**Acidiphilium organovorum** sp. nov., an Acidophilic Heterotroph Isolated from a *Thiobacillus ferrooxidans* Culture

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*Acidiphilium organovorum* sp. nov. is a gram-negative, heterotrophic bacterium that was isolated from a culture of *Thiobacillus ferrooxidans* which had been grown autotrophically on FeSO₄-basal salts medium for several years. Purification of *A. organovorum* was carried out on a 1.0% glucose-basal salts medium (pH 3.0) solidified with agarose. Growth was enhanced by adding high concentrations of glucose (0.5 to 2.0%) and by supplementing the medium with yeast extract and trace amounts of FeSO₄. However, these supplements were not necessary for growth. A wide variety of organic compounds were suitable substrates for growth, but inorganic forms of reduced sulfur or ferrous iron were not. Doubling times of 2.5 h and cell densities of >2 × 10⁴ cells per ml were obtained at the optimal temperature of 37°C and pH 3.0. The guanine-plus-cytosine content of the deoxyribonucleic acid was 64 mol%. *A. organovorum* contains at least three distinct plasmids; one of these plasmids is larger than 30 kilobase pairs, and two are smaller than 4.0 kilobase pairs. Homology studies in which we compared the total deoxyribonucleic acid of *A. organovorum* with the total deoxyribonucleic acids of *Acidiphilium cryptum* and several *Thiobacillus* species indicated that *A. organovorum* is most closely related to *A. cryptum*. *A. organovorum* can be differentiated from *Thiobacillus acidophilus* by its higher temperature optimum, its faster growth rate, and its inability to utilize reduced forms of sulfur or iron as energy sources. The abundant cell growth that occurs in a medium which either is rich in organic compounds or completely lacks nutritional supplements distinguishes *A. organovorum* from *A. cryptum*. The other physiological and genetic characteristics which we examined are in close agreement with the characteristics of members of the genus *Acidiphilium*. The type strain of *A. organovorum* is strain ATCC 43141.

The presence of acidophilic, heterotrophic bacteria in presumably pure cultures of *Thiobacillus ferrooxidans* has received increasing attention in recent years. There have been several reports of *T. ferrooxidans* strains which can grow heterotrophically on glucose and autotrophically by using ferrous iron as an energy source (20, 25, 27). Recently, there have been reports of two strains of *T. ferrooxidans* that can grow mixotrophically on glucose and ferrous iron (2, 23). However, in cases where a culture of *T. ferrooxidans* has been adapted to grow solely on glucose, it has been difficult to grow the organism autotrophically on ferrous iron (19, 24, 27). The inability to reverse growth capabilities has led to speculation about the validity of these reports. Until recently, it has been difficult to grow *T. ferrooxidans* on solid medium (12, 14, 15). Without pure-colony isolations, the acquisition of pure cultures of *T. ferrooxidans* was extremely difficult and often proved unreliable. Frequently, ostensibly pure cultures have contained acidophilic heterotrophs or more than one strain of *T. ferrooxidans* (7, 10). At least two new species of heterotrophic bacteria have been isolated from *T. ferrooxidans* cultures (4, 5, 29). The absence of growth on ferrous iron, in addition to several other characteristics, has indicated that these isolates are not *T. ferrooxidans*. One of these acidophilic heterotrophs, *Thiobacillus acidophilus*, reported was capable of autotrophic growth on elemental sulfur in addition to heterotrophic growth on a variety of organic compounds. Hence, it was assigned to the previously defined genus *Thiobacillus*. *T. acidophilus* was isolated by gradually increasing the glucose concentration from 0.01 to 1.0% with a concomitant decrease in the amount of FeSO₄ added to the medium (4). More recently, another heterotrophic bacterium which has been found in cultures of *T. ferrooxidans* was isolated. This isolate was assigned to a new genus and species, *Acidiphilium cryptum* (5). It was characterized as an acidophilic heterotroph which was incapable of autotrophic growth by using reduced sulfur compounds or ferrous iron as energy sources. *A. cryptum* reportedly required a very lean organic medium containing low concentrations of both glucose (0.1%) and organic supplements, such as yeast extract or Trypticase (0.01%). Higher concentrations of glucose or complex organic supplements, which are commonly used in most heterotrophic media, completely inhibited growth. Consequently, it was suggested that this microorganism could easily escape detection if isolation was attempted on a medium containing the normal amounts of glucose or organic supplements or both (5, 6).

