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Isolation and Characterization of an Acidophilic, Heterotrophic Bacterium Capable of Oxidizing Ferrous Iron

D. BARRIE JOHNSON,* M. AFZAL GHAURI, AND MAZIN F. SAID

School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, United Kingdom

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A heterotrophic bacterium, isolated from an acidic stream in a disused pyrite mine which contained copious growths of "acid streamers," displayed characteristics which differentiated it from previously described mesophilic acidophiles. The isolate was obligately acidophilic, with a pH range of 2.0 to 4.4 and an optimum pH of 3.0. The bacterium was unable to fix carbon dioxide but oxidized ferrous iron, although at a slower rate than either *Thiobacillus ferrooxidans* or *Leptospirillum ferrooxidans*. Elemental sulfur and manganese(II) were not oxidized. In liquid media, the isolate produced macroscopic streamerlike growths. Microscopic examination revealed that the bacterium formed long (>100 μm) filaments which tended to disintegrate during later growth stages, producing single, motile cells and small filaments. The isolate did not appear to utilize the energy from ferrous iron oxidation. Both iron (ferrous or ferric) and an organic substrate were necessary to promote growth. The isolate displayed a lower tolerance to heavy metals than other iron-oxidizing acidophiles, and growth was inhibited by exposure to light. There was evidence of extracellular sheath production by the isolate. In this and some other respects, the isolate resembles members of the *Sphaerotilus-Leptothrix* group of filamentous bacteria. The guanine-plus-cytosine content of the isolate was 62 mol%, which is less than that recorded for *Sphaerotilus-Leptothrix* spp. and greater than those of *L. ferrooxidans* and most *T. ferrooxidans* isolates.

Highly acidic environments formed by the oxidation of pyrite and other sulfidic minerals are known to be populated by a range of acidophilic and acid-tolerant prokaryotic and eukaryotic life forms (4, 9, 14). The most well studied of these are *Thiobacillus* spp., a genus of mesophilic bacteria that includes two species of iron-oxidizing acidophiles, *Thiobacillus ferrooxidans* and the halotolerant *Thiobacillus prosperus* (12). These are generally considered to be obligate chemolithotrophs, although one mixotrophic isolate of *T. ferrooxidans* has been described (1). *Thiobacillus acidophilus* and *Thiobacillus cuprinus* are mixotrophic acidophiles that can obtain energy from the oxidation of reduced sulfur compounds or organic substrates but not ferrous iron (7, 13). *Leptospirillum ferrooxidans* is an iron-oxidizing acidophilic bacterium which, in contrast to *T. ferrooxidans*, has not been shown to be capable of oxidizing reduced sulfur. Heterotrophic, acidophilic bacteria, often living in close association with chemolithotrophic primary producers, have also been isolated from extremely acidic environments. However, in contrast to *T. ferrooxidans* and *L. ferrooxidans*, these bacteria (many of which have been identified as *Acidiphilium* spp.) are unable to fix carbon dioxide and to oxidize ferrous iron (although about 40% of isolates were reported to be capable of reducing ferric iron [17]). In contrast to most mesophilic acidophiles, moderately thermophilic iron-oxidizing bacteria may live as chemolithoheterotrophs, obtaining energy from iron oxidation and carbon from organic substrates, or mixotrophically (6, 22). However, these are gram-positive rather than gram-negative bacteria, often forming endospores, and generally occur as single rods, although chain forms have been observed. Extreme thermophiles that oxidize ferrous iron are archaebacteria and have a distinct, often irregular morphology. Again, they are mixotrophic bacteria (11, 21).

There are other prokaryotes classified as "iron bacteria" that are not extreme acidophiles. The most well known of these are the filamentous iron bacteria that grow in more circumneutral pH environments. This group includes *Gallionella* sp., in which filaments are formed by the apical bean-shaped cells excreting an organic matrix in which ferric hydroxides precipitate (8), and the *Sphaerotilus-Leptothrix* group, rod-shaped bacteria that synthesize extracellular sheaths on which ferric and manganic compounds may accumulate (20). While there is evidence that *Gallionella* sp. is able to live autotrophically by using ferrous iron as its energy source, this is not the case for either *Sphaerotilus* or *Leptothrix* sp., both of which are obligate heterotrophs. The neutrophiles are therefore distinct from acidophilic iron-oxidizing mesophiles in their morphology and aspects of their physiology.

This article reports the isolation and initial characterization of a new class of iron-oxidizing acidophilic bacteria, which have morphological and behavioral characteristics similar to those of neutrophilic *Sphaerotilus-Leptothrix* spp.

