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Characterization of the acid stress response of *Acidithiobacillus ferrooxidans* ATCC 23270 based on the method of microarray

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Bioleaching has become a research hotspot, as a simple and effective technology for metal extraction from low-grade ores. During the process of bioleaching, the chemoautotroph organisms play a key role. However, their growth and activity are greatly restrained by the stress brought by over acidic extracellular environment in industrial practice. This research effort focused on the transciptome profile discrepancies of the typical bioleaching species *Acidithiobacillus ferrooxidans* ATCC 23270 when challenged by a pH downshift to pH 1.2 using the method of microarray. A total of 1853 genes were found to be significantly affected under acid stress challenge for 1 hr, and the array data revealed that *A. ferrooxidans* ATCC 23270 actively responses to acid stress from several levels: cell envelope level, cytoplasm level and transcription level. One remarkable change is that the ion transporters and the synthesis of glutamine and polyamines were regulated in chemoautotrophic *A. ferrooxidans* ATCC 23270 under acid stress, while in contrast, typical heterotrophic bacteria *Escherichia coli* depended on up-regulation of ATP synthase F0F1 and glutamate decarboxylase system. More specifically, the alkaline substances generated by *glnA*, *glnB*, *glnD*, *glnE* etc. were functional in buffering the cytoplasm pH, and certain proton pumps such as cytoplasm protons plasma-membrane proton transport P-type ATPase played an important role in proton expulsion to keep cytoplasm pH in certain range. The new information provided in this study could lead to a better understanding of the acid resistance mechanisms exhibited by autotrophic acidophilic bacteria.

Key words: *Acidithiobacillus ferrooxidans*, acid stress, microarray.

INTRODUCTION

Bioleaching is the extraction of specific metals from ores by using bacteria. The typical bioleaching strain *Acidithiobacillus ferrooxidans* utilizes energy from the oxidation of iron- and sulfur-containing minerals (Baker & Banfield, 2003) and grows around pH 2.0. As bacteria grow optimally within a specific narrow range of pH, the fluctuation of environmental acidity can bring certain disadvantageous impacts on both growth and activity of the bacteria. However, the extracellular environment of *A. ferrooxidans* is rarely optimal in industrial practice, usually over acid, leading to restrained growth and weakened activity. It was reported that the acidophiles require a neutral intracellular pH, just like the neutralophiles (Baker-Austin & Dopson, 2007), though acidophiles stand low pH, raising questions regarding their ability to

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function when facing a proton motive force across the inner membrane that is several orders of magnitude higher than the typical circumstance. As the reactions cells have to the stress could be reflected in the expression discrepancies, the study of gene expression of related genes after exposing to over acid environment could provide useful data for potential industrial application.

In general, the cytoplasmic pH should be within a certain range for surviving of both neutralophiles and acidophiles, and bacteria possess numerous mechanisms to cope with a sudden drop in pH (Cotter & Hill, 2003). The regulation mechanisms under acid stress have been studied in a variety of microorganisms, and the majority are heterotrophic bacteria, for instance, *Escherichia coli* (Gale & Epps, 1942), *Salmonella typhimurium* (Foster & Hall, 1990), *Staphylococcus aureus* (Bore *et al.*, 2007), *Lactobacillus reuteri* (Wall *et al.*, 2007), *Streptococcus mutans* (Hamilton & Buckley, 1991), *Listeria monocytogenes* (O'Driscoll *et al.*, 1996) and *Lactococcus lactis* (Rallu *et al.*, 1996).

Among these mechanisms, the most direct approach is the use of proton pumps, which literally pump protons out of the cytoplasm to keep the internal pH at an acceptable level (Foster & Hall, 1991). It has been reported that cytoplasmic pH of streptococci is regulated by changes in amount and activity of a proton-translocation ATPase (Kobayashi *et al.*, 1986). Acid tolerance of *Streptococcus bovis* and *Ruminococcus albus* appears to be related to the capacity of augmentation of the synthesis of H^+-ATPase responding to low pH (Miwa *et al.*, 2001). Amino acid (glutamate, arginine, or lysine) decarboxylase systems have been reported to play an important role in resisting acid stress. To be specific, the expression of GAD (glutamate decarboxylase activity) genes is positively regulated by acidic shock in *E. coli* (De Biase *et al.*, 1999), and survival of *L. monocytogenes* in gastric fluid is associated with the GAD system (Cotter *et al.*, 2001). Moreover, the protein product of *RpoS* (sigma-38) regulates a bank of genes acting in stationary phase, and some of them are involved in stress actions. The role of RpoS seems to have a certain impact upon stress, as it has been reported in many bacteria, such as *E. coli* (Hengge-Aronis, 2002) and *Pseudomonas aeruginosa* (Jorgensen *et al.*, 1999).

Although previous research has provided clues on the mechanisms existing in heterotrophic acidophiles upon acid stress, little is known about the acid stress response of autotrophic bacteria. The study of a chemoautotrophic and acidophilic organism such as *A. ferrooxidans* ATCC 23270 could enhance our knowledge and understanding of the acid resistance mechanisms. In addition, it is common practice to apply the methods that focus on certain genes (Chao *et al.*, 2008) such as real-time PCR in this area. Considering the fact that acid stress responses could induce complex and comprehensive cellular reactions, it is necessary to apply the microarray method that is a simple and natural vehicle for exploring the expression profile on genomic level in a systematic and comprehensive way (Brown & Botstein, 1999).

