J. Gen. Appl. Microbiol., **33**, 11–25 (1987)

ACIDOPHILIC HETEROTROPHIC BACTERIA ISOLATED FROM ACIDIC MINE DRAINAGE, SEWAGE, AND SOILS

NORIAKI KISHIMOTO AND TATSUO TANO*

Mimasaka Women's Junior College, Tsuyama, Okayama 708, Japan
**Department of Agricultural Chemistry, Faculty of Agriculture, Okayama University, Okayama 700, Japan*

(Received October 2, 1986)

Obligately acidophilic heterotrophic bacteria were isolated from weakly acidic environments such as sewage and soil as well as from acidic mine drainage. In the mine water, the bacterial populations ranged from 0 to 10^4 cells per ml of water or g of mud, moreover a large number of bacteria (10^4–10^6 cells per ml) were isolated from an aeration tank in which ferrous iron in the mine water was being oxidized to ferric iron by iron oxidizing bacteria. Although the population was small, 9 strains of bacteria of this type were found in 106 samples from the sewage and the soils. The bacterial characteristics of the strains isolated from acidic mine drainage were in good agreement with the genus *Acidiphilium*. However, the characteristics of the bacteria from weakly acidic environments differed from *Acidiphilium* in the pH growth range and sensitivity to organic substrates. The isolated bacteria were classified into 3 groups based on DNA base composition, cellular fatty acid composition, pH growth range and carbon sources for growth.

Acidophilic heterotrophic bacteria have been isolated from acidic mineral environments: coal mine drainage (*1*), coal refuse (*2, 3*), acidic swamp (*4*), acidic mine drainage (*5*), artificial coal spoil (*6*), and *Thiobacillus ferrooxidans* culture (*4, 7–10*).

WICHLACZ and UNZ (*5*) selected 37 strains among acidophilic heterotrophic bacteria isolated from acidic mine drainage, and classified them into 3 groups based on the ability to utilize organic acids. HARRISON (*9, 11*) characterized acidophilic heterotrophic bacteria from *T. ferrooxidans* cultures and acidic coal refuse, and proposed a new generic name, *Acidiphilium* gen. nov. (type species: *Acidiphilium cryptum*) for them. WICHLACZ et al. (*12*), recently, proposed *Acidiphilium rubrum*,

Address reprint requests to: Mr. N. Kishimoto, Mimasaka Women's Junior College, Kamigawara 32, Tsuyama, Okayama 708, Japan.

Acidiphilium angustum, and *Acidiphilium facilis* for each representative strain in 3 groups, and LOBOS et al. (*10*), *Acidiphilium organovorum* for the bacteria isolated from a culture of *T. ferrooxidans*.

All of the acidophilic heterotrophic bacteria reported to date were isolated from acidic (about pH 2.4–5.0) mineral environments. As far as we know there are no reports about their being isolated from neutral or weakly acidic (about pH 5.6–6.6) environments.

We isolated acidophilic heterotrophic bacteria from weakly acidic environments such as sewage, the soils of fields, and acidic mineral environments. In this paper we describe the isolation and the characteristics of the acidophilic heterotrophic bacteria isolated from strongly or weakly acidic environments.

MATERIALS AND METHODS

Collection of samples. Samples were collected from the Yanahara mine (Dowa Kogyo Co., Okayama, Japan) and from sewage, fields, and rivers in Tsuyama city (Okayama, Japan) from 1979 to 1985. Samples were collected in sterilized screw-cap test tubes, brought back to the laboratory and there plated on glucose-yeast extract plates within 8 hr after they were collected.

Media. Glucose-yeast extract medium (GYE medium) was used to isolate the bacteria, and the trypticase soy broth-glucose medium (TS-glucose medium) of HARRISON (*9*) was used to study the characteristics of the isolated bacteria. The GYE medium was composed of: (g/*l* of distilled water): $(NH_4)_2SO_4$, 2.0; KCl, 0.1; K_2HPO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; glucose, 1.0; yeast extract (Difco Laboratories), 0.1. The composition of the TS-glucose medium was: (g/*l* of distilled water): $(NH_4)_2SO_4$, 2.0; KCl, 0.1; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; glucose, 1.0; trypticase soy broth (BBL Microbiology Systems), 0.1. The pH of the medium was adjusted to 3.0 with H_2SO_4, except that the liquid medium for strain 29-R was adjusted to pH 5.0. The media were autoclaved at 121°C for 15 min. For the preparation of solid media, an agar suspension (30 g/*l*) containing double-strength glucose and yeast extract (or trypticase soy broth) was autoclaved separately to avoid hydrolysis of the agar. After the suspension was mixed aseptically with an equal volume of the other acidified double-strength ingredients, it was apportioned in standard plastic petri dishes. The agar was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan).

Isolation of acidophilic heterotrophic bacteria. The collected samples (0.1 ml) were spread on the GYE agar plates with a glass rod. The plates were incubated at 30°C for 4 to 10 days, and the colonies showing different morphologies were transferred on GYE agar slants. The bacteria grown on the slant were inoculated in GYE medium adjusted to pH 3.0 or 7.0, and the bacteria which could grow at pH 3.0 but not at pH 7.0 were selected as the acidophilic heterotrophic bacteria. Pure cultures were obtained by means of successive plating on GYE agar plate.

