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Regulation of CO_2 Fixation Gene Expression in *Acidithiobacillus ferrooxidans* ATCC 23270 by Lix984n Shock

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***Acidithiobacillus ferrooxidans* ATCC 23270 is an important model organism for bioleaching and bioremediation studies owing to its diverse metabolic capabilities, whereas lix984n is a widely used extractant. Little is known about the response of *cbb* genes in *A. ferrooxidans* to lix984n shock. Thus, to elucidate the response of the CO_2 fixation genes in *A. ferrooxidans* ATCC 23270 to the addition of lix984n, the gene expression of *cbb* genes was examined using a real-time PCR. Although a natural increase or decrease in the expression of most *cbb* genes was observed after 5 min of shock with 3% (v/v) lix984n, *sdhC* and *cbbR* exhibited quick responses to the shock. Ten min of shock had a greater effect on the *cbb* gene expression, yet 15 min of shock had a significant effect on the Calvin cycle in *A. ferrooxidans* ATCC 23270, as the expression of all the *cbb* genes reached a very high level. Therefore, after a short lix984n shock, a solution of *A. ferrooxidans* can be re-used for bioleaching.**

Keywords: *A. ferrooxidans* ATCC 23270, *cbb* genes, lix984n, real-time PCR

For several decades, bioleaching has been applied on an industrial scale to extractive metallurgy for low-grade ores (*i.e.*, containing gold, copper, manganese, nickel, or uranium at a concentration of less than 0.5 wt%), owing to its environmentally friendly nature, low cost, and flexibility as regards the raw material composition [3, 5, 30, 35, 29].

For copper extraction, the industrial application of biological metallurgy has already been performed in and outside China [22]. For example, *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*) found in acidic mine drainage can solubilize metals from ores, and is used as a valuable tool by the biomining industry worldwide [47].

A. ferrooxidans is a well-known acidophilic, chemolithoautotrophic, and Gram-negative bacterium involved in bioleaching and acid mine drainage. Under aerobic conditions, it gains energy mainly from the oxidation of ferrous iron, whereas CO_2 fixation occurs *via* the Calvin-Benson-Bassham reductive pentose phosphate cycle (Calvin cycle). Since the genes and biochemical reactions of the Calvin cycle are highly conserved between organisms, this has facilitated their discovery and prediction, respectively, in novel organisms both by DNA and experimentation. Early studies have already determined the rate of iron oxidation and CO_2 fixation in *A. ferrooxidans* [37, 41]. Moreover, several enzymes of the Calvin cycle have been detected, including the key enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) [8], which is composed of eight large and eight small subunits (L_8S_8, *cbbL* and *cbbS* genes, respectively) and catalyzes the formation of two molecules of 3-phosphoglyceric acid (PGA) from ribulose bisphosphate and CO_2 [1]. Whereas CbbL fulfills a catalytic function, the role of CbbS is not yet completely understood [24]. Although the small subunits possess no catalytic activity, their presence considerably increases the activity of the enzyme, likely due to the stabilization of the hexadecameric form and conformational shifts in the active center of the enzyme [38]. There are two sets of *cbbLS* genes in *A. ferrooxidans* ATCC 23270, and codon usage would seem to indicate that a lateral gene transfer mechanism gave rise to these two sets of genes, due to the catalytic inefficiency of RuBisCO [18]. CbbR plays an important role in the expression of the Calvin cycle enzyme [12, 27, 36], whereas CbbQ is thought to play an important role in the post-translational regulation of RuBisCO, as the coexpression of *cbbQ* with *cbbLS* in *Escherichia coli* affects the conformational state and activity of the RuBisCO of *H. thermoluteolus* [14, 15, 17]. The *cbbP* gene, which has been found as a different operon in *H. thermoluteolus* [42], has rarely been reported in *A. ferrooxidans*. The functions of the above genes are summarized in Table 1, obtained from The Institute for Genomic Research Web site at http://www.tigr.org.

Copper extraction from a bioleaching solution of low-grade copper ore has been successfully commercialized for

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Table 1. *Acidithiobacillus ferrooxidans* ATCC 23270 role report: Energy metabolism-chemoautotrophy.

