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Isolation of Exocellular Polymer from *Zoogloea* Strains MP6 and 106 and from Activated Sludge

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Exocellular polymer was isolated from zoogloeae of *Zoogloea* strains MP6 and 106 and from activated sludge flocs by blending samples with phosphate buffer and precipitation of solubilized polymer with cetyltrimethylammonium bromide. Samples of polymer from these sources were similar and yielded amino sugars as the principal components after acid hydrolysis.

Since flocculation of activated sludge and slime production in trickling filters are important aspects of aerobic wastewater treatment, the microorganisms involved in these processes have received considerable attention. Whereas some workers emphasized the activities of *Zoogloea ramigera* in flocculation of activated sludge (3, 14), other workers noted that floc-forming organisms other than *Z. ramigera* could be isolated from activated sludge (18, 19). In some studies *Zoogloea* spp. were found to be a minor component of, or were not isolated from, activated sludge and trickling filter slimes (1, 2, 16), whereas other studies indicated that *Zoogloea* spp. were a major component of the microflora of these systems (7, 15).

Flocculation of axenic *Zoogloea* cultures is generally associated with production of exocellular polymer (12; S. R. Farrah, Ph.D. thesis, The Pennsylvania State Univ., University Park, 1974), although polyhydroxybutyric acid has been implicated in flocculation (4). Therefore, analysis of activated sludge for the presence of exocellular polymer similar to that produced by *Zoogloea* spp. could help determine the role of *Zoogloea* spp. in flocculation. In this work, a procedure developed for isolation of polymer from axenic *Zoogloea* strains was applied to activated sludge flocs. Samples of exopolymer obtained from *Zoogloea* strains MP6 and 106 and from activated sludge were found to be similar in certain respects.

MATERIALS AND METHODS

Bacteria. Axenic cultures of the following bacteria were used: *Z. ramigera* 106 (ATCC 19544); *Zoogloea* MP6, which was isolated from activated sludge by using a sodium *m*-toluate medium (26); *Z. ramigera* I-16-M (ATCC 19623); and *Z. ramigera* 115, which was kindly supplied by P. R. Dugan, The Ohio State University. Stock cultures were maintained on Casitone-yeast autolysate medium containing 5.0 g of Casitone (Difco) and 1.0 g of yeast autolysate (Charles Pfizer and Co., Inc., New York) per liter of water.

Activated sludge. Fresh activated sludge was obtained from the wastewater treatment plants at State College, Pa., and Cambridge, Ohio.

Bacterial production and harvest. Mass production of the zoogloeal matrix required for chemical analysis of the polymer was accomplished by batch culturing zoogloea-forming bacteria in 1 liter of medium on a reciprocating shaker at 20°C. The bacteria were grown on Casitone-yeast autolysate medium or on a basal medium containing, per liter of distilled water: $(NH_4)_2SO_4$, 0.264 g; K_2HPO_4, 0.087 g; $MgSO_4$, 0.006 g; $CaSO_4$, 0.136 g; and sodium lactate, 1 g. Zoogloeal flocs were harvested from 48-h cultures by centrifugation and washed twice in distilled water.

Isolation of polymer. Fresh activated sludge flocs or zoogloeae of *Zoogloea* strains were washed twice with distilled water, suspended in an equal volume of 0.04 M K_2HPO_4 (final concentration, 0.02 M phosphate), and blended for 1 min. The samples were centrifuged for 10 min at 27,000 × *g*, the pellet was discarded, and cetyltrimethylammonium bromide (CTAB) was added to the supernatant to produce a final concentration of 0.8% (wt/vol). The mixture was allowed to stand at room temperature for 4 h before the precipitate was collected by centrifugation. The precipitate was mixed with 10 volumes of 0.5 M NaCl and centrifuged. Insoluble material was discarded and the clear supernatant was dialyzed against distilled water at 4°C for 24 h. The dialyzed sample was dried under a stream of air or in vacuo. The isolated polymer along with CTAB contamination was washed with 80% ethanol to remove the residual CTAB. Analysis of acid-hydrolyzed crude extract before precipitation and of hydrolyzed isolated polymer from *Zoogloea* MP6 for reducing substances indicated that approximately 85% of the polymer was recovered.

