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A PHENOTYPIC AND GENOTYPIC COMPARISON OF SIX STRAINS OF *THIOBACILLUS FERROOXIDANS*

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Abstract

Six strains of *Thiobacillus ferrooxidans* were grown with ferrous iron as the energy source. DNA was extracted from each strain, digested with restriction endonucleases, and the fragments separated in agarose gels and subsequently transferred to nitrocellulose. Restriction fragments containing either the form I or form II large subunit gene for ribulose bisphosphate carboxylase/oxygenase were elucidated by heterologous hybridization using specific gene probes. Two strains yielded identical fragments. The other four were readily identified by their hybridization patterns. Phenotypic properties were examined by extracting the lipids and determining both the phospholipid ester-linked fatty acid, and the lipopolysaccharide lipid A hydroxy fatty acid patterns. Complete linkage hierarchical cluster analysis of mole percent profiles of the lipid A hydroxy fatty acid patterns showed two groups, each with three strains. The two strains with apparently identical ribulose bisphosphate carboxylase/oxygenase restriction endonuclease fragments were in different groups. Analysis of the phospholipid ester-linked fatty acid profiles yielded considerably different groupings for the strains.

Introduction

Bacteria isolated from different sites and classified as the same species can show differences when characteristics which are used to define the species are analyzed carefully. These differences are useful in strain identification and may suggest trends in their evolution. In this study, six strains of the chemoautotroph *Thiobacillus ferrooxidans* were examined. The natural environment of this organism is largely limited to acidic mining discharges. The relationships of the strains based on restriction endonuclease fragment analysis of the ribulose bisphosphate carboxylase/oxygenase (RuBisCO) genes were compared to the phenotypic expression of multiple genes involved in phospholipid and lipopolysaccharide biosynthesis.

Materials and Methods

Six ATCC strains of *T. ferrooxidans* (13598, 13661, 14119, 21834, 23270, and 33020) were used in this study. The cultures were grown in batch to the maximum stationary growth phase in the medium of Tuovinen and Kelly (1). The cells were harvested by filtration and centrifugation, washed in 0.01 N H_2SO_4, and frozen as pellets. The cells were washed several times in 10 mM Tris-HCl, 1 mM EDTA buffer (pH 8.0) before beginning DNA isolation. All techniques (cell lysis, DNA purification, restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting, probe preparation, hybridization, washing, autoradiography) were accomplished as previously described (2). The heterologous DNA probes used in this study were a 1.5 kbp *Eco*RI-*Pst*I fragment from pANP1155 representing nearly the complete large subunit gene (*rbc*L) of RuBisCO from *Anacystis nidulans* (3) and two *Sal*I fragments from pRR116 representing about one-third of the RuBisCO gene (*rbp*L) from *Rhodospirillum rubrum* (4). These probes are for form I and form II RuBisCO, respectively. The probes do not cross hybridize under our experimental conditions.

All of the techniques and methods for lipid analysis have been described elsewhere in detail (5, 6, 7, 8, and 9). Ester-linked phospholipid fatty acids (PLFA) and lipopolysaccharide lipid A hydroxy fatty acids (LPS OH-FA) were extracted from pure isolates in an organic solvent system and the total lipid extract fractionated (5). The PLFA were recovered and then derivatized for identification and quantification by gas chromotography/mass spectrometry (GC/MS) (6,

7). Double bond positions in the monoenoic PLFA were verified by GC/MS (8). The residue from the original lipid extraction and subsequent fractionation was then treated as described to recover the LPS OH-FA (9). After forming trimethyl silyl derevatives by the addition of 100 µl N,O-bis (Trimethylsilyl)-trifluoroacetamide and heating at 60°C for 30 min, the OH-FA were quantified on a GC with structural verification by GC/MS (8). PLFA and LPS OH-FA mole percent profiles were then compared by a complete linkage hierarchical cluster analysis using the software package Ensight (Infometrix, Seattle, WA). The principal components analysis was accomplished using the same software package.

Results

Both probes gave positive results with all strains suggesting the presence of genes for both form I and II RuBisCO (Figures 1 and 2, Tables I and II). It is difficult to perfectly align the pictures of the different agarose gels and the autoradiograms. Therefore, the figures are presented only for visualization purposes and do not accurately represent the restriction fragment sizes. The approximate fragment sizes are given in Tables I and II. It should be noted that eventhough restriction fragments are indicated as the same size, they may not necessarily be the same fragment. This is especially true of the larger fragments where resolution of the agarose gel is poor. However, when the patterns exhibited by all five restriction enzymes are the same for two strains, one can be reasonably sure that the two strains are identical, or nearly so, for that gene.