MATERIALS AND METHODS

Bacterial origin and isolation. The bacterium described in this report (coded CCH7) was isolated from a disused pyrite mine (Cae Coch) situated in the Conwy Valley, North Wales. The most notable microbiological feature of the mine, which has been described in detail elsewhere (15), is the copious growth of gelatinous "acid streamers" that occur in its main drainage stream and on moist vertical and semivertical faces. Microscopic examination of these streamers has shown them to consist of a mixed community of unicellular and filamentous bacteria, with occasional protozoa and rotifera which may be observed grazing the bacteria (14). Stream water samples containing disrupted (lightly homogenized) acid streamers were streaked onto solid FeTSB medium (16) and incubated at 28°C for up to 4

* Corresponding author.

weeks. Bacterial colonies were then divided into those which were ferric iron encrusted (i.e., iron-oxidizing bacteria) and those which were not (i.e., acidophilic heterotrophs). Colonies, which were generally <1 mm in diameter, were carefully removed and put into liquid media. These media were 10 mM ferrous sulfate (pH 2.0) and 10 mM ferrous sulfate–0.025% (wt/vol) tryptone soy broth (pH 2.5) for iron-oxidizing and heterotrophic isolates, respectively. Purification of cultures was achieved by repeated plating and single-colony isolation.

Culture conditions. After initial isolation and purification, isolate CCH7 was routinely subcultured in 10 mM ferrous sulfate–0.02% (wt/vol) yeast extract liquid medium poised initially at pH 2.5. Growth was estimated visually by noting the production of macroscopic flocs or streamers (described below) and by monitoring ferrous iron oxidation (titration with 1 mM $KMnO_4$ in 1 M H_2SO_4). Cultures were incubated, shaken or unshaken, at 28°C. Ferrous sulfate concentrations varied in some experiments between 0 and 50 mM. The temperature range of CCH7 was assessed by growing the isolate in ferrous sulfate-yeast extract medium at between 10 and 45°C. To test for growth in acid mine drainage water, filter-sterilized Cae Coch stream water (pH 2.3; [Fe^{2+}], 6.9 mM) containing 0.02% yeast extract was inoculated with CCH7. Growth at different oxygen tensions was assessed by using a roll tube method; molten FeTSB medium in universal tubes was inoculated with exponentially growing CCH7.

To ascertain whether biomass of CCH7 in shake flask cultures was correlated with available ferrous iron, cells were grown in a lean organic medium (0.0025% tryptone soy broth [pH 2.0]) containing different concentrations of ferrous sulfate (0.025, 0.25, 2.5, and 25 mM), and, at stationary phase, the cultures were gently homogenized (to break bacterial filaments) and bacterial biomass was estimated by direct counting of acridine orange-stained membrane filtrates as described by Johnson and McGinness (18).

The ability of CCH7 to oxidize elemental sulfur and manganese(II) was tested by subculturing the isolate in 1% elemental sulfur or 10 mM manganese(II) sulfate media, both containing 0.02% yeast extract and poised initially at pH 2.5. Oxidation of pyrite and chalcopyrite (6) was assessed by subculturing into media containing 1% (wt/vol) ground ore, 10 mM ferrous sulfate, and 0.02% yeast extract, incubating cultures at 28°C for up to 50 days, and monitoring soluble iron and copper by atomic absorption spectrophotometry. Control cultures of *T. ferrooxidans* in sulfur, pyrite, and chalcopyrite media were also set up.

The effect of light on growth of isolate CCH7 was examined by inoculating ferrous sulfate-yeast extract media with actively growing cultures of the bacterium and incubating the cultures at room temperature (ca. 23°C) either in a darkened cupboard or on a laboratory bench (exposed to daylight but out of direct sunlight). After 7 days, the cultures were examined for growth and iron oxidation and transferred to a 28°C incubator for a further 10 days. Isolate CCH7 was also grown in a chemostat (LH series 500) in a culture vessel which was either shielded from (by using a black cardboard cover) or exposed to light.

