Microarray and bioinformatic analyses suggest models for carbon metabolism in the autotroph *Acidithiobacillus ferrooxidans*

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Abstract

Acidithiobacillus ferrooxidans is a chemolithoautotrophic bacterium that uses iron or sulfur as an energy and electron source. Bioinformatic analysis of the *A. ferrooxidans* draft genome sequence was used to identify putative genes and potential metabolic pathways involved in CO_2 fixation, 2P-glycolate detoxification, carboxysome formation and glycogen utilization. Microarray transcript profiling was carried out to compare the relative expression of the predicted genes of these pathways when the microorganism was grown in the presence of iron versus sulfur. Several gene expression patterns were confirmed by real-time PCR. Genes for each of the above-predicted pathways were found to be organized into discrete clusters. Clusters exhibited differential gene expression depending on the presence of iron or sulfur in the medium. Concordance of gene expression within each cluster suggested that they are operons. Most notably, clusters of genes predicted to be involved in CO_2 fixation, carboxysome formation, 2P-glycolate detoxification and glycogen biosynthesis were upregulated in sulfur medium, whereas genes involved in glycogen utilization were preferentially expressed in iron medium. These results can be explained in terms of models of gene regulation that suggest how *A. ferrooxidans* can adjust its central carbon management to respond to changes in its environment.

Keywords: Calvin cycle; Glycogen; Glycolate; Carboxysome; Genome of *Acidithiobacillus ferrooxidans*

1. Introduction

The acidophilic bacterium *Acidithiobacillus ferrooxidans* is an obligate chemolithoautotroph. It fixes CO_2 using energy and reducing power derived from the oxidation of iron or sulfur. CO_2 fixation in both chemolithoautotrophs and photoautotrophs occurs via the

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Calvin-Benson-Bassham reductive pentose phosphate cycle (Calvin cycle). The genes and biochemical reactions of the Calvin cycle are highly conserved between organisms facilitating their discovery and prediction in novel organisms both by DNA sequence analysis and by experimentation.

Early studies showed a relationship between the rate of iron and sulfur oxidation and the rate of CO_2 fixation in *A. ferrooxidans* [1,2]. Several enzymes of the Calvin cycle have been detected in *A. ferrooxidans* including the key enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) [3]. RuBisCO catalyzes the formation of two molecules of 3-phosphoglyceric acid (PGA) from ribulose bisphosphate and CO_2. A plant-type hexadecameric form of the enzyme, consisting of eight copies each of the small (CbbS) and large (CbbL) subunits, has been purified from *A. ferrooxidans*. This enzyme was shown to have K_m values for CO_2 and ribulose bisphosphate that are similar to those of RuBisCO from plants and green algae but are four- to fivefold lower than those typical of bacteria [4].

Two structurally distinct forms of RuBisCO (I and II), with different catalytic properties, are present in autotrophs [5]. Form I is composed of both large (catalytic) and small subunits in a hexadecameric structure, and form II is composed exclusively of multiples of the large subunit. It has been suggested that form I provides the cell with fixed carbon whereas form II functions primarily as a terminal electron acceptor, assisting in the maintenance of the redox balance of the cell [6]. In *A. ferrooxidans* (strain Fe1), two sets of identical genes, originally termed *rbcLS1* and *rbcLS2* but more correctly known as *cbbLS1* and *cbbLS2*, encoding the large and small subunits of form I RuBisCO have been cloned and characterized [7–9]. Co-transcription of the large and small subunit genes was shown to occur when *A. ferrooxidans* was grown on iron, but sequence identity between the two copies does not allow to distinguish between their expression. Located upstream of *cbbLS1*, and divergently transcribed from this gene cluster, is a well conserved LysR-type transcriptional regulator gene (*cbbR*) known to bind specifically to overlapping promoter elements in the intergenic sequence between *cbbR* and *cbbL1*. In addition, the presence of a putative CbbR binding site upstream of the second set of the *cbb* genes (*cbbLS2*) suggests that both RuBisCO form I gene clusters are under the control of the CbbR regulator [9].

