CHARACTERIZATION OF *ARTHROBACTER* ISOLATED FROM URANIUM MILL TAILINGS

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CONVERSION FACTOR

The inch-pound units used in this report may be converted to metric units by use of the following conversion factor:

Multiply inch-pound unit	*By*	*To obtain metric unit*
inch	25.40	millimeter (mm)

To convert degree Celsius (°C) to degree Fahrenheit (°F), use the following formula:

$$°F=9/5\ °C+32$$

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ABSTRACT

Ten bacterial cultures were isolated: seven from uranium-mill tailings, one from soil, and two from uraniferous peat; all cultures were classified by usual microbiological methods.

Nine of the ten cultures were classified as *Arthrobacter* species on the basis of morphology, biochemical characteristics, and nutritional requirements. These cultures grew well on nutrient agar and EYGA agar, and exhibited the rod-to-coccus transformation characteristic of *Arthrobacter* species. The other culture, originally isolated from peat, was identified as *Pseudomonas putida*. All 10 cultures were obligate aerobes, and all were prototrophic, requiring no vitamins or accessory growth factors.

INTRODUCTION

Earlier work by the present authors indicated that radium-226 is leached from uranium mill tailings by sulfate-reducing bacteria (Landa and others, 1986). Studies of the microorganisms present in such tailings showed that sulfate-reducing bacteria, *Bacillus* species and *Arthrobacter* species, could be isolated from the tailings (Miller and others, in press).

Arthrobacter was by far the predominant microbial genus in the sand fraction of the mill tailings; whereas, *Bacillus* and fungi predominated in the very fine slime fraction. This report discusses the methods of characterizing 10 bacterial isolates thought to be *Arthrobacter*.

METHODS AND MATERIALS

The methods used to collect the tailings samples and to obtain bacterial isolates are described in detail by Miller and others (1986). Tailings were collected using aseptic technique and stored in tightly sealed glass vials at 5 °C. Bacterial isolates were obtained from dilutions of tailings samples, plated on nutrient agar, and incubated at room temperature.

Stock cultures were maintained on nutrient-agar slants at 5 °C. All cultures were restreaked several times on nutrient-agar plates; well-isolated colonies were picked each time to ensure purity of the cultures. Then all colonies were transferred to nutrient-agar slants and incubated at 28 °C. All cultures also were grown on plates of EYGA agar, the medium recommended by Keddie and Jones (1981) for identification of *Arthrobacter* and other saprophytic-coryneform bacteria, and on King's agars A and B (Stolp and Gadkari, 1981), a medium used for the demonstration of pigment production.

Determination of Morphology

Cultures on nutrient-agar plates and slants and on EYGA-agar plates were examined at intervals of 6 hours, 24 hours, and 5 days by phase-contrast microscopy at 1000X to detect the rod-to-coccus transformation characteristic of *Arthrobacter*. Gram stains were prepared on all cultures from both young (rod-phase) cultures and older (coccoid-phase) cultures by the method described by Gerhardt (1981). The potassium hydroxide method of Buck (1982) also was employed to determine gram negative or positive character. Flagella stains were made on young cultures using the method of Mayfield and Innis (1977). Motility was evaluated by examining agar-slant and nutrient-broth cultures after 1, 2, 3, and 7 days incubation at 28 °C by phase-contrast microscopy at 1000X. Scanning electron microscopy was performed on a young culture of strain 4 growing on Stanier's mineral-salts agar, and on 13-day-old cultures of strains 1, 4, 9, and 10 grown on nutrient-agar plates at 28 °C, using a Cambridge model 250 scanning-electron microscope[1]. Good results were obtained by gently pressing an aluminum stub directly against a bacterial colony, air-drying the stub for a few minutes, coating the cells adhering to the stub with gold, and then examining the stub.

Biochemical Tests

The standard biochemical tests: anaerobic growth, glucose fermentation; catalase production; cytochrome-oxidase production; gelatine hydrolysis; and nitrate reduction were performed using methods described by Gerhardt (1981). Anaerobic-growth tests were performed by stab inoculation deep into nutrient-agar tubes. Glucose-fermentation tests were performed in phenol-red glucose broth containing 1 g/L (gram per liter) of beef extract, 10 g/L of proteose peptone no. 3, 5 g/L of sodium chloride, 0.01 g/L of phenol red, and 10 g/L glucose. Catalase production and cytochrome-oxidase tests were performed on colonies grown on nutrient-agar plates. The cytochrome-oxidase test was performed with Pathotec® cytochrome-oxidase test strips. Gelatine hydrolysis was studied by stab inoculation deep into nutrient-gelatine tubes. Nitrate-reduction tests were performed using a nutrient broth containing 1 g/L of potassium nitrate, and frequent tests were performed for nitrite by the sulfanilic acid and α-naphthylamine test. Nitrate was tested for by adding zinc dust prior to the addition of the previously listed reagents.