In this report, we describe another acidophilic, heterotrophic bacterium that was isolated from a culture of *T. ferrooxidans*. This organism possesses important characteristics which distinguish it from the two heterotrophic bacteria described above.

**MATERIALS AND METHODS**

**Isolation.** *Acidiphilium organovorum* was isolated from a *T. ferrooxidans* culture that had been grown in a basal salts-FeSO₄ medium for several years (21). Isolation was achieved by plating a presumably pure culture of *T. ferrooxidans* on a 1% glucose-basal salts medium solidified with 0.6% agarose (pH 3.0). Uniform colonies appeared on the glucose medium after incubation at 30°C for 5 days. The culture was purified by repeated subcloning on basal salts-glucose plates.

**Medium.** For growth studies, *A. organovorum* was cultured in NP-glucose medium containing the following basal salts (per liter of distilled water): (NH₄)₂SO₄, 3 g; KCl, 0.1 g; MgSO₄·7H₂O, 0.5 g; Ca(NO₃)₂·4H₂O, 0.02 g; NaH₂PO₄, 6.9 g; and glucose, 10 g. The pH was adjusted to 3.0 to 3.5 with H₃PO₄. Additions of FeSO₄ (10 μg/ml) and yeast extract (1.0 g/liter) were made as indicated below. For routine
cultivation, 50 mM citrate-phosphate-buffered glucose medium (CP-glucose) supported the best growth. This medium was the same as NP-glucose medium except that the NaH₂PO₄ was replaced with 9.6 g of citric acid per liter and 3.55 g of Na₂HPO₄ per liter (pH 3.0). Solid medium was prepared by combining equal volumes of double-strength basal salts medium (pH 3.5) and double-strength electrophoresis grade agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) after the solutions had been autoclaved separately. Glucose, yeast extract, and FeSO₄ were then added as described above. After autoclaving it was important to cool the basal salts and agarose solutions to 60°C before they were combined to prevent hydrolysis of the agarose.

**Growth studies.** The optimal and extreme conditions for growth (including pH, temperature, glucose concentration, and nutritional supplements) were determined in NP-glucose medium to which the appropriate additions had been made (see figure legends); 2% inocula of a freshly grown _A. organovorum_ culture were added to 25 ml of NP-glucose medium in 125-ml screw-cap bottles. The cultures were incubated at 37°C (unless indicated otherwise) with vigorous agitation (200 rpm) in a rotary shaker-incubator. Growth was monitored by measuring absorbance at 600 nm with a Spectronic 20 spectrophotometer. The total number of cells per milliliter was determined by using a Petroff-Hauser cell-counting chamber. In studies that tested the ability of organisms to use different organic compounds as substrates, the substrate solutions were prepared separately, filter sterilized, and added aseptically to NP-basal salts medium at concentrations ranging from 0.05 to 0.5%. Susceptibility to various antibiotics was tested on solid NP-glucose medium (pH 3.5) by using Sensi-Discs (diameter, 7 mm; BBL Microbiology Systems, Cockeysville, Md.). _A. organovorum_ was judged to be susceptible if a clear zone that was ≥15 mm in diameter appeared around a filter disk after 3 days of incubation at 37°C.

**Bacterial strains.** _A. organovorum_ was isolated from a _T. ferroxidans_ culture supplied by H. L. Ehrlich, who received it from the Diamond-Shamrock Corp., Painesville, Ohio. In addition to _A. organovorum_, the following bacterial strains were used in the deoxyribonucleic acid (DNA) analyses: _A. cryptum_ ATCC 334637 (T = type strain), _T. acidophilus_ ATCC 27807 and DSM 700, _T. ferroxidans_ ATCC 13661 and ATCC 33302, _Thiobacillus novellus_ ATCC 8093, and _Escherichia coli_ strain B (DNA received from Sigma Chemical Co., St. Louis, Mo.).

