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Exocellular Mucopolysaccharide Closely Related to Bacterial Floc Formation

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A bacterium isolated from activated sludge formed a visible floc and also produced an exoenzyme that could bring about deflocculation. Scanning electron microscopic examination revealed that the cells were embedded in a film mesh in the floc, which disappeared after treatment with the deflocculating enzyme. Polysaccharides isolated from the floc were fractionated into three fractions by diethylaminoethyl-Sephadex A-25 column chromatography, whereas those from the free cells were fractionated into only two fractions. The missing fraction was a mucopolysaccharide composed of glucosamine, glucose, mannose, galactose, and rhamnose and was hydrolyzed to oligosaccharides by the deflocculating enzyme. The other two fractions were resistant to the enzyme. These results show that the mesh structure of the floc is dependent on a mucopolysaccharide hydrolyzed by the deflocculating enzyme.

The mechanisms of microbial floc formation have been discussed recently, and a hypothesis that exopolymeric materials are responsible for floc formation has been widely supported (5). In activated sludge, exopolymeric materials such as polysaccharides, polyamino acids, polynucleic acids, and their complex polymers can usually be found (5). Floc formation would be caused by these polymers, and treatment with a suitable exopolymer-degrading enzyme would cause deflocculation of the floc. In pure culture, there are many bacteria that form flocs susceptible to cellulase and some that form flocs susceptible to protease or deoxyribonuclease (1, 3, 6, 9, 13, 17). The natural floc of activated sludge, however, could not be entirely deflocculated by these enzymes (11). Therefore, some other exopolymers, for example, heteropolysaccharides, which are more biologically stable than homopolysaccharides, may contribute to floc formation.

In a previous paper (14), we reported on a floc-forming bacterium isolated from activated sludge. It belongs to the genus *Pseudomonas*, although it is not classifiable into one of the described species. It forms a visible floc unsusceptible to cellulase, protease, and lysozyme and produces an exoenzyme capable of deflocculating its own floc. An extensive increase in the release of sugar was observed during the deflocculation by this enzyme. This paper deals with the isolation and fractionation of the polysaccharides of this bacterium to elucidate the materials concerned with floc formation.

MATERIALS AND METHODS

Organism. Bacterial strain no. 12 (15), isolated from a phenol-adapted activated sludge, was used throughout this work. This strain belongs to the genus *Pseudomonas* and forms a visible floc unsusceptible to various cellulases, proteases, lysozyme, and ethylenediaminetetraacetic acid.

Medium and cultivation conditions. Peptone medium containing 15 g of peptone, 1.0 g of K_2HPO_4, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 0.02 g of $FeCl_3 \cdot 6H_2O$ per liter (pH 7.2) was used for floc formation and for production of the deflocculating enzme. A 10-ml portion of a 3-day culture of strain no. 12 was inoculated into 1 liter of the same medium in a 5-liter flask and incubated on a rotary shaker at 20°C for 5 days.

Deflocculating enzyme. The deflocculating enzyme used here was isolated from the 5-day culture fluid of strain no. 12 and purified as described previously (14). This enzyme could deflocculate the floc of strain no. 12 without decreasing its growth. One "unit" of deflocculating activity is the amount of the enzyme required for a complete deflocculation of 0.2 mg of lyophilized floc in 0.01 M acetate buffer (pH 5.8) at 30°C in 30 min.

Preparation of floc from strain no. 12. The 5-day culture of strain no. 12 was allowed to stand for 30 min to settle all the floc. The settled floc was collected and washed several times with deionized cold water by decantation. This raw floc was lyophilized and stored in a desiccator.

Preparation of free cells from floc. One gram (dry weight) of raw floc was suspended in 75 ml of 0.01 M acetate buffer, pH 5.8, with 2,000 units of the deflocculating enzyme and incubated at 30°C for 4 h. The reaction mixture thus fully deflocculated was centrifuged at 10,000 × g for 15 min, and the residue

was washed twice with deionized water and used as free cells.

Scanning electron microscopy. For scanning electron microscopy, two samples were prepared. One was raw floc, and the other was free cells from floc packed by centrifugation. The floc and the packed free cells were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 h at room temperature, dehydrated with ethanol stepwise, dried by a critical point dryer before coating with gold by spatter coating, and observed in a Hitachi HHS-2R scanning electron microscope operating at 20 kV.

