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Characteristics of *Sulfobacillus acidophilus* sp. nov. and other moderately thermophilic mineral-sulphide-oxidizing bacteria

Paul R. Norris, Darren A. Clark, Jonathan P. Owen and Sara Waterhouse

Author for correspondence: Paul R. Norris. Tel: +44 1203 523733. Fax: +44 1203 523701. e-mail: pn@dna.bio.warwick.ac.uk

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Several isolates of Gram-positive, acidophilic, moderately thermophilic, ferrous-iron- and mineral-sulphide-oxidizing bacteria were examined to establish unequivocally the characteristics of *Sulfobacillus*-like bacteria. Two species were evident: *Sulfobacillus thermosulfidooxidans* with 48–50 mol% G+C and *Sulfobacillus acidophilus* sp. nov. with 55–57 mol% G+C. Both species grew autotrophically and mixotrophically on ferrous iron, on elemental sulphur in the presence of yeast extract, and heterotrophically on yeast extract. Autotrophic growth on sulphur was consistently obtained only with *S. acidophilus*.

Keywords: *Sulfobacillus*, iron oxidation, acidophiles

INTRODUCTION

Acidophilic bacteria that are most active in oxidation of ferrous iron and mineral sulphides at about 45–50 °C (Brierley & Brierley, 1986; Norris, 1990) have been isolated from geothermal environments, mineral sulphide mines, coal and mineral spoil heaps, and commercial metal leaching dumps. Their growth on ferrous iron and mineral sulphides in medium containing yeast extract was described (Brierley & Le Roux, 1977; Golovacheva & Karavaiko, 1979) before it was found this supplement could be replaced by separate, defined sources of organic carbon (some amino acids) and reduced sulphur (Brierley *et al.*, 1978; Norris *et al.*, 1980). Autotrophic growth on ferrous iron was demonstrated when culture atmospheres were enriched with CO_2 (Marsh & Norris, 1983a) and was confirmed by measurements of CO_2 incorporation (Wood & Kelly, 1983) and ribulose bisphosphate carboxylase/oxygenase activity (Wood & Kelly, 1985). Mineral sulphide dissolution during autotrophic growth of some strains (Marsh & Norris, 1983b) demonstrated a potential application of these bacteria in extraction of metals from mineral sulphide concentrates and indicated a capacity for significant biogeochemical activity in acidic environments.

One of the most studied strains has been named *Sulfobacillus thermosulfidooxidans* (Golovacheva & Karavaiko, 1979). Some variation in its morphology (rods and coryneforms) has been described (Golovacheva, 1979) but this has not been reported for otherwise apparently similar bacteria (Brierley, 1978; Ghauri & Johnson, 1991). Isolates from several locations have been examined and are described in this paper in order to establish unequivocally the characteristics of *Sulfobacillus*-like bacteria. The dissimilarity between the reported 16S rDNA sequences of the *S. thermosulfidooxidans* type strain (Tourova *et al.*, 1994) and of very similar bacteria is addressed.

METHODS

Bacterial strains. The strains examined are listed in Table 1. *Sulfobacillus thermosulfidooxidans* type strain VKM B-1269 was provided by G. Karavaiko (Institute of Microbiology, RAS, Moscow, Russia) in 1993. Strain TH1 was provided by N. W. Le Roux (then at DTI Warren Spring Laboratory, Stevenage, UK) in 1977. Strains YTF and THW were provided by D. B. Johnson (University of Wales, Bangor). All other strains were isolated in this laboratory. Strains LM1 and BC1 were referred to as strains LM and BC in the description of their isolation (Marsh & Norris, 1983a) and the number added subsequently as different types (e.g. strain LM2, BC13; Norris, 1990) were obtained from the original enrichment cultures or sample sites. Several strains were isolated from provided samples and details of sample site conditions were not available. Strain TH3 was isolated from a copper leaching dump sample that was provided from the New Mexico Institute of Technology, Socorro, by J. A. Brierley (current affiliation Newmont Metallurgical Services, Salt Lake City, UT, USA). It was designated strain TH3 because it appeared very similar (Norris & Barr, 1985) to another strain TH3 (Brierley, 1978) which was no longer available. Both of these TH3 strains were isolated from the same site. Strain ICP is another isolate of the TH3 type (Clark & Norris, 1996). Strain C-MT1 (Goebel & Stackebrandt, 1994) was not examined in this work, but is discussed. It was obtained from a mineral sulphide ore-leaching, continuous bioreactor and described as a

Table 1. Strains and sources of moderately thermophilic, ferrous-iron-oxidizing bacteria

The approximate date when laboratory cultures were established is indicated.

Strain	Source
S. thermosulfidooxidans	Mineral sulphide ore deposit, Armenia (1977)
TH1	Thermal spring, Iceland (1972)
BC1	Coal spoil heap, Birch Coppice colliery, UK (1981)
ALV	Coal spoil heap, near Alvecote, UK (1979)
NAL	Coal spoil heap, near Alvecote, UK (1988)
LM1	Thermal spring, Iceland (1981)
TH3	Copper leach dump, New Mexico, USA (1984)
2B, 3B, 3C	Thermal spring, Iceland (1989)
N	Thermal spring, Yellowstone National Park (1989)
YTF	Thermal spring, Yellowstone National Park (1989)
THW	Coal spoil heap, Wales (1988)
ICP	Thermal spring, Iceland (1993)

moderately thermophilic, iron-oxidizing, Gram-positive bacterium.