Locus	Gene symbol	Function	Other Roles
AFE_0057	*cbbQ-1*	CbbQ protein	(None)
AFE_0058	*cbbS-1*	Ribulose bisphosphate carboxylase, small subunit	(None)
AFE_0059	*cbbL-1*	Ribulose bisphosphate carboxylase, large subunit	(None)
AFE_0552	*sdhC*	Succinate dehydrogenase/fumarate reductase, C subunit	Energy metabolism: TCA cycle:
AFE_0941	*cbbQ-2*	CbbQ protein	(None)
AFE_0942	*cbbL2*	Ribulose bisphosphate carboxylase, large subunit 2	(None)
AFE_1393	*cbbR*	Rubisco operon transcriptional regulator	Regulatory functions: DNA interactions:
AFE_1394	*cbbL-2*	Ribulose bisphosphate carboxylase, large subunit	(None)
AFE_1395	*cbbS-2*	Ribulose bisphosphate carboxylase, small subunit	(None)
AFE_2502	*cbbP*	Phosphoribulokinase	(None)

decades, owing to the availability of copper-selective extractants [2]. Most commercially available copper-specific extractants are oxime types. Among them, the LIX series from Henkel Corporation have been extensively used [20, 49]. Generally, lix984 has a copper extraction rate above 97%, with an extraction concentration of 3% and extraction time of 3 min [20, 21]. However, studies on the cellular response of bioleaching bacteria to extractant shock are very rare. Accordingly, since *A. ferrooxidans* has already been extensively studied as a bioleaching microorganism, this study investigated the regulation of CO_2 fixation gene expression in *A. ferrooxidans* ATCC 23270 in response to lix984n shock at a transcriptional level using a real-time PCR. This study also illustrates ways in which these different systems can be exploited to further current knowledge of this important catalyst and its regulation of CO_2 fixation. Finally, the results can also contribute to a better understanding of the properties of bioleaching and bioremediation, with the ultimate purpose of developing an appropriate method to facilitate the optimization of bioleaching strategies.

Materials and Methods

Bacterial Strains and Growth Conditions

A. ferrooxidans ATCC 23270 was grown at 30°C under oxic conditions (170 rpm) in an ATCC medium 2039 that contained 0.4 g of K_2HPO_4, 20 g of $FeSO_4 \cdot 7H_2O$, 2.0 g of $MgSO_4 \cdot 7H_2O$, 0.8 g of $(NH_4)_2SO_4$, and 5.0 ml of Wolfe's Mineral Solution per liter. The Wolfe's Mineral Solution was composed of the following (g/l) components: 1.5 nitrilotriacetic acid; 3.0 $MgSO_4 \cdot 7H_2O$; 0.5 $MnSO_4 \cdot H_2O$; 1.0 NaCl; 0.1 $FeSO_4 \cdot 7H_2O$; 0.1 $CoCl_2 \cdot 6H_2O$; 0.1 $CaCl_2$; 0.1 $ZnSO_4 \cdot 7H_2O$; 0.01 $CuSO_4 \cdot 5H_2O$; 0.01 $AlK(SO_4)_2 \cdot 12H_2O$; 0.01 H_3BO_3; and 0.01 $NaMoO_4 \cdot 2H_2O$. The medium was adjusted to pH 2.3 with H_2SO_4 before the addition of $FeSO_4 \cdot 7H_2O$ (20.0 g/l), and then filter sterilized.

Sample Collection

Earlier studies indicated that *A. ferrooxidans* ATCC 23270 was able to grow aerobically in an ATCC medium 2039 in the presence of 3% (v/v) lix984n, yet showed severe growth inhibition at levels above that concentration (Fig. 1). Thus, preliminary experiments to determine the proper lix984n shock conditions were performed for 3, 5, 10, and 15 min with a 3% (v/v) lix984n concentration. Samples were removed from cultures grown with a 0% and 3% (v/v) concentration for 5, 10, 15 min, and then centrifuged for 10 min at 12,000 rpm at 4°C using a 5804R centrifuge (Eppendorf, Wesbury, NY, U.S.A.). The culture supernatant was removed instantly and the cell pellets immediately processed for RNA extraction.