Isolation of polysaccharides from floc and from the free cells prepared from floc. The free cells prepared from 1 g of the lyophilized floc, or 1 g of the lyophilized floc, were suspended in cold 0.5 N NaOH solution, and the suspension was stirred for 24 h at 4°C. With this treatment, the floc was entirely dispersed. The suspension was centrifuged at 10,000 × *g* for 15 min to remove insoluble materials. The supernatant fluid was neutralized with concentrated acetic acid and dialyzed against water at 4°C overnight. To this solution, sodium acetate was added to give a concentration of 3% before adding 5 volumes of ethanol with mechanical stirring, and the mixture was allowed to stand at 4°C overnight. The precipitate formed was collected by centrifugation at 10,000 × *g* for 15 min, dissolved in 20 ml of water, and stirred mechanically at 4°C overnight. Insoluble material was removed by centrifugation at 10,000 × *g* for 30 min. The supernatant fluid was dialyzed against water at 4°C for 2 days, lyophilized, and stored in a desiccator.

Fractionation of polysaccharides. The crude polysaccharides obtained as above from 1 g of floc and from the free cells prepared from 1 g of floc were each dissolved in 25 ml of 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, and 10 ml of each was applied to a column (1 by 20 cm) of diethylaminoethyl-Sephadex A-25 previously equilibrated with the same buffer and fractionated by stepwise elution with NaCl in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer by increasing the molarity from 0 to 0.5 M. Five-milliliter fractions were collected, and the sugar content was determined. Appropriate fractions were combined, dialyzed against water overnight, lyophilized, and stored in a desiccator.

Treatment of polysaccharides with the deflocculating enzyme. The fractionated polysaccharides were dissolved in 0.01 M acetate buffer, pH 5.8, containing 50 units of the deflocculating enzyme per ml, to give a concentration of 0.2%. The mixtures were incubated at 30°C, and the increase in reducing power was measured.

Analytical methods. Total sugar was determined by the method of Dubois et al. (2). Reducing power was measured by the method of Park and Johnson (10). Thin-layer chromatography was carried out on Kieselgel 60 with ammonia water-*n*-propyl alcohol-water (8:12:1) at 4°C for polysaccharide analysis (8). *p*-Anisaldehyde-sulfuric acid was used for staining (12). Amino acids and amino sugars were analyzed with a JEOL automatic amino acid analyzer, model JLC-6AH, after the polysaccharide preparations were hydrolyzed with 6 N HCl at 100°C for 24 h in sealed and evacuated tubes.

Sugars were identified mainly by gas chromatography as the reduced and acetylated derivatives (7, 8). Polysaccharide preparations were hydrolyzed with 3 N HCl for 3 or 15 h in sealed and evacuated tubes in a boiling-water bath. The reduction and acetylation of the hydrolysates were mainly made as reported by Yang and Hakomori (18). The reduced and acetylated derivatives were dissolved in 20 to 30 μl of acetone, and the solution (2 μl) was injected into a 4-foot (ca. 1.2-m) column of EGNSS-M coated on Gas Chrom Q and developed at 180°C for neutral sugar analysis and 210°C for amino sugar analysis on a Shimadzu GC-4B gas chromatograph.

Chemicals. Diethylaminoethyl-Sephadex A-25 was the product of Pharmacia, Uppsala. Kieselgel 60 was obtained from E. Merck, Darmstadt. EGNSS-M was obtained from Gas-Chro Kogyo Co., Tokyo. Other chemicals were of the purest grade commercially available.

RESULTS

Scanning electron microscopy. The presence of polymer on floc and its lack on free cells have been observed in electron micrographs of many floc-forming bacteria (1, 3, 9). If this is the case in the floc of strain no. 12, the presence of exopolymer on the floc and its lack on free cells should be observed. According to scanning electron microscopy, cells of floc were embedded in a film mesh (Fig. 1A and B), and the free cells were not (Fig. 2A and B). Flagella were often seen on the free cells. The film mesh was not separated from the flocculated cells by extensive centrifugation but was attached to cells more tightly than so-called slime was.