Culture conditions. All cultures were grown in shaken flasks in a mineral salts medium containing, per litre, $MgSO_4.7H_2O$, 0·5 g; $(NH_4)_2SO_4$, 0·4 g; K_2HPO_4, 0·2 g; and KCl, 0·1 g. When 50 mM ferrous iron was the substrate ($FeSO_4.7H_2O$, 13·9 g l^{-1}), the medium was initially adjusted with H_2SO_4 to pH 1·7. The medium was supplemented with tetrathionate ($K_2S_4O_6$, 0·15 g l^{-1}) for autotrophic growth on ferrous iron because some moderately thermophilic, iron-oxidizing acidophiles require a source of reduced sulphur for growth (Brierley *et al.*, 1978; Norris & Barr, 1985). The medium was adjusted initially to pH 2 when yeast extract (0·25 g l^{-1}) was the substrate, and to pH 3 when the substrate was elemental sulphur (5 g l^{-1}). Medium containing sulphur or yeast extract was supplemented with a trace of iron ($FeSO_4.7H_2O$, 10 mg l^{-1}). Medium containing pyrite (40%, w/v, iron; particle size diameter < 75 μm) was adjusted to pH 2 before inoculation. Pyrite was added in two stages, 1% (w/v) at inoculation and 4% (w/v) when growth was established. Cultures growing autotrophically on ferrous iron, sulphur or pyrite were gassed with 5% (v/v) CO_2 in air. All cultures were grown at 48 °C.

Growth assays. As described previously (Marsh & Norris, 1983a; Wood & Kelly, 1983, 1984), growth on ferrous iron was followed by titration of residual substrate using ceric sulphate and phenanthroline/ferrous sulphate as indicator. Pyrite dissolution was followed by measuring iron in supernatants of centrifuged samples by atomic absorption spectrophotometry. Heterotrophic growth on yeast extract was estimated as culture optical density, measured at 440 nm using 1 cm light path cells and a Beckman DU-70 spectrophotometer.

Microscopy. Photomicrographs were obtained using a Leitz Dialux 22/22 EB microscope and Vario Orthomat 2 automatic microscope camera. Cells were concentrated by centrifugation from exponentially growing cultures, or from cultures shortly after lag phase if an exponential phase was not obvious, as with some strains growing autotrophically on ferrous iron. A Joel JEM-100S transmission electron microscope was used to view thin sections of cells which had been fixed in glutaraldehyde and stained with uranyl acetate using standard techniques.

Electrophoresis. Cell lysates were prepared by incubation with lysozyme (5 mg ml^{-1}) at 37 °C for 15 min and subjected to SDS-PAGE (Laemmli, 1970) using a 10% (w/v) polyacrylamide gel.

DNA extraction and analyses. Cells were grown with yeast extract as substrate, harvested by centrifugation and washed with distilled water. Cell pellets from cultures (10 l) were resuspended in 6 ml buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8). EDTA (0·25 M, pH 8, 3·75 ml) and 50 mg lysozyme were added. After incubation at 37 °C for 15 min, 125 μl proteinase K (20 mg ml^{-1}) and 3·25 ml SDS (10% w/v) were added and incubation continued until the suspension cleared. DNA was isolated and purified following a modification of the Marmur protocol (Johnson, 1991) and a CsCl centrifugation step before dialysis.

DNA from moderate thermophiles and DNA (Sigma) from *Clostridium perfringens* (26·5 mol% G+C), *Escherichia coli* (52 mol% G+C) and *Micrococcus luteus* (72 mol% G+C) was dialysed three times against diluted standard saline citrate (i.e. 15 mM NaCl, 1·5 mM trisodium citrate, pH 7). Melting curve mid-points (T_m) were determined using a Hewlett Packard automated DNA melt testing system and 8452A spectrophotometer. Unknown base compositions were calculated from the formula mol% G+C = mol% G+C of $X + 2{\cdot}08(T_m - T_m$ of $X)$, where X was the DNA of known base composition (Owen & Hill, 1979). Means of duplicate T_m determinations were used in the calculations and the mol% G+C of each moderate thermophile was taken as the mean of the three values calculated with reference to values obtained for the three DNA standards.

DNA:DNA hybridization was carried out using a filter hybridization technique as described by Sharp & Williams (1988). The results are the means of duplicate hybridizations except for strain TH3, with which a single experiment was performed. Hybridization is expressed as percentage of homologous hybridization counts.

Analysis of 16S rDNA sequences. PCR products comprising 16S rDNA of strain BC1 and *S. thermosulfidooxidans* were generated using 27f and 1492r primers (Lane, 1991). Cloning was done using the TA Cloning Kit (Invitrogen). Sequencing was done using primer 357f (Lane, 1991), the downstream vector primer M13r (5′-CAGGAAACAGCTATGAC-3′) and an upstream vector primer (5′-GGCCCTCTAGATGCAT-3′). An Applied Biosystems model 373A was used for automatic sequencing. Percentage similarities were calculated for aligned sequences between bases 28–260, 371–617 and 1200–1460 (*E. coli* sequence numbering), a total of 741 bases. Included in

alignments were sequences previously determined by Lane *et al.* (1992) for strain BC1 (GenBank accession numbers M79380, M79381 and M79382) and strain ALV (M79375, M79376 and M80290), by Tourova *et al.* (1994) for *S. thermosulfidooxidans* (Z21979), and by Goebel & Stackebrandt (1994) for strain C-MT1 (X75270).

RESULTS

Whole-cell protein electrophoresis profiles

Comparative electrophoresis of whole-cell proteins of several isolates of ferrous iron-oxidizing moderate thermophiles revealed three groups of strains (Fig. 1). Further comparisons have shown that the protein profile of strain BC1 matched those of *Sulfobacillus thermosulfidooxidans* and strains TH1, LM1 and 3C (data not shown). Profiles of most of the other isolates (strains ALV, NAL, 2B, 3B, THW, YTF and N) did not match that of strain BC1 (Fig. 1) and were also different therefore from other representatives of the group containing *S. thermosulfidooxidans*. Within the strain ALV group, protein profiles were similar. There were a few, reproducible differences, particularly in the region showing polypeptides of about 25 kDa apparent molecular mass, where major bands were not aligned in the protein profiles of strains ALV and NAL, and were absent from profiles of strains THW, 2B and 3B. The SDS-PAGE protein profile of strain TH3 was very different from those of strain BC1 and strain ALV group bacteria (Fig. 1).

