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Preliminary Proteomic Analysis of *Thiobacillus ferrooxidans* Growing on Elemental Sulphur and Fe^{2+} Separately

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***Thiobacillus ferrooxidans* is one of the most important bacterium used in bioleaching, and can utilize Fe^{2+} or sulphide as energy source. Growth curves for *Thiobacillus ferrooxidans* have been tested, which show lag, logarithmic, stationary and aging phases as seen in other bacteria. The logarithmic phases were from 10 to 32 hours for *Thiobacillus ferrooxidans* cultivated with Fe^{2+} and from 4 to 12 days for *Thiobacillus ferrooxidans* cultivated with elemental sulphur. Differences of protein patterns of *Thiobacillus ferrooxidans* growing on elemental sulphur and Fe^{2+} separately were investigated after cultivation at 30°C by the analysis of two-dimensional gel electrophoresis (2-DE), matrix-assisted laser desorption/ ionization (MALDI)-Mass spectrometry and ESI-MS/MS. From the 17 identified protein spots, 11 spots were found more abundant when growing on elemental sulphur. By contrast 6 protein spots were found decreased at elemental cultivation condition. Among the proteins identified, cytochrome C have been previously identified as necessary elements of electron-transfering pathway for *Thiobacillus ferrooxidans* to oxidize Fe^{2+}; ATP synthase alpha chain and beta are expressed increased when *Thiobacillus ferrooxidans* cultivated with Fe^{2+} as energy source. ATP synthase Beta chain is the catalytic subunit, and ATP synthase alpha chain is a regulatory subunit. The function of ATPase produces ATP from ADP in the presence of a proton gradient across the membrane.**

Keywords: ESI-MS/MS, Ionization-mass spectrometry, Mass spectrometry, Matrix assisted laser desorption, *Thiobacillus ferrooxidans*, Two-dimensional gel electrophoresis

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Introduction

Thiobacillus ferrooxidans is a Gram-negative, acidophilus, chemolithotrophic bacterium which is able to derive energy for growth from the oxidation of ferrous to ferric ion and elemental sulfur or reduced inorganic sulfur compounds to sulfate using oxygen as electron acceptor. This ability is particularly suited for leaching of minerals, since the bacterium can oxidize metal sulphides to acid-soluble metal sulphates. It is one of the most important microorganisms involved in bioleaching of sulphidic ores (Brierley, 1978; Rojas-Chapana *et al.*, 1995, 1996; Tributsch, 1998). Both direct and indirect mechanisms are involved in this process. The direct dissolution of minerals is caused by the attack on sulphide ions by the enzymatic system of bacteria oxidizing them to sulphates. In the indirect mechanism (Nemati and Webb, 1996) the ferric iron produced by *Thiobacillus ferrooxidans* from oxidation of ferrous iron serves as a leaching agent that reacts chemically with the minerals. The bacterial oxidation of ferrous iron is based on the reaction:

$$4FeSO_4 + O_2 + H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O \quad (1)$$

There are a lot of reports on the mechanism of bio-leaching of sulfide minerals. The elucidation of the oxidation mechanisms by the bacterium of Fe^{2+} and sulfur compounds will be both important for the development of microbial physiology and promote the effective application of the bacteria in technology. Genetic problems (Rawlings and Kusano, 1994) and application to the bacterial leaching (Leduc and Ferroni, 1994; Yamanaka and Fukumori 1995) of the bacterial have already been reviewed.

Many metabolic pathways and physiological/genetic characteristics of *Thiobacillus ferrooxidans* have attracted attention and are worthy of further evaluation by whole proteome analysis. These include pathways and regulatory mechanisms for the oxidation of Fe(II), reduced sulfur compounds, formate and hydrogen. Other aspects of interest

include, for example, chemotaxis and CO_2 fixation etc. In this report, we focus only on the oxidation of Fe(II) and elemental sulfur. This information might give some help to reconstruct metabolic pathways "in silico" and further unravel the often complicated and multilevel regulation of cellular functions.

Materials and methods

Strains and growth conditions *Thiobacillus ferrooxidans* was isolated from Chengmenshan Mine China Which then was cultured in 9 K base medium additionally adding Fe^{2+} or elemental sulphur respectively as energy resource at 30°C (Amaro and Chamorro *et al.*, 1998; Schippers and Rohwerder *et al.*, 1999). The pH of 9 k basic medium was adjusted to 2.0 with 2 N H_2SO_4.The reactors were 500-ml Erlenmeyer flasks mounted to a HZQ-C constant temperature vibrator agitated at 200 rpm, and the initial liquid volume of each reactor was 200 ml.Sterility was ensured by autoclaving the flasks and liquid solutions at 121°C for 25 min, covering the opening with cotton plugs. Bioreactors were confirmed to maintain dissolved O_2 concentrations in excess of 6 mg/liter during the experimented periods. The number of cells were counted in a Helber chamber.

