***ACIDIPHILIUM MULTIVORUM* SP. NOV., AN ACIDOPHILIC CHEMOORGANOTROPHIC BACTERIUM FROM PYRITIC ACID MINE DRAINAGE**

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Six strains of acidophilic chemoorganotrophic bacteria from acid mine drainage were studied in their taxonomic aspects. They were gram negative, aerobic, mesophilic, oxidase negative, catalase positive, urease positive, nonsporeforming, and rod-shaped. Carotenoid and bacteriochlorophyll *a* were formed. Two strains had a polar flagellum and other two strains fimbriae. They used a wide variety of organic compounds for growth, but did not use ferrous iron, elemental sulfur, and thiosulfate as the sole energy source. Acetate was inhibitory to growth. Growth was enhanced by adding high concentrations of glucose or complex organic compounds such as trypticase soy (BBL) and yeast extract. Methanol was utilized as the sole source of carbon and energy. The major ubiquinone was Q-10. The major cellular fatty acid was straight-chain unsaturated C_{18:1} acid. The hydroxy acid was 3-OH C_{14:0} acid. The DNA base composition was 66.2 to 68.1 mol% guanine plus cytosine. The isolates showed relatively low levels of genetic similarity to *Acidiphilium cryptum* and *Acidiphilium organovorum*. On the basis of the phenotypic, chemotaxonomic, and genotypic characters, we conclude the isolates as a new species, for which we propose *Acidiphilium multivorum* sp. nov. The type strain is AIU 301 (JCM 8867).

There have been some reports on acidophilic, mesophilic, and chemoorganotrophic bacteria which were isolated from acid mine drainage (14,35), coal refuse

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and coal mine drainage (20,27), and cultures of *Thiobacillus ferrooxidans* (5–9,11,18,36,37). Harrison (6) studied systematically acidophilic, mesophilic, heterotrophic bacteria derived directly from coal strip-mine refuse and also as contaminants in cultures of *T. ferrooxidans*, and proposed the designation of the genus *Acidiphilium* for these bacteria. The genus *Acidiphilium* now consists of five species, *A. cryptum* (6), *A. organovorum* (18), *A. angustum* (36), *A. facilis* (36), and *A. rubrum* (36). Recently, Kishimoto et al. (13) have reported *Acidobacterium capsulatum*, a new acidophilic heterotrophic bacterium isolated from acidic mineral environments. A facultatively methylotrophic bacterium designated as *Acidomonas methanolica* is also a mesophilic acidophile (28–30).

Previously, we isolated six strains of acidophilic heterotrophic bacteria from acid mine drainage (pH 2–3) discharged from the abandoned Matsuo sulfur-pyrite mine area in Iwate Prefecture (32). These bacteria were found to be genuine acidophiles that require high acidity of pH 3. In this report we describe the phenotypic, chemotaxonomic, and genotypic characteristics of these isolates and their taxonomic relationship to other acidophilic heterotrophic bacteria from similar environments.

MATERIALS AND METHODS

Bacterial strains. The six strains of isolates and five reference strains investigated are listed in Table 1. They were maintained on agar slants, and subcultured every three months.

Media and cultivation. Basal salts (BS) medium used for the isolates consisted of 0.2% $(\text{NH}_4)_2\text{SO}_4$, 0.01% KCl, 0.01% K_2HPO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{Ca}(\text{NO}_3)_2$. The culture media were adjusted to pH 3.0 with H_2SO_4 , unless otherwise noted. BS medium (pH 3.0) was supplemented with either 0.5%

Table 1. Strains of acidophilic heterotrophic bacteria used.

Strain/Species	Source
Isolate AIU 301 ^a	Acid mine drainage
Isolate AIU 302 ^a	Acid mine drainage
Isolate AIU 303 ^a	Acid mine drainage
Isolate AIU 304 ^a	Acid mine drainage
Isolate AIU 305 ^a	Acid mine drainage
Isolate AIU 306 ^a	Acid mine drainage
<i>Acidiphilium cryptum</i> DSM 2389 ^b	<i>Thiobacillus ferrooxidans</i> culture
<i>Acidiphilium organovorum</i> ATCC 43141 ^b	<i>Thiobacillus ferrooxidans</i> culture
<i>Acidiphilium angustum</i> ATCC 35903 ^b	Acid mine drainage
<i>Acidiphilium facilis</i> ATCC 35904 ^b	Acid mine drainage
<i>Acidiphilium rubrum</i> ATCC 35905 ^b	Acid mine drainage

^a The strains were isolated from acid mine drainage at the abandoned Matsuo sulfur-pyrite mine in Iwate Prefecture (32).

