Phylogenetic and genetic variation among Fe(II)-oxidizing acidithiobacilli supports the view that these comprise multiple species with different ferrous iron oxidation pathways

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Autotrophic acidophilic iron- and sulfur-oxidizing bacteria of the genus *Acidithiobacillus* constitute a heterogeneous taxon encompassing a high degree of diversity at the phylogenetic and genetic levels, though currently only two species are recognized (*Acidithiobacillus ferrooxidans* and *Acidithiobacillus ferrivorans*). One of the major functional disparities concerns the biochemical mechanisms of iron and sulfur oxidation, with discrepancies reported in the literature concerning the genes and proteins involved in these processes. These include two types of high-potential iron–sulfur proteins (HiPIPs): (i) Iro, which has been described as the iron oxidase; and (ii) Hip, which has been proposed to be involved in the electron transfer between sulfur compounds and oxygen. In addition, two rusticyanins have been described: (i) rusticyanin A, encoded by the *rusA* gene and belonging to the well-characterized *rus* operon, which plays a central role in the iron respiratory chain; and (ii) rusticyanin B, a protein to which no function has yet been ascribed. Data from a multilocus sequence analysis of 21 strains of Fe(II)-oxidizing acidithiobacilli obtained from public and private collections using five phylogenetic markers showed that these strains could be divided into four monophyletic groups. These divisions correlated not only with levels of genomic DNA hybridization and phenotypic differences among the strains, but also with the types of rusticyanin and HiPIPs that they harbour. Taken together, the data indicate that Fe(II)-oxidizing acidithiobacilli comprise at least four distinct taxa, all of which are able to oxidize both ferrous iron and sulfur, and suggest that different iron oxidation pathways have evolved in these closely related bacteria.

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INTRODUCTION

The majority of obligately chemolithoautotrophic acidophilic bacteria that can oxidize ferrous iron [Fe(II)], sulfur

and reduced inorganic sulfur compounds (RISCs) have for many years been considered a priori to be strains of the well-documented species *Acidithiobacillus ferrooxidans* (*At. ferrooxidans*). The genus *Acidithiobacillus* has generally been considered to be a member of the Gammaproteobacteria, though recent phylogenetic analysis suggests it arose after divergence from the Alphaproteobacteria but before the Betaproteobacteria/Gammaproteobacteria split (Williams *et al.*, 2010). There have been numerous reports suggesting that Fe(II)-oxidizing acidithiobacilli are a heterogeneous collection of bacteria with sufficient genetic variability to warrant classification as more than one species (Harrison, 1982, 1984; Novo *et al.*, 1996; Amils *et al.*, 1998; Selenska-Pobell *et al.*, 1998; Paulino *et al.*, 2001; Karavaiko *et al.*, 2003; Mitchell *et al.*, 2003; Bergamo *et al.*, 2004; Akbar *et al.*, 2005; Waltenbury *et al.*, 2005; Peng *et al.*, 2006; Ni *et al.*, 2007, 2008a, b). For instance, a

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Abbreviations: BV, bootstrap value; HiPIP, high potential iron–sulfur protein; MLSA, multilocus sequence analysis; PP, posterior probability; RISC, reduced inorganic sulfur compound.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are provided in Figs 2–4 and Supplementary Table S2.

A supplementary figure, showing determination of the presence or absence of *rusA* and *rusB* in Fe(II)-oxidizing acidithiobacilli that reportedly contain both genes, and two supplementary tables, listing oligonucleotides used and database accession numbers of the nucleotide sequences analysed, with supplementary references, are available with the online version of this paper.

recent study using microarrays of the whole genome of the *At. ferrooxidans* type strain (ATCC 23270) showed that 12 strains isolated from different locations, and presumptively identified as *At. ferrooxidans*, were highly diverse at the genomic level (Luo *et al.*, 2009). In addition to genetic variability, several phenotypic differences have been observed among Fe(II)-oxidizing acidithiobacilli, such as their motility and the presence/absence of flagella (Dispirito *et al.*, 1982; Valdés *et al.*, 2008; Hallberg *et al.*, 2009, 2010; Li *et al.*, 2010), as well as their optimal pH and temperature for growth (Harrison, 1982; Kupka *et al.*, 2007; Dave *et al.*, 2008; Ni *et al.*, 2008b; Hallberg *et al.*, 2009, 2010). On the basis of several of these differentiating characteristics, a new species (*Acidithiobacillus ferrivorans*; *At. ferrivorans*) has recently been proposed to circumscribe some of the Fe(II)-oxidizing acidithiobacilli (Hallberg *et al.*, 2009, 2010).

