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ORIGINAL PAPER

Sulfobacillus benefaciens sp. nov., an acidophilic facultative anaerobic *Firmicute* isolated from mineral bioleaching operations

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Abstract Gram-positive bacteria found as the sole *Firmicutes* present in two mineral bioleaching stirred tanks, and a third bacterium isolated from a heap leaching operation, were shown to be closely related to each other but distinct from characterized acidophilic iron- and sulfur-oxidizing bacteria of the genus *Sulfobacillus*, to which they were affiliated. One of the isolates (BRGM2) was shown to be a thermo-tolerant (temperature optimum 38.5°C, and maximum 47°C) obligate acidophile (pH optimum 1.5, and minimum 0.8), and also noted to be a facultative anaerobe, growing via ferric iron respiration in the absence of oxygen. Although isolates BRGM2 and TVK8 were able to metabolize many monomeric organic substrates, their propensity for autotrophic growth was found to be greater than that of *Sulfobacillus thermosulfidooxidans* and the related acidophile, *Sb. acidophilus*. Faster growth rates of the novel isolates in the absence of organic carbon was considered to be a major reason why they, rather than *Sb. thermosulfidooxidans* (which shared many physiological characteristics) more successfully exploited conditions in the stirred tanks. Based on their phylogenetic and phenotypic characteristics, the isolates are designated strains of the proposed novel species, *Sulfobacillus benefaciens*, with isolate BRGM2 nominated as the type strain.

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Introduction

The ability of some acidophilic prokaryotes to accelerate the oxidative dissolution of sulfide minerals is exploited in an expanding area of biotechnology, generally referred to as "biomining" (Rawlings and Johnson 2007a). Analysis of mineral leachate liquors in stirred-tank and bioheap operations has shown that microbial communities establish in these open, non-sterile operations. These appear, almost invariably, to include primary iron- and sulfur-oxidizing chemolithotrophs, and heterotrophic (or mixotrophic) acidophiles, some of which also are capable of the dissimilatory oxidation of iron and/or sulfur (Okibe et al. 2003; Mikkelsen et al. 2006; Rawlings and Johnson 2007b). While the identities of many of the primary (iron-oxidizing) prokaryotes frequently found in commercial biomining operations are known (e.g., *Leptospirillum ferriphilum* in tanks, and *Acidithiobacillus ferrooxidans* in heaps) it is probable that many (novel) species and genera present have not yet been identified. One reason for this is that the compositions of microbial bioleaching communities appear to vary from site to site, and are determined by a number of factors such as the mineralogical composition of the ore or concentrate being processed, operating temperatures and pH.

The genus *Sulfobacillus* was first described in 1978 (Golovacheva and Karavaiko 1978). Currently, there are four classified species in this genus—*Sb. thermosulfidooxidans, Sb. acidophilus, Sb. thermotolerans* and *Sb. sibiricus.* They are all *Firmicutes* (spore-forming Gram-positive bacteria with chromosomal DNA of low G + C content) that oxidize ferrous iron and reduced forms of sulfur (such as tetrathionate and elemental sulfur) and

which can assimilate both inorganic and organic carbon. These bacteria can be differentiated from each other by phylogenetic (e.g., 16S rRNA gene sequences) and some physiological traits (Norris et al. 1996; Bogdanova et al. 2006).

During the course of determining the microbial composition of a pilot-scale mineral (cobaltiferous pyrite) leaching stirred tank system operated in France, a *Firmicute*, later shown to be a novel *Sulfobacillus* sp., was isolated and identified as a major component of the bioleaching consortium. Subsequently, similar bacteria were isolated from a stirred tank system operated in South Africa, and a heap leaching operation in Finland. Here, the physiological and phylogenetic characteristics of this new species, for which we propose the name "*Sulfobacillus benefaciens*", are described.

Materials and methods

Isolation and cultivation of BRGM2 and related *Firmicutes*

Bacteria were isolated from three pilot-scale mineral bioleaching systems: (1) isolate BRGM2 from a cobaltiferous pyrite concentrate processed at 40°C in stirred tank bioreactors at the Bureau de Recherches Géologiques et Minières (BRGM), Orleans, France (d'Hugues et al. 2008); (2) TKV8 from a polymetallic black schist ore processed in a 50,000 ton bioheap at Talvivaara, Finland (Riekkola-Vanhanen 2007); and (3) MT606 from a polymetallic nickel–copper concentrate processed at 45°C in stirred tank bioreactors at Mintek, Randberg, South Africa. Samples were streaked onto a variety of "overlay" solid media that support the growth of different species of acidophilic prokaryotes (Johnson and Hallberg 2007) and incubated at 37°C for up to 2 weeks. Plates were examined and colonies with distinct morphologies were sub-cultured on solid media (for culture purification). All *Sulfobacillus* strains were routinely cultivated in liquid medium containing 10 mM ferrous sulfate and 0.02% yeast extract in basal salts at pH 1.8, unless otherwise indicated. The basal salts contained (mg l^{-1}): $Na_2SO_4{\cdot}10H_2O$ (150); $(NH_4)_2SO_4$ (450), KCl (50), $MgSO_4{\cdot}7H_2O$ (500), KH_2PO_4 (50), and $Ca(NO_3)_2{\cdot}4H_2O$ (14). The type strain of *Sb. thermosulfidooxidans* (DSM 9293), and *Sb. acidophilus* strain ALV (which is identical to the type strain, NAL) were used as reference bacteria.