Hydrolysis of polymer. Isolated polymer was sus-

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pended in distilled water, mixed with concentrated HCl to produce a final concentration of 6 N, and dispersed in screw-capped test tubes. The tubes were heated in a boiling water bath for 45 min. After hydrolysis, HCl was removed by drying samples under a stream of air, adding distilled water, and again drying with air.

Chemical analyses. Hexosamine was determined by the modified Elson-Morgan method as described by Kabat and Mayer (17), using D-glucosamine hydrochloride as the standard. Total reducing substance was analyzed by the procedure of Nelson (21). Uronic acids were determined by the method of Dische (8). Hexoses were measured by the phenol-sulfuric acid method of Dubois et al. (9).

Ion-exchange chromatography. Zoogloeal matrix hydrolysates were fractionated on columns (0.9 by 33.0 cm) containing Dowex 50-X8, H^+ form, according to the method of Gardell (13).

Paper chromatography. One-dimensional descending paper chromatography was performed by using Whatman no. 1 filter paper and one of the following solvent systems: butanol-acetic acid-water (12:3:5); butanol-pyridine-water (3:2:1:5); or isopropanol-water (4:1). Chromatograms were treated with silver nitrate reagent (23), 3.0% *p*-anisidine hydrochloride in butanol (20), or 0.3% ninhydrin in acetone to reveal spots. Areas on untreated chromatograms corresponding to spots detected on parallel, treated chromatograms were eluted with 5 ml of water. The eluates were tested for amino sugars and reducing substances.

Infrared spectroscopy. Infrared spectra were determined by using a Beckman model IR-20R infrared spectrophotometer. Samples (0.5 mg) were mixed with KBr (99.5 mg) for analysis.

Ultraviolet spectroscopy. Ultraviolet spectra were determined by using a Beckman model DB spectrophotometer.

RESULTS

Exopolymer of *Z. ramigera* 106 and *Zoogloea* MP6 was found to be soluble in 0.1 N NaOH but not in 0.1 N HCl or in lipid solvents (chloroform, acetone, or ethanol). Bacterial cells of zoogloeae treated with 0.1 N NaOH appeared largely distorted, and it was feared that the intracellular contents of damaged bacteria might seriously contaminate the exopolymer. Therefore, milder polymer recovery methods are desired to reduce the possibility of intracellular contamination of the polymer. It was found that mechanical blending of zoogloeae in 0.02 M potassium phosphate, pH 10.0, solubilized the polymer without damaging the contained cells. In contrast with cells treated with 0.1 N NaOH, cells released from polymer by blending with potassium phosphate retained their motility and were morphologically similar to untreated cells when examined in wet mounts with phase optics or in Gram-stained preparations. Blending with 0.02 M potassium phosphate did not reduce the total or viable cell counts. Treatment with 0.05 M potassium phosphate reduced the viable count by 50%.

Preliminary experiments indicated that exopolymer extracted from *Zoogloea* strains by blending with potassium phosphate buffer was precipitated by either CTAB or 80% ethanol. Analysis of the extracts and the precipitates for reducing substances after acid hydrolysis indicated that more than 95% of the polymer was precipitated by either reagent. Since ethanol precipitation is less specific and would likely result in a mixture of polymers being isolated from activated sludge, the isolation procedure using CTAB and described in Materials and Methods was selected for use with both *Zoogloea* strains and activated sludge. Dried polymer obtained from *Zoogloea* strains MP6 and 106 and from activated sludge is a brittle, flaky, hydroscopic material that rapidly adsorbs water to produce a white, amorphous gel.

Ultraviolet spectra of polymer solubilized with 0.1 N NaOH or potassium phosphate buffer at pH 10.0 revealed no adsorption peaks between 300 and 220 nm.

Paper chromatography of hydrolyzed exopolymer from *Zoogloea* MP6, *Z. ramigera* 106, and activated sludge using each of the solvent systems described in Materials and Methods revealed two major reducing and ninhydrin-positive spots. A third minor spot near the origin was also reducing and ninhydrin positive. One of the spots co-chromatographed with D-glucosamine. $R_{\text{D-glucosamine}}$ values for the different spots observed during chromatography using isopropanol-water as the solvent system are presented in Table 1. Eluates from areas on parallel, unstained chromatograms that corresponded to spots A and B contained approximately equal amounts of amino sugars. Eluates from the area corresponding to spot C contained less than 10% of the amino sugars associated with the other two spots.