**DNA isolation.** Total DNA was prepared by a modification of the method of Blin and Stafford (3). Approximately 10⁸ cells were collected by centrifugation, suspended in 10 ml of residual supernatant, and added dropwise to 50 ml of 0.4 M ethylene diaminetetraacetate–0.2 M tris(hydroxymethyl)aminomethane (pH 9.2) at 65°C. The cells were lysed by adding 3 ml of a 20% sodium dodecyl sulfate solution and incubating the preparation at 65°C for 15 min. The cell lysate was then cooled to 50°C, and crystalline proteinase K (Beckman Instruments, Inc., Fullerton, Calif.) was added to a final concentration of 500 µg/ml before the preparation was incubated overnight at 50°C. The cell lysate was extracted five or six times with phenol-chloroform (1:1) equilibrated with 10 mM tris(hydroxymethyl)aminomethane–1 mM ethylene diaminetetraacetate (pH 7.5). The DNA was precipitated with 2 volumes of isopropanol at −18°C, spooled on a glass rod, and suspended in 10 mM tris(hydroxymethyl)aminomethane–1 mM ethylenediaminetetraacetate (pH 7.5). The total DNA was purified by cesium chloride-ethidium bromide buoyant density centrifugation. Plasmids from the acidophilic heterotrophs and recombinant plasmids from _E. coli_ were isolated by a modified rapid-boiling procedure (8), followed by two rounds of cesium chloride-ethidium bromide buoyant density centrifugation. Plasmids from _T. ferroxidans_ strains were prepared by the procedure of Martin et al. (13).

**DNA homology studies.** All radioactive DNA probes were generated by nick translation, using a Nick Translation Reagent Kit (Bethesda Research Laboratories) and [α-³²P]deoxyctydine triphosphate (800 Ci:mmol; Amersham Corp., Arlington Heights, Ill.). The incorporation of ³²P-labeled deoxyctydine triphosphate was such that approximately 10⁶ cpm/µg of DNA was routinely obtained. Total DNA-DNA hybridization was carried out by using the dot blot procedure and Genescreen hybridization transfer membranes (New England Nuclear Corp., Boston, Mass.) according to the recommendations of the manufacturer. The plasmid homology studies were done by using the Southern blot method of DNA-DNA hybridization (22). Capillary blots were done on GeneScreen hybridization transfer membranes by the specifications of the manufacturer.

**DNA base composition.** The guanine-plus-cytosine (G+C) content of DNA was determined by reverse-phase high-performance liquid chromatography (26, 28). DNA (100 µg) was hydrolyzed to the four DNA bases in 2 ml of 88% formic acid at 125°C for 2 h (11). A high-performance liquid chromatography system (Waters Associates, Inc., Milford, Mass.) consisting of two model S10 pumps, a Lambda Max 481 variable-wavelength detector operated a 254 nm, a 20 mm sodium acetate buffer (pH 4.7) for 10 min at a flow rate of 2 ml/min. Standards of the four DNA bases were prepared in triplicate, both separately and as mixtures. Multiple determinations of the G+C content of each organism from independent DNA isolations were made. The standard deviation was ±0.6% in every case.

**RESULTS**

**Cultivation and cell and colony morphology.** _A. organovorum_ cells grown in liquid basal salts-glucose medium were short, motile bacilli or coccobacilli that were ~0.6 by 1.0 µm. Cells in logarithmic growth occurred in pairs or chains and were gram negative. No endospores were detected under any growth conditions. Growth on solid medium was best when 0.6% electrophoresis grade agarose was used as the gelling agent. The colonies initially appeared to be glossy, entire, and white and turned pink after 2 to 2 weeks.

**Response to pH and temperature.** Figure 1 shows the effect of pH on cell growth in NP-glucose medium prepared at four different initial pH values. The medium was only weakly buffered, so changes in pH were measured along with cell growth. The growth rate was very slow when the initial pH was 4.5 or 5.5 (Fig. 1C and D). When the initial pH was lowered to 3.5 or 2.5 (Fig. 1A and B), a significant increase in the growth rate was observed. When the initial pH was 3.5 or greater (Fig. 1B through D), a continuous decrease in pH was observed during cell growth. However, when the initial pH was 2.5 (Fig. 1A), no change in pH occurred over the
growth period. The optimum pH was 3.0, and no growth occurred below pH 2.0 or above pH 5.5.