Fixation of carbon dioxide and utilization of organic substrates. To test for autotrophic growth of CCH7, the isolate was grown in 25 mM ferrous sulfate medium (with or without yeast extract) containing $^{14}CO_2$. Cultures were supplemented with 0.5 M $NaH^{14}CO_3$ in sealed flasks, at the rate of 1 ml/100 ml of culture and at a specific activity of 20,000 cpm/μmol. Growth of CCH7 on single organic substrates was assessed by using ferrous sulfate medium supplemented with glucose, mannitol, glycine, glutamic acid, citric acid, glycerol, or ethanol (all at 0.05% [wt/vol]), with or without added yeast extract (at 0.002% [wt/vol]). Growth on glucose and glycine was monitored by adding [U-^{14}C]glucose or [U-^{14}C]glycine, both at 1 mM (final concentration) and with specific activities of 90,000 cpm/μmol, to ferrous sulfate media. Because of the macroscopic nature of CCH7 growth, it was necessary to harvest whole cultures, homogenize them (by using a Silverson blender), and take 2-ml subsamples for analysis of radiolabel. Bacterial growths were filtered onto 0.2-μm cellulose nitrate membrane filters (Whatman Ltd.), washed with 1 M H_2SO_4 to remove ferric precipitates, repeatedly rinsed, and finally air dried. Membranes were then dissolved in cocktail T "Scintran" before scintillation counting (6).

Tolerance of heavy metals. The tolerance to some heavy metals of isolate CCH7, in comparison with that of other acidophilic bacteria, was monitored by subculturing into ferrous sulfate-tryptone soy broth medium containing varying concentrations of $CuSO_4$, UO_2SO_4, Ag_2SO_4, Na_2MoO_4, and $Fe_2(SO_4)_3$. The metal concentrations used were as follows: copper, 5, 10, 50, 100, 200, 300, and 400 mM; uranium, 0.5, 2, and 5 mM; silver, 0.1, 0.5, 1, 5, and 10 μM; molybdenum, 0.01, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 mM; iron, 10, 25, 50, 100, 200, 300, 500, and 800 mM. To avoid precipitation, the concentration of ferrous sulfate was lowered to 1 mM, and all chlorides were omitted from the medium when silver tolerance was assessed. Growth of CCH7 in cultures containing up to 800 mM ferrous iron was also monitored. In metal tolerance experiments, bacterial growth was recorded as either positive or negative.

Miscellaneous analyses. Transmission electron micrographs were taken on a Corinth series 273 transmission electron microscope by using culture homogenates adsorbed onto carbon-coated copper grids and stained with 1% (wt/vol) tungstophosphoric acid. For scanning electron microscopy, bacteria were fixed in 5% glutaraldehyde overnight, dehydrated in acetone, and critical point dried in liquid CO_2 before being viewed with a Hitachi S-120 scanning electron microscope. For DNA isolation and determination of base composition, isolate CCH7 was grown in a 1 mM ferrous sulfate–0.02% yeast extract medium (1 liter). Bacteria were harvested and acid washed. The methods used for DNA extraction and determination of guanine-plus-cytosine (G+C) content (melting-point analysis) have been described elsewhere (6). The carbon, nitrogen, and hydrogen contents of isolate CCH7 (grown in 0.025% tryptone soy broth–1 mM ferrous sulfate) were determined by using a Carlo-Erbo elemental analyser (model 1106).

Other acidophilic bacteria. The following were used as reference microorganisms: *T. ferrooxidans* (type strain, NCIB 11820), *L. ferrooxidans* (the original Markosyan isolate), and *Acidiphilium cryptum*. *T. ferrooxidans* and *A. cryptum* were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, and *L. ferrooxidans* was provided by Paul Norris of Warwick University, Warwick, United Kingdom.

RESULTS

Isolate CCH7 was originally observed as a small, off-white or cream-colored colony on FeTSB media. As such, it was classified as an acidophilic heterotroph, but unlike other such isolates it formed macroscopic, gelatinous growths when grown in liquid media and oxidized ferrous iron. In batch cultures of CCH7, filamentous growths resembling

small acid streamers were usually evident after 2 to 4 days. These were initially creamy white in color, but became voluminous and increasingly encrusted with ferric iron precipitates. After about 7 days of incubation, the macroscopic growths began to break up and become less evident. Microscopic examination revealed that young cultures were composed of entwined bacterial filaments of >100 μm average length but that in older cultures these had, for the most part, disintegrated, producing unicellular motile rods (ca. 4 by 0.5 μm; Fig. 1 and 2). Within the filaments of CCH7, spaces corresponding to unit cell lengths were frequently observed, as shown in Fig. 2. Thin, tubular, and smooth sheathlike structures were found to bridge these voids. The unicellular rods of CCH7 were gram negative and did not produce endospores. Ferric iron deposits did not appear to form on the filaments themselves (as with the sheathed neutrophilic bacteria) but occurred as discrete globular deposits within the bacterial matrix. Isolate CCH7 grew in media poised initially between pH 2.0 and 4.4 (optimum at 3.0), but no growth was observed at pH 1.7 or 5.7. The isolate was mesophilic in that it grew between 10 and 30°C but not at 37°C or above. Growth and iron oxidation were observed in yeast extract-enriched acid mine drainage. Colonies of isolate CCH7 developed both on the surface and within the top 2 cm (although no lower) of inoculated roll tubes, indicating that the bacterium was capable of growth under reduced oxygen tensions but not anaerobically.