ATCC 13661, 21834, and 23270 yielded identical restriction endonuclease fragment patterns using the form I RuBisCO probe (Figure 1 and Table I). The ATCC 13598 pattern also appeared identical except an additional small (~1.0 or 1.4 kbp) hybridizing fragment was present in each digest. This probably represents a small cryptic plasmid carried by ATCC 13598 which shows some homology with the vector carrying the form I gene insert, i.e. pBR322. *Bam*HI, *Hind*III, and *Pst*I hybridizing fragments of ATCC 33020 were also the same as those in the above mentioned strains, however the hybridizing fragments of the *Eco*RI and *Sal*I digests were different. ATCC 14119 shows both similarities and differences with the other strains.

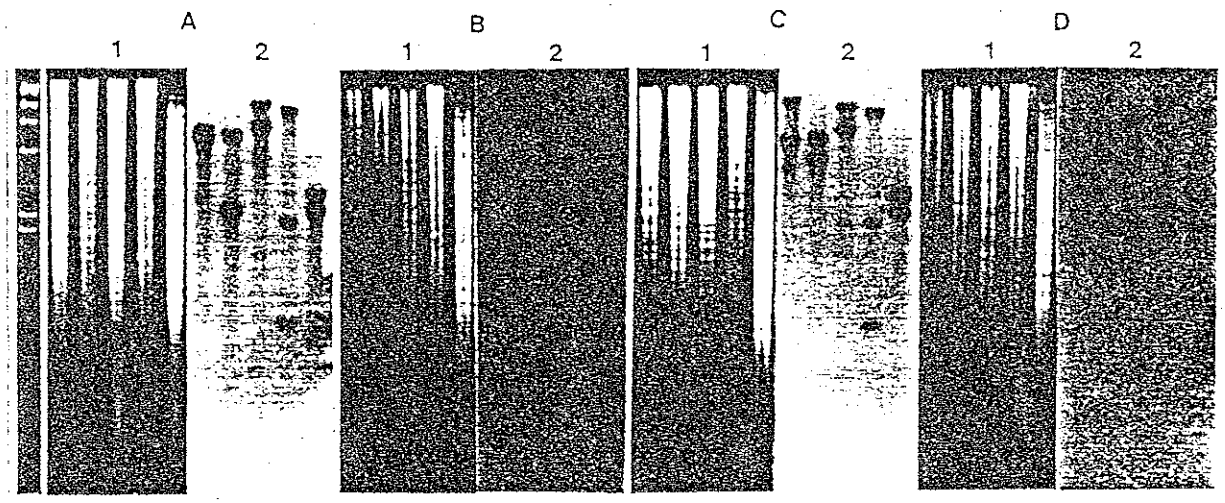

Figure 1. Hybridization of the form I RuBisCO probe to *T. ferrooxidans* DNA. Panel A, ATCC 13661, 21834, and 23270; Panel B, ATCC 13598; Panel C, ATCC 33020; and Panel D, ATCC 14119. Subpanels labelled 1 are ethidium bromide stained agarose gels. Subpanels labelled 2 are autoradiograms of Southern blots. The DNA in each panel was digested (from left to right) with *Eco*RI, *Sal*I, *Bam*HI, *Hind*III, and *Pst*I. The far left lane of the figure is lambda DNA digested with *Hind*III.