Characterization of the isolated strains. *Acidiphilium cryptum* strain Lhet2

(ATCC 33463) served as the characterization control. The characteristics of the isolated strains were determined using cells grown in TS-glucose medium at 30°C for 2 to 3 days. Gram staining was carried out by the modified HUCKER's method (*13*). Motility at a late exponential phase was observed with a microscope. Cell shape, size, and flagellar morphology were determined by electron microscopy with a preparation negatively stained with uranium acetate. Catalase activity was detected in terms of the production of bubbles in a 3% hydrogen peroxide solution. The oxidase reaction was tested with a cytochrome oxidase test paper (Eiken Chemical Co., Tokyo). Growth temperature and generation time were determined using an L-type culture tube with shaking in a water incubator. Assimilation of carbon compounds was tested using liquid TS-glucose medium supplemented with various carbon compounds (0.1%) instead of glucose, at 30°C for 3 days. Utilization of ferrous iron or sulfur as an energy source was determined after 7 days of growth in liquid TS-glucose medium supplemented with ferrous sulfate (4.4%) or powdered sulfur (2%) instead of glucose and trypticase soy broth. Production of hydrogen sulfide was tested using a liquid TS-glucose medium containing L-cysteine (0.1 g/*l*). Hydrogen sulfide was detected with lead acetate test paper. Urease effects on the pH of cultures were observed in TS-glucose medium containing urea (0.1 g/*l*). The ability to grow on complex organic medium was determined by the development of colonies on plates of nutrient broth (Oxoid Limited), heart infusion broth (Eiken Chemical Co.), casamino acids (Nissui Seiyaku Co.), and bactopeptone (Difco Laboratories) medium containing 1.5% agar. Susceptibility to antibiotics was detected with the sensitivity disk method (Showa Yakuhin Kako Co., Tokyo) applied on a TS-glucose plate.

Quinone system. Cells grown at a late exponential phase were collected by centrifugation, washed with distilled water and lyophilized. Quinones were extracted and purified by the method of COLLINS and LANGWORTHY (*14*). Purified quinones were analyzed by high-performance reverse-phase thin layer chromatography using Merck HPTLC RP-18F_{254} plates (10 × 10 cm) with acetone/water (90 : 10 v/v) as the developing solvent. Spots on the thin layer were detected by irradiating ultraviolet light at 254 nm. The isoprene units of the purified ubiquinones were identified by comparing with the rate of flow of authentic CoQ_9 and CoQ_{10} purchased from Merck Co.

DNA base composition. Cells were cultivated in TS-glucose medium at 30°C and harvested at the late-exponential phase. DNA was extracted and purified by the phenol method (*15*). After DNA was hydrolyzed into nucleosides with enzyme, DNA base composition was determined by reversed-phase high-performance liquid chromatography (*16*).

Cellular fatty acid composition. Cells were cultivated in TS-glucose medium at 30°C and collected by centrifugation at a late-exponential phase. The harvested cells were washed with distilled water, lyophilized, then methylated with 5% HCl-methanol at 100°C for 1 hr. After methanolysis, the reaction mixture was extracted with *n*-hexane. The hexane fraction was concentrated under nitrogen current, and

the fatty acids were analyzed with a Shimadzu Model 4CM-PF gas chromatograph equipped with a hydrogen-flame ionization detector. Samples were analyzed on a glass column (3 mm × 2 m) packed with 10% diethylene glycol succinate coated with Chromosorb W. The temperature at the injector and detector was 205°C and the column temperature was 170°C. Nitrogen was used as a carrier gas at a flow rate of 30 ml/min.

Hydroxy fatty acids were analyzed by gas chromatography after separation of 2-hydroxy and 3-hydroxy fatty acids from non-polar fatty acids using thin layer chromatography. Bromination and hydrogenation were used for the identification of unsaturated fatty acids (*17*). Fatty acids were primarily identified by comparing the relative retention time of their methyl esters with standard fatty acids. 19-Cyclopropane acids contained in the samples were identified by comparison with the relative retention time of cellular fatty acids extracted from *Pseudomonas aeruginosa* (IAM 1514). *P. aeruginosa* was cultivated in nutrient broth (Oxoid Limited No. 2) for 40 hr at 30°C. The cellular fatty acids of *P. aeruginosa* were prepared according to the above method and identified by comparison with the relative retention time reported by IKEMOTO et al. (*18*).

RESULTS

Isolation of bacteria

Acidophilic heterotrophic bacteria were isolated from a wide variety of sources including bubbles and water of the mine drainage being treated in an aeration tank, acidic mine water, mine soils, river water, sewage, and field soils (Table 1). All of the 121 strains isolated from very acidic sources were found to be acidophilic. On the other hand, among those bacteria isolated from weakly acidic environments (pH 5.6–6.6) such as river water, sewage, and field soils, only 37 strains from 106

Table 1. Habitats, pH, and bacterial populations.

