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Are there multiple mechanisms of anaerobic sulfur oxidation with ferric iron in *Acidithiobacillus ferrooxidans*?

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Abstract

To clarify the pathway of anaerobic sulfur oxidation coupled with dissimilatory ferric iron reduction in *Acidithiobacillus ferrooxidans* strain CCM 4253 cells we monitored their energy metabolism gene transcript profiles. Several genes encoding electron transporters that are involved in the aerobic iron and sulfur respiration were induced during anaerobic growth of ferrous iron-grown cells. Most sulfur metabolism genes were either expressed at basal level or their expression declined. However, transcript levels of genes assumed to be responsible for the processing of elemental sulfur and other sulfur intermediates were elevated at the beginning of the growth period. In contrast, genes with predicted functions in the formation of hydrogen sulfide and sulfate were significantly repressed. The main proposed mechanism involves: the outer-membrane protein Cyc2 (assumed to function as a terminal ferric iron reductase); periplasmic electron shuttle rusticyanin; c_4-type cytochrome CycA1; inner-membrane cytochrome bc_1 complex I; and the quinone pool providing connection to the sulfur metabolism machinery, consisting of heterodisulfide reductase, thiosulfate:quinone oxidoreductase and tetrathionate hydrolase. However, an alternative mechanism seems to involve a high potential iron-sulfur protein Hip, c_4-type cytochrome CycA2 and the inner-membrane cytochrome bc_1 complex II. Our results conflict with findings regarding the type strain, indicating strain- or phenotype-dependent pathway variation.

Keywords: *Acidithiobacillus ferrooxidans*; anaerobic respiration pathway; sulfur metabolism; ferric iron reduction; real-time quantitative PCR

1. Introduction

Since various forms of iron and sulfur have played important roles in the evolution of the Earth, studies on iron- and sulfur-dependent extremophiles under anaerobic conditions might provide important insights into the primordial microbial processes that occurred on our planet. Anaerobic bacterial activity indicates that some bacteria can survive without molecular oxygen (O_2) in sulfide-rich environments where elemental sulfur (S^0) is available and can be (bio)oxidized by ferric iron (Fe^{3+}) to sulfuric acid. The process may affect such environments, and biomining possibilities, under O_2-limited or anaerobic conditions [1]. In extremely acidic environments, the ability to catalyze the dissimilatory reduction of Fe^{3+} using inorganic and organic electron donors is relatively widespread in diverse species of acidophilic prokaryotes. Nevertheless, the use of Fe^{3+} as an electron acceptor to support cell growth is generally facultative and occurs when availability of the primary electron acceptor O_2 becomes limited [2]. The microbial mechanisms of Fe^{3+} reduction under neutral conditions (where most forms of Fe^{3+} are insoluble) have been relatively well described [3]. However, there is little knowledge of mechanisms operating when the pH is below 3, even though Fe^{3+} is a more bioavailable and thermodynamically favorable electron acceptor under these conditions [4]. Mesophilic and acidophilic ferrous iron (Fe^{2+})- and S^0-oxidizing *Acidithiobacillus* spp. (*A. ferrooxidans*, *A. ferrivorans*, *A. ferridurans*) and thermo-tolerant *Acidiferrobacter thiooxydans* (formerly *Thiobacillus ferrooxidans* strain M-1) can couple dissimilatory Fe^{3+} reduction to S^0 oxidation, while *A. ferrooxidans* and *A. ferridurans* can also use hydrogen (H_2) as an electron donor. In addition, the moderate thermophiles *Sulfobacillus thermosulfidooxidans* and *S. benefaciens* grow anaerobically via Fe^{3+} reduction with H_2 as an electron donor [5].