*Acidiphilum organovorum* grew over a wide range of temperatures. The optimal temperature was 37°C, although good growth was obtained from 30 to 42°C. No growth occurred at temperatures above 45°C, and much slower growth was observed at 20°C or below. Doubling times of 2.5 h and cell densities of \(2 \times 10^{10}\) cells per ml were achieved in citrate-phosphate-buffered medium at 37°C (pH 3.0).

**Glucose and other organic substrates.** The effects of various concentrations of glucose on cell growth are shown in Fig. 2. No significant effect on cell growth was observed when the glucose concentration was varied between 0.5 and 2.0%. However, at 0.1% glucose the substrate became limiting, and both growth rate and final cell density were substantially reduced. Some inhibition of cell growth occurred at very high glucose concentrations (4.0%).

In addition to glucose, a wide variety of organic compounds supported growth as sole sources of carbon and energy. These included a number of sugars (fructose, sucrose, galactose, arabinose, ribose, and xylose). No growth was observed on cellobiose, lactose, or maltose. Several carboxylic acids, including malate, succinate, fumarate, gluconate, and especially citrate, were used as sole carbon and energy sources, but acetate and pyruvate did not support growth and were completely inhibitory at concentrations of 0.1%. Mannitol, xylitol, sorbitol, and glycerol were also good substrates. Of the naturally occurring L-amino acids tested, only proline and glutamic acid supported growth.

**Nutritional supplements.** The addition of yeast extract at concentrations ranging from 0.01 to 0.5% enhanced cell growth significantly, with the best growth occurring at concentrations of 0.05 to 0.2%. Tryptone also improved cell growth, but to a lesser extent. Addition of Trypticase or a vitamin mixture did not improve cell growth. Addition of either trace metals of FeSO₄ greatly increased both the specific growth rate and the final cell density.

Stimulation of cell growth by FeSO₄ was explored in greater detail. Growth rates and final cell densities were compared at FeSO₄ concentrations ranging from 0 to 100 μg/ml. Addition of more than 1 μg of FeSO₄ per ml only slightly increased the growth rate, but caused a remarkable improvement in the final cell density. Exponential growth occurred for a longer time when 100 μg of FeSO₄ per ml was added, and the final cell density was almost 10-fold greater than the density in the absence of added FeSO₄. The energy available from the oxidation of this amount of Fe²⁺ is insignificant compared with the energy required to explain the observed increase in growth. Therefore, Fe²⁺ appears to be a growth factor rather than an energy source.

**DNA analyses.** The base composition of DNA isolated from *A. organovorum* was determined by high-performance liquid chromatography to be 64 mol% G+C. Using the same analytical procedure, we found that *A. cryptum* ATCC 33463T and *T. acidophilus* ATCC 27807 DNAs had G+C contents of 64 and 62 mol%, respectively. In addition, *E. coli* strain B DNA had a G+C content of 50 mol%.

![FIG. 1. Effect of pH on cell growth. The growth of *A. organovorum* in 50 mM NP-glucose medium was determined at initial pHs of 2.5 (A), 3.5 (B), 4.5 (C), and 5.5 (D). The pH was adjusted by adding either H₃PO₄ or Na₂HPO₄, and the medium was supplemented with yeast extract (0.05%) and FeSO₄ (10 μg/ml). Symbols: ●, cell density (absorbance at 600 nm [A₆₀₀ nm]); ○, pH.](image-url)