^b DSM, Deutsche Sammlung von Mikroorganismen; ATCC, American Type Culture Collection; type strain.

polypeptone (Daigo Eiyo Co., Tokyo, Japan), 0.5% glucose, 0.2% vitamin-free casamino acids (Difco Laboratories, Detroit, MI, U.S.A.) plus 0.5% glucose, 0.2% trypticase soy (BBL Microbiology Systems, Cockeysville, MD, U.S.A.), or 0.2% trypticase soy plus 1% glucose; these media were designated as BS-P, BS-G, BS-CG, BS-T, and BS-TG media, respectively. BS-N medium (pH 3.0) without nitrogen source contained 0.01% KCl, 0.01% K_2HPO_4 , 0.01% $MgSO_4 \cdot 7H_2O$, and 0.01% $CaCl_2 \cdot 2H_2O$. Peptone medium (pH 3.5) was composed of 0.1% polypeptone.

For *Acidiphilium* species, BS-A medium (pH 3.5) was used, which contained 0.2% $(NH_4)_2SO_4$, 0.01% KCl, 0.05% K_2HPO_4 , 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% trypticase soy, and 0.1% glucose.

Agar solid medium of BS-CG or BS-TG contained 2% agar. In order to prevent the hydrolysis of agar by autoclaving at low pH conditions, agar and other components were autoclaved separately and combined before solidified.

Incubation temperature was 30°C, unless otherwise stated. Liquid cultures were grown aerobically either in 50 ml of medium in a 500-ml flat-bottom flask or 5 ml in a test tube (16.5 × 165 mm) on a reciprocal shaker. Bacterial growth was determined by counting cells with a bacterial counting chamber (Erma Optics Co., Tokyo, Japan) or by measuring absorbance at 660 nm spectrophotometrically.

Morphological and cultural characteristics. Cell morphology was investigated under a phase-contrast microscope and type of flagellation electron microscope. Gram staining was performed by the modified Hucker's method (26). Poly- β -hydroxybutyrate granules in cells were stained by using Nile Blue A method (23) and examining an excitation wavelength of 490 nm under a Nikon Labophot Microscope (Nikon Co., Tokyo, Japan) with an episcopic fluorescence attachment. Metachromatic granules were stained by Albert's method as modified by Laybourn (17).

Physiological, nutritional, and biochemical tests. Effect of pH on growth was determined on BS-P (0.1% polypeptone) media adjusted to pH values between 1.5 and 6.0 at 0.5 pH unit intervals. Growth temperature was determined in BS-P medium in the range of 7 to 48°C at 1.6°C intervals by the use of Temperature Gradient Incubator TN-3 (Toyo Scientific Co., Tokyo, Japan).

Salt tolerance was determined in peptone medium with 1, 2, 3, 4, 5, and 7% sodium chloride after 2 weeks incubation. Growth with potassium cyanide was examined in BS-CG medium (pH 3.5) with filter-sterilized 0.1% potassium cyanide. Heat resistance was determined by inoculating strains in BS-P medium, exposing to 67°C for 2 min, and then incubating at 30°C for a week.

Utilization of nitrogen compounds was observed in BS-N medium supplemented with 0.5% glucose plus either 0.2% $(NH_4)_2SO_4$, 0.3% KNO_3 , or 0.1% urea after a week incubation. Utilization of carbon sources was determined with BS medium (pH 3.5) supplemented with filter-sterilized organic compounds. Final concentration of the organic additives was 0.1%, unless otherwise stated. Growth was examined after 10 days incubation. Utilization of L-amino acids and a related

compound as the sole source of carbon and nitrogen was tested on BS-N medium. The filter-sterilized substances were added to the medium to a final concentration of 0.1%, unless otherwise stated. Growth was read at intervals for 20 days.

Growth on complex organic compounds was determined on BS and BS-G (0.1% glucose) media supplemented with trypticase soy (BBL), trypticase peptone (BBL), bacto-peptone (Difco), bacto-tryptone (Difco), vitamin-free casamino acids (Difco), tryptose (Difco), yeast extract (Difco), nutrient broth (Difco), polypeptone, and meat extract (Kyokuto Seiyaku Co., Tokyo, Japan). Final concentrations of each substance were 1, 0.1, 0.01, and 0.001%. Susceptibility to organic substances was examined on BS medium (pH 3.5) containing 0.05–7% trypticase soy, 0.05–0.7% yeast extract, or 0.05–10% glucose.

Oxidation/fermentation test was made in BS-G medium (pH 4.0) after 10 days of both aerobic and anaerobic cultivations. Acid production from glucose was determined as a decrease in the measured pH of cultures by at least 0.2 pH units. Acid production from other carbohydrates was also examined (10, 19).