The most widely studied chemolithotrophic acidophile *A. ferrooxidans* has been shown to anaerobically oxidize S^0 with Fe^{3+} according to the stoichiometry shown below [6].

$$8\ Fe^{3+} + 5\ Fe_2(OH)_2^{4+} + 3\ S^0 + 11\ SO_4^{2-} + 2\ H_2O \rightarrow$$

$$\rightarrow 10\ Fe^{2+} + 8\ FeSO_4^0 + 6\ HSO_4^- + 8\ H^+ \quad (1)$$

While the ability of *A. ferrooxidans* to anaerobically reduce Fe^{3+} has been known for many years [7–10], the molecular mechanism involved remains unclear. The first model of the anaerobic respiratory pathway assumed electron transport from S^0 via the bc_1 complex and periplasmic transporters of the iron-oxidizing system to the terminal acceptor Fe^{3+} [11]. The proposed mechanism was supported by inhibition studies [8] and partially by an increase in the abundance of rusticyanin (Rus) and c_4-type cytochrome Cyc1 during anaerobic incubation of resting Fe^{2+}-grown *A. ferrooxidans* CCM 4253 cells [12]. Involvement of the iron-oxidizing system in anaerobic respiration was indirectly supported by dissimilarities in kinetic traits of two *A. ferrooxidans* CCM 4253 phenotypes. Fe^{2+}-grown cells of this strain could anaerobically reduce Fe^{3+}, but not cells that had been maintained on S^0 for several generations, most likely because they lacked some parts of the iron-oxidizing system. Additionally, Fe^{2+}-grown cells lost their Fe^{3+}-reducing activity after transition and subsequent passaging on S^0 [12]. Our recent broad-range proteomic analysis of these cells lacking the Fe^{3+}-reducing capacity revealed down-regulation of energy metabolism proteins. In some cases, these proteins were even absent. Among the repressed and missing proteins, Cyc2, Rus, heterodisulfide reductase (Hdr), thiosulfate:quinone oxidoreductase (Tqo) and sulfide:quinone reductase (Sqr) were identified [13].

High levels of a *c*-type cytochrome were observed in *A. ferrooxidans* JCM 7811 grown anaerobically on S^0 or H_2 in the presence of Fe^{3+} as an electron acceptor [14]. The reduced form of this soluble acid-stable 27.4-kDa protein was re-oxidized by Fe^{3+}. Immunostaining also revealed the presence of Rus in cells anaerobically grown on H_2 with Fe^{3+} as an electron acceptor [14].

In contrast to the above observations, different expression patterns of energy metabolism genes and proteins were observed in an RNA microarray- and proteomics-based study of S^0-grown *A. ferrooxidans* ATCC 23270 (the type strain) cells grown anaerobically on S^0 as an electron donor and Fe^{3+} as an electron acceptor. These included increases in abundance under anaerobic conditions of an iron-sulfur binding subunit of sulfur reductase (SreB) and Tat, the twin-arginine translocation pathway signal sequence domain protein [15]. Tat was recently described as a tetrathionate-forming thiosulfate dehydrogenase [16]. In addition, transcript-level upregulation of *tusA* and *dsrE* encoding sulfur-relay enzymes (parts of the *hdr* operon) and *petII* operon (*petA2*, *petB2*, and *cycA2*) genes encoding the bc_1 complex II (PetA2B2C2) and cytochrome CycA2 was detected in anaerobic cells. Furthermore, anaerobic induction of the *sre* operon encoding four subunits of putative sulfur reductase (Sre) was confirmed using real-time PCR. In contrast, reductions in the abundance of Cyc2, an outer-membrane iron oxidase, and heterodisulfide reductase subunits HdrA and HdrB2, were detected in anaerobic cells of the type strain. Genes encoding heterodisulfide reductase subunit B (*hdrB2*), which is separated from the *hdr* operon, tetrathionate hydrolase (*tth*) and iron oxidation system-encoding *rus* operon (*cyc1*, *cyc2*, *coxA*, *coxC* and *rus*) were downregulated at the transcript level in the type strain under anoxic conditions. Thus, a second model of the anaerobic respiratory pathway has been suggested, which includes S^0 disproportionation, whereby hydrogen sulfide (H_2S) is formed via the action of Sre, and sulfate (SO_4^{2-}) via the actions of Hdr and ATP sulfurylase (Sat) [15]. Under this proposed mechanism, Fe^{3+} reduction is mediated, at least in part, by an indirect chemical reaction with H_2S in the acidic medium. A direct mechanism within this model, involving electron transfer from S^0 to Fe^{3+} via a respiratory chain consisting of the bc_1 complex II and c_4-type cytochrome CycA2 was also postulated. However, the terminal Fe^{3+} reductase and other possible electron carriers during anaerobic S^0 oxidation remain undiscovered [15].