Total RNA Extraction, Purification, and cDNA Generation

The TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) was used to extract the total cellular RNA, which was then treated with RNase-free DNase I (Qiagen, Valencia, CA, U.S.A.) to digest any residual chromosomal DNA and subsequently purified using a Qiagen RNeasy Mini kit according to the manufacturer's instructions. The total cellular RNA was quantified at OD_{260} [23] and OD_{280} using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.), and the purified RNA from each sample was used as a template to generate cDNA.

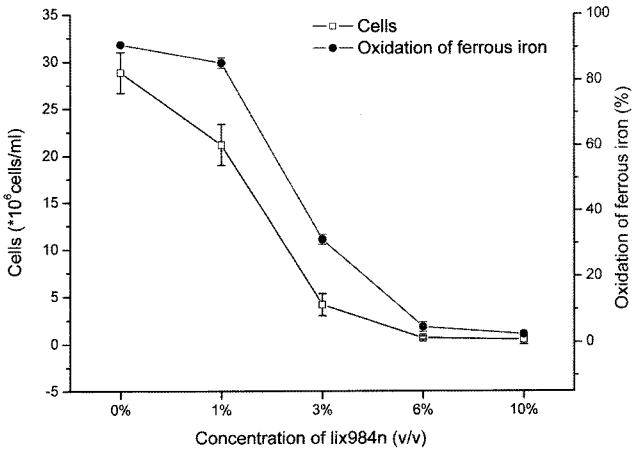

Fig. 1. Dose-response growth curve showing inhibitive effect of lix984n on *A. ferrooxidans* ATCC 23270.
The effects were determined in an ATCC medium 2039 containing different lix984n concentrations under aerobic growth conditions at 30°C based on measuring the cells and oxidation of ferrous iron in triplicate after about 48 h. The graph shows the mean±standard error (bars) for three independent dose-response curves for *A. ferrooxidans* ATCC 23270.

Table 2. Primer pairs used for real-time PCR.

Gene symbol	Sequence		Size of product (bp)
	Forward primer (5'–3')	Reverse primer (5'–3')	
cbbQ-1	AGCAGTCCACCAAGCAGC	CCTTCGCCTCAACACCCT	231
cbbS-1	AACGCCTTCGGCAACTA	GTACCCTGGGACTGCTTG	158
cbbL-1	GGTCGCTTCCGGTGGTAT	GCCTTGCCTTCCTTCTCG	201
sdhC	TTGTCGGATTCGGATACTTC	TCGGGAATGCCTACCTT	233
cbbQ-2	TCTCAAGCAATCCACCAAGC	TCATCCAGACCGTGACCCT	171
cbbL2	TCCCGACGCCGATAAGTT	GAATACCGGACGCACCCT	206
cbbR	CCTCCTGCTCTGTCCATT	TGCCCTTCTCCAGTCCTTG	190
cbbL-2	GGATTTCACCAAGGACGAT	GAACTCTGCCCGCTCAT	178
cbbS-2	ATGGCTGACATTCAAGACTACGA	AACGGTGTCCACTGACTGCTC	216
cbbP	AACCATCAGCCATTTCGG	CGTCTTCACGCCACCAT	223
16S rDNA	AATCCAAGAAGAAGCACCG	CCACTGATGTTCCTCCAG	238

Primer Pair Design

Primer pairs were designed using Primer Premier 5 software and synthesized by Shanghai Sangon. The primer pairs for the *cbb* genes are summarized in Table 2.

The specific primers were amplified using the following cycling conditions: 30 s denaturation at 95°C, 1 min annealing at 60°C, and 1.5 min extension at 72°C, along with an initial 5 min denaturation at 95°C, and a final extension reaction at 75°C for 7 min. All the PCR products were purified using a QIAquick 96-well purification kit (Qiagen, Valencia, CA, U.S.A.).

