Journal of General Microbiology (1979), **112**, 261–268. *Printed in Great Britain*

Ultrastructure of *Cytophaga johnsonae* and *C. aquatilis* by Freeze-etching

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(*Received* 6 *February* 1979)

Cytophaga johnsonae and *C. aquatilis* (ATCC 29551), examined by frozen-surface replica and freeze-etch techniques, displayed undulated and particulated surfaces. They produced copious amounts of extracellular slime when cultured. *Cytophaga aquatilis* contained large, complex mesosomes in freeze-etched cells whereas *C. johnsonae* contained none. In non-cryoprotected freeze-etch preparations, *C. aquatilis* contained two major fracture planes whereas the *C. johnsonae* cell envelope fractured in three distinct planes. Linear fibres, such as the peripheral fibrils previously described in *Flexibacter columnaris*, were not observed in the outer membrane of freeze-etched *C. aquatilis*. *Cytophaga johnsonae* contained periplasmic linear fibrils; however, these appeared to be associated with the outer surface of the cytoplasmic membrane or an intermediate layer and not with peripheral fibrils.

INTRODUCTION

The general ultrastructure of the cytophagas (inclusive of the genera *Cytophaga*, *Flexibacter* and *Sporocytophaga*) has been investigated by several groups (Christensen, 1977). Of the single cell type, however, only *Flexibacter columnaris* (formerly *Chondrococcus* and *Cytophaga columnaris*) has been studied extensively for its ultrastructure (Pate & Ordal, 1967*a*, *b*; Pate *et al.*, 1967; Burchard & Brown, 1973; Kuhrt & Pate, 1973). Freeze-etch preparations of the cytophagas have been limited to *F. columnaris* (Burchard & Brown, 1973) and *C. flevensis* (van der Meulen *et al.*, 1974).

Freeze-etch and frozen-surface replica techniques were used in this study to describe further the ultrastructure of *C. johnsonae* and *C. aquatilis*. *Cytophaga johnsonae* is a well-defined species of *Cytophaga* whereas *C. aquatilis* has only recently been described (Strohl & Tait, 1978).

METHODS

Organisms and culture conditions. Strain N of *Cytophaga aquatilis* (ATCC 29551) and strain G (Group II; Strohl & Tait, 1978) of *C. johnsonae*, used for this investigation, were isolated from the gills of freshwater salmonoids at the Platte River Fish Hatchery, Honor, Michigan, in 1974 (Strohl & Tait, 1978). Stock cultures and cultures used for electron microscopy were grown at room temperature (22 to 24 °C) on a medium of 2 % (w/v) Casitone supplemented with Chu no. 10 basal salts (Shilo, 1970). Frozen-surface replica preparations were made using cells grown for 24 to 48 h on plates of basal medium solidified with 1·5 % (w/v) agar.

Electron microscopy. Cells harvested from the liquid basal medium by centrifuging (3000 *g* for 20 min) were placed directly into complementary specimen holders (Steere, 1973) or recentrifuged on a cryoprotective glycerol/sucrose density gradient (Steere *et al.*, 1975) before placing into the specimen holders. Frozen-surface replicas were prepared according to Steere (1973) using cultures that had been prefixed for 1 h on plates of basal medium with 3 % (v/v) glutaraldehyde. The agar was sliced approximately 3 mm below the surface and was then put on metal stubs for insertion into the freeze-etch module (Steere, 1973). Both freeze-etch and frozen-surface replicas were etched for 2 min at −98 °C, shadowed at a 45° angle with

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platinum/carbon and then carbon coated. A modified Denton DFE-2 freeze-etch module was used for the freeze-etch and the frozen-surface replicas. A JEOL JEM 100B electron microscope equipped with a 60° top entry goniometer stage was used at 80 kV acceleration voltage for all micrographs.