As outlined above there are interesting, and poorly understood, variations in outcomes of end-point screening across various strains and proposed mechanisms [12,15]. Thus, the purpose of this study was to verify and extend previous observations by real-time analysis of energy metabolism genes that could be involved in the anaerobic pathway of S^0 oxidation coupled with dissimilatory Fe^{3+} reduction in *A. ferrooxidans* CCM 4253 cells. To meet these aims, gene transcript profiles were monitored throughout cells' growth phases. The results of this and previous studies indicate that there may be more mechanisms of the anaerobic respiratory pathway.

2. Materials and methods

2.1 Bacteria and culture conditions

A. ferrooxidans strain CCM 4253 (Czech Collection of Microorganisms) was used in this study. This strain was shown to be highly related (100% identity) to the type strain of *A. ferrooxidans* ATCC 23270 by 16S rRNA gene sequencing (EF465493)[17]. Fe^{2+}-grown cells were cultured in 9K medium [18]. For anaerobic growth, *A. ferrooxidans* CCM 4253 was cultivated in a 10 L bioreactor (Biostat B-DCU; B. Braun Biotech International) with agitation by stirring (200 rpm) at 28°C. The bioreactor was charged with a basal salts-trace element medium [14] containing 89.5 mM of Fe^{3+} in the form of filter-sterilized ferric sulfate hexahydrate and 1% (w/v) S^0 sterilized by boiling (Sulfur Extra Pure, Riedel-deHaën). Fe^{2+}-grown cells were harvested by centrifugation at 15,000 × *g* for 10 min and then inoculated into the bioreactor at a final density of around 10^8 per mL. The pH of the inoculated medium was adjusted to 1.9-2.0 with ammonia, and an Ar/CO_2 (9:1) gas mix was bubbled through the culture to eliminate dissolved O_2 and provide a source of carbon. On the basis of optimization studies, the influx speed was set to 1 L min^{-1} for 15 min on each day of cultivation. Culture samples of 300-500 mL were collected for transcript analyses at 24-h intervals over 8 days

(all the Fe^{3+} in the medium had been consumed after this period). The remaining S^0 was removed from the samples by paper filtration, and the biomass was harvested by centrifugation (15,000 × *g* for 10 min at 4°C). The resulting cell pellets were then carefully washed once with a fresh basal salts medium without added trace elements and frozen at -70°C.

For studies on the anaerobic culture growth kinetics and optimization of the cultivation conditions, 250-mL flasks with outlets for gas supply and sampling were charged with Fe^{2+}-grown cells in 200 mL of the basal salts-trace element medium containing 89.5 mM Fe^{3+} and 1% (w/v) S^0. The pH was adjusted to 1.9-2.0 and the temperature was maintained at 28°C. The cultures were agitated by stirring at 200 rpm, and an Ar/CO_2 (9:1) gas mix was bubbled through the cultures at an influx speed of 1 L min^{-1} at various frequencies.

2.2 Analytical procedures for kinetic studies

Cell densities were monitored turbidimetrically at 450 nm [19] and microscopically using a Cyrus chamber and an Olympus BX50 optical microscope. Concentrations of Fe^{2+} in cultures were determined by the o-phenanthroline method [20] and H^+ concentrations were determined with a pH electrode (Radiometer Copenhagen). O_2 levels were measured using a dissolved O_2 sensor (Mettler-Toledo) and sulfide concentrations in culture samples were quantified using a modification of an established method [21] based on a reaction with the thiol-specific derivatization agent monobromobimane, followed by reversed-phase fluorescence HPLC (Agilent Technologies). The method's limit of detection was determined from a calibration curve [22].