In this study, we investigated the effects of acid stress on gene expression profiles of autotrophic *A. ferrooxidans* ATCC 23270 by using the microarray technique. The results indicated that acid stress significantly modulated the expression of genes related to cytoplasm pH buffering, proton transport, and transcription. Besides, the microarray analysis suggested that acid stress inhibited energy metabolism, thus growth was not favored under acid stress conditions. Our results also provided evidence that acid stress promotes biosynthesis of glutamate, which would be conducive in stabilizing cytoplasm pH.

MATERIALS AND METHODS

Strains and growth conditions

The strain ATCC 23270 of *A. ferrooxidans* was obtained from American Type Culture Collection (ATCC). It was incubated in 9K medium with S^0 on a rotary platform with initial pH 2.3, temperature 30°C, and rotation speed 170 r min^{-1}. The cell concentration in the culture with original pH 2.3 was monitored using hemocytometer, in order to decide the magnitude of pH stress applied in the experimental groups. The pH of culture (original pH 2.3) declined consistently dur-

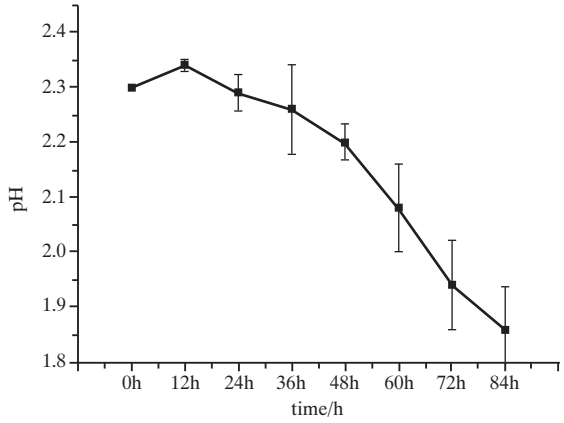

FIG. 1. pH curve of *A. ferrooxidans* ATCC 23270 cultured in 9K medium with original pH 2.3.

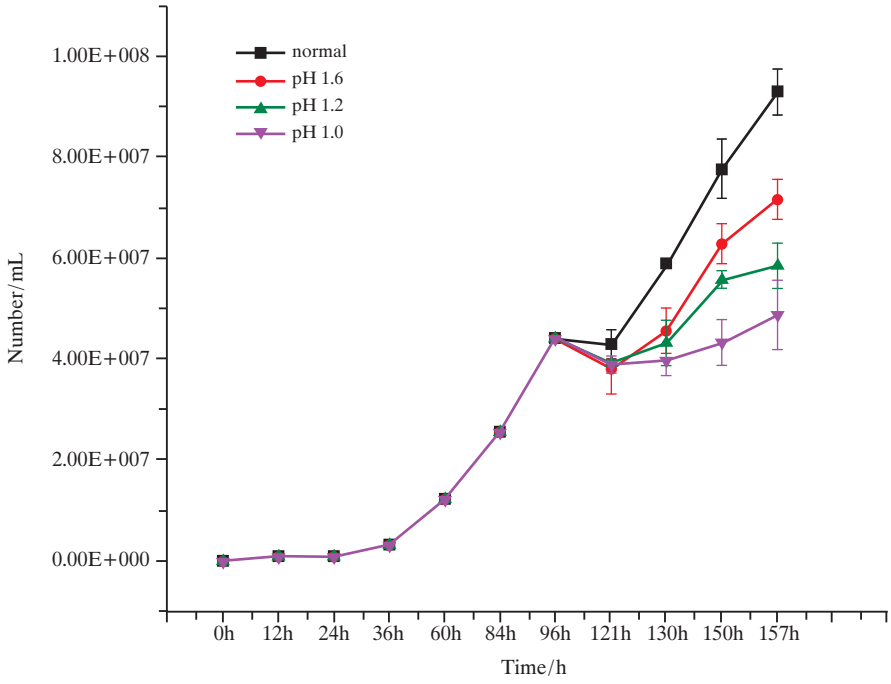

FIG. 2. Growth curve of *A. ferrooxidans* ATCC 23270 measured by hemacytometry, adjusting pH of exponential phase cultures to pH 1.6, 1.2, 1.0 or not, respectively.

ing the incubation and it dropped to pH 1.86 at the middle of log phase (84 hrs), thus the appropriate pH applied to the experimental group could be chosen among pH 1.6, 1.2 and 1.0 (Fig. 1).

Cells in late-exponential phase (96 hrs) were respectively stressed with the above pH, and the number of cells in the experimental groups decreased upon the treatment, then restarted to increase after different periods (Fig. 2). Among the experimental groups, the group of pH 1.6 was the first to continue growing (about 34 hrs after the treatment), and then the one of pH 1.2 (about 44 hrs later); however, the number of cells under pH 1.0 showed no obvious growth. Based on the pH curves, an appropriate acid stimulus should be decided. Considering the fact that the magnitude of transcriptome discrepancies observed between the experimental and control groups could be greater once the pH was lower, and both the obvious discrepancies and the growth should be ensured, pH 1.2 was chosen.

Experimental stimuli

Cells (that were contained in ~300 ml culture) were collected at late-exponential phase (96 hrs) for each sample, centrifuged at 5000 r min^{-1} for 20 min, adding cell-free culture media with pH 1.2 to resuspend. Once the duration of acid treatment reached 30 min, 60 min, 120 min and 240 min, cells in experimental group and control group were collected and transferred to 1.5 ml tubes, respectively. Other parameters like temperature and rotation speed were kept constant as the treatment continued. All the cells collected were stored in liquid nitrogen in preparing for the next step.