Many CO_2-fixing microorganisms, including chemolithoautotrophic bacteria and cyanobacteria, contain polyhedral inclusion bodies known as carboxysomes [10]. These structures were originally isolated from *Halothiobacillus neapolitanus* (previously *Thiobacillus neapolitanus*) [11] and found to contain substantial amounts of RuBisCO type I [12]. In addition to RuBisCO, up to seven additional polypeptides are associated with the carboxysome; five of these have been identified as shell proteins [13]. These polypeptides are encoded by genes within the *cso* gene cluster, an apparent operon that also includes *cbbL* and *cbbS* RuBisCO subunits in several *Thiobacillus* species [14] and in cyanobacteria [15]. Additionally, low levels of carbonic anhydrase (CA) appear to co-purify with the particles from *Synechococcus* [16] and *Synechocystis* [17]. Recently, the carboxysomal shell protein, CsoS3 from *H. neapolitanus*, was shown to constitute a novel evolutionary lineage of CAs (ε class) [18]. The location of the carboxysomal CA in the shell suggests that it could supply the active sites of RuBisCO in the carboxysome with the high concentrations of CO_2 necessary for optimal RuBisCO activity.

In the obligate, chemolithoautotroph *T. neapolitanus* carboxysome synthesis appears to be regulated by the concentration of CO_2 in the growth medium, with large quantities appearing under CO_2-limiting conditions [19]. In the facultative organism *T. intermedius*, the observed number of carboxysomes under mixotrophic growth conditions varied in approximate proportion to the specific activity of RuBisCO, suggesting that the synthesis of these structures is under metabolic control [20]. Whether the regulation of carboxysome formation is linked to the control of RuBisCO synthesis is yet to be determined.

A deleterious side product of the Calvin cycle is 2-phosphoglycolate (2P-glycolate) which is typically metabolized to innocuous compounds in CO_2 fixing organisms. No information regarding this pathway has been published for *A. ferrooxidans*. Information regarding other aspects of CO_2 fixation in *A. ferrooxidans* is also lacking. For example, nothing is known regarding the reduction of Calvin cycle intermediates, nor how ribulose 1,5-bisphosphate (RuBP) is regenerated. In addition, there is a lack of information as to how carbon derived from the Calvin cycle is channelled into subsequent pathways for intermediate metabolism. Challenged by these deficiencies, we undertook a bioinformatic analysis of potential pathways involved in CO_2 fixation, carboxysome formation, 2P-glycolate detoxification and in the synthesis and utilization of glycogen in *A. ferrooxidans*. In addition, microarray transcript profiling of genes in these pathways was carried out in order to advance our understanding of

how these pathways might be regulated when cells are grown in either iron or sulfur.

2. Materials and methods

Bioinformatic analysis of candidate genes, from the TIGR draft genome, and metabolic reconstruction of *A. ferrooxidans* ATCC 23270 (type strain) were carried out as previously described [21]. Microarray transcript profiling was carried out as described in the accompanying paper [22]. Sequences deposited in GenBank: *cbbR*[a]: B49698; *cbbL1*[b]: RKBCLT, S18315; *cbbS1*[c]: B41323, S18316.

3. Results and discussion

Putative genes were identified in the genome of *A. ferrooxidans* predicted to be involved in the Calvin cycle, 2P-glycolate detoxification, the formation of carboxysomes and glycogen metabolism (Table 1). Several of these candidate genes potentially encode proteins that exhibit conserved motifs and predicted folds characteristic of the proposed function (Table 1). The relative level of expression of these genes in cells grown in either iron (Fe) or sulfur (S) medium was evaluated by microarray analysis and confirmed in several cases by real-time PCR (Table 1).

3.1. Calvin cycle

Two enzymes that are unique to the Calvin cycle are ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and phosphoribulokinase (PRK). RuBisCO catalyzes the first step of the Calvin cycle, the carboxylation of ribulose 1,5-bisphosphate (RuBP) with CO_2. Phosphoribulokinase (PRK) catalyzes the last step of the cycle, which is the regeneration of the CO_2 acceptor molecule, RuBP, via the phosphorylation of ribulose 5-phosphate with ATP. All other steps of the Calvin cycle are catalyzed by enzymes common to other pathways of intermediary metabolism. Briefly, these steps fulfill (i) the reduction of 3-phosphoglyceric acid, (ii) the formation of fructose-6-phosphate and (iii) the regeneration of the CO_2 acceptor RuBP. For convenience, the reactions of the Calvin cycle can be grouped into four main events: CO_2 uptake, CO_2 fixation, intermediate reduction and regeneration of the CO_2 acceptor molecule. We have identified candidate genes in *A. ferrooxidans* for each of these functions (Table 1). The key genes and reactions are depicted in the metabolic scheme presented in Fig. 1. Also included in Fig. 1 is an indication of whether the expression of each gene is enhanced when cells are grown in media containing iron versus sulfur.