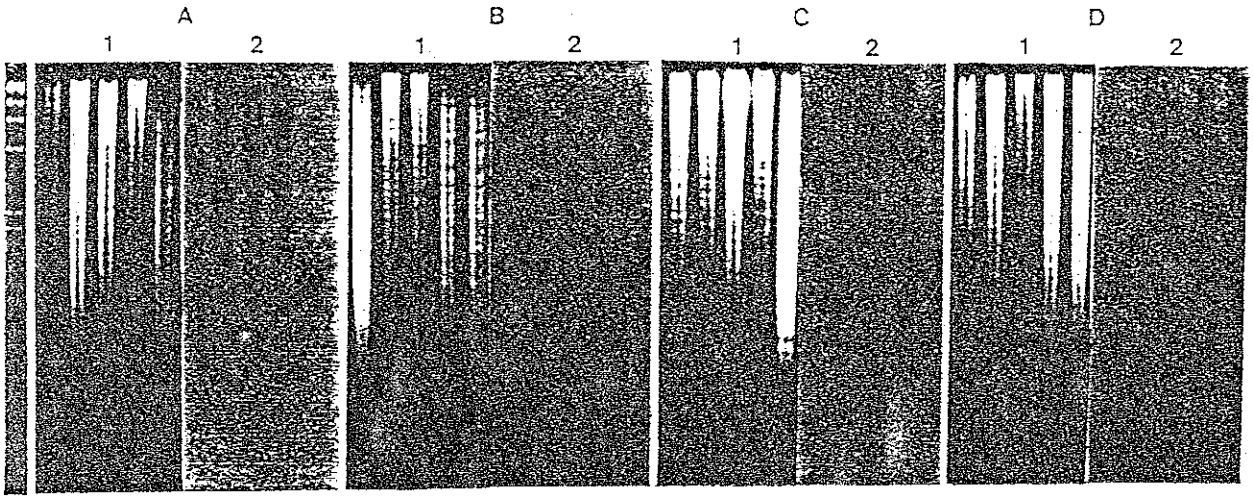

Figure 2. Hybridization of the form II RuBisCO probe to *T. ferrooxidans* DNA. Panel A, ATCC 13598, 21834, and 23270; Panel B, ATCC 13661; Panel C, ATCC 33020; and Panel D, ATCC 14119. The rest of the legend is the same as Figure 1.

Table I. Estimated sizes of the hybridizing *T. ferrooxidans* restriction endonuclease fragments revealed using the form I RuBisCO probe.

Enzyme	ATCC #			
	23270 21834 13661	13598	33020	14119
*Eco*RI	5.8	5.8 1.4	12.0 5.8	5.8 4.5
*Sal*I	5.9 2.8	5.9 2.8 1.4	5.9 4.6	5.9 5.5
*Bam*HI	13.4 6.8	13.4 6.8 1.4	13.4 6.8	6.1 5.8 3.9 1.8
*Hin*dIII	9.3 2.5 1.1	9.3 2.5 1.1 1.0	9.3 2.5 1.1	9.3 6.0 5.2 3.2
*Pst*I	3.2 2.8	3.2 2.8 1.4	3.2 2.8	6.7 4.6 3.2 2.2

Table II. Estimated sizes of the hybridizing *T. ferrooxidans* restriction endonuclease fragments revealed using the form II RuBisCO probe.

Enzyme	ATCC #			
	23270 21834 13598	13661	33020	14119
*Eco*RI	3.5	1.2	3.5	3.5
*Sal*I	2.6	2.6	0.6	2.6
*Bam*HI	13.4	13.4	13.4 10.4	3.8
*Hin*dIII	7.0	1.3	16.3	9.3
*Pst*I	1.2	1.2	1.2	13.0

ATCC 13598, 21834, and 23270 yielded identical hybridizing fragments using the form II RuBisCO probe (Figure 2 and Table II). ATCC 21834 and 23270 had also been shown to be the same with the form I probe (above), and ATCC 13598 differed only with respect to the presence of the small hybridizing fragment in each digest. The other strains showed both similarties and differences to each other and to the other first strains.

The complete (mole percent) PLFA and LPS OH-FA analyses are presented in Tables III and IV. A principal components analysis for the PLFA is presented in Figure 3. All six strains are tightly clustered. Principal component (PC) #1 is a linear combination of variables (fatty acids) which explains the greatest amount of varibility between all

Table III. The complete (mole percent) PLFA analysis of the various *T. ferrooxidans* strains.

