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CHARACTERIZATION OF THE EXOCELLULAR POLYSACCHARIDES FROM *Azotobacter chroococcum*

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ABSTRACT

Azotobacter chroococcum produces appreciable amounts of exocellular polysaccharide. Produced by *A. chroococcum* strain NRRL B-14341 are at least two distinct polysaccharides which can be separated by ion-exchange chromatography on DEAE-Sephadex A-25. Exopolysaccharide I (EPS-I) consists of rhamnose, mannose, and galactose in the molar ratios of 1:2:2, along with a trace of glucose. EPS-I also contains pyruvic acetal and acetic ester groups. Exopolysaccharide II (EPS-II) consists mainly of mannuronic acid, with much smaller proportions of guluronic acid. Acetic ester groups are also present in EPS-II. Methylation analysis of reduced EPS-II shows it to be (1→4)-linked, and ^{13}C-nuclear magnetic resonance spectroscopy indicates that it is mainly (1→4)-β-D-mannuronan. EPS-II is evidently a low-guluronate form of alginate. Batch-to-batch variation was observed in the relative amounts of the two polysaccharides, the acetyl and pyruvic contents of EPS-I, and the degree of acetylation of EPS-II.

INTRODUCTION

Azotobacter chroococcum is an amylolytic, nitrogen-fixing bacterium with an extremely high rate of respiration. It is quite common in neutral and slightly alkaline soils, and is believed to contribute significantly to non-symbiotic nitrogen fixation in these soils. Because of its importance to agriculture, *A. chroococcum* has been the subject of numerous studies over the years. Of particular interest has been the exocellular slime that it produces. This slime, which is mainly polysaccharide in nature, has been implicated in a number of biological, ecological, and physical phenomena. Microbial polysaccharides in general are known to play a role in adhesion to surfaces[1,2], and are important in soils, where they help bind soil particles together and contribute to soil stabilization and retention of micro-

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

nutrients[3–5]. The exopolysaccharide material from *A. chroococcum* has also been implicated in iron and molybdenum uptake by this organism[6].

Previous studies regarding the chemical composition of the exopolysaccharide from *A. chroococcum* presented conflicting results. The early literature pertaining to this material has been reviewed by Rubenchik[7]. In 1968, Lopez and Backing[8] used paper chromatography to identify rhamnose, xylose, glucose, galactose, and galacturonic acid from the exopolysaccharide of *A. chroococcum* grown on a nitrogen-free D-glucose medium. Wurst *et al.*[9] analyzed by gas–liquid chromatography the trimethylsilyl derivatives of the constituents of acid-hydrolyzed exopolysaccharide, and found galactose, glucose, galacturonic acid, rhamnose, ribose, xylose, and glucuronic acid. In another paper-chromatographic study, Foda *et al.*[4] identified glucuronic acid, galactose, glucose, and a trace of mannose from the exopolysaccharide. Most recently, Ferrala *et al.*[10] have analyzed the exopolysaccharide from *A. chroococcum* strain B-8 by gas–liquid chromatography of the alditol acetates. They found galactose, mannose, glucose, arabinose, mannuronic acid, glucuronic and/or guluronic acid, and inositol to be present when the organism was grown on a solid, nitrogen-free medium.

It has been recognized by most of these investigators that the amount of polysaccharide, and possibly its composition, may vary, depending on the medium and culture conditions. In light of this reported variability in exopolysaccharide composition, we began to suspect that more than one exopolysaccharide may actually be produced by this organism.

EXPERIMENTAL

Organism. — *Azotobacter chroococcum* NRRL B-14341 is this Laboratory's strain designation for ACTT 7491, which is the same as the USDA strain B-8 of Burk and co-workers[11]. It was grown on a nitrogen-free, liquid medium (B_6) described by Dalton and Postgate[12], with D-glucose as a carbon source, rather than D-mannitol. Growth was achieved at 28°, with aeration by shaking at 180 r.p.m. Exopolysaccharide was isolated from the culture medium of 4–5-day-old culture, which coincided with the end of log-phase growth and the beginning of the stationary phase.

Isolation of crude exopolysaccharide (EPS). — Cells were removed from the culture medium by centrifugation at 20,000*g* for 30 min at 5–10°. Acidic polysaccharide was precipitated from the cell-free supernatant fluid with 0.1 vol. of 5% (w/v) hexadecylpyridinium chloride (Sigma Chemical Co., St. Louis). The precipitate was mainly dissolved in 1.5M KCl at 35–45°, the suspension filtered, and EPS precipitated from the filtrate by the addition of ethanol (2 vol.). This precipitate was dissolved in distilled water, reprecipitated with 2 vol. of ethanol, and redissolved in water. After removal of acidic EPS by precipitation with a cationic detergent, no neutral polysaccharide could be precipitated from the cell-free, culture-supernatant fluid by the addition of three vol. of ethanol.

Separation of exopolysaccharides by ion-exchange chromatography. — The aqueous solution of crude EPS was dialyzed against distilled water (4 L), and diluted with 0.1M potassium phosphate buffer, pH 7.0, to give a final buffer concentration 10mM in phosphate. A solution (100 mL) of EPS in 10mM buffer (~25–75 mg EPS) was applied to a column (2.5 × 10 cm) of DEAE-Sephadex A-25 (Pharmacia, Inc., Piscataway, NJ) pre-equilibrated with the same buffer. Fractions (10 mL each) were collected. After the sample had been applied, the column was washed with 100 mL of buffer. This was followed by a linear gradient of 0 to 1.5M NaCl in 10mM phosphate buffer. The salt gradient was monitored conductimetrically. Individual fractions were analyzed for total carbohydrate content by the phenol–sulfuric acid method[13], using 0.5 mL of each fraction, 0.5 mL of 5% (w/v) phenol, and 2.5 mL of sulfuric acid. Uronic acid content was determined by the method described by Knutson and Jeanes[14] for mannuronic acid, using 2.5 mL of boric acid–sulfuric acid reagent, 0.4 mL of each fraction, 0.1 mL of carbazole reagent, and an incubation temperature of 55°. Fractions that contained the polysaccharides of interest were pooled, concentrated by ultrafiltration using an Amicon ultrafiltration cell (Amicon Corp., Danvers, MA) having a PM-10 membrane, dialyzed against distilled water (3 × 4 L), and lyophilized.

Structural analyses. — Acetic ester content was measured by the hydroxymate method[15], using acetylcholine standards. Pyruvic acetal (1-carboxyethylidene) content was determined by the lactate dehydrogenase method[16]. Both pyruvic acetal and acetyl content are reported as per cent by weight of the sodium salt form of the polysaccharide, which had been dried for 24 h in a vacuum oven at 45°.

Uronic acid-containing polysaccharide was reduced according to the method of Taylor and Conrad[17], using sodium borodeuteride to reduce uronic acid residues that had been activated with 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide.

The monosaccharide composition of neutral polysaccharides was determined by thin-layer chromatography (t.l.c.) of acid-hydrolyzed polysaccharide and by capillary gas–liquid chromatography (g.l.c.) of the alditol acetates from acid-hydrolyzed polysaccharide. For t.l.c. the polysaccharide (~2–5 mg) was suspended in 2 mL of 2M trifluoroacetic acid (TFA) in a glass ampule. The ampule was sealed and hydrolysis was conducted for 2 h at 110°. The TFA was evaporated under a stream of nitrogen at 45°, the residue dissolved in water (2 mL), and a 5-μL sample spotted on a t.l.c. plate of Whatman LK5 silica gel (Whatman Laboratory Products, Inc., Clifton, NJ). The plate was developed for four ascents in 9:1 (v/v) acetonitrile–water. Sugars were visibilized with *N*-1-(naphthyl)ethylenediamine dihydrochloride (Aldrich Chemical Corp., Milwauke, WI) in methanol containing 3% sulfuric acid[18]. For quantitative analysis, the method of Blakeney *et al.*[19] was used to prepare the alditol acetates. Gas–liquid chromatography was performed with a Spectra Physics model 7100 capillary g.l. chromatograph equipped with flame-ionization detectors and a 15-m, fused capillary column of Supelco SP 2330 (Supelco, Inc., Bellefonte, PA). Hydrogen was the carrier gas. Separation of alditol acetates was accomplished, with complete baseline separation, in[20] 12 min. The