Cell morphology

Bacteria were viewed routinely by phase contrast microscopy (Leitz Labolux) at ×400 magnification. To determine cell size, cultures were grown in liquid medium, fixed with glutaraldehyde, critical point-dried and gold-coated, ahead of viewing with an Hitachi S-520 scanning electron microscope. The lengths and widths of several cells were measured and mean dimensions recorded.

Determination of temperature and pH optima of isolate BRGM2

Isolate BRGM2 was grown in a liquid medium containing 20 mM ferrous sulfate, 0.02% (w/v) yeast extract and basal salts, in a 2 l bioreactor (Electrolab Ltd., UK) with a working volume of 1.5 l. Cultures were aerated (0.5 l min^{-1}) and stirred at 100 rpm. The bioreactor temperature was set at varying temperatures (30–50°C) at a constant pH of 1.85, or varying pH values (0.4–2.0) at a constant temperature of 38.5°C. Samples were withdrawn at regular intervals and ferrous iron concentrations determined by titrating against 1 mM potassium permanganate. Semi-logarithmic plots of ferrous iron oxidized against time were used to identify exponential growth phases, and from them specific growth rates were calculated. Growth and iron oxidation by isolate BRGM2 and *Sb. thermosulfidooxidans*^{T} in liquid media with initial pH of 0.7–1.5 were also tested in shake flask cultures.

Dissimilatory oxidation of ferrous iron, sulfur and tetrathionate, and oxidative dissolution of pyrite

To test growth on ferrous iron, isolates BRGM2 and TVK8 were grown in shake flasks (38.5°C, pH 1.8; in triplicate) in a liquid medium containing 25 mM ferrous sulfate/basal salts ± yeast extract at 0.02% (w/v), alongside either *Sb. thermosulfidooxidans*^{T} and *Sb. acidophilus* (strain ALV). Similar tests were carried out using elemental sulfur (0.5%, w/v) or potassium tetrathionate (5 mM) except that the initial pH of liquid cultures was set at 3.0. Ferrous iron concentrations were determined by titration with permanganate, and growth and oxidation of sulfur and tetrathionate were assessed by monitoring numbers of cells and measuring culture pH. Oxidative dissolution of cobaltiferous pyrite (which contained ca. 80% FeS_2 and 1.3% Co) by BRGM2 at 38.5°C was assessed using 0.2% (w/v) suspensions of sterile pyrite in basal salts, poised initially at pH 2.0. Concentrations of total soluble iron were determined by atomic absorption spectrometry.

Specific rates of ferrous iron oxidation

Isolate BRGM2 was grown in shake flasks in different liquid media: (1) 50 mM ferrous sulfate (pH 1.7); (2) 20 mM ferrous sulfate/0.02% yeast extract (pH 1.8); (3) 20 mM ferrous sulfate/0.02% yeast extract/5 mM glycerol

(pH 1.8); (4) 0.5% (w/v) fine ground elemental sulfur/0.02% yeast extract (pH of 2.5). Cells were harvested by centrifugation, washed and resuspended in acidified basal salts. The protein contents of the cell suspensions were determined using the Bradford assay (Bradford 1976), and aliquots were dispensed into shake flasks containing 20 ml of 1 mM ferrous sulfate/basal salts (pH 1.8) medium. Iron oxidation was monitored by measuring concentrations of ferrous iron (using the ferrozine assay; Lovley and Phillips 1987). The specific rates of iron oxidation by *Sb. thermosulfidooxidans*^{T} and *Sb. acidophilus* (strain ALV) grown in medium (2) were compared under the same conditions of pH (1.8) and temperature (37°C).

Ferric iron respiration

Isolate BRGM2 was grown aerobically in 20 mM ferrous sulfate/5 mM glycerol/0.002% yeast extract/basal salts (pH 1.8, 37°C). When the iron was completely oxidized, glycerol was added (to give a concentration of 5 mM) and aliquots transferred to completely fill sterile universal bottles, which were sealed and re-incubated. When the cultures had bleached (indicating reduction of ferric iron), aliquots were inoculated into de-oxygenated liquid medium containing 5 mM glycerol/0.002% yeast extract/basal salts (pH 2.0) and varying concentrations of ferric sulfate. Cultures were incubated under anaerobic conditions (AnaeroGen^{TM}; Oxoid, UK) for 7 days, and ferrous iron concentrations (ferrozine assay) and cell yields (optical densities at 600 nm and enumeration using a Thoma counting chamber) were determined.