Attempts at growing isolate CCH7 in the open laboratory were unsuccessful until it was noted that the bacterium was inhibited by exposure to light. Batch cultures exposed to daylight neither grew nor oxidized ferrous iron, in contrast to control cultures kept in the dark. When light-exposed cultures were subsequently incubated in the dark, they again failed to grow, indicating that the light effect was lethal rather than merely inhibitory. When grown in continuous culture, isolate CCH7 produced long streamerlike growths (up to 10 cm) which attached to various supports within the fermentor vessel but only when light was excluded from the growth chamber. Macroscopic growths were not iron encrusted when CCH7 was grown in continuous culture.

On solid media, isolate CCH7 produced small (<1 mm), raised colonies, which became ferric iron stained with prolonged incubation. However, growth was sporadic and only successful when fresh medium was used. Plating efficiency was improved by prewashing the gelling agent (agarose) with distilled water prior to medium preparation.

The rate of ferrous iron oxidation by isolate CCH7 was very much slower than that by either *T. ferrooxidans* or *L. ferrooxidans* (Fig. 3). No ferrous iron oxidation was observed in uninoculated controls. The data shown correspond to mean generation times of 6.5, 11, and 30 h for *T. ferrooxidans*, *L. ferrooxidans*, and CCH7, respectively. However, iron oxidation by CCH7 was much faster when the isolate was grown in media initially poised between pH 2.75 and 3.0 (mean generation times of between 12 and 15 h), and it was noted that similar growth rates could be achieved at pH 2.0 after continued subculturing at this pH. When grown in the absence of an organic substrate, ferrous iron oxidation did not go to completion (Fig. 3) and was insignificant when further subcultured in inorganic media. Isolate CCH7 demonstrated a requirement for iron which was greater than that observed for other acidophilic heterotrophs such as *A. cryptum*. Continued subculturing in media devoid of added iron was not successful but was achieved when either ferrous or ferric sulfate (at 250 μM and greater) was added. The iron-oxidizing acidophiles *T. ferrooxidans* and *L.*

