Structure of Exocellular Polymers and Their Relationship to Bacterial Flocculation

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Several gram-negative, polarly flagellated rods were isolated on the basis of their flocculent growth habit. Some of the isolates possessed a capsular matrix which is composed of exocellular fibrils. Other isolates did not appear to have a capsular matrix when examined with a bright-field microscope with or without the aid of stains. However, these latter type isolates did possess exocellular material which can be demonstrated by adsorption of a fluorescent dye under an ultraviolet microscope. Electron microscopic examination demonstrated that the exocellular material around all isolates examined is fibrillar. The fibrils were susceptible to cellulase although all fibrils did not appear to be identical. It is postulated that the exocellular polymers were responsible for the flocculent growth habit of the bacteria, and that the process of bacterial flocculation produced by synthetic polyelectrolytes was essentially the same as that caused by naturally produced exopolymers.

Several bacteria have been isolated in this laboratory and elsewhere on the basis of their flocculent growth habit. Floc-forming bacteria grow primarily as clumps of large numbers of cells which may be surrounded by an exocellular polymer matrix (12). Because flocs settle out of suspension in the absence of agitation the medium rarely becomes turbid.

A distinction is made in this paper between "floc formation," which implies growth in a clumped arrangement, and "flocculation," which implies aggregation from a turbid suspension after the cells have grown. This latter phenomenon is often referred to in the literature as "flocculation" (2, 3, 15, 18, 20, 22) and is often an effect caused by the addition of chemical agents (e.g., blood sera, bentonite, polyvalent ions). On a physical-chemical basis the causes of flocculation and floc formation may be similar with the differences being based on the origin and time of involvement of the substance causing the clumping phenomenon.

Floc-forming bacteria are indigenous to natural water and the flocculent growth habit is exploited in biological waste treatment processes. Bacterial flocculation has been discussed recently by Crabtree et al. (8) and an explanation of the flocculation based upon induction by poly-beta-hydroxybutyric acid (PHB) storage granules has been proposed (6).

Tenney and Stumm (21) and Busch and Stumm (4) have described bio-flocculation as "an agglomeration of cells resulting from specific adsorption of polymer segments and from bridging of polymers between cells." These authors presented chemical data based upon the ability of synthetic polyelectrolytes to cause aggregation of bacteria. They were able to show that polymers extracted from Aerobacter aerogenes behaved in the same manner as synthetic polyelectrolytes.

The zoogloal matrix of Zoogloea ramigera strain 115 was shown to be composed of polysaccharide susceptible to cellulase and therefore believed to contain β-1-4 linkages (12). The polymer also possesses properties of a polyelectrolyte (10), as it binds high concentrations of metallic ions. Although the polymer is not readily observable around all Zoogloea isolates by techniques such as capsule stains, observation by means of electron microscopy revealed the presence of exocellular fibrils. These observations led us to examine several floc-forming bacterial isolates for the presence of exocellular polymer. We present microscopic evidence involving exocellular polymers as a cause of bacterial flocculation.

MATERIALS AND METHODS

 Cultures. The following cultures which have been described elsewhere were used in this study: Z.
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ramigera isolate 115 and Pseudomonas C-3 (11), a neotype strain Z. ramigera I-16-M, Z. filipendula P-8-4, Pseudomonas denitrificans P-93-5 and an unidentified gram-negative rod C-22-4 (7, 8, 11), and Z. ramigera Z-SC-38 (13).

Bright-field and ultraviolet microscopy. A 0.1% aqueous solution of Paper White BP (a diamine stilbene disulfonic acid dye which has a high affinity for cellulose; E. I. du Pont de Nemours and Co., Inc., Chicago) was used to stain 72-hr cultures of Z. ramigera 115 and Z. ramigera I-16-M in a manner similar to that described by Harrington and Raper (14). The stained cell flocs were examined by both bright-field and ultraviolet microscopy. Photomicrographs were taken on Kodak Plus-X film. Exposure times were 5 to 8 sec in visible light and 15 to 90 sec in ultraviolet light.

Preparation for electron microscopy. Z. ramigera isolates 115 and I-16-M were grown in a modification of the arginine salts medium of Crabtree and McCoy (8), which has the following composition per 100 ml: arginine hydrochloride, 0.05 g; MgSO4·7H2O, 0.02 g; K2HPO4, 0.2 g; KH2PO4, 0.1; glucose, 0.5 g; B15, 1.5 × 10-2 g. Proteose Peptone yeast extract, 0.05% (Difco), was used to obtain flocculent growth of cultures other than Z. ramigera 115 and Z. ramigera I-16-M. The bacteria were cultured in Erlenmeyer flasks containing 100 ml of growth medium and were either incubated on a reciprocal shaker (100 strokes/min) at 28 C until flocculent growth occurred, or held stationary at 28 C until a pellet formed. Flocs or pellets were washed twice with distilled water. In some experiments, we then boiled the cell-flocs in 1 N NaOH for 20 min (17) or allowed them to stand for 24 hr at 28 C to remove alkali-soluble material.