Real-Time PCR

The *cbb* gene products amplified by the PCR using genomic DNA as the template were used to construct standard curves. The reactions were performed with 40 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C and monitored using an iCycler iO Real-time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.). The standard curves were derived from the PCR products representing each *cbb* gene with genomic DNA as the template, and used to convert threshold crossings to log copy numbers. The expression of each gene was determined from three replicates of a single real-time PCR experiment. The expression ratio was recorded as the fold difference in quantity of the real-time PCR product from the treated samples versus the control concentration. The relative abundance of each gene versus the constitutively expressed gene (16S rDNA) was also determined.

RESULTS

Physiological Effect of lix984n on Aerobic Growth of *A. ferrooxidans* ATCC 23270

Direct cell counting in a Neubauer chamber [48] and Standard Methods [12] were used to assess the physiological effect of various lix984n concentrations on the aerobic growth of *A. ferrooxidans* ATCC 23270. In three independent dose-response experiments, *A. ferrooxidans* ATCC 23270 exhibited the same cell viability during the first 30 min with 0%, 1%, 3%, and 6% (v/v) lix984n concentrations. In the presence of a 1% (v/v) lix984n concentration, *A. ferrooxidans* ATCC 23270 was able to grow; however, its growth became highly inhibited above this concentration (Fig. 1). Thus, with a 3% (v/v) lix984n concentration, *A. ferrooxidans* ATCC 23270 only grew slowly, whereas it showed no viability with 6% and 10% (v/v) lix984n concentrations (Fig. 1). Therefore, a 3% lix984n concentration (v/v) was selected for a time-series gene expression analysis in response to a short lix984n shock, since this dose did not affect cell growth during the first 30 min, yet produced a high inhibition after 48 h, plus the dose is also the optimal extraction concentration for copper extraction [20, 21].

Testing of Primer Pairs

The quality of the amplified products was checked by 1.5% agarose gel electrophoresis and ethidium bromide staining. The amplified DNA fragments were considered correct if the PCRs contained a single product of the expected size (Fig. 2).

Expression of *cbb* Genes of *A. ferrooxidans* ATCC 23270 Under lix984n Shock

To gain a general understanding of the cellular response of *A. ferrooxidans* ATCC 23270 to lix984n shock, the *cbb* genes expression ratios were obtained using a real-time PCR (Table 3).

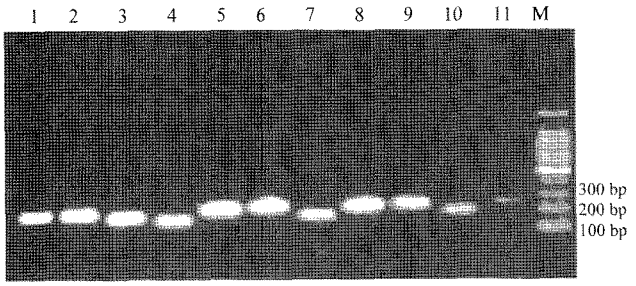

Fig. 2. Electrophoresis analysis of primer pairs.
Lanes: lane 1, *cbbL2*; lane 2, *cbbL*-1; lane 3, *cbbL*-2; lane 4, *cbbS*-1; lane 5, *cbbS*-2; lane 6, *cbbQ*-1; lane 7, *cbbQ*-2; lane 8, *cbbP*; lane 9, *sdhC*; lane 10, *cbbR*; lane 11, 16S rDNA; lane M, DNA marker.

Table 3. Expression ratio of *cbb* genes of *A. ferrooxidans* ATCC 23270 with 3% (v/v) lix984n shock for 5, 10, and 15 min.