Growth on organic compounds

Isolates BRGM2 and TVK8 were grown in a liquid medium containing 5 mM glycerol, 1.0 mM ferrous sulfate, 0.002% yeast extract and basal salts at pH 1.8. Cells were harvested, washed and resuspended in basal salts solution, and aliquots served as inocula of cultures in the same medium (pH 1.8) containing one of the test organic substrates listed in Table 2, in place of glycerol. The concentrations of potential organic substrates were varied in order to provide similar amounts of carbon equivalents. Cultures containing organic substrates, together with inoculated controls containing only ferrous sulfate and yeast extract were incubated, shaken (150 rpm) at 37°C for 7 days. Protein concentrations were used to estimate bacterial biomass.

Tolerance of transition metals

Isolates BRGM2 and TVK8, *Sb. thermosulfidooxidans*^{T} and *Sb. acidophilus* (strain ALV) were grown at 37°C for 1 week in the standard liquid medium containing varying concentrations (in 10 mM increments) of transition metals (added as sulfate salts from stock solutions at pH 2.0) listed in Table 3. Growth of the bacteria was assessed visually and as oxidation of iron by measuring ferrous iron concentrations (permanganate titration), or in the case of cobalt-containing medium (which is pink) by measuring ferric iron as a yellow colored chloride complex (Schnaitman et al. 1969).

Analysis of chromosomal DNA base composition

Isolates BRGM2 and TVK8 were grown in shake flasks incubated at 37°C. Bacteria were harvested by centrifugation and were washed in TE buffer (10 mM Tris, 1 mM EDTA at pH 8). Cells in TE were incubated with lysozyme at 37°C for 30 min before chromosomal DNA was extracted using proteinase K and SDS and purified by cesium chloride gradient centrifugation (Wilson 1987). The G + C content of purified chromosomal DNA was determined by melting point analysis as described previously (Okibe et al. 2003).

Phylogenetic analysis

The 16S rRNA genes from isolates BRGM2, TVK8 and MT606 were amplified by PCR and sequenced as described previously (Okibe et al. 2003; Hallberg et al. 2006). The resulting gene sequences (GenBank accession numbers EF679212, EU099988 and EU495236) were aligned using ClustalX (Thompson et al. 1997) with selected sequences obtained from GenBank. This alignment was used to construct a phylogenetic tree by the neighbor joining method (Saitou and Nei 1987). The reliability of the phylogenetic relationships inferred was tested by bootstrap analysis on 100 samples (Felsenstein 1985).

DNA–DNA hybridization

DNA–DNA hybridization was carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The technique used was that described by De Ley et al. (1970) as modified by Huss et al. (1983).

Results

Isolation of BRGM2 and similar bacteria

All three isolates that were subsequently identified as strains of a novel *Sulfobacillus* sp. were isolated on ferrous iron/tetrathionate solid medium, where they formed distinct "fried-egg"-like colonies (round orange-centered colonies

with off-white margins). BRGM2 was isolated from mineral leachate from cobaltiferous pyrite concentrate, TVK8 from polymetallic black schist ore, and MT606 from nickel–copper concentrate. No other *Sulfobacillus* spp. were isolated from the stirred tank bioreactors, but both *Sb. thermosulfidooxidans* and *Sb. acidophilus*, in addition to TVK8, were isolated from the heap leaching operation (K. B. Hallberg and D. B. Johnson, unpublished). Cells were straight rods, 2.5 ± 0.5 μm long and 0.6 ± 0.05 μm wide, that formed terminal oval endospores, and were observed periodically to be motile (especially in early stages of growth). All three isolates grew in liquid media containing 10 mM ferrous iron and 0.02% yeast extract (poised initially at pH 1.8). Isolate MT606 was subsequently lost, and further characterization (apart from sequencing of 16S rRNA genes) was carried out only with isolates BRGM2 and TVK8.

Temperature and pH characteristics

Isolates BRGM2 and TVK8 were sub-cultured routinely in ferrous iron/yeast extract liquid medium (pH 1.7) at 37°C. When grown in the bioreactor, the temperature optimum for growth of isolate BRGM2 was 38–39°C (Fig. 1a), and the maximum temperature for growth and iron oxidation was 47°C (no growth occurred at 48°C). The pH optimum for growth of isolate BRGM2 was about 1.5 (Fig. 1b) and the minimum pH at growth was observed was 0.8. Growth at pH above 2.2 was not tested in iron-containing media, due to inherent problems of ferric iron hydrolysis and precipitation at these higher pH values. The maximum growth rate determined for isolate BRGM2 under conditions of optimum pH and temperature was 0.22 h^{-1}, corresponding to a culture doubling time of 3.1 h. Growth and iron oxidation by both isolate BRGM2 and *Sb. thermosulfidooxidans*^{T} occurred in shake flask cultures with initial pH values of 0.8–1.5 (and growth of the latter also at pH 0.7). The pH of these cultures showed little change during incubation, particularly those with initial pH values of <1.0.