2.3 Transcript analysis

Gene expression was analyzed by real-time quantitative PCR (RT-qPCR) using the

fluorescent intercalating dye SYBR-Green and a Light Cycler 480 (Roche). Total RNA was isolated from two biological replicates of 300-500 mL using the TRI Reagent® (Sigma) with spin column purification by Direct-zol™ RNA MiniPrep (Zymogene Research) including in-column DNase I treatment (Thermo Scientific) according to the manufacturer's instructions. Reverse transcription and cDNA amplification by qPCR were carried out as previously described [23]. Briefly, the cDNA for each gene of interest was amplified in triplicate by qPCR using gene-specific primers (we used the available *A. ferrooxidans* ATCC 23270 genomic sequence and annotation; details are given in Table S1 in the Supplementary material) and GoTaq qPCR Master Mix (Promega) according to the manufacturer's instructions. The qPCR reaction conditions consisted of 35 cycles of DNA denaturation at 95 °C for 20 s followed by annealing and extension at 60 °C for 40 s. The transcript level of each gene was normalized using a normalization factor (defined by transcript levels of the reference genes *rrs*, *map* and *alaS*) to facilitate evaluation of gene expression in relation to an endogenous control by the $\Delta\Delta C_T$ method [24,25]. The normalized values at different time points during the culture periods were compared with those obtained at time zero (when the inoculum was added). A 0.7-fold deviation in $\log_2$ ratio was regarded as an indicator of significant differential gene expression [15], and each gene expression value was evaluated by the *t*-test, with the significance threshold set at $P < 0.05$.

3. Results and Discussion

3.1 Growth kinetics of an anaerobic A. ferrooxidans CCM 4253 culture

The optimal CO_2 supply rate for *A. ferrooxidans* under anaerobic growth conditions is not known, so in preliminary tests we grew the test strain with several supply rates. Our results indicated that a daily CO_2 supply of 0.75 L, corresponding to 5.59 ± 0.65 nL CO_2 per cell (mean ± SD), was sufficient for unlimited cell growth in the 10-L bioreactor (see

illustrative Fig. S1 in the Supplementary material). In additional experiments to identify potentially growth-inhibiting dosages cell growth was not inhibited by CO_2 doses up to forty times this level (30 L/day). Overall, we concluded that a CO_2 supply of 1.5 L every 24 h was sufficient to sustain unlimited growth.

According to the model reaction stoichiometry (1), the ratio of changes in H^+ and Fe^{2+} concentrations should be 0.444 [6]. The experimentally determined slope (see Fig. S2 in the Supplementary material), corresponding to the H^+/Fe^{2+} ratio, was 0.438 ± 0.036 (mean ± SD), insignificantly different from the model ratio ($P > 0.05$). Thus, the stoichiometry of anaerobic S^0 oxidation during cell growth corresponded to that determined for resting cells [6], indicating that the reaction mechanism was the same.

3.2 Transcript analysis of A. ferrooxidans CCM 4253 under anaerobic conditions

Real-time qPCR was used to monitor the transcript profiles of energy metabolism genes in anaerobic *A. ferrooxidans* CCM 4253 cultures that were performing dissimilatory Fe^{3+} reduction coupled with S^0 oxidation (Figs. 1 and 2). The acquired transcript profiles demonstrated that transcriptional levels of genes might vary substantially throughout the process, and thus affect results of end-point analyses, responses of translational and protein turnover machinery, and (hence) both transcript and protein profiles.

Generally, during anaerobic growth of Fe^{2+}-grown cells, transcript levels of iron metabolism-related genes (represented by the *res*, *petI*, and *rus* operons) were the same and/or higher than their basal levels in the aerobic Fe^{2+}-grown cells. Several sulfur metabolism-related genes were induced at the beginning of the anaerobic growth period, but subsequently most of them were expressed at their basal level or significantly repressed. The *petII* operon involved in electron transport during aerobic sulfur respiration showed a similar

transcript profile during anaerobic growth to the *petI* operon (Fig. 2). However, the *petI* operon was more heavily transcribed than the *petII* operon (Table 1).

