An Acid-tolerant Iron-oxidizing Metallogenium

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SUMMARY

A filamentous iron bacterium similar to some reported forms of *Gallionella* and *Metallogenium* has been isolated in pure culture from a number of widely distributed iron-bearing streams. This organism grows in multi-branching colonies of iron-encrusted filaments (diameter: 0.1 to 0.4 μ m) without a conventional cell body. Differentiation of this bacterium from *Gallionella* and other forms of *Metallogenium* is based upon morphology, growth requirements, and ability to catalyse iron oxidation under meso-acidic conditions.

INTRODUCTION

Gallionella ferruginea has been characterized as a bean-shaped cell with twisted non-living stalks exuded from side or ends (Cholodny, 1924). These stalks are the site of precipitation of ferric iron compounds as demonstrated by a strong Prussian blue reaction with ferrocyanide. Pringsheim (1949) classified *G. ferruginea* as an iron bacterium because of its ability to precipitate iron from iron-bearing waters. Both Pringsheim (1949) and Vatter & Wolfe (1956) concluded that the stalks were non-living exudates of the cell. More recently, van Iterson (1958), Balashova (1967, 1968, 1969), Engel & Hanert (1967) and Hanert (1968, 1970) have suggested that the stalks of *Gallionella* may be living elements.

The question of the viability of these stalks has been complicated by Zavarin's (1961) suggestion that the forms of *Gallionella* which have viable stalks may be *Metallogenium* spp. He described *Metallogenium symbioticum* as a filamentous organism growing in iron-rich environments with *Leptothrix* which oxidizes and precipitates manganese. The organism *M. symbioticum* grew in two forms: as filaments without cells and as cells which sprouted filaments. Zavarzin observed (1964) that *M. symbioticum* grew symbiotically with an unspecified fungus. Dubinina (1969) reported that *Metallogenium* sp. grown on horse serum, could be obtained in pure culture and did not lose its ability to oxidize or precipitate manganese. She also noted (1970) a number of pleomorphic filamentous forms in older cultures grown on manganese which were of uneven thickness and contained electron-dense granules.

The organism described here grows as filaments encrusted with ferric iron compounds. It is morphologically similar to the rose garland form of *Metallogenium* described by Dubinina (1970). It oxidizes and precipitates ferrous iron compounds, lacks a conventional cell body and grows as filaments which are self-reproducing units. We have classified it as an iron-oxidizing strain of *Metallogenium*.

METHODS

Organism sources. The iron content and pH of samples from five widely separated iron-bearing ground waters, less than 1 week old when received, were measured. The samples comprised: coal mine drainage water from Illinois (50 mg iron/l, pH 3.5); ditch water

Table 1. *Growth of the iron-oxidizing organism on various media*

Isolates were initially grown on the minimal medium (MM) buffered with phthalate and containing formalin (see text).

Medium	Initial pH	Ferrous iron concentration (mg/l)				
		0	10	40	150	300
Minimal medium (MM)	6.8	—	+	(+)	—	—
Minimal medium	4.0	—	+	+	—	—
MM + 0.4 % phthalate	4.1	—	(+)	++	+++	+++
MM + 0.2 % phthalate	4.1	—	(+)	++	++	(+)
MM + agarose (1.5 %)	6.5	—	—	(+)	++	(+)
MM + glass beads	6.5	—	—	+	(+)	—
MM + glass beads	4.2	—	—	+	+	—

from New Hampshire (1.5 mg iron/l, pH 6.7); coal mine drainage water from W. Virginia (125 mg iron/l, pH 2.5); zinc mine drainage water from New Jersey (27 mg iron/l, pH 5.7); and stream water from Vermont (2.5 mg iron/l, pH 6.8). The first two samples were obtained in October, the third in September, the fourth in August and the last in May.

Organism isolation. The isolation medium contained: $(\text{NH}_4)_2\text{SO}_4$, 0.1 %; CaCO_3 , 0.01 %; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 %; KH phthalate, 0.4 %; KH_2PO_4 , 0.001 %. This medium was autoclaved before addition of ferrous iron, which was prepared as a 9 % $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution acidified to pH 1 with conc. H_2SO_4 before autoclaving. The isolation medium was finally adjusted to pH 4.1 with 0.2 N-NaOH. During initial isolation of organisms, but not for routine cultivation, 0.4 % formalin was added. Nunley & Krieg (1968) used 0.4 % formalin to obtain cultures of *Gallionella ferruginea* free from heterotrophic contaminants. We observed that this concentration of formalin inhibited growth of the acid-tolerant (pH 3.5) non-filamentous iron bacterium *Thiobacillus ferrooxidans*.