Autotrophic oxidation of iron and reduced sulfur

Isolates BRGM2 and TVK8 could be successfully subcultured in "inorganic" ferrous sulfate or sulfur-containing liquid media. Successful growth of isolate BRGM2 on tetrathionate required the addition of ferrous iron (at ~100 μM). Although growth yields of isolate BRGM2 were greatly enhanced by the inclusion of yeast extract, culture doubling times (based on rates of iron oxidation) were very similar in shake flask (pH 1.7; 38°C) cultures containing either ferrous iron medium (6.51 ± 0.13 h) or ferrous iron/yeast extract medium (5.95 ± 0.46 h). In contrast, although *Sb. thermosulfidooxidans* and *Sb. acidophilus* grew more rapidly than isolate BRGM2 in yeast extract-amended media (data not shown), growth of both of these bacteria in "inorganic" ferrous iron medium was slower than that of BRGM2 (Fig. 2) and more sporadic. The greater propensity for autotrophic growth displayed by isolate BRGM2 than other *Sulfobacillus* spp. was also apparent during growth on cobaltiferous pyrite, where addition of yeast extract had little effect on the overall rate of mineral dissolution (Fig. 3) in contrast to previous results reported for strains of *Sb. thermosulfidooxidans* and *Sb. acidophilus* (Ghauri and Johnson 1991).

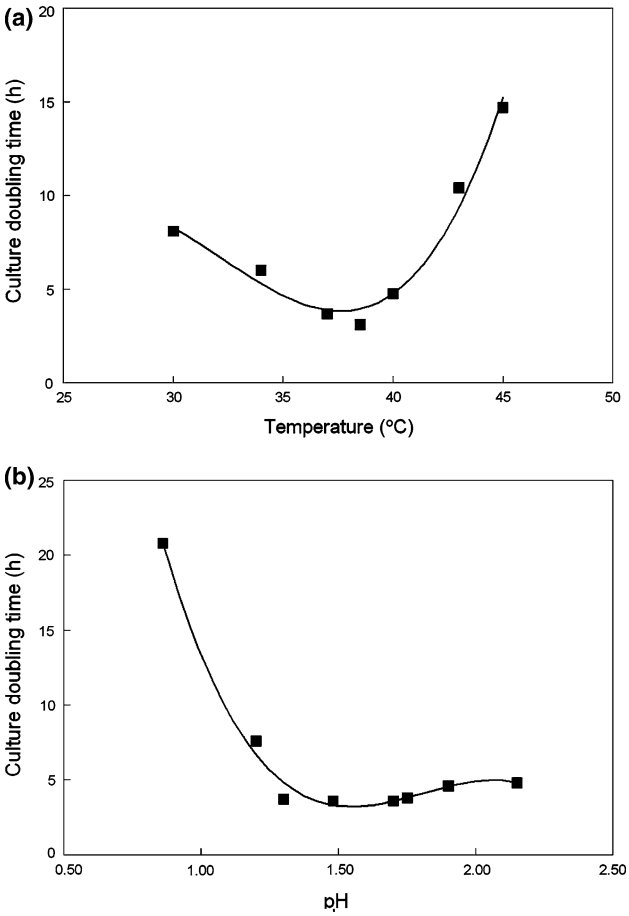

Fig. 1 Effect of **a** temperature and **b** pH on the growth of isolate BRGM2

Specific rates of ferrous iron oxidation

Specific rates of ferrous iron oxidation by isolate BRGM2 grown in iron/yeast extract medium and tested at 37°C were significantly faster than of *Sb. acidophilus* grown under the same conditions, but slower than that of *Sb. thermosulfidooxidans* (Table 1). More rapid specific rates of iron oxidation by BRGM2 were found when the bacteria were grown in glycerol-supplemented ferrous iron medium. Although rates of iron oxidation were slower

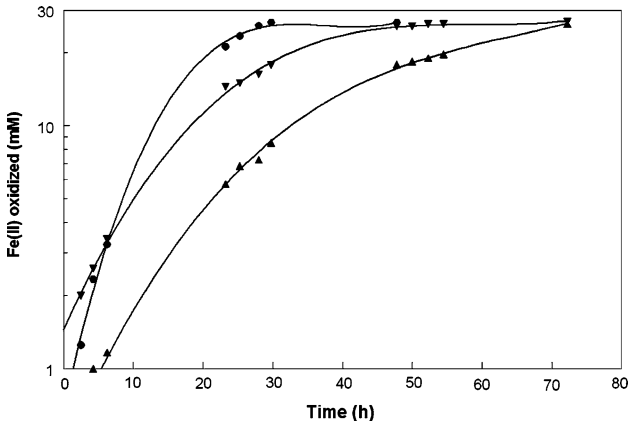

Fig. 2 Semi-logarithmic plot of iron oxidation against time by *Sulfobacillus* spp. grown autotrophically on ferrous iron: *inverted filled triangle Sb. thermosulfidooxidans*^{T}, *filled triangle Sb. acidophilus* ALV, *filled circle* isolate BRGM2

