm.