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Polymer-Producing Species of *Arthrobacter*

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ABSTRACT

GASDORF, HELEN J. (Northern Regional Research Laboratory, Peoria, Ill.), R. G. BENEDICT, M. C. CADMUS, R. F. ANDERSON, AND R. W. JACKSON. Polymer-producing species of *Arthrobacter*. J. Bacteriol. **90**:147–150.1965.—Two slime-producing microorganisms, designated as NRRL B-1973 and NRRL B-1797, were isolated from a Guatemalan soil sample. Their morphological and physiological characteristics permit their assignment to the genus *Arthrobacter*. Both cultures produce a large amount of extracellular polysaccharide, the maximal amount being 1.4 g per 3 g of glucose. The carbohydrate constituents of B-1973 polysaccharide are galactose, glucose, and mannuronic acid; those of B-1797 are galactose, glucose, and glucuronic acid. The organisms are morphologically and physiologically alike. The differences between these two cultures and previously described species of *Arthrobacter* appear sufficient to designate a new species. The name *Arthrobacter viscosus* sp. n. is proposed.

In the course of a survey to find polymer-producing microorganisms, two unusual cultures isolated from soil collected at the Guatemala City Airport in 1956 were selected for further study. Their morphological and physiological characteristics permit their assignment to the genus *Arthrobacter* (Conn and Dimmick, 1947), and it is proposed that they constitute a new species.

MATERIALS AND METHODS

The survey that revealed the new *Arthrobacter* forms was conducted on 34 samples of soil originating from Illinois; Indiana; New York; Arizona; Ontario, Canada; and Central and South America. The samples were plated on the synthetic basal medium of Pridham and Gottlieb (1948). This medium contained 0.26% $(NH_4)_2SO_4$ as the sole nitrogen source and was supplemented with various carbon sources (glucose, maltose, lactose, galactose, sucrose, arabinose, mannitol, glycerol, raffinose, α-methyl-D-glucoside). After an incubation period of 4 days at 30 C, plates with viscous colonies were segregated for further processing. Isolates picked from the original plates and incubated in nutrient broth were then streaked on agar media containing various organic nitrogen sources.

The following *Arthrobacter* cultures from the Agricultural Research Service Culture Collection were selected for comparison by both routine and specific tests: *A. globiformis*, NRRL B-2880; *A pascens*, NRRL B-1814; *A. pascens* (mucoid), NRRL B-2884; *A. aurescens*, NRRL B-2879; *A. citreus*, NRRL B-1258 and NRRL B-2882; *A. tumescens*, NRRL B-2881; *A. atrocyaneus*, NRRL B-2883; *A. simplex*, NRRL B-3157 and NRRL B-3158; and *A. ramosus*, NRRL B-3159. All cultures were maintained on yeast-malt slants (Haynes, Wickerham, and Hesseltine, 1955) and were grown at 25 C. Weekly transfers were made, and the cultures were stored at 10 C.

The characteristics of B-1973 and B-1797 differed sufficiently from the published descriptions of *A. nicotianae* (Giovannozzi-Sermanni, 1959), *A. duodecadis* (Lochhead, 1958), *A. flavescens* (Lochhead, 1958), *A. terregens* (Lochhead and Burton, 1953), *A. oxydans* (Sguros, 1955), *A. ureafaciens* (Krebs and Eggleston, 1939), and *A. helvolum* (Conn and Dimmick, 1947) that laboratory comparisons were not undertaken.

Carbohydrate fermentation and acid production were determined by use of phenol red broth containing 1% of various carbohydrates. The utilization of cellulose was tested by inoculating strips of filter paper immersed in a yeast-malt broth; cultures were grown in 300-ml flasks on a reciprocal shaker at 25 C for 5 days. The standard media for other identification tests were prepared as described in the *Difco Manual*. Identification procedures as described in the *Manual of Microbiological Methods* (Society of American Bacteriologists, 1957) were used, with additional specific procedures as needed.

Soil extract for use in media to characterize members of *Arthrobacter* was made in the following way. Soil (500 g) was suspended in 1,300 ml of an

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aqueous 0.1% Na_2CO_3 solution and boiled for 1 hr. After the suspension was filtered through cheesecloth, the filtrate was centrifuged at 10,000 × *g* for 20 min. The supernatant fluid was decanted, and 25% of this was incorporated into Tryptone-glucose-yeast extract-agar.

Optimal *p*H for growth was determined as follows. Cells were grown in a medium containing 3% glucose, 0.25% peptone, 0.4% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$; and trace amounts of $MnSO_4$, NaCl, and $Fe_2(SO_4)_3$ with *p*H values of 3 through 10 at 25 C on a rotary shaker. Samples were taken at 4-hr intervals, and the amount of growth was estimated by measurement of light transmission at 605 mμ.

Optimal *p*H for polymer production was also investigated. Cultures were grown in a medium shown by Cadmus et al. (1963) to give optimal polymer production by B-1973. The medium consisted of 3% commercial corn sugar, 0.25% enzyme-hydrolyzed casein (EHC, Amber Laboratories, Inc., Milwaukee, Wis.), 0.4% K_2HPO_4, 0.08% $MgSO_4 \cdot 7H_2O$, and 0.005% $MnSO_4 \cdot 4H_2O$ at *p*H 5 through 10. The fermentations were carried out on a rotary shaker at 25 C for 3 days. The polymers were then precipitated from 10 ml of crude centrifuged culture liquor with 2.5 volumes of ethyl alcohol and 3 drops of saturated KCl. Precipitated polymers were air-dried for 2 hr at 110 C and weighed. Yields were calculated as grams per 100 ml of culture fluid.

Temperature relationships were determined at 10 and 37 C. Cultures were grown on yeast-malt slants and were serially transferred daily for 3 days while being maintained at 10 or 37 C. Growth was not considered to take place at these temperatures unless cultures survived and grew during at least three serial transfers.

Gram staining was done according to the Hucker modification and flagella staining by the Leifson (1951) method.

Purified polymer from NRRL B-1973 and B-1797 was prepared for physical and chemical studies in the following manner. The viscous culture liquor was diluted 1:4 with water; 0.5 volume of ethyl alcohol was added to expedite the centrifugation of cells, and the mixture was centrifuged at 20,000 × *g* for 30 min. The supernatant liquid was decanted, and the polysaccharide was precipitated from this by addition of 1 g of KCl, per 100 ml of solution, and 2.5 volumes of ethyl alcohol. The entire precipitate was removed and dissolved in a volume of water equal to the original volume of the supernatant fluid, and then reprecipitated. Finally, the purified precipitate was dissolved in water and lyophilized to obtain a white, dry product.

For chromatographic analysis, 2% solutions of the polymers were hydrolyzed with 2 N HCl for 1 hr at 100 C. Hydrolysates were then spotted on Whatman no. 1 paper, and the components were separated by descending chromatography with the solvent system, ethyl acetate-acetic acid-pyridine-water, in the ratio of 5:1:5:3, v/v (Gee and McCready, 1957). This procedure chromatographically separates galacturonic, glucuronic, and mannuronic acids. The lactones corresponding to the latter acids are also separated. Spots were developed with the *o*-aminodiphenyl spray reagent (Gordon, Thornburg, and Werum 1956).

RESULTS AND DISCUSSION

The two isolates, NRRL B-1973 and B-1797, from Guatemalan soil produced extremely viscous growth on media containing various carbon sources. Cells from an 18-hr culture on yeast-malt agar were highly pleomorphic and gram-negative. These cells changed from short-branched, curved and straight rods at 8 to 12 hr to staphyloccocus-like forms at 24 to 48 hr. A definite life cycle (Chaplin, 1957) seemed to take place. Many of the cells exhibited uneven staining. The cultures failed to initiate growth in synthetic medium with phenol as a carbon source and also produced nitrites from nitrates; consequently, *Mycoplana* was eliminated as a generic possibility. Growth did not occur on nitrogen-free synthetic media; thus, the genus *Azotobacter* was eliminated. Growth occurred on the mannitol-calcium-glycero-phosphate-agar of Riker et al. (1930) without browning of the medium. Large circular colonies were produced on nutrient gelatin. These characteristics eliminated *Agrobacterium radiobacter* (Hofer, 1941) as a possibility. Since cellulose was not utilized, the genus *Cellulomonas* could be excluded.

The occasional formation of "Y," "T," and "U" forms with uneven staining appeared typical of one of the soil diphtheroids, whose taxonomy was reviewed by Clark (1952). Utilization of inorganic nitrogen and the gram-negative staining reaction ruled out *Corynebacterium* and pointed toward *Arthrobacter* as the probable genus.

On the basis of morphological and physiological comparisons, NRRL B-1973 and B-1797 do not appear to fit the description of any of the known species of *Arthrobacter*. They most closely resemble the nonchromogenic, vitamin non-requiring species *A. globiformis* (Conn and Dimmick, 1947), *A. pascens* (Lochhead and Burton, 1953), *A. simplex*, (Jenson, 1934), and *A. ramosus*, (Jensen, 1960), but are distinguished from each of them by several characteristics. They differ from *A. globiformis* and *A. pascens* by their urease activity, lack of gelatin and starch hydrolysis, and lack of hydrogen sulfide production; from *A. simplex* by their urease activity, lack of gelatin hydrolysis, and lack of growth at 37 C; and from *A. ramosus* in ability to reduce nitrates and lack of gelatin hydrolysis. Furthermore, they produce large amounts of extracellular polysaccharide in medium consisting of various organic nitrogen

sources, potassium phosphate buffer, and inorganic salts. None of the comparison cultures produced extracellular polysaccharide under these conditions. Although Mulder and Anthenuisse (1963) stated that some *Arthrobacter* strains are able to produce large amounts of extracellular polysaccharide, he gave data only on *A. globiformis*. The polymer produced by this organism contains glucose and glucuronic acid. Although B-1797 also produces a polymer containing glucuronic acid, it differs from *A. globiformis* in several respects; the strain B-1973, which elaborates a polymer containing mannuronic acid, also differs considerably otherwise from *A. globiformis*.

The maximal amount of polysaccharide produced by B-1973 in flasks was 1.24 g per 3 g of glucose; that of B-1797 was 1.30 g per 3 g of glucose. Final viscosity of cultures was 10,000 to 12,000 centipoises. Cadmus et al. (1963) showed that the maximal amount of polymer produced by B-1973 is 1.4 g per 3 g of glucose on a pilot-plant scale. The optimal *p*H for polymer production by both B-1973 and B-1797 was 7.0. Reduced production occurred at *p*H 5.0, 6.0, 8.0, and 9.0, with none below 4.0 or above 10.0.

The polymer from these organisms is gelatinous, fibrous, and somewhat cohesive when first precipitated from crude culture liquor. The lyophilized material is white and spongelike. Aqueous solutions of the polysaccharides are very viscous; a 1% solution gives a viscosity of 10,000 centipoises.

The carbohydrate constituents of the polysaccharide from B-1973 are galactose, glucose, and mannuronic acid; those of B-1797 are galactose, glucose, and glucuronic acid. The composition and structure of the polymer from B-1973 were determined by Sloneker et al. (1963). The polysaccharide was found to possess a linear structure composed of repeating units having equal proportions of D-mannuronic acid, D-glucose, and D-galactose. These three sugar residues make up 75% of the dry weight of the polysaccharide; the remaining 25% is in the form of *O*-acetyl groups. The structure of the B-1797 polysaccharide has not been determined.

In addition to the uronic acid components of their extracellular polysaccharide, B-1973 and B-1797 differ in that the latter produces much more mucoid growth on solid medium. However, these cultures are alike in all other morphological and physiological characteristics. They may, therefore, be considered to constitute a new species of the genus *Arthrobacter*, and B-1973 is designated as the type strain. Morphological and physiological descriptions follow.

Morphology. At 12 to 18 hr, short gram-negative rods were present. Some stained evenly, but the majority stained unevenly. Some "Y," "X," "U," and "T" forms present were also unevenly stained. The short rods exhibited bipolar staining. The longer rods appeared to have three to six heavily stained granules in a more lightly stained cell.

After 24 to 48 hr, micrococcus-like cell groupings were predominant. These cells were unevenly stained but appeared to be gram-negative.

Flagella staining of cells grown on yeast-malt slants and suspended in distilled water showed no flagella, but the highly pleomorphic "Y," "X," and "U" cell forms were made plainly visible by this technique.

Colonial characteristics. Agar streak: filiform, raised, glistening, viscous. Agar colonies: 3 mm, circular, raised, glistening, viscous, white, opaque. Soil extract-agar: filiform, moderate growth, glistening, soft, viscous, raised, cream-colored. Gelatin colonies: circular 2 to 3 mm, smooth, raised, glistening, opaque, no liquefaction. Asparagine-agar slant: growth moderately abundant, light cream, slightly mucoid, glistening, wrinkled edge. Asparagine-agar colonies: 1 mm, circular, translucent, white, raised, slightly mucoid. Potato: growth moderately abundant, glistening, soft, filiform, cream-buff. Yeast-malt slant: viscous, profuse white growth, slightly mucoid in 24 hr. Mannitol-calcium-glycerophosphate colonies: 3 to 3.5 mm, raised, glistening, mucoid, circular; $CaCO_3$ formed around colonies; no browning of medium.

Physiology. Aerobic, weakly catalase-positive. No acid production in glucose, sucrose, lactose, or mannitol. Final *p*H of fermentation of glucose was 7.7; sucrose and lactose, 7.5; and mannitol, 7.3. Slight acid production was noted in glycerol (*p*H 6.2). Basic products resulted from fermentations of arabinose, raffinose, and α-methyl glucoside (*p*H 8.3 to 8.4). No gelatin liquefaction shown after 3 months of incubation. Neither indole nor acetylmethylcarbinol was produced. Nitrites produced from nitrates. No starch hydrolysis. Litmus milk completely cleared in 1 month with no curd formation; reduction in 23 days and complete by 25 days; a hard pellicle formed on milk in about 21 days; reaction slightly basic. Urease produced. Citrate utilized as the sole source of carbon. H_2S not produced from either cysteine or thiosulfate. Inorganic nitrogen utilized as sole source of nitrogen. Optimal temperature, 25 to 28 C. No growth at 37 C and little growth after 3 to 4 days of incubation at 10 C. The optimal *p*H, 6.1; growth sharply reduced at *p*H values below 5.0 and above 7.5.

These organisms differ from any previously

described *Arthrobacter* species and constitute a new species, *Arthrobacter viscosus* sp. n.

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