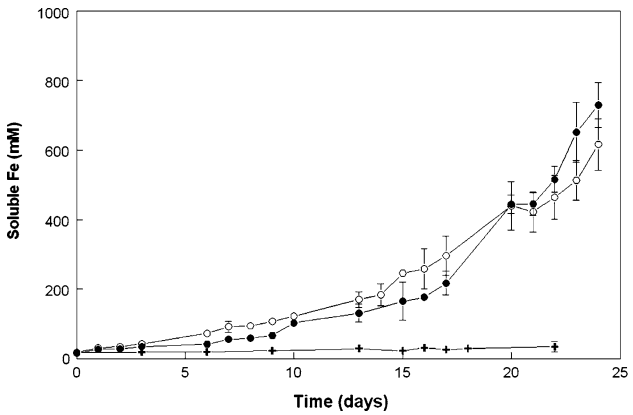

Fig. 3 Oxidative dissolution of cobaltiferous pyrite by isolate BRGM2 in cultures amended with yeast extract (at 0.02%, w/v, *filled circle*), yeast extract-free cultures (*open circle*), and in sterile controls (*plus symbol*). Data points show means of three replicate cultures (two in the case of the controls) and *error bars* show standard deviations

when the isolate was grown in sulfur/yeast extract medium, the values recorded were still about 30% of those measured with ferrous iron/yeast extract-grown cells (Table 1).

Utilization of organic compounds

Growth of both isolates BRGM2 and TVK8 was enhanced by a number of monosaccharides, and also some other small molecular weight organic compounds, though there were some differences between the two strains (Table 2). Mannose, citric acid and glutamic acid were found to significantly enhance the growth yields of both isolates.

Anaerobic growth of isolate BRGM2

Isolate BRGM2 was able to grow in an oxygen-free liquid medium using ferric iron as terminal electron acceptor and glycerol as electron donor. There was a strong correlation between biomass yield and the amount of ferric iron reduced, whether the former was measured as optical densities ($r = 0.991$) or cell numbers ($r = 0.978$; Fig. 4). No growth was observed in anaerobic cultures that contained 25 mM ferrous in place of ferric iron.

Metal tolerance

All of the sulfobacilli tested were able to grow in media containing high concentrations of iron (Table 3), though *Sb. acidophilus* strain ALV was relatively more sensitive to ferric iron than the rest. Strains BRGM2 and TVK8 exhibited comparable tolerance to other metals as *Sb. thermosulfidooxidans*, though the latter was able to grow in medium containing twice as much copper and cobalt as the two novel strains (Table 3). In contrast, *Sb. acidophilus* was much more sensitive to all other metals tested.

Genotypic characteristics

The 16S rRNA genes from BRGM2, TVK8 and MT606 shared 100% identity with each other, and with a clone (uncultured *Sulfobacillus* sp. K55, GenBank accession AF460984) obtained in an earlier study on the microbial population oxidizing the cobaltiferous pyrite (Battaglia-Brunet et al. 2002). In addition, the gene sequences from

Table 1 Specific rates of ferrous oxidation (as μg Fe^{2+} oxidized/minute/mg protein) by isolate BRGM2, and comparison with values obtained with two other *Sulfobacillus* spp.

Growth medium	Isolate BRGM2	*Sb. thermosulfidooxidans*	*Sb. acidophilus*
Fe^{2+}	230 ± 36	ND	ND
$Fe^{2+}/S_4O_6^{2-}$	94 ± 27	ND	ND
Fe^{2+}/YE	341 ± 7	449 ± 4	236 ± 11
Fe^{2+}/YE/glycerol	494 ± 16	ND	ND
S^0/YE	108 ± 7	ND	ND

Cultures were grown in 25 mM ferrous iron/0.02% yeast extract (YE), with or without 5 mM glycerol, and in 0.5% elemental sulfur/0.02% yeast extract. Specific rates of iron oxidation by harvested cells were determined at 37°C and pH 1.8 (*ND* not determined). Data are means (±standard deviations) of triplicates

Table 2 Utilization of organic compounds by *Sulfobacillus* isolates BRGM2 and TVK8, assayed in media supplemented with 100 μM ferrous iron and 0.002% (w/v) yeast extract

Substrate (concentration in mM)	BRGM2	TVK8
(i) Monosaccharides		
Glucose (5 mM)	++	+
Fructose (5 mM)	+	++
Arabinose (5 mM)	−	+
Mannose (5 mM)	++	++
Galactose (5 mM)	+	++
Glucosamine (5 mM)	−	+
Glucuronic acid (5 mM)	−	−
(ii) Alcohols		
Glycerol (10 mM)	+	+
Mannitol (5 mM)	+	++
Ethanol (15 mM)	−	−
(iii) Organic acids		
Citric acid (10)	++	++
Acetic acid (10)	−	−
(iv) Amino acids		
Glycine (10 mM)	−	−
Glutamic acid (5 mM)	++	++