Locus	Gene	Expression ratio		
		5 min	10 min	15 min
AFE_0057	*cbbQ-1*	1.11	2.76	2.44
AFE_0058	*cbbS-1*	1.32	3.52	10.30
AFE_0059	*cbbL-1*	0.63	1.40	13.90
AFE_0552	*sdhC*	4.42	8.89	39.70
AFE_0941	*cbbQ-2*	0.99	13.60	16.60
AFE_0942	*cbbL2*	0.79	1.42	17.60
AFE_1393	*cbbR*	2.64	12.40	5.82
AFE_1394	*cbbL-2*	0.60	9.84	4.47
AFE_1395	*cbbS-2*	0.63	3.61	7.92
AFE_2502	*cbbP*	1.53	1.15	81.30

After 5 min of lix984n shock, the *cbb* gene expression by *A. ferrooxidans* ATCC 23270 was not significantly changed, except for *sdhC* and *cbbR*, suggesting a natural increase or decrease in the expression of these *cbb* genes under unfavorable growth conditions. Thus, 5 min of lix984n shock produced little effect on most *cbb* genes, although *sdhC* and *cbbR* exhibited a quick response.

After 10 min of lix984n shock, most *cbb* genes were upregulated 2.5- to 14-fold, except for *cbbL-1*, *cbbL2*, and *cbbP*, suggesting that the expression of *cbbR* after 5 min of shock may have affected the other *cbb* genes, yet had little influence on *cbbL-1*, *cbbL2*, and *cbbP*. Thus, the slightly longer shock seemed to have an effect on the Calvin cycle.

After 15 min of lix984n shock, all the *cbb* genes showed a high level of induction, especially *cbbP*, which was upregulated by above 80-fold. Thus, 15 min of lix984n shock clearly had a significant effect on the Calvin cycle in *A. ferrooxidans* ATCC 23270.

Discussion

Regulation of *cbb* Genes of *A. ferrooxidans* ATCC 23270 Under lix984n Shock

To identify the relationship of the *cbb* genes, they were classified into three groups according to their sequence alignment (Fig. 3).

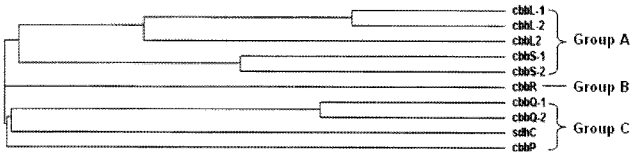

Fig. 3. Alignment of *cbb* gene sequences.
This was performed online using a CLUSTAL W (1.83) multiple sequence alignment at http://www.ebi.ac.uk/Tools/clustalw.

Group A contained the genes encoding the subunits of RuBisCO: *cbbL-1*, *cbbL-2*, *cbbL2*, *cbbS-1*, and *cbbS-2*, which are located together in the bacterial genome (*cbbS* downstream of *cbbL*), separated by 50–200 base pairs, and cotranscripted [13, 40]. Similar to other bacterial strains carrying more than one copy of form I RuBisCO genes [12, 16, 27, 28, 32, 36, 44, 45], *A. ferrooxidans* ATCC 23270 has two copies of *cbbLS*. The *cbbLS-1* genes are transcripted in the same direction (Table 3), where *cbbL-1* is 1,422 bp in length and encodes a 473-amino-acid peptide, and *cbbS-1* is 357 bp in length and encodes a 118-amino acid peptide. The *cbbLS-2* genes are also transcripted in the same direction, where *cbbL-2* is 1,422 bp in length and encodes a 473-amino-acid peptide, and *cbbS-2* is 333 bp in length and encodes a 110-amino-acid peptide. The deduced amino acid sequences for the two CbbL and CbbS polypeptides exhibit a 77.6% and 57.8% identity, respectively. The other large subunit of RuBisCO in *A. ferrooxidans* ATCC 23270 is CbbL2, where the *cbbL2* gene, which is 1,380 bp in length and encodes a 459-amino-acid peptide, is located away from the two sets of *cbbLS* genes and transcripted in the same direction as *cbbLS-1*, yet in the divergent direction from *cbbLS-2*. The deduced amino acid sequence for *cbbL2* exhibits a 32.5% and 33.1% identity with CbbL-1 and CbbL-2, respectively. Consequently, since the results showed that the *cbbLS-1* gene expression ratios for 5, 10, and 15 min of lix984n shock were more similar than the *cbbLS*-2 gene expression ratios (Table 3), this confirmed the cotranscription of *cbbLS-1* in *A. ferrooxidans* ATCC 23270. When *A. ferrooxidans* ATCC 23270 was shocked for 5 min, the *cbbLS* genes showed little difference in expression. When it was shocked for 10 min, *cbbS-1*, *cbbS-2*, and *cbbL-2* showed a high expression compared with *cbbL-1* and *cbbL2*. When it was shocked for 15 min, all the *cbbLS* genes were upregulated more than 4-fold. Therefore, whereas a short shock with 3% lix984n had little effect on the *cbbLS* genes, a 15 min shock produced high activity by all the *cbbLS* genes. Another interesting finding was that the transcription of the *cbbLS* genes affected their downstream gene expression, especially *cbbQ-1*.

