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Electron microscopic examination of the extracellular polymeric substances in anaerobic granular biofilms

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Scanning electron microscopy revealed that collapsed extracellular polymeric substances (EPS) surrounded bacteria present in granular sludge. Treatment of granular sludge with whole-cell antiserum and staining with polycationic ferritin demonstrated that bacteria were enveloped by extensive EPS. Antibody stabilization permitted a visualization of the EPS which more closely resembled its natural hydrated state. The EPS was seen to completely fill the intercellular spaces in the microcolonies. Both pure and mixed microcolonies were observed to be enclosed by EPS. The presence of these large amounts of EPS indicates that this extracellular layer is important in maintaining the structural integrity of granular sludge.

Key words: Anaerobic granule, electron microscopy, extracellular polymeric substances.

Successful operation of upflow anaerobic sludge blanket (UASB) reactors in wastewater treatment depends upon the large accumulation of well-settling bacterial aggregates (De Zeeuw 1988). The aggregates produced in this process generally range from 1 to 3 mm in diam. (Beeftink & Van Den Heuvel 1988; Guiot *et al.* 1988) and are referred to as bacterial granules. The stability of anaerobic granules is based upon the firm attachment of the cells to each other. The irreversible adhesion of bacterial cells to both inert and living surfaces is mediated by a number of surface structures, including pili (Svanborg-Eden *et al.* 1977), flagella (Sjoblad & Doltsch 1982), distinct holdfasts (Poindexter 1964) and extracellular polymeric substances (EPS). EPS appear to be ubiquitous in natural ecosystems (Costerton *et al.* 1981a, b) and their fibrous nature makes them ideal for irreversible adhesion, since it is very unlikely that, once adsorbed, all the fibres would desorb at the same time. Adhesion mediated by EPS results in the development of an aggregate in which a large number of cells is bound in a 'biological cement'. The high stability of the bacterial granules produced in upflow wastewater treatment systems indicates that bacterial EPS may be involved in the development and maintenance of the granular structure.

As bacterial EPS may consist of 99% water (Sutherland 1972) they become severely condensed during the dehydration which is required for examination by standard electron microscopy (Costerton *et al.* 1981a). To prevent this loss of structure, specific antibodies (Bayer & Thurow 1977; Mackie *et al.* 1979) have been used to cross link the polymers of the EPS. The application of stains which bind to the EPS, such as ruthenium red (Luft 1971) and polycationic ferritin (Danon *et al.* 1972) can then be used to demonstrate the dimensions of the stabilized EPS. In the present study these stabilization techniques have been used to determine the importance of EPS in the structure of granular sludge.

Materials and Methods

Anaerobic Granules

The anaerobic granules used came from a 13.5-l upflow sludge bed filter (UBF) operated at 35°C and pH 7.2 ± 0.1 and fed a synthetic solution containing (g/l): sucrose, 4.4; yeast extract, 0.044; KH_2PO_4, 0.088; K_2HPO_4, 0.118; $(NH_4)_2SO_4$, 0.221; $KHCO_3$, 5.0; and $NaHCO_3$, 4.0. The reactor design and operation details are described by Arcand *et al.* (1994). The rate of dilution was 3.0 vol/vol. day. A recirculation to feed flow ratio of 10:1 produced a

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liquid upflow velocity of 0.9 m/h. The substrate removal efficiency was 82%, with a specific organic loading rate of 1.3 g chemical oxygen demand (COD)/g volatile suspended solid (VSS) day.

Bacterial Antigens

For preparation of bacterial antigens, 0.5 g sludge was homogenized for 30 s, in a commercial homogenizer (Kinematica; Brinkmann), in 15 ml phosphate-buffered saline (PBS) containing (g/l): NaCl, 8.5; KH_2PO_4, 0.61; and K_2HPO_4, 0.96 (pH 7.0). The samples were then diluted with 35 ml PBS and 50 ml 0.6% formalin and left at room temperature for 48 h. Bacterial death was determined with acetate and glucose activity tests, as described by Guiot *et al.* (1988). After bacterial death, the samples were shaken and the clumps were allowed to settle before aliquots containing the suspended cells were removed. The bacteria were then centrifuged at 4000 × *g* for 15 min, washed three times in PBS, and finally resuspended to give 200 μg protein/ml, determined with a bicinchonic acid protein assay. The immunogens were quick-frozen in liquid N_2 and stored in 1-ml lots at −80°C. For preparation of antisera, a New Zealand white rabbit was initially given two intramuscular injections totalling 0.5 ml, of a 1:1 (v/v) mixture of immunogen and Freund's complete adjuvant (Difco). Two weeks later, 0.5 ml of a 1:1 (v/v) mixture of immunogen and Freund's incomplete adjuvant (Difco) was given, followed by weekly injections each of 0.5 ml immunogen alone. Three days after the 13th weekly injection, 15 ml of whole blood was obtained by marginal ear bleeding. The serum was collected, quick frozen in liquid N_2 and stored at −80°C. Before use, the serum was heat-inactivated at 56°C for 0.5 h.