The nomenclature proposed by Branton *et al.* (1975) was adopted for the description of the freeze-etched cell envelopes: the exoplasmic or extracellular half of the fractured membrane is designated *E* and the inside or protoplasmic half of the fractured membrane is designated *P*. Therefore, the abbreviations used for the fractured faces were: *ECM* and *PCM* for the *E* and *P* faces of the cytoplasmic membrane, respectively; *EOM* and *POM* for the *E* and *P* faces of the outer membrane, respectively; and *PS* for the etched outer surface (*P* face) of the cell.

RESULTS AND DISCUSSION

Cytophaga aquatilis exhibited a typical Gram-negative cell envelope (Figs 1, 2) comprising a cytoplasmic membrane (6·5 nm), a peptidoglycan layer (4·5 nm) and an outer membrane (7·5 nm). The thickness of the entire cell envelope, including the periplasmic space, averaged 32 nm. The *C. johnsonae* cell envelope (Fig. 3) was similar.

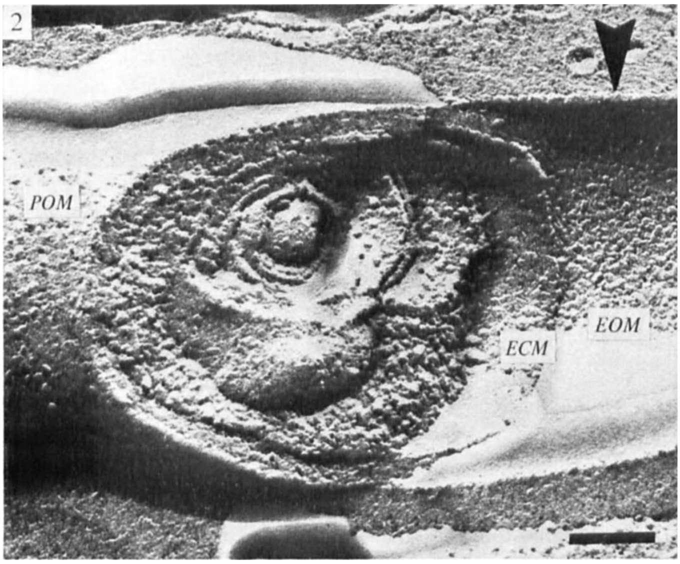
Dividing cells of *C. aquatilis* formed a septum (Figs 4, 5; L. Tait, personal communication) and mesosomes were always associated with septum formation (Fig. 5). Several filamentous gliding bacteria have been shown to possess septa during division (Poos *et al.*, 1972; Ridgeway *et al.*, 1975; Reichenbach & Golecki, 1975; Strohl & Larkin, 1978). The non-filamentous gliding bacterium *Chondromyces crocatus* (MacRae & McCurdy, 1975) also possesses septa during division.