Growth was determined by assessments of optical densities and microscopic examination of cultures, and scored as: ++ good growth, + limited growth, − no growth

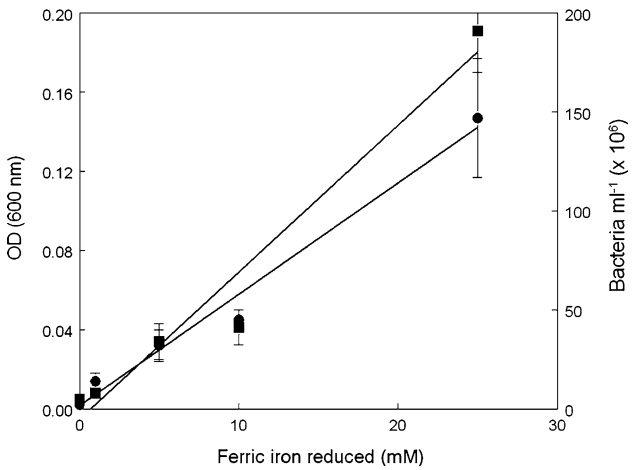

Fig. 4 Correlation between growth of isolate BRGM2 (*filled square* as cell numbers and *filled circle* from measurements of optical densities) and ferric iron reduction in anaerobic cultures. Data points are means of triplicate cultures, and *error bars* depict standard deviations

these microbes was over 99% identical to cloned genes from a copper mine in Australia (Goebel and Stackebrandt 1994) and to a microbe detected in Iron Mountain as a clone (Bond and Banfield 2001). The closest cultured relatives to isolates BRGM2 and TVK8 and MT606 are

Table 3 Metal tolerance of *Sulfobacillus* strains

Strain	MIC[a] (mM)					
	Fe^{2+}	Fe^{3+}	Cu	Ni	Zn	Co
BRGM2	>100	>100	120	120	360	110
TVK8	>100	>100	130	130	360	120
Sb. thermosulfidooxidans	>100	>100	230	160	>400	200
Sb. acidophilus	>100	50	<5	<5	110	30

Growth was assessed as iron oxidation after 1 week incubation at 37°C. Concentrations listed as ">" are the highest concentrations tested where growth was obtained, and those as "<" are the lowest concentrations tested

[a] Minimum inhibitory concentration

species of the genus *Sulfobacillus* (Fig. 5), with 16S rRNA gene sequence identities ranging from 90.3 to 97.1% (Table 4).

The GC content (±standard deviation, $n = 3$) of the chromosomal DNA from isolate BRGM2 is 50.63 ± 0.18 mol% and that of TVK8 is 50.09 ± 0.50 mol%, which differentiates this group from classified *Sulfobacillus* spp. (Table 5). Results of DNA–DNA hybridization analysis showed that isolates BRGM2 and TVK8 shared 78%

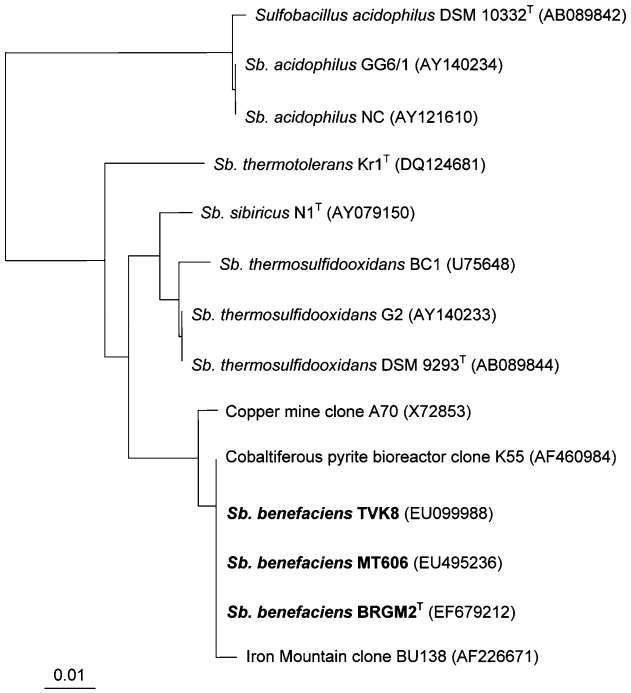

Fig. 5 Phylogenetic tree, showing the relationship of the three novel isolates (in *bold*) to known *Sulfobacillus* spp. and some related bacteria detected as cloned 16S rRNA genes. The tree was rooted with the 16S rRNA gene sequence from *Alicyclobacillus acidocaldarius* strain DSM 446^T (GenBank accession number AJ496806, not shown). GenBank accession numbers for other bacteria are given in *parentheses*, and the *scale bar* represents 1 nucleotide substitution per 100 nucleotides

