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Phylogeny and photosynthetic features of *Thiobacillus acidophilus* and related acidophilic bacteria: its transfer to the genus *Acidiphilium* as *Acidiphilium acidophilum* comb. nov.

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Phylogenetic analyses based on 16S rDNA sequences and genomic DNA–DNA relatedness showed that the sulphur-oxidizing facultative chemolithotroph *Thiobacillus acidophilus* was closely related to members of the genus *Acidiphilium*, which is a group of strictly aerobic, heterotrophic acidophiles now categorized into aerobic photosynthetic bacteria. Lipophilic pigment analyses revealed that zinc-chelated bacteriochlorophyll *a* and carotenoids occurred in appreciable amounts in *T. acidophilus* and all established species of the genus *Acidiphilium*. PCR experiments showed that *T. acidophilus* as well as *Acidiphilium* species contained *puf* genes, encoding the photosynthetic reaction centre proteins and the core light-harvesting complex of the purple bacteria. There were high similarities between *T. acidophilus* and *Acidiphilium* species in the primary structure of their reaction centre proteins deduced from the nucleotide sequence data. The phylogenetic tree of the reaction centre proteins was in agreement with the 16S rDNA sequence-based phylogenetic tree in the relationship between *T. acidophilus* and *Acidiphilium* species and between the *Acidiphilium* cluster and other purple photosynthetic bacteria. Based on these results, together with previous phylogenetic and phenotypic information, it is proposed to reclassify *T. acidophilus* (Guay and Silver) Harrison 1983 as *Acidiphilium acidophilum* comb. nov. The type strain is ATCC 27807^T (= DSM 700^T).

Keywords: *Thiobacillus acidophilus*, zinc-bacteriochlorophyll, photosynthetic reaction centre, phylogeny, *Acidiphilium acidophilum* comb. nov.

INTRODUCTION

The genus *Thiobacillus* is a group of obligately or facultatively chemolithotrophic aerobic proteobacteria that are capable of growing with reduced inorganic sulphur compounds as sole energy source, but, at this time, is quite heterogeneous with members exhibiting a wide range of physiological, chemotaxonomic and genetic characteristics (Katayama-Fujimura *et al.*, 1982, 1983; Kelly & Harrison, 1989; Lane *et al.*, 1985, 1992). This group encompasses a number of acidophilic species (Harrison, 1984; Pronk *et al.*, 1990), including *Thiobacillus acidophilus*, in addition to neutrophilic thiobacilli. The name *T. acidophilus* was proposed by Guay & Silver (1975) for some strains of facultatively chemolithotrophic acidophilic thiobacilli, but did not appear on the Approved Lists of Bacterial Names (Skerman *et al.*, 1980). Harrison (1983) revived the name *T. acidophilus* following his confirmation that this organism grew equally well with elemental sulphur or glucose as a sole energy source. Phylogenetic analyses based on 5S rRNA sequences (Lane *et al.*, 1985) and partial 16S rRNA sequences (Lane *et al.*, 1992), however, indicated that *T. acidophilus* was far distant from any other *Thiobacillus* species and was closely related to species of the genus *Acidiphilium*, which includes aerobic

Abbreviations: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin.

The DDBJ accession numbers for the 16S rDNA sequences determined in this paper are D86508, D86509, D86511, D86513 and AB006712; for the *puf* gene the number is AB013379.

acidophilic chemo-organotrophic bacteria that are unable to use reduced sulphur compounds as energy source (Harrison, 1989; Kishimoto *et al.*, 1995b). Despite the dissimilarity in sulphur metabolism between *T. acidophilus* and *Acidiphilium* species, the available phylogenetic information has strongly suggested that the former species should be positioned among members of the genus *Acidiphilium*.

Recent research has shown that *Acidiphilium* species can be categorized into a group of aerobic photosynthetic bacteria (Shimada, 1995) because of their production of bacteriochlorophyll (BChl) only under aerobic growth conditions and their photosynthetic activity (Kishimoto *et al.*, 1995a; Wakao *et al.*, 1993, 1994). Interestingly, it has been shown more recently that a representative member of *Acidiphilium*, *Acidiphilium rubrum*, contains a fully active photosynthetic system with zinc-chelated bacteriochlorophyll (Zn-BChl) *a* as the major pigment (Wakao *et al.*, 1996). This was the first demonstration of the existence of natural photosynthesis using (bacterio-)chlorophylls containing a metal other than magnesium. Further studies have shown that all established species of the genus *Acidiphilium* contain the *puf* operon (Nagashima *et al.*, 1997b), which is an assemblage of genes encoding the proteins of the photosynthetic reaction centre (L, M and C subunits) and the core light-harvesting complex (α and β subunits) of the purple photosynthetic bacteria. The structure of the *puf* operon is well-conserved among species of these bacteria, and thus the PCR technique is applicable for detection of a conserved region of *puf* genes (Nagashima *et al.*, 1997a, b).

These recent findings motivated us to re-evaluate the phylogenetic relationships between *T. acidophilus* and members of the genus *Acidiphilium* and to determine whether *T. acidophilus* has photosynthetic properties. A previous study undertaken to detect photopigments in *T. acidophilus* gave negative results (Kishimoto *et al.*, 1995a), but our attempts to find Zn-BChl *a* and *puf* genes in *T. acidophilus*, as well as in all *Acidiphilium* species, have been successful. Phylogenetic analyses based on 16S rDNA sequences, genomic DNA–DNA relatedness and the L and M subunit proteins of the reaction centre demonstrated that there were close relationships between *T. acidophilus* and *Acidiphilium* species. These results led us to conclude that *T. acidophilus* should be transferred to the genus *Acidiphilium* as *Acidiphilium acidophilum* comb. nov.