amount of gulose present in reduced EPS-II was calculated from the total combined areas of the peaks representing gulitol hexaacetate and 2,3,4-tri-*O*-acetyl-1,6-anhydrogulose[21]. Gulose was unequivocally identified and quantitated by Dionex ion chromatography (Dionex Corp., Sunnyvale, CA) of acid-hydrolyzed, carboxyl-reduced EPS-II, utilizing an HPIC-AS6A 5-μm column and a pulsed, amperometric detection-system. Elution was achieved with either 150mM NaOH or 48.5mM NaOH + 1.5mM acetate; each system was capable of clearly distinguishing gulose from glucose; this overcomes the ambiguity resulting from the fact that, when reduced with borohydride, both D-glucose and L-gulose give rise to the same aldohexitol.

For methylation analysis of EPS-II, the polysaccharide was converted into the acid (protonated, uncharged) form, and the carboxyl groups were activated by reaction with ethylene oxide, and reduced with[22] NaB^2H_4. The complete reduction of EPS-II was accomplished in this manner, and was verified by carbazole analysis. Methylation was carried out by a modification of the Hakomori method[23,24]. Per-*O*-acetylated methylated sugars were analyzed by g.l.c.–m.s., using a Perkin–Elmer Sigma 3B capillary gas–liquid chromatograph equipped with a Hewlett–Packard 5790 Series Mass Selection Detector[25].

^{13}C-Nuclear magnetic resonance spectroscopy was performed with a Bruker WM-300 spectrometer operated in the ^1H-decoupled mode, with a probe temperature of 60°. Solutions of ~10 mg of sample in 1–2 mL of deuterium oxide were used. Chemical shifts, referenced to tetramethylsilane, were measured from internal 1,4-dioxane (δ 67.4 at 60°).

Gel-filtration chromatography was conducted on samples (~1–2 mg) in 1 mL of 10mM potassium phosphate buffer, pH 7.0, which was also used as the eluant. Sepharose 4B (Pharmacia) in a column (1 × 45 cm) was used. Fractions were assayed for total carbohydrates or uronic acid as already described.

RESULTS

Fig. 1 shows the results of DEAE-Sephadex chromatography of the acidic EPS mixture from *A. chroococcum* NRRL B-14341. Two distinct polysaccharide fractions are evident: EPS-I, which is eluted with 0.3M NaCl, and EPS-II, which is eluted with 0.55M NaCl. Although Fig. 1 shows the relative quantities of I and II to be approximately equal, this was not always the case; the ratio of I to II was found to vary greatly from batch to batch. The amount of polysaccharide recovered from the column depended on the amount applied, but generally ranged between 60 and 80%. Yields were enhanced by filtering the EPS through a glass-fiber filter prior to chromatography. Additional polysaccharide could be extracted from the column by removing the packing material and washing it with 0.5M potassium hydroxide. Generally, polysaccharide removed in this way consisted of a mixture of materials that could not be completely dissolved in water. Its composition was not studied further.

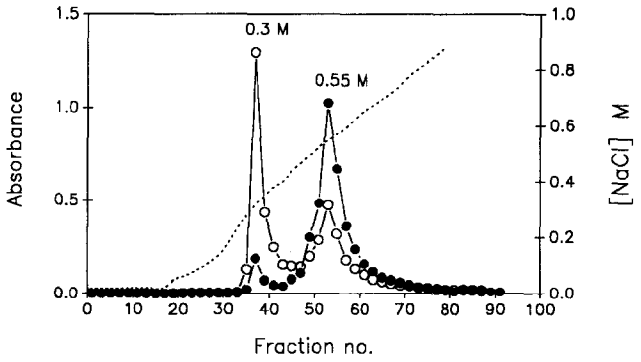

Fig. 1. DEAE-Sephadex A-25 chromatography of crude, acidic EPS mixture. —○—○—, Absorbance at 485 nm in phenol–H_2SO_4 carbohydrate assay; —●—●— absorbance at 530 nm in carbazole–H_2SO_4 uronic acid assay; ---- NaCl concentration.

The molecular size of EPS-I, as determined by gel-filtration chromatography, was greater than that of EPS-II. Fig. 2 shows the elution profiles of both polymers from Sepharose 4B. EPS-I is eluted in the void volume, indicating a molecular size $>20 \times 10^6$, whereas EPS-II is significantly smaller than this.