3.2.1 Anaerobic respiratory pathway – Sulfur metabolism

The mechanism of sulfur metabolism involving S^0 and other reduced inorganic sulfur compounds (RISC) in *A. ferrooxidans* differs from that in most prokaryotes. The sulfur-oxidizing systems encoded by the *sox* and *sor* genes that are found in most bacteria and archaea have not been identified in the *A. ferrooxidans* genome sequence [26]. On the other hand, a cluster of six co-transcribed genes (*hdr* operon) encoding a heterodisulfide reductase complex has been found to be greatly upregulated during growth on S^0 [27,28]. In *A. ferrooxidans* under both aerobic and anaerobic conditions, Hdr has been postulated to work in reverse, using the naturally existing proton gradient to drive the oxidation of disulfide compounds – probably glutathione sulfane-sulfur (GSSH) – to sulfite (SO_3^{2-}). S^0 is poorly soluble in water and it reacts non-enzymatically with glutathione (GSH) to form GSSH. However, it is still unknown how S^0 is incorporated in to the cell. The collected electrons from GSSH oxidation are then delivered to an inner-membrane quinone pool (Q/QH_2) [15,28].

In our experiments, two genes of the *hdr* operon that were significantly overexpressed in Fe^{2+}-grown cells during anaerobic S^0 oxidation were *hdrA* and *hdrB* (Fig. 2), which encode FAD-dependent pyridine nucleotide-disulfide oxidoreductase (heterodisulfide reductase subunit A) and heterodisulfide reductase subunit B, respectively. This conflicts with previous findings that the corresponding HdrA protein declines in abundance under anaerobic conditions [12,15]. However, the apparent reduction in abundance of RISC metabolism proteins under anaerobic conditions may have occurred because the end-point analysis focused on a level of the late active S^0 oxidation phase [12,15]. We detected no significant

increase in the transcript levels in this phase (Fig. 2). Use of an aerobic S^0-grown culture (which would be expected to exhibit strong sulfur respiration activity with O_2 as an electron acceptor) in the comparison may also have contributed to the reported reduction in abundance of RISC metabolism proteins under anaerobic conditions. Despite these caveats, the overexpression of *hdrA* and *hdrB* in Fe^{2+}-grown cells during anaerobic S^0 oxidation supports the hypothesis that Hdr is involved in the anaerobic process, since the RISC metabolism genes are expressed weakly in aerobic Fe^{2+}-grown cells. However, this raises questions regarding the enzyme complex's ability to fulfill this function because, given its recognized role in two-electron reduction in anaerobes, even acting in reverse it may be unable to catalyze four-electron transfer from S^0 to SO_3^{2-}.

No significant induction of *dsrE* and *tusA* transcription was detected in any monitored anaerobic growth phase (Fig. 2) in contrast to previous reports [15], where their function in the H_2S transport associated with S^0 reduction by Sre was assumed. However, their transcript profiles in *A. ferrooxidans* CCM 4253 rather indicate functions in sulfur transport associated with S^0 oxidation by Hdr, implying their localization in the *hdr* operon. Rhodanese-like protein encoded by *p11*, and other genes (*hdrB1* and *hdrC1*) included in the *hdr* operon, were repressed after three days of anaerobic growth (Fig. 2) and are probably not involved in S^0 oxidation or transport under anaerobic conditions. On the other hand, our preliminary quantitative proteomic analysis of whole cell lysates of the culture that lost anaerobic Fe^{3+}-reducing activity during passage on S^0 indicated that it contained no pyridine nucleotide-disulfide oxidoreductase (HdrA) or iron-sulfur cluster-binding protein (HdrC1), while the preceding culture retained these proteins [13]. Proteomic data together with transcription profiles in this work strongly support involvement of Hdr in the anaerobic S^0 oxidation. Nevertheless, the role of HdrC1 in the anaerobic process remains ambiguous with respect to the current transcript-level results.

ATP sulfurylase (Sat), which is encoded by *sat* and catalyzes production of SO_4^{2-} and ATP from adenosine-5'-phosphosulfate (APS) and pyrophosphate [28], was significantly repressed at the transcript level during anaerobic growth (Fig. 2). Its role in the anaerobic mechanism of SO_4^{2-} formation may not be essential and the anaerobic SO_4^{2-}-forming mechanism still remains unclear. The depression of certain genes involved in RISC metabolism during later phases of anaerobic growth (Fig. 2) may mirror the concentration of Fe^{2+}, which gradually increases over time in cultures growing anaerobically (Fig. 1). This is indicative of regulation at the transcription level by the RegBA system, and more specifically overexpression of the *regA* gene might be responsible for the repression of some RISC metabolism genes at low redox potential [29].