A 0.5 ml inoculum was added to 100 ml of this phthalate isolation medium with formalin and incubated at 22 °C. Isolates of a filamentous iron bacterium were obtained from all five sources after three subculture transfers.

Two other media (Table 1), both without phthalate but with the concentrations of salts listed above, were used. The first was identical with the isolation medium except that instead of phthalate it contained 10 g of 0.1 mm glass beads/100 ml. In the second, 100 ml of the salts solution without phthalate or iron was added to 50 ml of solidified 1.5 % agarose in a 250 ml flask to which the ferrous iron had been added just before solidification.

Preparation for morphological observation. Samples (20 ml) of 3-day cultures from the phthalate growth medium were centrifuged at 13000 g. The resultant pellet was washed gently with 0.05 % oxalic acid solution and with the sterile growth medium (without iron). This washing was repeated three times and the filaments observed by phase contrast and electron microscopy (Hitachi HU 11). Incomplete removal of the ferric iron from the filaments was shown by a positive Prussian blue reaction with potassium ferrocyanide. We tried washing with other organic and inorganic acids but found that concentrations sufficient to dissolve the ferric iron precipitate lysed the filaments, as did higher concentrations of oxalic acid.

Organism-growth. We measured oxygen uptake, DNA and protein production, biomass and ferrous iron oxidation catalysis. A Gibson micro-respirometer was used to measure oxygen uptake. DNA and cell-protein production were followed by using the diphenylamine method (Burton, 1956) and Folin's phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951), respectively. Both DNA and protein determinations were made on washed bacteria

from 10 ml samples because high ferrous iron concentrations were found to interfere. Ferrous iron oxidation catalysis was measured by following ferrous iron concentrations by acid permanganate titration of a 1 ml sample using an *o*-phenanthroline indicator; the concentration of iron in solution, measured by atomic absorption at 247.7 nm did not change significantly during the test period.

A 0.5 ml inoculum was used in the catalysis experiments. Determination of the number of active filaments in this inoculum was difficult. Estimation of cell numbers by plate counts was impossible because the organism does not form conventional colonies on solid media. We estimated the number of filaments by a most-probable-number technique in the phthalate-iron medium, using pipettes with especially broad tips. By this method we observed that 5-day cultures reached a relatively stable population size of 10^5 viable filaments/ml, provided the initial ferrous iron concentration was 250 mg/l. This method was not reliable because shaking broke the filaments into smaller viable units. The protein concentration of the 5-day cultures was constant at 0.02 mg/ml.

For biomass determination, which was hampered by the ferric iron precipitate on the filaments, the pellet obtained by centrifuging 20 ml of a 5-day culture was weighed after drying overnight at 70 °C; biological material should not have been lost at this temperature. However, not all of the water of hydration may have been driven from the iron precipitate. The weighed pellet was suspended in 0.1 N-H₂SO₄ and the total iron content measured by atomic absorption spectrometry. The iron salts on the filaments may have been any combination of the hydrated forms of ferric hydroxide, carbonate, phosphate, or sulphate. Of these an equivalent of iron would weigh most as ferric sulphate and least as ferric hydroxide. The difference between the pellet weight (average 702 mg/l) and the calculated weight of ferric sulphate (average 621 mg/l) or ferric hydroxide (average 334 mg/l) gave the range of biomass produced in 5 days. The percent biological material in the pellet thus ranged from 12 to 48 %.

Sensitivity to antibiotics. The filamentous iron bacterium was tested for sensitivity to penicillin and chloramphenicol in the phthalate medium at pH 4.1. The penicillin strength varied from 8 to 331 units/ml; the chloramphenicol from 0.0025 to 0.025 mg/ml. Penicillin at 83 units/ml, and chloramphenicol at 0.005 mg/l, inhibited growth of acid mine water heterotrophs which were cultured routinely on the phthalate medium.

Filterability. Cultures of the filamentous iron bacterium were passed sequentially through a series of six Millipore filters with pore sizes from 1.2 to 0.025 μ m. Inocula from each of the filtrates were added to 100 ml of fresh phthalate medium. The original culture was also passed through each filter-size separately, the only difference being that cultures passed through only one filter-size grew more quickly.