The first step of carbon fixation is carboxylation of the acceptor molecule, ribulose-1,5-bisphosphate (RuBP), by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO; EC 4.1.1.39). Since the substrate for RuBisCO is CO_2, a carbonic anhydrase (CA; EC 4.2.1.1) that catalyzes the reversible hydration of CO_2 is needed to ensure rapid conversion of cytosolic HCO_3^- to CO_2 at concentrations that support optimal RuBisCO activity. HCO_3^- is accumulated in the cytosol by the operation of a number of active CO_2 and HCO_3^- transporters.

3.2. 2P-Glycolate detoxification

RuBisCO is a bifunctional carboxylase/oxygenase able to utilize both CO_2 and O_2 as substrates depending on their availability. While the carboxylase reaction initiates CO_2 fixation, the oxygenase reaction initiates the C2 oxidative carbon cycle that results in the poisonous intermediary, 2-phosphoglycolate (2P-glycolate) [23]. Unless this product is dephosphorylated by a 2-phosphoglycolate phosphatase (PGP; EC 3.1.3.18) to yield glycolate, the accumulation of the compound as a dead-end metabolite results in the inhibition of triose-phosphate isomerase [24]. Further breakdown of glycolate by a peroxisomal glycolate oxidase (GOX; EC 1.1.3.15) allows carbon from 2-phosphoglycolate to be recycled into the Calvin cycle.

Candidate genes predicted to encode enzymes that participate in the detoxification of 2P-glycolate were detected in the genome of *A. ferrooxidans* (Table 1) and a scheme illustrating the possible carbon-salvaging 2P-glycolate of this organism is shown in Fig. 1. All the genes in this pathway were upregulated in cells grown in sulfur medium.

3.3. Carboxysome formation

Obligate chemolithotrophic sulfur-oxidizing bacteria, nitrifying bacteria and cyanobacteria, have a polyhedral protein microcompartment that contains RuBisCO together with a carboxysomal carbonic anhydrase (CA). CA converts an accumulated cytosolic pool of HCO_3 into CO_2 within the carboxysome, elevating the CO_2 around the active site of RuBisCO. Carboxysomes are not present in facultative autotrophs, despite the fact that when these organisms grow as photoautotrophs they use the Calvin cycle to fix CO_2. Although the detailed biochemical mechanism by

Table 1
Candidate genes of *A. ferrooxidans* suggested to be involved in carbon metabolism and the $\log_2$ relative level of expression in cells grown in iron (Fe) or sulfur (S) medium