Shadow-casting and carbon replicas. The cell-flocs were placed on Formvar-coated grids and shadowed with carbon platinum or they were shadowed and then carbon coated. Carbon replicas were prepared by the technique of Bradley and Williams (1).

Freeze-etching. The flocs were prefrozen and frozen-etched as previously described (12).

Cellulase assay. Cellulase [Aspergillus niger, type II (Sigma Chemical Co., St. Louis, Mo.), 30 mg per 15 ml of 0.05 M sodium citrate (pH 4.0)] was added to whole-cell flocs and incubated at 24 ± 1 C for periods up to 120 min. At desired time intervals, 1.0 ml of Nelson’s alkaline copper reagent was added to each tube and the solution was assayed colorimetrically for sugar as described by Nelson (16).

Carboxymethylcellulose (12.0 mg/ml) was added to buffered enzyme in place of cells and served as a positive control.

RESULTS

Individual cells and flocs of Z. ramigera I-16-M were observed under bright-field and ultraviolet illumination (Fig. 1–3).

Z. ramigera isolate I-16-M has been described as a nonzoogloeal matrix-producing organism (7). An isolate I-16-M floc was stained with 1% aqueous crystal violet and photomicrographed (Fig. 1). No exocellular material was observed by this technique, and many of the cells appeared to be free from the main clump of cells. When the isolate I-16-M flocs were examined in a wet mount without staining, they also appeared as dispersed cells without an identifiable exocellular matrix as shown in Fig. 2. However, when the same flocs as shown in Fig. 2 are stained with the fluorescent dye Paper White BP and photographed through an ultraviolet microscope, the dye can be seen to be concentrated between the cells (Fig. 3). This suggested the presence of substances which have a high affinity for the dye (e.g., cellulose or other polysaccharides).

Since the association of the fluorescent dye with cell-flocs was quite rapid, wet mounts could be examined immediately after preparation. Fluorescence with I-16-M was initially quite intense, but gradually lessened during a 5-min interval. Z. ramigera 115 fluoresced weakly compared to I-16-M or particles of cellulose used as controls.

Figure 4 is an electron micrograph of isolate I-16-M cells which were treated in 1 N NaOH for 20 min at 100 C. Exocellular fibrils present in both shaken and stationary cultures were clearly evident. The presence of exocellular fibrils which appear to entangle the cells was also revealed in a frozen-etched preparation of the I-16-M isolate (Fig. 5). The larger fibrils (Fig. 4) are approximately 12 to 14 nm in diameter and appear to be formed from elementary fibrils which are 2 to 5 nm in diameter. The large fibrils shown in Fig. 5 are about 40 nm in diameter. This suggested that in frozen-etched preparations, material adheres to the fibrils, whereas it is removed when treated with 1 N NaOH (Fig. 4). A granule which is presumed to be PHB is also present. The report that these cells contained PHB (8) was confirmed in our laboratory.

We have previously reported the presence of a fibrillar polysaccharide matrix around Z. ramigera isolate 115 which was susceptible to cellulase, indicating that the fibril is a polysaccharide containing β-1-4 linkages. However, comparison of electron micrographs of isolates 115 to I-16-M and comparison of the physical and chemical properties of the polymers show that the two polymers are not identical (12).

Several other isolates which have been partially described previously (11) were also examined for the presence of exocellular polymers. Pseudomonas C-3 was examined by several techniques (Fig. 6–9) all of which showed the presence of fibrils surrounding the cells. A shadow-cast preparation showed fibrils obtained by placing the cell-flocs in 1 N NaOH for 24 hr at 28 C (Fig. 6). Ghosts of several cells remain.

A carbon replica of isolate C-3 was prepared...
FIG. 1. Cell-floc of *Z. ramigera* isolate 1-16-M stained with 1% aqueous crystal violet and photographed in a bright-field microscope. Individual cells appear to be detached from the floc, and no exocellular material is evident.

FIG. 2. Cells of *Z. ramigera* isolate 1-16-M photographed in a wet mount under a bright-field microscope.

FIG. 3. Same cells shown in Fig. 2 after addition of the fluorescent dye Paper White BP and photographed in a wet mount under an ultraviolet microscope.

FIG. 4. Shadow-cast preparation of *Z. ramigera* isolate 1-16-M after cells were boiled for 20 min in 1 M NaOH. Fibrils (f) surrounding cell ghosts can readily be seen. The encircled arrow on all electron micrographs indicates the direction of the shadow.

FIG. 5. Frozen-etched preparation of *Z. ramigera* isolate 1-16-M. The cell wall (CW), cytoplasmic membrane (CM), and cytoplasm (CY) of several cells is shown. A granule presumed to be PHB as well as fibrils (f) are present.
Fig. 6. Shadow-cast preparation of Pseudomonas isolate C-3 after cells were held for 24 hr at 28 C in 1 N NaOH. Fibrils (f) and cell ghosts (CG) are present.