METHODS

Bacterial strains and cultivation. *Thiobacillus acidophilus* ATCC 27897^T (^{T} = type strain) was studied. The following strains were used as the reference organisms: *Acidiphilium angustum* ATCC 35903^T, *Acidiphilium cryptum* ATCC 33463^T, *Acidiphilium multivorum* AIU301^T, *Acidiphilium rubrum* ATCC 35905^T, *Acidiphilium organovorum* ATCC 43141^T and *Acidiphilium* sp. strains St1-5 and St1-7. All strains with ATCC numbers were obtained from the American Type Culture Collection (Rockville, MD, USA). *A. multivorum* AIU301^T was isolated from acidic mine drainage (Wakao *et al.*, 1994). All other strains were isolated newly by us from acidic mine water. All test organisms were grown aerobically at 30 °C in GYS medium, a chemically defined medium (pH 3·5) which consisted of a mineral base RM2 (Hiraishi & Kitamura, 1984), 15 mM glucose as the sole carbon source and 0·03 % (w/v) yeast extract as the growth factor. *T. acidophilus* was also grown chemolithotrophically with elemental sulphur as the energy source as described by Harrison (1983). For chemical and genetic testing, cells were harvested by centrifugation from a culture at the late-exponential phase of growth, washed twice with sterile 50 mM phosphate buffer (pH 6·8) and pelleted. The cell pellets were used immediately for analysis or stored at −20 °C until they were analysed.

Analysis of BChls. Lipophilic pigments were extracted from fresh wet cells with acetone-methanol (7:2, v/v), evaporated in vacuum and analysed by reverse-phase HPLC with a Shimadzu Liquid Chromatograph LC-10A equipped with a Beckman Ultrasphere ODS column (4·6 i.d. × 250 mm) in a column oven at 30 °C. Samples were eluted with methanol at a flow rate of 1 ml min^{-1} and monitored with a photodiode array detector, Shimadzu SPD-10A, in a wavelength range of 350–800 nm. Post-run data analysis was performed with the Shimadzu CLASS-M10A program. For the identification and quantification of BChls, parameters for peak identification and calibration of detector response factors were set in the program on the basis of HPLC data on a known concentration of *A. rubrum* Zn-BChl *a* which had been determined spectrophotometrically (Wakao *et al.*, 1996). BChl *a* and bacteriopheophytin (BPhe) purified from a purple phototrophic bacterium, *Rhodobacter sphaeroides* DSM 158^T, were also used as the standard pigments. Although zinc-chelated BChl should be called zinc-BPhe more precisely, we used herein the term Zn-BChl for convenience.

Analysis of carotenoids. Pigments extracted as noted above were analysed by HPLC equipped with a μBondapak C18 column (8 mm i.d. × 100 mm) (Waters). Carotenoid components were eluted with methanol at flow rate of 2 ml min^{-1} and detected with a MCPD-3600 photodiode array detector (Otsuka Electronics) in a wavelength range of 250–600 nm (Takaichi & Shimada, 1992). For spectrophotometric measurement of carotenoids, the following extinction coefficients in methanol were used: 150 mM^{-1} cm^{-1} at 492 and 480 nm for spirilloxanthin and rhodovibrin, respectively. Major carotenoids were also purified by column chromatography on silica gel 60 (Merck). Molecular masses were determined by field-desorption mass spectrometry with a double-focusing gas chromatograph-mass spectrometer equipped with a field desorption apparatus (Hitachi) (Takaichi, 1993). Spirilloxanthin purified from a purple phototrophic bacterium, *Rhodospirillum rubrum* ATCC 11170^T, was used as the standard.

DNA–DNA hybridization. Genomic DNA was extracted and purified by the method of Marmur (1961). DNA–DNA pairing studies were performed by the quantitative dot-blot hybridization method with biotin labelling and colorimetric detection as reported previously (Hiraishi *et al.*, 1991).

Analysis of 16S rDNA. 16S rRNA gene fragments that corresponded to positions 8 to 1510 of *Escherichia coli* 16S rRNA (Brosius *et al.*, 1978) were amplified directly from the cell lysate by PCR with *Taq* DNA polymerase (Takara

Shuzo) and a pair set of eubacterial universal primers 27f and 1492r (Lane, 1991). PCR products were treated with a chloroform/isoamyl alcohol mixture and purified by the PEG precipitation method (Kusukawa *et al.*, 1991; Hiraishi *et al.*, 1995). 16S rDNA was sequenced with a SequiTherm Long-Read Cycle sequencing kit (Epicentre Technologies) with fluorescent primers and analysed with a Pharmacia ALF DNA sequencer as described previously (Hiraishi *et al.*, 1994). RFLP analysis of 16S rDNA was performed as previously reported (Hiraishi *et al.*, 1995).

Analysis of *puf* genes. PCR amplification of *puf* genes was performed as described previously (Nagashima *et al.*, 1997a, 1997b). A 2·1 kb fragment that corresponded to a continuous nucleotide stretch between *pufB* and *pufM* was amplified with a pair set of primers, B140F (5′-TGGCASTGGCGY-CCGTGG-3′) and MR (5′-CCATSGTCCAGCGCCAGA-3′). PCR experiments were also performed with other two pair sets of primers, B140f vs L810R (5′-TTGAGCCAC-CAGCTCACACA-3′) and L810F (5′-TGGTGGAGCYG-GTGGCTCAA-3′) vs MR, resulting in generation of a 1·2 kb fragment between *pufB* and *pufL* and of a 0·9 kb fragment between *pufL* and *pufM*. PCR products were treated with chloroform/isoamyl alcohol, purified by agarose gel electrophoresis and glass binding with a Takara EasyTrap version 2 kit and sequenced directly by fluorescent cycle sequencing with primers previously described (Nagashima *et al.*, 1997b). The two fragments of 1·2 and 0·9 kb were also subcloned by the TA cloning method (Marchuk *et al.*, 1991) with a pT7Blue T-Vector kit (Novagen). Transformation of *E. coli* JM109 was carried out according to a standard manual of molecular cloning (Sambrook *et al.*, 1989). Plasmid DNA was isolated and purified by using a Pharmacia FlexiPrep kit according to the manufacturer's instructions. The subcloned DNA sequences were determined by cycle sequencing with pUC/M13 and T7 promoter universal primers. All reactions were analysed with a Pharmacia ALF DNA sequencer and a Perkin-Elmer ABI 373A DNA sequencer.