Habitat	Number of samples	pH of samples	Bacterial colonies per ml or g	Acidophilic heterotrophic bacterial strains
Yanahara mine				
aeration tank treating acid mine drainage	6	2.3–2.4	10^4–10^6	81
mine water	11	2.5–5.0	0–10^4	21
soils in the mine	8	2.6–5.3	0–10^3	19
Tsuyama city				
Yoshii river	3	5.8–6.2	0–2	0
Kamo river	7	5.6–6.2	0	0
Miya river	7	5.6–6.6	0–4	0
sewage	78	5.8–6.4	0–7	8
soils in the fields	11	5.6–6.4	0–16	1

Table 2. Acidophilic heterotrophic bacterial strains studied.

Strain	Isolated from habitat	pH of sample
63	mine water in Yanahara mine	3.1
A	aeration tank treating acid mine drainage in Yanahara mine	2.4
16-R	sewage in Tsuyama city	6.3
24-R	sewage in Tsuyama city	6.4
22-M	sewage in Tsuyama city	6.2
29-R	soil of field in Tsuyama city	5.6
Acidiphilium cryptum Lhet2	ATCC 33463	

samples were able to grow optimally under acidic conditions. Moreover 9 of 37 strains were acidophilic and the other strains were thought to be acid-tolerant.

Six representative strains were selected from the collection of isolates, and tentatively named strains 63, A, 16-R, 24-R, 22-M, and 29-R (Table 2). Strains 63 and A from Yanahara mine inhabited acidic environments, but strains 16-R, 24-R, 22-M, and 29-R from sewage and soils inhabited weakly acidic environments (Table 2).

Phenotypic characteristics

The phenotypic characteristics of the six strains isolated are shown in Tables 3 through 6.

All six strains were gram-negative, non-sporeforming, aerobic, rod-shaped bacteria. *A. cryptum* and strain 29-R were smaller than the other bacteria. The shape of strain 24-R depended on the pH of the medium. They were straight rods at pH 5.0, but filamentous at pH 3.0. *A. cryptum* and strain 22-M were motile with lateral flagella. Colonies of the bacteria were circular, entire, smooth, opaque, and white or cream color except that strain 29-R was irregular, undulate, rugose, opaque, and white. Colonies of *A. cryptum* and strain A were smaller than the other strains.

All six strains were weakly catalase-positive and oxidase-negative. Growth was depressed in the presence of 3% sodium chloride and stopped with 7% sodium chloride. Cells were killed within 2 min at 67°C. All six strains grew well between 28°C and 35°C, but no growth occurred at 47°C. The generation time at 25°C was between 3.9 hr and 8.2 hr. In TS-glucose medium, strains 16-R, 24-R, and 22-M grew faster than the other strains.

All six strains produced hydrogen sulfide from TS-glucose medium containing L-cysteine, but the gas production of *A. cryptum* and strain 29-R were weakly positive. None of the strains hydrolyzed urea. Acid was produced oxidatively from glucose. Tetracycline inhibited the growth of all six strains, and all but 29-R were inhibited with chloramphenicol. Strains 16-R, 24-R, and 22-M were also inhibited

Table 3. Morphological characteristics of the acidophilic heterotrophic bacterial strains.

	63	A	*A. cryptum*	16-R	24-R	22-M	29-R
I. Microscopic morphology on TS-glucose medium at pH 3.0 (cells at late exponential phase)							
shape	rod	rod	rod	rod	rod	rod	rod
size (μm)	0.9–1.0 × 1.1–1.6	0.8–0.9 × 1.8–2.1	0.4–0.6 × 0.7–0.9	0.9–1.0 × 1.2–1.6	0.7–0.8 × 3.3–14.7	0.8–0.9 × 1.7–2.0	0.3–0.4 × 0.7–0.8
motility	–	–	+	–	–	+	–
gram stain	–	–	–	–	–	–	–
formation of endospore	–	–	–	–	–	–	–
II. Colonial morphology on TS-glucose agar medium at pH 3.0 (7 days at 30 C)							
shape	circular	circular	circular	circular	circular	circular	irregular
edge	entire	entire	entire	entire	entire	entire	undulate
surface	smooth	smooth	smooth	smooth	smooth	smooth	rugose
density	opaque	opaque	opaque	opaque	opaque	opaque	opaque
color	white-cream	white-cream	white-cream	white-cream	white-cream	white-cream	white
size (ϕ, mm)	2.0–3.0	0.4–0.5	0.5–0.6	2.0–3.0	2.0–3.0	2.0–3.0	4.0–6.0

Table 4. Physiological characteristics of the acidophilic heterotrophic bacterial strains.