Isolation and fractionation of polysaccharides. In a previous paper (14), it was shown that extensive release of sugar from the floc of strain no. 12 occurred concurrently with its deflocculation by the deflocculating enzyme. In this study, a film mesh was observed on the floc, but not on the free cells, by scanning electron microscopy. Therefore, this deflocculating enzyme might be a polysaccharide hydrolase, and polysaccharides sensitive to this enzyme might conjugate cell to cell and form the floc.

Polysaccharides were isolated from the floc of strain no. 12 and the free cells. By alkali treatment (shown in Table 1), 42.6 and 32.3 mg of sugar were obtained from 1 g of the floc and free cells corresponding to 1 g of the floc, respectively. By treatment with the deflocculating enzyme, 10.9 mg of sugar was released from 1 g of the floc. This value is quite similar to the difference between the amounts of the above two sugar fractions.

Crude polysaccharides isolated from the floc

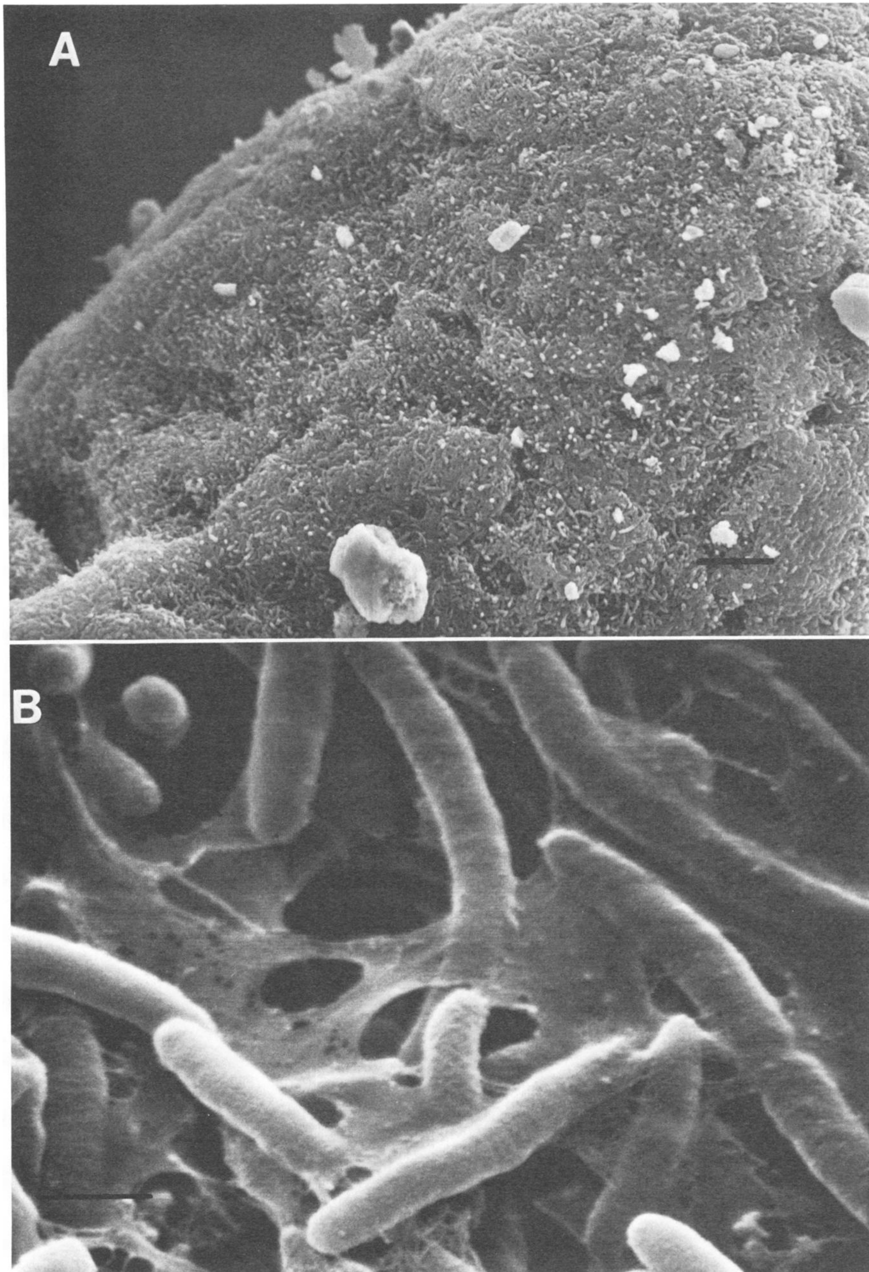

FIG. 1. *Scanning electron micrographs of floc of strain no. 12. (A) Bar = 10 μm; (B) bar = 0.5 μm.*