One of the major functional disparities among the Fe(II)-oxidizing acidithiobacilli concerns their propensities and mechanisms for oxidation of Fe(II) and RISCs. Different strains have been reported to display very different specific rates of Fe(II) oxidation (e.g. Suzuki *et al.* 1990). Harvested biomass of some strains is able to oxidize Fe(II) immediately, irrespective of the substrate on which they have been grown, while others only do so when grown on Fe(II) (Suzuki *et al.*, 1990), suggesting either constitutive or inducible expression of the genes involved. Differences concerning RISC oxidation have also been observed, including (i) low RISC-oxidizing activities of some strains compared with others (Wakao *et al.*, 1991; Ni *et al.*, 2008b; Hallberg *et al.*, 2010) and (ii) the absence in at least one strain of the thiosulfate ubiquinone oxidoreductase that is found in the type strain (Wakai *et al.*, 2004). Furthermore, some strains oxidize Fe(II) and sulfur simultaneously, while others oxidize Fe(II) prior to sulfur (Suzuki *et al.*, 1990; Yarzabal *et al.*, 2004).

In addition to these observations, inconsistencies have been reported in the literature regarding the genes encoding redox proteins involved in Fe(II) and RISC oxidation pathways. For instance, the unique high-potential iron-sulfur protein (HiPIP) present in *At. ferrooxidans* has been proposed to be involved in the sulfur oxidation respiratory chain in a number of strains, including the type strain and ATCC 33020 (Bruscella *et al.*, 2005, 2007; Quatrini *et al.*, 2006, 2009; Valdés *et al.*, 2008), while it was described as the first electron acceptor from Fe(II) in some other strains (e.g. JCM 7811) (Fukumori *et al.*, 1988; Kusano *et al.*, 1992; Cavazza *et al.*, 1995) (Fig. 1a). The HiPIP has been referred to as Hip in the former case, and Iro (iron-oxidizing enzyme) in the latter. Also, several strains presumptively identified as *At. ferrooxidans* have been reported to have two distinct types of the blue copper protein rusticyanin (Sasaki *et al.*, 2003). The first (RusA) was detected in some *At. ferrooxidans* strains including the type strain and ATCC 33020 (see references in Holmes & Bonnefoy, 2007), whereas the second (RusB) was found in some other strains (e.g. JCM 3865, JCM 7811 and IFO 14246) (Sasaki *et al.*,

2003) and recently in most strains of *At. ferrivorans* (Hallberg *et al.*, 2009, 2010). While most strains carry only one type of rusticyanin gene, some strains have been reported to possess both (JCM 7811, JCM 3865 and IFO 14246) (Sasaki *et al.*, 2003). The *rusA* gene belongs to the *rus* operon, which encodes two cytochromes *c* and the *aa₃* cytochrome oxidase (Fig. 1b), all of which have been shown to be involved in Fe(II) oxidation (see references in Holmes & Bonnefoy, 2007; Quatrini *et al.*, 2009). While RusA is considered to play a central role in this process, the function of RusB, which has a lower kinetic rate constant for electron transfer from Fe(II) (Ida *et al.*, 2003), has not been established. Interestingly, neither *rus* nor rusticyanin has been detected in one strain (CF27) of *At. ferrivorans*, though this strain is capable of growth with Fe(II) as electron donor (Blake & Johnson, 2000; Hallberg *et al.*, 2009, 2010). In addition, strain AF2, presumptively identified as *At. ferrooxidans*, contains neither the *rusA* nor the *coxC* (*aa₃*-type cytochrome oxidase subunit III) genes from the *rus* operon (Chen *et al.*, 2009). This suggests that both of these strains use a different Fe(II) respiratory chain from that of *At. ferrooxidans* ATCC 23270^T.

All of these observations support the hypothesis that the Fe(II)-oxidizing acidithiobacilli encompass distinct species, including *At. ferrooxidans* and *At. ferrivorans*, that are metabolically similar but taxonomically distinct. In this paper, we address two questions. (i) Is there a correlation between phylogenetic relationships and genetic heterogeneity among the Fe(II)-oxidizing acidithiobacilli, in particular concerning *rusA/B*, *hip/iro* genes? (ii) Could such heterogeneity be useful in delineating different species of these bacteria? To answer these questions we have reconstructed the phylogeny of 21 Fe(II)-oxidizing acidithiobacilli based on a multilocus sequence analysis (MLSA), and investigated the presence of genes encoding the two rusticyanins (*rusA/B*) and the two HiPIPs (*hip/iro*).

METHODS

Strain selection and cultivation conditions. The Fe(II)-oxidizing acidithiobacilli used in this study are listed in Table 1, of which the genome sequences of two (ATCC 23270^T and ATCC 53993) are in the public domain. Strains CC1, CB5 and B20 were isolated from arsenic-rich mining sites, while strains R1, A1 and A2 have been described as producing more extracellular polysaccharide (EPS) than *At. ferrooxidans* ATCC 23270^T (Harneit *et al.*, 2006).