Preparation of protein extracts When the culture reached the later exponential phase of growth, it was centrifuged at 5,000 × *g* for 10 min, and the bacterial sediment was firstly washed three times with the same basic culture medium without ferrous ions, then (from 1000 mL of culture) were washed three times with ice-cold water and resuspended in 50 μL of 7.5 M urea, 2.5 M thiourea, 1.25 mM EDTA, 1.75 mg/mL Pepstatin A, protease inhibitor cocktail. Cells were disrupted by sonicatted with a sonicater six times for 30 s with 1 min on ice in between. Samples were vortexed thoroughly, and shaken. Cell debris were removed by centrifugation at 10,000 × *g* for 10 min at 4°C, and the resulting supernatant stored at −70°C. Protein concentrations were measured using a Coomassie protein assay kit with BSA as the standard protein (Pierce Biotechnology, Rockford, USA).

Two-dimensional ampholyte gel electrophoresis About 900 μg of protein were loaded in triplicate on Immobiline. Dry Strip gels (18 cm, pH 4-7 linear; Amersham Biosciences) and run on an Ettan. IPGphor isoelectric focusing unit (Amersham Biosciences) for 57900 Vh. Precast SDS 12.5% polyacrylamide gels (26620 cm; Amersham Biosciences) were used for the second dimension (initially run for 1 h at 30 W, then for 4.5 h at 180 W, for a set of 2 gels) using an Ettan DALT System (Amersham Biosciences).

Staining with visible dyes Proteins were visualized by "blue silver" colloidal Coomassie Blue staining."blue silver" Colloidal Coomassie Blue staining was carried out according to Giovanni Candiano (Giovanni and Maurizio *et al.*, 2004), a modified Neuhoffs colloidal Coomassie Blue G-250 stain. The main modified modifications, as compared to Neuhoffs protocol, were: a 20% increment in dye concentration (from 0.1% up to 0.12%) and a much higher level of phosphoric acid in the recipe (from 2% up to 10%). The blue silverexhibits a much faster dye uptake (80% during the first hour of coloration, *vs.* none with a commercial preparation from Sigma). Even at equilibrium (24 h staining), the blue silver exhibits a much higher sensitivity than all other recipes, approaching (but lower than) the one of the classical silver stain. Colloidal Coomassie Blue staining is well compatible with mass spectrometry analysis.

Image analysis Triplicate gels were scanned using a Hewlitt Packard Scanjet 5370C, and the images analyzed using PDQuest analyzing software to identify features that were reproducibly up-regulated or down-regulated. Spot volumes were normalized against total spot volume and total spot area. Features that displayed statistically significant changes in mean normalized spot volume on at least two out of three replicate gels ($p < 0.05$; Student's *t*-test) were excised for protein identification.

In-gel digestion In-gel digestion was performed using a modified version of previously published protocol (Gamble and Dunn *et al.*, 1999). Briefly, protein spots were excised from 2-D gels stained with Coomassie Blue G-250 and were destained by washing in 200 ml aliquots of 50 mm ammonium bicarbonate in 50% v/v acetonitrile for 30 min. The gel pieces were then dried in a SpeedVac Vacuum (Savant Instruments, Holbrook, NY, USA) and rehydrated at 0°C for 30 min in 10 μl digestion solution (25 mm ammonium bicarbonate and 0.01 mg/ml modified sequence-grade trypsin), 20 μl of digestion solution without trypsin was then added to keep the gel pieces wet during the digestion. After overnight incubation at 37°C, the digestion was stopped with 5% trifluoroacetic acid (TFA) for 20 min. Peptides were extracted by 20 μl 5% TFA for 1 h at 37°C and then by 20 μl 2.5% TFA/50% acetonitrile for 1 h at 37°C. The combined supernatants were evaporated in the SpeedVac Vacuum and dissolved in 4 μl 0.5% aqueous TFA for mass spectrometric analysis.

Protein identification by peptide mass fingerprinting All mass spectra of MALDI-TOF-MS were obtained on a Bruker REFLEX III MALDI-TOF-MS (Bruker-Franzen, Bremen, Germany) in positive ion mode at an accelerating voltage of 20 kV with the matrix of A-cyano-4-hydroxy cinnamic acid. The spectra were internally calibrated using trypsin autolysis products. PMF obtained was used to search through the SWISS-PROT and NCBInr database by the Mascot search engine (http://www.matrixscience.co.uk) with a tolerance of 7B 0.1 D and one missed cleavage site.