PLFA	#33020	#23270	#13661	#14119	#21834	#13598
15:0	1.59	0.26	0.58	0.21	1.68	0.25
16:1w7c	10.30	17.39	17.55	17.73	19.14	12.43
16:0	13.37	10.31	8.90	11.29	13.19	8.61
17:1w8	1.50	1.13	2.01	0.73	1.09	0.98
17:1w6	2.68	1.31	3.28	0.95	3.92	0.63
cy17	1.71	0.22	1.06	2.84	2.06	1.76
17:0	6.11	1.14	1.84	1.10	2.85	0.98
18:1w7c	22.04	58.8	42.94	36.28	31.31	34.01
cy18	2.38	0.22	0.86	0.57	0.77	0.39
18:0	2.92	1.30	0.98	1.46	1.23	1.04
cy19	26.65	5.42	14.33	21.27	18.26	18.38
19:1	1.96	1.53	2.68	1.85	1.48	8.29
brcy20	6.79	0.98	2.99	3.73	3.03	12.24
Total	100.00	100.00	100.00	100.00	100.00	100.00
pmol/mgdw	24535	96549	82529	105319	70574	34537

Table IV. The complete (mole percent) LPS OH-FA analysis of the various *T. ferrooxidans* strains.

LPS-OHFA	#33020	#23270	#13661	#14119	#21834	#13598
3-OH13:0	5.80	2.11	3.53	1.20	4.06	1.58
3-OH14:0	77.24	89.39	80.66	92.12	79.16	88.28
3-OH15:0	5.70	1.02	2.43	0.64	3.13	0.94
3-OH16:0	6.91	5.21	6.32	1.86	6.62	4.67
9-OH16:0	0	0	0.52	0.36	0	0
10-OH16:0	0	0	0.55	0.37	0	0
11-OH18:0	1.00	0.71	1.89	0.88	1.84	1.15
12-OH18:0	0.48	0	1.20	0.63	1.17	0.75
11-OH19:0	1.58	0.88	1.61	1.05	2.19	1.34
13-OH19:0	1.30	0.68	1.29	0.89	1.83	1.30
Total	100.00	100.00	100.00	100.00	100.00	100.00
pmol/mgdw	5752	3877	5518	7818	4257	6207

replicates (samples) in the data set (in this set 72.46% of the varibility). PC #2 is also a linear combination of variables explaining the greatest amount of variability after that explained by PC #1 (in this set 12.13% of the variability). One way to define the PC is by the loadings (fatty acids) which are assigned the highest positive value (cofficient) after running the PC alogrithim. In this set PC #1 was defined by 18:1w7c (0.79), 16:1w7c (0.37), 16:0 (0.33), and cy19:0 (0.31). PC #2 was defined by i15:0 (0.51), 15:0 (0.47), a15:0 (0.23), and i16:0 (0.21). The values in parentheses are the coefficients. A hierarchical cluster analysis (complete linkage method) of the PLFA data is shown in Figure 4. ATCC 13598, 21834, 14119, and 13661 possess a 0.79 similarity index. ATCC 33020 and 23270 show a 0.65 and 0.42 similarity index to the other strains, respectively. The hierarchical cluster analysis (complete linkage method) of the LPS OH-FA data is presented in Figure 5. Strains 13598, 14119, and 23270, form one cluster group with a similarity index of 0.72 and strains 13661, 21834, and 33020 a second group with a similarity index of 0.68.