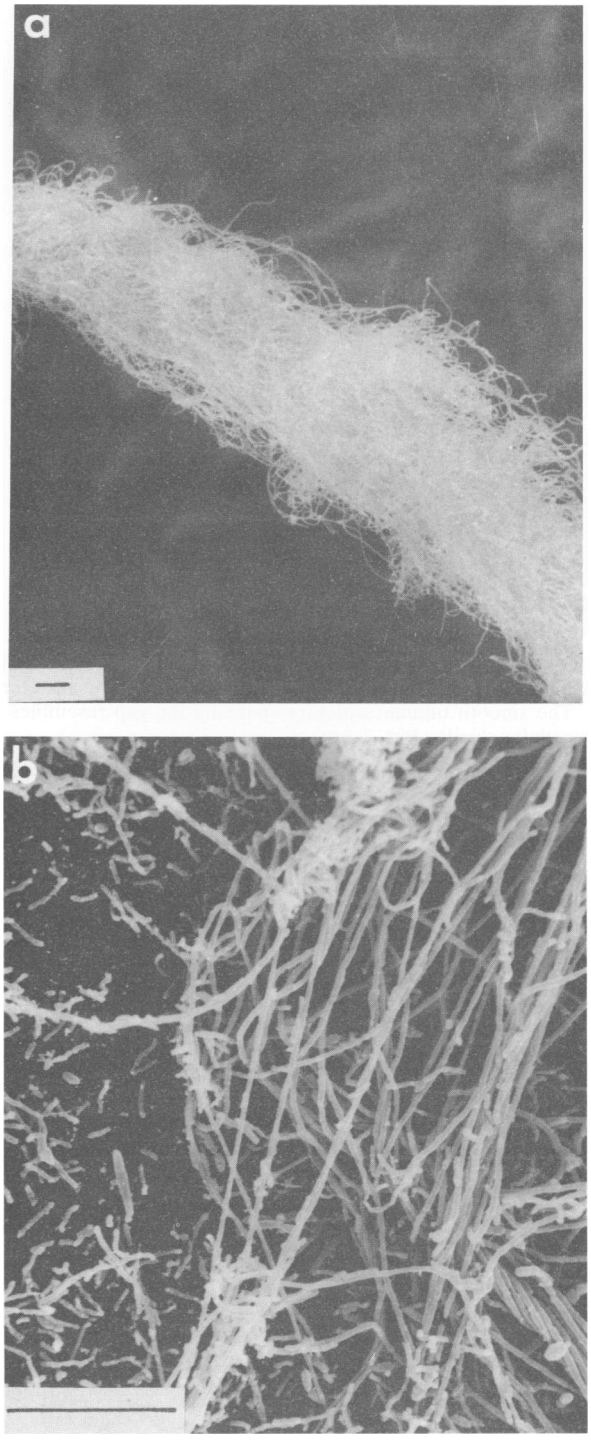

FIG. 1. Scanning electron micrographs of isolate CCH7. The streamerlike structure, formed by entwined bacterial filaments, was sampled at the exponential growth phase (a) and at the stationary growth phase, at which time the filaments have begun to degenerate and single cells have become more abundant (b). Cells were grown in a 1 mM ferrous sulfate–0.02% (wt/vol) yeast extract liquid medium at pH 2.3. Bar, 10 μm.

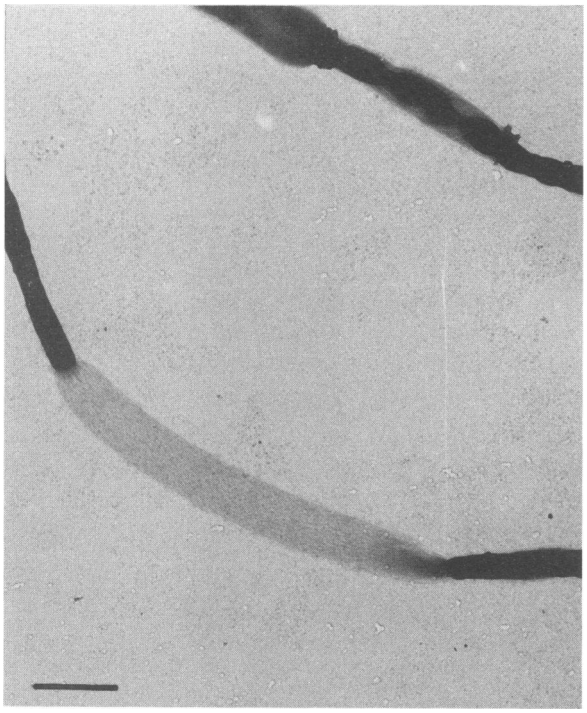

FIG. 2. Transmission electron micrograph of isolate CCH7 showing an area of a filament which has been vacated by a bacterial cell. The smooth tubular structure spanning the gap resembles an extracellular sheath. Bar, 1 μm.

ferrooxidans utilize the energy from ferrous iron oxidation for biomass production. However, increasing concentrations of ferrous sulfate did not result in increased cell biomass of isolate CCH7 (Fig. 4). The filamentous nature of CCH7 and the practical problems encountered in dispersing the gelatinous growths without disrupting and destroying bacterial cells produced large standard errors in direct cell counts.

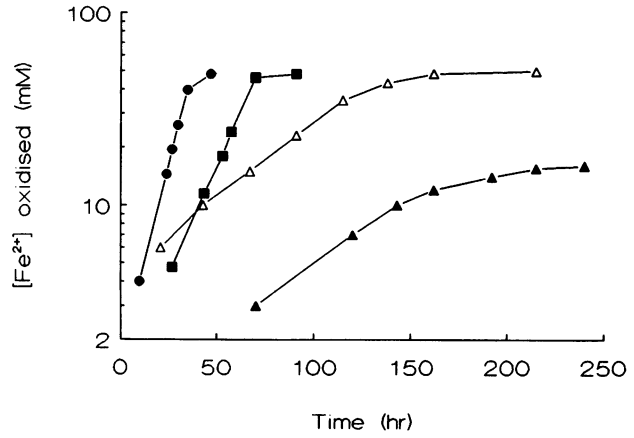

FIG. 3. Ferrous iron oxidation by isolate CCH7 in comparison with those by *T. ferrooxidans* and *L. ferrooxidans*. Cultures were grown in 50 mM ferrous sulfate (closed symbols) or 50 mM ferrous sulfate–0.02% (wt/vol) yeast extract (CCH7 only; open symbol). Both media were poised initially at pH 2.0, and ferrous iron concentrations were monitored by regular titration with 1 mM potassium permanganate. No oxidation occurred in uninoculated controls. Symbols: △, CCH7 grown in ferrous sulfate-yeast extract medium; ▲, CCH7 grown in ferrous sulfate medium; ●, *T. ferrooxidans*; ■, *L. ferrooxidans*.