Phylogenetic analysis. Sequence data were compiled with the GENETYX-MAC program package (Software Development). Multiple alignment of sequence was performed with the CLUSTAL W program (Thompson *et al.*, 1991). Evolutionary distances were calculated by using Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were reconstructed by the neighbour-joining method (Saitou & Nei, 1987), and the topology of trees was evaluated by bootstrapping with 1000 resamplings (Felsenstein, 1985). Alignment positions with gaps and unidentified bases were excluded from the calculations.

RESULTS

16S rDNA sequence comparisons

The phylogenetic relationships between *T. acidophilus* and *Acidiphilium* species were re-examined based on 16S rDNA sequences. Although the sequence data on *T. acidophilus* was already available from the DDBJ, EMBL and GenBank databases before this study, there were large numbers of undetermined positions. To ascertain the exact phylogenetic position of *T. acidophilus*, therefore, we determined nearly complete sequences of the 16S rDNA of *T. acidophilus* and related acidophilic organisms. Binary sequence comparisons indicated that the 16S rDNA of *T. acidophilus* was most closely related to the 16S rDNAs of *A. angustum* and *A. rubrum* at a similarity level of 98·3% (corrected distance = 0·0168). A neighbour-joining phylogenetic tree was constructed on the basis of the distance matrix data on the test strains and several reference bacteria (Fig. 1). The tree showed that *T. acidophilus* fell into a cluster of the genus *Acidiphilium* with *A. angustum* and *A. rubrum* as its closest relatives. The monophyly of the cluster of the genus *Acidiphilium* and of the subcluster of *T. acidophilus* with *A. angustum* and the related strains as the sister group was supported by nearly 100% levels of bootstrap confidence. The results of our phylogenetic studies supported the previous results on the phylogeny of *Acidiphilium* species and related acidophiles (Lane *et al.*, 1985, 1992; Sievers *et al.*, 1994; Kishimoto *et al.*, 1995b).

Genomic DNA relatedness

Interrelationships between *T. acidophilus* and *Acidiphilium* species were also studied by genomic DNA–DNA hybridization assays (Table 1). The DNA of *T. acidophilus* had 18–24% binding levels to those of *A. angustum* and *A. rubrum* and 9–11% to those of other *Acidiphilium* strains tested. The low but significant levels of DNA–DNA relatedness between *T. acidophilus* and *A. angustum* or *A. rubrum* were in accordance with the results of the 16S rDNA-based phylogenetic analysis. A high level of similarity between *A. angustum* and *A. rubrum* in the 16S rDNA structure and genomic DNA relatedness, as reported here and elsewhere (Wakao *et al.*, 1994; Kishimoto *et al.*, 1995b), suggest synonymy of the two species names. In this study, '*Thiobacillus organoparus*' (Markosyan, 1973), an acidophilic facultative chemolithotroph similar to *T. acidophilus*, was not used, since the former species is probably a synonym of the latter in view of the high level of DNA–DNA homology between the two (Katayama-Fujimura *et al.*, 1983). The results of DNA–DNA hybridization assays indicate that *T. acidophilus* is phylogenetically related to, but distinct from, the previously known species of the genus *Acidiphilium*.

Photosynthetic pigments

Comparative HPLC assays of the lipid extracts of *T. acidophilus* and *A. rubrum* demonstrated that the former organism produced much smaller but appreciable amounts of photopigments than the latter (Fig. 2). By comparing HPLC elution times and absorption spectrum patterns (not shown) of the separated components, the main pigment of *T. acidophilus* was identified as Zn-BChl *a*. In all other test strains of *Acidiphilium*, Zn-BChl *a* as well as Mg-BChl *a* and BPhe were detected by HPLC. The contents of these photopigments varied significantly among the strains tested, whereas Zn-BChl *a* always predominated in all