Gene	EC No.	Assigned function	Conserved domains	Gene expression ($\log_2$) in S or Fe	
Calvin cycle					
cbbO		RuBisCO activator		S*	2.7
cbbQ		RuBisCO activator		S	0.9
cbbR[a]		RuBisCO transcriptional regulator		Fe	0.9
fba1	4.1.2.13	Fructose-1,6-bisphosphate aldolase	COG1830, PD008351	Similar	0.0
fba2	4.1.2.13	Fructose bisphosphate aldolase	pfam01116, COG0191	S	0.5
fba3	4.1.2.13	Fructose-bisphosphate aldolase	pfam01116, COG0191	S	1.7
fba4	4.1.2.13	Fructose-bisphosphate aldolase		S	0.8
g6pl	5.3.1.9	Glucose-6-phosphate isomerase		Fe	0.4
gapA	1.2.1.12	Glyceraldehyde-3-P dehydrogenase	pfam02800, COG0057	Similar	0.1
glpx1	3.1.3.11	Fructose-1,6-bisphosphatase	pfam02800, COG0057	Fe	0.4
glpx2	3.1.3.11	Fructose-1,6-bisphosphatase	pfam00316, COG0158	S	0.4
pgk	2.7.2.3	3-Phosphoglycerate kinase	PD000619	S	0.4
prkB	2.7.1.19	Phosphoribulokinase	pfam00485, COG3954	S	0.7
rpe	5.1.3.1	Ribulose-phosphate 3-epimerase	pfam00834, COG0036	Fe	0.5
rpiA	5.3.1.6	Ribose 5-P isomerase A	pfam06026, COG0120	S	0.7
tkt1	2.2.1.1	Transketolase 1	pfam00456, COG3959	Fe	1.1
tkt2	2.2.1.1	Transketolase 2		S	0.4
tpiA	5.3.1.1	Triosephosphate isomerase	pfam00121, COG0149	Similar	0.0
xfp	4.1.2.9	Phosphoketolase	pfam03894, COG3957	S	2.6
zwf	1.1.1.49	Glucose-6-P dehydrogenase	pfam02781, TIGR00871	Fe	1.4
cbbL1[b]	4.1.1.39	RuBisCO (large subunit)		S	0.8
cbbS1[c]	4.1.1.39	RuBisCO (small subunit)		S	2.4
cbbL2	4.1.1.39	RuBisCO (large subunit)		S	0.7
cbbS2	4.1.1.39	RuBisCO (small subunit)		S	0.4
cbbL3	4.1.1.39	RuBisCO (large subunit)		S	0.5
cbbM	4.1.1.39	RuBisCO (large subunit), form II		Similar	0.1
Carboxysome formation					
csoS2		Carboxysome shell		S	2.2
csoS3		Carboxysome shell, carbonic anhydrase		S	1.3
pepA		Carboxysome shell		S	0.8
pepB		Carboxysome shell		S	1.6
csoS1A		Carboxysome shell		S	1.3
csoS1B		Carboxysome shell		S	1.6
csoS1C		Carboxysome shell		S	0.7
Glycolate detoxification					
glcD	1.1.3.15	Glycolate oxidase		S	1.0
glcE	1.1.3.15	Glycolate oxidase		S	0.6
glcF	1.1.3.15	Glycolate oxidase		S	1.5
gph1	3.1.3.18	Phosphoglycolate phosphatase		Fe	1.6
gph2	3.1.3.18	Phosphoglycolate phosphatase		S	0.7
Glycogen biosynthesis					
glgA	2.4.1.21	Glycogen synthase	COG0297, TIGR02095	S	0.6
glgB1	2.4.1.18	1,4-α-Glucan branching enzyme	pfam02922, COG0296	S*	0.8
glgB2	2.4.1.18	1,4-α-Glucan branching enzyme	pfam02922, COG0296	Similar	0.0
glgC	2.7.7.27	ADP-glucose pyrophosphorylase	pfam00483, COG0448	S	0.7
pgm1	5.4.2.2	Phosphoglucomutase	pfam02878, COG0033	Similar*	0.2
pgm2	5.4.2.2	Phosphoglucomutase	pfam02878, COG0033	Fe	0.9
Glycogen utilization					
cga-1	3.2.1.3	Glucoamylase	pfam00723, COG3387	Fe	0.6
cga-2	3.2.1.3	Glucoamylase	pfam00723, COG3387	S	1.4
hxk	2.7.1.1	Hexokinase		S	1.3

Table 1 (*continued*)

Gene	EC No.	Assigned function	Conserved domains	Gene expression (log_2) in S or Fe	
malQ	2.4.1.25	4-α-Glucanotransferase	pfam03065, COG1449	S*	1.0
glgP1	2.4.1.1	Glucan phosphorylase	pfam00343, COG0058	Fe	0.8
glgP2	2.4.1.1	Glucan phosphorylase	pfam00343, COG0058	S	0.4
Other functions					
pyk	2.7.1.40	Pyruvate kinase	pfam00224,TIGR01064	Fe	0.9
eno	4.2.1.11	2-Phosphoglycerate dehydratase	PR00148	Fe	1.0

Enzyme commission (EC) numbers and conserved domains and motifs are shown for predicted protein products where appropriate.
* Microarray data validated by real-time PCR.

which carboxysomes enhance autotrophic CO_2 fixation is not well understood, collective evidence suggests that the unique structural organization and the bounding shell of carboxysomes provide a distinct catalytic advantage for this process. Seven candidate genes potentially involved in carboxysome formation have been discovered in the genome of *A. ferrooxidans* (Table 1).