	63	A	*A. cryptum*	16-R	24-R	22-M	29-R
Requirement for oxygen	aerobic	aerobic	aerobic	aerobic	aerobic	aerobic	aerobic
Heat-tolerance at 67°C, 2 min	−	−	−	−	−	−	−
Salts-tolerance							
3% NaCl	±	±	±	±	±	±	±
7% NaCl	−	−	−	−	−	−	−
Catalase activity	+[w]	+[w]	+[w]	+[w]	+[w]	+[w]	+[w]
Oxidase reaction	−	−	−	−	−	−	−
pH range for growth	2.0–5.8	2.0–5.8	1.9–5.8	2.6–6.2	2.6–6.0	2.6–6.0	2.6–6.4
Optimum pH for growth	2.5–5.5	3.0–5.0	2.5–5.0	3.0–5.8	3.0–5.5	3.0–5.5	4.0–6.2
Temperature for growth	20–42	20–42	20–42	20–37	20–39	20–39	20–37
Optimum temp. for growth	28–39	28–39	35–41	28–35	28–37	28–37	28–35
Generation time at 25°C (hr)	5.1	8.2	6.0	3.9	4.0	4.9	6.7
H_2S production from cysteine	+	+	+[w]	+	+	+	+[w]
Ability to hydrolyze urea	−	−	−	−	−	−	−
OF-test	oxidation	oxidation	oxidation	oxidation	oxidation	oxidation	oxidation
Acid production from glucose	+	+	+	+	+	+	+
Sensitivity for antibiotic							
Tetracycline	+	+	+	+	+	+	+
Chloramphenicol	+	+	+	+	+	+	−
Ampicillin	−	−	−	+	+	+	−
Sulbenicillin	−	−	−	−	−	−	+
Penicillin	−	−	−	−	−	−	−
Streptomycin	−	−	−	−	−	−	−
Fladiomycin	−	−	−	−	−	−	−
Cell growth inhibited by 0.01% acetate	+	+	+	+	+	+	−
TS conc. inhibited cell growth	0.4%	0.4%	0.1%	1.0%	1.0%	1.0%	>4.0%
YE conc. inhibited cell growth	0.5%	0.3%	0.15%	2.0%	1.5%	1.5%	>4.0%
Utility of Fe^{2+} or S^0 as a energy source	−	−	−	−	−	−	−

[w]: weakly.

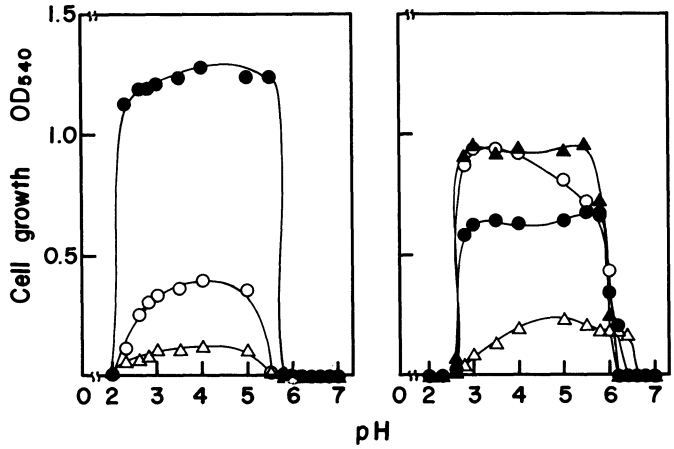

Fig. 1–1 (left), Growth of strains 63, A, and *A. cryptum* at various pHs.

An aliquot of actively growing culture was diluted with the medium to 1×10^8 cells/ml. The resulting cell suspension was inoculated into 100 ml of TS-glucose medium, which was adjusted to the various pHs indicated in the figure, in a 500-ml shaking flask and incubated with shaking at 30°C for 3 days. ○, 63; ●, A; △, *A. cryptum*.

2 (right), Growth of strains 16-R, 24-R, 22-M, and 29-R at various pHs.

The cultural conditions were the same as in Fig. 1–1. ○, 16-R; ●, 24-R; ▲, 22-M; △, 29-R.

with ampicillin, and strain 29-R with sulbenicillin. None was inhibited by streptomycin, fladiomycin, or penicillin. Acetate (0.01%) strongly inhibited the growth of five strains but not 29-R. None of the strains could utilized sulfur or ferrous iron as a source of energy.

Strains 63, A, and *A. cryptum* grew between pH 2.0 and 5.8, strains 24-R and 22-M grew between pH 2.6 and 6.0, strain 16-R between pH 2.6 and 6.2, and strain 29-R between pH 2.6 and 6.4 (Fig. 1–1, 2). In general, inhabitants of acidic environments were able to grow under more acidic conditions than those from weakly acidic environments.

Organic substances such as yeast extract and trypticase soy broth stimulated the growth of these acidophilic bacteria at a low concentration, but they inhibited the growth at high concentrations. The growth of strains 63, A, and *A. cryptum* were inhibited in the presence of lower concentration of yeast extract compared with strains 16-R, 24-R, and 22-M. However, the growth of strain 29-R was not inhibited by the addition of 4.0% yeast extract or trypticase soy broth (Fig. 2–1, 2).

The bacteria from mine water could not grow on peptone and tissue-extract agar plates at the concentrations commonly employed in dehydrated media. All strains from sewage formed colonies on 1% bactopeptone agar plates adjusted to pH 3.0, but they did not grow on the other tissue-extract plate. Strain 29-R could grow on tissue-extract plates adjusted to pH 3.0 (Table 5).