Purity of cultures was tested by streaking onto both ferrous sulfate and yeast extract overlay solid media (Johnson & Hallberg, 2007) to screen for the presence of single-colony morphologies of Fe(II)-oxidizing bacteria and heterotrophic contaminants, respectively. Following the inference that JCM 7811 comprises more than one strain of Fe(II)-oxidizing acidophile, the freeze-dried biomass obtained was suspended in 200 µl acidified (pH 2.5) basal salts solution and portions were inoculated into a 20 mM ferrous sulfate/basal salts/trace elements medium (Wakeman *et al.*, 2008) that had been adjusted to either pH 1.6 or 2.2. When the cultures had oxidized most of the ferrous iron (as indicated by a colour change from colourless to orange), they were streak-inoculated onto ferrous iron overlay plates, and representative colonies of differing morphologies

Fig. 1. Genetic organization of the rusticyanin- and HiPIP-encoding genes and the pathways in which they are involved. (a) *hip* in *At. ferrooxidans* ATCC 23270^T and ATCC 33020 involved in the electron transfer chain between RISCs and O₂; *iro* in JCM 7811 proposed to be involved in the electron transfer chain between Fe(II) and O₂. (b) *rusA* in *At. ferrooxidans* ATCC 23270^T and ATCC 33020 involved in the Fe(II) oxidation pathway; *rusB* in JCM 3865, JCM 7811 and IFO 14246, for which a role is unknown. Solid arrows represent transcriptional units; dotted arrows represent electron transport.

were picked off and maintained as separate cultures (denoted as JCM 7811-P1 and JCM 7811-P4).

Most of the strains were grown under aerobic conditions at 30 °C in acidified (pH 1.6) ferrous sulfate/basal salts liquid medium, as described previously (Yarzabal *et al.*, 2003). Strains of *At. ferrivorans* (which is less acidophilic than *At. ferrooxidans*) were also grown at 30 °C but at pH 2.2 (Hallberg *et al.*, 2010).

DNA extraction, amplification and sequencing. Genomic DNA from most of the *Acidithiobacillus* strains was prepared with the NucleoSpin Tissue kit (Macherey Nagel). *At. ferrivorans* cells and those from the original JCM and IFO strain suspensions (100 µl) or from liquid cultures of the latter (1 ml) were harvested, and crude DNA was extracted as described previously (Okibe *et al.*, 2003).

Primers used for PCR amplification of each targeted gene and the sizes of the expected PCR products are described in Supplementary Table S1 available with the online version of this paper. The PCR

program consisted of an initial denaturation step at 94 °C for 2 min 30 s, followed by 30 (or 40) cycles of 30 s at 94 °C, 30–60 s at primer annealing temperature (see Supplementary Table S1), 30–90 s at 72 °C, with a final extension step at 72 °C for 7 min. PCR products were analysed by electrophoresis in a 1% agarose gel, then concentrated and purified using Microcon PCR (Millipore) or QIAquick (Qiagen) reagents.

Nucleotide sequences of the amplified DNA were determined by Cogenics or Eurofins MWG operon, or as described previously (Hallberg *et al.*, 2006).

Phylogenetic analysis. The sequences of the phylogenetic markers used in this study [*rrs* (16S rRNA gene), 16S–23S ITS1, *atpD*, *nifH*, *recA*, *rusA/B* and *iro/hip*] were obtained from the published genomes of *At. ferrooxidans* ATCC 23270^T and strain ATCC 53993, or, for other strains, were determined as described above. For the analysis of the *rrs* gene, we used sequences from two closely related species (*Acidithiobacillus thiooxidans* and *Acidithiobacillus albertensis*) as