Fig. 7. Carbon replica of Pseudomonas C-3 cells. Cells (C), fibrils (f) and what appear to be flagella (fl) are present.

Fig. 8. Frozen-etched preparation of Pseudomonas C-3 cells. Cells are shown which appear to be in cross section. Cytoplasm (CY) and fibrils (f) extending outward from cells are present.

Fig. 9. Frozen-etched preparation similar to that shown in Fig. 8. Fibrils (f) can be seen as well as cell wall (CW), cell membrane (CM) and cytoplasm (CY).
FIG. 10. Shadow-cast preparation of Z. filipendula isolate P-8-4 cells demonstrating the presence of exocellular fibrils.

FIG. 11. Shadow-cast preparation of Pseudomonas denitrificans isolate P-95-5 cells, also showing exocellular fibrils.

FIG. 12. Shadow-cast preparation of an unidentified gram-negative rod reported as isolate C-22-4, again showing exocellular fibrils.

FIG. 13. Shadow-cast preparation of an organism reported to be Z. ramigera isolate Z-SC-38, again illustrating exocellular fibrils.
(Fig. 7). Fibrils are quite common and crisscross over the cells. Thicker fibrils which may be flagella (f) can be seen. In frozen-etched preparations of isolate C-3 (Fig. 8–9), fibrils extend outwardly (Fig. 8) as rays (f) from the cross sections. These rays may be analogous to the polymer matrix observed with frozen-etched preparations of Z. ramigera isolate 115 (12), and may represent a microcapsule surrounding the cells.

In Fig. 9 is shown an entire cell with elementary fibrils (f) adhering to the outer cell surface. The cell wall (CW), cell membrane (CM), and cytoplasm (CY) can be seen.

Shadow-cast preparations of other floc-forming bacteria, Z. fillpendula isolate P-8-4 (7, 8, 10; Fig. 10), Pseudomonas denitrificans isolate P-95-5 (7, 8, 10; Fig. 11), an unidentified gram-negative rod isolate C-22-4 (7, 8, 10; Fig. 12), and a gram-negative rod which was identified as a Z. ramigera isolate Z-SC-38 (13; Fig. 13), also showed the presence of fibrils attached to cells.

DISCUSSION

During a morphological and biochemical study of zoogloeal and floc-forming bacteria, it was observed that several of our isolates as well as several from other investigators, although they did not possess the characteristic zoogloeal matrix upon examination with capsular staining, nevertheless did form flocs (7, 8, 10, 13). Routine electron microscopy studies revealed the presence of fibrils in all preparations examined.

Our data suggest that bacteria which have been isolated on the basis of their characteristic floculent growth habit all possess exocellular fibrillar polymers. Entanglement of cells among fibrils, or adsorption of cells to fibrils is a plausible explanation of the flocculation phenomenon. This conclusion supports the opinions of Tenney and Stumm (21), Busch and Stumm (4), Clark (5), and, to some extent, those of Finstein (9) and Heukelekian (15). The physical and chemical properties of the specific exocellular fibrillar polymers will determine the extent to which water is bound to the polymer and will also determine the solubility properties of the polymers and their affinity for stain. Therefore some bacterial polymers will appear as observable capsules, whereas others will not.

The purified polymer from Z. ramigera isolate 115 was reported to have properties similar to synthetic polyelectrolytes (12), and flocculation of bacteria caused by synthetic polyelectrolytes would be analogous to the naturally occurring process. We further postulated that polyvalent ions can complex with functional groups on two different elementary fibrils and thereby cause an effective bridge and alteration of the polymer charge. The association of exocellular polymers produced by one of the isolates (C-22-4) with submicroscopic inorganic particles has been reported (19). We wish to point out the analogy of polymer bridges among bacterial cells to similar polymer bridges among inorganic or synthetic materials (e.g., polystyrene latex balls) such as reported by Ries and Meyers (20).

Floc formation from other types of polymer should also be considered. Wessman and Miller (23) found that clumping of Pasteurella pestis was caused by the polymerization of extracellular nucleic acids that were excreted by the cells. The role of PHB reported by Crabtree et al. (6) should also be further considered as an explanation of bacterial flocculation.

One additional point concerns the taxonomic status of the floc-forming isolates described. All of the flocs were susceptible to cellulase and showed a positive hexose response within 15 to 120 min. Quantitative values are not presented here because the polymers were not always obtained in pure form and the quantitative variations probably reflect varying amounts of polymer surrounding individual cell isolates.

All of the isolates are polarly flagellated rods which do not resemble Acetobacter xylinum or Sarcina ventriculi. Few of the isolates are acid producers and most do not closely resemble Gluconobacter or Acetomonas. If the polymer around certain isolates proves to be cellulose, a taxonomic reappraisal would be in order.

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LITERATURE CITED