All six strains assimilated glucose, fructose, sucrose, and glycerol, but not

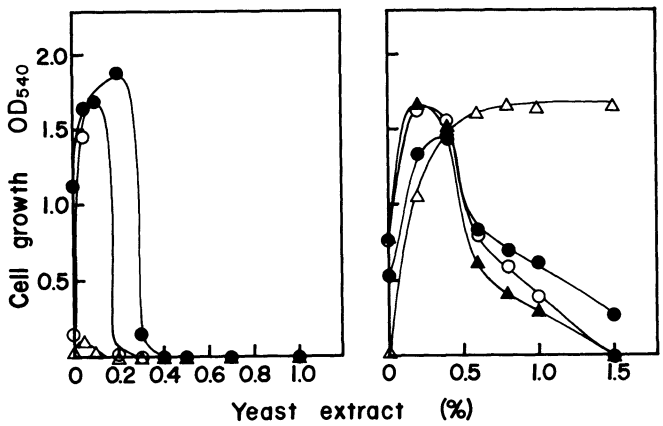

Fig. 2–1 (left), Effect of concentration of yeast extract on the growth of strains 63, A, and *A. cryptum*.

An aliquot of actively growing culture was diluted with the medium to 1×10^8 cells/ml. The resulting cell suspension was inoculated into 100 ml of GYE medium, which was adjusted to the various concentration of yeast extract in the figure, in a 500-ml shaking flask, and incubated with shaking at 30°C for 3 days. ○, 63; ●, A; △, *A. cryptum*.

2 (right), Effect of concentration of yeast extract on the growth of strains 16-R, 24-R, 22-M, and 29-R.

Strain 29-R was cultured at pH 5.0 and the other strains were cultured at pH 3.0. The other cultural conditions were the same as in Fig. 2–1. ○, 16-R; ●, 24-R; ▲, 22-M; △, 29-R.

methanol, ethanol, acetate, lactate, pyruvate, glyconate, oxalacetate, lactose, glycine, and L-cysteine. All of the strains except 29-R utilized ribose, arabinose, glutamate, citrate, and α-ketoglutarate as a carbon and energy source. In addition, strains 16-R, 24-R, and 22-M could assimilate fumarate and succinate. Strain 29-R assimilated maltose and raffinose (Table 6).

DNA base composition

The DNA base compositions of the six strains were in the range of 63 to 68 mol% G+C (Table 7). Strains 63, A, and *A. cryptum* from acidic mineral environments contained 67.6 to 68.0 mol% G+C, whereas strains 16-R, 24-R, and 22-M from sewage contained 63.0 to 65.2 mol% G+C.

Quinone system

TLC analysis of the lipid extracts of the six strains and *A. cryptum* revealed ubiquinones as the quinones. All the six strains and *A. cryptum* contained Q_{10} as their major respiratory quinones (Table 7). Q_9 was a minor component in all the strains.

Cellular fatty acid composition

The six strains and *A. cryptum* were divided into three groups on the basis of

Table 5. Cultural characteristics of the acidophilic heterotrophic bacterial strains.

		63	A	*A. cryptum*	16-R	24-R	22-M	29-R
1.0% casamino acids plate	pH 3.0	+	+	+	+	+	+	−
	pH 7.0	−	−	−	−	−	−	−
1.0% bactopetone plate	pH 3.0	−	−	−	+	+	+	+
	pH 7.0	−	−	−	−	−	−	−
2.5% nutrient broth plate	pH 3.0	−	−	−	−	−	−	+
	pH 7.0	−	−	−	−	−	−	−
2.5% heart infusion broth plate	pH 3.0	−	−	−	−	−	−	+
	pH 7.0	−	−	−	−	−	−	−
TS-glucose plate	pH 3.0	+	+	+	+	+	+	+
	pH 7.0	−	−	−	−	−	−	−

The plates were incubated at 30°C for 7 days. +; Colony was formed. −; Colony was not formed.

Table 6. Growth of the acidophilic heterotrophic bacteria on various carbon sources.

C-source	63	A	*A. cryptum*	16-R	24-R	22-M	29-R
Glucose	+++	+	+	+++	+++	+++	+
Fructose	++	+	+	++	++	++	+
Sucrose	+	+	+	+	+	+	+
Ribose	++	++	+	+	+	+	−
Arabinose	+++	++	+	+	++	++	−
Lactose	−	−	−	−	−	−	−
Maltose	−	−	−	−	−	−	+
Raffinose	−	−	−	−	−	−	+
Methanol	−	−	−	−	−	−	−
Ethanol	−	−	−	−	−	−	−
Glycerol	+++	+	+	+++	+++	+++	+
Mannitol	+++	+	+	+	+++	++	+
Glycine	−	−	−	−	−	−	−
L-cysteine	−	−	−	−	−	−	−
L-glutamate	++	+	+	++	++	++	−
Citrate	++	+	+	+	++	++	−
Malate	++	+	+	+	++	++	−
α-ketoglutarate	++	+	+	+	++	++	−
Tartarate	−	−	−	−	−	−	−
Fumarate	−	−	−	+	++	++	−
Succinate	−	−	−	++	++	++	−
Acetate	−	−	−	−	−	−	−
Lactate	−	−	−	−	−	−	−
Pyruvate	−	−	−	−	−	−	−
Glycolate	−	−	−	−	−	−	−
Oxalacetate	−	−	−	−	−	−	−

Growth of the bacteria was measured by the turbidity at 540 nm. Symbols: +++, very good growth ($\geqq 1.0$); ++, good growth (0.5–1.0); +, slight growth (0.05–0.5); −, no growth ($\leqq 0.05$).