Group B consisted of a single copy gene, *cbbR*, which is 999 bp long and possibly translates into a 332-amino-acid protein. It is located upstream in a divergent direction to *cbbLS-2* with a 72-bp intergenic sequence. CbbR has already been identified, and in some cases has been shown to control *cbb* expression in autotrophic organisms that employ the Calvin cycle pathway, including *C. vinosum* [45], *R. rubrum* [7], *X. flavus* [31], *R. eutropha* (*A. eutrophus*) [46], and *N. vulgaris* [39]. The deduced *A. ferrooxidans* ATCC 23270 CbbR protein shares a high level of sequence identity with *C. vinosum* RbcR (45% identity), *A. eutrophus* CfxR (38% identity), and *X. flavus* ORFD (36% identity) (Fig. 4), plus it has already been reported that *C. vinosum* RbcR [44] and *A. eutrophus* CfxR [31] belong to the LysR

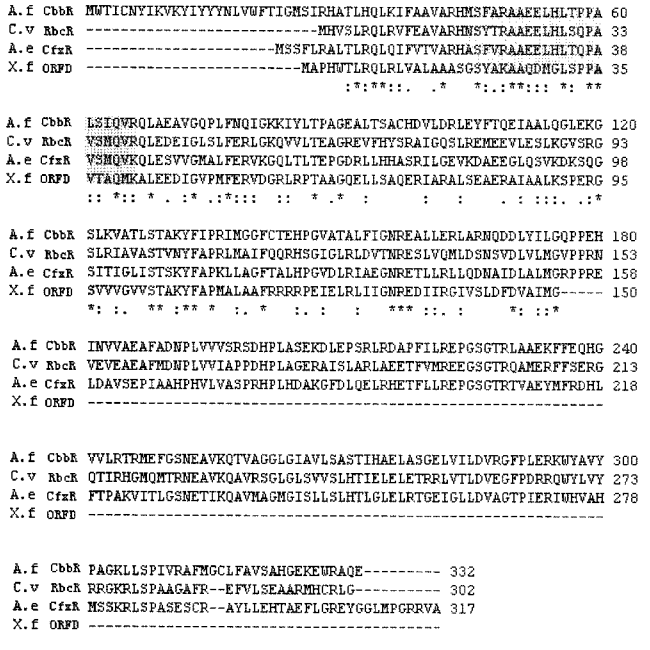

Fig. 4. Comparison of deduced amino acid sequences for putative regulatory proteins of *cbb* (*rbc* or *cfx*) operons in *A. ferrooxidans* ATCC 23270 (*A.f*) (TIGR Web site), *C. vinosum* (*C.v*) [46], *A. eutrophus* (*A.e*) [29], and *X. flavus* (*X.f*) [34].
The putative helix-turn-helix DNA-binding motif is indicated by the shaded box.

