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Original article

Acidithrix ferrooxidans gen. nov., sp. nov.; a filamentous and obligately heterotrophic, acidophilic member of the *Actinobacteria* that catalyzes dissimilatory oxido-reduction of iron

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Abstract

A novel acidophilic member of the phylum *Actinobacteria* was isolated from an acidic stream draining an abandoned copper mine in north Wales. The isolate (PY-F3) was demonstrated to be a heterotroph that catalyzed the oxidation of ferrous iron (but not of sulfur or hydrogen) under aerobic conditions, and the reduction of ferric iron under micro-aerobic and anaerobic conditions. PY-F3 formed long entangled filaments of cells (>50 μm long) during active growth phases, though these degenerated into smaller fragments and single cells in late stationary phase. Although isolate PY-F3 was not observed to grow below pH 2.0 and 10 °C, harvested biomass was found to oxidize ferrous iron at relatively fast rates at pH 1.5 and 5 °C. Phylogenetic analysis, based on comparisons of 16S rRNA gene sequences, showed that isolate PY-F3 has 91–93% gene similarity to those of the four classified genera and species of acidophilic *Actinobacteria*, and therefore is a representative of a novel genus. The binomial *Acidithrix ferrooxidans* is proposed for this new species, with PY-F3 as the designated type strain (=DSM 28176^T, =JCM 19728^T).

Keywords: Acidophile; *Actinobacteria*; Iron oxidation; Iron reduction; Novel species

1. Introduction

Extremely acidic environments, generally considered as those with pH values of <3, can be populated by a physiologically and phylogenetically diverse range of prokaryotic and eukaryotic microorganisms [2,14]. Many very low pH environments are associated with microbially-accelerated oxidation of sulfur and sulfide minerals, for example in areas impacted by mining [25]. Although chemolithotrophic iron- and sulfur-oxidizing bacteria and archaea are the most widely studied acidophilic microorganisms, obligate and facultative heterotrophic acidophiles live in the same environments, sometimes forming intimate associations with acidophilic algae and autotrophic bacteria (e.g. [7]). "Mixotrophic" nutrition, in which an inorganic electron donor is used as an energy source, and an organic substance as a carbon source, is exhibited by a number of acidophilic prokaryotes, such as *Acidiphilium acidophilum* [9] and *Sulfobacillus* spp. [16,22].

Gram-positive extremely acidophilic bacteria currently comprise two phyla: the low GC, spore-forming *Firmicutes* (*Sulfobacillus* and *Alicyclobacillus* spp.) and the non-sporulating *Actinobacteria*. The latter includes four validated genera, all of which currently contain a single species: *Acidimicrobium (Am.) ferrooxidans* [5], *Aciditerrimonas (Act.) ferrireducens* [13] *Ferrimicrobium (Fm.) acidiphilum* and *Ferrithrix (Fx.) thermotolerans* [20], and one candidate genus ("*Acidithiomicrobium*" [6]). All of these are either facultative or obligate heterotrophs. Another unifying characteristic is their abilities to catalyze the dissimilatory reduction of ferric

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iron, though this trait has apparently not yet been tested for with "*Acidithiomicrobium*". While all species, except *Act. ferrireducens*, also oxidize ferrous iron, other characteristics such as cell morphologies and temperature characteristics are more variable between the acidophilic *Actinobacteria*.

Acid mine drainage (AMD) streams that flow from mines and mine wastes are typical of the kind of environment that is populated by acidophilic prokaryotes [3]. A study of growth of macroscopic "acid streamers" in an pH 2.5 AMD stream in Wales found that the dominant prokaryotes present from the stream's inception through to nine years later were the iron-oxidizer "*Ferrovum (Fv.) myxofaciens*" [19], the iron/sulphur oxidizer *Acidithiobacillus (At.) ferrivorans* [10] and an uncharacterized actinobacterium [23]. An iron-oxidizing isolate identified (from restriction enzyme fragment length polymorphism analysis) as corresponding to this actinobacterium was obtained on solid media and given the proposed name "*Acidithrix (Acx.) ferrooxidans*". Here we provide a detailed description of this novel acidophile, and confirm that it is the first and currently sole species of a novel genus of the phylum *Actinobacteria*.