RNA extraction, cDNA labeling

Samples were thawed and RNA from each sample was extracted by using the Trizol reagent, and purified according to Qiagen RNeasy Mini Kit protocol (RNeasy Mini Handbook, 04/2006). Samples of RNA were spectrophotometrically quantified (NanoDrop ND-1000 UV-Vis). RNA samples were quality checked by A260/A280 ratios (which were generally ≥ 2.00), and yielded a range of more than 200 ng μl^{-1}. Also, electrophoretic analysis and DNAse treatment of RNA samples were performed. In each sample, 2.0 μg RNA is required for next steps.

RNA samples were labeled with Cy3-labeled dUTP during reverse transcription using Superscript® reverse transcriptase (Invitrogen), following the manufacturer protocol. The pool of nucleotides in the labeling reaction was 0.5 mM each of dATP, dTTP, dGTP and 0.25 mM dCTP (New England Biolabs, Beverly, USA). Fluorescent nucleotides, Cy3-dUTP (Amersham Pharmacia Biotech, Piscataway, USA), were present at 0.1 mM. The labeled probes were purified in accordance with QIAquick PCR Purification Kit protocol (QIAquick Spin Handbook, 11/06).

Microarray fabrication

Oligonucleotide probes spotted on the microarray were specifically designed for certain sequence, as the whole genome sequence of *A. ferrooxidans* ATCC 23270 (Accession number: NC_011761) has been determined (http://www.ncbi.nlm.nih.gov/). The whole-genome oligonucleotide array was developed based on the 3217 ORFs of *A. ferrooxidans* ATCC 23270 genome, including the genes related to 20 categories: biosynthesis of cofactors, prosthetic groups, and carriers; central intermediary metabolism; signal transduction; mobile and extrachromosomal element function; amino acid biosynthesis; cell envelop; cellular processes; disrupted reading frame; DNA metabolism; energy metabolism; fatty acid and phospholipid metabolism; protein fate; protein synthesis; purines, pyrimidines, nucleosides and nucleotides; regulatory functions; transcription; transport and binding proteins; hypothetical proteins; hypothetical proteins-conserved; unknown.

Microarray hybridization and image processing

Whole-genome oligonucleotide array for *A. ferrooxidans* ATCC 23270 was applied in this study; details of its designing and implementation have been reported in Li *et al.* (2008). Formamide and RNase-free water was used to dissolve the labeled cDNA after concentrating for about 30 min, and herring sperm DNA (Promega, USA), 5% SDS, 20×SSC was added into the solutes of each sample. The mixture was kept in 98°C for 3 minutes, and then the hybridization was carried out at 42°C overnight in a hybridization chamber (Corning, USA). The hybridized microarray was then washed sequentially for 5 min in 1×SSC-0.2% SDS, for 10 min in 0.1×SSC-0.2% SDS and for 30 s in 0.1×SSC at ambient temperature before being dried.

The microarrays were scanned by GenePix 4000B Array Scanner (AXON instruments, USA) at a resolution of 10 μm, and the emitted fluorescent signal was detected by a photomultiplier tube (PMT) at 532 nm (Cy3) or 635 nm (Cy5). Each hybridization spot in the image was quantified by the software GenePix Pro version 6.0 (AXON instruments, USA), and exported as Excel data file for further processing. A grid of individual circles defining the location of each DNA spot on the array was superimposed on the image to designate each fluorescent spot to be quantified. The mean signal intensity was determined for each spot. The local background signal was subtracted automatically from the hybridization signal of each separate spot, also computed for each spot to discriminate true signals from noise. The SNR ratio was applied to judge the validity of each data, calculated through the following formula (Verdnick *et al.*, 2002): SNR = (Signal Intensity – Background) / Background Standard Deviation, in which "Background" referred to the local spot background intensity and "Background Standard Deviation" was calculated across all pixels measured by the ImaGene software. In this study, the criterion for the minimum signal (threshold) that can be accurately quantified is SNR ≥ 2. The SNRs from three replicate data sets were then averaged to represent the SNR for a particular probe. Spots that appeared to be lower than the threshold value were removed from the data set. The time-course changes of genes during acid stress were determined using hierarchical cluster analysis (CLUSTER) and visualized with TREEVIEW (Eisen *et al.*, 1998).

RESULTS

General overview of transcriptomic response of A. ferrooxidans ATCC 23270 to acid stress

Whole-genome DNA microarrays were used to obtain a comprehensive, general description of the molecular response mounted by *A. ferrooxidans* ATCC 23270 when challenged by a pH downshift (Fig. 3). In total, 1949 genes (898 induced and 1051 repressed) at 30 min, 1853 genes (1086 and 767) at 60 min, 1938 genes (946 and 992) at 120 min, and 2424 genes (1050 and 1374) at 240 min exhibited significantly differential expression at a twofold or greater level.

Genes involved in cell envelope (106/61, induced/repressed), transcription (16/6), DNA metabolism (37/21), and hypothetical proteins (266/151) were up-regulated at 60 min, whereas genes encoding cell process (39/47, induced/repressed), energy metabolism (50/60), and protein fate (23/31) were repressed. The down-regulated genes involved in energy metabolism outnumbered the up-regulated ones at all four durations (30 min: 32/81; 60 min: 50/60; 120 min: 49/75; 240 min: 53/105; induced/repressed), which was similar to the results of cold shock response of *Shewanella oneidensis* MR-1 (Gao *et al.*, 2006). Besides, some genes related to acid stress response are listed in Tables 2 and 3, and they are separately involved in cell envelope, transport and binding proteins, central intermediate metabolism, amino acid