Protein identification by ESI-MS/MS Proteins were identified by ESI-MS/MS spectrometry of their tryptic peptides essentially as previously reported (Young and Eugene *et al.*, 2003). Trypsin-digested peptides were analyzed by μLC-ESI-MS/MS using an LCQ-DECA mass spectrometer equipped with a C18 trap ESI-emitter/micro-LC column. Trypsin-digested peptides (2 μg) were loaded to a Hewlett Packard/Agilent 1100 Series high-pressure LC system using a Famos Autosampler (Dionex, San Francisco, USA). The peptides bound to the C18 matrix were eluted by acetonitrile gradient (5% to 35%) by mixing acetonitrile with 0.4% acetic acid in water. The eluted peptides were injected into the mass spectrometer by nano-ESI (Figeys and Ducret *et al.*, 1996; Ducret and Van *et al.*, 1998). Mass spectra were acquired by data-dependent ion selection from a full range as well as discrete and narrow survey scan *m/z* ranges to increase the number of

identifications. Proteins were identified from tandem mass spectra using the SEQUEST (Eng and Yates *et al.*, 1994) database search engine to search against the bacteria database.

Results

The measurement of growth curve for *Thiobacillus ferrooxidans* To explore the growth characteristics of *Thiobacillus ferrooxidans* for the purpose of performing the comparative proteome analysis of this organism, we experimented the growth curves of *Thiobacillus ferrooxidans*. As showing in Fig. 1, both growth curves followed the same lag, logarithmic, stationary and aging phase as seen in other bacteria. The logarithmic phases was from 10 to 32 hours for *Thiobacillus ferrooxidans* cultivated with Fe^{2+} and from 4 to 12 days for *Thiobacillus ferooxidans* cultivated with elemental sulphur. When *Thiobacillus ferrooxidans* cultivated with Fe^{2+} for bout 36 hrs, the number of cells would reach the highest (about $1 \times 10^{6.5}$ cells/ml). While *Thiobacillus ferrooxidans* grew on elemental sulphur, the lag phase was greatly longer than that of *Thiobacillus ferrooxidans* grown with Fe^{2+}, and the number of cells reach maximum (about $1 \times 10^{7.5}$ cells/ml) after 14 days cultivation.

Two-dimensional proteome maps identify changes in protein expression of *Thiobacillus ferrooxidans* growing with elemental sulphur and Fe^{2+} separately To identify changes in protein expression in response to different energy resource (Fe^{2+} and elemental sulphur), we compared proteome of *Thiobacillus ferrooxidans* growing on elemental sulphur with proteome of *Thiobacillus ferrooxidans* growing on Fe^{2+}. Cells were collected at respective later half-logarithmic. Cell lysates were resolved by 2-DE. We reproducibly detected about 630 protein spots after "blue silver" colloidal Coomassie Blue staining (Fig. 2: a and b). Although the number of protein spots displayed by Colloidal Coomassie Blue staining (900 μg protein loaded) is lower than that displayed by silver staining (data and figures not shown in this paper), colloidal Coomassie Blue staining is well compatible with mass spectrometry analysis than silver staining. We ran and analyzed at least three gels for each experimental condition.

From the gels, we noticed all of the spots showed in Fig. 2 were distributed in PI 5-7 and their molecular massed ranged 10-140 kD. The 30 spots selected, which were obviously changed in their expression in response to different energy resources (elemental sulphur and Fe^{2+}), were subjected to in-gel trypsin digestion and analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and nanoelectrospray randem mass spectrometry (ESI-MS/MS) (Table 1). As a result, 17 protein spots identified and two got sequence tag. Among the 17 protein spots identified, 11 spots were found more abundant when growing on elemental sulphur. By contrast 6 protein spots were found decreased under the condition of elemental cultivation. results identified by peptide mass fingerprinting (PMF) was evaluated by MOSCOW values and sequence coverage.

From a differential expression protein spot 1 (That was lately identified as ATP synthase beta chain as showing in Fig. 4) highlighted from three pares of gels, We found that The amount of this protein expressed when *Thiobacillus ferrooxidans* growing on Fe^{2+} is about five folds of expressed when growing on elemental sulphur in each parallel experiment (As shown in Fig. 3: a compared with b; c compared with d; e compared with f).The protein spots identified by MALDI-TOF-MS and ESI-MS showed that three proteins: ATP sythase alpha chain (as Fig. 5 shown), ATP synthase beta chain (as Fig. 4 shown) and Cytochrome C have already found in *Thiobacillus ferrrooxidans* bacterium, while other proteins are from other strains (as show in table 1), which included PcaR protein, conserved hypothetical protein, Acyl-coA dehydrogenase, RNA polymerase beta, Membrane protease subunite stomatin/ rohibitin homologs, NAP/NADP transhydrogenase, Enoyl-CoA hydratase/carnithine, Ribose-5-phosphate Isomerase, short chain dehydrogenase, Polyribonucleotide nucletidyltransferase, cytochrome oxidase II, which are due to the incomplete genomic sequencing of *Thiobacillus ferrooxidans*.