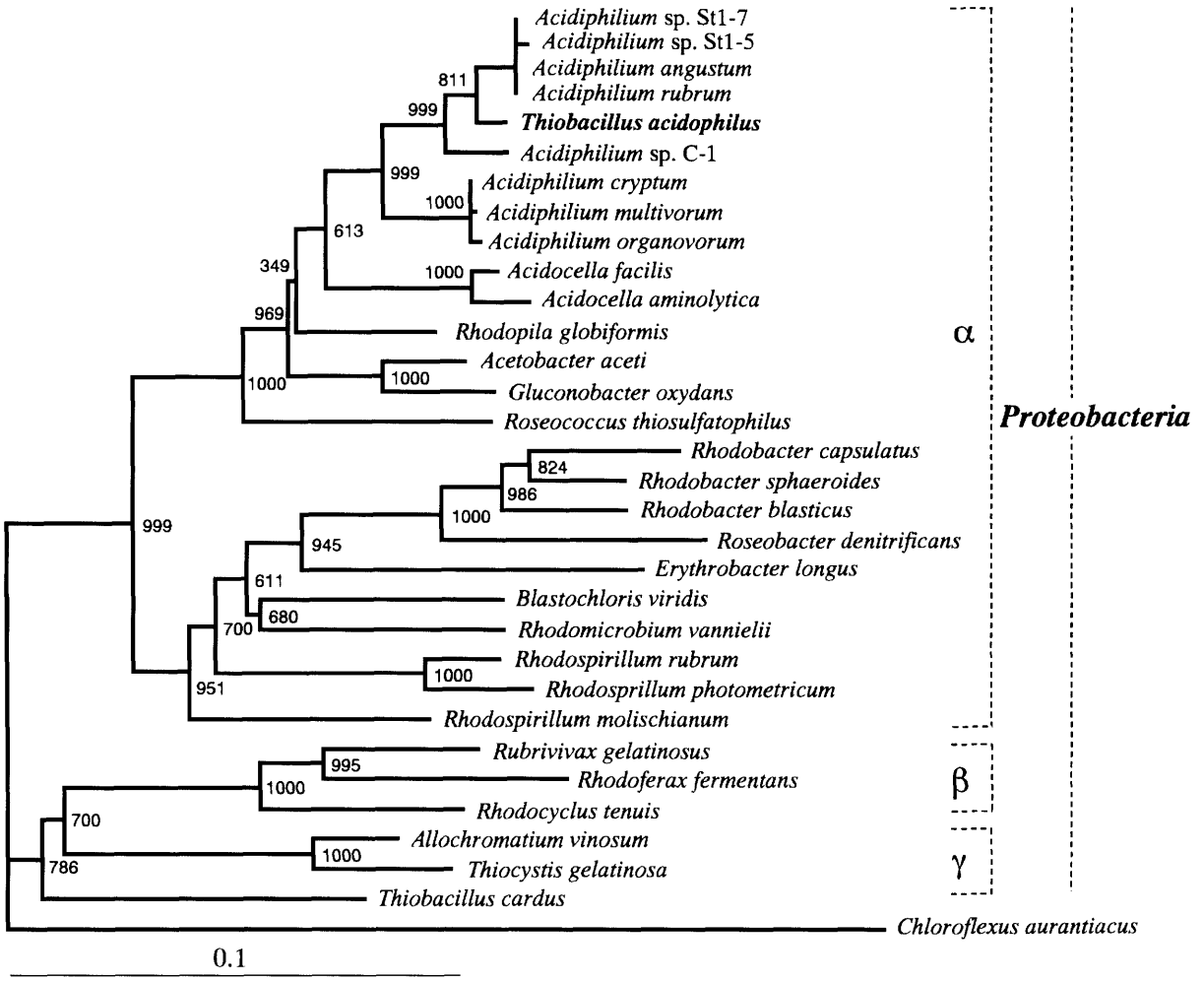

Fig. 1. Distance matrix tree showing phylogenetic affiliations of *Thiobacillus acidophilus* and other species of *Proteobacteria* based on 16S rDNA sequences. *Chloroflexus aurantiacus* was used as a member of outgroups to root the tree. Bootstrap values obtained with 1000 bootstrap resamplings are given at branching points of interest. Bar, 10 nucleotide substitutions per 100 nucleotides. The organisms and their sequences used in the analysis are as follows: *Acetobacter aceti*, D30768; *Acidiphilium angustum*, D30772; *Acidiphilium cryptum*, D30773; *Acidiphilium multivorum*, AB006712; *Acidiphilium organovorum*, D30775; *Acidiphilium rubrum*, D30776; *Acidiphilium* sp. C-1, D30769; *Acidiphilium* sp. St1-5, D86508; *Acidiphilium* sp. St1-7, D86509; *Acidocella aminolytica*, D30771; *Acidocella facilis*, D30774; *Blastochloris viridis* (formerly *Rhodopseudomonas viridis*), D25314; *Chloroflexus aurantiacus*, D32255; *Allochromatium vinosum* (formerly *Chromatium vinosum*), M26629; *Erythrobacter longus*, M59062; *Gluconobacter oxydans*, X73820; *Rhodobacter blasticus*, D16429; *Rhodobacter capsulatus*, D16428; *Rhodobacter sphaeroides*, D16425; *Rhodocyclus tenuis*, D16208; *Rhodoferax fermentans*, D16211; *Rhodomicrobium vannielii*, M34127; *Rhodopila globiformis*, D86513; *Rhodospirillum molischianum*, M59067; *Rhodospirillum photometricum*, D30777; *Rhodospirillum rubrum*, D30778; *Roseobacter denitrificans*, M59063; *Roseococcus thiosulfatophilus*, X72908; *Rubrivivax gelatinosus*, D16213; *Thiobacillus acidophilus*, D86511; *Thiobacillus caldus*, X29975; *Thiocystis gelatinosa*, D50655.

test organisms (Table 2). The molar ratio of Zn-BChl *a*:Mg-BChl *a*:BPhe in the test organisms was relatively constant; for example, the mean ratio of the pigments with a molar basis of BPhe was 13:2:1 in the high Zn-BChl producers *A. angustum* and *A. rubrum*. In the strains showing low contents of the pigments (i.e. *A. cryptum*, *A. multivorum* and *A. organovorum*), the relative contents of Mg-BChl *a* was lowered, and those of BPhe were increased compared to the high Zn-BChl producers. These may be due to pheophytinization of a small amount of the Mg-BChl *a* during the extraction of pigments or HPLC analysis. Possibly for this reason, Mg-BChl *a* was hardly detected in *T. acidophilus*.

HPLC experiments also revealed that *T. acidophilus* and all *Acidiphilium* species contained spirilloxanthin as the sole or predominant carotenoid component (Fig. 2 and Table 2). The major carotenoid component purified from these bacteria had absorption maxima at 317, 384, 464, 491 and 524 nm in methanol. This component showed an HPLC retention time corre-

Table 1. Genomic DNA relatedness between *Thiobacilus acidophilus* and members of the genus *Acidiphilium*

Strain	DNA G+C content (mol%)	Hybridization (%) with labelled DNA from:	
		ATCC 27897^T	ATCC 35905^T
Thiobacillus acidophilus ATCC 27897^T	63·5*	100	24
Acidiphilium angustum ATCC 35903^T	63·4†	18	98
Acidiphilium cryptum ATCC 33463^T	67·3†	8	11
Acidiphilium multivorum AIU301^T	67·6†	10	10
Acidiphilium organovorum ATCC 43141^T	67·4†	10	9
Acidiphilium rubrum ATCC 35905^T	63·2†	22	100

* Cited from Katayama-Fujimura *et al.* (1983).

† Cited from Wakao *et al.* (1994).