Electron Microscopy

Granules were collected under anaerobic conditions and washed three times in anaerobic cacodylate buffer, pH 7.2 ± 0.1, containing (g/l): cacodylic acid, 16.0; cysteine-HCl, 0.125; $Na_2S.9H_2O$, 0.125; and resazurin, 0.001. Complete granules or those that were cleaved with a scalpel were emersed in undiluted serum for 1 h with frequent mixing. The granules were then allowed to settle and were washed three times with anaerobic cacodylate buffer. The antibody-treated samples, as well as control samples without antibody stabilization, were fixed overnight at 4°C in anaerobic cacodylate buffer containing 5% glutaraldehyde. After glutaraldehyde fixation the remaining preparatory steps were performed under aerobic conditions. Fixation was followed by three washes in cacodylate buffer (16 g cacodylic acid/l, pH 7.2 ± 0.1). The samples were then exposed to polycationic ferritin (Sigma) at a final concentration of 1.0 mg/ml, for 30 min at 20°C (Weiss *et al.* 1979). The samples were diluted 10-fold and then washed three times in cacodylate buffer. Each granule was immobilized in 4% agar and washed five times with buffer. Post-fixation was performed with 2% osmium tetroxide for 2 h. The samples were again washed five times with buffer, and were then dehydrated in a graded acetone series (30% to 100%), followed by two exposures to propylene oxide. After embedding in Spurr's low-viscosity resin, thin sections were cut, stained with 1% uranyl acetate (pH 5.0) and lead citrate (Reynolds 1963) and examined with a Hitachi 601 transmission electron microscope operated at an accelerating voltage of 50 kV. Granule aliquots for scanning electron microscopy were prepared as described by MacLeod *et al.* (1990) and examined with a JEOL T220 scanning electron microscope operated at an accelerating voltage of 15 kV.