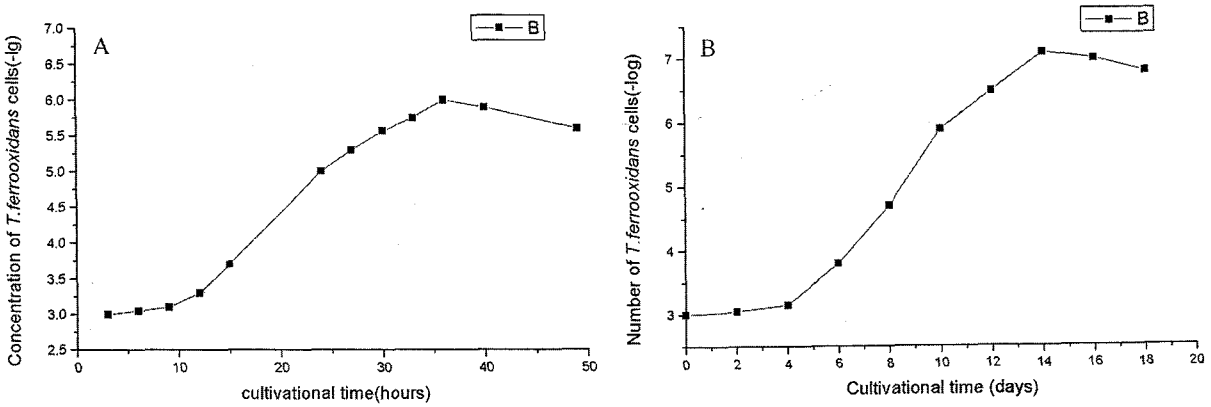

Fig. 1. Growth kinetics of *Thiobacillus ferrooxidans* cultured with elemental sulphur and Fe^{2+} separately. (A) *Thiobacillus ferrooxidans* bacterium grown on Fe^{2+}, (B) *Thiobacillus ferrooxidans* bacterium grown on elemental sulphur.

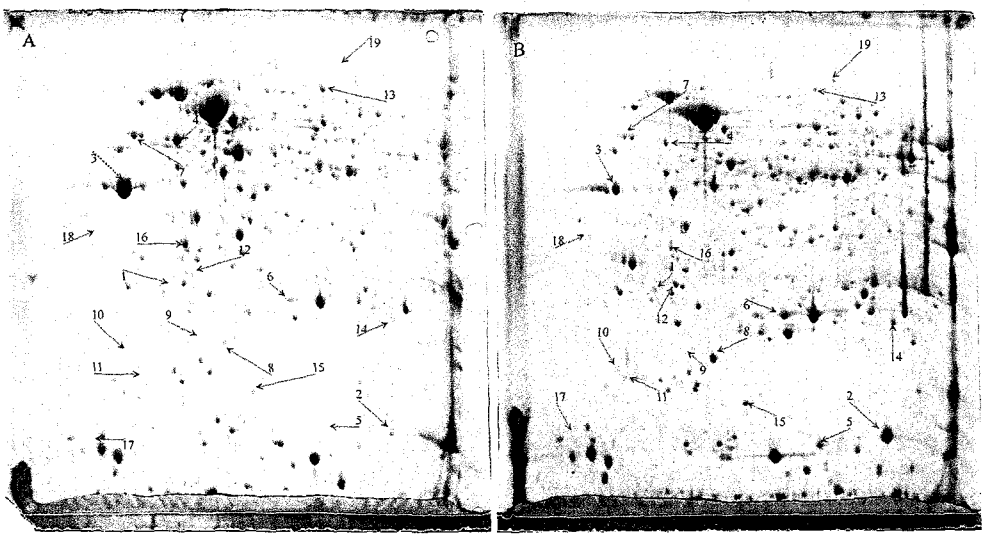

Fig. 2. (A) 2-D SDS-PAGE of proteins isolated from cells of *Thiobacillus ferrooxidans* grown on 9 K base medium adding Fe^{2+}. The amount of protein loaded was 900 μg. (B) 2-D SDS-PAGE of protein isolated from cells of *Thiobacillus ferrooxidans* grown on 9 K base medium adding elemental sulphur, and the amount of protein loaded was 850 μg. No. 18 and No. 19 haven't been identified by ESI-MS/MS, but have a sequence tag respectively as following: **LTPADFAGTGPTEK, TDPDAVNFCK.**