Nitrate reduction was determined on the BS-N medium supplemented with 0.2% glucose and 0.1% potassium nitrate by adding the Griess-Ilosvay reagent (26) at intervals during incubation for 10 days. Denitrification was determined in BS-N medium with 0.2% glucose and 0.1% potassium nitrate by observing bubbles in Durham tubes after 10 days anaerobic cultivation. Both nitrate reduction and growth were also checked.

Chemolithotrophic growth was determined after 30 days incubation in BS medium (pH 3.0) with either 0.1% ferrous sulfate or 1% elemental sulfur and BS medium (pH 5.0) with 1% sodium thiosulfate as the sole source of energy. Growth inhibition by thiosulfate was determined on BS-P or BS-G (0.2% glucose) medium (pH 3.5) containing 0.1% sodium thiosulfate. Growth inhibition by monocarboxylic acids was determined in BS-T or BS-G (0.2% glucose) medium (pH 3.5) containing 0.01% filter-sterilized acids. For *Acidiphilium* species, BS-A medium supplemented with 0.01% yeast extract and 0.1% glucose was used. Effect of ferrous iron on growth was determined with BS-G medium (0.1% glucose) and BS-T medium (0.1% trypticase soy) supplemented with ferrous sulfate.

Indole production was observed after 3, 5, and 10 days incubation in BS-P medium with 0.03% tryptophan in a test tube by the addition of Kovacs' reagent (2) and by Indole test paper strips (Nissui Seiyaku Co., Tokyo, Japan). Hydrogen sulfide production was investigated in BS-G medium (pH 3.5) with 1% cysteine hydrochloride by detecting the blackening of lead acetate paper strips after 5 days incubation.

Catalase was detected by using a 3% hydrogen peroxide solution that was added to a smear of washed-concentrated cells. Oxidase was detected by using cytochrome oxidase test paper (Nissui Seiyaku) (15). Urease activity was determined by a modification of NCTC micromethod (2). Washed cell suspension was prepared from 4 days BS-CG cultures. The cell suspension (1 ml) was mixed with 4% urea solution (0.5 ml) and 0.005 M McIlvaine buffer (pH 4.0) (0.5 ml) and

incubated at 35°C for 3 h, followed by measurement of pH of the mixture. Evidence of the activity was taken as a change in the pH from an initial value (pH 4.0) to at least 4.5. Starch hydrolysis was determined by growing strains on BS-P agar supplemented with 1% soluble starch, and by flooding the plates with iodine solution after 2 weeks incubation. Tween hydrolysis was observed after 2 weeks incubation on BS-P agar (pH 3.5 and 4.5) supplemented with filter-sterilized Tweens 40, 60, and 80 at a final concentration of 1%. Gelatin liquefaction was determined in BS-CG gelatin (12%) stab by incubating at 22 and 27°C for a month. Amino acid decarboxylase was detected by NCTC micromethod (2) using heavy cell suspension prepared from BS-CG agar slant cultures. Deoxyribonuclease (DNase) activity was determined by growing strains on BS-P agar (pH 3.5) supplemented with filter-sterilized 0.2% DNA (salmon sperm, Sigma Chemical Co., MO, U.S.A.), and by flooding the plates with 1 N HCl after 2 weeks incubation at 20, 27, and 35°C. Arsenite oxidation was determined in BS-T medium containing 50 mg/l As(III) in the form of NaAsO₂. Arsenic was determined by the iodometric method (32).

Susceptibility of various antibiotics was tested by the sensitivity disk method (Showa Yakuhin Kako Co., Tokyo, Japan) on BS-TG agar (pH 3.5) for the isolates and BS-A agar for *A. cryptum* and *A. organovorum*.

Pigment formation. The pigments were extracted from washed cell pellets with chloroform-methanol (1:1 v/v) and absorption spectra of the extracts were recorded. The pigments obtained were also inspected for fluorescence under UV-lamp.

Quinones and fatty acids. Both quinone and cellular fatty acid compositions were determined as described previously (31).

DNA base composition and DNA-DNA hybridization. Cells were harvested from cultures at the late exponential phase of growth. DNA was prepared according to the method of Marmur (21) and guanine-plus-cytosine (G+C) content of DNA (mol%) was determined by high-performance liquid chromatography as described by Kaneko et al. (12). DNA-DNA hybridization was carried out by the dot blot hybridization method with photobiotin labeling and colorimetric detection (4,22). The color intensity of the spots was measured with Dual-Wavelength Flying-Spot Scanner CS-9000 (Shimadzu Co., Kyoto, Japan) to calculate homology levels. DNA-DNA hybridization was also performed by the membrane filter method (3) with labeling of DNA with ³H-deoxycytidine triphosphate by nick translation (24), and the radioactivity of filters was measured with a Packard 300C Tri-Carb Liquid Scintillation Counter (Packard Instrument Co., Inc., Lockville, MD, U.S.A.), as described by Akagawa and Yamasato (1).