RESULTS

Isolation. The isolates obtained from the five water samples were morphologically similar and had similar abilities to catalyse ferrous iron oxidation under meso-acidic conditions (pH 4.1). They would not grow in any medium without ferrous iron. Culture purity of the isolates was indicated by the absence of contaminant growth on the following media: Chlorella agar (Difco); Nitrosomonas medium (Pramer & Schmidt, 1964); nutrient agar (Difco); Sabouraud's dextrose agar (Difco); Thiobacillus agar (Burnett, Pelczar & Conn, 1957); thioglycollate medium (Difco); *Thiobacillus ferrooxidans* 9K medium (Silverman & Lundgren, 1959); trypticase soy broth supplemented with 0.5 % yeast extract (Difco); Wolfe's medium with ferrous sulphide (Kucera & Wolfe, 1957); our culture media (Table 1) without ferrous iron.

Fig. 1. Electron micrograph of filaments taken directly from phthalate-buffered growth medium and washed with 0.05 % oxalic acid.

Fig. 2

Fig. 3

Fig. 2. Electron micrograph of a single filament washed three times with 0.05 % oxalic acid solution.

Fig. 3. Electron micrograph of a mass of filaments, morphologically similar to the rose garland form of *Metallogenium* reported by Dubinina (1970).

Morphology. The bacterial growth in the liquid phthalate medium appeared as a rusty flocculent precipitate. When this was washed carefully with 0.05 % oxalic acid solution, the filamentous organisms (diameter: 0.1 to 0.4 μm) could be seen under phase contrast. We have never observed conventional cell body structures. Fig. 1 is an electron micrograph of acid-washed filaments from the phthalate medium. Fig. 2 shows a single filament almost free of iron precipitate. The electron-dense granules at irregular intervals along the filament may mark the sites of future filament growth. With the electron microscope a partially cleaned bacterial mass appeared to contain a number of interwoven branching filaments of various lengths and diameters without a central locus of radiation (Fig. 3).

Acid tolerance. The organism has a wide pH tolerance (3.5 to 6.8) with growth optimum

Fig. 4. Effect of initial ferrous iron concentration on respiration rates. Data corrected for abiotic oxygen uptake. Initial pH 4.1 buffered with 0.024 M-phthalate. Δ — Δ , 40 mg ferrous iron/l; $\bullet$ — $\bullet$, 300 mg ferrous iron/l; $\circ$ — $\circ$, 900 mg ferrous iron/l.

at pH 4.1. Phthalate (0.024 M) was used to buffer the medium at this pH value; significant growth did not occur when acetate, citrate or phosphate were substituted for phthalate, or at less than pH 3.5. The organism did not grow in the phthalate medium in the absence of ferrous iron.

Ferrous iron inhibition. In the phthalate (0.024 M) at pH 4.1 good growth of the organism was observed for initial ferrous iron concentrations as high as 400 mg/l. In its absence, growth was inhibited at initial ferrous iron concentrations greater than 100 mg/l with a growth optimum of 40 mg/l. Growth at ferrous iron concentrations as high as 150 mg/l in the absence of phthalate could be obtained when the medium contained glass beads or when the iron was incorporated in an agarose layer. The phthalate seemed to form a reversible phthalate-iron complex which limited the ferrous iron available.

The rate of oxygen uptake by the filamentous bacterium was dependent on initial ferrous iron concentrations (Fig. 4). Ferrous iron concentrations greater than 450 or less than 50 mg iron/l decreased oxygen uptake rates below the maximum uptake rates observed at 300 mg iron/l. The inhibitory effect of initial ferrous iron concentrations as high as 600 mg iron/l was decreased by increasing the phthalate concentration to 0.045 M, probably due to the formation of a phthalate-iron complex.

Iron oxidation rate. At an initial pH 4.1 buffered with 0.024 M-phthalate and an initial ferrous iron concentration of 400 mg/l, we observed that the rate of ferrous iron oxidation in the presence of the iron bacterium was 22 mg/l day for the first 50 h (Fig. 5) while the abiotic rate was measured at 0.9 mg/l day. In this experiment an inoculum size of 0.01 mg bacterial protein/100 ml was used.

The rate of DNA and cell protein production mirrored the rate of iron oxidation. The cell-protein concentration increased to 22 mg/l and the DNA level to more than 5.4 mg/l in 50 h for the iron oxidation rate of 22 mg/l day reported above. During the same time period, biomass production was between 81 and 368 mg/l depending on whether the hydrated ferric iron precipitate on the filaments was predominantly a hydroxide or a sulphate. The theoretical content of cell protein in the biomass was thus between 6 and 27 %.