Nutritional Requirements

Nutritional requirements were determined by adding organic nutrients to a mineral-salts medium based on the medium discussed by Stanier and others (1963) containing the following constituents: 1 g/L of ammonium chloride; 1 g/L of potassium phosphate; 0.2 g/L of magnesium sulfate (seven hydrate); 0.01 g/L of ferrous sulfate (seven hydrate); 0.01 g/L of calcium chloride; 0.1 mg/L (milligram per liter) of the following trace elements: sodium

[1]The use of trade or product names in this report is for identification purposes only, and does not constitute endorsement by the U.S. Geological Survey.

molybdate (dihydrate), manganese chloride (tetrahydrate), cuprous sulfate, zinc sulfate (seven hydrate), and cobaltous chloride.

Salt-tolerance tests were performed using a nutrient broth containing 1-, 2-, 4-, or 8-percent sodium-chloride. To test pH tolerance, nutrient agar at pH 4.8, 7.0, and 8.7 (after autoclaving) was employed. For the purposes of assessing oligotrophic growth and utilization of humic acid, Stanier's mineral salts with 1.5 percent purified agar both with and without the addition of 0.1 percent purified humic acid, was used, as well as distilled water containing 1 percent purified agar (Fisher Scientific, Denver). Temperature effects were studied as follows: the cultures were streaked on nutrient-agar plates; one series of plates was incubated at 28 °C; another at 35 °C; a third series was incubated at 42 °C.

RESULTS

The origin of the 10 isolates, including a description of the environments from which they were isolated, is described in table 1.

All cultures except culture 9 exhibited the rod-coccus transformation, as illustrated for *Arthrobacter* species by Cure and Keddie (1973). Some cultures (2 and 3) developed rudimentary branching; other cultures were short, irregular-shaped rods after 16 to 24 hours incubation on nutrient agar; all cultures changed to coccoid morphology after an additional 2 to 6 days incubation. All cultures grew well on nutrient agar, forming smooth, circular, opaque convex colonies, with an entire edge 2 to 5 mm in diameter after 1 to 3 days growth. Cultures 5 and 10 turned pale yellow after 4 to 6 days growth on nutrient agar at 28 °C; all other cultures remained white. All cultures also grew well on EYGA agar; all cultures completed the rod-to-coccus transformation much more rapidly than cultures grown on nutrient agar. All cultures consisted of mostly coccoid forms after 24 hours at 28 °C. Gram stains indicated Gram-negative rods in very young cultures and Gram-positive coccoid forms in older cultures; the KOH testing method of Buck (1982) indicated a Gram-positive type of test (no viscous slime) for all cultures except culture 9, after 2 days or more incubation at 28 °C. Cultures 1 through 9 grew profusely on King's agars A and B; culture 10 died before it could be tested on King's agars. Culture 9 produced abundant yellow fluorescent pigment after 2 days incubation. None of the other cultures produced pigment in 2 days. Motility was shown only by cultures 9 and 10. Flagella stains succeeded only with culture 9, which consisted of short rods, motile by 1 or 2 polar flagella. Electron micrographs of the old cultures growing on nutrient agar were of poor quality and showed only irregular coccoid cells, but electron micrographs of young cultures growing on mineral-salt agar were of excellent quality and showed the rod-coccus transformation as illustrated in figure 1.

Reactions of cultures to standard biochemical tests are presented in table 2. All cultures grew well on glucose mineral-salts agar and are therefore prototrophic, requiring no accessory-growth factors. All cultures grew only at or near the surface of the agar in stab-inoculated nutrient-agar tubes; hence, they are obligate aerobes. No cultures fermented glucose to acid or gas. All cultures produced catalase, although culture 9 produced

Table 1.--*Origin of isolated cultures*

[--, no data]

Culture number	Original isolate number	Site[1]	Type of sample	pH of sample	Depth of sample (inches)	Moisture content (percent)	Organic carbon content (percent)
1	E1	Edgemont, S.Dak.	Uranium mill tailings.	6.6	15	1.8	0.06
2	E3	Edgemont, S.Dak.	Uranium mill tailings.	6.6	21	4.6	.32
3	R12	Rifle, Colo.	Uranium mill tailings.	7.6	15	7.8	.14
4	R13	Rifle, Colo.	Uranium mill tailings.	7.3	15	13	<.01
5	R15	Rifle, Colo.	Uranium mill tailings.	7.5	15	9.0	<.01
6	M18	Maybell, Colo.	Uranium mill tailings.	7.2	15	3.0	<.01
7	M20	Maybell, Colo.	Soil	8.1	15	4.6	.23
8	M22	Maybell, Colo.	Uranium mill tailings.	4.8	15	3.1	<.01
9	FC83-30,P	Flodelle Creek, Wash.	Uraniferous peat.	--	56 to 58	--	--
10	FC83-3,Q	Flodelle Creek, Wash.	Uraniferous peat.	--	50.5 to 52.5	--	--