RESULTS

Morphological and cultural characteristics

The cells of six isolates (Table 1) were Gram negative, nonsporeforming,

straight rods with rounded ends, occurring singly. The cellular morphology appeared to be changed depending on culture conditions such as medium composition and pH. The cell sizes on BS-CG medium ranged from 0.5 to 0.9 μm by 1.5 to 3.8 μm , but were smaller, measuring 0.7 to 1.2 μm by 0.9 to 1.8 μm , under limited nutritional conditions (32). The cells occurred often in long chains composed of five or more cells when cultivated at a pH of 2 and below. The cells of strains AIU 301 and AIU 306 had a polar flagellum (Fig. 1a). The cells of strains AIU 302 and AIU 305 had fimbriae (Fig. 1b). The cells with both fimbriae and flagella were also observed (Fig. 1b). Poly- β -hydroxybutyrate granules were observed in all isolates, but metachromatic granules were not present.

On BS-CG agar medium the isolates formed slightly convex, entire, smooth, weakly glistening colonies with 2–3 mm diameter. The color of colonies was variable, being creamy white to pale pinkish. The growth of colonies and formation of the pigment were enhanced in complex media supplemented with glucose plus either peptone or casamino acids as compared with in a simple inorganic salts medium (32).

Physiological and nutritional characteristics

The isolates were obligately aerobic and acidophilic bacteria that grew at pH 1.9 to 5.6. No growth occurred at pH 1.7 or 6.0. Slight differences in pH optima for growth were observed among the test strains; pH 3.2 for AIU 303 and AIU 304, pH 3.5 for AIU 301, AIU 302, and AIU 305, and pH 3.8 for AIU 306. The strains grew at 17 and 42°C but not at 15 or 45°C. Best growth occurred at 27 to 35°C. All isolates were nonhalophilic but grew at moderate concentrations of sodium chloride up to 3%, with the exception of strain AIU 306, which was able to grow sparsely in 4% sodium chloride. Their growth was negative with potassium cyanide. The isolates were not heat-resistant, indicating nonsporeforming.

Growth of the isolates occurred in complex media containing peptone and

Fig. 1. Electron micrographs of cells of the isolates AIU 301 (a) and AIU 305 (b). Bar marker = 0.5 μm .

mineral media supplemented with a simple organic compound as the sole carbon source and yeast extract as the growth factor. All isolates assimilated the following compounds as the sole sources of carbon and energy in BS medium: D-ribose, D-xylose, D-arabinose, L-arabinose, D-glucose, D-galactose, D-fructose, L-sorbose, L-rhamnose, D-fucose, L-fucose, sucrose, raffinose, melezitose, glycerol, adonitol, sorbitol, *meso*-inositol, mannitol, galacturonic acid, glucuronic acid, mucic acid, saccharic acid, arbutin, inulin, α -ketogluconate, methanol, ethanol, *n*-propanol, citrate, gluconate, succinate, α -ketoglutarate, *cis*-aconitate, L-malate, fumarate, tyramine, and putrescine. The following compounds did not support growth: D-mannose, maltose, cellobiose, lactose, trehalose, melibiose, *meso*-erythritol, galactitol, esculin, salicin, dextran, soluble starch, glycogen, *n*-butanol, DL- β -hydroxybutyrate, salicylate (0.05%), *p*-hydroxybenzoate, *m*-hydroxybenzoate, betaine, pyruvate, lactate, tartarate, glycine, cysteine, oxalacetate, glycolic acid, oxalic acid, malonate, acetate, formate, propionate, butyrate, *n*-valeric acid, caproic acid, 2,3-butanediol, maleate, creatine, monomethylamine, acetamide, *o*-toluic acid, *p*-anisic acid, glyoxylic acid, and cadaverine.

The isolates used the following amino acids as the sole carbon and nitrogen sources in BS-N medium: alanine, leucine, proline, aspartic acid, asparagine, glutamic acid, glutamine, histidine, arginine, lysine, ornithine, citrulline. The following amino acids and a related compound did not support the growth: glycine, valine, isoleucine, serine, threonine, phenylalanine, tyrosine (ca. 0.04%), tryptophan, cysteine, methionine, hydroxyproline, and taurine.

Ammonium sulfate, nitrate, and urea were assimilated as nitrogen sources in glucose. Among these, ammonium salts were the best nitrogen source for growth.