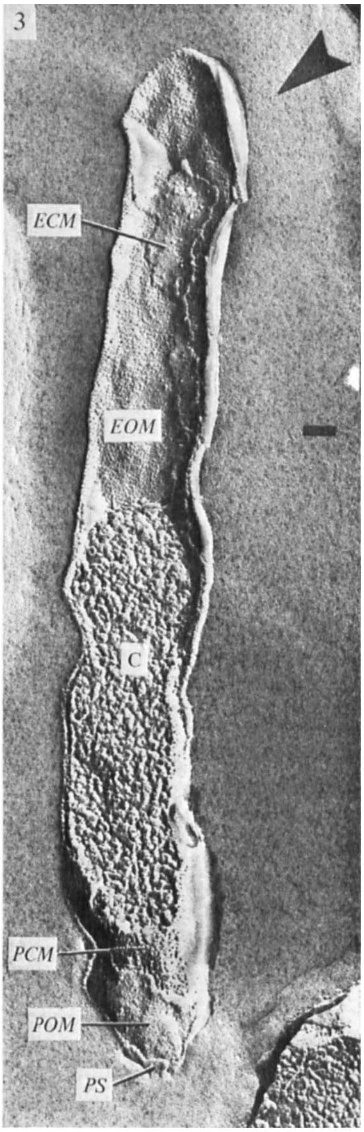
Mesosomes were observed in most *C. aquatilis* cells. These mesosomes were complex and composed of several membrane folds or layers (Figs 2, 5). Doughnut-shaped, mesosome-like structures were observed in a few cells (Fig. 6) and a connected series of sack-like mesosomes was observed in one cell (Fig. 1). Several mesosome membranes were morphologically similar to the cytoplasmic membrane (Fig. 2) and some mesosome surfaces were smooth or pitted (Fig. 5). The complex mesosomes observed in most of the freeze-etched cells of *C. aquatilis* appeared similar in complexity to the mesosomes observed in thin sections of *F. columnaris* (Pate & Ordal, 1967*a*) and of *Myxococcus xanthus* (Voelz, 1965). The morphological similarity of freeze-etched mesosomal membranes to the cytoplasmic membrane has been reported for *Bacillus subtilis* and a variety of other organisms (Nanninga, 1973),. In both thin sections (Strohl & Tait, 1978) and freeze-etch (no cryoprotection or prefixation) preparations (Fig. 2), the mesosomes found in *C. aquatilis* appeared to be more complex than those of most Gram-negative bacteria. Unlike *C. aquatilis*, no mesosome-like structures were detected in freeze-etch preparations of *C. johnsonae*. Because both organisms were freeze-etched simultaneously, the differences observed in their internal membrane structures were probably due to differences in the organisms themselves rather than to fixation artefacts.

The surfaces of both cytophagas were irregularly undulated in both the frozen-surface replicas (Fig. 4) and the freeze-etch micrographs (Figs 6, 7), although the dimensions of the undulations were different in different preparations. The surface of *C. johnsonae* has been described as deeply and irregularly undulated due to the production of slime within the outer membrane (Follett & Webley, 1965; Schmidt-Lorenz & Kühlwein, 1969). Pate & Ordal (1967*b*) also found a 'large amount of ruthenium red-positive material located in the space between the plasma membrane and the outer unit membrane' in damaged cells of *F. columnaris*.

The cell envelope membranes of freeze-etched *C. johnsonae* appeared to be very porous with 4 nm pores randomly distributed throughout (Fig. 8). Moreover, Follett & Webley (1965) suggested that pores existed in the outer membrane of *C. johnsonae* that allowed slime to be extruded from the cells.

The extracellular slime of *C. aquatilis* was fibrillar in nature and was often associated with the cell surfaces (Fig. 4). The *C. aquatilis* slime appeared similar to the mucopoly-





Large arrows designate the direction of shadow. Bar markers represent 0·1 μm.

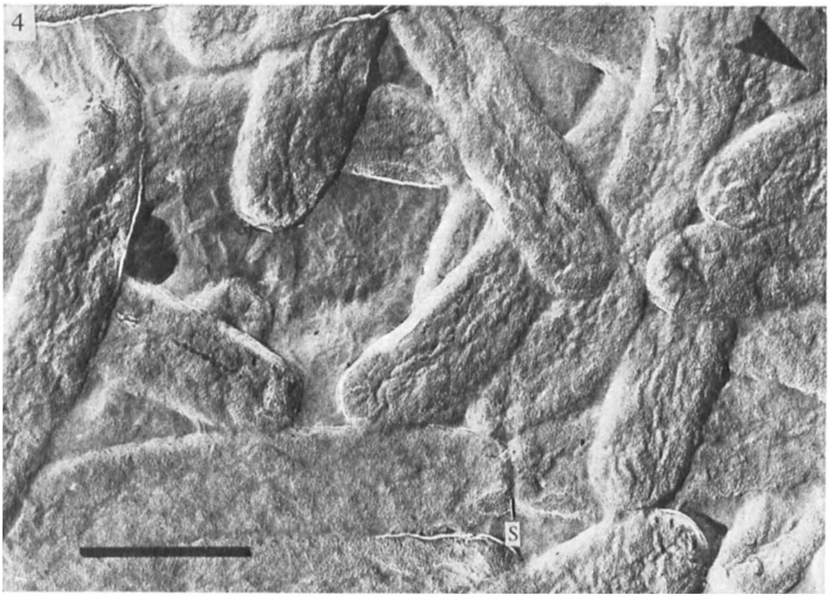
Fig. 1. Freeze-etched *C. aquatilis* showing a series of mesosomes, the partitioned cell envelope (CE), the cytoplasm (C) and the *P* face of the fractured outer membrane (*POM*).