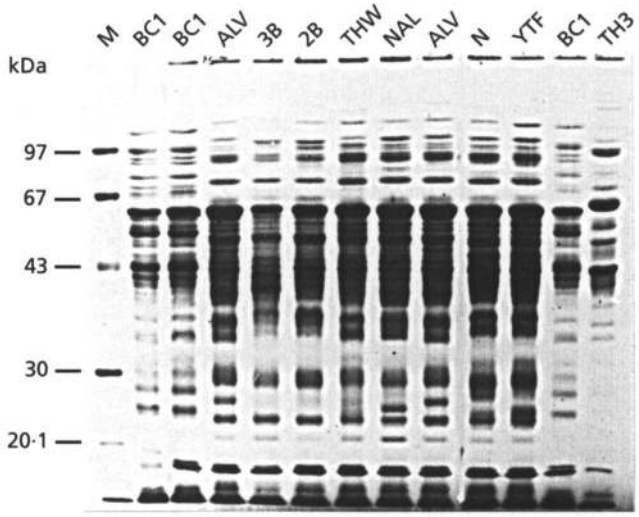

Fig. 1. SDS-PAGE whole-cell protein profiles of strains of moderately thermophilic acidophiles. All cultures were grown on ferrous iron in the presence of yeast extract. Molecular mass markers are in lane M.

DNA:DNA hybridization

Three groups of moderate thermophiles were evident among the strains examined (Table 2), confirming the divisions seen with the protein profiles. Only one species in each group was indicated. There was generally well over 70% DNA:DNA hybridization among isolates of the strain ALV group. Further experiments with DNA from strains NAL, 2B, THW and 3B (not performed in duplicate; data not shown) showed high levels of DNA:DNA hybridization among these strains, so the latter two can also be included in the single species of the strain ALV group. There was over 80% hybridization between DNA from strain BC1 and *S. thermosulfidooxidans*.

DNA G+C content

The range of G+C mol% values for *S. thermosulfidooxidans* (Table 3) could reflect the variety of subspecies as well as different procedures in laboratories. The value obtained for the type strain in this study, and all values given for the similar strains BC1 and TH1 (Table 3), indicated a G+C content of between 48 and 50 mol%

Table 2. DNA:DNA relatedness among moderately thermophilic, ferrous-iron-oxidizing acidophiles

Results are expressed as percentages of the homologous hybridizations. ND, Not determined.

Filter-bound DNA from:	^{3}H-labelled DNA from:							
	*S. th.**	BC1	ALV	NAL	2B	3B	N	TH3
*S. th.**	100	81	ND	11	ND	ND	ND	2
BC1	90	100	8	11	7	8	8	4
ALV	ND	13	100	93	88	69	83	ND
NAL	12	10	86	100	93	83	79	5
2B	ND	10	82	96	100	96	83	ND
3B	ND	14	81	92	95	100	94	ND
N	ND	13	75	82	73	83	100	ND
TH3	3	4	ND	2	ND	ND	ND	100

* *S. thermosulfidooxidans*.

Table 3. Chromosomal DNA base composition of moderately thermophilic, ferrous-iron-oxidizing acidophiles

Strain	mol% G+C	
	This study	Other values†
*S. th.**	48·4	53·6[a], 47·2[b], 49·3[c], 45·5[d]
BC1	48·6	50·4[e], 48·2[f]
TH1	ND	48[g], 49·7[e], 48[h]
ALV	55·3	57·0[e]
NAL	56·2	54·9[f]
N	55·1	
TH3	67·7	68·5[e], 68·0[f]

ND, Not determined.

* *S. thermosulfidooxidans.*

† *a*, Golovacheva & Karavaiko (1979); *b*, Karavaiko *et al.* (1988); *c*, subspecies *thermotolerans*, Kovalenko & Malakhova (1984); *d*, subspecies *asporogenes*, Vartanyan *et al.*(1988); *e*, Harrison (1986); *f*, Clark & Norris (1996); *g*, Brierley *et al.* (1978); *h*, Ghauri & Johnson (1991).

for *S. thermosulfidooxidans*. The G+C content range of the strain ALV group bacteria (ALV, NAL and N) was 55–57 mol% (Table 3). A value of approximately 68 mol% G+C has been consistently obtained for the unrelated strain TH3.

16S rDNA analysis

The partial 16S rDNA sequence of strain BC1 determined in this work had an overall similarity of 99·5% with the sequence of the same strain previously determined by Lane *et al.* (1992). Comparison of the strain BC1 sequence (obtained in this work) with the aligned sequences from *S. thermosulfidooxidans* (obtained in this work) and strain C-MT1 (database sequence) showed similarities of 97·3% and 97·6%, respectively. The similarity between the sequence of strain BC1 (obtained in this work) and the *S. thermosulfidooxidans* database sequence (Tourova *et al.*, 1994) was 73%. The similarity between the partial sequences of strain BC1 and strain ALV was 88·6%.

Morphology

Strains of the three groups of moderate thermophiles defined by whole-cell protein electrophoresis, DNA G+C content and DNA:DNA hybridization could be similarly grouped on the basis of their size and their morphological variation in response to growth conditions. Strain ALV was in part an exception to the pattern, growing as chains of sometimes distorted cells when oxidizing ferrous iron (Fig. 2c), but reverting to regular rods during autotrophic growth on sulphur (not shown) and during heterotrophic growth on yeast extract. Other isolates of the strain ALV group varied little in form (0·5–0·8 × 3–5 μm). In contrast, *S. thermosulfidooxidans* strains tended to increase in size from about 0·6 × 2–3·5 μm during autotrophic growth on ferrous iron to 0·8–1·8 × 3–6·5 μm during growth on ferrous iron plus yeast extract and heterotrophic growth with yeast extract as the sole substrate (Fig. 2). The length of individual cells observed by light microscopy was difficult to measure, with most of the longer forms, including the filamentous forms of strain ALV, comprising dividing cells which had not separated (electron microscopy observations, not shown). With yeast extract as sole substrate, some *S. thermosulfidooxidans* strains became shorter and swollen, particularly strain BC1 (Fig. 2b) and, as reported previously, strain TH1 (Norris *et al.*, 1980). Although flagella were not observed with the cells prepared for microscopy, limited motility of most *Sulfobacillus*-like strains was evident, but only when they were growing autotrophically on ferrous iron.