Table 7. Biochemical characteristics of the acidophilic heterotrophic bacterial strains.

	63	A	*A. cryptum*	16-R	24-R	22-M	29-R
DNA base composition (G+C mol%)	6.80	67.8	67.6	63.0	65.2	64.9	67.3
Quinone system major component	Q_{10}	Q_{10}	Q_{10}	Q_{10}	Q_{10}	Q_{10}	Q_{10}
minor component	Q_9	Q_9	Q_9	Q_9	Q_9	Q_9	Q_9
Fatty acid							
Straight-chain acids							
12:0	2.8	3.5	3.7	—	—	—	—
14:0	tr	tr	tr	tr	—	tr	tr
16:0	1.2	3.4	4.2	9.8	8.4	8.3	13.0
18:0	4.8	6.1	8.6	1.3	1.9	1.8	20.8
18:1	56.9	55.8	63.1	21.7	29.9	32.0	46.0
Cyclopropane acid							
19 cyc	24.1	18.1	11.3	34.1	34.8	36.9	2.6
Hydroxy acids							
3-OH 10:0	tr	tr	tr	tr	tr	tr	—
2-OH 14:0	—	—	—	—	—	—	2.4
3-OH 14:0	1.1	1.2	tr	2.1	2.8	2.1	1.8
2-OH 16:0	—	—	—	4.3	5.8	5.1	10.3
Unknown number of peaks	4	4	3	4	4	4	1
rrt 2.02	3.0	2.6	2.3	5.4	2.9	2.7	—
rrt 2.57	tr	tr	—	1.3	tr	tr	—
rrt 4.93	5.9	6.9	5.6	13.5	8.7	7.4	—
rrt 6.32	—	—	—	—	—	—	2.7
rrt 6.76	tr	tr	tr	3.8	3.4	2.1	—

rrt: relative retention time. tr: trace amounts (less than 1%).

cellular fatty acid composition. Strains 63, A, and *A. cryptum* contained straight-chain unsaturated acids of $C_{18:1}$ as a major component, and cyclopropane $C_{19:0}$. This group was characterized by the presence of a small amount of acids of $C_{12:0}$ and the absence of 2-OH fatty acid. Strains 16-R, 24-R, and 22-M mainly contained straight-chain unsaturated acid of $C_{18:1}$ and cyclopropane $C_{19:0}$. This group was characterized by the presence of 2-OH $C_{16:0}$. Strain 29-R mainly contained straight-chain acids of $C_{18:1}$ and $C_{18:0}$ and was characterized by the presence of 2-OH $C_{14:0}$, 2-OH $C_{16:0}$, and a small content of cyclopropane $C_{19:0}$ (Table 7).

DISCUSSION

The acidophilic heterotrophic bacteria were wide-spread in acidic mineral environments. Many bacteria of this type (10^2–10^4 cells/ml or g) were found in mine water and mud formed by the precipitation of ferric hydrates, and a few (0–10 cells/ml or g) were also isolated from clear mine water and soils in the mine. TUTTLE et al. (*3*) isolated 15–19 cells/ml from acidic coal refuse drainage, and WICHLACZ and

UNZ (5) obtained 20–250 cells/ml from natural mine waters. However, it is difficult to compare those bacterial numbers because the number of isolated bacteria depended on the culture medium and the isolation sources.

In the Yanahara mine, the acidic mine drainage contained high concentrations of ferrous iron (1,500 ppm Fe^{2+}) and sulfate. It was treated in the aeration tank using iron-oxidizing bacteria which oxidized ferrous iron to ferric iron and precipitated them as ferric hydrates. Therefore, the aeration tank in the Yanahara mine plays a kind of open, continuous culture system of iron-oxidizing bacteria.

Many more acidophilic heterotrophic bacteria were isolated from the aeration tank than from the mine water. In the mineral environments, especially, in the aeration tank, a large number of autotrophic bacteria provided the nutritive organic compounds for the heterotrophic bacteria. HARRISON (*11*) reported helpful interaction between iron-oxidizing bacteria and acidophilic heterotrophic bacteria in the course of a study using pyruvate. So the aeration tank using iron-oxidizing bacteria constitutes an enrichment culture system not only for iron-oxidizing bacteria but also for acidophilic heterotrophic bacteria.

Ten times as many bacteria were found in the bubbles formed on the surface of the water in the aeration tank as in the water. Apparently the bacteria are adsorbed and concentrated on the bubbles in a manner similar to froth flotation. Small numbers of yeasts and fungi, other than bacteria, were isolated from the aeration tank and the acidic mine drainage.