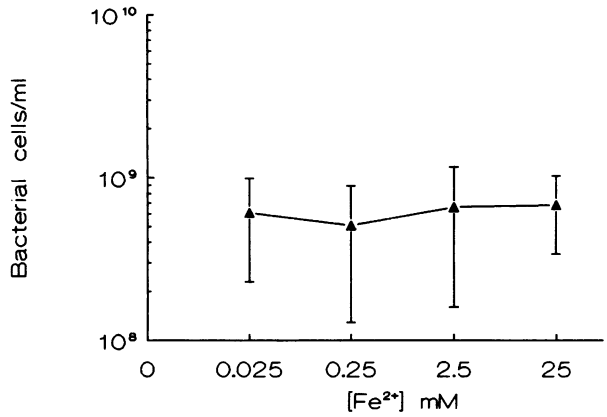

FIG. 4. Effect of different ferrous sulfate concentrations on biomass of isolate CCH7. The bacterium was grown in a lean organic liquid medium (0.0025% tryptone soy broth [pH 2.0]) containing ferrous sulfate at different concentrations. At stationary phase, whole cultures were harvested and homogenized to disperse the gelatinous bacterial growths. Cell numbers were estimated by using acridine orange direct counts. The bars represent standard deviations.

No oxidation of pyrite, chalcopyrite, elemental sulfur, or manganese(II) by isolate CCH7 occurred in yeast extract-free or yeast extract-amended cultures, although oxidation of sulfur, pyrite, and chalcopyrite was observed in control cultures of *T. ferrooxidans* (data not shown). In contrast to some other heterotrophic acidophiles (17), isolate CCH7 was unable to reduce ferric iron in microaerophilic or anoxic cultures.

Isolate CCH7 did not display any capacity to grow autotrophically when oxidizing ferrous iron (Fig. 5). There was no evidence of carbon dioxide fixation when grown in ferrous sulfate-yeast extract or ferrous sulfate medium (growth in the latter being limited, as described above). In

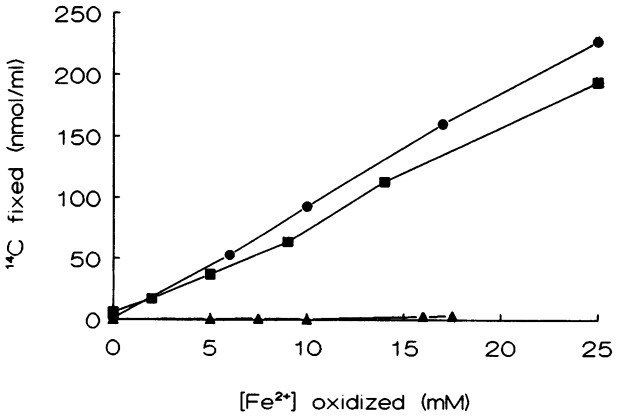

FIG. 5. Incorporation of $^{14}CO_2$ by isolate CCH7 during oxidation of ferrous sulfate in comparison with those by *T. ferrooxidans* and *L. ferrooxidans*. Cells were grown in 25 mM ferrous sulfate medium (pH 2.0) containing 5 mM $NaH^{14}CO_3$ (specific activity, 20,000 cpm/μmol), and the amount of ^{14}C in biomass was assessed at various intervals. Symbols: ▲, CCH7; ●, *T. ferrooxidans*; ■, *L. ferrooxidans*.

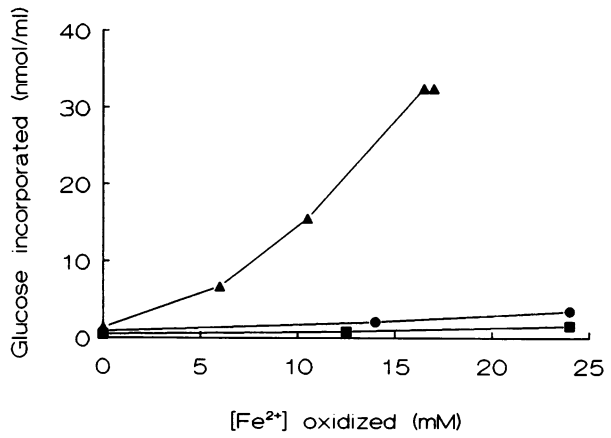

FIG. 6. Incorporation of [U-^{14}C]glucose by isolate CCH7 during oxidation of ferrous sulfate, in comparison with those by *T. ferrooxidans* and *L. ferrooxidans*. Cells were grown in 25 mM ferrous sulfate medium (pH 2.0) containing 1 mM glucose (specific activity, 90,000 cpm/μmol), and the amount of ^{14}C in biomass was assessed at various intervals. Symbols: ▲, CCH7; ●, *T. ferrooxidans*; ■, *L. ferrooxidans*.