![FIG. 2. Effect of glucose concentration on cell growth. *A. organovorum* was grown in NP-glucose medium containing different concentrations of glucose. Symbols: ○, 0.1% (wt/vol) glucose; △, 0.5% and 1.0% (wt/vol) glucose; ▽, 2.0% (wt/vol) glucose; △, 4.0% (wt/vol) glucose. A₆₀₀ nm, Absorbance at 600 nm.](image-url)
DNA sequence homology studies were done by hybridizing the total DNA of A. organovorum to the total DNAs of A. cryptum and several Thiobacillus species, using the DNA dot blot technique. The intensities of the dots on the autoradiogram shown in Fig. 3 indicate the extent of DNA hybridization that occurred when 1.0 μg of A. organovorum 32P-labeled probe DNA was hybridized to 1.0, 0.1, and 0.01 μg of target DNA. The amount of target DNA fixed to the filter was decreased in 10-fold increments so that estimates of the extent of homology between the two different organisms could be made. Considerable hybridization of A. organovorum DNA to A. cryptum DNA was observed (Fig. 3, column 2). However, a comparison of these hybridization results with the results obtained when A. organovorum DNA was hybridized to itself (Fig. 3, column 1) clearly indicated that A. cryptum is not completely homologous to A. organovorum. Substantial hybridization also occurred between A. organovorum DNA and the DNAs of two T. acidophilus strains (Fig. 3, columns 3 and 4) and T. novellus (Fig. 3, column 5). Strikingly, there was little or no homology to either of the T. ferrooxidans strains tested (Fig. 3, columns 6 and 7).

As determined by gel electrophoresis, A. organovorum contains at least three distinct plasmids. Using restriction enzyme analysis, we found that these plasmids are 3.3, 3.8, and ≥30 kilobase pairs long. The two small plasmids, designated pAO1 and pAO2, were cloned intact into E. coli plasmid pBR322 and used as probes to look for homologous DNA sequences on plasmids present in a number of other acidophilic heterotrophs and T. ferrooxidans. Considerable sequence homology was observed between the two small plasmids in A. organovorum. Also, a plasmid from T. acidophilus DSM 700 had significant sequence homology with pAO2, but not with pAO1. Plasmids isolated from several other acidophilic heterotrophs and T. ferrooxidans strains showed no sequence homology to either pAO1 or pAO2.

**DISCUSSION**

Acidophilic heterotrophs are associated with T. ferrooxidans strains isolated from a wide variety of sources (10, 15, 16). Certain conditions which support the growth of T. ferrooxidans (e.g., acidic, mesophilic, aerobic, ferrous iron or other mineral sulfide substrate) are suitable for the perpetuation of some heterogeneous consortia. The way in which these acidophilic heterotrophs are able to coexist with T. ferrooxidans in a medium lacking added organic energy sources is not completely understood. It has been shown that T. ferrooxidans cells excrete organic compounds, such as pyruvate, glutamate, aspartate, serine, glycine, and other amino acids, which these heterotrophs can use as substrates for growth (1, 9, 18). Alternatively, lysed cells of T. ferrooxidans may provide another source of organic matter. In either case, the amount of available organic material in iron-grown T. ferrooxidans cultures is extremely small. Therefore, the acidophilic heterotrophs must be efficient scavengers of these organic compounds. Since some of these compounds have been shown to be inhibitory to T. ferrooxidans (6, 9, 24), it is likely that the association is symbiotic in nature.

Two distinct types of acidophilic heterotrophs, exemplified by T. acidophilus and A. cryptum, have been recovered previously from T. ferrooxidans enrichments. Both of these heterotrophs are gram-negative, acidophilic, motile bacilli which grow aerobically on a wide variety of organic compounds. However, T. acidophilus has been reported to be a facultative autotroph that is capable of growth by using elemental sulfur as an energy source, whereas the growth of A. cryptum is strictly heterotrophic (4, 5). A. organovorum represents a new species of bacterium which possesses characteristics that distinguish it from both T. acidophilus and A. cryptum. The inability of A. organovorum to utilize any form of reduced sulfur or ferrous iron as an energy source distinguishes it from the acidophilic thiothiobacilli. The increased growth rate, the different substrate specificities, and the high cell yields observed at high concentrations of certain organic substrates (e.g., 1.0 to 4.0% glucose) with and without organic supplements (e.g., yeast extract) distinguish A. organovorum from A. cryptum (5). Also, the growth rate of A. organovorum on solid medium is much greater than the growth rate reported for either A. cryptum or T. acidophilus (4, 5).

The G+C contents of A. organovorum and A. cryptum DNAs were determined to be the same by using the high-performance liquid chromatography method. The value of 64 mol% G+C determined for A. cryptum ATCC 33463T was lower than the value reported previously but is still within the range of values reported for the genus Acidiphilium (6). There are several possible explanations for this difference, such as the presence of modified nucleotides (26). DNAs from T. acidophilus and E. coli produced values of 62 and 30 mol% G+C, respectively. Both of these values are very similar to values reported previously (4, 17).