2. Materials and methods

2.1. Isolation and maintenance of strain PY-F3

Strain PY-F3 was isolated from acid streamer growths found in the AMD stream (the Afon Goch) that drains the abandoned Mynydd Parys copper mine in Anglesey, north Wales [23] by streaking disrupted streamers onto a solid medium containing ferrous iron and tryptone soya broth ("FeTSB" [15]). Following repeated single colony isolation using the same solid medium composition, the isolate was transferred into a liquid medium containing 0.025% (w/v) tryptone soya broth and 5 mM ferrous sulfate (pH 2.5), and from there to a medium containing 5 mM glucose, 0.01% (w/v) yeast extract and 100 μM ferrous sulfate and basal salts [24], pH 2.5 ("GYEFe" medium). Cultures stored for periods of up to 6 months at 4 °C remained viable.

2.2. Optimization of a solid medium for cultivating strain PY-F3

Although strain PY-F3 was initially isolated on FeTSB medium, growth tended to be inconsistent and colonies varied greatly in size. A new overlay solid medium was devised (designated FeTSB-Po), a double layer variant of FeTSB medium in which the acidophilic heterotroph used to inoculate the gel underlayer was the type strain (PFBC) of *Acidocella (Ac.) aromatica* [21] rather than *Acidiphilium* sp. SJH (which is used in standard overlay media). While both of these heterotrophic acidophiles can degrade pyruvic acid (formed via acid hydrolysis of the gelling agent, and which is highly toxic to most acidophiles), *Ac. aromatica* (unlike *Acidiphilium* SJH) does not metabolize either glucose or casein hydrolysate (both of which are components of TSB) and these are therefore available to support the growth of heterotrophs in the inocula. A second overlay variant, a gelled version of GYEFe liquid medium in which *Ac. aromatica* was again inoculated into the gel underlayer, was also tested. All solid media were inoculated aerobically at 30 °C.

2.3. Effect of pH and temperature on growth of isolate PY-F3

The effect of temperature on the growth of isolate PY-F3 was determined by growing the bacterium in modified GYEFe liquid medium (containing 20 mM ferrous iron; adjusted to pH 2.5) in shake flasks, at between 8 °C and 38 °C. To determine its optimum and minimum growth pH values, PY-F3 was grown in the same medium in a stirred (100 rpm) and aerated (1 L/min) bioreactor, maintained at 25 °C, with pH maintained between 1.9 and 3.6 by automated addition of 1 M sulfuric or 1 M sodium hydroxide. In both cases, samples were withdrawn at regular intervals to determine concentrations of ferrous iron [26], and culture doubling time obtained from exponential phases in the growth cycle (logarithms of ferrous iron oxidized versus time). Growth of isolate at pH values above 3.6 was tested using GYEFe medium containing 100 μM ferrous iron in shake flasks.

2.4. Specific rates of ferrous iron oxidation

Specific rates of ferrous iron oxidation by strain PY-F3 were determined using a modified version of the technique described elsewhere [17]. Bacteria were grown in GYEFe liquid medium containing either 10 mM or 100 μM ferrous iron, at 30 °C and an initial pH of 2.5. Biomass was harvested at late exponential/early stationary phase by centrifugation, suspended in 50 ml of pH 3.0 basal salts and gently homogenized using a Scientific Pro 200 homogenizer. The protein concentration of the homogenized biomass was measured [4] and aliquots added to 10 ml of a reaction mixture (in 20 ml glass bottles) containing 1 mM ferrous iron and basal salts. Prior to this, the pH of reaction mixtures had been adjusted to between pH 1.0 and 3.5 with sulfuric acid and pre-incubated at between 5 °C and 40 °C. Aliquots were withdrawn at regular intervals for up to 1 h to determine concentrations of ferrous iron. Triplicate analyses were carried out at each temperature and pH, and specific rates determined as the amount of ferrous iron oxidized in unit time per unit quantity of protein.

2.5. Reduction of ferric iron by isolate PY-F3 and screening for growth under microaerobic and anaerobic conditions

Isolate PY-F3 was grown in GYEFe liquid medium containing 5 mM ferrous iron at pH 2.2 and 30 °C. When ferrous iron concentrations had declined to <0.5 mM, the concentration of total soluble iron was determined (by reducing soluble ferric iron to ferrous using ascorbic acid, and repeating the ferrozine analysis). Additional glucose was then added to the culture (to 5 mM), which was shaken to disrupt the streamer biomass, and 20 ml aliquots placed into six sterile 100 ml