When corrected for the color reaction due to neutral sugars, the carbazole reaction showed that EPS-I contained little or no uronic acid. On the other hand, EPS-II was found to consist primarily of uronic acids. Furthermore, the reactivity of EPS-II in the carbazole reaction at different temperatures and in the presence and absence of borate[14] indicated that the main component was mannuronic acid.

The ^{13}C-n.m.r. spectra of the two EPS fractions show several characteristic features. The spectrum of EPS-I (Fig. 3a) contains three resonances in the 16–26-p.p.m. region. The resonance at 17.9 p.p.m. is attributable to C-6 of a 6-deoxy-hexose. That seen at 21.6 p.p.m. arises from the methyl carbon atom of an acetyl

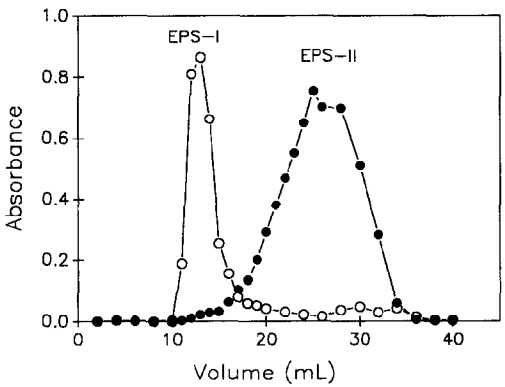

Fig. 2. Sepharose 4B chromatography of purified EPS fractions I and II. Symbols are the same as in Fig. 1. Phenol–H_2SO_4 assay was used for EPS-I, and carbazole–H_2SO_4 assay for EPS-II. Void volume, 12 mL; void plus included volume, 43 mL.

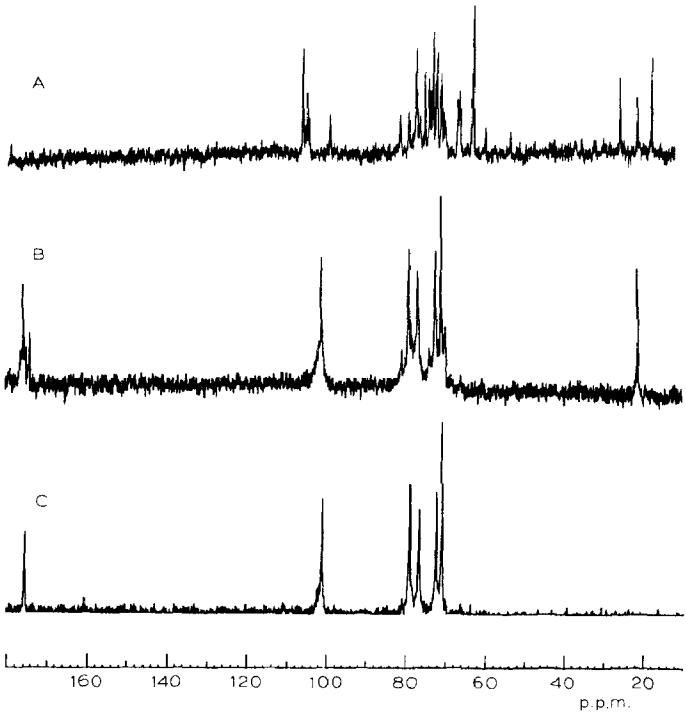

Fig. 3. ^{1}H-Decoupled, ^{13}C-n.m.r. spectra of polysaccharides. A, EPS-I; B, EPS-II; C, deacetylated EPS-II.

group, while the resonance which appears at 25.7 p.p.m. is due to the methyl carbon atom of a 1-carboxyethylidene group. The same region of the spectrum of EPS-II (Fig. 3b) shows only a single peak, at 21.5 p.p.m., which is due to an acetyl group. When EPS-II was deacetylated[26], the peak at 21.5 p.p.m. disappeared entirely (Fig. 3c), as did the minor resonances at 173.9 and 69.9 p.p.m., which were respectively due to the carbonyl carbon atom of the acetyl group and an unidentified carbon atom of the sugar ring. The anomeric region (95–110 p.p.m.) of the spectrum in Fig. 3a shows that EPS-I contains three or more distinct types of anomeric carbon atoms, while the spectrum of deacetylated EPS-II contains only one anomeric resonance, indicating the presence of only a single major type of sugar linkage. It should be pointed out that sugars present at a level of less than about one in ten are seldom detected by ^{13}C-n.m.r. spectroscopy.