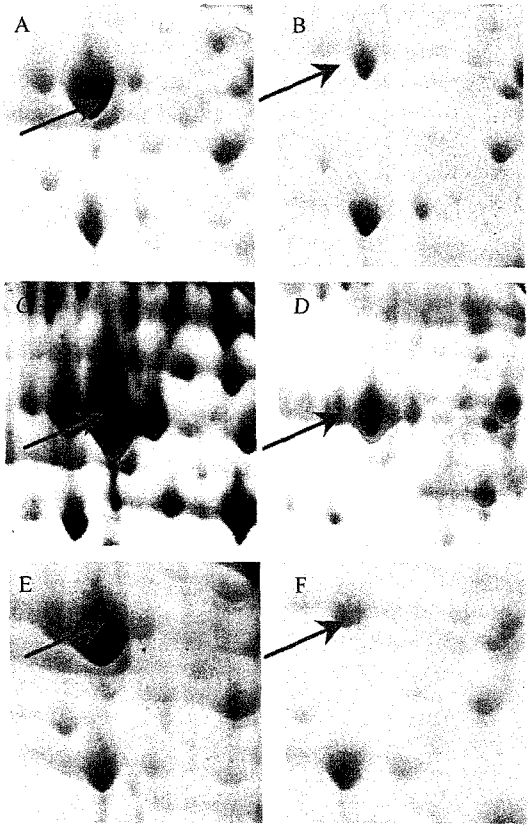

Fig. 3. A reproducible expression change in the *Thiobacillus ferrooxidans* proteome that occurs in response to different energy resource: Fe^{2+} and elemental sulphur. The highlighted protein is ATP synthase beta chain. A, C and E are *Thiobacillus ferrooxidans* cultivated with Fe^{2+}, B, D, and F are *Thiobacillus ferrooxidans* cultivated with elemental sulphur.

Discussion

Bioleaching is the solubilization of metals from sulfide-containing minerals by micro-organisms, among which *Thiobacillus ferrooxidans* is one of the most thoroughly studied. This Gram-negative acidophilic chemolithoautotrophic eubacterium derives the energy required for its growth from the oxidation by oxygen of ferrous iron (Fe^{2+}) or reduced sulfur compounds present in ores. Various redox proteins involved in electron transfer have been already identified and some have been characterized (Corinne and Abderahmane *et al.*, 1998). These include the blue copper rusticyanin, iron-sulfur proteins, two different cytochrome oxidases and several c-type cytochromes. Most of these electron carriers have been supposed to belong to the iron respiratory chain even if their exact role is always subject to controversy.

In order to further unravel the mechanisms by which *Thiobacillus ferrooxidans* oxidize Fe^{2+} or elemental sulphur, we completed an analysis of the global effect of different energy resources on *Thiobacillus ferrooxidans* proteome. The proteomic approach possibly provides us the changes of protein expressions and modifications in panoramic view and paves the way for a more detailed characterization of protein function. In our experiment, Since the total genomic sequence of *Thiobacillus ferrooxidans* still doesnt published, so among the tested 30 protein spots changed in response to different energy resources, only 17 have been identified.

Cytochrome C (Table 1) has been previously identified and regarded as an essential element of iron respiratory chain which is an atypical periplasmic cytochrome. It co-transcribed with the *cyc1* gene and with a gene a periplasmic protein of unknown function. The co-synthesis of the two cytochromes

Table 1. Identified *Thiobacillus ferrooxidans* proteins that were regulated by different energy resources