[1]For a more thorough description of these sites, please refer to Miller and others, in press.

only a very weak catalase test. Only culture 9 produced a very strongly positive reaction for cytochrome oxidase. All cultures except 7 and 9 liquefied gelatine, but very slowly. Liquefaction commenced after 5 days or more at room temperature (22-24 °C) at the top of the tube, then slowly proceeded downward in a stratified pattern. Nitrate was reduced to nitrite by cultures 4, 5, 9, and 10. Culture 9 reduced nitrate to nitrite very rapidly; the nitrite then disappeared within another 24 hours. None of the cultures produced gas (denitrification reaction).

Results of the salt-tolerance test are presented in table 3. All cultures were moderately tolerant of salt; nine cultures grew in 4-percent sodium-chloride solution and three in an 8-percent sodium-chloride solution.

All cultures grew on Stanier's mineral-salts agar with or without 0.1 percent purified humic acid; all cultures also produced slight growth on 1-percent purified agar. Several species of *Pseudomonas*, streaked on the 1-percent purified agar, gave no growth. Thus, the cultures are all oligotrophs, as defined by Poindexter (1981) and by Kuznetsov and others (1979). Culture 9 is *Pseudomonas putida*, and differs from the other *Pseudomonas* cultures tested, growing under oligotrophic conditions.

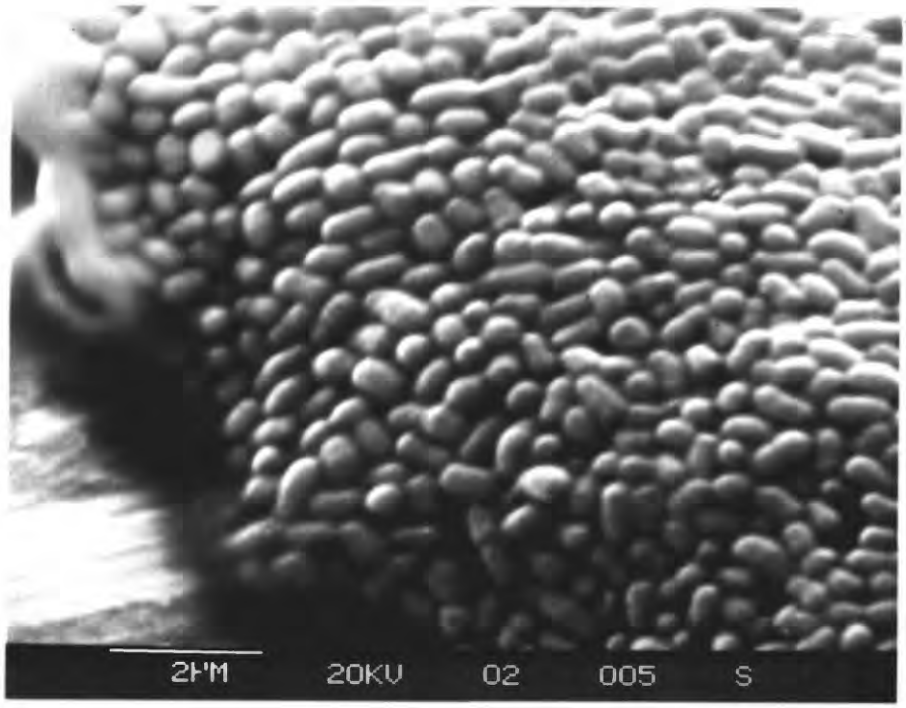

Figure 1.--Scanning electron micrograph of mixed coccoid and rod-phase *Arthrobacter* sp., culture 5. Incubated on Stanier's mineral-salts agar for 5 days at 28 °Celsius.

All cultures grew rapidly at 28 and 35 °C; only culture 1 grew at 42 °C. All cultures grew well on nutrient-agar plates at pH 7.0 and pH 8.7; four cultures grew at pH 4.8. Thus, these cultures tolerate a considerable range of environmental pH values.