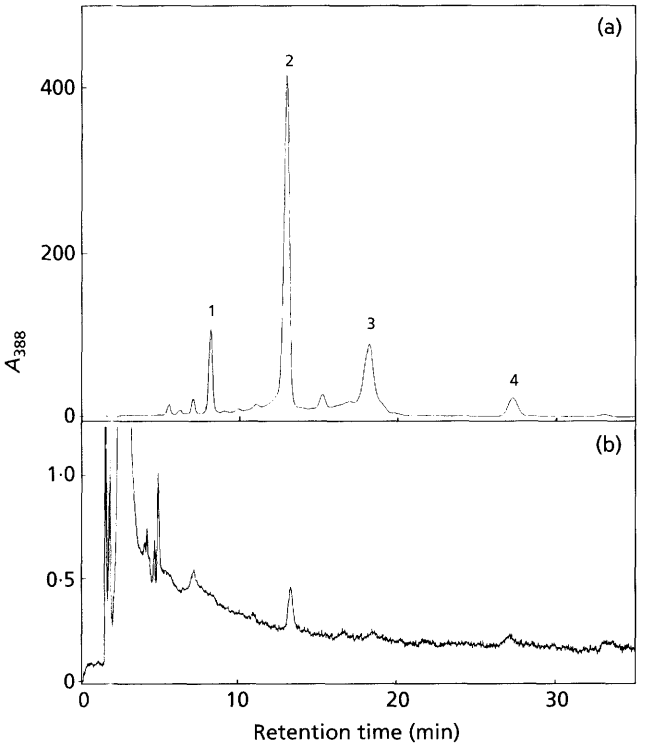

Fig. 2. HPLC analysis of the pigment extract of *Thiobacillus acidophilus* (b) compared with the extract of *Acidiphilium rubrum* (a). Peaks: 1, Mg-BChl *a*; 2, Zn-BChl *a*; 3, spirilloxanthin; 4, BPhe.

sponding to that of the authentic spirilloxanthin from the purple phototrophic bacterium *Rhodospirillum rubrum*. Mass spectroscopy showed that the carotenoid component from *A. rubrum*, the highest pigment producer, had a molecular mass of 596, thereby confirming it to be spirilloxanthin. *A. cryptum* and *A. organovorum* also produced a minor carotenoid component, which was identified as rhodovibrin on the basis of its HPLC retention time and absorption maxima at 465, 489 and 521 nm. This component is known to be an intermediate in spirilloxanthin biosynthesis in the purple bacteria.

While the liquid culture of *T. acidophilus* looked colourless, the massive cell pellet harvested by centrifugation was pale brown, probably due to the production of the photopigments. Direct spectrophotometric measurement of the acetone/methanol extract (3 ml) from the cell pellet (wet wt 1 g) detected only a weak peak with an absorption maximum at 763 nm, which is characteristic of Zn-BChl *a* (Wakao *et al.*, 1996).

Structure of *puf* genes

In the purple bacteria, the genes encoding the photosynthetic reaction centre proteins and the core light-harvesting complex form an operon called *puf*. This gene construction has been shown to be well-conserved not only among the species of the anaerobic photosynthetic bacteria but also in *Acidiphilium* species (Nagashima *et al.*, 1997a, b). Using the PCR technique, DNA fragments between *pufB* and *pufM* that covered a continuous 2·1 kb stretch of the *puf* genes were successfully amplified from *T. acidophilus* cells and sequenced.

The primary sequence of the *puf* genes of *T. acidophilus* contained three ORFs corresponding to *pufA*, *pufL* and *pufM* (data not shown). The levels of amino acid sequence similarity between *T. acidophilus* and *Acidiphilium* species were 88–93% in the L and M subunits, whereas much lower levels of similarity were found between *T. acidophilus* and other photosynthetic bacteria (Fig. 3). It has been shown that the L and M subunits of *Acidiphilium* species have one characteristic replacement of an amino acid in the region around the special pair when the three-dimensional structures of the reaction centres of *Blastochloris viridis* (formerly *Rhodopseudomonas viridis*) (Deisenhofer *et al.*, 1995) and *Rhodobacter sphaeroides* (Allen *et al.*, 1987) are

Table 2. Bacteriochlorophyll and carotenoid contents of *Thiobacillus acidophilus* and *Acidiphilium* species

Rhod, Rhodovibrin; Spir, spirilloxanthin.

Test organism	Pigment content (nmol g^{-1} dry wt)				
	Zn-BChl *a*	Mg-BChl *a*	BPhe	Spir	Rhod
Thiobacillus acidophilus ATCC 27897^T	2·3	0	0·8	3·3	0
Acidiphilium angustum ATCC 35903^T	180	26	14	115	0
Acidiphilium cryptum ATCC 33463^T	57	5·1	5·1	37	1·5
Acidiphilium multivorum AIU 301^T	67	8·8	5·9	54	0
Acidiphilium organovorum ATCC 43141^T	6·7	0·4	0·9	6·0	0·4
Acidiphilium rubrum ATCC 35905^T	810	120	61	510	0
Acidiphilium sp. St1-5	170	25	13	100	0
Acidiphilium sp. St1-7	130	20	11	82	0