and from the free cells were fractionated with diethylaminoethyl-Sephadex A-25 column chromatography, and the sugar content in each fraction was determined. Polysaccharides from the floc were eluted mainly at NaCl concentrations of 0, 0.05, and 0.2 M, whereas those from the free cells were mainly eluted at 0 and 0.05 M but not at 0.2 M (Fig. 3). About 10% of the total polysaccharides from the floc was observed in the 0.2 M NaCl fraction. From these results, it is clear that the floc contains a polysaccharide fraction eluted with 0.2 M NaCl (fraction 3), but the free cells do not contain this fraction. These results are in agreement with the presence of a film mesh on the floc and its absence on free cells. This fraction alone must

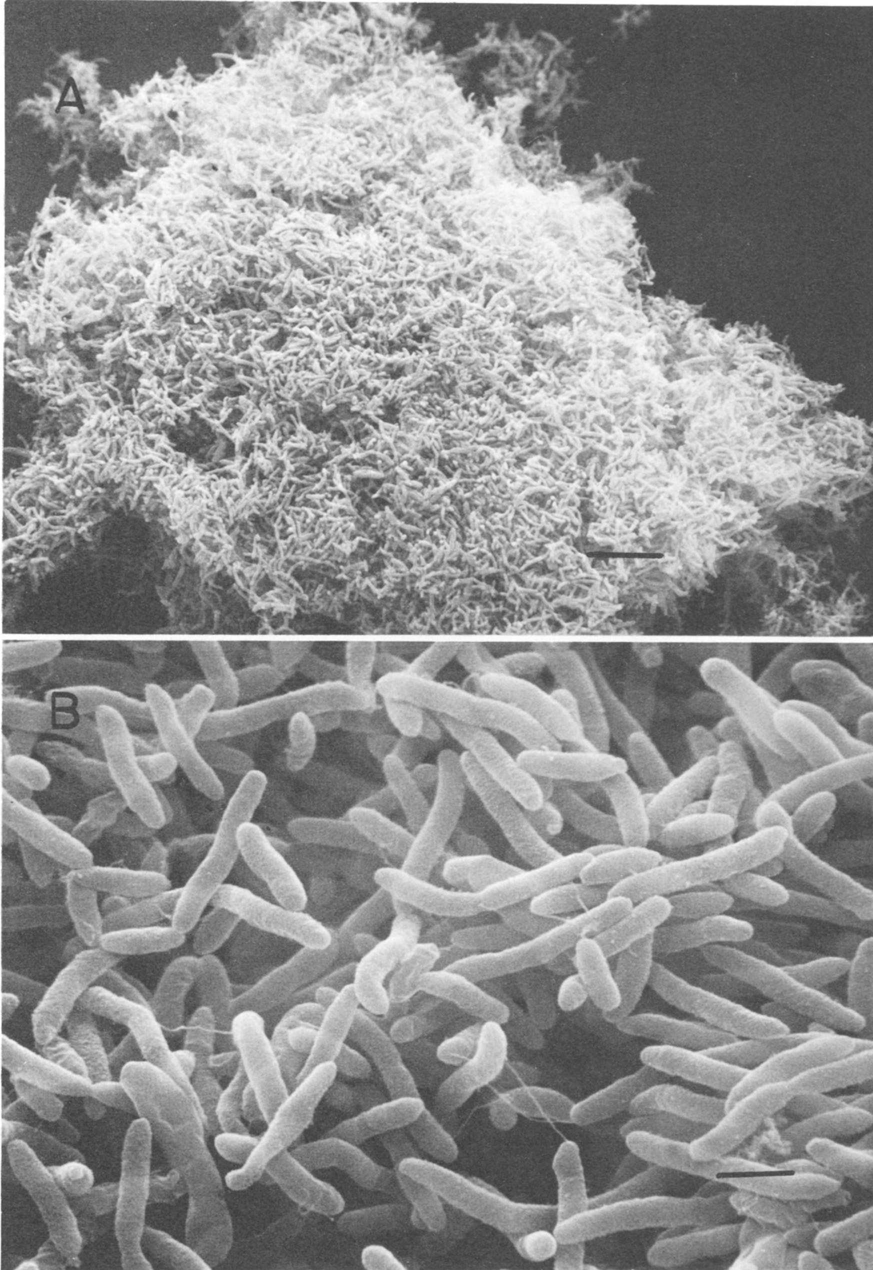

FIG. 2. *Scanning electron micrographs of free cells of strain no. 12 packed by centrifugation. (A) Bar = 10 μm; (B) bar = 1 μm.*

be hydrolyzed by the deflocculating enzyme.

Susceptibility of each polysaccharide fraction to the deflocculating enzyme. To examine which polysaccharide fraction is hydrolyzed by the deflocculating enzyme, the polysaccharide of each fraction was incubated with the enzyme and the increase in reducing power was measured. The reducing power of fraction 3 increased linearly with incubation time, whereas those of other fractions did not (Fig. 4).

Components of fraction 3. The chemical properties of fraction 3 were investigated in detail. Elementary analysis showed no phosphorus and no sulfur in fraction 3. Therefore,

TABLE 1. *Comparison of rhamnose equivalents solubilized from lyophilized floc and free cells by deflocculating enzyme and by alkali*