SUMMARY AND CONCLUSIONS

Conn and Dimmick (1947) described the characteristics of the genus *Arthrobacter* as: (1) Irregular-shaped Gram-negative rods in young cultures, changing to Gram-positive coccoid cells on further incubation; (2) aerobic; (3) liquefying gelatine slowly; and (4) common soil bacteria. Our cultures 1 through 8 and culture 10 clearly have the characteristics of this genus. However, culture 9 clearly has the characteristics of the genus *Pseudomonas* as described by Stolp and Gadkari (1981): (1) Small, non-sporulating Gram-negative rods motile by means of one or a few polar flagella; (2) aerobic; (3) all members of the genus use molecular oxygen as a terminal oxidant, and some can use denitrification as an alternative, anaerobic-respiratory mechanism; (4) chemo-organotrophs; (5) catalase positive; and (6) oxidase positive. Culture 9 was readily identified using the characteristics provided by Stolp and Gadkari (1981) as *Pseudomonas putida*.

Table 2.--*Biochemical tests on* Arthrobacter *cultures*

[0=negative reaction; ±=slight positive reaction; +=moderate positive reaction; and ++=strongly positive reaction]

Culture number	Nitrate reduced to nitrite	Nitrate remaining	Glucose fermentation; final pH	Utilization of glucose	Gelatine liquefaction	Catalase	Cytochrome oxidase
1	0	++	7.3	+	+	+	0
2	0	++	7.3	+	+	+	0
3	0	++	6.6	+	+	+	0
4	+	++	6.6	+	+	+	0
5	++	++	6.6	+	+	+	0
6	0	++	6.7	+	+	+	0
7	0	++	6.8	+	0	+	0
8	0	++	6.8	+	+	+	0
9	+	0	6.7	+	0	±	++
10	+	++	7.3	+	+	+	0

Table 3.--*Effect of sodium chloride on the growth of cultures in nutrient broth*

[0=no growth; and +=substantial growth as evidenced by turbidity]

Culture number	Growth at salt concentration indicated (percent)				
	0	1	2	4	8
1	+	+	+	+	0
2	+	+	+	+	0
3	+	+	+	0	0
4	+	+	+	+	0
5	+	+	+	+	+
6	+	+	+	+	0
7	+	+	+	+	0
8	+	+	+	+	+
9	+	+	+	+	+
10	+	+	+	+	0

The *Arthrobacter* cultures studied cannot be assigned species names because the described species are, in many cases, based on a single, isolated strain, and are poorly characterized in other cases. Keddie and Jones (1981) divided the genus *Arthrobacter* into three species groups: the *globiformis* group; the *simplex/tumescens* group; and the *terregens/flavescens* group. They further proposed that the *terregens/flavescens* group be excluded from the genus, thus defining a smaller group, *Arthrobacter sensu stricto*. The distinction among these groups was made primarily on the basis of deoxyribonucleic acid (DNA) base composition and the composition of the peptidoglycan-cell wall. The *globiformis* group have guanine plus cytosine (G+C) values of 60 to 71 percent and the peptidoglycan contains lysine and no L-diaminopimelic acid or ornithine. The *simplex/tumescens* group has a guanine plus cytosine value of 72 to 74 percent and the peptidoglycan contains L-diaminopimelic acid and no lysine or ornithine. The *terregens/flavescens* group has a guanine plus cytosine of 69 percent, and the peptidoglycan contains ornithine and no lysine or L-diaminopimelic acid. The principal base was erroneously reported as lysine in Keddie (1974).

The members of the *terregens/flavescens* group require a growth factor, the *terregens* factor. All of our cultures are prototrophic, requiring no accessory-growth factors; therefore, they do not belong to the *terregens/flavescens* group. Since we have not determined the amino-acid composition of the cell walls, we cannot determine whether our cultures belong to the *globiformis* group or the *simplex/tumescens* group.

Arthrobacter species have been reported to constitute a very large proportion of the bacterial flora in many sedimentary and soil environments. Mulder and Antheunisse (1963) reported that more than 60 percent of the colonies growing on soil-extract casein agar, inoculated from Dutch and Nigerian soils, were *Arthrobacter*; de Haan (1977) isolated *Arthrobacter* cultures on benzoic-acid medium from a Dutch lake, and showed that it was capable of partially degrading fulvic acids; Updegraff and Douros (1972) found that 72 percent of the cultures isolated from organic-rich sedimentary uranium ores were *Arthrobacter*; Gong-Collins and Read (1985) recently described a new species of *Arthrobacter* from a microbial mat at Laguna Figueroa, Baja, California, that was capable of withstanding wide variations in salinity and in dehydration of wetting cycles.

The characteristics of *Arthrobacter* that contribute to its abundance in aerobic-soil and sediment habitats, including uranium mill tailing, include: (1) An oligotrophic nature, (2) an ability to utilize a broad range of organic compounds as a source of energy and carbon, (3) relatively wide salinity limits, and (4) a resistance to dessication.

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