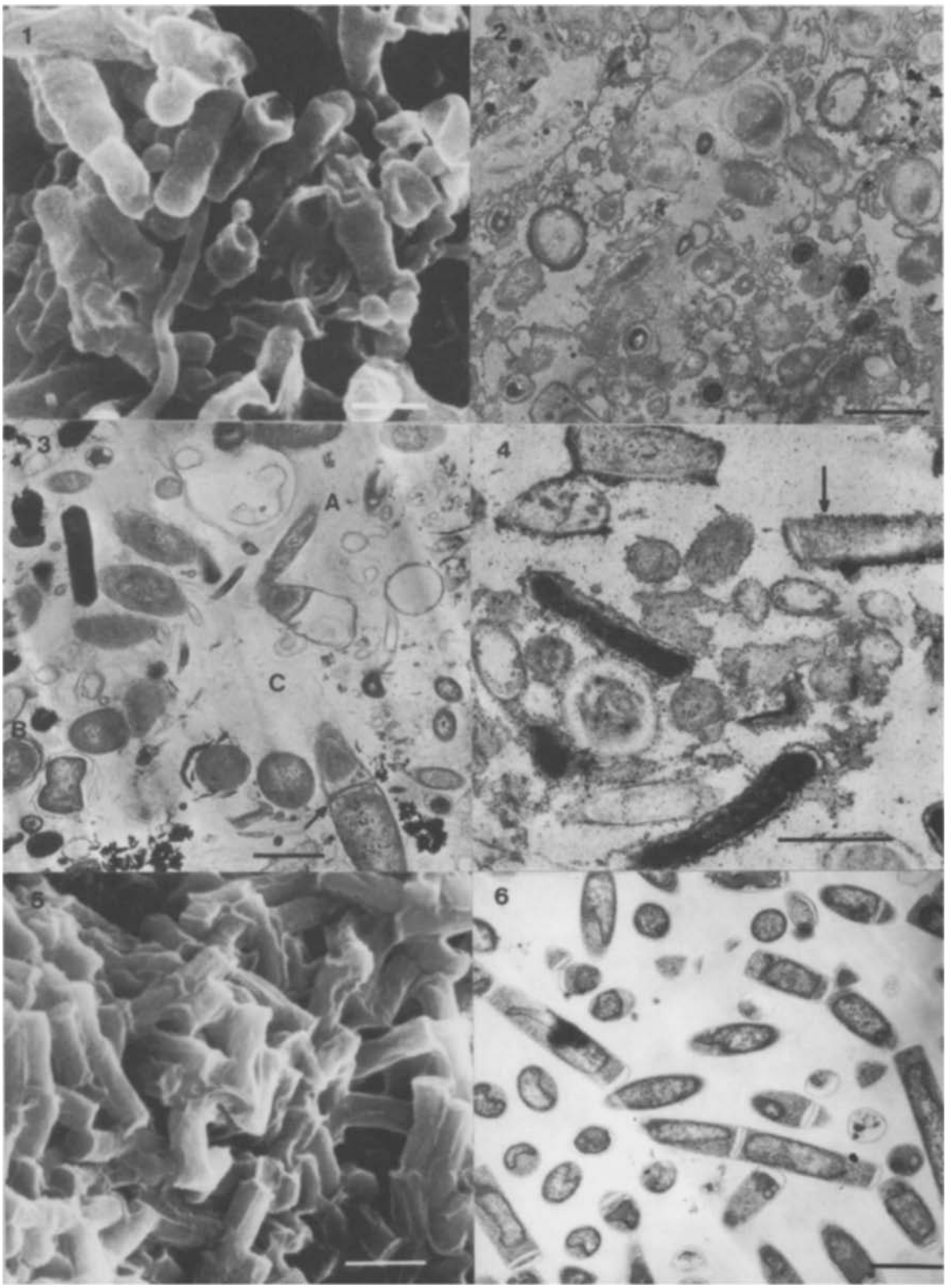
Results and Discussion

Scanning electron microscopy provided a three-dimensional picture of the bacterial associations which developed in the granular sludge (Figures 1 and 5). Due to the dehydration required for specimen preparation, the EPS collapsed upon the surface of the cells. However, it was often still apparent that this extracellular structure formed an enveloping layer which linked each cell to its neighbouring organisms. Transmission electron microscopy of thin sections of granular sludge that had been stained with ruthenium red sometimes revealed the presence of EPS around certain cells. This extracellular layer appeared as either a diffuse or a coarse fibrillar structure (Figure 3, areas A and B, respectively). Occasionally this material was seen to bridge the space between adjacent cells. Generally, however, EPS were very difficult to detect and most of the bacterial cells were seen to be separated from each other by electron translucent zones (Figure 3, area C). Thus, even with the use of a specific stain, EPS were difficult to detect in samples of granular sludge due to the collapse of their delicate structure during the preparative dehydration required for conventional electron microscopy. To overcome this problem, the granular sludge was treated with antiserum before fixation and stained with polycationic ferritin. After this treatment, EPS were observed to form thick, electron-dense structures

Figure 1. Scanning electron micrograph showing the large number of cell morphologies present at the surface of the granular sludge. The mixed microbial population is seen to be embedded in collapsed EPS. Bar = 1 μm.

Figure 2. Transmission electron micrograph near the surface of a bacterial granule which had been treated with whole-cell antiserum and polycationic ferritin. The various morphotypes were enveloped by very extensive EPS which often completely filled the intercellular space. Bar = 1 μm.

Figures 3 and 4. Transmission electron micrographs at an approximate depth of 30 to 40 μm into the granule interior. Figure 3 is a preparation treated with ruthenium red. Electron dense rods and cocci are present. Comments on areas A, B, C are given in the text. A large flat-ended *Methanosaeta*-like rod is visible at the lower right (arrow). Figure 4 is of a preparation treated with antiserum and polycationic ferritin. The EPS are seen to form an incomplete matrix around an aggregate of cocci, electron-dense rods, and *Methanosaeta*-like cells. The arrow points to a *Methanosaeta*-like cell. Bar = 1 μm.