Fig. 2. Freeze-etched *C. aquatilis* showing a fractured lamellar mesosome containing several layers. Note the *P* and *E* faces of the fractured outer membrane (*POM* and *EOM*) and the *E* face of the fractured cytoplasmic membrane (*ECM*).

Fig. 3. Freeze-etched *C. johnsonae* showing the *E* and *P* faces of the cytoplasmic (*ECM* and *PCM*) and the outer (*EOM* and *POM*) membranes, the surface (*PS*) and the cytoplasm (C).

saccharide slime of other gliding bacteria (Pate & Ordal, 1967*b*; Verma & Martin, 1967; Follett & Webley, 1965; Schmidt-Lorenz & Kühlwein, 1969).

Two fracture planes were observed within the cell envelope of *C. aquatilis*, the cytoplasmic and outer membranes appearing to have been fractured through the lipophilic regions (Fig. 9). The external surfaces of some *C. aquatilis* cells were also observed (Fig. 6). Three fracture planes were detected in the freeze-etch preparations of *C. johnsonae*; these were (i) a fracture of the cytoplasmic membrane through its hydrophobic region (Figs 3, 10),



Large arrows designate the direction of shadow. Bar markers represent 1 μm in Fig. 4 and 0·1 μm in Fig. 5.

Fig. 4. Frozen-surface replica of *C. aquatilis* cells showing fibrillar slime and the undulated surfaces of the cells. Note the septation between two dividing cells (S) and the flexibility evident in some cells.

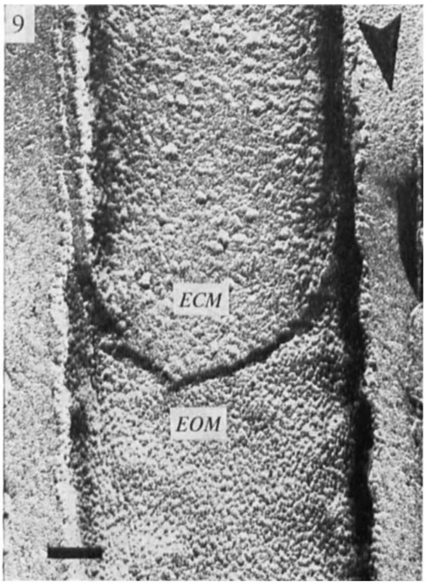
Fig. 5. Freeze-etched *C. aquatilis* showing septation with associated mesosomes.

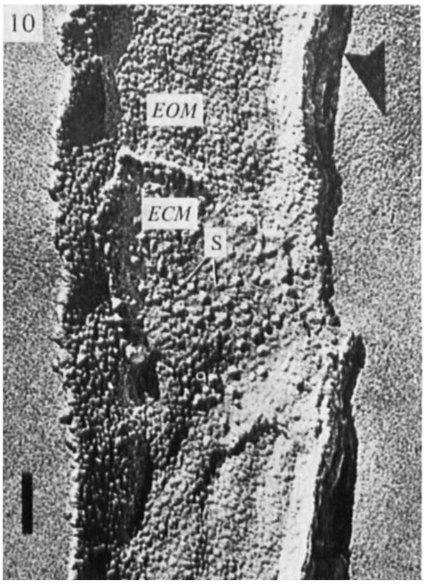
Fig. 6. Freeze-etched *C. aquatilis* showing the cell surface (*PS*), the *E* face of the outer membrane (*EOM*), several mesosomes (including a doughnut-shaped mesosome) and the 6 to 8 nm protrusions (S) associated with the surface of the cells.