The isolates utilized neither ferrous iron nor elemental sulfur as the sole source of energy. Sodium thiosulfate did not support chemolithotrophic growth and prevented completely heterotrophic growth on glucose. The isolates grew in the presence of high concentrations of ferrous iron (less than 10,000 mg/l). Furthermore, the addition of ferrous iron enhanced the cell growth at the early stages of the culture. The specific growth rate (μ) at the logarithmic growth phase with ferrous iron (100 mg/l) was about 0.14, which was 4 times larger than that for the control culture ($\mu=0.035$). Therefore, ferrous iron appears to be a growth-stimulating factor rather than an energy source.

Growth responses to organic compounds

Growth responses to different concentrations of organic compounds have been used as important criteria for identification and characterization of *Acidiphilium* species (6, 14, 18, 36). Therefore our isolates were studied in these respects.

The isolates assimilated methanol ($\leq 0.5\%$ v/v) (pH 3.5) as the sole source of carbon and energy, as described above, but did not use other single-carbon compounds as formate and monomethylamine. The bacterial cells (3×10^6 cells/ml) inoculated in 0.2% (v/v) methanol increased to 8×10^7 cells/ml after 13 days of incubation (Table 2). The specific growth rate (μ) was calculated to be about

Table 2. Growth of the isolate on methanol as the sole source of carbon and energy.^a

Medium	Cell number/ml of culture		μ (h ⁻¹) ^b
	Initial	Final	
Methanol	3×10^6	8×10^7 (13 days)	0.011
Yeast extract	6×10^6	2×10^7 (13 days)	0.004
Methanol + yeast extract	7×10^6	8×10^8 (13 days)	0.015
Glucose	5×10^6	2×10^9 (4 days)	0.062

^a AIU 301 was cultivated by shaking in 50 ml BS medium (pH 3.5) in a 500-ml culture flask.

The concentrations of methanol, yeast extract, and glucose were 0.2% v/v, 0.0001% w/v, and 0.2% w/v, respectively. Parentheses indicate cultivation periods.

^b Specific growth rate (μ) was calculated at logarithmic growth phase.

0.011, which was smaller than that (0.062) on BS-G (0.2% glucose). Growth on methanol was enhanced by the addition of a small amount of yeast extract. *A. cryptum* and *A. organovorum* did not grow on methanol, ethanol, formate, or monomethylamine.

The effects of various concentrations of glucose in BS medium on cell growth were almost similar in the isolates. As shown in Fig. 2, they grew well on glucose concentrations of 0.1–0.5%. However, above 1% glucose, the growth rate and final cell yield were reduced successively with its increasing concentrations. Cell growth was suppressive at glucose concentrations of more than 6% and completely inhibited at 10%.

Fig. 2. Effect of glucose concentrations on cell growth of the isolate AIU 301.

The isolate was grown at 30°C in BS medium (pH 3.5) with various concentrations of glucose.

A yeast extract concentration of 0.05–0.5% in BS medium was stimulatory to growth of the isolates; and with 0.6% yeast extract, the cell growth was inhibited. The addition of trypticase soy at the concentrations ranging from 0.05–5% enhanced growth, with the best growth occurring at concentrations of 1–4%. All isolates failed to grow at 6% trypticase soy. The growth of *A. organovorum* was inhibited by the presence of trypticase soy above 3%.

Luxuriant growth of the isolates occurred in the presence of 1% bacto-tryptone, vitamin-free casamino acids, trypticase peptone, polypeptone, and tryptose. No growth occurred in the presence of 1% bacto-peptone, yeast extract, and nutrient broth. At the 0.1% concentration of these compounds sparse growth occurred and the addition of 0.1% glucose together with these compounds stimulated extremely the growth. No growth was observed with 0.01–0.1% meat extract and better growth with the coexistence of meat extract and 0.1% glucose. However, meat extract, bacto-peptone, yeast extract, and nutrient broth at the concentration of 1% were strongly inhibitory to growth even with the supplement of 0.1% glucose.

Growth of the isolates was completely inhibited by the presence of 0.01% formate, acetate, propionate, butyrate, valeric acid, caproic acid, and pyruvate in both glucose and trypticase soy. These monocarboxylic acids (0.01%) inhibited completely also the growth of *A. cryptum* and *A. organovorum* in both glucose and yeast extract.

Biochemical characteristics

The isolates did not grow by anaerobic fermentation of glucose. Acid was produced oxidatively from glucose, D-xylose, D-fructose, L-sorbose, D-fucose, L-fucose, sucrose, melezitose, adonitol, mannitol, and arbutin. No acid was produced oxidatively from galacturonic acid, glucuronic acid, α -ketogluconate, and gluconate.

In nitrate-containing BS-N medium, aerobic growth of the isolates and nitrate reduction to nitrite was observed. No growth occurred and no bubbles were produced under anaerobic condition, indicating the absence of denitrification.