Treatment	mg of rhamnose equivalents solubilized by:	
	Lyophilized floc	Free cells
Deflocculating enzyme[a]	10.9[b]	
Cold 0.5 N NaOH[c]	42.6	32.3

[a] One gram of lyophilized floc was suspended in 75 ml of 0.01 M acetate buffer, pH 5.8, with 2,000 units of the deflocculating enzyme and incubated at 30°C for 4 h.

[b] The value is the sum of sugars released by deflocculating enzyme and sugars released without the enzyme. When heat-treated floc (50°C, 10 min) was incubated in acetate buffer at 30°C for 4 h in the absence of the enzyme, about 5.8 mg of sugars was released, but deflocculation was not observed.

[c] The free cells obtained from 1 g of the lyophilized floc or 1 g of the lyophilized floc were suspended in 30 ml of cold NaOH solution, and the suspension was stirred at 4°C for 24 h.

nucleic acids were not present in fraction 3. No amino acids were detected by amino acid analysis. On thin-layer chromatography, fraction 3 did not move from its original spot, whereas enzyme-treated fraction 3 moved to give a single spot at an R_f value of 0.29 (Fig. 5). This suggests that fraction 3 was hydrolyzed to a homogeneous oligosaccharide. Gas chromatograms of the sugars in fraction 3 are shown in Fig. 6A and B. This fraction was composed of glucosamine, glucose, mannose, galactose, and rhamnose, and their molar ratio was 1:2:1:0.6:0.9. These results are summarized in Table 2.

DISCUSSION

Floc formation is an integral part of the activated sludge process, and the mechanisms involved have been discussed (5). Previous investigators have discussed a relationship between floc formation and the synthesis of exopolymers, such as so-called gelatinous matrix, slime, capsule, etc. Recently, Deinema and