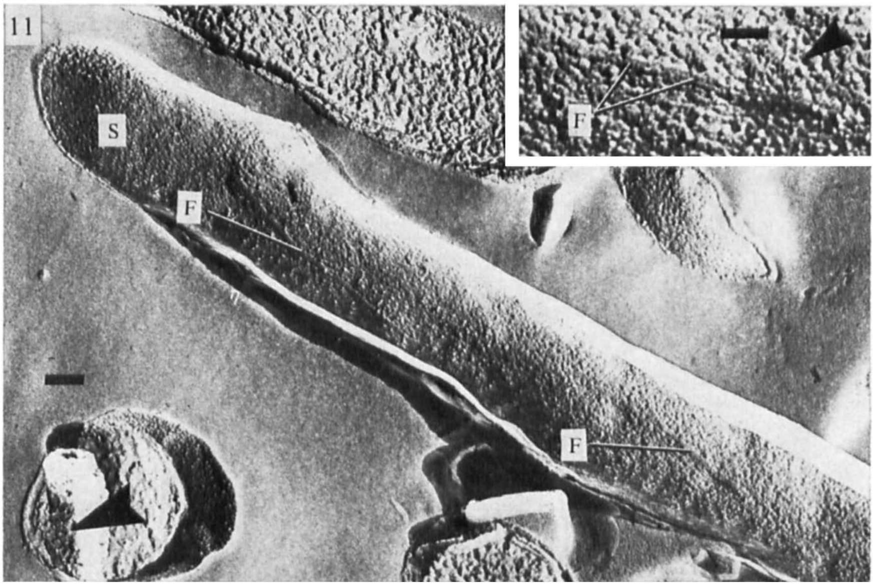
Fig. 7. Glycerol/sucrose cryoprotected freeze-etched *C. johnsonae* cell showing the outer surface (*PS*) and the 8 nm protrusions associated with it. Also shown is the *E* face of the fractured outer (lipopolysaccharide) membrane (*EOM*).

Fig. 8. The tangentially fractured *C. johnsonae* cell shows the *P* face of the cytoplasmic and outer membranes (*PCM* and *POM*) and a layer sandwiched between them (ML), presumably the peptidoglycan layer. The longitudinal cell shows the *P* face of the cytoplasmic membrane (*PCM*), the cytoplasm (C), the membrane associated pores (P; see inset) and a pitted surface (S), presumably located between the cytoplasmic membrane fracture and the outer membrane, which has been fractured away.

Large arrows designate the direction of shadow. Bar markers represent 0·1 μm (20 nm in Fig. 8 inset).







Large arrows designate the direction of shadow. Bar markers represent 0·1 μm (50 nm in Fig. 11 inset).

Fig. 9. Freeze-etched *C. aquatilis* showing the *E* surfaces of the fractured cytoplasmic (*ECM*) and outer (*EOM*) membranes.

Fig. 10. High magnification of *C. johnsonae* cell envelope showing the *E* faces of the cytoplasmic (*ECM*) and the outer (*EOM*) membranes. The 6 to 8 nm protrusions (S) associated with the fractured cytoplasmic membrane and the 4 to 6 nm beads in the outer membrane are visible.

Fig. 11. Freeze-etched *C. johnsonae* cell showing the pitted surface (S) of an intermediate layer, the double-stranded longitudinal fibrils (F; see inset) and remnants of the cell wall which has been fractured away.

(ii) a similar fracture through the outer membrane (Figs 3, 7, 10) and (iii) a fracture which appeared between the cytoplasmic and the lipopolysaccharide membranes, exposing a pitted, morphologically distinct surface (Figs 8, 11). This third fracture plane may be located at the outer surface of the cytoplasmic membrane or it may be a fracture between the outer portion of the peptidoglycan layer and the inner surface of the outer membrane. The surface of some *C. johnsonae* cells was also preserved when freeze-etched in the presence of the glycerol/sucrose cryoprotectant (Fig. 7).