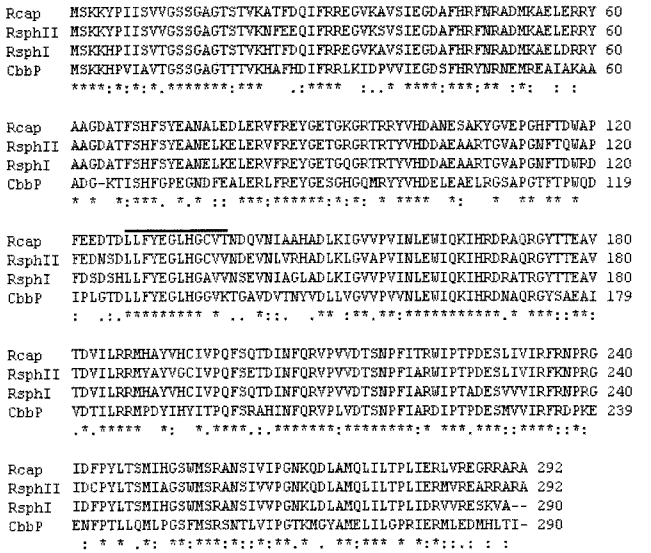

Fig. 5. Comparison and alignment of deduced amino acid sequences with sequences of known proteins.
The *A. ferrooxidans* ATCC 23270 PRK amino acid sequence (CbbP) is aligned with the amino acid sequences for the *R. capsulatus* PRK (*Rcap*) [9] and *R. sphaeroides* PRK I [11] and PRK II [4] (*RsphI* and *Rsp*II). The putative ATP binding domain is indicated by the shaded box, and the pyridine nucleotide binding site is indicated by the bar.

family of bacterial transcription regulatory proteins [19]. All the members of this family include a putative DNA-binding domain with a helix-turn-helix motif. In this study, the expression of *cbbR* in *A. ferrooxidans* ATCC 23270 increased immediately after 5 min of lix984n shock (Table 3), exhibited a maximal fold change after 10 min of shock, and then decreased in induction after 15 min of shock, which was also consistent with the change in expression of *cbbL-2* located just downstream of *cbbR*. Therefore, *cbbR* may have a direct control over *cbbL-2* in *A. ferrooxidans* ATCC 23270.

Group C was composed of the genes that have special functions in the Calvin cycle, including *cbbQ-1*, *cbbQ-2*, *cbbP*, and *sdhC*. Two sets of *cbbQ* encode the CbbQ protein, which is thought to play an important role in the posttranslational regulation of RuBisCO, as the coexpression of *cbbQ* with *cbbLS* in *Escherichia coli* affects the conformational state and activity of the RuBisCO of *H. thermoluteolus* [14, 15, 17]. In this study, *cbbQ-1* expression was induced with time, yet only to a slight degree, whereas *cbbQ-2* expression was upregulated to a high degree after 10 min of shock. Therefore, *cbbQ-2* was more active than *cbbQ-1* in *A. ferrooxidans* ATCC 23270 in response to lix984n shock. The *cbbP* gene is located downstream in a divergent direction to *cbbLS-2* and encodes a 290-amino-acid protein, PRK, which is one of the key enzymes unique to the Calvin cycle. PRK has been suggested to be the target enzyme for *in vivo* control of the rate of CO_2 fixation [6]. The present investigation strongly indicated a potential regulatory role of a metabolite generated by the PRK function (either ADP, RuBP, or a derivative thereof). Presumably, this compound may act as a specific effector in mediating the ability of CbbRI and CbbRII to influence *cbb* expression in SBI/II. *A. ferrooxidans* ATCC 23270 PRK is highly similar to *R. capsulatus* PRK (58% identity) [9], *R. sphaeroides* PRK I (58% identity) [10], and PRK II (56% identity) [4] (Fig. 5). The domains involved in ATP [25, 26] and pyridine nucleotide [4, 9] binding are indicated in Fig. 5. In this study, the expression of *cbbP* suddenly increased to a high level after 15 min of shock, indicating that *cbbP* may play an important role in the Calvin cycle in *A. ferrooxidans* ATCC 23270 after 15 min of lix984n shock. The *sdhC* gene is located independently of the *cbb* genes and encodes a 303-amino-acid protein. SdhC is the C subunit of succinate dehydrogenase/fumarate reductase in *Acidithiobacillus ferrooxidans* ATCC 23270, known as a regulator in the Calvin cycle, and also involved in the TCA cycle. However, little is known about the putative electron acceptor subunit in *Acidithiobacillus ferrooxidans* ATCC 23270. In this study, the expression of *sdhC* increased slightly after 5 min of shock, and exhibited higher expression levels after 10 min and 15 min of shock. Therefore, *sdhC* was upregulated in *Acidithiobacillus ferrooxidans* ATCC 23270 over time under lix984n shock.