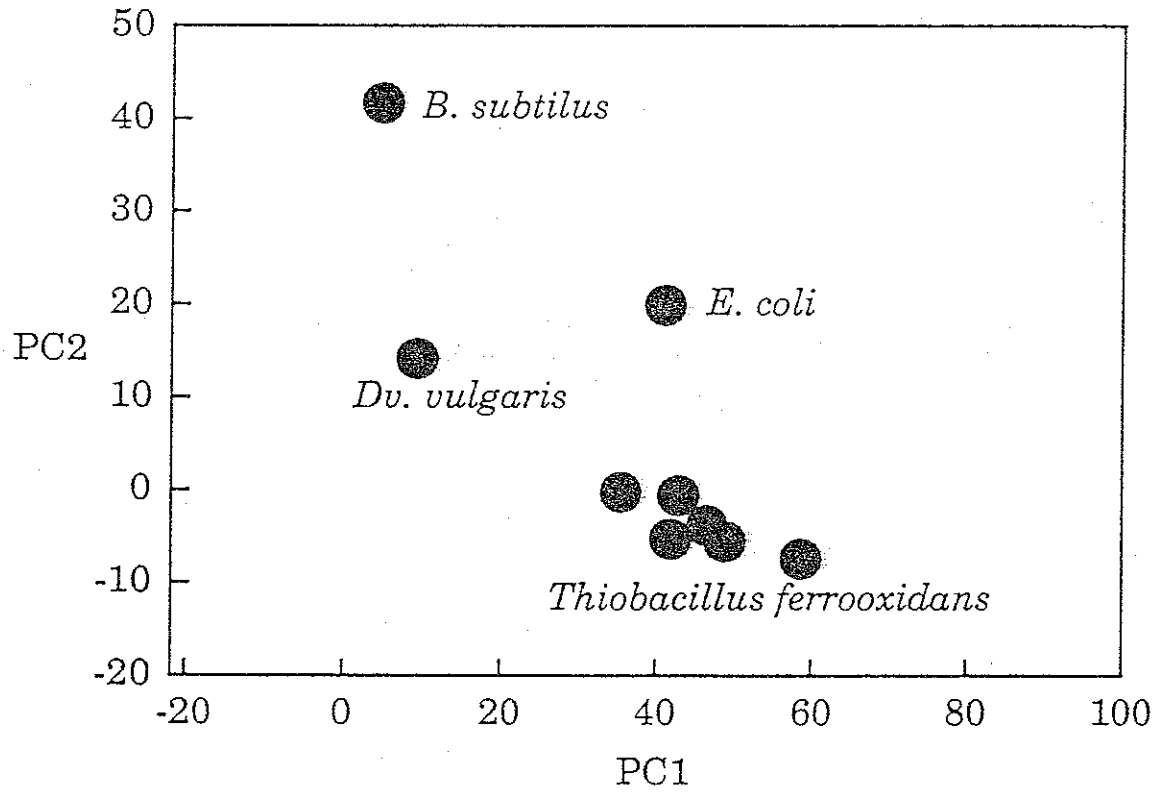

Figure 3. Principal component analysis of *T. ferrooxidans* PLFA.

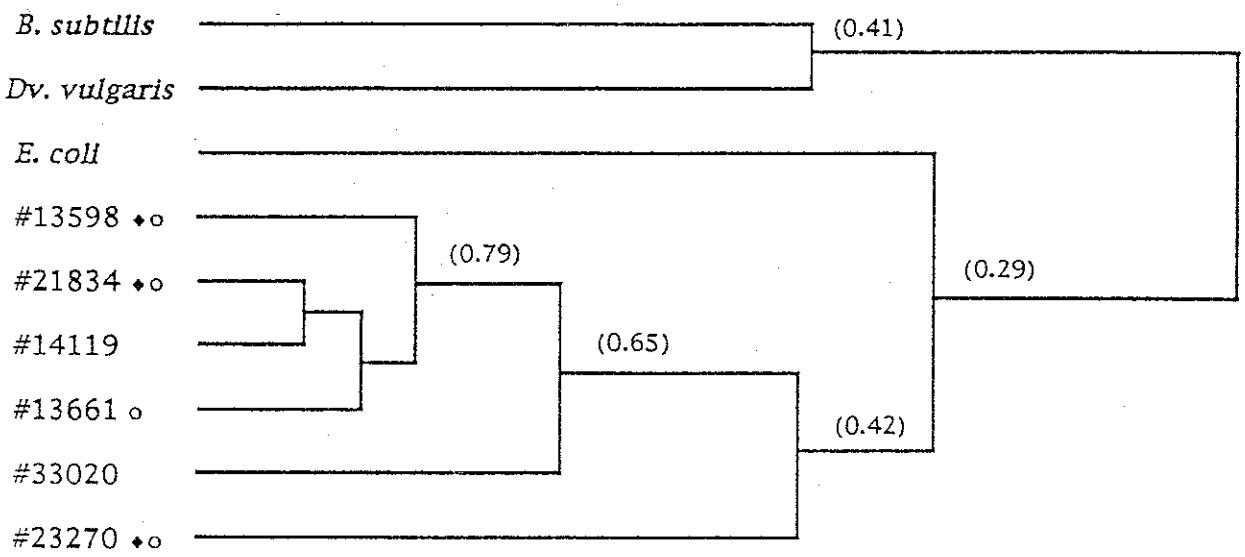

Figure 4. Hierarchical cluster analysis (complete linkage method) of *T. ferrooxidans* PLFA. # is ATCC strain designation. (#) = similarity index. o and ♦ designate strains with identical form I and II RuBisCO restriction fragments, respectively.