The existence of three forms of CAs, α, β and γ has been known for some time. Candidate genes for the β and γ forms have been detected in the genome *A. ferrooxidans* (data not shown). It was demonstrated recently that the carboxysomal shell protein, CsoS3, from *H. neapolitanus* is a novel form of CA [18]. This shell-localized CA is proposed to supply CO_2 to the active sites of RuBisCO in the carboxysome to support optimal CO_2 fixation activity by catalyzing the reversible hydration of CO_2. *A. ferrooxidans* has a candidate gene for this form of CA (Table 1).

All seven predicted genes for carboxysome formation are upregulated when *A. ferrooxidans* is grown in S medium (Table 1).

3.4. Glycogen metabolism

Glycogen is produced and accumulates in many bacteria where it is thought to be used as a stored source of energy and carbon [25,26]. Little is known about glycogen synthesis and breakdown in *A. ferrooxidans*.

Candidate genes and predicted enzymes and pathways for the glycogen biosynthesis and breakdown have been detected in the genome of *A. ferrooxidans* (Table 1). Metabolic reconstruction (Fig. 2) suggests that ADP-glucose provides the donor sugar nucleotide, whose synthesis is catalyzed by the enzyme glucose-1-phosphate adenylyltransferase (*glgC*; EC 2.7.7.27). The glucosyl moiety of ADP-glucose is transferred, in

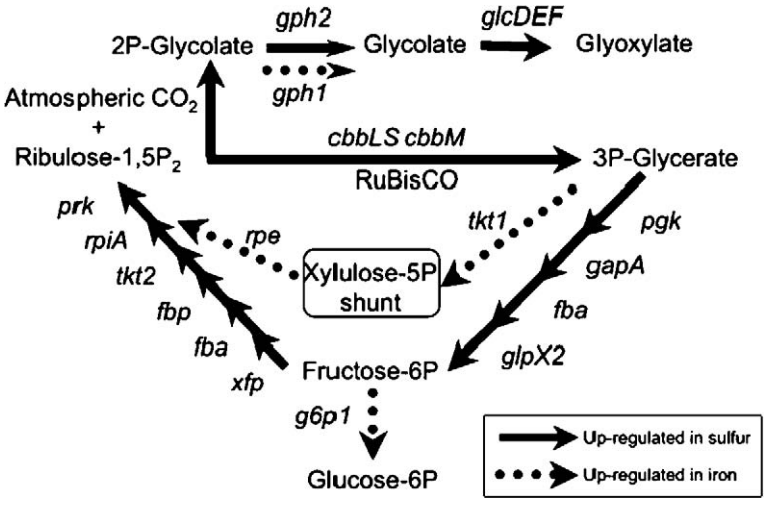

Fig. 1. Representation of the candidate genes and predicted enzymes and pathways for the Calvin cycle and for 2P-glycolate detoxification in *A. ferrooxidans*.

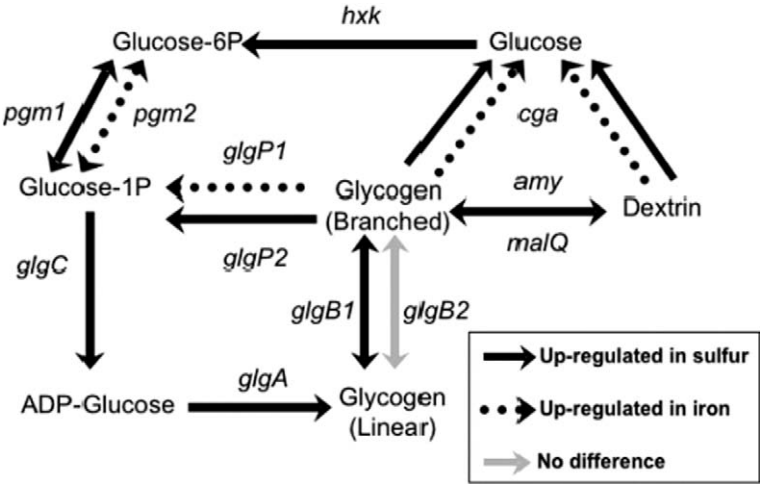

Fig. 2. Representation of the candidate genes and predicted enzymes and pathways for the biosynthesis and degradation of glycogen in *A. ferrooxidans*.