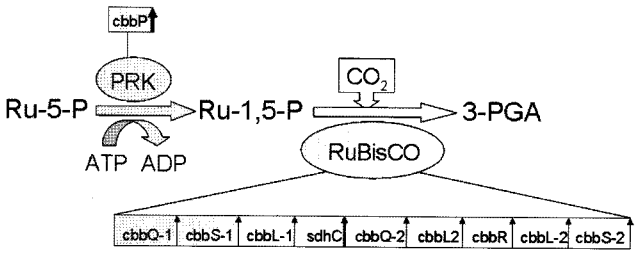

Fig. 6. Part of the Calvin cycle involving *cbb* genes.
Metabolites: 3-PGA, 3-phosphoglycerate; PRK, phosphoribulokinase; Ru-1,5-P, ribulose-1,5-bisphosphate; Ru-5-P, ribulose-5-phosphate; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase. Upward arrows denote significant upregulation in expression.

Metabolic Regulation of Calvin Cycle Pathway in *A. ferrooxidans* ATCC 23270 by lix984n Shock

Insight on the metabolic factors that may control the function of the Calvin cycle is limited because of the complexity of the system, since the Calvin cycle involves up to 13 enzymes acting on 16 metabolites in a complex network of reactions, plus it is also dependent on a ready source of ATP and reducing equivalents, as well as the removal of metabolites to be used in further biosynthetic pathways involved in cell growth [34]. However, this study provided a general view of the regulation of the *cbb* genes in *A. ferrooxidans* ATCC 23270 in response to lix984n shock (Fig. 6). The enzyme responsible for the actual fixation of CO_2, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), catalyzes the carboxylation of ribulose-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate. Meanwhile, the other enzymes involved in the Calvin cycle are dedicated to the regeneration of RuBP. The final step in the regeneration of RuBP is catalyzed by the unique Calvin cycle enzyme phosphoribulokinase (PRK), which phosphorylates ribulose-5-phosphate at the expense of ATP. Under 15 min of shock, all of the *cbb* gene expression was upregulated.

To know more about the *cbb* genes, the BDGP Web site (http://www.fruitfly.org/seq_tools/promoter.html) was used to search for potential upstream promoters of the *cbb* genes, and Table 4 shows that some *cbb* genes were found to share one promoter, plus the expression of the *cbb* genes with one promoter was very similar (Table 3).

Based on a comparison of these promoters, no similar region was identified. Thus, the overall regulation of the CO_2 fixation genes in *A. ferrooxidans* ATCC 23270 would appear to be quite complex and remains to be resolved.

Acknowledgments

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Table 4. Promoter sequence analyzed at BDGP.

Gene symbol	Promoter sequence (5'–3')
cbbQ-1	GATTTTAAGCCCCTATGAAAAGAGCCCTGTACAATACCAAAAAATCCCGG
cbbS-1	
cbbL-1	
sdhC	ATTTGCCTTGCTGTTCATACTTTTCCTGATAGAGTCTTGCAGGTAGTTTT
cbbQ-2	AATATTTGTGCCATCATGATCCGATCCGGTAACAGCCTTTTTCTGTACAG
cbbL2	
cbbR	CCTGGTTGTGGCGTAATTTTGAAACAGGCTAAGATATCAGAGTCCAAAGG
cbbL-2	GGTTGCATGACGGATAGACATGCCAATAGTAAACCATACTAAATTATAAT
cbbS-2	
cbbP	GACTTGTGGTAAATGTATCTAAACCGGGCTATGATCATAACAGGTTTTAT

ribulose 1,5-bisphosphate carboxylase/oxygenase deletion strain of *Rhodospirillum rubrum*. *J. Bacteriol.* **175:** 5066–5077.
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