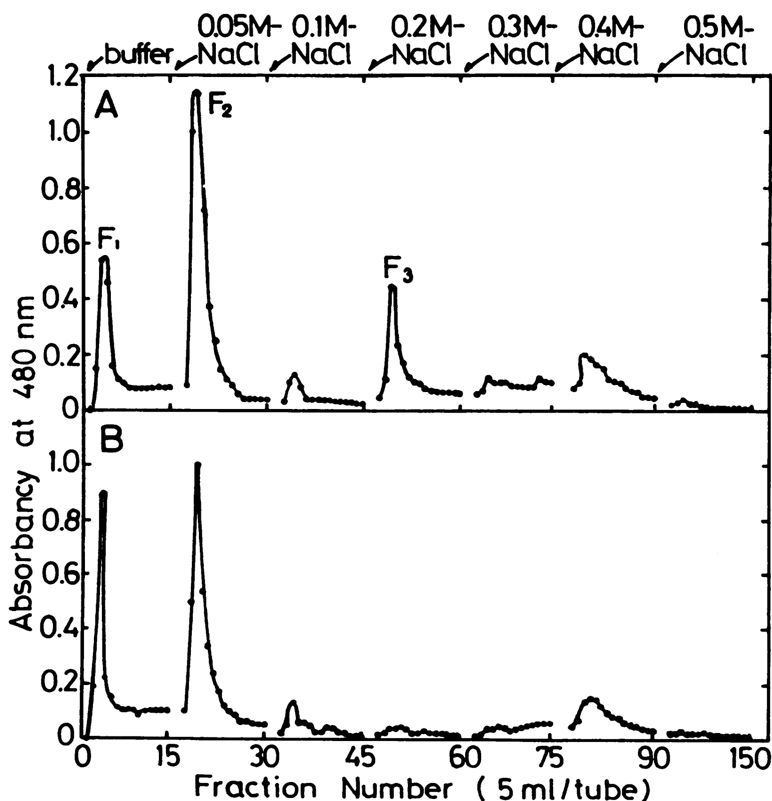

FIG. 3. *Diethylaminoethyl-Sephadex A-25 column chromatography of the polysaccharides extracted from floc (A) and from free cells (B) of strain no. 12. Forty percent of each crude polysaccharide, obtained as described in the text, was applied to a column (1 by 20 cm) (8.0 mg in A and 6.3 mg in B, as rhamnose). Stepwise elution was made with NaCl in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer by increasing the molarity from 0 to 0.5.*

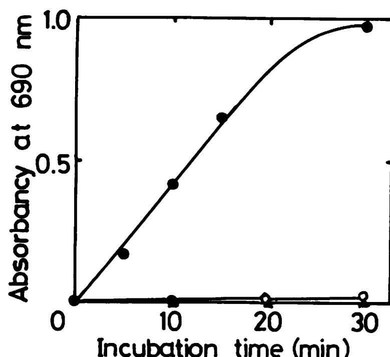

FIG. 4. *Hydrolysis of the fractionated polysaccharides by the deflocculating enzyme. A reaction mixture containing 2 mg of fractionated polysaccharide per ml and 50 units of the deflocculating enzyme per ml was incubated at 30°C, and the increase in reducing power was measured. Symbols: 0 M NaCl-eluted fraction* (▲); *0.05 M NaCl-eluted fraction* (○); *0.2 M NaCl-eluted fraction (fraction 3)* (●).

Zevenhuizen (1) and Napoli et al. (9) showed that cellulose fibrils were responsible for floc formation. Deinema and Zevenhuizen, however, reported that the amount of fibrils in activated sludge was considerably lower than in the pure culture of the isolated floc-forming bacteria (1). In activated sludge, many microorganisms that are able to degrade cellulose exist (4). This evidence suggests that the cellulose fibrils and other exopolymers that are easily degraded by many microorganisms usually found in activated sludge are not the main cause of floc formation in activated sludge.

Farrah and Unz reported that a mucopolysaccharide from *Zoogloea* sp. contained amino sugars as major components (S. R. Farrah and R. F. Unz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, G260, p. 63) and that the floc formation was accompanied by the production of a mucopolysaccharide exopolymer (16). However, they did not show that the exopolymer was obligately related to floc formation.

The present study showed that a mucopolysaccharide composed of glucosamine, glucose, mannose, galactose, and rhamnose, which was only 10% of the total polysaccharides isolated from floc of strain no. 12, contributed to the floc formation, whereas the other 90% of the polysaccharides could not be shown to play a role in floc formation. According to scanning electron microscopy, a film mesh embedding cells was seen on the floc, whereas it was not seen on the free cells obtained from the floc after treatment with the deflocculating enzyme. The absence of the film mesh was coincident with the lack of the above-mentioned mucopolysaccharide. From these results, it is concluded that this