Spots	Protein name	NCBI ID no.	MASS	Mr (kDa)	Theo-retical pI	Sequence Coverage (%)	Score	Protein expression	strain
1	PcaR protein	AJ 252090	MALDI-TOF-MASS	29.613	8.76	36	72	↑	*Agrobacterium tumefaciens (strain C58,cereon)*
2	hypothetical protein	gi|39590092	MALDI-TOF-MASS	38.535	6.5	37	77	↑	*Caenorhabditis briggsae*
3	Acyl-coA dehydrogenase	gi|21225225	MALDI-TOF-MASS	43.391	5.92	31	71	↓	*Streptomyces coelicolor*
4	ATP synthase Beta chain	gi|728923	MALDI-TOF-MASS ESI-MS/MS	50.749	5.06	39	78	↓	*Thiobacillus ferrooxidans*
5	conserved hypothetical protein	CH75580	MALDI-TOF-MASS	34.936	6.0	35	72	↑	*Deinococcus Radiodurans (strain1)*
6	RNA polymerase beta prime subunit	gi|15606950	MALDI-TOF-MASS	179.30 2	5.64	35	74	↑	*Aquifes aellicus VF5*
7	Membrane protease subunite, stomatin/ rohibitin homologs	gi|23118383	MALDI-TOF-MASS	18.998	6.0	36	74	↓	*Desulfitobact erium hafniense*
8	NAP/NADP transhy-drogenase alpha subunit subunit	gi|48728529	MALDI-TOF-MASS	39.21	6.11	34	73	↑	*Pseudomonas Fluorescens Pfo-1*
9	Enoyl-CoA hydratase/ carnithine racemase	gi|23469147	MALDI-TOF-MASS	40.517	5.50	35	72	↑	*Pseudomonas syringae pv. Syringae B728a*
10	Ribose-5-phosphate Isomerase	gi|23200282	ESI-MS/MS	22.766	5.06	7	79	↑	*E.coli*
11	ATP synthase alpha chain	gi|3282057	ESI-MS/MS	55.737	5.27	8	912	↑	*Thiobacillus ferrooxidans*
12	short chain dehydrogenase	gi|36958924	ESI-MS/MS	27.843	4.66	4	83	↑	*Rhozobium SP.NGR234*
13	Polyribonucleotide nucletidyltransferase	gi|46311354	ESI-MS/MS	177	5.35	14	177	↓	*Burkholderia cepacia R18194*
14	unnamed protein product	gi|48339	ESI-MS/MS	50.838	4.75	2	93	↑	*Vibrio alginolyticus*
15	unnamed protein product	gi|51623	ESI-MS/MS	26.413	8.13	17	70	↑	*Mus musculus*
16	Cytochrome C	gi|3282057	ESI-MS/MS	52.889	5.54	4	79	↓	*Thiobacillus ferrooxidans*
17	cytochrome oxidase II	gi|5924188	ESI-MS/MS	25.865	4.57	4	50	↓	*Chalcites lucidus*

The expression ratio for " unique "spots" only detected under one condition was calculated by dividing the normalized peak volume by the lowest detectable normalized volume. Differences in expression in the sulphur-grown bacteria are relative to the Fe^{2+}-grown bacteria. Protein spots that were differentially regulated (>3 folds) are indicated by arrows. (↑) indicates that protein expression was increased, while (↓) indicates a decrease in protein expression. Another two protein spots which haven't been identified by ESI-MS/MS arent shown in the table, and a sequence tag for each protein is as following: **LTPADFAGTGPTEK**, **TDPDAVNFCK**. No. 4 and No. 11 have sequence tags respectively as following: **TLLSETGSFLTLLK**, **SKPALGKPVDGK**. Only when the sequence coverage for each protein is greater than 30%, can protein identified by MALDI-TOF-MS be meaningful.

means that the belong to the same electron transfer chain (Yamanaka and Fukumori *et al.*, 1997). The putative protein it encodes might be translocated to the pariplasm as it has a typical amino-terminal signal sequence (residues 1-31). No significant homologies were detected in database banks. However, a typical haem-binding site, C-A-A-C-H (residue 43-47), present in they amino-terminal part of the putative mature protein (Giudici-Orticoni and Nitschke *et al.*, 1997). The corresponding gene has accordingly been called *cyc2*, The amino-acid composition of the mature Cyc-2 cytochrome matchs that of the membrane-bound 46kDa cytochrome identified in *Thiobacillus ferrooxidans* BRGM strain (Elbehti and Asso *et al.*, 1997). Furthermore, the two NH_2 amino-acid sequences are 66.7% identical and 85.7% similar, with the

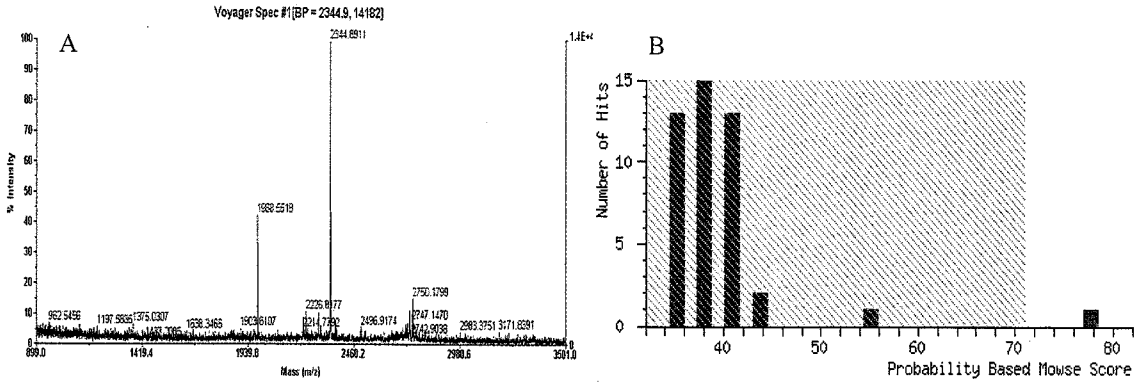

Fig. 4. Analysis of differently expressed No. 1 protein and the masses indicated were matched to ATP synthase β band. a: MALDI-TOF-MS spectra obtained for ATP synthase â band. Monoisotopic peptide were used to search protein databases to match and subsequently identify individual protein spots. The 2211.7792 were is a trypsin auto-digestion peptide used for mass calibration. b: Probability Based Mowse Score. Score is −10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 71 are significant ($p < 0.05$).