The acetyl content of both exopolysaccharides varied from batch to batch. The range of values obtained for EPS-I was 2.4 to 3.4% (by weight) of the sodium salt form of the polysaccharide, with an average value of 2.7%. The acetyl content of EPS-II was higher, ranging from 4.0 to 11.5%, with an average of 6.4%. The ranges reported here represent variation in the composition of the polysaccharide samples from batch to batch, rather than experimental error in the determination of acetyl content.

TABLE I

STRUCTURAL FEATURES OF *A. chroococcum* NRRL B-14341 EXOPOLYSACCHARIDES

Parameter	*EPS-I*	*EPS-II*
Monomer composition	Man:Gal:Rha = 2:2:1	ManA:GulA = 9:1[a]
Pyruvic acetal content[b]	3.6–5.7	none
Acetic ester content[b]	2.4–3.4	4.0–11.5

[a]Variable from batch to batch; observed range, 7.3:1–11.5:1. [b]Percent (by weight) of the sodium salt form of the polysaccharide.

The pyruvic acetal content of EPS-I also varied from batch to batch, ranging from 3.6 to 5.7%, with an average value of 4.6% (by weight) of the sodium salt form of EPS-I. Polysaccharide EPS-II contained no pyruvic acetal.

When analyzed by acid hydrolysis and t.l.c., EPS-I was found to contain mainly rhamnose, galactose, and mannose, with a trace of glucose. G.l.c. analysis of the alditol acetates showed rhamnose, galactose, and mannose to be present in the molar ratios of 1:2:2, along with traces of glucose and arabinose. No arabinose could be detected by t.l.c.

Prior to acid hydrolysis, EPS-II was reduced to a neutral polysaccharide[17]. The carboxyl-reduced polysaccharide was then hydrolyzed, the products were converted into the alditol acetates, and these were analyzed by g.l.c. The major component of the reduced polysaccharide was found to be mannose, which constituted ~90% of the carbohydrate present. Gulose and arabinose were also detected, at ~9 and 1% by weight, respectively. The mannose and gulose were shown by g.l.c.–m.s. to have arisen from sodium borodeuteride reduction of their respective uronic acids. The values given in Table I indicate both the average monomer composition for EPS-II and the range of values observed, because batch to batch variation was encountered. It should be pointed out that both L-guluronic acid and D-glucuronic acid give rise to the same hexitol hexaacetate by this method, but can be distinguished by the fact that acid treatment of gulose affords an equilibrium mixture of 42% of gulose and 58% of 1,6-anhydrogulose, whereas the comparable equilibrium mixture for glucose contains only a trace of anhydro sugar[21]. Gulose was also positively identified by ion-exchange chromatography of acid-hydrolyzed, carboxyl-reduced EPS-II. Methylation analysis and g.l.c.–m.s. showed that reduced EPS-II gave rise to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl mannitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgulitol. Thus, the mannuronic and guluronic acid residues in EPS-II were shown to be (1→4)-linked.

DISCUSSION

This is the first definitive characterization of more than one exopolysaccharide from *Azotobacter chroococcum*. Most other studies have treated the EPS as a single polysaccharide. The one notable exception to this was the study

conducted by Lawson and Stacey[27], in which they reported that some strains of *A. chroococcum* produce both a neutral polysaccharide and a glucuronic acid-containing exopolysaccharide. The methods used in that study would not have detected pyruvic acetal as an acidic functional group, and may not have been able to differentiate among glucuronic acid and some of other hexuronic acids. Also, in their study, it was stated that the acidic portion was only a minor component of the total exopolysaccharide, whereas under our growth conditions, we found it to be one of two major constituents. It is not known why others have detected either glucuronic or galacturonic acid as the major uronic acid, because both our results and those of Ferrala *et al.*[10], using g.l.c.–m.s. of the alditol acetates of carboxyl-reduced EPS, clearly demonstrate that the major uronic acid component is mannuronic acid, along with lesser amounts of glucuronic or guluronic acid, or both. One possibility is that the paper-chromatographic systems used in some of the earlier studies may not have been able to resolve these uronic acids from one another.