Figures 5 and 6. Electron micrographs at an approximate depth of 100 μm into the granule interior. Figure 5 is a scanning electron micrograph of a large microcolony of *Methanosaeta*-like cells. The EPS have collapsed around the cells but are still seen to span the space between cells. Figure 6 is a transmission electron micrograph in which EPS cannot be detected, even though the preparation depicted was treated with antiserum and polycationic ferritin. Bar = 1 μm.

which enveloped the bacterial cells and often completely filled the intercellular spaces in the granule structure (Figures 2 and 4). The EPS were observed to form a structural matrix around the diverse variety of morphotypes which was present near the granule surface. Figure 2 reveals that a number of regular and irregular coccoid morphotypes, 0.3 to 1.0 μm in diam., as well as several rod morphotypes, possessed stabilized EPS. Transmission electron micrographs of a mixed microbial community do not permit one to state non-equivocally that all these coccoid morphologies are indeed coccoid forms rather than cross sectional views of rods. However, the scanning electron micrograph at this depth (Figure 1) indicates that a certain percentage of the cells are indeed coccoid. EPS were also observed to form an enveloping layer around the mixed populations of morphotypes present at a depth of approximately 40 μm below the granule surface (Figure 4). At this depth, electron-dense rods, approximately 0.25 × 2.0 μm, displayed extracellular material. Figure 4 also shows a number of coccoid morphotypes approximately 0.9 μm in diam., as well as flat-ended rods, approximately 0.4 to 0.5 × 1.4 to 1.7 μm, which exhibited layers of EPS. This latter morphotype was similar to that of cells which were present almost exclusively in pure microcolonies in the central core of the granule. The distinct rectangular shape and unique cell envelope structure of this organism are identical to the morphology presented for *Methanosaeta concilii* (Patel 1984) and *Methanosaeta soehngenii* (Huser *et al.* 1982). It was difficult to obtain antibody-stabilized preparations of the central core region. EPS were not observed in transmission electron micrographs of the granule core as the intercellular space was electron translucent (Figure 6). In all probability, an antibody-stabilized layer was not detected in the granule core because the serum and polycationic ferritin could not penetrate that far into the granule matrix. However, scanning electron microscopy revealed that the *Methanosaeta*-like cells in the central core region are enmeshed in a collapsed layer of EPS (Figure 5). Furthermore, Figure 4 indicates that antibodies against the EPS of the *Methanosaeta*-like cells were present in the serum.

The presence of bacterial EPS in granular sludge has been reported previously (Dolfing *et al.* 1985). However, Dolfing *et al.* (1985) did not use an EPS stabilization technique and consequently only observed the presence of EPS in certain samples and did not detect any extracellular material associated with *Methanosaeta*-like cells. In the present study, use antibody stabilization and staining with polycationic ferritin, together with scanning electron microscopy, demonstrated that EPS were associated with all observed cell morphologies, including the *Methanosaeta*-like cells. It was not possible to determine which morphologies actually produced the EPS although EPS production appears to be an ubiquitous phenomenon (Costerton *et al.* 1981a; Costerton 1985). It remains possible that only a few of the morphologies present in the granules produced the EPS, which then entrapped the other morphologies.

A three-layered structure has already been described for the granules by MacLeod *et al.* (1990), who proposed that a nutrient cascade was in operation within the granule structure. It was suggested that fermentative bacteria present in the outer layer supply acetogens in the middle layer with volatile fatty acids. This middle group then converts the volatile acids to acetate and this is utilized by the *Methanosaeta* present in the third layer, the central core. The results of the present study (Figures 1 to 6) indicate that EPS are important in maintaining the structural integrity of the granular sludge in regions representative of each of these three layers. Evidence for the structural role of EPS in consortia has also been presented for bacterial populations involved in cellulose digestion (Cheng *et al.* 1984) and the oxidation of saturated fatty acids (McInerney *et al.* 1981).

That bacterial EPS may play a major role in maintaining the structural integrity of the anaerobic granules is not surprising because the design of the UASB reactor provides a selective force for bacteria with strong adhesive tendencies. The upward flow of the wastewater washes out poorly settling microbial aggregates (Lettinga *et al.* 1980). Thus, with time, the only organisms which will remain in the reactor will be those which can adhere to form aggregates which possess a density that will negate the force of the upflow velocity. The physical nature of the EPS provides the means to form such aggregates.

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References

Arcand, Y., Guiot, S.R., Desrochers, M. & Chavarie, C. 1994 Impact of the reactor hydrodynamics and organic loading on the size and activity of anaerobic granules. *Chemical Engineering Journal* **56**, B23–B35.

Bayer, M.E. & Thurow, H. 1977 Polysaccharide capsule of *Escherichia coli*: microscope study of its size, structure, and site of synthesis. *Journal of Bacteriology* **130**, 911–936.

Beeftink, H.H. & Van Den Heuvel, C. 1988 Physical properties of bacterial aggregates in a continuous flow reactor with biomass retention. In *Granular Anaerobic Sludge; Microbiology and Technology*, eds Lettinga, G., Zehnder, A.J.B., Grotenhuis, J.T.C. & Hulshoff Pol, L.W. pp. 162–169. Wageningen: Puduc.