Table 4 Relatedness of 16S rRNA genes from all type strains of the genus *Sulfobacillus* and isolate BRGM2

Strain	% identity of 16S rRNA gene sequence				
	1	2	3	4	5
Sb. thermosulfidooxidans DSM 9293^T (1)	–				
Sb. acidophilus NAL^T (2)	90.5	–			
Sb. sibiricus N1^T (3)	98.7	89.6	–		
Sb. thermotolerans Kr1^T (4)	95.6	90.6	95.6	–	
BRGM2 (5)	97.1	90.3	96.8	95.4	–

homology and that BRGM2 shared 45% homology with *Sb. thermosulfidooxidans*^{T}. These data confirm that isolates BRGM2 and TVK8 are strains of a separate species within the genus *Sulfobacillus.*

Discussion

Two of the three novel *Sulfobacillus* strains were isolated from pH- and temperature-controlled stirred tanks in which sulfide mineral concentrates were being oxidized in order to solubilise base metals (cobalt in one case, and nickel and copper in the other). The third strain was also isolated from mineral leachate, though in this case the polymetallic ore was being leached in a large heap where, as is typical in such situations, pH and temperature were subject to wide variation (Rawlings and Johnson 2007b). Other acidophilic bacteria were also present in all three mineral leachates (K. B. Hallberg and D. B. Johnson, unpublished data) though a far more restricted diversity of acidophiles (only 2–3 different species) was found in stirred tank leachates than in the heap leachate, as is commonly the case (Rawlings and Johnson 2007b). The fact that three very closely related sulfobacilli were important members of the bioleaching consortia in three geographically distant locations, and where different sulfide minerals were being processed, suggests a possible generic role for such bacteria in biomining operations. It is worth noting in this regard that clones closely related to BRGM2 had previously been obtained from a copper mine in Australia (Goebel and Stackebrandt 1994) as well as from an abandoned polymetallic mine (at Iron Mountain) in California (Bond and Banfield 2001).

Although phylogenetic analysis clearly delineates bacteria represented by isolates BRGM2, TVK8 and MT606 as a separate group from previously classified *Sulfobacillus* spp., phenotypically they have many similar phenotypic traits, such as tolerance to the transition metals tested, in common with both *Sb. thermosulfidooxidans* and the closely related (and possibly identical) species, *Sb. sibiricus* (Table 5). The question arises, therefore, why BRGM2-like bacteria were the only sulfobacilli detected in significant numbers in both the BRGM and Mintek (copper–nickel) stirred tank leachates. Isolate BRGM2 was found to be less thermo-tolerant than *Sb. thermosulfidooxidans*, but the published literature would suggest that it is more acidophilic, as the pH minimum for growth of the type strain of *Sb. thermosulfidooxidans* was claimed to be 1.5 (Golovacheva and Karavaiko 1978). However, Watling et al. (2008) found that *Sb. thermosulfidooxidans*^{T} can grow at pH 1.3, and experiments carried out in the current investigation showed clearly that this *Firmicute* can grow at even lower pH (at least pH 0.7). The pH values of the leach liquors from which isolates BRGM2 and MT606 were isolated were pH 1.4 and 1.6, respectively. The reason why these strains of a novel *Sulfobacillus* sp. rather than *Sb. thermosulfidooxidans*^{T} established in these "open" bioreactors was therefore probably not due to pH, temperature or inhibitory concentrations of soluble metals. Isolate BRGM2 was found to display much greater propensity for autotrophic growth than both *Sb. thermosulfidooxidans*^{T} and *Sb. acidophilus* ALV. Stirred tanks in which mineral concentrates are bioleached are essentially inorganic systems, in that the only materials added to stimulate microbial growth are inorganic salts of nitrogen, phosphorus, and (in some cases) carbonates (as a source of CO_2). Dissolved organic carbon compounds originate in bioleach liquors from primary iron- and/or sulfur-oxidizing autotrophs, such as *L. ferriphilum* and *At. caldus*, and can support the growth of smaller numbers of heterotrophic and mixotrophic acidophiles (Rawlings and Johnson 2007b). The faster growth rate of isolate BRGM2 than *Sb. thermosulfidooxidans*^{T} when growing as autotrophs implies that the latter would be far more prone to washout in a stirred tank operated, as most are, in continuous feed mode, even though the specific rate of ferrous iron oxidation at 37°C was found to be slightly greater for *Sb. thermosulfidooxidans*^{T} than for BRGM2. Elsewhere it was found that, when the same inoculum used to process the cobaltiferous pyrite in the BRGM bioreactor was used to bioleach a copper-rich black shale concentrate, *Sb. thermosulfidooxidans* was generally found to be the dominant *Firmicute* present, though BRGM2 again emerged as the dominant *Firmicute* when the residence time in the stirred tank was