After two days of anaerobic growth of Fe^{2+}-grown cells, the *p14.3*, *p21* and *tth* genes were also overexpressed (Fig. 2). The rhodanese-like protein P21, a putative thiosulfate sulfurtransferase, was upregulated more strongly in *A. ferrooxidans* ATCC 19859 cultures grown on different RISC and metal sulfides than in Fe^{2+}-grown cells [30]. The *p21* and *p14.3* genes cluster into the *tqo* operon, which encodes the Tqo complex [28]. We did not detect any increase in expression of *tat* transcripts, encoding a protein that has thiosulfate-dehydrogenase activity [16] and is reportedly abundant in anaerobic cells [15]. Expression of another gene (*tdt*) of the *tqo* operon was reduced after half of the anaerobic growth period (Fig. 2). Moreover, Tat pathway signal sequence domain protein encoded by the *tqo* operon was downregulated in the cells unable to reduce Fe^{3+} under anaerobic conditions. Two other proteins encoded by the same operon - rhodanese-like protein P21 and periplasmic solute-binding protein (Psb) have not been found in these cells [13]. Therefore, we concluded that Tqo contributes to RISC metabolism under anaerobic conditions. The low level of *sat* gene RNA transcripts (for sulfite oxidation) and increased *tqo* gene transcripts could indicate that sulfite abiotically reacts with elemental sulfur to form thiosulfate which is oxidized by Tqo.

Like HdrA, reductions in abundance of Tth in anaerobic cells have been previously reported [15], and the discrepancy may be due to increases in transcript level at the beginning of the anaerobic process (Fig. 2) and anaerobic S^0/Fe^{3+} respiration being less efficient than aerobic S^0/O_2 respiration. Tetrathionate hydrolase (Tth), encoded by *tth*, disproportionately hydrolyzes tetrathionate to thiosulfate, S^0 and SO_4^{2-} [31]. Tth was also proposed to be a candidate final electron donor to Fe^{3+} [32]. However, no direct connection of Tth to the respiratory chain has been demonstrated as yet. Therefore, Tth-mediated Fe^{3+} reduction is a relatively minor process compared to respiratory Fe^{3+} reduction. Moreover, since *tth* has a transcript profile similar to that of the other RISC metabolism genes (Fig. 2), Tth is more likely to be primarily involved in RISC metabolism than to serve as a terminal Fe^{3+} reductase.

Expression of the *sre* operon, which encodes a putative sulfur reductase, was significantly repressed during anaerobic growth (Fig. 2). Its transcript profiles suggest that little or no S^0 disproportionation to H_2S and SO_4^{2-} occurred in Fe^{2+}-grown *A. ferrooxidans* CCM 4253 cells, in contrast to previously proposed S^0 disproportionation mechanism via the action of Sre in S^0-grown *A. ferrooxidans* ATCC 23270 cells [15]. Sulfur reductase reduces S^0 with H_2 as electron donor in the presence of [NiFe] hydrogenase, with which it participates in membrane-bound multienzyme complex in the sulfur-dependent hyperthermophilic archaeon *Acidianus ambivalens* [33] and bacterium *Aquifex aeolicus* [34]. Moreover, sulfur reductase cannot be reportedly separated from the hydrogenase during purification without loss of activity [33]. Transcript levels of *hynD* and *hynS*, which encode the membrane-bound respiratory [NiFe] hydrogenase [35], were reportedly reduced under anaerobic conditions in the *A. ferrooxidans* type strain [15]. Hence, the mechanism of H_2S formation from S^0 and proposed role of the Sre in *A. ferrooxidans* under anaerobic conditions require further investigation. Our qualitative test for the presence of H_2S using lead acetate yielded negative

results. The maximum soluble sulfide concentrations determined in the cultures monitored in this study were consistently below the limit of detection (7.1 μM) and therefore taken to be zero or trace levels at most. In contrast to a previous study [15], we obtained no conclusive evidence of H_2S formation. Our results with *A. ferrooxidans* CCM 4253 did not confirm the model of the anaerobic sulfur-oxidizing system that includes S^0 disproportionation to form H_2S and SO_4^{2-}. They are more consistent with earlier observations and conclusions [8,11]. However, the reduction of S^0 cannot be completely excluded. We assume that the process will be minor in RISC metabolism of *A. ferrooxidans* CCM 4253 in the absence of H_2 as an electron donor under anaerobic conditions.