Table 1. Strains of Fe(II)-oxidizing acidithiobacilli used in this study

Strain*	Origin	Percentage DNA : DNA hybridization (against the strain listed)	Source or reference
<i>At. ferrooxidans</i> ATCC 23270^T	Acid bituminous coal mine effluent, USA	94 (ATCC 19859)†, 91 (A1)†	American Type Culture Collection (Leathen & Braley, 1954)
ATCC 53993	Derived from <i>Leptospirillum ferrooxidans</i> DSM 2705 ^T (ex Markosyan 1972)		Hippe (2000)
ATCC 19859	Acid copper mine leaching water, Canada	81 (A2)†	American Type Culture Collection (Razzell & Trusell, 1963)
A1	Disused sulfidic ore mine, Rammelsberg, Germany	99 (A2)†	Brauckmann (1985)
A2	Disused sulfidic ore mine, Rammelsberg, Germany		Brauckmann (1985)
ATCC 33020 (11Fe)	Uranium mine, Japan	63 (<i>At. ferrooxidans</i> ATCC 23270 ^T)‡, 60 (ATCC 19859)§	American Type Culture Collection (Tomizuka <i>et al.</i> , 1976)
CC1	Acid mine drainage, Carnoulès, France		Duquesne <i>et al.</i> (2003)
CB5	Acid mine drainage, Carnoulès, France		O. Bruneel and others, unpublished data
B20	Acid mine drainage, Carnoulès, France		Egal <i>et al.</i> (2009)
BRGM1	Isolate from BRGM consortium (sulfide ore mine, South Africa)		Liu <i>et al.</i> (2000)
<i>At. ferrivorans</i> (DSM 22755^T)	Copper mine spoil drainage, Norway	37 (<i>At. ferrooxidans</i> ATCC 23270 ^T)	Hallberg <i>et al.</i> (2010)
Peru6	Abandoned mine drainage in the Andes, Peru		Hallberg <i>et al.</i> (2010)
OP14	Abandoned Cae Coch pyrite mine, North Wales, UK		Hallberg <i>et al.</i> (2010)
CF27	Abandoned copper/cobalt mine drainage, ID, USA		Hallberg <i>et al.</i> (2010)
SS3-P1	Clonal isolate obtained in this study from SS3 culture, Norilsk mining area, Russia		Kupka <i>et al.</i> (2007)
JCM 3865 (THI135)	Acid mine water, Shimokawa, Japan		Japan Collection of Microorganisms (Takamori <i>et al.</i> , 1983)
JCM 7811-P1	Clonal isolate obtained in this study from the JCM 7811 culture originating in the Matsuo sulfur and iron sulfide mine, Japan		Japan Collection of Microorganisms (Wakao <i>et al.</i> , 1991)
JCM 7811-P4	<i>ibid</i>		
JCM 7812 (Fe2)	Matsuo sulfur and iron sulfide mine, Japan		Japan Collection of Microorganisms (Wakao <i>et al.</i> , 1991)
IFO 14246 (BA-4)	Settling pond, Belle Ayr coal mine, USA	24 (ATCC 19859)§	NITE Biological Resource Center (Harrison, 1982)
R1	Sulfidic copper–zinc ore (Ilba mine), Romania	40 (<i>At. ferrooxidans</i> ATCC 23270 ^T)†	Sand <i>et al.</i> (1992)

*Type strains are indicated in bold type.

†W. Sand, University of Duisberg-Essen, personal communication.

‡This study.

§Harrison (1982).

||Hallberg *et al.* (2009, 2010).

outgroups. These sequences were retrieved from the *nr* database at the National Center for Biotechnology Information (NCBI) (accession numbers AJ459803 and AJ459804, respectively). For each marker, sequences were aligned using CLUSTAL W2 default parameters (Larkin *et al.*, 2007). The resulting alignments were inspected using the MUST software (Philippe, 1993). Regions where the alignment was ambiguous were removed prior to phylogenetic analysis using MUST. The concatenation based on the alignments of *atpD*, *nifH* and *recA* was constructed using in-house software.

For each marker, and for the concatenated sequences, phylogenetic trees were reconstructed using maximum-likelihood and Bayesian methods

implemented, respectively, in TREEFINDER (Jobb *et al.*, 2004) and MrBayes (Ronquist & Huelsenbeck, 2003). For maximum-likelihood analyses, the evolutionary models used were selected according to the 'propose model' tool implemented in TREEFINDER. The branch robustness of the resulting trees was estimated by the non-parametric bootstrap procedure implemented in TREEFINDER using the same parameters. For Bayesian analyses we used the GTR model and a gamma distribution to account for evolutionary site rate variations (four categories and an estimated alpha-parameter). MrBayes was run with four chains for 1 million generations and trees were sampled every 100 generations. To construct the consensus tree, the first 1500 trees were discarded as 'burn-in'.

DNA–DNA hybridization. Cells from 80 l cultures of Fe(II)-grown *At. ferrooxidans* ATCC 23270^T and strain ATCC 33020 were lysed using a French pressure cell (Thermo Spectronic), and genomic DNA from these lysates was purified by chromatography on hydroxyapatite, as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out by the Deutsche Sammlung von Mikroorganismen und Zellkulturen, as described by De Ley *et al.* (1970) with the modifications of Huß *et al.* (1983), using a Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian).

RESULTS

MLSA-based phylogeny

While the *rrs* gene sequences of the strains examined showed less than 2 % divergence, which is less than the often-cited threshold of 3 % recommended to differentiate species (Stackebrandt & Goebel, 1994), phylogenetic analysis clearly placed them into four distinct clusters (I, II, III and IV, Fig. 2a). Group I is represented by *At. ferrooxidans* ATCC 23270^T and also includes ATCC 53993, ATCC 19859 and strains A1 and A2. Group II is represented by ATCC 33020, and also includes strains CC1, CB5, B20 and BRGM1. Group III is composed exclusively of strains of *At. ferrivorans*, and Group IV of strains JCM 7812, JCM 3865, IFO 14246 and R1. However, while these four clusters were well supported by high bootstrap values (BVs) ($\geq 96\%$) and posterior probabilities (PPs) (≥ 0.98), the relationships to each other are not fully resolved. Indeed, while the link between Groups III and IV was well supported (BV=96 % and PP=1.0), their

relationship with Group II strains was less clear (BV=78 % and PP=0.74, Fig. 2a). This lack of resolution is likely to be linked to the high similarity between *rrs* gene sequences, which also confounds the classification of the Fe(II)-oxidizing acidithiobacilli strains based on this gene sequence alone.