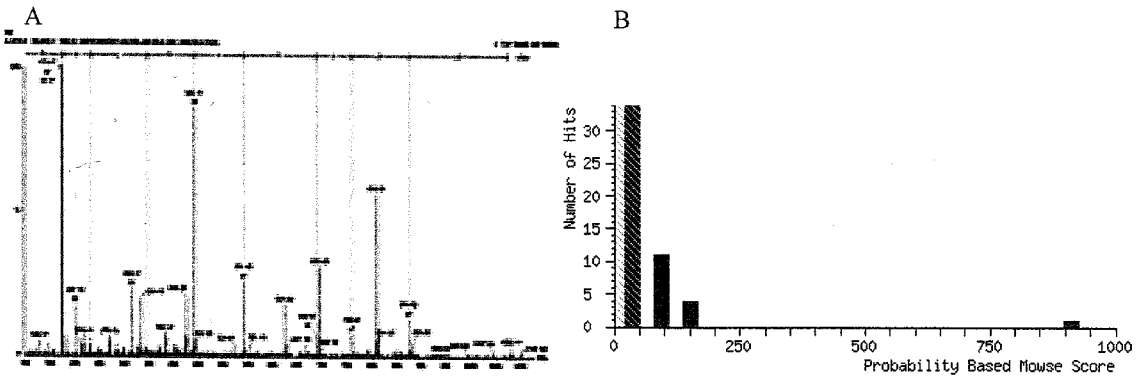

Fig. 5. The protein identified by ESI-MS/MS is ATP synthase alpha chain. Probability Based Mowse Score. Score is −10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 60 are significant ($p < 0.05$).

first nine residues being identical. These data, and the similarity in the molecular masses of the mature cytochromes, obviously tells that the cyc2 gene encodes the 46-kDa cytochrome equivalent of ATCC 33020 strain.

F-type ATPase have 2 components, CF(1)-the catalytic core and CF(0)-the membrane proton channel. CF(1) has five subunits: ALPHA(3), BETA(3), GAMMA(1), DELTA(1), EPSILON(1). CF(0) has three main subunits: A, B and C. ATP synthase Beta chain is the catalytic subunit, and ATP synthase alpha chain is a regulatory subunit. The function of ATPase produces ATP from ADP in the presence of a proton gradient across the membrane. Catalytic activity of ATPase as following:

$$ATP + H(2)O + H(+)\ (In) = ADP + phosphate + H(+)\ (Out).$$

An atp gene cluster from the extreme acidophile *Thiobacillus ferrooxidans* was able to complement Escherichia coli F1 unc mutants for growth on minimal medium plus succinate (Brown *et al.*, 1994). Complementation with all four *E. coli* F1 mutants tested was observed and subunits for the F1 portion of the *Thiobacillus ferrooxidans* ATP synthase formed a functional association with the F0 subunits of the *E. coli* enzyme. In addition, a hybrid F1 enzyme in which some units were derived from *E. coli* and some from *Thiobacillus ferrooxidans* was partially functional. No clones capable of complementing *Escherichia coli* F0 unc mutants were isolated. The nucleotide sequence of the gene cluster was determined and the genes for the F0 and the F1 ATP synthase subunits were found to be physically linked. Our work found that ATP synthase Beta chain and ATP synthase alpha chain are expressed when grown on Fe^{2+} outgoing those expressed when grown on elemental sulphur, mechanism of which still need further research.

The rest protein spots identified are all belonging to other strains just like *Escherichia coli* etal. Their exact roles in the mechanisms of utilization of $Fe^{2\pm}$ or elemental sulphur by *Thiobacillus ferrooxidans* need for further research.

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References

Amaro, A. M., Chamorro, D., Seeger, M., Arredondo, R., Peirano, I. and Jerez, C. A. (1999) Effect of external pH perturbations on in vivo protein synthesis by the acidophilic bacterium *Thiobacillus ferrooxidans*. *J. Bacteriol.* **173**, 910-915.

Brierley, C. L. (1978) Bacterial leaching. *Crit. Rev. Microbiol.* **6**, 207-262.