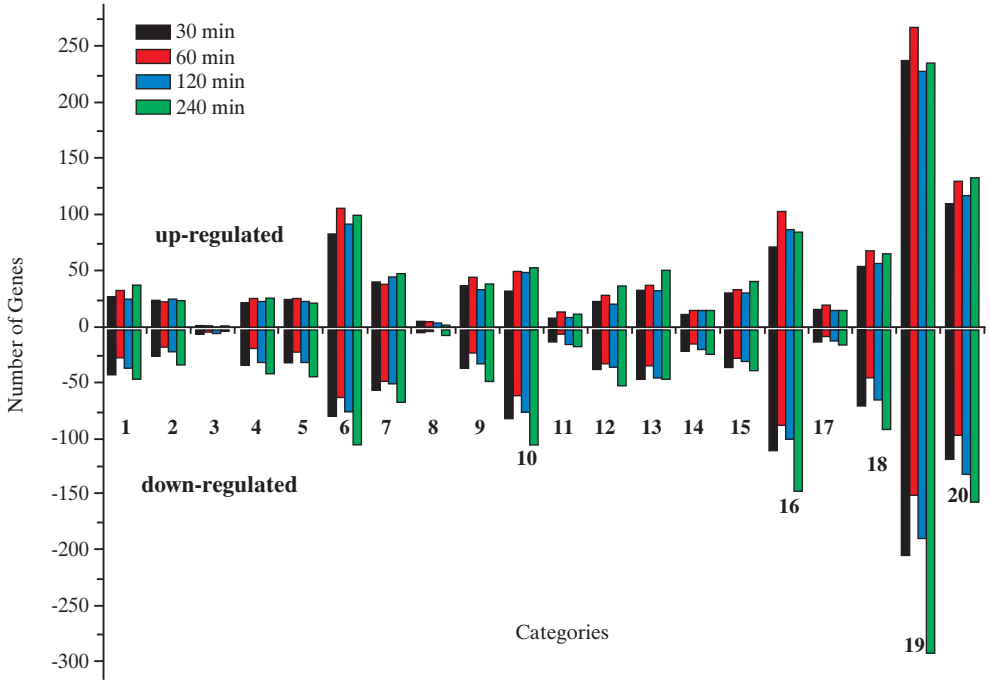

FIG. 3. Differentially expressed genes grouped by functional classification according to the TIGR *A. ferrooxidans* ATCC 23270 genome database. Columns: 1. Biosynthesis of cofactors, prosthetic groups, and carriers; 2. Central intermediary metabolism; 3. Signal transduction; 4. Mobile and extrachromosomal element function; 5. Amino acid biosynthesis; 6. Cell envelope; 7. Cellular processes; 8. Disrupted reading frame; 9. DNA metabolism; 10. Energy metabolism; 11. Fatty acid and phospholipid metabolism; 12. Protein metabolism; 13. Protein synthesis; 14. Purines, pyrimidines, nucleosides and nucleotides; 15. Regulatory functions; 16. Unknown; 17. Transcription; 18. Transport and binding proteins; 19. Hypothetical proteins; 20. Hypothetical proteins-conserved.

biosynthesis, energy metabolism, transcription and regulatory functions.

Cell envelope-associated proteins and transporters

Cell envelope is in charge of support and transportation. The exposure of *A. ferrooxidans* ATCC 23270 to acid stress could result in its change of structure, function, or both. Based on the microarray results, several genes related to capsule polysaccharide were significantly down-regulated under acid stress, while the genes related to glycosyl-transferase and tight adherence protein were induced (Table 1). Glycosyl transferase is reported to be responsible for synthesis of lipopolysaccharide (Pollock, 1998), and Tad secretion system is dedicated to the assembly and export of Flp pili in *Aggregatibacter actinomycetemcomitans* (Clock *et al.*, 2008). In addition, the majority of the membrane proteins and the outer membrane proteins were repressed. Significantly, two genes of the outer membrane proteins *Omp85 family protein* (AFE1452) and *OmpH family protein* (AFE1453) showed high expression under the stress (Table 1), and Omp85 is required for integrating proteins into the outer membrane of *Neisseria meningitidis* (Voulhoux *et al.*, 2003) and effective insertion of lipids (Genevrois *et al.*, 2003). According to the above results, *A. ferrooxidans* ATCC 23270 could probably change the percentage of some substance in the envelope under the acid stress. To be specific, induced glycosyl-transferase increased the synthesis of lipopolysaccharide.

Related ion transporters could be regulated upon the acid stress. Several genes related to transport of potassium and ammonium were significantly down-regulated upon acid stress (Table 2); these include *potassium-efflux system protein* (AFE1968), *potassium uptake protein* (AFE1753) and *amt-1* (AFE2916), *amt-2* (AFE2911). Interestingly, *large conductance mechanosensitive channel protein* (*mscL*/AFE3260: 203.06 folds) was induced upon the acid stress, which is closely related to K^+ sensitivity of *E. coli* (Cui & Adler, 1996) (Table 2). It is reported that Na^+/H^+ antiporters were important for Na^+ efflux and salt tolerance in the fungus *Schizosaccharomyces pombe* (Jia *et al.*, 1992), and the Na^+/H^+ antiporter cytoplasmic

TABLE 1. The significantly regulated genes related to cell envelope by acid stress