Four additional markers were analysed to refine the phylogeny of Fe(II)-oxidizing acidithiobacilli. Importantly, these markers are located in different regions of the chromosome, at least in those of *At. ferrooxidans* ATCC 23270^T and strain ATCC 53993, limiting biases due to single horizontal gene transfer (HGT) of adjacent loci. The unrooted phylogenies based on each of these genes were congruent with the tree based on *rrs* gene sequences. In particular, Groups I to IV were recovered and well supported in all trees (Figs 2b and 3a–c). The only exceptions were Group I and Group IV, in ITS1 and *nifH* trees, respectively. In the former case, ITS1 sequences from strains A1 and A2 did not cluster with other Group I sequences (Fig. 2b), while in the latter case, the JCM 3865 *nifH* sequence emerged as a sister of Group I, albeit with moderate statistical support (BV=82 % and PP=0.69, Fig. 3b). Interestingly, in the ITS1 tree, the grouping of the strains correlated with sequence lengths, as observed by Ni *et al.* (2007, 2008a). The size of the ITS1 was 441, 454, 442–443 and 452 bp for strains from Groups I–IV, respectively. Exceptions were strains A1 and A2, which harboured an atypical and divergent ITS1 resulting from the insertions of short runs of nucleotides, which also might skew the phylogenetic analysis, and resulted in the separa-

Fig. 2. Bayesian phylogenetic trees of the Fe(II)-oxidizing *Acidithiobacillus rrs* gene (a) and the 16S–23S rRNA ITS1 (b) (1387 and 577 positions, respectively). The *rrs* tree is rooted with sequences from *Acidithiobacillus thiooxidans* (At) and *Acidithiobacillus albertensis* (Ata). The ITS1 tree is unrooted. Italicized numbers at nodes represent PPs, whereas numbers in bold type represent BVs. For clarity, only PPs greater than 0.5 and BVs greater than 50 % are shown. The scale bars represent the average number of substitutions per site. *At. ferrooxidans* ATCC 23270^T and *At. ferrivorans* DSM 22755^T are in Group I and Group III, respectively. Accession numbers of the nucleotide sequences are given in parentheses. Type strains are indicated in bold type.

Fig. 3. Bayesian unrooted phylogenetic trees of Fe(II)-oxidizing *Acidithiobacillus atpD* (a) (1256 positions), *nifH* (b) (346 positions) and *recA* (c) (805 positions) genes and of their concatenation (d) (2407 positions). Italicized numbers at nodes represent PPs, whereas numbers in bold type represent BVs. For clarity, only PPs greater than 0.5 and BVs greater than 50 % are shown. The scale bars represent the average number of substitutions per site. *At. ferrooxidans* ATCC 23270^T and *At. ferrovorans* DSM 22755^T are in Group I and Group III, respectively. Accession numbers of the nucleotide sequences are given in parentheses. Type strains are indicated in bold type.

tion of these two strains from the rest of the Group I strains with a very long branch (Fig. 2b). The close relationship between Group III and Group IV based on *rrs* analysis was also observed in the *atpD* and *recA* trees (BV=100 and 82 %, and PP=1.0 and 0.89) (Fig. 3a, c).

These results support the hypothesis that Fe(II)-oxidizing acidithiobacilli comprise at least four distinct taxa. However, as with the *rrs* gene phylogeny, the branching pattern within each group was poorly supported (most BVs <90 %, Figs 2 and 3), indicating that the phylogenetic signal carried by these individual markers is not sufficient to resolve the relationships between the corresponding strains, possibly due in part to their relatively small size but also to the great similarity between sequences.

To improve the resolution of the relationships within each group, sequences of the *recA*, *atpD* and *nifH* genes were concatenated, leading to a supermatrix of 2407 nt positions. The resulting phylogenetic tree supported the split of the Fe(II)-oxidizing acidithiobacilli into four clades (BV ≥98 % for groups I, II and III and 66 % for group IV, Fig. 3d). Interestingly, whereas most strain relationships within groups remained poorly resolved, some well-supported groupings emerged: A1 and A2 strains within Group I (BV=100 % and PP=0.99); the four remaining strains of Group I (BV=100 % and PP=1.0); BRGM1 and ATCC 33020 strains within Group II (BV=100 % and PP=1.0); OP14 and CF27 strains in Group III (BV=92 % and PP=0.92); Peru6 and SS3-P1 strains, together with *At. ferrooxidans* ATCC 23270^T in Group III (BV=83 % and

98 %, and PP=0.72 and 0.79, respectively); and IFO 14246, JCM 7812 and JCM 7811-P4 (BV=88 %), together with R1 (BV=83 % and PP=0.94), within Group IV.