We have found that both the relative amounts of the two polysaccharides produced by this organism and some of their structural details are variable. Although the DEAE-Sephadex elution-profile in Fig. 1 shows a case in which the relative proportions are approximately equal, this often was not the case. In some instances, for example, the organism produced mainly EPS-II, and only a small amount of EPS-I. Fifteen other strains of *A. chroococcum* were screened for the production of EPS, and it was found that they all exhibited this same property; *i.e.*, some produced mainly EPS-I, while others produced mainly EPS-II. The relative proportions in each of those cases was also variable, depending on culture conditions, stage of growth, *etc.* The overall yield of polysaccharide was variable from batch to batch, and decreased with repeated subculturing of the organism. This phenomenon has been noted by others[7,28], and may be due to the fact that bacteria removed for transfer are taken from the liquid, suspended phase, while those which produce high amounts of EPS may preferentially adhere to the walls and neck of the vessel as a biofilm. The loss of ability to produce EPS is not observed with bacteria grown on solid media, which tends to support this hypothesis.

It was also found that the chemical constitution of the exopolysaccharides could vary from batch to batch. For EPS-I, this was observed in the percentage of acetylation and pyruvic acetalation, as described in the Results section. For EPS-II, as for EPS-I, the degree of acetylation was found to vary from batch to batch. In addition, we have some evidence that the monomer composition of EPS-II can vary. In some instances, the ^{13}C-n.m.r. spectra of EPS-II exhibited additional peaks, most notably one in the anomeric region, which could be attributable to α-L-guluronic acid residues; this is supported by g.l.c.–m.s., which indicated that guluronic acid may be present in some samples at levels >10% (w/w) (see footnote to Table I).

The chemical and chromatographic data indicate that EPS-II is a low-

guluronate type of alginate, and the ^{13}C-n.m.r. spectrum shown in Fig. 3c supports this. The spectrum shown is identical to that of (1→4)-β-D-mannuronan[29]. The presence of alginate in *A. chroococcum* has not been demonstrated prior to this, although it is well known that *Azotobacter vinelandii* produces exocellular alginate. Several species of *Pseudomonas* are also known to produce exocellular alginate rich in D-mannuronic acid[28,30]. The structure of bacterial alginate is known to be dependent on the culture conditions, especially with respect to the relative proportions of D-mannuronate and L-guluronate[31,32], acetyl content[32], molecular weight[33], and certain physical properties[34,35]. Ferrala *et al.*[10] found that the relative proportions of some of the monosaccharide components of *A. chroococcum* total exopolysaccharide are dependent on the concentration of iron in the medium.

The production of two different exopolysaccharides by *A. chroococcum* is not unusual. *A. vinelandii* produces both exocellular alginate[31] and a heteropolysaccharide, reported by various workers to contain glucose, rhamnose, galaturonic acid, other uronic acids, and *O*-acetyl groups, as well as other components[8,35,36]. Exopolysaccharide I from *A. chroococcum* B-14341 is somewhat unusual, in that it is an acidic polysaccharide whose carboxylic acid groups are found solely as pyruvic acetal functionalities; EPS-I contains no uronic acids or other acidic carbohydrate groups.

Although viscosity studies were not carried out, it is evident from working with these two polysaccharides that solutions of EPS-I are generally more viscous than solutions containing similar concentrations of EPS-II. The lower viscosity of EPS-II could be due to the structural features of this polymer, or to enzymic degradation of the polysaccharide after biosynthesis, as has been reported[37] for another strain of *A. chroococcum*.

The results described herein, while strictly applicable only to *A. chroococcum* NRRL B-14341 grown under the conditions described, show that this bacterium is capable of producing two exocellular polysaccharides, one which resembles alginate (EPS-II), and one which contains rhamnose, mannose, galactose, pyruvic acetal, and acetate. The results described by Ferrala *et al.*[10] for this same strain grown on solid media are consistent with our findings. Our preliminary studies on other strains of *A. chroococcum* show that the strain described here is representative of this entire species. It should be mentioned that *A. chroococcum* grown on starch produces the same exopolysaccharides as when grown on D-glucose; this is not surprising, as we have found that this species produces mainly D-glucose from soluble starch (data not shown).

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