The most commonly fractured planes in Gram-negative bacteria appear to be the lipophilic regions of the cytoplasmic and the lipopolysaccharide membranes (Van Gool & Nanninga, 1971). Thus, the observation of a third fracture plane in *C. johnsonae* at a location between the two unit membranes is of interest, as it appears to be unusual.

Several 6 to 8 nm protrusions, considered to be globular protein molecules (Costerton *et al.*, 1974) or enzyme groups (Moor, 1966), were randomly located in the external faces of the fractured and etched cytoplasmic membranes of both cytophagas (Figs 9, 10). The protoplasmic face (*PCM*) of the cytoplasmic membrane in *C. johnsonae* was very granular (Fig. 8). Closely packed 4 to 6 nm beads were observed on the external face of the fractured outer membranes of both organisms (Figs 6, 7, 9, 10). The external surfaces of both organisms contained 8 nm protrusions (Figs 6, 7).

Although 6 nm particles occurred in a position similar to the peripheral fibrils found in glutaraldehyde-prefixed freeze-etched *F. columnaris* (Burchard & Brown, 1973), no peripheral fibrils were observed in *C. aquatilis*. Double-stranded longitudinal fibres, 10 to 12 nm in width, were observed on the surface of the intramembrane fracture in *C. johnsonae* in non-prefixed freeze-etched cells (Fig. 11). The number, structure, location and fixation of the *C. johnsonae* fibrils suggest that they are different from the *F. columnaris*-type peripheral fibrils. No function has been attributed to the *C. johnsonae* fibrils.

I wish to thank Dr Russell Steere and Mr Eric Erbe, Plant Virology Laboratory, Plant Protection Institute (U.S.D.A., Beltsville, Md., U.S.A.) for their excellent technical assistance in the preparation of the freeze-etch micrographs and Dr Steere for the use of his laboratory and equipment. I also thank Mr Larry R. Tait and Drs Ellis R. Brockman and James R. Lampky (Central Michigan University, Mt Pleasant, Mich., U.S.A.) for their stimulation of thoughtful discussion throughout this investigation.

REFERENCES

BRANTON, D., BULLIVANT, S., GILULA, N. B., KARNOVSKY, M. J., MOOR, H., MÜHLETHALER, K., NORTHCOTE, D. H., PACKER, L., SATIR, B., SATIR, P., SPETH, V., STAEHLIN, L. A., STEERE, R. L. & WEINSTEIN, R. J. (1975). Freeze-etching nomenclature. *Science* **190**, 54–56.

BURCHARD, R. P. & BROWN, D. T. (1973). Surface structure of gliding bacteria after freeze-etching. *Journal of Bacteriology* **114**, 1351–1355.

CHRISTENSEN, P. J. (1977). The history, biology, and taxonomy of the *Cytophaga* group. *Canadian Journal of Microbiology* **23**, 1599–1653.

COSTERTON, J. W., INGRAM, J. M. & CHENG, K. J. (1974). Structure and function of the cell envelope of gram-negative bacteria. *Bacteriological Reviews* **38**, 87–110.

FOLLETT, E. A. C. & WEBLEY, D. M. (1965). An electron microscope study of the cell surface of *Cytophaga johnsonii* and some observations on related organisms. *Antonie van Leeuwenhoek* **31**, 361–382.

KUHRT, M. F. & PATE, J. L. (1973). Isolation and characterization of tubules and plasma membranes from *Cytophaga columnaris*. *Journal of Bacteriology* **114**, 1309–1318.

MACRAE, T. H. & MCCURDY, H. D. (1975). Ultrastructural studies of *Chondromyces crocatus* vegetative cells. *Canadian Journal of Microbiology* **21**, 1815–1826.

MOOR, H. (1966). Use of freeze-etching in the study of biological ultrastructure. *International Review of Experimental Pathology* **5**, 179–216.