As described previously (Brierley, 1978), strain TH3 was morphologically unique among the isolates. The cells were characteristically narrower (0·4 μm wide) than *Sulfobacillus* strains and often in filaments (Fig. 2f). However, strain TH3 was occasionally observed to grow as pairs of relatively short cells, particularly during exponential growth on yeast extract in well-agitated cultures, when the cells were also motile.

Endospore formation

Endospores were observed in *S. thermosulfidooxidans* and in the strain ALV group bacteria. They were more commonly observed in cells under the relatively poor nutritional conditions of autotrophic growth on ferrous iron (Fig. 2), with only strain N also showing endospores during heterotrophic growth (Fig. 2e). Whether sporulation is more prevalent towards the stationary phase of growth in the presence of yeast extract has not been examined. Spores appeared mostly spherical and terminal in the strain ALV group bacteria and possibly slightly more oval with less swelling of the cell in the *S. thermosulfidooxidans* strains. Sections of sporulating cells of each type revealed a typical *Bacillus* forespore development and mature spore structure, with clearly visible cortex and spore coat layers (Fig. 3).

Growth and ferrous iron oxidation

The three groups of moderate thermophiles had different capacities for autotrophic growth on ferrous iron, assuming that iron oxidation was directly related to growth. Ferrous iron oxidation by strain TH1 growing on ferrous iron and yeast extract has previously been correlated with growth (cell carbon and protein) (Marsh & Norris, 1983a). Iron oxidation by autotrophically growing strains BC1 and ALV has been correlated with CO_2 fixation (Wood & Kelly, 1983). *S. thermosulfidooxidans* isolates (e.g. the type strain and strain LM1; Fig. 4) were able to maintain an initially high rate of oxidation of 50 mM ferrous iron. A gradual decline in the rate of oxidation was more evident with strains ALV and NAL and there was virtually no exponential phase of iron oxidation during

growth of strains TH3 and ICP. In the presence of yeast extract, strains of all three groups oxidized 50 mM ferrous iron rapidly and completely (data not shown), as shown previously with strains BC1, ALV and TH3 (Norris & Barr, 1985).

Growth and sulphur oxidation

Growth of all isolates in the presence of yeast extract and sulphur resulted in acidification of the medium, though this was relatively weak in cultures of strains TH3 and ICP. Transfer through many serial cultures in medium containing sulphur but no yeast extract resulted in consistent, autotrophic growth of strains ALV, NAL, THW, N and YTF, with, typically, pH 1·5 being reached, as seen previously with strain ALV (Norris *et al.*, 1986). Only strain 2B of the strain ALV type did not readily switch to autotrophic growth on sulphur. In contrast, growth of *S. thermosulfidooxidans*, strain BC1 and strain TH3 became progressively weaker through serial culture on sulphur in the absence of yeast extract, and autotrophic cultures could not be maintained.

Growth on pyrite

Growth of some bacteria on mineral sulphides is inhibited by agitation with high mineral concentrations unless cultures are allowed to become established under less severe conditions (unpublished results). Strains BC1 and N, and the pyrite enrichment culture from which strain N was isolated, were therefore grown with only 1 % (w/v) pyrite initially (Fig. 5). The concentration of pyrite was then increased so that concentrations of potentially growth-inhibiting end products of pyrite oxidation, H_2SO_4 and ferric iron, were reached. Extensive pyrite dissolution occurred during autotrophic growth of all *Sulfobacillus*-like isolates tested (*S. thermosulfidooxidans*, strains BC1, LM1, N, NAL, THW, YTF) except strain ALV. After addition of 4 % (w/v) pyrite to established cultures (Fig. 5), iron solubilization was more rapid by strain BC1 (60 mg l^{-1} h^{-1}) than by strain N (41 mg l^{-1} h^{-1}).

Heterotrophic growth

Bacteria of the strain ALV group generally tended to grow more readily than *S. thermosulfidooxidans* strains when switched from autotrophic growth and they were also easier to maintain subsequently with yeast extract as the sole substrate. The mean doubling times (estimated from culture optical density increase) were between 6 and 8 h for the strain ALV group (strains ALV, NAL, 2B and N) and between 8 and 12 h for the *S. thermosulfidooxidans* strains (i.e. the type strain, strains BC1, 3C and LM1). The yields of strains of both groups (estimated from culture optical density) were approximately proportional to the yeast extract concentration between 0·1 g l^{-1} and 0·5 g l^{-1}. However, the maximum yield of *S. thermosulfidooxidans* strains growing on yeast extract (0·25 g l^{-1}) was on average one-third less (OD_{440} 0·15–0·2) than that of strain ALV group bacteria (OD_{440} 0·3). The optimum pH for growth of most strains on yeast extract was 2–2·2, but the precise optima were not determined. The growth rate and yield of strain TH1 were considerably reduced at pH 1·5 and pH 3·0 in comparison to growth at pH 2 (data not shown). In contrast, acidophilic, moderately thermophilic, heterotrophic *Bacillus*- or *Alicyclobacillus*-like isolates from the same environments as some of the iron-oxidizing bacteria generally grew with estimated doubling times of 1·5–2 h and with a higher optimum pH (P. R. Norris, unpublished results).

DISCUSSION

The *Sulfobacillus*-like bacteria examined were clearly divided into two groups on a range of criteria (protein profiles, G+C content, DNA:DNA hybridization, morphology, characteristics of autotrophic growth on ferrous iron and sulphur, and heterotrophic growth yield). Taking into account all of these features, the extreme similarity of isolates in the first group, with 48–50 mol % G+C, indicated that they belonged to a single species. It is proposed that this should be *Sulfobacillus thermosulfidooxidans*, notwithstanding the incompatible rDNA sequences of the type strain as given in the database and as determined in this work. A phylogenetic tree derived from database sequences has placed *S. thermosulfidooxidans* in a cluster with the acidophilic heterotroph *Alicyclobacillus* rather than with strains BC1 and ALV (Tourova *et al.*, 1994), with which it appears to have much more in common. Subspecies of *S. thermosulfidooxidans* have been described as *thermotolerans* (Kovalenko & Malakova, 1984) and *asporogenes* (Vartanyan *et al.*, 1988). The relatively low mol % G+C contents of these strains (Table 3) places them with *S. thermosulfidooxidans* rather than the strain ALV group of bacteria. The 81 % DNA:DNA hybridization between sub-species *asporogenes* and *S. thermosulfidooxidans* confirms this placement (Vartanyan *et al.*, 1988).