Taxonomy of Fe(II)-oxidizing acidithiobacilli

MLSA showed that *At. ferrooxidans* ATCC 23270^T and strain ATCC 33020 belong to two different phylogenetic groups (I and II), though their *rrs* genes have 99 % sequence identity. The same gene of *At. ferrooxidans* ATCC 23270^T and Group IV strain JCM 7812 shared 98.5 % sequence identity. All DNA–DNA hybridization values (DDHVs) for strains identified by MLSA to be in Group I were >80 % (W. Sand, personal communication) (Table 1), confirming that these do indeed belong to the same species as *At. ferrooxidans* ATCC 23270^T. By contrast, Group II strain ATCC 33020 and *At. ferrooxidans* ATCC 23270^T were shown to have a DDHV of only 63 % (Table 1), a value similar to that (60 %) previously reported for strain ATCC 33020 and another Group I strain (ATCC 19859) (Harrison, 1982). Elsewhere, it has been found that *At. ferrooxidans* ATCC 23270^T and Group IV strain R1 have a DDHV of 40 % (W. Sand, personal communication), while Harrison (1982) had earlier reported an even smaller DDHV (24 %) for Group I strain ATCC 19859 and Group IV strain IFO 14246 (Table 1). Since DDHVs of <70 % are generally considered to indicate different species (Wayne *et al.*, 1987), it is apparent that neither Group II nor Group IV bacteria should be regarded as strains of *At. ferrooxidans*, like those in Group III, which are strains of *At. ferrivorans*.

Representative isolates from Groups I–IV revealed some phenotypic differences from each other (Table 2). These

included: (i) a lesser propensity for growth on sulfur or tetrathionate (as evidenced by protracted lag phases) in strains of Groups III and IV compared with strains of Groups I and II; (ii) similar cell sizes to each other of bacteria of Groups I, II and IV, although notably smaller than that of *At. ferrivorans* (Group III); (iii) non-motility of cells of Groups I and IV, and motility of Groups II and III; (iv) growth at 4 °C of Group III acidithiobacilli alone; and (v) sensitivity to low pH and absence of growth at pH 1.6 of Group III strains.

Differences in the ferrous iron oxidation pathway among acidithiobacilli

The biochemical mechanism by which *At. ferrooxidans* mediates Fe(II) oxidation has been the subject of considerable debate and some controversy for a number of years. To ascertain whether some of the disparate results reported correlate with the phylogenetic clustering of strains revealed in the present study, PCR amplification of the *hip*, *iro*, *rusA* and *rusB* genes in the strains examined was performed. The results revealed a strong correlation between the distribution of *rusA/rusB* and *hip/iro* among the strains and the four phylogenetic groups detected by MLSA (Fig. 4). Notably, the presence of a *rusA* gene and the absence of a *rusB* gene were detected in strains from Groups I and II, while one, two or no copies of *rusB*, and no *rusA* gene were observed in strains from Groups III and IV with the primers used. Furthermore, whereas the *hip* gene was amplified from all strains in Groups I and II, only the *iro* gene was detected in strains from Groups III and IV with our primers. Phylogenies based on the *rusA/rusB* and *hip/iro* genes were in agreement with those obtained by MLSA (Fig. 4).

Table 2. Differentiation of Fe(II)-oxidizing *Acidithiobacillus* species and isolates based on selected phenotypic traits

All strains grew at 30 and 37 °C and in medium of pH 2.2. The main differences are indicated in bold type.

Strain	MLSA group	Growth at 4 °C	Growth at pH 1.6	Motility	Sulfur oxidation
<i>At. ferrooxidans</i> ATCC 23270 ^T	I	—*	+†	—	+
JCM 7811-P1	I	—	+	—	+
ATCC 33020	II	—	+	+	+
CC1	II	—	+	+	+
<i>At. ferrivorans</i> DSM 22755 ^{T*}	III	+	—	+	–/+‡
CF27*	III	+	—	+	–/+‡
JCM 3865	IV	—	+	—	–/+‡
JCM 7811-P4	IV	—	+	—	–/+‡
JCM 7812	IV	—	+	—	–/+‡
R1	IV	—	+	—	–/+‡

*Data from Hallberg *et al.* (2010).

†Data from Kelly & Wood (2005).

‡These strains exhibited a long lag phase before growth with sulfur or tetrathionate as electron donor occurred. Upon subsequent subculturing into medium with these electron donors, lag phases were less pronounced.

Fig. 4. Bayesian unrooted phylogenetic trees of Fe(II)-oxidizing *Acidithiobacillus iro/hip* (a) and *rus* (b) genes (358 and 392 positions, respectively). Italicized numbers at nodes represent PPs, whereas numbers in bold type represent BVs. Stars highlight the two different copies of *rusB* amplified from JCM 3865. For clarity, only PPs greater than 0.5 and BVs greater than 50 % are shown. The scale bars represent the average number of substitutions per site. *At. ferrooxidans* ATCC 23270^T and *At. ferrivorans* DSM 22755^T are in Group I and Group III, respectively. Accession numbers of the nucleotide sequences are given in parentheses. Type strains are indicated in bold type.