Hydrogen sulfide, but not indole, was produced in all isolates. The isolates were positive for catalase and urease and negative for oxidase and DNase. Tweens 40 and 60 but not Tween 80 were hydrolyzed. Neither gelatin nor starch was hydrolyzed. Decarboxylase activities of arginine, lysine, and ornithine were negative in all isolates.

The isolates could grow in the medium containing 50 mg/l As(III) and had arsenite-oxidizing activity, which was dependent on the presence of plasmids in the cells (data not shown). However, very sparse growth of *Acidiphilium* strains occurred in the presence of As(III) and no arsenite oxidation was detected.

Sensitivity to antibiotics

Growth of all isolates was inhibited by tetracycline, doxycycline, ampicillin,

Fig. 3. Absorption spectra of the crude pigments extracted with chloroform-methanol (1:1 v/v) from cells of the isolate (AIU 301) (A), *Acidiphilium cryptum* DSM 2389 (B), and *Acidiphilium organovorum* ATCC 43141 (C).

chloramphenicol, cefatrizine, cloxacillin, streptomycin, cephaloridine, penicillin, and 5-hydroxytetracycline. Their growth was not inhibited by lincomycin, tobramycin, josamycin, fradiomycin, erythromycin, kanamycin, and polymyxin B. Growth of *A. cryptum* and *A. organovorum* was inhibited by tetracycline, doxycycline, ampicillin, chloramphenicol, cefatrizine, cloxacillin and not by streptomycin, lincomycin, tobramycin, and josamycin. Penicillin was sensitive to *A. organovorum* but not to *A. cryptum*.

Pigment formation

The crude pigments extracted from the isolate AIU 301 and *A. cryptum* gave identical absorption spectra with four absorption peaks at 465, 495, 528, and 770 nm (Fig. 3). The absorption peaks at approximately 500 and 770 nm indicated carotenoid and bacteriochlorophyll (Bchl) *a*, respectively (16,33). The same absorption spectra were observed for the other five isolates, *A. angustum*, and *A. rubrum*, but not detected for *A. organovorum* and *A. facilis*.

Quinone and fatty acid compositions

All isolates contained ubiquinone Q-10 as their major quinones. Ubiquinones Q-9 and Q-8 occurred as minor components (Table 3). The cellular fatty acids of

Table 3. Ubiquinone system and cellular fatty acid composition of the strains of isolates and *Acidiphilium* species.

	Isolates						<i>A. cryptum</i>	<i>A. organovorum</i>
	AIU301	AIU302	AIU303	AIU304	AIU305	AIU306	DSM 2389	ATCC 43141
Ubiquinone composition (%) ^a								
Q-8	1	1	2	4	3	1	2	2
Q-9	6	6	7	7	10	6	6	7
Q-10	93	93	91	89	87	93	92	91
Fatty acid composition (%) ^b								
Non-hydroxylated acid								
C _{10:0}	tr	tr	tr	tr	tr	tr	tr	tr
C _{12:0}	4	4	4	5	4	5	6	3
C _{14:0}	tr	tr	tr	tr	tr	1	tr	tr
C _{16:0}	1	1	1	1	1	1	1	1
C _{18:0}	4	3	3	3	2	4	6	3
C _{14:1}	tr	tr	tr	tr	tr	tr	tr	tr
C _{16:1}	tr	tr	tr	tr	tr	tr	tr	tr
C _{18:1}	83	84	85	81	89	82	71	78
ΔC _{19:0}	5	6	6	8	3	6	13	12
Hydroxy acid								
3-OH C _{14:0}	1	1	1	1	1	1	2	1
3-OH C _{16:0}	tr	tr	tr	tr	tr	tr	tr	tr

^a The abbreviations used for ubiquinones indicate the number of isoprene units in a side chain.

^b In fatty acid designations, the first number indicates the number of carbon atoms and the second the number of double bonds; Δ indicates cyclopropane fatty acid. tr indicates trace amount (less than 1%).

the isolates consisted of a large amount of straight-chain unsaturated C_{18:1} acid and small amounts of straight-chain saturated C_{12:0} and C_{18:0} acids and cyclopropane C_{19:0} acid. Hydroxy fatty acids with 3-OH C_{14:0} was also predominantly present (Table 3). Similar profiles were found in *A. cryptum* and *A. organovorum*.

DNA base composition and DNA-DNA homology

The base composition of DNAs from the isolates was 66.2–68.1 mol% G + C (Table 4). The isolates showed very high levels of similarity to each other, indicating their belonging to the same group (Table 4). The mean homology values of the isolates with *A. cryptum* and *A. organovorum* ranged from 48.6±3.8% and 49.0±3.7%, respectively. *A. facilis*, *A. rubrum*, and *A. angustum* showed very low homology with the isolates and two other *Acidiphilium* species.