It is proposed that the second group of *Sulfobacillus*-like bacteria, with 55–57 mol % G+C, represent a new species, *Sulfobacillus acidophilus*. The two species shared little DNA:DNA relatedness but strain BC1 (*S. thermosulfidooxidans*) and strain ALV (*S. acidophilus*) are more closely related phylogentically to each other than to any other species (Lane *et al.*, 1992) and they share a similar general physiology. Similar difference spectra indicated principally *b*-type and aa_3 cytochromes in strain TH1 (*S. thermosulfidooxidans*) and strain ALV (*S. acidophilus*) (Barr *et al.*, 1990). The mixotrophic behaviour of strains ALV and BC1 was also generally similar, with simultaneous utilization of glucose and CO_2 during growth on ferrous iron (Wood & Kelly, 1983), although yeast extract depressed CO_2 fixation by strain BC1 more than that by strain ALV and, during growth in the absence of an enhanced CO_2 concentration, glucose stimulation of ferrous iron oxidation by strain ALV was stronger than with strain BC1. The difference in stimulation by glucose under air was also seen with strains NAL (*S. acidophilus*) and LM1 (*S. thermosulfidooxidans*), the latter showing much less improvement in growth (Clark & Norris, 1996). Glucose was utilized primarily by the oxidative pentose phosphate pathway in strain ALV (Wood & Kelly, 1984). Strain

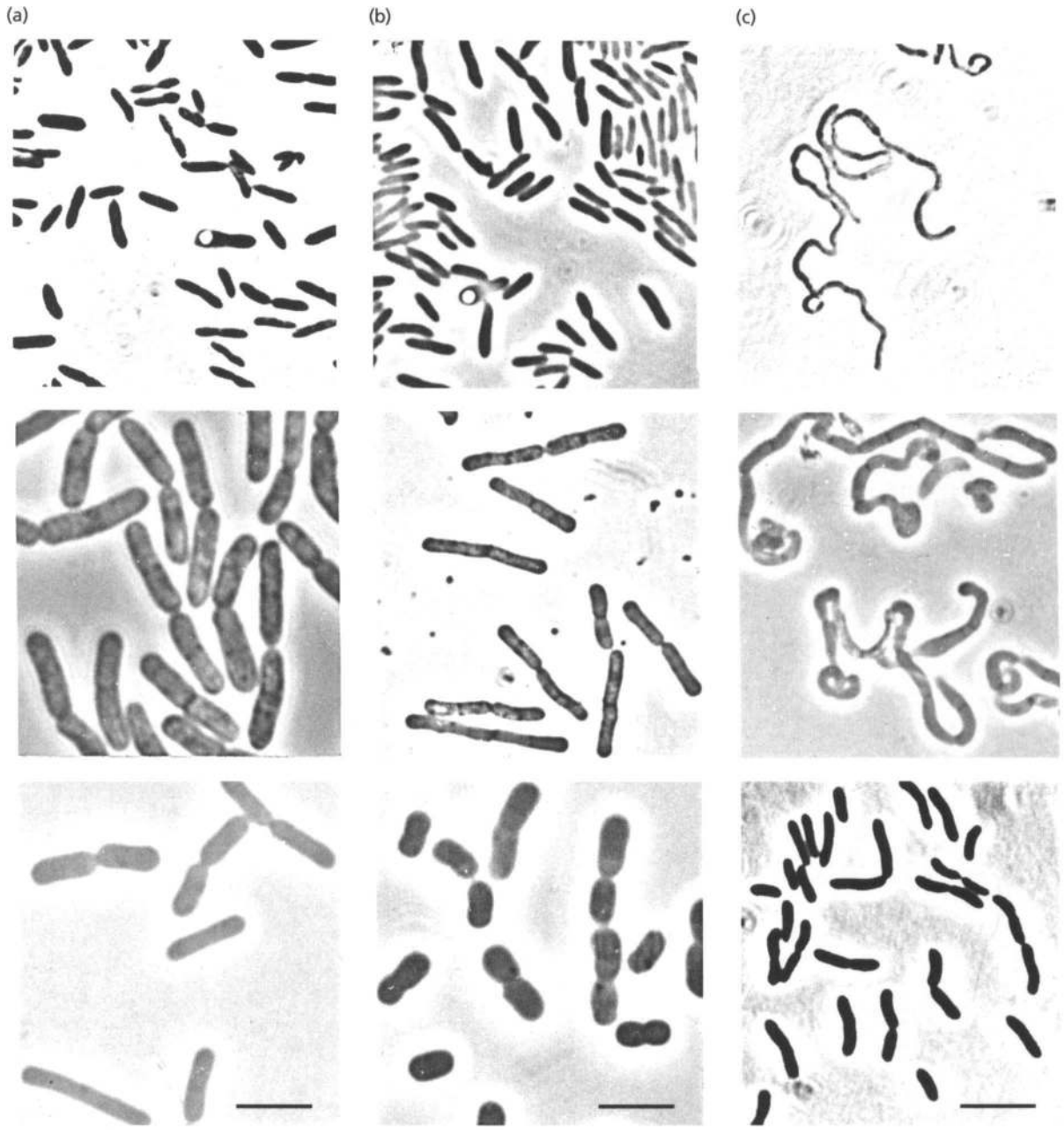

Fig. 2. For legend see facing page.

ALV has received most study (Marsh & Norris, 1983b; Wood & Kelly, 1983, 1984; Norris *et al.*, 1986; Harrison, 1986), but this comparison of several isolates has shown that its morphology during growth on iron and its poor growth on pyrite were not typical of the species. Strain NAL, isolated from the same site as strain ALV, is proposed as the type strain.