Locus_Tag	Ratio (1 hr)	Description
		capsule polysaccharide
AFE3013	0	capsule polysaccharide modification protein
AFE2962	–1.000	*ctrC*, capsule polysaccharide exporter, inner-membrane protein CtrC
AFE2963	–2.400	*ctrD*, capsule polysaccharide exporter, ATP-binding protein
AFE2975	–35.00	capsule polysaccharide export protein, BexD/CtrA/VexA family
AFE2961	–15.300	capsule polysaccharide export inner-membrane protein
AFE3012	1.090	capsule polysaccharide biosynthesis protein
AFE3016	–2.079	capsular polysaccharide biosynthesis protein, putative glycosyl-transferase
		glycosyl-transferase
AFE1776	12.500	*glycosyl-transferase*
AFE3008	0	*glycosyl-transferase, group 1*
AFE2976	–1.450	*glycosyl-transferase, group 1*
AFE2967	–28.600	*glycosyl-transferase, group 1*
AFE2083	17.900	*glycosyl-transferase, group 1*
AFE1763	–15.400	*glycosyl-transferase, group 1*
AFE1357	125.000	*glycosyl-transferase, group 1*
AFE0615	13.200	*glycosyl-transferase, group 1*
AFE1354	199.000	*glycosyl-transferase, group 1 family protein*
AFE1427	0	*glycosyl-transferase, group 1 family protein*
AFE1425	1.350	*glycosyl-transferase, group 1 family protein*
AFE1355	61.400	*glycosyl-transferase, group 1 family protein*
AFE1351	0	*glycosyl-transferase, group 1 family protein*
AFE1349	0	*glycosyl-transferase, group 1 family protein*
AFE0230	0	*glycosyl-transferase, group 1 family protein*
AFE0792	18.100	*glycosyl-transferase, group 2*
AFE2121	10.500	*glycosyl-transferase, group 2 family protein*
AFE1851	1.100	*glycosyl-transferase, group 2 family protein*
AFE1345	22.600	*glycosyl-transferase, group 2 family protein*
AFE0266	6.020	*glycosyl-transferase, group 2 family protein*
AFE0233	145.000	*glycosyl-transferase, group 2 family protein*
AFE0228	2.330	*glycosyl-transferase, group 2 family protein*
AFE0078	256.000	*glycosyl-transferase, group 2 family protein*
AFE0616	12.700	*glycosyl-transferase, group 1*
AFE0079	130.000	*glycosyl-transferase, putative*
		tight adherence protein
AFE2702	50.000	tight adherence protein TadA
AFE2701	1.510	tight adherence protein TadB, putative
AFE2700	4.800	tight adherence protein TadC
		outer membrane protein
AFE1453	92.400	outer membrane protein, OmpH family
AFE1452	23.400	outer membrane protein, OMP85 family

TABLE 2. Genes related to i) transport and binding proteins and ii) central intermediate metabolism, significantly regulated by acid stress

Locus_Tag	Ratio (1 hr)	Description
	transport and binding proteins	
	Na^+/H^+ antiporter	
AFE2454	2.640	$Na^{\pm}/H^{\pm}$ antiporter
AFE2205	0	*nhaA*, Na^+/H^+ antiporter NhaA
AFE2245	2.700	Na^+/H^+ antiporter, putative
AFE0791	12.100	Na^+/H^+ antiporter, putative
	potassium	
AFE1968	–31.100	potassium-efflux system protein
AFE1753	–1.410	*kup*, potassium uptake protein
	ammonium transporter	
AFE2916	–6.540	*amt-1*, ammonium transporter
AFE2911	–1.760	*amt-2*, ammonium transporter
	others	
AFE3260	203.000	*mscL*, large conductance mechanosensitive channel protein
AFE0818	23.000	plasma-membrane proton-efflux P-type ATPase
	central intermediate metabolism	
	nitrogen fixation	
AFE1538	40.300	nitrogen fixation protein, putative
AFE1536	27.400	NifZ domain protein
AFE1535	97.200	NifZ domain protein
AFE1527	26.500	*nifA*, Nif-specific regulatory protein
AFE1524	0	NAD(+)—dinitrogen-reductase ADP-D-ribosyltransferase
AFE1522	19.600	*nifH*, nitrogenase iron protein
AFE1521	1.460	*nifD*, nitrogenase molybdenum-iron protein, alpha subunit
AFE1520	44.700	*nifK*, nitrogenase molybdenum-iron protein, beta subunit
AFE1515	16.300	*nifX*, nitrogen fixation protein NifX
AFE1511	17.100	nitrogen fixation protein NifQ
AFE1508	28.300	nitrogen fixation protein nifU
AFE1504	0	nifW protein, putative
AFE1503	0	nitroreductase, putative
AFE1480	0	*fixA*, electron transfer flavoprotein, beta subunit
AFE1479	0	*fixB*, electron transfer flavoprotein, alpha subunit
AFE1477	0	*fixX*, ferredoxin-like protein
	polyamine biosynthesis	
AFE1471	12.9	*speA*, arginine decarboxylase
AFE0157	6	s-adenosylmethionine decarboxylase proenzyme
AFE0156	55.8	*speE*, spermidine synthase
	others	
AFE0287	160	carbonic anhydrase

domain mediated growth factor signals and controlled "H^+-sensing" (Wakabayashi *et al.*, 1992). In this study, DNA microarray results indicated that the gene encoding Na^+/H^+ antiporter (*NhaA*/AFE2205: 0 fold) was not significantly modulated under the acid stress, but putative *Na^+/H^+ antiporters* (AFE2454; AFE2245; AFE0791) were induced (Table 2). Besides, acid stress did cause significant increases in *plasma-membrane proton-efflux P-type ATPase* (AFE0818, Table 2) that its product functioned in evicting protons (Axelsen & Palmgren, 1998).