Brown, L. D., Dennehy, M. E. and Rawlings, D. E. (1994) The F1 genes of the F1F0 ATP synthase from the acidophilic bacterium *Thiobacillus ferrooxidans* complement *Escherichia coli F1* unc mutants. *FEMS Microbiol Lett.* **122**, 19-25.

Corinne, A. A. and Abderrahmane, B. (1998) Characterization and expression of the co-transcribed cyc1 and cyc2 genes encoding the cytochrome c4(c552) and a high-molecular-mass cytochrome c from *Thiobacillus ferrooxidans* ATCC 33020. *FEMS Microbiol. Lett.* **167**, 171-177.

Ducret, A., Van Oostveen, I., Eng, J. K., Yates, J. R. 3rd and Aebersold, R. (1998) High throughput protein characterization by automated reversephase chromatography/electrospray tandem mass spectrometry. *Protein Sci.* **7**, 706-719.

Elbehti, A., Asso, M., Guigliarelli, B. and Lemesle-Meunier, D. (1997) Characterization of the three membrane bound metallo enzymes required to postulate the existence of a bc-type complex in *T. ferrooxidans*. *Biomine.* **2**, 1-9.

Eng J. K., McCormack, A. L. and Yates, J. R. 3rd. (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5**, 976-989.

Figeys, D., Ducret, A., Yates, J. R. 3rd and Aebersold, R. (1996) Protein identification by solid phase microextraction-capillary zone electrophoresis-microelectrospray-tandem mass spectrometry. *Nat. Biotechnol.* **14**, 1579-1583.

Gamble, S. C., Dunn, M. J., Wheeler, C. H., Joiner, M. C., Adu-Poku, A. and Arrand, J. E. (2000) Expression of proteins coincident with inducible radioprotection in human lung epithelial cells. *Cancer Res.* **60**, 2146-2151.

Giovanni, C., Maurizio, B., Luca, M., Laura, S., Gian, M., Ghiggeri, B., Carnemolla, P. O., Luciano, Z. and Pier, G. R. (2004) Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* **25**, 1327-1333.

Giudici-Orticoni, M.-T,. Nitschke, W., Cavazza, C. and Bruschi, M. (1997) Characterization and functional role of a cytochrome c4 involved in the iron respiratory electron transport chain of *Thiobacillus ferrooxidans*. *Biomine.* **4**, 1-10.

Leduc, L. G. and Ferroni, G. D. (1994) The chemolithotrophic bacterium *Thiobacillus ferrooxidans*. *FEMS Microbiol. Rev.* **14**, 103-120.

Nemati, M. and Webb, C. (1996) Effect of ferrous iron concentration on the catalytic activity of immobilized cells of *Thiobacillus ferrooxidans*. *Appl. Microbiol. Biotechnol.* **46**, 250-255.

Rojas-Chapana, J. A., Giersig, M. and Tributsch, H. (1995) Sulphur colloids as temporary energy reservoirs for *Thiobacillus ferrooxidans* during pyrite oxidation. *Arch. Microbiol.* **163**, 352-356.

Rojas-Chapana, J. A., Giersig, M. and Tributsch, H. (1996) The path of sulfur during the bio-oxidation of pyrite by *Thiobacillus ferooxidans*. *Fuel.* **75**, 923-930.

Schippers, A., Rohwerder, T. and Sand, W. (1999) Intermediary sulfur compounds in pyrite oxidation: implications for bioleaching and biodepyritization of coal. *Appl. Microbiol. Biotechnol.* **52**, 104-110.

Tributsch, H., Rojas-Chapana, J. A., Bartels, C. C., Ennaoui, A. and Hofmann, W. (1998) Role of transient iron sulfide films in microbial corrosion of steel. *Corrosion* **54**, 216-227.

Rawlings, D. E. and Kusano. T. (1994) Molecular genetics of *Thiobacillus ferrooxidans*. *Microbiol. Rev.* **58**, 39-55.

Tateo, Y. and Yoshihiro, F. (1995) Molecular aspects of the electron transfer system which participates in the oxidation of ferrous ion by *Thiobacillus ferrooxidans*. *FEMS Microbiol. Rev.* **17**, 401-413.

Yamanaka, T. and Fukumori, Y. (1997) Molecular aspects of the electron transfer system which participates in the oxidation of ferrous ion by *Thiobacillus ferrooxidans*. *FEMS Microbiol. Rev.* **17**, 401-413.

Young, A. G., Eugene, C., Nitin, S. B., Weiguo, A. T., Min, P., Ruedi, A., David, R. G, Leroy, H. and Wailap, V. N. (2003) Proteomic analysis of an extreme halophilic archaeon, *Halobacterium sp.* NRC-1. *Mol. Cell. Proteomics* **2**, 506-524.