Several *S. acidophilus* strains catalysed extensive and rapid dissolution of pyrite, though with strain N at least, the rate was still slower than with an *S. thermosulfidooxidans* strain (Fig. 5). There was also a tendency for the rate to decline more rapidly as the acidity and the ferric iron concentration increased during the mineral dissolution (Fig. 5). A difference in susceptibility of the two *Sulfobacillus* species to ferric iron end-product inhibition of ferrous iron oxidation was also indicated by the more rapidly declining oxidation rate during growth of *S. acidophilus* (Fig. 4). Much greater inhibition of strain ALV, in comparison to strain BC1, occurred when ferric iron was added to growth medium (Norris *et al.*, 1988). The least extensive ferrous iron oxidation was seen during growth of strains TH3 and ICP (Fig. 4), and this has also been correlated with an increased sensitivity to ferric iron

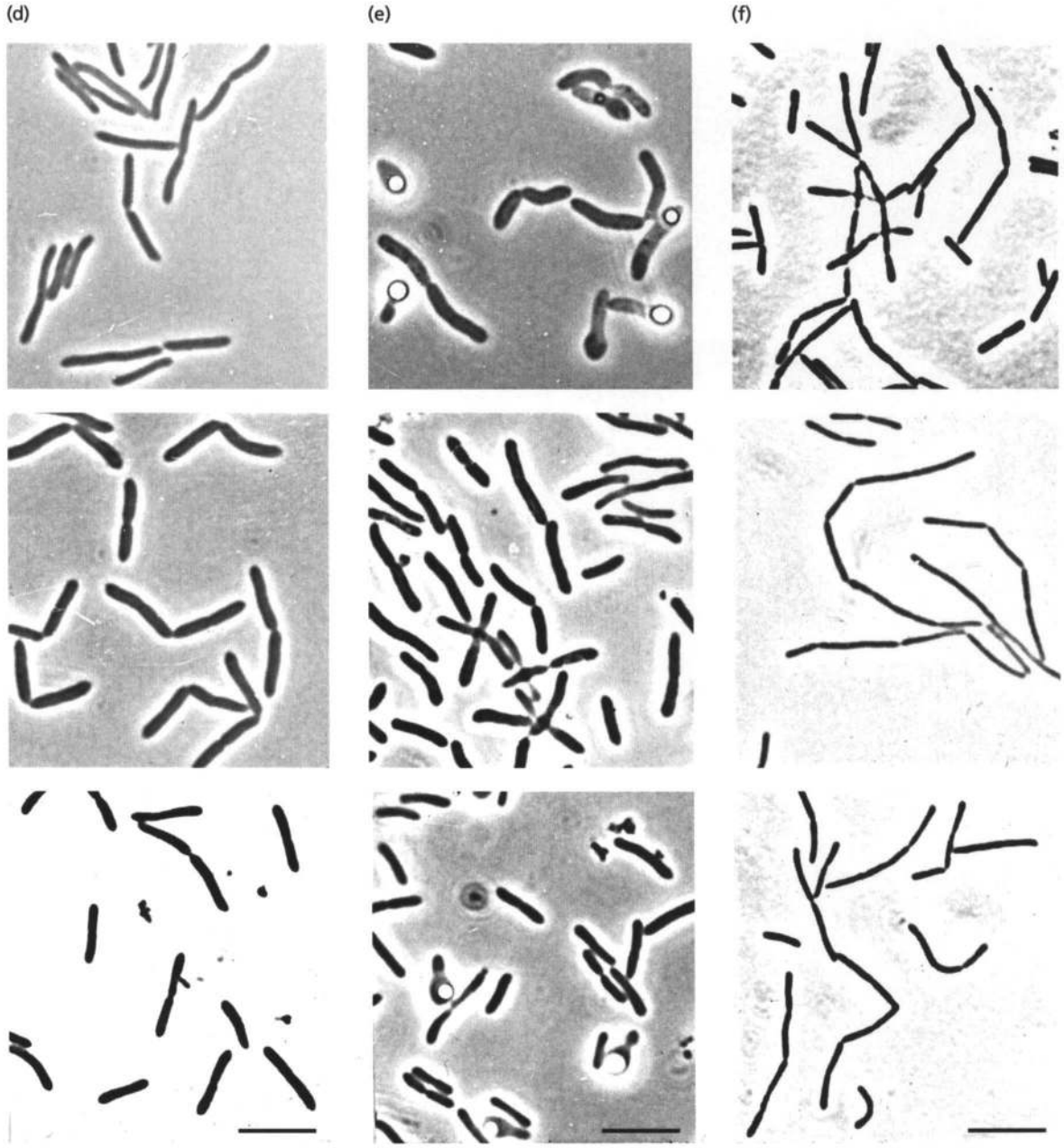

Fig. 2. Moderately thermophilic, acidophilic bacteria observed by light microscopy. Growth was on ferrous iron autotrophically (top row), on ferrous iron plus yeast extract (middle row), or heterotrophically on yeast extract (bottom row). All to same scale; bars 5 μm. Column (a) *S. thermosulfidooxidans*, (b) strain BC1, (c) strain ALV, (d) strain NAL, (e) strain N, (f) strain TH3.

in comparison with that of *S. thermosulfidooxidans* strains (D. A. Clark & P. R. Norris, unpublished).

Strain TH3 was clearly not related to the *Sulfobacillus* species. A new genus has been proposed, *Acidimicrobium*, with strain TH3 and strain ICP as isolates of its single species, *A. ferrooxidans* (Clark & Norris, 1996). Moderately thermophilic, ferrous-iron-oxidizing bacteria that appear distinct from *Sulfobacillus* and *Acidimicrobium* species have also been isolated (e.g. strain LM2: Norris, 1990; Ghauri & Johnson, 1991) but remain to be fully characterized.

Description of *Sulfobacillus acidophilus* sp. nov.

Sulfobacillus acidophilus (a.ci.do'phi.lus) sp. nov. ML n. *acidum* an acid; Gr. adj. *philus* loving; ML adj. *acidophilus* acid-loving.