Genes related to metabolism

The majority of the genes related to nitrogen fixation showed to be up-regulated (10/16, induced/detected, Table 2). Also, several genes related to cytoplasm pH buffering were significantly upregulated by acid stress (Table 2); these include *carbonic anhydrase* (AFE0287) and polyamine biosynthesis (*speA*, *arginine decarboxylase*/AFE1471; *s-adenosylmethionine decarboxylase proenzyme*/AFE0157; *speE*, *spermidine synthase*/AFE0156). Carbonic anhydrase plays an important part in buffering the periplasm under acidic pH (Pastorekova *et al.*, 2006; Wen *et al.*, 2007). The products of polyamine biosynthesis were alkaline, which is reported to stabilize membranes by associating with negatively charged phospholipids (Velikova *et al.*, 2000). Besides, arginine decarboxylase is an elemental component for the acid resistance system (Lin *et al.*, 1996) in *E.coli*.

The majority of genes related to amino acid biosynthesis were repressed upon acid stress. On the contrary, few up-regulated genes drew our attention (Table 3 and Fig. 4), such as *glutamine synthetase* (type I, *glnA*/AFE0466), *glutamate-ammonia-ligase adenylyltransferase* (*glnE*/AFE0924), *glutamate synthase* (large subunit, *glt B*/AFE0729), *glutamate synthase* (small subunit, *glt D*/AFE0730), *asparagine synthase* (glutamine-hydrolyzing, AFE1353). Among the above, glutamate synthase and glutamine synthetase (type I) convert other substances to glutamate along with consuming ammonia, and the induced asparagine synthase (glutamine-hydrolyzing, AFE1353) functions by converting asparage to glutamate.

Genes of *A. ferrooxidans* ATCC 23270 involved in energy metabolism were significantly down regulated under the acid stress. The majority of ATP synthase F0F1 subunits (Table 3) were down-regulated, which is responsible for the synthesis of ATP using the energy stored in the transmembrane proton and potential gradients. The experimental results (Fig. 5) revealed that all the subunits of ATP synthase F0F1 were down-regulated or unaffected when the duration of acid stress treatment reached 30 min. However, alpha subunit (*atpA*/AFE3205) and epsilon subunit (AFE2423, putative) of ATP synthase F1 were induced as the duration extended to 60 min, while other subunits were still repressed or unaffected under

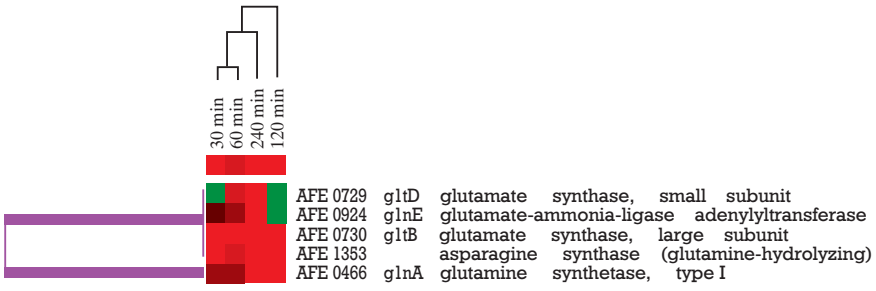

FIG. 4. Expression of the genes related to amino acid biosynthesis exhibiting altered mRNA levels upon acid stress. Columns represent time points. Red and green indicate genes that are induced and repressed, respectively.

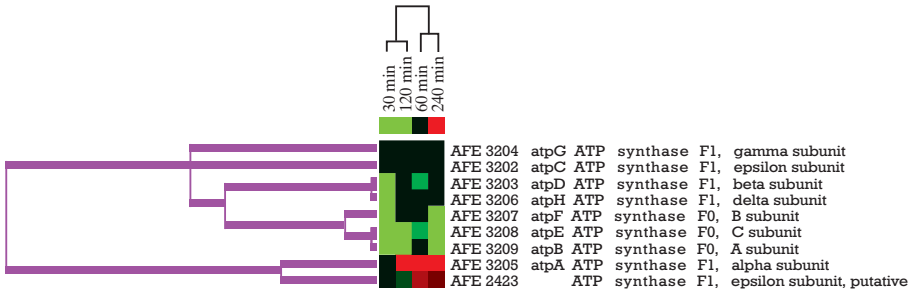

FIG. 5. Expression of the genes of the ATP synthase F0F1 exhibiting altered mRNA levels upon acid stress. Columns represent time points. Red and green indicate genes that are induced and repressed, respectively.

TABLE 3. Genes related to i) amino acid biosynthesis, ii) energy metabolism, and iii) transcription and regulatory functions, significantly regulated by acid stress

Locus_Tag	Ratio (1 hr)	Description
	amino acid biosynthesis	
AFE0730	28.800	*gltB*, glutamate synthase, large subunit
AFE0729	17.800	*gltD*, glutamate synthase, small subunit
AFE0924	9.830	*glnE*, glutamate-ammonia-ligase adenylyltransferase
AFE0466	8.430	*glnA*, glutamine synthetase, type I
AFE1353	16.100	asparagine synthase, glutamine-hydrolyzing
	energy metabolism	
	ATP-proton motive force interconversion	
AFE3202	0	*atpC*, ATP synthase F1, epsilon subunit
AFE3203	–12.500	*atpD*, ATP synthase F1, beta subunit
AFE3204	0	*atpG*, ATP synthase F1, gamma subunit
AFE3205	22.400	*atpA*, ATP synthase F1, alpha subunit
AFE3206	0	*atpH*, ATP synthase F1, delta subunit
AFE3207	0	*atpF*, ATP synthase F0, B subunit
AFE3208	–14.400	*atpE*, ATP synthase F0, C subunit
AFE3209	0	*atpB*, ATP synthase F0, A subunit
AFE2423	14.600	ATP synthase F1, epsilon subunit, putative
	Ferredoxin	
AFE0014	174.000	ferredoxin
AFE0984	12.700	ferredoxin
AFE1512	14.500	ferredoxin
AFE1519	28.400	ferredoxin
AFE1530	0	ferredoxin
AFE1844	2.460	ferredoxin
AFE3238	13.600	ferredoxin, putative
AFE1518	18.100	ferredoxin, putative
AFE1541	37.900	ferredoxin, putative
AFE1953	0	ferredoxin, putative
AFE1656	1.290	ferredoxin/oxidoreductase, FAD/NAD-binding
	transcription and regulatory functions	
AFE0470	56.90	*rpoS*, RNA polymerase sigma-38 factor
AFE1658	196.000	transcriptional regulator, Crp/Fnr family
AFE0857	14.700	transcriptional regulator, Crp/Fnr family
AFE0270	61.700	transcriptional regulator, Crp/Fnr family
AFE0119	9.430	transcriptional regulator, Crp/Fnr family
AFE1467	11.700	transcriptional regulator, Fur family
AFE2607	2.140	transcriptional regulator, MerR family
AFE2509	14.300	transcriptional regulator, MerR family
AFE1431	9.820	transcriptional regulator, MerR family