Cheng, K.J., Stewart, C.S., Dinsdale, D. & Costerton, J.W. 1984 Electron microscopy of bacteria involved in the digestion of plant cell walls. *Animal Feed Science and Technology* **10**, 93–120.

Costerton, J.W. 1985 The role of bacterial exopolysaccharides in nature and disease. *Developments in Industrial Microbiology* **26**, 249–261.

Costerton, J.W., Irvin, R.T. & Cheng, K.J. 1981a The bacterial glycocalyx in nature and disease. *Annual Review of Microbiology* **35**, 299–324.

Costerton, J.W., Irvin, R.T. & Cheng, K.J. 1981b The role of bacterial surface structures in pathogenesis. *CRC Critical Reviews in Microbiology* **8**, 303–338.

Danon, D., Godstein, L., Marikovsky, Y. & Skutelsky, E. 1972 Use of cationized ferritin as a label of negative charges on cell surfaces. *Journal of Ultrastructure Research* **38**, 500–510.

De Zeeuw, W.J. 1988 Granular sludge in UASB-reactors. In *Granular Anaerobic Sludge; Microbiology and Technology*, eds Lettinga, G., Zehnder, A.J.B., Grotenhuis, J.T.C. & Hulshoff Pol, L.W. pp. 132–145. Wageningen: Puduc.

Dolfing, J., Griffioen, A., Van Neerven, A.R.W. & Zevenhuizen, L.P.T.M. 1985 Chemical and bacteriological composition of granular methanogenic sludge. *Canadian Journal of Microbiology* **31**, 744–750.

Erdos, G.W. 1986 Localization of carbohydrate-containing molecules. In *Ultrastructural Techniques for Microorganisms*, eds Aldrich, H.C. & Todd, W.J. pp. 399–420. New York: Plenum.

Guiot, S.R., Gorur, S.S. & Kennedy, K.J. 1988 Nutritional and environmental factors contributing to microbial aggregation during upflow anaerobic sludge bed-filter (UBF) reactor start-up. In *Proceedings of the 5th International Symp. on Anaerobic Digestion, Bologna, Italy*, eds Hall, E.R. & Hobson, P.N. pp. 47–53. Oxford: Pergamon Press.

Huser, B.A., Wuhrmann, K. & Zehnder, A.J.B. 1982 *Methanothrix soehngenii* gen. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. *Archives of Microbiology* **132**, 1–9.

Lettinga, G., Van Velsen, A.F.M., Hobma, S.W., De Zeeuw, W.J. & Klapwijk, A. 1980 Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnology and Bioengineering* **22**, 699–734.

Luft, J.H. 1971 Ruthenium red and ruthenium violet. I. Chemistry, purification, methods of use for electron microscopy, and mechanisms of action. *Anatomical Record* **171**, 347–368.

Mackie, E.B., Brown, K.N., Lam, J. & Costerton, J.W. 1979 Morphological stabilization of capsules of group B streptococci, types Ia, Ib, II and III, with specific antibody. *Journal of Bacteriology* **138**, 609–612.

MacLeod, F.A., Guiot, S.R. & Costerton, J.W. 1990 Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. *Applied and Environmental Microbiology* **56**, 1598–1607.

McInerney, M.J., Bryant, M.P., Hespell, R.B. & Costerton, J.W. 1981 *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium, *Applied and Environmental Microbiology* **41**, 1029–1039.

Patel, G.B. 1984 Characterization and nutritional properties of *Methanothrix concilii* sp. nov., a mesophilic, aceticlastic methanogen. *Canadian Journal of Microbiology* **30**, 1383–1396.

Poindexter, J.S. 1964 Biological properties and classification of the *Caulobacter* group. *Bacteriological Reviews* **28**, 231–295.

Reynolds, E.S. 1963 The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–242.

Sjoblad, R.D. & Doltsch, R.N. 1982 Adsorption of polarly flagellated bacteria to surfaces. *Current Microbiology* **7**, 191–194.

Sutherland, I.W. 1972 Bacterial exopolysaccharides. *Advances in Microbial Physiology* **8**, 143–213.

Svanborg-Eden, C., Eriksson, B. & Hansen, L.A. 1977 Adhesion of *Escherichia coli* to human uroepithelial cells 'in vitro'. *Infection and Immunity* **18**, 767–774.

Weiss, R., Schiefer, H.G. & Krauss, H. 1979 Ultrastructural visualization of *Klebsiella* capsules by polycationic ferritin. *FEMS Microbiology Letters* **6**, 435–437.

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