NANNINGA, N. N. (1973). Freeze-fracturing of micro-organisms. Physical and chemical fixation of *Bacillus subtilis*. In *Freeze-etching techniques and applications*, pp. 151–179. Edited by E. L. Benedetti & P. Favard. Paris: Société Française de Microscopie Électronique.

PATE, J. L. & ORDAL, E. J. (1967*a*). The fine structure of *Chondrococcus columnaris*. I. Structure and formation of mesosomes. *Journal of Cell Biology* **35**, 1–13.

PATE, J. L. & ORDAL, E. J. (1967*b*). The fine structure of *Chondrococcus columnaris*. III. The surface layers of *C. columnaris*. *Journal of Cell Biology* **35**, 37–51.

PATE, J. L., JOHNSON, J. L. & ORDAL, E. J. (1967). The fine structure of *Chondrococcus columnaris*. II. Structure and formation of rhapidosomes. *Journal of Cell Biology* **35**, 15–35.

POOS, J. C., TURNER, F. R., WHITE, D., SIMON, G. D., BACON, K. & RUSSELL, C. T. (1972). Growth, cell division, and fragmentation in a species of *Flexibacter*. *Journal of Bacteriology* **112**, 1387–1395.

REICHENBACH, H. & GOLECKI, J. R. (1975). The fine structure of *Herpetosiphon*, and a note on the taxonomy of the genus. *Archives of Microbiology* **102**, 281–291.

RIDGEWAY, H. F., WAGNER, R. M., DAWSEY, W. T. & LEWIN, R. A. (1975). Fine structure of the cell envelope layers of *Flexibacter polymorphus*. *Canadian Journal of Microbiology* **21**, 1733–1750.

SCHMIDT-LORENZ, W. & KÜHLWEIN, H. (1969). Beitrage zur Kenntnis der Myxobakterienzelle. 2. Mitteilung Oberflächenstrukturen der Schwarmzellen. *Archiv für Mikrobiologie* **68**, 405–426.

SHILO, M. (1970). Lysis of blue-green algae by myxobacter. *Journal of Bacteriology* **104**, 453–461.

STEERE, R. L. (1973). Preparation of high-resolution freeze-etch, freeze-fracture, frozen-surface, and freeze-dried replicas in a single freeze-etch module, and the use of stereo electron microscopy to obtain maximum information from them. In *Freeze-etching techniques and applications*, pp. 223–255. Edited by E. L. Benedetti & P. Favard. Paris: Société Française de Microscopie Électronique.

STEERE, R. L., MOSELEY, J. M. & ERBE, E. F. (1975). Use of sucrose-glycerol density gradients in the preparation of living cells for high resolution complementary freeze-etch specimens. In *33rd Annual Proceedings of the Electron Microscopy Society of America*, pp. 616–617. Edited by G. W. Bailey. Baton Rouge, U.S.A.: Claitor's Publ. Div.

STROHL, W. R. & LARKIN, J. M. (1978). Cell division and trichome breakage in *Beggiatoa*. *Current Microbiology* **1**, 151–155.

STROHL, W. R. & TAIT, L. R. (1978). *Cytophaga aquatilis* sp. nov., a facultative anaerobe isolated from the gills of freshwater fish. *International Journal of Systematic Bacteriology* **28**, 293–303.

VAN DER MEULEN, H. J., HARDER, W. & VELDKAMP, H. (1974). Isolation and characterization of *Cytophaga flevensis* sp. nov., a new agarolytic flexibacterium. *Antonie van Leeuwenhoek* **40**, 329–346.

VAN GOOL, A. P. & NANNINGA, N. N. (1971). Fracture faces in the cell envelope of *Escherichia coli*. *Journal of Bacteriology* **108**, 474–481.

VERMA, J. P. & MARTIN, M. M. (1967). Chemistry and ultrastructure of surface layers in primitive myxobacteria. *Folia microbiologica* **12**, 248–253.

VOELZ, H. (1965). Formation and structure of mesosomes in *Myxococcus xanthus*. *Archiv für Mikrobiologie* **51**, 60–70.