Table 5 Comparison of phenotypic characteristics of *Sulfobacillus* species

Characteristic	1	2	3	4	5
Cell size (μm)	2.5 ± 0.5 × 0.6 ± 0.05	2.0 ± 1.4 × 0.7 ± 0.14	4.0 ± 1.4 × 0.65 ± 0.21	2.0 ± 1.4 × 0.9 ± 0.28	3.0 ± 2.1 × 1.0 ± 0.28
Growth pH range (optimum)	0.8–2.2 (1.5)	1.5–5.5[a] (1.7–2.4)	(~2.0)	1.1–2.6 (2.0)	1.2–2.4 (2.0)
Growth temperature range (optimum) (°C)	30[b]–47 (38.5)	20–60 (50–55)	(45–50)	17–60 (55)	20–60 (40)
Minimum culture doubling time (h)[c]	3.1	2.5	3.5	1.4	2.0
G + C content (mol%)	50.6 ± 0.2	47.2–47.5	56 ± 1	48.2 ± 0.2	48.2 ± 0.5
Chemolithotrophic growth with:					
Fe^{2+}	+	+	+	+	+
S^0	+	+	+	+	+
Tetrathionate	+	+	ND	+	+
Sulfide minerals	+	+	+	+	+
Anaerobic growth with ferric iron as electron acceptor	+	+[d]	+[d]	ND	ND
Utilization of:					
Yeast extract	+	+	+	+	+
Fructose	+	+	+	+	+
Glucose	+	+	+	+	+
Mannose	+	+	−	ND	−
Glutamate	+	+	−	ND	−

+ growth, − no growth, *ND* indicates not determined. Strains include: 1, BRGM2; 2, *Sb. thermosulfidooxidans* DSM 9293^T (Golovacheva and Karavaiko 1978); 3, *Sb. acidophilus* NAL^T (Norris et al. 1996); 4, *Sb. sibiricus* N1^T (Melamud et al. 2003); and 5, *Sb. thermotolerans* Kr1^T (Bogdanova et al. 2006)

[a] Published values, though data from current experiments show that *Sb. thermosulfidooxidans*^{T} can grow in media poised at pH 0.7

[b] Lowest temperature tested

[c] Using ferrous iron as electron donor

[d] Data from Bridge and Johnson (1998)

reduced from three to two days (P. d'Hugues and C. Joulian, unpublished data).

On the basis of their distinct phylogenies, it is proposed that bacteria presented by isolates BRGM2 and TVK8 are recognized as a novel species *of Sulfobacillus*, and that BRGM2 is designated as the type strain. The species name *"benefaciens"* is proposed to highlight the fact that all three strains of this acidophile that have been isolated, to date, have been obtained from biomining operations, where they appear to be important members of the microbial communities that accelerate the oxidative dissolution of metal sulfides.

Emended description of genus *Sulfobacillus* Golovacheva and Karavaiko 1978

The description of the genus *Sulfobacillus* (Golovacheva and Karavaiko 1978) is emended to reflect the novel phenotypic trait described here and previously (Bridge and Johnson 1998). Those species tested are facultative anaerobes, capable of anaerobic growth with ferric iron as electron acceptor.

Description of *Sulfobacillus benefaciens* sp. nov

Sulfobacillus benefaciens (ben.e.fac.iens L. part. adj. *benefaciens*, doing a good action, imparting benefits).

Cells are motile, Gram-positive, straight rods that form terminal, oval endospores. Cells are 2.5 ± 0.5 μm long and 0.6 ± 0.05 μm wide. Form "fried-egg"-like colonies (round orange-centered colonies with off-white margins) on acidic iron/tetrathionate overlay medium. Facultative autotrophs capable of autotrophic growth with S°, tetrathionate, ferrous iron, and sulfide minerals. Facultative anaerobe, capable of anaerobic growth with ferric iron as electron acceptor. Growth yields are enhanced in the presence of 0.02% yeast extract or other organic compounds. Organotrophic growth is supported by a range of monosaccharides, glycerol, mannitol, citric acid and glutamic acid. Thermo-tolerant; optimum temperature for growth is 38–39°C, and growth occurs from 30°C (the lowest tested) up to 47°C. Acidophilic; the optimum pH for growth is 1.5 and the pH range for growth is from 0.8 to 2.2 (highest pH tested). The DNA G + C content is 50.6 ± 0.2 mol%. Sequencing of 16S rRNA gene indicates that *Sb. benefaciens* belongs to the family *Alicyclobacillaceae* in the *Firmicutes*.

The type strain, BRGM2^T (= DSM 19468^T = ATCC BAA–1648^T), was isolated from a bioreactor treating cobaltiferous pyrite. Two other isolates of *Sulfobacillus benefaciens* were obtained from pilot scale bioreactors (stirred tank and heap) treating other sulfide concentrates or ores, and bacteria with identical 16S rRNA gene sequences have also been detected in association with sulfidic ores.

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