DNA hybridization studies in which the dot blot technique was used demonstrated that A. organovorum has extensive homology with both A. cryptum and the facultative heterotrophs T. acidophilus and T. novellus. We found little or no DNA homology between A. organovorum and any of biological agars were used. A positive catalase reaction was observed, but the organism was oxidase negative. Growth was inhibited by a number of antibiotics, including ampicillin, tetracycline, chloramphenicol, novobiocin, and rifampin.
the *T. ferrooxidans* strains tested. These results are in agreement with the results of a previous study, in which workers found no homology between *T. ferrooxidans* and either *A. cryptum* or *T. acidophilus*, but found significant homology between *A. cryptum* and *T. acidophilus* (6, 7).

The discovery of sequence homology between at least one of the plasmids in *A. organovorum* and one plasmid in *T. acidophilus* may be relevant to the function of these plasmids. However, this homology cannot be used to determine phylogenetic relationships because of the possibility of horizontal transfer of plasmid DNA.

Unlike isolation of *T. acidophilus*, isolation of *A. organovorum* did not require gradual adaptation to glucose with a concomitant reduction in the FeSO₄ concentration (4). *A. organovorum* could easily be isolated from an iron-growth *T. ferrooxidans* culture by plating cells directly onto 1% glucose–minimal salts medium solidified with 0.6% agarose. Conventional bacteriological agar was unsuitable for the growth of this microorganism. Therefore, it is very important to use agarose when attempting to isolate these microorganisms on solid medium. It is not clear whether it is the high concentrations of bacteriological agars normally used to form suitable gels or the impurities present within the agars that are inhibitory to *A. organovorum* and other acidophilic bacteria (10, 12).

On the basis of both physiological characteristics and DNA analyses, *A. organovorum* best conforms with the genus *Acidiphilium*. It represents a new species that is not sensitive to high concentrations of organic compounds (e.g., glucose and yeast extract). Instead, growth is stimulated by the addition of high concentrations of glucose and yeast extract as well as certain other nutritional supplements. Also, growth is quite satisfactory in the absence of any nutritional supplement. Why growth of *A. organovorum* is so greatly enhanced by the addition of FeSO₄ is not known. It is possible that long-term exposure to high concentrations of iron in *T. ferrooxidans* cultures results in a gradual loss of the ability to bind and transport iron or leads to a greater metabolic requirement for iron.

*Acidiphilium organovorum* sp. nov. *Acidiphilium organovorum* (or. ga. no’ vor. um. N. L. n. organum organic compound; L. v. voro eat, consume; N. L. adj. organon organisms which use organic compounds), cells are gram-negative, aerobic, mesophilic bacilli or cocobacilli (0.6 by 0.8 to 1.0 μm). Motile, heterotrophic, obligately acidophilic. A variety of organic compounds support growth as sole carbon and energy sources. These include simple sugars, sugar alcohols, carboxylic acids, and amino acids. No growth occurs on lactose, maltose, or any form of reduced sulfur or iron. Acetic acid and pyruvic acid completely inhibit cell growth at concentrations of 0.01 and 0.1%, respectively. Increased growth occurs at glucose concentrations (0.5 to 2.0%) higher than those previously used for culturing other *Acidiphilium* species (5). Nutritional supplements of yeast extract and trace amounts of FeSO₄ enhance growth. Cell doubling times of 2.5 h and cell densities of ≥2 × 10¹⁰ cells per ml are obtained in basal salts-glucose medium supplemented with yeast extract and FeSO₄. For growth on solid medium, low amounts (0.5 to 0.8%) of high-purity agarose must be used. White, glossy colonies ≥2 mm in diameter form in 3 to 4 days. This bacterium gives positive catalase and negative oxidase reactions. No endospores are formed, and cells die rapidly at temperatures of ≥50°C. Susceptible to the antibiotics tetracycline, ampicillin, chloramphenicol, and rifampin. The G+C content of the DNA is 64 mol%. Of the bacteria tested, DNA homology studies indicate that *A. organovorum* is most closely related to *A. cryptum*. The type strain of *A. organovorum* is strain ATCC 43141.

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