Rusticyanin in strains JCM 7811, JCM 3865 and IFO 14246

As mentioned above, some Fe(II)-oxidizing acidithiobacilli reportedly have both *rusA* and *rusB* genes, and JCM 3865 has been reported to have two copies of the latter (Sasaki *et al.*, 2003). The presence of two copies of *rusB* in JCM 3865 could be explained by a single and specific gene duplication event in this strain. However, since none of the confirmed pure cultures of Fe(II)-oxidizing acidithiobacilli analysed in this study was found to contain both *rusA* and *rusB*, we examined the possibility that the cultures previously examined might have comprised more than a single strain.

DNA extracted from the original lyophilized culture of JCM 7811 gave a strong *rusA* and a weak *rusB* PCR product, whereas DNA from subcultures grown at either pH 1.6 or 2.2 gave relatively stronger *rusB* PCR products (Supplementary Fig. S1). The two colony variants obtained from the original culture, strains JCM 7811-P1 and JCM 7811-P4, matched the description of those of strains Fe1 and Fe2 (Wakao *et al.*, 1991), which were deposited as JCM 7811 and 7812, respectively. MLSA showed that JCM 7811-P1 belongs to Group I, and it was found to contain *hip* and *rusA* (but not *rusB*) genes (Figs 2–4). Like other Group I strains, it was not motile, grew at pH 1.6 but not at 4 °C, and oxidized RISCs efficiently (Table 2). In contrast, the second isolate, JCM 7811-P4 belongs to Group IV, which also includes JCM 7812. It harbours *rusB* (but not *rusA*) and *iro* genes (Figs 2–4). In addition, it shares physiological similarities with other representatives of Group IV; in particular, it exhibited a long lag phase prior to growth on RISCs (Table 2). It was thus apparent that the original JCM

7811 culture was mixed and contained at least two distinct strains of Fe(II)-oxidizing acidithiobacilli.

In contrast, the JCM 3865 culture was confirmed to be pure. However, while it was initially described as having two copies of *rusB* and one of *rusA*, we detected only the two different *rusB* genes (stars in Fig. 4b) and no *rusA* (Supplementary Fig. S1). The same result was obtained with a few pure colonies isolated from the original culture supplied. MLSA (Figs 2–4) and physiology analysis (Table 2) placed JCM 3865 in Group IV, and like other strains in this group it also contained the *iro* gene (Fig. 4).

We failed to get growth from two lyophilized cultures of IFO 14246 provided independently by the National Institute of Technology and Evaluation (NITE) Biological Resource Center, Japan. However, DNA was extracted directly from one of the lyophilized cultures, and this served as template for PCR and subsequent sequencing. Each of the DNA fragments amplified was highly identical to those of JCM 3865, JCM 7812 and JCM 7811-P4. MLSA placed IFO 14246 unambiguously in Group IV (Figs 2–4). As with all strains of Group IV, *rusB* and *iro* genes were amplified from the lyophilized IFO culture, but *rusA* and *hip* were not (Supplementary Fig. S1).

DISCUSSION

DNA–DNA hybridization and phylogenetic analysis of *rrs* gene sequences have been the main techniques used in recent decades to determine bacterial taxonomy (Stackebrandt & Goebel, 1994). More recently, MLSA, which has a number of advantages over more traditional

approaches (Maiden, 2006), has been used to clarify areas where phylogenetic relationships are unclear, allowing the refinement of the taxonomy of composite bacterial groups such as the *Geobacteraceae* (Holmes *et al.*, 2004), *Xanthomonas* (Young *et al.*, 2008), *Streptomyces* (Guo *et al.*, 2008) and *Bradyrhizobium* (Rivas *et al.*, 2009). In the current work, MLSA has been used to differentiate strains of Fe(II)-oxidizing acidithiobacilli and has provided firm evidence of the existence of multiple species within this bacterial group.

Collectively, the results of the *rrs*, ITS1, *recA*, *atpD*, *nifH*, *rusA/B* and *hip/iro* phylogenetic analyses showed that the 21 strains analysed fell into four distinct groups (Figs 2–4), which currently comprise only two recognized species. Group I includes strains of *At. ferrooxidans* while Group III is composed exclusively of *At. ferrivorans* strains. Group II includes ATCC 33020, a strain that has previously been suggested to be a distinct species (Selenska-Pobell *et al.*, 1998). This grouping is similar to that of Karavaiko *et al.* (2003), who found that 17 strains of '*At. ferrooxidans*' also fell into four distinct phylogenetic groups based on 16S DNA gene sequences, though Groups III and IV are reversed in the present study. DNA–DNA hybridization data confirmed that strains of Groups II and IV, like those of *At. ferrivorans* (Group III), do not belong to the species *At. ferrooxidans* (Group I) (Table 1). Furthermore, MLSA (Figs 2–4) suggested that Groups III and IV are related and distinct from Groups I and II. Group IV strains, however, were different from Group III bacteria in some physiological traits, including growth at low temperatures, sensitivity to low pH, cell size and motility (Table 2). We therefore suggest that phylogenetic Groups II and IV represent novel species of *Acidithiobacillus*, though more in-depth physiological analysis is necessary before these can be officially designated novel species.