acid stress. Besides, the majority of genes encoding *ferredoxin* (AFE1512, AFE1519, AFE0984, AFE0014, AFE1844, AFE1541, AFE1518, AFE3238 and AFE1656) were up-regulated upon the acid stress, as well as genes of certain subunits of the proton-transporting NADH-quinone oxidoreductase (Table 3).

Transcription and regulatory functions

Acid stress, like chemical stimuli, induces many signaling pathways to modulate stress resistance systems. The information provided by the microarray method demonstrated that the majority of transcription factors were up-regulated by acid stress, of which *RNA polymerase sigma-38 factor* (*rpoS*/AFE0470) is reported to be an essential component for initializing certain transcription of numerous σ^s-dependent genes, which is considered to be inducible under stress conditions and has been proved to act upon more than 100 promoters (Eisenstark *et al.*, 1996) involved in stress resistance (Jorgensen *et al.*, 1999) (Table 3). Besides, the up-regulation of *rpoS* is consistent with acid stress response of other bacteria, such as *E. coli* (Hengge-Aronis, 2002) and *P. aeruginosa* (Jorgensen *et al.*, 1999), suggesting that *A. ferrooxidans* ATCC 23270 adopted active defense under acid stress.

Some genes related to regulatory functions were induced based on the microarray results, and these include some transcription regulators (Table 3): *Crp/Fnr family transcription regulators* (AFE1658, AFE0857, AFE0270, AFE0119), *Fur family transcription regulator* (AFE1467), and *MerR family transcription regulators* (AFE2607, AFE2509, AFE1431).

DISCUSSION

The method of microarray provided a powerful and efficient way to monitor the expression level of thousands of genes simultaneously within a single reaction and enabled a systematic analysis of cellular gene programming responses to experimental conditions. The quantitative results of microarray allow the deduction of information on relevant cellular functions for the pursuit of in-depth studies on selected genes. Information on gene expression profile of *A. ferrooxidans* ATCC 23270 obtained from microarray studies can further direct our attention to previously unknown or ignored genes, thus enabling us to perform further analysis to decipher their potential function.

Various hypotheses have been proposed to explain how acidophiles are able to survive and thrive in such extreme acid conditions. Baker-Austin & Dopson (2007) explained the role of acidophiles pH homoeostasis system plays in maintaining the cytoplasm pH under low external pH conditions. The acid stressed *A. ferrooxidans* ATCC 23270 performed some of the mechanisms previously reported (Baker-Austin & Dopson, 2007), however, some other strate-

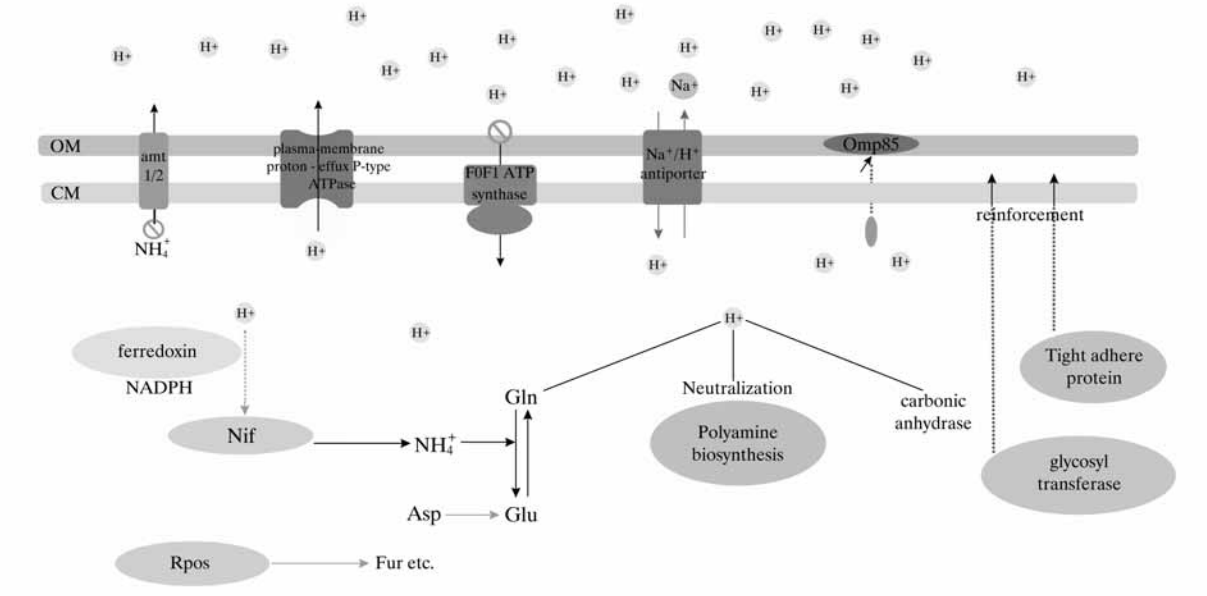

FIG. 6. Hypothetical metabolic mechanisms performed by *A. ferrooxidans* ATCC 23270 under acid stress for 1 hr.

gies exist as well (Fig. 6). Based on the experimental results, *A. ferrooxidans* ATCC 23270 reacted to acid stress mainly in the following aspects.