Various suggested mechanisms of anaerobic S^0 oxidation could essentially involve one of two key processes, reflecting the pathway variation across *A. ferrooxidans* strains or cellular phenotypes [12,15]. However, both mechanisms include Hdr mediating S^0 oxidation and passing electrons to the inner-membrane quinone pool, which further transfers electrons to downstream components of the Fe^{3+} respiratory chain. Tqo also contributes to the delivery of electrons to the quinone pool.

3.2.2 Anaerobic respiratory pathway – Dissimilatory Fe^{3+} reduction

The iron-oxidizing system contains several high-redox potential proteins. The proteins of the direct and reverse electron transport pathways are encoded by the *rus* and *petI* operons, respectively [36,37]. Moreover, a comparative analysis of whole genome sequences from various mesophilic acidophiles [38,39] has indicated that only *A. ferrooxidans*, *A. ferridurans*, and *A. ferrivorans* strains whose genome sequences contain the iron-oxidizing system encoded by the *rus* and *petI* operons and additional *petII* operon as a part of sulfur respiration are capable of anaerobic Fe^{3+} reduction using S^0 as an electron donor. Previous authors found that the *rus* operon was strongly expressed in aerobic Fe^{2+}-grown cells, but

weakly transcribed (with correspondingly low abundance of its translation products) in active S^0-grown cells [40]. We found that one of the *rus* operon genes, *cyc2*, was overexpressed throughout the anaerobic growth period (Fig. 2). This is interesting because *cyc2* encodes the *c*-type cytochrome Cyc2, which is located in the outer membrane and is the initial electron acceptor in Fe^{2+} oxidation. Another gene of the *rus* operon, *cyc1*, was significantly overexpressed during the first three days of anaerobic growth, and *coxBAC* genes were mildly induced, possibly as a result of the gene cluster's co-transcription (Fig. 2). Furthermore, Cyc2 has been downregulated and Rus has not been detected in cells unable to anaerobically reduce Fe^{3+} [13].

We hypothesize that the terminal Fe^{3+} reductase might be the outer-membrane cytochrome Cyc2, operating in reverse mode and reducing Fe^{3+} to Fe^{2+} under anaerobic conditions. Electrons required for this reduction are probably delivered from periplasmic Rus. Increases in protein levels of Rus and cytochrome Cyc1 have been previously observed in anaerobic resting cells [12], and Rus has been detected during anaerobic Fe^{3+} reduction with H_2 as the electron donor [14]. Rusticyanin is a stable and highly abundant protein in Fe^{2+}-grown cells, in which it represents about 5% of soluble protein [41], and will probably still be able to fulfill the role of an electron transporter in the respiratory chain, even after strong reduction of its content in a cell. Apparently, there is no need for its further overexpression at the transcript level during anaerobic growth (Fig. 2). However, upregulation of Rus at the protein level has been observed in anaerobic resting cells [12] relative to aerobic S^0-grown cells, which exhibit only transient overexpression of the *rus* operon during the early exponential phase (and even then its level is weaker than in Fe^{2+}-grown cells) [40]. A direct interaction between Rus and Cyc2 has been previously demonstrated, but no interaction has been detected between Cyc2 and Cyc1 or CycA1 [42]. This may explain the need for Rus in the anaerobic electron transport pathway. Whether or not cytochrome Cyc1 also participates

in the process is unclear since increases in its transcript and protein levels under anaerobic conditions could be due to *rus* operon co-transcription and/or the relatively slow turnover of both this and other iron respiratory proteins. On the other hand, roles of Cyc2 and Rus in the anaerobic respiratory pathway have been substantially supported by our recent study on cells that lost their Fe^{3+}-reducing activity [13] and results presented here.