One of the most significant findings from this work concerns the observed distribution of rusticyanin- and HiPIP-encoding genes in these four groups. *rusA* and *hip* genes were found only in Groups I and II, while *rusB* and *iro* were present only in Groups III and IV, which correlates with the close phylogenetic relationship between these respective groups (Figs 2–4). These data further support the view that Group II bacteria belong to a species different from those in Groups III and IV, and also that Group IV bacteria are distinct from *At. ferrooxidans* (Group I strains) (Figs 2–4). Also, the failure to detect any strain that harboured copies of both *rusA* and *rusB*, coupled with the finding that one of these cultures contained both Group I and Group IV strains, implies that the results of Sasaki *et al.* (2003) were erroneous and due to the use of mixed cultures. However, the existence of two *rusB* genes in strain JCM 3865 was confirmed. The differences in the distribution of *rusA/B* and *hip/iro* genes suggest that pathways of ferrous iron oxidation in bacteria in Groups I and II are different from those in Groups III and IV, which probably accounts for the disparate results obtained previously by different research groups who have investigated this subject (e.g. Fukumori *et al.*, 1988; Appia-Ayme *et al.*,

1999; Sasaki *et al.*, 2003; Bruscella *et al.*, 2005). The upstream and downstream regions of the two rusticyanin genes are also completely different. The *rusA* gene (in Groups I and II) belongs to the *rus* operon (Fig. 1b; Bengrine *et al.*, 1998; Appia-Ayme *et al.*, 1999; Valdés *et al.*, 2008), which is more highly expressed when cells are grown with ferrous iron than with sulfur (Yarzabal *et al.*, 2004; Quatrini *et al.*, 2006, 2009). In contrast, analysis of the DNA sequence data from JCM 3865 (Sasaki *et al.*, 2003) revealed that upstream of *rusB* there is a gene that encodes a putative VagC toxin component of a toxin–antitoxin system (Fig. 1b). The location of the *rusB* gene in strains of *At. ferrivorans* (Group III) is currently unknown, and no information is available about the regulation of the expression of this gene in any *Acidithiobacillus* strain. The absence of *rusB* (as well as *rusA*) in *At. ferrivorans* strain CF27, which nonetheless grows by iron oxidation, suggests strongly that rusticyanin is not a core component of the Fe(II)-oxidizing apparatus in Group III or (by inference) Group IV strains.

All strains in Groups III and IV have been shown to contain the *iro* gene, which encodes a HiPIP proposed to be an iron oxidase (Fukumori *et al.*, 1988; Kusano *et al.*, 1992), and which is monocistronic and located between the *purA* and leucyl tRNA genes (Kusano *et al.*, 1992) (Fig. 1a). The *iro* gene was not detected in the genome of *At. ferrooxidans* ATCC 23270^T and ATCC 53993 (Bruscella *et al.*, 2005; Valdés *et al.*, 2008; this study), and could not be amplified from any strain in Groups I and II (Bruscella *et al.*, 2005; this study). While neither *At. ferrooxidans* ATCC 23270^T nor Group II strain ATCC 33020 contains the *iro* gene, they both possess a homologue of *iro*, that is the *hip* gene. The *hip* gene has been shown to be located in a genetic context different from that of the *iro* gene, and is part of the *petII* operon encoding the *bc₁* complex (Fig. 1a). Transcription of this gene is higher when cells are grown with sulfur than with Fe(II) (Quatrini *et al.*, 2006, 2009; Bruscella *et al.*, 2007), implying that, in contrast to *iro*, *hip* participates in the oxidation of RISCs rather than Fe(II).

The results of this study strongly suggest that at least two different pathways for Fe(II) oxidation have evolved in the four highly related taxa of Fe(II)-oxidizing acidithiobacilli identified, and reconcile the models (Fig. 1) that had previously been considered to be conflicting. In *At. ferrooxidans* ATCC 23270^T, other Group I strains and Group II strains, proteins encoded by *rusA* and the corresponding operon have a central role, while in Group III (*At. ferrivorans*) and Group IV strains, the iron oxidase encoded by *iro* is probably a key component. Other questions remain, such as the role of *rusB* in *At. ferrivorans* (in those strains which possess it) and *Acidithiobacillus* Group IV strains, and this will be addressed in future work.

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