Both arginine-dependent system and glutamate-dependent system, the characterized acid resistance systems in *E.coli* (Lin *et al.*, 1996), were not complete in *A. ferrooxidans* ATCC 23270. However, the up-regulated glutamine synthetase (type I, *glnA*/AFE0466), glutamate-ammonia-ligase adenylyltransferase (*glnE*/AFE0924), glutamate synthase (large subunit, *glt B*/AFE0729) and glutamate synthase (small subunit, *glt D*/AFE0730) convert other substances to glutamate while consuming ammonia (Fig. 6), leading to glutamate accumulation and lowered dissociated ammonia concentration. Meanwhile, the induced asparagine synthase (glutamine-hydrolyzing, AFE1353) converts asparage to glutamate. The role accumulated glutamate performed could be more than pH buffering concerning the fact that *E. coli* taking the advantage of glutamate decarboxylase-dependent system (GDS) to survive in over-acid environment (Tucker *et al.*, 2002), and the consumed ammonia could derive from nitrogen metabolism since nitrogenase (*nifH*, *nifD*, *nifK*) was induced under acid stress (Fig. 6). By converting nitrogen to ammonia, nitrogenase could help to consume the extra cytoplasm protons as the NADPH that it used to fix nitrogen derived from the proton gradient indirectly. Considering the fact that ammonia transporters (*amt1*/AFE2916; *amt2*/AFE2911) seemed to shut down upon the stress, nitrogenase (*nifH*, *nifD*, *nifK*) and glutamine synthetase (type I, *glnA*/AFE0466) participate in transforming ammonia cycle, conducting acid stress resistance mechanisms (Fig. 6).

The ion transporters were actively regulated by acid stress (Fig. 6). Potassium-efflux system protein (AFE1968) and potassium uptake protein (AFE1753) were down-regulated, preventing potassium from depletion to maintain membrane potential. Meanwhile, the large conductance mechanosensitive channel protein (mscL/AFE3260) which related to K^+ sensitivity in *E. coli* (Cui & Adler, 1996) was induced suggesting that *A. ferrooxidans* ATCC 23270 tended to keep the membrane potential in certain range by selectively inducing or repressing the transporters under acid stress. Significantly, two putative Na^+/H^+ antiporters (AFE2245; AFE0791) were induced upon acid stress, which are reported to be important for Na^+ efflux and salt tolerance in the fungus *S. pombe* (Jia *et al.*, 1992). Thus, *A. ferrooxidans* ATCC 23270 under acid stress tended to expel Na^+ and keep K^+. Importantly, some transporters sited in the membrane acted actively to counteract the decrease of pH brought by induced Na^+/H^+ antiporters; the highly induced plasma-membrane proton-efflux P-type ATPase (AFE0818) functioned in evicting protons (Axelsen & Palmgren, 1998), which was crucial for keeping intercellular pH consistent.

The acid stress could be harmful to growth according to our findings considering the down-regulation of a number of genes related to energy metabolism, including certain subunits of ATP synthase F0F1 (Fig. 6). It is reported that *atpD* is up-regulated at alkaline pH in *Corynebacterium glutamicum* ATCC 13032 (Barriuso-Iglesias *et al.*, 2008). Among subunits of ATP synthase, down-regulation of *atpD* under acid stress in *A. ferrooxidans* ATCC 23270 was in accordance with this research effort. Up-regulation of ATP synthase F0F1 appeared to be the primary mechanism for removing protons in Gram-positive heterotrophic streptococci at acidic pH (Belli & Marquis, 1991). On the contrary, the repression of ATP synthase F0F1 held down the decline of intracellular pH in *A. ferrooxidans* ATCC 23270, possibly owning to the fact that *A. ferrooxidans* ATCC 23270 is Gram-negative autotrophic acidophile.

Based on the method of microarray, the study revealed that the transcript profiles of *A. ferrooxidans* ATCC 23270 changed significantly under acid stress, and the acid stress responses performed by chemoautotrophic *A. ferrooxidans* ATCC 23270 involved mainly the following aspects. Proton consuming and buffering reactions performed significantly to decrease the intercellular proton concentration, through up-regulation of glutamate synthase (*glnB*, *glnD*), glutamine synthetase (*glnA*, type I), glutamate-ammonia-ligase adenylyltransferase (*glnE*), arginine decarboxylase (*speA*), spermidine synthase (*speE*) and s-adenosylmethionine decarboxylase proenzyme under acid stress in *A. ferrooxidans* ATCC 23270, while the typical heterotrophic bacterium *E. coli* is reported to depend on glutamate and arginine decarboxylase systems to resist acid stress. Moreover, plasma-membrane proton-efflux P-type ATPase (AFE0818) was induced, while ATP synthase F0F1 was repressed, in order to keep the cytoplasm proton concentration in certain range. Based on the above results, this study revealed future research directions and the clues to a better understanding of acid stress response in acidophiles.

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