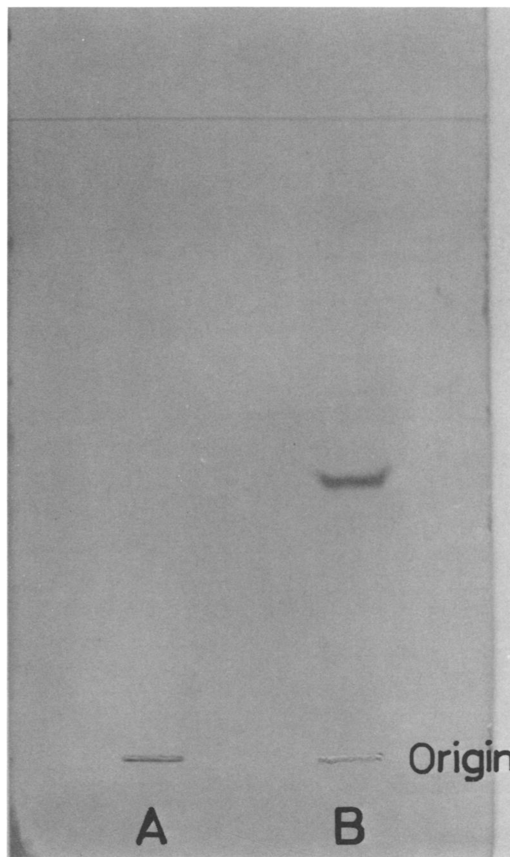

FIG. 5. *Thin-layer chromatogram of fraction 3 polysaccharide. (A) Fraction 3 polysaccharide; (B) fraction 3 polysaccharide treated with the deflocculating enzyme. The loading amount was 20 μg of rhamnose equivalent each for (A) and (B) and developing was with ammonia water-n-propyl alcohol-water (8:12:1) at 4°C. The staining was made with p-anisaldehyde-sulfuric acid.*

mucopolysaccharide comprises the film mesh bridging cell to cell and form the floc, and that the other polysaccharides are not directly concerned with floc formation. This is the first time that a mucopolysaccharide has been assigned an obligate role in floc formation by a bacterium isolated from activated sludge.

The mucopolysaccharide isolated from floc of strain no. 12 amounted to 5.6 mg (dry weight)/g (dry weight) of the floc. This polymer was soluble in water, the solution was highly viscous, and when the polymer was precipitated with ethanol, it appeared gelatinous. The viscosity of the aqueous solution of the polymer decreased by treatment with the deflocculating enzyme, and the product migrated as one spot on thin-layer chromatography, suggesting that

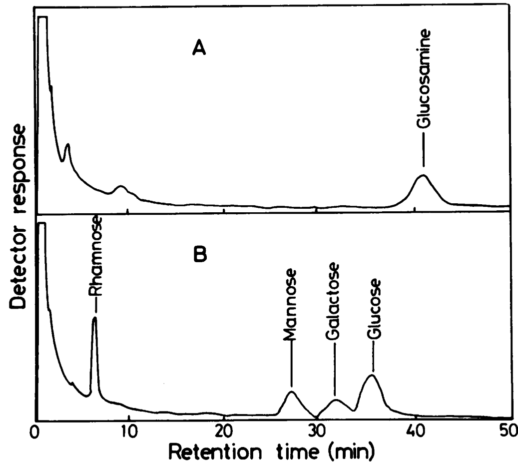

FIG. 6. *Gas chromatograms of the sugars in fraction 3 polysaccharide developed at 210°C (A) and 180°C (B). The detector temperature was 270°C, and injection port temperature was 300°C. The total amount of sugars injected was about 9 to 10 μg. Xylose and galactosamine were used as internal standards.*

TABLE 2. *Molar ratio of sugars in fraction 3*

Sugar	μg/100 μg of fraction 3	Molar ratio (to glucosamine)
Glucosamine	18.0	1.0
Glucose	38.5	2.1
Mannose	18.0	1.0
Galactose	10.5	0.6
Rhamnose	14.5	0.9

this mucopolysaccharide was composed of a single repeating unit.

The attempt to deflocculate floc from strain no. 12 with enzymes other than the deflocculating enzyme failed, as reported in a previous paper (14). Heteropolysaccharides, such as mucopolysaccharides, are biologically more stable than cellulose, and it is reasonable to consider that such polysaccharides are obligately related to floc formation of activated sludge.

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