contrast to *T. ferrooxidans* and *L. ferrooxidans*, CCH7 displayed pronounced incorporation of glucose (Fig. 6) and glycine (data not shown). Other organic substrates (mannitol, glutamic acid, citric acid, and ethanol) were also metabolized by CCH7, although, in each case, growth was substantially less when yeast extract was omitted from the medium. This is illustrated in Fig. 6, where, in a glucose-ferrous sulfate medium, both iron oxidation and glucose incorporation halted when approximately 60% of available ferrous iron had been oxidized.

The tolerance to some heavy metals of isolate CCH7, in comparison with those of other acidophilic bacteria, is shown in Table 1. The filamentous isolate tended to be more sensitive to the metals tested than other acidophiles. The greatest degree of tolerance recorded was for ferrous iron, although, in contrast, ferric iron was relatively toxic to CCH7.

The elemental composition of ferric iron-free CCH7 was 45% carbon, 12.3% nitrogen, and 8.1% hydrogen. The G+C content of its chromosomal DNA was 62 mol%. No plasmids were detected in isolate CCH7 during extraction and processing of its DNA.

DISCUSSION

The isolate described in this paper, CCH7, would seem to be distinct from previously described acidophilic bacteria. In common with *T. ferrooxidans* and *L. ferrooxidans*, CCH7 has the capacity to oxidize ferrous iron, but, in contrast to them, it appears not to utilize the albeit relatively small amount of energy released from the oxidation and is unable to fix carbon dioxide. The heterotrophic growth of *T. ferrooxidans* reported earlier is now generally accepted to have been the result of the undetected presence of heterotrophic contaminant bacteria in cultures. It is conceivable that ferrous iron oxidation by an acidophilic heterotrophic bacterium could also be due to the inadvertent use of mixed cultures. In the work described, cultures have been routinely screened throughout for the presence of *T. ferrooxidans* and *L. ferrooxidans* by using a number of techniques such as plating on media which are both selective and highly efficient for all autotrophic iron-oxidizing acidophiles (18). Further evidence that cultures of CCH7 did not contain acidophilic chemolithotrophic bacteria came from the observations of the lack of autotrophic capacity, the inability to completely oxidize iron in organic matter-free media, and the much slower rates of iron oxidation than exhibited by either *T. ferrooxidans* or *L. ferrooxidans*.

TABLE 1. Tolerance of isolate CCH7 to some heavy metals and comparison of CCH7 with other acidophiles[a]

Bacterium	MIC of:					
	$[Fe^{2+}]$ (mM)	$[Fe^{3+}]$ (mM)	$[Cu^{2+}]$ (mM)	$[UO_2^{2+}]$ (mM)	$[MoO_4^{2-}]$ (mM)	$[Ag^+]$ (μM)
Isolate CCH7	300	50	20	2.0	0.2	1.0
T. ferrooxidans	800	800	400	2.0	0.2	10
L. ferrooxidans	500	500	5.0	5.0	0.2	2.0
A. cryptum	300	300	50	0.5	0.1	0.5

[a] Cultures were grown in liquid media (1 mM ferrous sulfate–0.025% tryptone soy broth for CCH7 and *A. cryptum*; 10 mM ferrous sulfate for *T. ferrooxidans* and *L. ferrooxidans*). Results were recorded as positive (growth) or negative (no growth).