DISCUSSION

Harrison (6) first isolated and assigned acidophilic heterotrophic bacteria from *T. ferrooxidans* cultures as a new genus and species *Acidiphilium cryptum* (6). After then, *A. organovorum* (18), *A. facilis*, *A. angustum*, and *A. rubrum* (36) have been recovered from similar acidic mineral environments. Our isolates and these

Table 4. DNA base composition of and DNA homology among the strains of isolates and *Acidiphilium* species.

Strain/Species	G + C content (mol%)	% Homology with biotin-labeled DNA from							
		AIU 301	AIU 303	<i>A. cryptum</i> DSM 2389	<i>A. organovorum</i> ATCC 43141	<i>A. rubrum</i> ATCC 35905	<i>A. angustum</i> ATCC 35903	<i>A. facilis</i> ATCC 35904	
AIU 301	67.6	100 (100)	91	53 (63)	50	22	23 (10)	12	
AIU 302	66.2	87	83	44	44	24	21	9	
AIU 303	67.4	94	100	50	47	21	23	10	
AIU 304	66.8	85	96	47	49	19	23	12	
AIU 305	67.0	96	95	49	47	16	25	10	
AIU 306	68.1	98	83	43	46	18	29	15	
<i>A. cryptum</i> DSM 2389	67.3	55 (61)	48	100 (100)	64	26	30 (11)	9	
<i>A. organovorum</i> ATCC 43141	67.4	53	56	65	100	15	26	17	
<i>A. rubrum</i> ATCC 35905	63.2	9 (16)	24	11 (14)	10	100	92 (71)	14	
<i>A. angustum</i> ATCC 35903	63.4	8 (14)	23	11 (14)	9	92	100 (100)	14	
<i>A. facilis</i> ATCC 35904	64.2	10 (13)	13	10 (9)	11	9	11 (12)	100	

Nonlabeled DNAs were spotted onto nitrocellulose membrane filters to give a concentration of 50 ng/dot, baked at 80°C for 2 h, incubated at 42°C for 2 h in a prehybridization solution and then 16 h in a hybridization solution containing labeled DNA (0.2 µg/10 ml), 5 × SSC, and 50% formamide, followed by detection with the BlueGENE Nonradioactive Nucleic Acid Detection System. Figures in parentheses indicate % homology with ³H-labeled DNA from three strains.

Acidiphilium species have many common characteristics. They are Gram negative, aerobic, mesophilic, acidophilic, catalase positive, nonsporeforming, and rod-shaped bacteria. They are incapable of chemolithotrophic growth by using ferrous iron and reduced sulfur compounds as the energy sources. They utilize citrate and aspartic acid but not lactate, pyruvate, formate, propionate, butanol, and soluble starch. Growth of these bacteria, except *A. facilis* (36), is completely inhibited by acetate. The ubiquinone systems are Q-10. The G+C contents of DNAs of these bacteria are in the range of 63.2 to 68.1 mol%. Straight-chain C_{18:1} fatty acid exists as major cellular fatty acid component and 3-OH C_{14:0} acid as hydroxy fatty acid. Considering these features described above, it is apparent that the isolates belong to a species of the genus *Acidiphilium*.

The isolates and *Acidiphilium* species (6, 9, 11, 18, 36) are definitely distinguished from *Acidobacterium capsulatum* in cellular fatty acid composition, quinone system, G+C content of DNA, and genetic relatedness (13).

Acidomonas methanolica (28–30) is similar to our isolates and *Acidiphilium* species in ubiquinone type and some other respects. However, the former can be differentiated from the latter in oxidase positive, hydroxy fatty acids with 3-OH C_{16:0} and 2-OH C_{16:0} acids, and low G+C content of DNA and DNA homology (13, 30), and further in peculiar utilization pattern of the sole carbon and energy sources and requirement of yeast extract or pantothenic acid as a growth factor.

Among the isolates and *Acidiphilium* species, *A. angustum*, *A. facilis*, and *A. rubrum* are distinguished from the isolates and two other species by phenotypic characteristics and genetic relatedness (Table 4) (36). DNA-DNA hybridization studies show that the isolates exhibit relatively low level ($49 \pm 4\%$) of similarity in DNA homology with *A. cryptum* and *A. organovorum* (Table 4). Therefore, the isolates are genetically differentiated from the known two *Acidiphilium* species (34). It is noteworthy to note that, although *A. angustum* and *A. rubrum* are the separate species validly described, high levels of genomic DNA hybridization were obtained between the two (Table 4). These results may be supported by the finding in our preliminary experiments that the two species have a 99.9% sequence similarity of the 16S rRNA gene (data not shown).