Acidophilic heterotrophic bacteria were also isolated from weakly acidic environments such as the sewage and field soils. When the sewage and soil samples were spread on agar plates adjusted to pH 3.0, yeasts, fungi, and acid-tolerant bacteria formed a large number of colonies on the plates, but there were few colonies of acidophilic heterotrophic bacteria (only 9 strains from 106 samples). The reason may be that the pH of the sewage and soils was beyond the growth pH ranges of those bacteria, and that organic compounds (acetic acid, α-keto acids etc.) which inhibited those bacteria were present in high concentrations.

HORIKOSHI and AKIBA (*19*) reported that alkalophilic bacteria had been found in soils of pH 4, although they grew best at pH 9 to 10 and could not grow below pH 7. Acidophilic heterotrophic bacteria might also be widely distributed in environments extending from acidic to neutral pH, but it was considered that the population size depended on the amount of organic compounds inhibiting bacterial growth.

The environmental conditions of mine water differ from sewage in pH, and in the amounts of organic materials and heavy metals. These different conditions of the environments affected the characteristics of bacteria isolated from them. The bacteria from mine water could grow under more acidic conditions than the bacteria from sewage. The growth pH ranges did not change in media supplemented with citrate, glycerol, or glutamate instead of glucose. Small amounts of organic compounds such as yeast extracts and trypticase soy broth had more inhibitory effects on the growth of the bacteria from mine water. The bacteria from mine water

Table 8. Differences in characteristics of the acidophilic heterotrophic bacterial strains and their grouping.

	Group 1 A		Group 1 B	Group 2			Group 3
	Strain 63	Strain A	*A. cryptum* Lhet2	Strain 16-R	Strain 24-R	Strain 22-M	Strain 29-R
Size (μm)	0.9–1.0 × 1.1–1.6	0.8–0.9 × 1.8–2.1	0.4–0.6 × 0.7–0.9	0.9–1.0 × 1.2–1.6	0.7–0.8 × 3.3–14.7	0.8–0.9 × 1.7–2.0	0.3–0.4 × 0.7–0.8
Motility	–	–	+	–	–	+	–
Filamentous shapes at pH 3.0	–	–	–	–	+	–	–
Growth on the complex organic plate (pH 3)							
1.0% casamino acids plate	+	+	+	+	+	+	–
1.0% bactopeptone plate	–	–	–	+	+	+	+
2.5% nutrient broth plate or heart infusion broth plate	–	–	–	–	–	–	+
TS conc. inhibited the growth (%)	0.4	0.4	0.1	1.0	1.0	1.0	>4.0
Cell population at the stationary phase in TS-glucose medium (cells/ml)	3×10^9	7×10^8	2×10^8	1×10^9	1×10^9	1×10^9	5×10^8
pH range for growth	2.0–5.8	2.0–5.8	1.9–5.8	2.6–6.2	2.6–6.0	2.6–6.0	2.6–6.4
Growth inhibited by 0.01% acetate	+	+	+	+	+	+	–
Utilization of succinate and fumarate	–	–	–	+	+	+	–
Utilization of citrate, α-ketoglutarate, malate, glutamate, ribose, arabinose	+	+	+	+	+	+	–
Utilization of maltose and raffinose	–	–	–	–	–	–	+
DNA base composition (G+C mol%)	68.0	67.8	67.6	63.0	65.2	64.9	67.3
Fatty acid (%)							
18 : 1	56.9	55.8	63.1	21.7	29.9	32.0	46.0
19 cyc	24.1	18.1	11.3	34.1	34.8	36.9	2.6
2-OH 14 : 0	–	–	–	–	–	–	2.4
2-OH 16 : 0	–	–	–	4.3	5.8	5.1	10.3

could not grow on 1% bactopeptone, but the bacteria from sewage could do so. The bacteria isolated from mine water could grow in medium containing 4.4% $FeSO_4$, but the sewage bacteria could not.

Acidophilic methanol-utilizing bacteria were isolated recently by URAKAMI et al. (*20*), BABEL (*21*), and UHLIG et al. (*22*) proposed for them the new species *Acetobacter methanolicus*. However, the acidophilic heterotrophic bacteria isolated in this study differed from them in having no ability to assimilate methanol.

The acidophilic heterotrophic bacteria from acidic mine drainage and sewage are classified in the genus *Acidiphilium*, because most their characteristics fit that genus (*11*). The characteristics of strains 16-R, 24-R, and 22-M differed from the genus description in growth pH ranges and sensitivity to organic substrates. However, these differences may be attributable to the different habitat conditions since the definition of the genus *Acidiphilium* was proposed on the basis of investigation of the bacteria isolated from acidic mineral environments. Strain 29-R could grow in media containing tissue extracts or organic substrates at high concentration (4%). These characteristics differed greatly from the description of genus *Acidiphilium*. Therefore, it must be carefully considered whether strain 29-R belongs to the genus *Acidiphilium*.

The six strains and *A. cryptum* isolated in this study were classified into 3 groups based on their characteristics (Table 8). Among the three groups, there are differences in habitat, DNA base composition, cellular fatty acid composition, growth pH region, organic materials utilized for growth, and sensitivity to organic substrates.