a reaction catalyzed by a specific ADP-glucose-glycogen synthetase (*glgA*; EC 2.4.1.21), to a glycogen primer to form a new α-1,4-glucosidic bond. Subsequently, a branching enzyme (*glgB1*, *glgB2*; EC 2.4.1.18) catalyzes the formation of branched α-1,6-glucosidic linkages. The release of energy and carbon stored in glycogen is initiated by the enzyme glucan phosphorylase (*glgP*, *glgP2*; EC 2.4.1.1), which releases glucose-1-phosphate from the nonreducing terminus of the α-1,4 chain [27].

3.5. Organization and expression of candidate genes involved in the Calvin cycle, carboxysome formation, 2P-glycolate detoxification and glycogen metabolism

The proposed organization of selected candidate genes predicted to be involved in the Calvin cycle, carboxysome formation, 2P-glycolate detoxification and glycogen metabolism in *A. ferrooxidans* is shown in Fig. 3. Not all genes listed in Table 1 or indicated in Figs. 1 and 2 have been included in this figure for simplicity. Superimposed on the gene clusters is the microarray expression data for cells grown in iron versus sulfur (Fig. 3). Each experiment included appropriate controls and statistical validation. In some cases, microarray data was validated by real-time PCR analysis (Table 1).

Several general observations can be made.

- Genes with related functions tend to be clustered together, for example, those encoding carboxysome formation and glycolate detoxification (clusters I and III, respectively, Fig. 3).
- Given that the putative genes are densely packed within the clusters, allowing little space for the presence of individual promoters between genes, and since each cluster contains at least some genes with a common function, it is proposed that the clusters correspond to operons. The observation that genes within individual clusters are co-expressed in either iron or sulfur supports the contention that each cluster is an individual transcription unit.
- There are several instances where genes involved in the same function are found in different clusters, for example, those involved in the Calvin cycle are distributed in at least five clusters, two of which are shown as clusters I and II in Fig. 3. Since the majority of genes involved in the Calvin cycle also carry out other metabolic functions, having multiple clusters allows their regulation to be uncoupled from the Calvin cycle when required. There are several instances of duplicate genes. For example, several genes of the Calvin cycle are duplicated including *glpX* encoding fructose-1,6-bisphosphatase 1 and 2, *cbbLS* encoding the duplicated RuBisCO form I complex, *dnhA* 1 and 2 encoding fructose-1,6-bisphosphate aldolase, and *tkt* 1 and 2 encoding transketolase. Except for RuBisCO, these genes are also required for other metabolic functions. Duplicated genes are probably controlled by different promoters and regulatory signals. This would permit differential expression of the two gene copies in response to distinct triggering signals, extending the response capacities of the microorganism or even bypassing conditions of full repression of one of the isozyme forms. The importance of redundancy