Fig. 5. Ferrous iron oxidation at pH 4.1 in the presence of the stalked iron bacterium. Ferrous iron concentration: in the presence of the bacterium, ●—●; in the sterile control, ○—○.

Typical characteristics. The filamentous iron bacterium was insensitive to less than 150 units of penicillin/ml, but some filament growth was observed at concentrations as high as 331 units/ml. Growth of heterotrophs isolated from acidic mine drainage samples was entirely inhibited by concentrations greater than 83 units penicillin/ml in the phthalate medium. The iron bacterium and the heterotrophs were sensitive to chloramphenicol, with strong inhibition at concentrations as low as 0.005 mg/ml.

The smallest viable unit of the filamentous iron bacterium has not been identified. However, filaments producing growth passed through a 0.22 μm pore-size filter but not through a 0.025 μm filter. The smallest viable filaments thus had a diameter between 0.025 to 0.22 μm , although their minimal length is still undetermined.

DISCUSSION

The bacterium described in this study differs from *Gallionella ferruginea* as described by Cholodny (1924), Pringsheim (1949) and Vatter & Wolfe (1956) in its absence of a conventional cell body and in its growth optimum of pH 4.1. It resembles the unusual *Gallionella* forms of van Itersen (1958), Balashova (1968) and Hanert (1968) in that the filaments are viable units. It differs from *Thiobacillus ferrooxidans* in morphology and in its ability to grow in the presence of 0.4 % formalin. It also differs from *Caulobacter* (Poindexter, 1964) in its requirement of ferrous iron for growth and in the encrustation of the filaments with ferric iron compounds. It is morphologically similar to the rose garland form of the manganese bacterium, *Metallogenium*, described by Dubinina (1970). However, we observed only infrequent growth of this filamentous iron bacterium in the *Metallogenium symbioticum* medium described by Zavarzin (1964) which contains starch and manganese. Thus, while our filamentous iron bacterium is similar to some of the previously reported forms of *Metallogenium*, it appears to be sufficiently different to be considered a new strain (Table 2).

The role of ferrous iron in the metabolism of this filamentous iron bacterium is not clear.

Table 2. Comparison of the characteristics of our filamentous iron bacterium with those of *Gallionella* and *Metallogenium*

	Filamentous iron bacterium	<i>Metallogenium</i>	<i>Gallionella</i>
Viable stalk or filament	+	+	—
Conventional cell body	—	(+)*	+
Filterability	+	+	(—)†
Penicillin sensitive (< 150 units/ml)	—	—	+
Utilizes iron	+	—	+
Utilizes manganese	(+)‡	+	—

* Pleomorphic.

† 'Unusual' forms of Hanert (1968), Balashova (1968) and van Itersen (1958) reported as filterable.

‡ Very slow growth.

The organism apparently requires some iron for growth but is inhibited by higher iron concentrations. This dependence on, yet inhibition by, ferrous iron is similar to the response to manganese reported by Zavarzin (1964) for *Metallogenium symbioticum*. Further work by Dubinina (1969, 1970) has not explained the dependence of *M. symbioticum* on manganese but has shown that growth of the organism is dependent on some organic growth factor which can be supplied by symbiotic growth with fungi. Similarly, we consider the response of our filamentous iron bacterium to iron to be that of an organism which may use either iron or organic material oxidation as energy source. However, our data are not enough to identify the relative importance to it of autotrophic or heterotrophic energy-yielding metabolisms in our filamentous bacterium.

Our results, taken with the recent work of Hanert (1970) and Balashova (1967, 1968, 1969), suggest that those filamentous iron bacteria which lack a conventional cell body may be *Metallogenium* rather than *Gallionella* strains. The presence or absence of a conventional cell body and the filamentous form are but two genus characterizing factors. Species differentiation in *Metallogenium* can be based on acid tolerance, ferrous-iron tolerance, oxygen and carbon-dioxide requirements, growth medium, pH value requirement and ferrous iron (or manganese) oxidation catalysis rate. The filamentous iron bacterium isolated in the present study significantly catalysed iron oxidation in the range pH 3.5 to 5.0 with an optimum at pH 4.1 and was sensitive to concentrations of non-complexed ferrous iron above 50 mg/l.

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