Morphologically, isolate CCH7 is again distinct from other mesophilic, acidophilic heterotrophic bacteria that have been described. The ability to form long chains and filaments is not in itself an unusual prokaryotic feature. The entangled filaments superficially resemble actinomycete growths, but CCH7 has not been found to produce sporangia (or indeed endospores); filaments are unbranched (or show false branching), and individual cells can be seen within them. Isolate CCH7 shows some superficial resemblance to an autotrophic iron-oxidizing bacterium isolated by Cameron et al. (2) and to a moderately thermophilic spore-forming isolate described by Emtiazi et al. (5). However, although both of these isolates were acidophilic and filamentous, they differed from the present isolate in a number of fundamental aspects (autotrophic capacity and temperature response, etc.). In its morphology and physiology, isolate CCH7 would seem to resemble most closely the iron bacterium members of the *Sphaerotilus-Leptothrix* group. In common with isolate CCH7, these bacteria are filamentous, form macroscopic slimy streamerlike growths, and consist of motile rods which vacate the filaments when conditions become suboptimal (as in the late growth phase of CCH7). There are significant differences, however. Isolate CCH7 is obligately acidophilic and has been shown, unequivocally, to catalyze the oxidation of ferrous iron, whereas all documented strains of *Sphaerotilus* and *Leptothrix* spp. are neutrophilic and are thought to accumulate oxidized iron compounds on their extracellular slime layer or sheath rather than directly oxidize ferrous iron (although differentiation of biological and chemical ferrous iron oxidation is difficult at circumneutral pH values). A characteristic feature of the *Sphaerotilus-Leptothrix* group is the sheath which envelopes bacterial cells during filamentous growth. Evidence for sheath production by isolate CCH7 is not as yet conclusive and requires further, detailed work on the ultrastructure of the filaments. However, microscopic evidence indicates that the sheath in CCH7 is smooth rather than rough, which is in common with *Sphaerotilus* sp. rather than *Leptothrix* sp. Like *Sphaerotilus natans*, isolate CCH7 was unable to oxidize manganese(II), grew at low pO_2 values, and is an obligate heterotroph. It would appear, therefore, that isolate CCH7 may be an acidophilic species of the genus *Sphaerotilus*. However, while its G+C content is significantly greater than that of either *T. ferrooxidans* or *L.*

ferrooxidans (10), it is less than the 69 to 71 mol% reported for the *Sphaerotilus-Leptothrix* group (20).

Unlike the chemolithotrophic iron oxidizers *T. ferrooxidans* and *L. ferrooxidans*, CCH7 appears unable to utilize the energy released from iron oxidation. The free energy associated with ferrous iron oxidation is about 100 times less (in terms of kilojoules per mole) than that of glucose catabolism to carbon dioxide and water (19). Therefore, the design of the experiment which sought to elucidate whether isolate CCH7 utilized the energy from iron oxidation necessarily had to use media with a high ferrous iron/organic substrate ratio. In the 25 mM ferrous sulfate–0.0025% tryptone soy broth medium, the calculated free energy yields were about 0.75 kJ from iron oxidation and 0.3 kJ from catabolism of the organic moiety (calculated as glucose, which is a component of tryptone soy broth). The stoichiometry of ferrous iron oxidized to glucose metabolized found in another experiment (Fig. 6) was about 300:1, if it is assumed that 50% of the glucose was assimilated into biomass and 50% of the glucose was respired. Since cell numbers in this medium were not significantly ($P < 0.05$) different from those in media containing the same concentrations of tryptone soy broth but lower concentrations of ferrous sulfate, it was concluded that there was no evidence from this experiment to suggest that isolate CCH7 is a chemolithoheterotroph, making simultaneous use of energy from iron oxidation and the availability of organic substrates.

In its natural environment, CCH7 lives in association with a range of other acidophilic bacteria, including *T. ferrooxidans*, *L. ferrooxidans*, and *Acidiphilium*-like bacteria (14). Its contribution to total iron oxidation in situ is likely to be small in comparison to that catalyzed by chemolithotrophic bacteria. The inability of CCH7 to oxidize pyrite and chalcopyrite would imply that its potential as a biomining bacterium is negligible. Since isolate CCH7 is able to oxidize ferrous iron, it should theoretically be able to degrade pyrite by the indirect pathway (i.e., by regenerating ferric iron), and its observed inability to do so may be due to some other, unidentified factors (e.g., metal toxicity). In acidic, metalliferous environments, however, isolate CCH7 may be a highly significant microorganism. It is the only acidophilic isolate from acid streamers that forms large streamerlike growths in vitro in a relatively short time (14), and it is considered to be a major contributor to total acid streamer biomass, at least in Cae Coch. In an earlier work, Dugan et al. (3) isolated a bacilluslike isolate from mine water containing acid streamers, which also produced gelatinous macroscopic growths in vitro. However, in contrast to isolate CCH7, the isolate of Dugan et al. (3) was obligately neutrophilic and was not reported to oxidize ferrous iron. Bacterial filaments, similar morphologically to those of CCH7, are very common in native Cae Coch streamers. Future research will attempt to quantify the significance of CCH7 and other isolates in the acid-streamer community, by using such techniques as 16S RNA sequencing.

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