Column chromatography of hydrolyzed polymer from *Zoogloea* MP6 yielded two fractions with $R_{\text{D-glucosamine}}$ values of 0.95 and 1.77. The fractions contained approximately equal amounts of amino sugars. Column chromatography of hydrolyzed polymer from activated sludge was not done.

Chemical analyses of hydrolyzed polymer from *Zoogloea* MP6 and activated sludge are shown in Table 2. Reducing substances accounted for 20 and 25%, respectively, of the dry weight of isolated *Zoogloea* MP6 and activated sludge polymer. Amino sugars accounted for the major portion and uronic acids were a minor component of the reducing substances in

TABLE 1. *Paper chromatography of hydrolyzed exopolymer obtained from Zoogloea ramigera 106, Zoogloea MP6, and activated sludge*[a]

Source of exopolymer	$R_{D\text{-glucosamine-HCl}}$ values of exopolymer components[b]		
	Spot A	Spot B	Spot C
Zoogloea ramigera 106 ..	1.25[c]	1.00	0.07
Zoogloea MP6	1.27	1.00	0.14
Activated sludge	1.20	1.05	0.05

[a] *Zoogloea* strains were cultured on sodium lactate medium; activated sludge was obtained from the Cambridge, Ohio, sewage treatment plant.
[b] Solvent system: isopropanol-water (4:1).
[c] Mean of three determinations.

TABLE 2. *Chemical composition of hydrolyzed exopolymer from Zoogloea MP6 and activated sludge*[a]

Analytical test	Percent (wt/wt) of unhydrolyzed polymer from:	
	Zoogloea MP6	Activated sludge
Reducing substances	20[b]	25
Amino sugar	17	15
Hexose	2	7
Uronic acid	1	2
Ether-soluble material	0	0

[a] *Zoogloea* MP6 was cultured on sodium lactate medium; activated sludge was obtained from the Cambridge, Ohio, sewage treatment plant.
[b] Mean of triplicate determinations.

hydrolyzed polymer from both sources. Hexoses comprised 7% of the activated sludge polymer but only 2% of the *Zoogloea* MP6 polymer. Ether-soluble substances were not detected in hydrolyzed polymer from either source.

Infrared spectra of polymer from *Zoogloea* MP6 and activated sludge were similar (Fig. 1). These spectra differed from the spectrum obtained from polymer isolated from *Z. ramigera* 115 (12).

In a comparative study, the exopolymer isolation procedure described in Materials and Methods was applied to *Zoogloea* strains and activated sludge. The mucopolysaccharide obtained from activated sludge (measured as amino sugar released from hydrolyzed polymer per dry weight of initial sample) was only 1% of that obtained from *Zoogloea* MP6 (Table 3). Amino sugar-containing polymer could be obtained from *Z. ramigera* 106 but not from flocs of *Z. ramigera* I-16-M or *Z. ramigera* 115.

DISCUSSION

Since *Z. ramigera* 106 was isolated directly from natural finger-like zoogloeal projections (25) and is antigenically related to organisms within certain natural, finger-like zoogloeae (10), characterization of the zoogloeal matrix surrounding this organism was of particular interest. Unfortunately, repeated culturing of this organism on laboratory media resulted in a reduction of its ability to produce exopolymer, and difficulties were encountered in obtaining sufficient polymer for analyses. It was noted that freshly isolated *Zoogloea* strains produced relatively large amounts of exopolymer, and an activated sludge isolate, *Zoogloea* MP6, was used as a source of exopolymer for most work. *Zoogloea* MP6 was found similar to *Z. ramigera* 106 in most respects (26), and paper chromatography of polymer from both strains revealed two major spots that were reducing and ninhy-