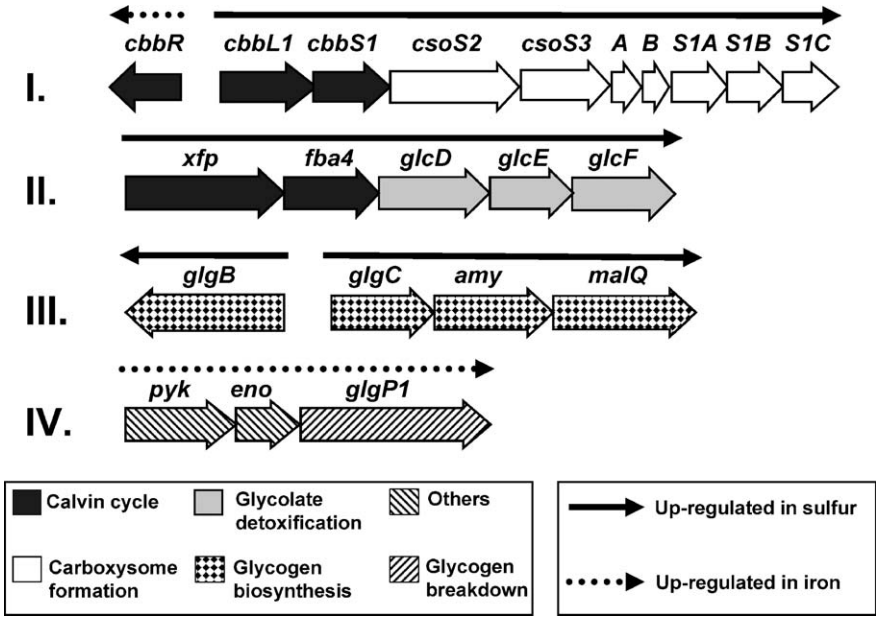

Fig. 3. Proposed genetic organization of the candidate genes of *A. ferrooxidans* involved in the Calvin cycle, glycolate detoxification, carboxysome formation and glycogen metabolism. Arrows indicate proposed transcription.

becomes obvious in the case of facultative heterotrophs where Calvin cycle genes are completely turned off in the presence of a metabolizable carbon compound [5].

- With the exception of *cbbR* which is divergently expressed, clusters I and II are upregulated in sulfur medium (Table 1 and Figs. 1 and 3). This also applies to other genes of the Calvin cycle as shown in Table 1 (but not illustrated in Fig. 3). This suggests that the Calvin cycle is more active when cells are grown in sulfur versus iron. Since a more active Calvin cycle would generate more poisonous 2P-glycolate, it is consistent that the 2P-glycolate detoxification pathway is also upregulated in sulfur medium (Table 1 and Figs. 1 and 3). These proposed models of gene organization will help focus future efforts to detect common regulatory elements in these clusters that respond to environmental signal(s) when sulfur but not iron is present in the medium.Although genes of the Calvin cycle are upregulated in sulfur medium, two genes *tkt1* and *rpe* (Table 1 and Fig. 1) are upregulated in iron medium. These genes classically represent an alternate way to regenerate ribulose-5P from glyceraldehyde-3P via xylulose-5P (xylulose-5P shunt, Fig. 1). Why they are specifically upregulated in iron medium remains to be investigated.
- Microarray data indicates that copies 1 and 2 of RuBisCO form I and their cognate post-translational modulator genes *cbbOQ* had higher ratios in cells grown in sulfur compared to iron (Table 1). In addition, real-time PCR experiments using primers specifically designed to distinguish between *cbbO* copies suggest that only the major cluster encoding CbbLS1 is upregulated in S medium and that CbbLS2 and the putative operon encoding the CbbM RuBisCO form II are expressed approximately at the same level in both iron and sulfur medium. These results are consistent with those reported for facultative autotrophs where RuBisCO form I is predominant under autotrophic growth conditions, whereas form II is expressed under all growth conditions [6]. It is speculated that form II RuBisCO functions primarily as a terminal electron acceptor, assisting in the maintenance of the redox balance of the cell whereas the function of the form I enzyme in *A. ferrooxidans* growing in sulfur is to provide the cell with fixed carbon.The observation that the major gene cluster encoding for CbbLS1 is upregulated in S medium as judged by real time PCR is in potential conflict with proteomic data that suggest that both subunits of RuBisCO encoded by the major *cbb* operon, and the modulator CbbQ (P30) are down-regulated in sulfur [28]. It is possible that expression at the level of RNA of the major cluster (*cbbI1*) is enhanced in sulfur medium but that the levels of the respective proteins increase in iron medium, suggesting that important translation regulatory mechanisms remain to be discovered.

- Genes involved in glycogen biosynthesis and the glycogen branching/debranching system tend to be upregulated in sulfur medium (Table 1 and cluster III, Fig. 3), whereas those proposed to be required for glycogen breakdown are upregulated in iron medium (Table 1 and cluster IV, Fig. 3). The other two genes, *pyk* and *eno*, associated with the glycogen breakdown gene *glgP1* in cluster IV are predicted to be involved in sugar metabolism, suggesting that this cluster is involved in the recovery of energy and carbon from glycogen. Theoretically, sulfur should yield more energy than iron. Therefore, perhaps, it is metabolically favorable to fix CO_2 and to channel some of the fixed carbon to stored glycogen when sulfur is available as an energy source. The glycogen can be broken down later to yield carbon and energy in leaner times, for example, when only iron is available as an energy source.

Note added in proof

The complete sequence of *A. ferrooxidans* has now been released (TIGR. org).

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