Gram-positive rods (0·5–0·8 × 3·0–5·0 μm) with spherical endospores. Optimum growth is at 45–50 °C and approxi-

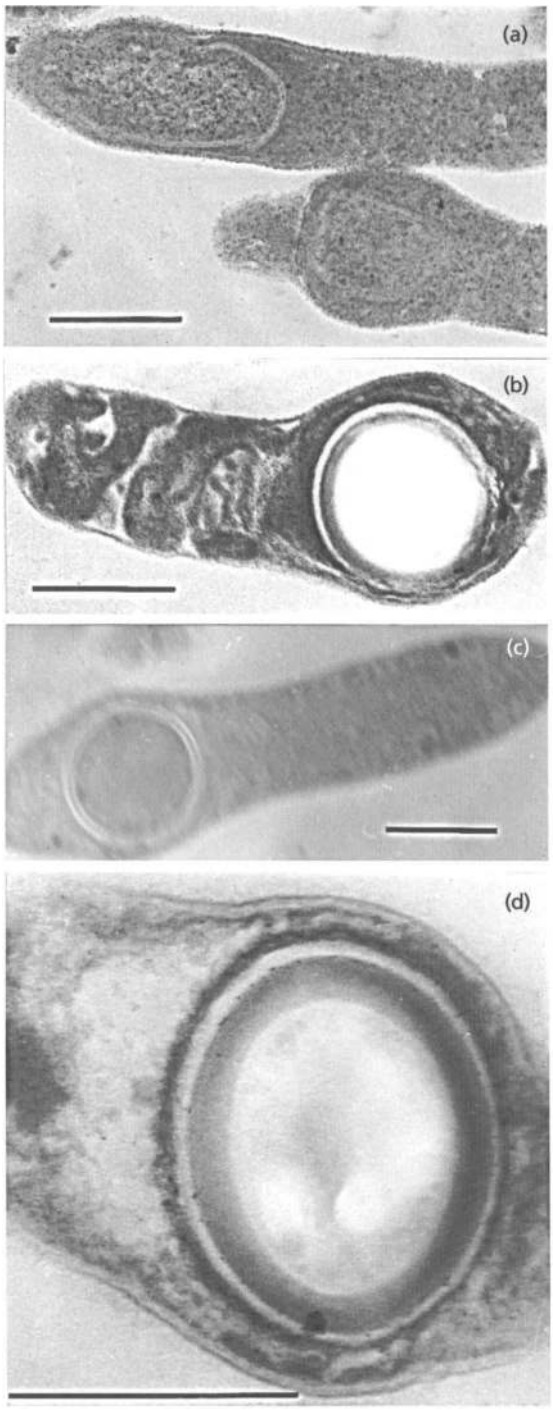

Fig. 3. Endospore formation in strain NAL (a, b) and strain BC1 (c, d). Bacteria were grown autotrophically on ferrous iron. Bars, 0·5 μm.

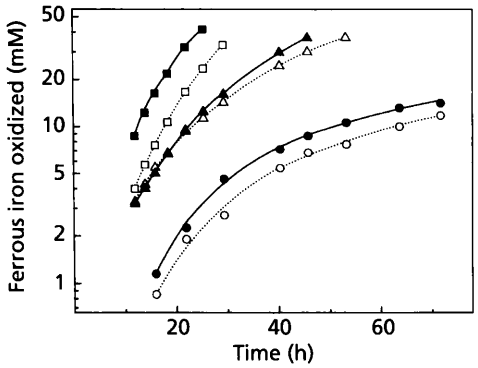

Fig. 4. Ferrous iron oxidation during autotrophic growth of acidophiles at 48 °C. ■, *S. thermosulfidooxidans*; □, strain LM1; ▲, strain ALV; △, strain NAL; ●, strain ICP; ○, strain TH3.

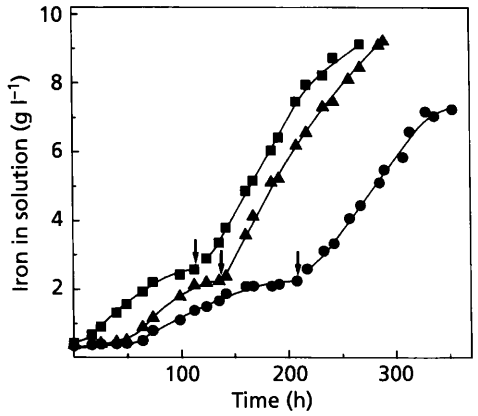

Fig. 5. Dissolution of pyrite at 48 °C during growth of strain BC1 (■), strain N (●) and a mixed enrichment culture from which strain N was isolated (▲). The concentration of pyrite, initially 1 % (w/v), was increased with addition (arrowed) of 4 % (w/v) mineral to each culture.

mately pH 2. Autotrophic growth occurs with ferrous iron and elemental sulphur as substrates. Growth on ferrous iron can also be mixotrophic with simultaneous utilization of glucose and CO_2. Heterotrophic growth occurs with yeast extract as substrate. Chromosomal DNA base composition is between 55 and 57 mol % G+C. Source: various acidic environments rich in iron, sulphur or mineral sulphides. Type strain: strain NAL (German Collection of Micro-organisms, DSM 10332).

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References

Barr, D. W., Ingledew, W. J. & Norris, P. R. (1990). Respiratory chain components of iron-oxidizing, acidophilic bacteria. *FEMS Microbiol Lett* **70**, 85–90.

Brierley, J. A. (1978). Thermophilic iron-oxidizing bacteria found in copper leaching dumps. *Appl Environ Microbiol* **36**, 523–525.

Brierley, J. A. & Brierley, C. L. (1986). Microbial mining using thermophilic microorganisms. In *Thermophiles: General, Molecular and Applied Microbiology*, pp. 279–305. Edited by T. D. Brock. New York: Wiley.

Brierley, J. A. & Le Roux, N. W. (1977). A facultative *Thiobacillus*-like bacterium: oxidation of iron and pyrite. In *GBF Conference: Bacterial Leaching*, pp. 55–66. Edited by W. Schwartz. New York: Verlag Chemie.