	Sequence similarity (%)	160 ▼170
Zn-BChl producers		
Thiobacillus acidophilus	100.0	HLDWASNTGYNYINFEYNPMHMVAVT
Acidiphilium angustum	93.3	HLDWVSNTGYNYINFEYNPMHMVAVT
Acidiphilium rubrum	93.3	HLDWVSNTGYNYINFEYNPMHMVAVT
Acidiphilium cryptum	88.3	HLDWVSNTGYAYLNFEYNPMHMVAVT
Acidiphilium multivorum	87.9	HLDWVSNTGYAYLNFEYNPMHMVAVT
Acidiphilium organovorum	88.1	HLDWVSNTGYAYLNFEYNPMHMVAVT
BChl producers		
Rhodopila globiformis	70.6	HLDWVSNTGYQYLHFHYNPAHMIAIS
Rhodospirillum rubrum	68.2	HLDWVSNTGYQYANFHYNPAHMLGIT
Rhodospirillum photometricum	64.3	HLDWVSNIGYQFLHFHYNPAHMIGIT
Rhodospirillum molischianum	68.3	HLDWVSNVGYQFLHFHYNPAHMIAIS
Rubrivivax gelatinosus	72.3	HLDWVSNVGYQYLHFHYNPAHMLAIT
Rhodoferax fermentans	70.9	HLDWGSNVGYQYLHFHYNPAHMLAIS
Rhodocyclus tenuis	69.7	HLDWVSNTGYQYLHFHYNPGHMLGIA
Allochromatium vinosum	68.7	HLDWVSNVGYQFLNFHYNPAHMLAIT
Thiocystis gelatinosa	69.3	HLDWVSNVGYQFLHFHYNPAHMLAIT
Blastochloris viridis	61.6	HLDWVNNFGYQYLNWHYNPGHMSSVS
Rhodomicrobium vannielii	70.3	HLDWVSNTGYQYLHFHYNPAHMLAIS
Rhodobacter capsulatus	61.6	HLDWVSNTGYTYGNFHYNPFHMLGIS
Rhodobacter sphaeroides	63.6	HLDWVSNTGYTYGNFHYNPAHMIAIS
Rhodobacter blasticus	61.8	HLDWVSNTGYTYGNFHYNPAHMLGIS
Roseobacter denitrificans	65.5	HLDWVSNVGYAYLHFHYNPAHMLAVT
Erythrobacter longus	62.1	HLDWVSYTGYNYVNFHYNPVHMLAIT
Chloroflexus aurantiacus	45.7	HLDWVSNFGYRYNNFFYNPFHAIGIT
		**** ** *** *

Fig. 3. Amino acid sequence similarity in the L and M subunits of the photosynthetic reaction centre between *Thiobacillus acidophilus* and other photosynthetic bacteria and alignment of a part of the L subunit amino acid residues around the special pair. Asterisks mark identical residues in all species compared. One characteristic replacement of amino acid specific to *Acidiphilium* is shown by an arrow.

taken into account for comparison (Nagashima *et al.*, 1997b). This is at Glu L168, at which histidine occurs in the purple bacteria in general. The occurrence of glutamic acid at this position was also the case in *T. acidophilus* (Fig. 3).

A phylogenetic tree of the reaction centre proteins was constructed on the basis of the present data (Fig. 4). The topography of the tree was similar to that of 16S rDNA sequence-based tree in the relationships between *T. acidophilus* and *Acidiphilium* species and between the *Acidiphilium* cluster and the phototrophic bacteria of the α-subclass of the *Proteobacteria*. The fact that the phototrophic bacteria of the β- and γ-subclasses were positioned among members of the α-subclass of the phototrophs has been explained by considering possible lateral gene transfer (Nagashima *et al.*, 1997a).

Analysis of lithotrophically growing cells

Since all established species of the genus *Acidiphilium* and the related new genus *Acidocella* (Kishimoto *et al.*, 1995b) have been reported to be unable to grow with reduced sulphur compounds as energy sources (Harrison, 1989), it seemed curious that the sulphur-oxidizing bacterium *T. acidophilus* has close phylogenetic relationships to those acidophilic chemo-organotrophic bacteria. This situation led us to re-examine whether *T. acidophilus* truly can oxidize reduced sulphur compounds as energy sources for growth, and this gave positive results in agreement with the previous reports (Guay & Silver, 1975; Harrison, 1983; Norris *et al.*, 1986; Pronk *et al.*, 1990; Meulenberg *et al.*, 1992).

We compared the 16S rDNA structure of the sulphur-grown cells with that of the heterotrophically grown cells of *T. acidophilus* by studying RFLP patterns with *Hae*III, *Hha*I, *Msp*I and *Rsa*I. The resulting 16S rDNA-RFLP patterns of the two cultures were identical and matched completely with the computer-predicted RFLP profiles (not shown). The DNA–DNA binding level between chemo-organotrophically grown and chemolithotrophically grown cells of *T. acidophilus* was 100%. PCR amplification of the *puf* gene from the sulphur-grown cells was also successful. These findings excluded the possibility that *T. acidophilus* is a mixed culture of a chemo-organotrophic species and a chemolithotrophic one.