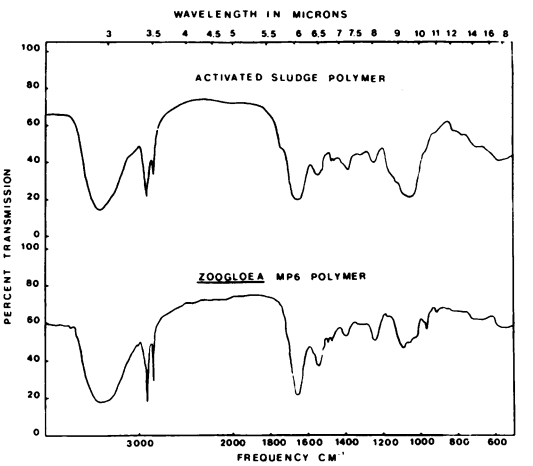

FIG. 1. *Infrared spectra of exopolymer from activated sludge and Zoogloea MP6.*

TABLE 3. *Removal of mucopolysaccharide from Zoogloea strains and activated sludge*[a]

Sample	Initial sample (mg [dry wt])	Exopolymer recovered (as amino sugar) (μg)	μg of amino sugar in exopolymer recovered/mg (dry wt) of sample
Zoogloea MP6	15.6[b]	545	35
Z. ramigera 106	18.4	39	2.1
Z. ramigera 115	46.5	0	0
Z. ramigera I-16-M ..	54.4	0	0
Activated sludge ...	660.0	205	0.31

[a] *Zoogloea* strains were harvested after 48 h of culture on Casitone-yeast autolysate medium; activated sludge was obtained from the Cambridge, Ohio, sewage treatment plant.
[b] Mean of duplicate determinations.

drin positive and had similar mobilities in the solvent system used.

Although additional chemical and immunological characterization of polymer from *Zoogloea* strains and activated sludge is required, certain observations can be made. Unhydrolyzed samples of polymer from *Zoogloea* MP6 and activated sludge have similar infrared spectra and are indistinguishable by macroscopic or microscopic observation. Hydrolyzed samples of polymer from both sources produce the same pattern on paper chromatography and are chemically similar. It appears that amino sugars are the principal constituents of hydrolyzed polymer. Amino and nucleic acids were not detected on paper chromatography or by ultraviolet analysis, and no ether-soluble material was detected. The fact that amino sugars account for less than 25% of the dry weight of hydrolyzed polymer is likely the result of decomposition of polymer during acid hydrolysis. Decomposition of mucopolysaccharide during hydrolysis has been reported elsewhere (6, 22). Paper and column chromatography indicate that one of the amino sugars is likely glucosamine, whereas the elution pattern from column chromatography of the other amino sugar is suggestive of a methyl-pentose amine (6, 22).

Amino sugars have been detected in extracts from other organisms described as *Z. ramigera*. A pentose and a hexosamine were isolated from an organism resembling *Z. ramigera* (R. Anderson and E. McCoy, Bacteriol. Proc., p. 162, 1963). Crabtree et al. (4) obtained hexosamine in hot-water extracts of flocs and cells of *Z. ramigera* I-16-M. Tezuka (22) found two amino sugars in the exopolymer of *Z. ramigera* and identified the compounds as glucosamine and possibly fucosamine. The polymer isolated by Tezuka appears similar to the polymer isolated from *Zoogloea* strains MP6 and 106 and from activated sludge in this work. Other workers have failed to find amino sugars in the polymer from *Z. ramigera*. Friedman and Dugan (11) reported that the exopolymer associated with *Z. ramigera* 115 was composed of glucose and galactose. Wallen and Davis (27) found glucose, mannose, and galactose in the polymer from their *Z. ramigera* strain designated NRRL B-3669M.

The different descriptions of exopolymer associated with organisms described as *Z. ramigera* are likely a result of the taxonomic confusion surrounding the genus *Zoogloea* rather than a result of different isolation and characterization procedures being used by different investigators. Organisms that differ in morphology, physiology, and in the ability to produce zoogloeae that are visible by light microscopy have been named *Z. ramigera* (5, 11, 25). The taxonomy of the genus *Zoogloea* and of *Z. ramigera* in particular has been considered in other works (5, 24, 28).

It would seem that an understanding of the role of *Z. ramigera* in the activated sludge process requires a clearer description of the genus *Zoogloea* and establishment of an acceptable neotype strain of *Z. ramigera*. It may then be possible to obtain information of the number of viable *Z. ramigera* cells, and the amount of exopolymer produced by these cells, in activated sludge.

Future work should be aimed at determining whether the polymer isolated from activated sludge is unquestionably associated with *Zoogloea* bacteria or if other resident sludge bacteria could be the source. A quantitative assay for the polymer in activated sludge is also required.

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