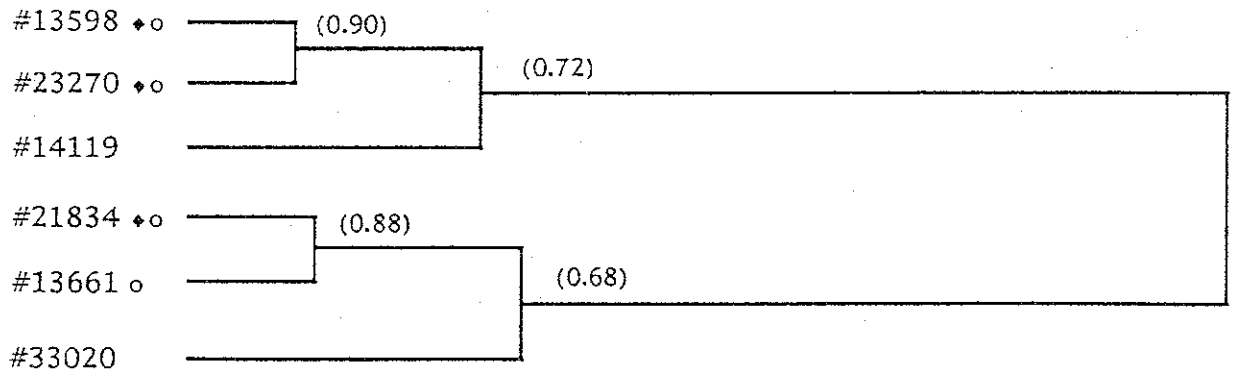

Figure 5. Hierarchical cluster analysis (complete linkage method) of *T. ferrooxidans* LPS OH-FA. # is ATCC strain designation. (#) = similarity index. o and ♦ designate strains with identical form I and II RuBisCO restriction fragments, respectively.

Discussion

Hybridization of probes for both types of RuBisCO large subunit genes (*rbc*L and *rbp*L) suggests the presence of both forms of the enzyme in *T. ferrooxidans*. However, all attempts in this labroatory to demonstrate the presence of the form II enzyme have thus far failed. We have also failed to demonstrate the expression of a form II gene in *Thiobacillus intermedius* (10). In this case we confirmed the presence of the gene by isolation and expression in *Escherichia coli*. This will also have to be accomplished with the *T. ferrooxidans* gene. Two forms of RuBisCO are produced by *Thiobacillus denitrificans* (11). It is interesting to note that two *rbc*L genes have been demonstrated in *T. ferrooxidans* Fe1 (12). The multiple hybridizing restriction fragment in certain digests of ATCC 14119 suggests that this might also be true in this strain.

Classification of the six strains based on the restriction endonuclease fragment analysis of RuBisCO genes showed ATCC 21834 and 23270 to be the same. ATCC 13598 is probably the same except it may harbor a small plasmid. The other three strains show both similarties and differences to each other and to the first three strains. The hybridizing RuBisCO restriction fragments for ATCC 23270 have remained constant after five years of culturing.

The PLFA hierarchical cluster analysis showed that ATCC 21834 and 14119 were most closely related to each other and then to ATCC 13661 and 13598. The type strain, 23270 was the least related to the other species. However, principal components analysis showed all the strains to be tightly clustered. It is interesting that the 16S rRNA sequences for *T. ferrooxidans* F221, *T. ferrooxidans* ATCC 19859, *T. ferrooxidans* ATCC 23270, and *T. thiooxidans* ATCC 19337 show a high degree of similarity (13). DNA-DNA hybridization measurements on the other hand showed the *T. ferrooxidans* strains to be closely related, but that the *T. thiooxidans* strain was very unrelated (13). The LPS OH-FA analysis demonstrated two groups of related strains. These groups more closely paralled the DNA relationships except that ATCC 21834 and 23270, the two strains determined to be identical by Southern blot analysis, fall into different groups. In this study, the LPS OH-FA appears to be the more useful phylogenetic characteristic since the PLFA would likely be more affected by environmental changes, e.g. changes in growth temperature result in marked changes in fatty acid composition of the PLFA.

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It is clear from this study that multiple analyses of genotype and phenotypic expression will be important in understanding the phylogeny and evolution of *T. ferrooxidans*. The requirement for multiple analyses was clearly the case with the methane oxidizing bacteria (14).

Acknowledgements

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