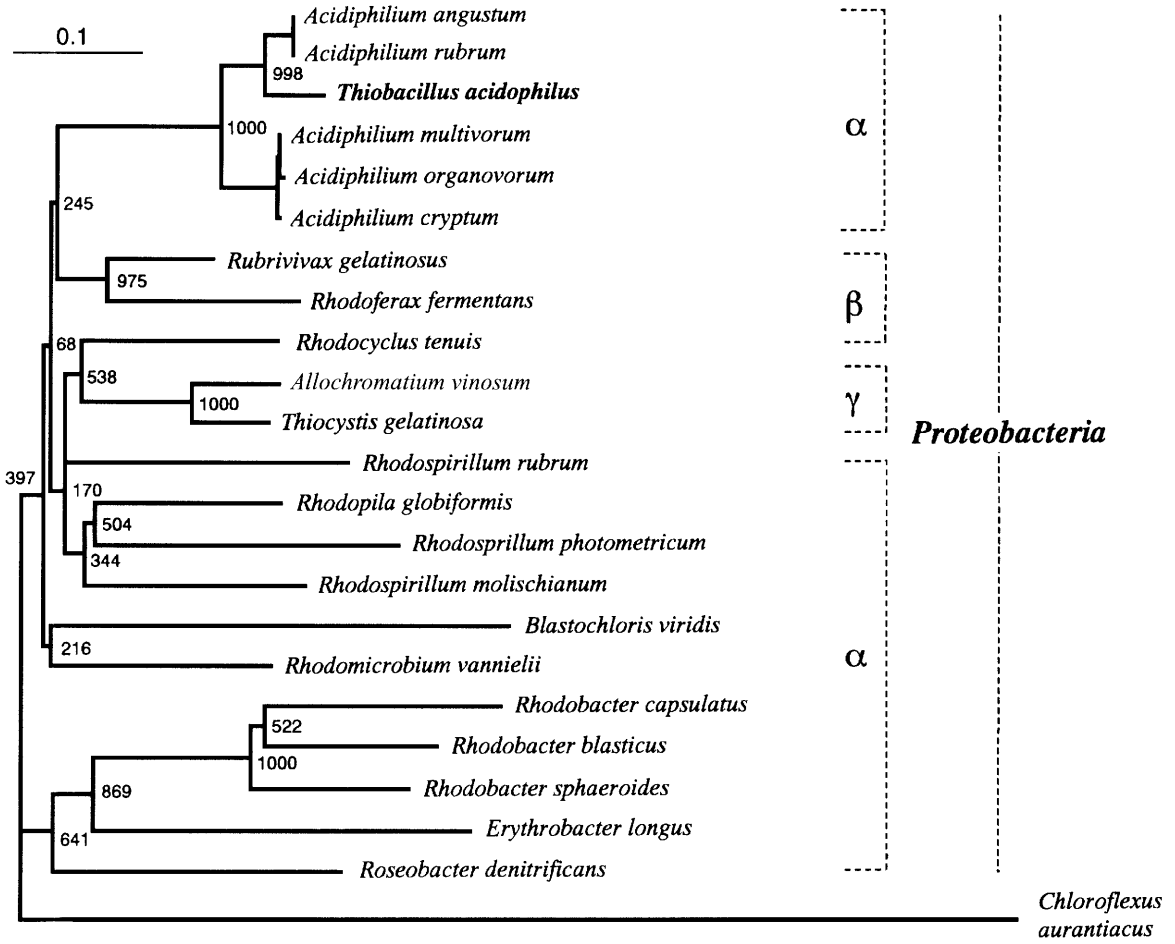

Fig. 4. Distance matrix tree showing phylogenetic relationships between *T. acidophilus* and photosynthetic proteobacteria based on continuous amino acid sequences of the L and M subunit proteins of the *puf* gene. *Chloroflexus aurantiacus* was used as a member of outgroups to root the tree. Bootstrap values obtained with 1000 bootstrap resamplings are given at branching points of interest. Bar, 10% amino acid substitutions. The organisms and their sequences used in the analysis are as follows: *Acidiphilium angustum*, AB005219; *Acidiphilium cryptum*, AB005220; *Acidiphilium multivorum*, AB005221; *Acidiphilium organovorum*, AB005222; *Acidiphilium rubrum*, AB005218; *Blastochloris viridis* (formerly *Rhodopseudomonas viridis*), X03915; *Chloroflexus aurantiacus*, X14979; *Allochromatium vinosum* (formerly *Chromatium vinosum*), D50647; *Erythrobacter longus*, D50648; *Rhodobacter blasticus*, D50649; *Rhodobacter capsulatus*, Z11165; *Rhodobacter sphaeroides*, X63404 and K00827; *Rhodocyclus tenuis*, D50651; *Rhodoferax fermentans*, D50650; *Rhodomicrobium vannielii*, D50652; *Rhodopila globiformis*, unpublished data (A. Hiraishi); *Rhodospirillum molischianum*, D50654; *Rhodospirillum photometricum*, D50681; *Rhodospirillum rubrum*, J03731; *Roseobacter denitrificans*; X57597; *Rubrivivax gelatinosus*, D16822; *Thiobaciilus acidophilus*, AB013379; *Thiocystis gelatinosa*, D50653.

DISCUSSION

As reported here, the molecular genetic analysis based on 16S rDNA sequence and DNA–DNA relatedness clearly demonstrates that *T. acidophilus* falls into a cluster of the genus *Acidiphilium* with *A. angustum* and *A. rubrum* as its nearest phylogenetic neighbours. These molecular data are in agreement with the previous findings as to the phylogeny of *T. acidophilus*, *Acidiphilium* species and related acidophiles (Lane *et al.*, 1985, 1992; Sievers *et al.*, 1994; Kishimoto *et al.*, 1995b). Although *T. acidophilus* is most closely related to *A. rubrum*, it is clear that this bacterium represents a distinct species within the genus *Acidiphilium*, in view of the DNA–DNA hybridization data. These results unequivocally warrant reclassification of *T. acidophilus* as a new distinct species of this genus from a phylogenetic point of view.

The genus *Acidiphilium* was first proposed to accommodate strictly aerobic, chemo-organotrophic, acidophilic bacteria that grow under strongly acidic conditions (pH 2–6) (Harrison, 1983). Later some members of this genus have been shown to produce BChls during aerobic growth (Wakao *et al.*, 1993, 1994; Kishimoto *et al.*, 1995a). This finding, together with 16S rDNA-based phylogenetic information, has led to the emendation of the genus *Acidiphilium* concurrently with the transfer of the non-pigment-producing species to the new genus *Acidocella*

Table 3. Differential characteristics of *Acidiphilium acidophilum* comb. nov. and other *Acidiphilium* species

Symbols and abbreviations: +, positive; (+), weakly positive; −, negative; W, white; PB, pale brown to pink for old cultures. Information from Guay & Silver (1975), Harrison (1983), Katayama-Fujimura *et al.* (1983), Lobus *et al.* (1986), Wakao *et al.* (1994) and this study.