The phenotypic differentiating features between the isolates and both *A. cryptum* and *A. organovorum* are the utilization of alcoholic compounds such as methanol, ethanol, and propanol, sensitivity to streptomycin, and arsenite oxidation (Table 5). The ability of the isolates to assimilate methanol as the sole source of carbon and energy suggests that the isolates have facultatively methylotrophic properties.

The isolates, *A. cryptum*, *A. angustum*, and *A. rubrum* form photosynthetic pigments carotenoid and Bchl *a*, which has been ascertained to be Bchl *a*_p containing phytol as esterifying alcohol (33). These Bchl-containing acidophilic bacteria are aerobic phototrophic bacteria but do not grow anaerobically in the light (25, 33). The isolates are similar to *A. cryptum* but different from *A. organovorum* in respect of the formation of Bchl *a*.

Table 5. Differentiating characteristics for the strains of the isolates, *Acidiphilium cryptum*, and *Acidiphilium organovorum*.

	Isolates ^a	<i>A. cryptum</i> DSM 2389	<i>A. organovorum</i> ATCC 43141
Utilization of sole carbon and energy sources			
D-Mannose	—	+	—
Methanol	+	—	—
Ethanol	+	—	—
Propanol	+	—	—
Acetate	—	—	—
Succinate	+	—	+
Fumarate	+	—	+
α -Ketoglutarate	+	+	—
Gluconate	+	—	+
Glucose conc. inhibited growth (%)	10	0.2	4
TS conc. inhibited growth (%) ^b	6	0.1	3
YE conc. inhibited growth (%) ^b	0.6	0.15	0.5
Hydrolysis of urea	+	—	+
Pigmentation			
Carotenoid	+	+	—
Bchl <i>a</i>	+	+	—
Sensitivity to			
Streptomycin	+	—	—
Penicillin	+	—	+
Arsenite oxidation	+	—	—
G + C content (mol%)	66.2–68.1	67.3	67.4

^a Strains AIU 301, 302, 303, 304, 305, and 306 were included.

^b TS, trypticase soy; YE, yeast extract; +, positive; —, negative.

On the basis of the phenotypic, chemotaxonomic, and genotypic characteristics, the isolates can best be allocated to the genus *Acidiphilium* but should be placed in a new species of the genus. We propose the new species described below.

Acidiphilium multivorum sp. nov. *Acidiphilium multivorum* (mul. ti. vo'rum. L. adj. *multus* many; L. trans. v. *varare* to swallow; M. L. adj. *multivorum* devouring many kinds of substances). Cells are nonsporeforming, polarly flagellated, rod-shaped (0.5–0.9 by 1.5–3.8 μ m), and Gram negative. Poly- β -hydroxybutyrate is formed. Colonies are circular with entire margins, slightly convex, smooth, opaque, and creamy white or pale pinkish. Carotenoid pigments and Bchl *a* are formed under aerobic conditions. They grow at pH 1.9–5.6. Good growth pH, 3.2–4.0. They grow at 17–42°C. Good growth temperature, 27 to 35°C. Growth occurs with 3% sodium chloride but not with potassium cyanide. Aerobic chemoorganotroph having a strictly respiratory type of metabolism with oxygen as terminal electron acceptor. Nitrate is reduced to nitrite. No denitrification. Ammonium, nitrate, and urea are utilized as nitrogen sources. No autotrophic growth occurs on ferrous iron, elemental sulfur, and thiosulfate. Utilizes as the sole carbon and energy source a wide variety of organic compounds. Good growth

occurs with high concentrations of glucose, trypticase soy, and yeast extract. Methanol, ethanol, and propanol are utilized as the sole carbon and energy source. Acid is formed from D-glucose, D-xylose, D-fructose, L-sorbose, D-fucose, sucrose, melezitose, adonitol, and mannitol. Growth is inhibited by many kinds of monocarboxylic acids. Catalase and urease positive. Oxidase, DNase, and amino acid decarboxylases negative. Hydrogen sulfide is formed. Indole is not formed. Gelatin and starch are not hydrolyzed. Tweens 40 and 60 are hydrolyzed. Arsenite oxidation positive. The major ubiquinone is Q-10. The major cellular fatty acid is straight-chain C_{18:1} acid and hydroxy acid is 3-OH C_{14:0} acid. The DNA base composition is 66.2 to 68.1 mol% G + C.

Sources: Pyritic acid mine drainage.

Type strain: Strain AIU 301, which has been deposited with the Japan Collection of Microorganisms as *Acidiphilium multivorum* JCM 8867. Strains examined: AIU 301 (JCM 8867), AIU 302 (JCM 8868), AIU 303 (JCM 8869), AIU 304 (JCM 8870), AIU 305 (JCM 8871), and AIU 306 (JCM 8872).

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