Group 1 organisms were isolated from acidic mineral environments. From the same environments, *A. cryptum* (*9*), *A. organovorum* (*10*), *A. facilis* (*12*), *A. angustum* (*12*), and *A. rubrum* (*12*) were previously isolated. The DNA base composition and the organic materials utilized for growth indicate that strains 63 and A are related to *A. cryptum* or *A. organovorum*. But these strains differ from *A. cryptum* in bacterial size, mobility, yield of cells, and sensitivity to organic substrates. Although the cells of strain 63 and A amount to $6–9 \times 10^9$ cells/ml in the medium containing TS-base, 0.2% glucose, 0.05% yeast extract, and 0.01% $FeSO_4$ at 30°C (pH 3.5), the yield of *A. cryptum* was less than $4–5 \times 10^8$ cells/ml in the same medium. Strain 63 and A also differed from *A. organovorum* in forming the colonies on solid medium using conventional agar and in utilizing succinate and fumarate.

Group 2 organisms were isolated from sewage, the DNA base composition indicates that strain 16-R is related to *A. rubrum* and that strains 24-R and 22-M are related to *A. facilis*. But strain 16-R did not produce a reddish violet pigment as *A. rubrum* did, and could assimilate glycerol and fumarate. Strains 24-R and 22-M also differed from *A. facilis* in growth inhibition by acetate, urea hydrolysis, and utilizing ethanol and lactose.

The group 3 organism was isolated from field soils. DNA base composition and organic materials utilized for growth indicate that strain 29-R is related to *A. angustum*. But it did not produce a pink pigment as *A. angustum* doses, and also

differed from that species in its growth in tissue-extract medium.

Further research is needed to establish the taxonomic position of the acidophilic heterotrophic bacteria isolated from acidic mine drainage, sewage, and soils.

We thank Dr. K. Komagata, The Institute of Applied Microbiology, University of Tokyo and Dr. T. Sugio, University of Okayama, for their helpful discussion and encouragement, and Mr. J. Tamaoka, University of Tokyo, for kindly determining the DNA base composition of the strains. We also thank Dr. H. Kagawa, University of Okayama, for kindly photographing electron micrograph of the strains. We further thank Mr. K. Ooida and Mr. Y. Fujiwara, Dowa Kogyo Co. for their help and discussion regarding the Yanahara mine collection site.

REFERENCES

1) H. L. Manning, *Appl. Microbiol.*, **30**, 1010 (1975).
2) R. T. Belly and T. D. Brock, *J. Bacteriol.*, **117**, 726 (1974).
3) J. H. Tuttle, C. I. Randles, and P. R. Dugan, *J. Bacteriol.*, **95**, 1495 (1968).
4) G. A. Zavarzin, *Microbiology*, **41**, 323 (1972).
5) P. L. Wichlacz and R. F. Unz, *Appl. Environ. Microbiol.*, **41**, 1254 (1981).
6) A. P. Harrison, Jr., *Appl. Environ. Microbiol.*, **36**, 861 (1978).
7) A. P. Harrison, Jr., B. W. Jarvis, and J. L. Johnson, *J. Bacteriol.*, **143**, 448 (1980).
8) D. B. Johnson and W. I. Kelso, *J. Gen. Microbiol.*, **129**, 2969 (1983).
9) A. P. Harrison, Jr., *Int. J. Syst. Bacteriol.*, **31**, 327 (1981).
10) J. H. Lobos, T. E. Chisolm, L. H. Bopp, and D. S. Holmes, *Int. J. Syst. Bacteriol.*, **36**, 139 (1986).
11) A. P. Harrison, Jr., *Annu. Rev. Microbiol.*, **38**, 265 (1984).
12) P. L. Wichlacz, R. F. Unz, and T. A. Langworthy, *Int. J. Syst. Bacteriol.*, **36**, 197 (1986).
13) T. Hasegawa, *In* Classification and Identification of Microorganisms, Gakkai Shuppan Center, Tokyo (1978), p. 217.
14) M. D. Collins and T. A. Langworthy, *Syst. Appl. Microbiol.*, **4**, 295 (1983).
15) H. Saito and K. Miura, *Biochim. Biophys. Acta*, **72**, 619 (1963).
16) J. Tamaoka and K. Komagata, *FEMS Microbiol. Lett.*, **25**, 125 (1984).
17) T. Suto, Y. Fujimura, and H. Kuraishi, *In* Chemotaxonomic Methods of Microorganisms, ed. by K. Komagata, Gakkai Shuppan Center, Tokyo (1982), p. 162.
18) S. Ikemoto, H. Kuraishi, K. Komagata, R. Azuma, T. Suto, and H. Murooka, *J. Gen. Appl. Microbiol.*, **24**, 199 (1978).
19) K. Horikoshi and T. Akiba, *In* Alkalophilic Microorganisms, Japan Scientific Societies Press, Tokyo (1982), p. 10.
20) T. Urakami, I. Terao, and I. Nagai, *J. Ferment. Technol.*, **60**, 287 (1982).
21) W. Babel, *Acta Biotechnol.*, **4**, 369 (1984).
22) H. Uhlig, K. Karbaum, and A. Steudel, *Int. J. Syst. Bacteriol.*, **36**, 317 (1986).