Character	*A. acidophilum*	*A. angustum*/ *A. rubrum*	*A. cryptum*	*A. multivorum*	*A. organovorum*
Colour of colonies	W, PB	Pink to red	W, PB	W, PB	W, PB
Growth factor required	−	+	+	+	+
Chemolithotrophic growth with:					
Sulphur	+	−	−	−	−
Thiosulphate	+	−	−	−	−
Carbon source utilization:					
Fumarate	−	−	−	+	−
Succinate	−	−	−	+	+
Ethanol	+	−	−	+	−
Methanol	(+)	−	−	+	−
Glutamate	+	−	+	+	+
DNA G+C content (mol%)	62·9–63·5	63·2–63·4	67·3–68·3	66·2–68·1	67·4

(Kishimoto *et al.*, 1995b). Recently, the main component of photopigments in *A. rubrum* has been found to be Zn-BChl *a* (Wakao *et al.*, 1996). Moreover, molecular genetic analyses have shown that all previously known species of the genus *Acidiphilium* emend. contain *puf* genes encoding proteins of the photosynthetic reaction centre and the core light-harvesting complex (Nagashima *et al.*, 1997b). Thus, *Acidiphilium* species are now recognized as aerobic photosynthetic bacteria that are unique in containing Zn-BChl *a*.

In this context, we examined the photosynthetic properties of *T. acidophilus* compared with those of all established species of the genus *Acidiphilium*. *T. acidophilus* as well as all *Acidiphilium* species produced detectable amounts of Zn-BChl *a* and carotenoids as reported here. The structural genes encoding the photosynthetic reaction centre of *T. acidophilus* were also detected and found to be similar to those of *Acidiphilium* species in primary structures and amino acid replacement around the special pair in the L subunit. The phylogenetic tree deduced from amino acid sequences of the L and M subunits demonstrates close relationships of *T. acidophilus* to *Acidiphilium* species with *A. angustum* and *A. rubrum* as its closest relatives, being consistent with the topography of the tree based on 16S rDNA sequences noted above.

Thiobacillus acidophilus differs from all known *Acidiphilium* species in the chemolithotrophic metabolism with reduced sulphur compounds. Nevertheless, the ability of *T. acidophilus* to produce the photopigments with Zn-BChl *a* as the major component demonstrates its phenotypic similarity to members of the genus *Acidiphilium*, and this provides a firm basis for the taxonomic reassignment of *T. acidophilus* in that genus. Concurrent phenotypic studies have also shown that *T. acidophilus* and *Acidiphilium* species share a number of common characteristics useful for the circumscription of the genus but are distinguishable from each other in some phenotypes as described below. Thus, based on the phylogenetic and phenotypic information described here, we propose transfer of *T. acidophilus* (Guay and Silver) Harrison 1983 to the genus *Acidiphilium* as *Acidiphilium acidophilum* comb. nov. Diagnostic characteristics of this organism and other *Acidiphilium* species are shown in Table 3.

Following this proposal, the situation may call for emendation of the genus *Acidiphilium*, because this genus has previously been defined to accommodate only 'non-sulphur' chemo-organotrophic acidophiles (Kishimoto *et al.*, 1995b). However, it is our view, at this time, that all members of the genus *Acidiphilium* need re-examining more thoroughly in terms of sulphur metabolism. A previous study suggested that *A. cryptum* was unable to use sulphur as the energy source for growth but that it had the capacity for sulphur oxidation (Harrison, 1983). More effective data on sulphur metabolism should be helpful for the emendation of the genus *Acidiphilium* in the future.

Description of *Acidiphilium acidophilum* [*Thiobacillus acidophilus* (Guay and Silver) Harrison 1983] comb. nov.

Acidiphilium acidophilum (a.ci.do'phi.lum. L. adj. *acidus* sour; M.L. neut. n. *acidum* acid; Gr. adj. *philus* loving; M.L. adj. *acidophilum* acid-loving).

The description of this species is based on information from Guay & Silver (1975), Harrison (1983), Katayama-Fujimura *et al.* (1982, 1983, 1984), Norris *et al.* (1986), Mason *et al.* (1987), Pronk *et al.* (1990) and this study. Cells are rod-shaped, 0·5–0·8 μm wide

by 1·0–1·5 μm long, occurring singly, pairs and rarely chains. Non-spore-forming. Motile or non-motile. Gram-negative. Colonies on agar media are round, regular, convex, slightly translucent, and white to cream; cell pellets harvested by centrifugation shows pale brown. Strictly aerobic facultative chemolithotrophs and mixotrophs growing with elemental sulphur as an energy source and with oxygen as the terminal electron acceptor. Thiosulphate, trithionate and tetrathionate also serve as electron donor. Neither sulphite, sulphide nor ferrous iron serves as electron donor. Do not denitrify. Polyhedral inclusion bodies (carboxysomes) are present in elemental sulphur-grown cells. Optimal growth occurs at 25–30 °C (range, 10–35 °C) and at pH 3·0–3·5 (range, pH 1·5–6·0). No growth factor is required. Usable carbon sources are: L-arabinose, D-xylose, D-ribose, D-glucose, D-galactose, D-fructose, sucrose, glycerol, D-mannitol, ethanol, gluconate, L-malate, citrate, L-glutamate, L-histidine, L-proline, DL-aspartate. Methanol and *n*-propanol support weak growth. Not utilized are: L-sorbose, L-rhamnose, D-mannose, D-maltose, lactose, cellobiose, trehalose, D-melibiose, raffinose, n-butanol, cyclohexanol, ascorbic acid, formate, acetate, propionate, *n*-butyrate, lactate, pyruvate, succinate, fumarate, α-ketoglutarate, glutarate, glyoxylate, oxalate, adipate, pimerate, benzoate, *p*-hydroxybenzoate, *p*-aminobenzoate, L-alanine, L-serine, L-leucine, L-isoleucine, L-phenylalanine, L-tryptophan, L-cysteine and tyrosine. Ammonium salts but not nitrate salts are utilized as nitrogen source. Zn-BChl *a* (bacteriochlorophyll *a* chelated with zinc as the central metal) occurs in a very small amount. Ubiquinone-10 is the major respiratory quinone. The G+C content of genomic DNA is 62·9–63·5 mol%. Source: originally isolated from an iron-oxidizing culture of *Thiobacillus ferrooxidans* and found in strongly acidic environments including mine water. Type strain: ATCC 27807^T (= DSM 700^T).

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