Brierley, J. A., Norris, P. R., Kelly, D. P. & Le Roux, N. W. (1978). Characteristics of a moderately thermophilic and acidophilic iron-oxidizing *Thiobacillus*. *Eur J Appl Microbiol* **5**, 291–299.

Clark, D. A. & Norris, P. R. (1996). *Acidimicrobium ferrooxidans* gen. nov., sp. nov.: mixed-culture ferrous iron oxidation with *Sulfobacillus* species. *Microbiology* **142**, 785–790.

Ghauri, M. A. & Johnson, D. B. (1991). Physiological diversity amongst some moderately thermophilic iron-oxidising bacteria. *FEMS Microbiol Ecol* **85**, 327–334.

Goebel, B. M. & Stackebrandt, E. (1994). Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Appl Environ Microbiol* **60**, 1614–1621.

Golovacheva, R. S. (1979). Characteristics of *Sulfobacillus thermosulfidooxidans* morphogenesis. *Microbiology* (English translation of *Mikrobiologiya*) **48**, 705–709.

Golovacheva, R. S. & Karavaiko, G. I. (1979). *Sulfobacillus* – a new genus of spore-forming thermophilic bacteria. *Microbiology* (English translation of *Mikrobiologiya*) **48**, 658–665.

Harrison, A. P., Jr. (1986). Characteristics of *Thiobacillus ferrooxidans* and other iron-oxidizing species, with emphasis on nucleic acid analyses. *Biotechnol Appl Biochem* **8**, 249–257.

Johnson, J. L. (1991). Isolation and purification of nucleic acids. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 1–19. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Karavaiko, G. I., Golovacheva, R. S., Pivovarova, T. A., Tzaplina, I. A. & Vartanyan, N. S. (1988). Thermophilic bacteria of the genus *Sulfobacillus*. In *Biohydrometallurgy, Proceedings of the International Symposium*, pp. 29–41. Edited by P. R. Norris & D. P. Kelly. Kew: Science and Technology Letters.

Kovalenko, E. V. & Malakova, P. T. (1984). The spore-forming iron-oxidizing bacterium *Sulfobacillus thermosulfidooxidans*. *Microbiology* (English translation of *Mikrobiologiya*) **52**, 760–763.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Lane, D. J., Harrison, A. P., Jr, Stahl, D., Pace, B., Giovannoni, S. J., Olsen, G. J. & Pace, N. R. (1992). Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. *J Bacteriol* **174**, 269–278.

Marsh, R. M. & Norris, P. R. (1983a). The isolation of some thermophilic, autotrophic iron- and sulphur-oxidizing bacteria. *FEMS Microbiol Lett* **17**, 311–315.

Marsh, R. M. & Norris, P. R. (1983b). Mineral sulphide oxidation by moderately thermophilic acidophilic bacteria. *Biotechnol Lett* **5**, 585–590.

Norris, P. R. (1990). Acidophilic bacteria and their activity in mineral sulfide oxidation. In *Microbial Mineral Recovery*, pp. 3–27. Edited by H. L. Ehrlich & C. L. Brierley. New York: McGraw-Hill.

Norris, P. R. & Barr, D. W. (1985). Growth and iron oxidation by acidophilic moderate thermophiles. *FEMS Microbiol Lett* **28**, 221–224.

Norris, P. R., Brierley, J. A. & Kelly, D. P. (1980). Physiological characteristics of two facultatively thermophilic mineral-oxidising bacteria. *FEMS Microbiol Lett* **7**, 119–122.

Norris, P. R., Marsh, R. M. & Lindström, E. B. (1986). Growth of mesophilic and thermophilic acidophilic bacteria on sulfur and tetrathionate. *Biotechnol Appl Biochem* **8**, 318–329.

Norris, P. R., Barr., D. W. & Hinson, D. (1988). Iron and mineral oxidation by acidophilic bacteria: affinities for iron and attachment to pyrite. In *Biohydrometallurgy, Proceedings of the International Symposium*, pp. 43–59. Edited by P. R. Norris & D. P. Kelly. Kew: Science and Technology Letters.

Owen, R. J. & Hill, L. R. (1979). The estimation of base compositions, base pairing and genome sizes of bacterial deoxyribonucleic acids. In *Identification Methods for Microbiologists* (SAB Technical Series no. 14), pp. 277–296. Edited by F. A. Skinner & D. W. Lovelock. London: Academic Press.

Sharp, R. J. & Williams, R. A. D. (1988). Properties of *Thermus ruber* strains isolated from Icelandic hot springs and DNA:DNA homology of *Thermus ruber* and *Thermus aquaticus*. *Appl Environ Microbiol* **54**, 2049–2053.

Tourova, T. P., Poltoraus, A. B., Lebedeva, I. A., Tsaplina, A. I., Bogdanova, T. I. & Karavaiko, G. I. (1994). 16S ribosomal RNA (rDNA) sequence analysis and phylogenetic position of *Sulfobacillus thermosulfidooxidans*. *Syst Appl Microbiol* **17**, 509–512.

Vartanyan, N. S., Pivovarova, T. A., Tsaplina, I. A., Lysenko, A. M. & Karavaiko, G. I. (1988). New thermoacidophilic bacterium of the genus *Sulfobacillus*. *Microbiology* (English translation of *Mikrobiologiya*) **57**, 216–221.

Wood, A. P. & Kelly, D. P. (1983). Autotrophic and mixotrophic growth of three thermoacidophilic iron-oxidizing bacteria. *FEMS Microbiol Lett* **20**, 107–112.

Wood, A. P. & Kelly, D. P. (1984). Growth and sugar metabolism of a thermoacidophilic iron-oxidizing mixotrophic bacterium. *J Gen Microbiol* **130**, 1337–1349.

Wood, A. P. & Kelly, D. P. (1985). Autotrophic and mixotrophic growth and metabolism of some moderately thermoacidophilic iron-oxidizing bacteria. In *Planetary Ecology*, pp. 251–262. Edited by D. E. Caldwell, J. A. Brierley & C. L. Brierley. New York: Van Nostrand Reinhold.

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