The cytochrome *bc*$_1$ complex plays an important role in the anaerobic respiratory pathway connecting S^0 oxidation and dissimilatory Fe^{3+} reduction under anaerobic conditions, according to the first proposed model based on studies using respiratory chain inhibitors [8,11]. Two operons encoding different inner-membrane cytochrome *bc*$_1$ complexes have been found in the *A. ferrooxidans* ATCC 23270 genome sequence. Both *bc*$_1$ complexes consist of a *c*$_1$-type cytochrome encoded by *petC*, a *b*-type cytochrome encoded by *petB* and a Rieske protein encoded by *petA* [43]. The cytochrome *bc*$_1$ complex I (PetA1B1C1) has been shown to be activated only in Fe^{2+}-grown cells and to participate in reverse electron flow during Fe^{2+} respiration, delivering electrons to the NADH complex (NDH-1) via the quinone pool [44]. The cytochrome *bc*$_1$ complex II (PetA2B2C2), which is induced mainly in S^0-grown cells and temporarily to a lesser extent in Fe^{2+}-grown cells, is part of the sulfur respiration system, transmitting electrons from the quinone pool to the terminal oxidase via periplasmic transporters such as the high potential iron-sulfur protein (Hip) and cytochrome CycA2 [44].

In anaerobic conditions, the situation appears to be more complicated and both types of *bc*$_1$ complex seem to be present. During anaerobic oxidation of S^0 with Fe^{3+}, reduction in abundance of the SdrA2 protein has been observed in Fe^{2+}-grown *A. ferrooxidans* CCM 4253 cells [12], and increases in *petA2*, *petB2*, and *cycA2* transcript levels in S^0-grown *A. ferrooxidans* ATCC 23270 cells [15]. SdrA2, like SdrA1 may promote electron transfer from the quinone pool to the NADH complex [28]. In this study, we observed significant

overexpression of *petA1*, *petC1*, *sdrA1*, *petC2*, *petB2*, and *hip* during anaerobic growth (Fig. 2). Under anaerobic growth conditions, the *petI* operon was expressed much more strongly than the *petII* operon (Table 1), in accordance with previous observations of Fe^{2+}-grown cells under aerobic conditions [44]. Thus, the bc_1 complex I seems to play a more dominant role during anaerobic electron transport pathway than the bc_1 complex II in the Fe^{2+}-grown *A. ferrooxidans* CCM 4253 cells. In the anaerobic Fe^{3+} respiratory chain, the bc_1 complex I would operate in a direct energetically favorable exergonic mode in contrast to the reverse endergonic mode required in the aerobic Fe^{2+} respiratory chain. In anaerobic mode, H^+ translocation by the cytochrome bc_1 complex I would be directed from the cytoplasm to the periplasm, thereby generating the proton motive force required for ATPase activity.

During anaerobic growth, *resB* and *hyp* were induced (Fig. 2). The *res* operon is located next to the *petI* operon in the *A. ferrooxidans* ATCC 23270 genome sequence, and the proteins it encodes are involved in maturation of *petI* operon products [45,46]. Increased levels of the *res* operon transcripts under anaerobic conditions support involvement of the *petI* operon in anaerobic Fe^{3+} respiration. Therefore, we assume that the cytochrome bc_1 complex I is part of the main anaerobic respiratory pathway in Fe^{2+}-grown cells that accepts electrons from S^0 oxidation via the inner-membrane quinone pool and passes them to c_4-type cytochrome.

One of two possible respiratory c_4-type cytochromes, Cyc1, interacts with Rus and cupredoxin "acidophile cytochrome *c* oxidase partner" (AcoP), and also binds to an integral inner-membrane terminal oxidase in a process facilitated by physical interaction [47]. No evidence of interaction between Cyc1 and the cytochrome bc_1 complex I has been found so far. Therefore, Cyc1 probably does not participate in the electron transport between the bc_1 complex I and Rus. The other candidate, CycA1, interacts with and is reduced by Rus [47]. Since CycA1 and the cytochrome bc_1 complex I are both encoded by the *petI* operon, they are

generally assumed to interact, although no clear evidence for this interaction has been presented yet. Based on the gene transcript profiles, we assume that electrons delivered to the cytochrome bc_1 complex I from sulfur oxidation via the quinone pool are further transported to Rus (which functions as a periplasmic electron shuttle) via cytochrome CycA1 to the outer-membrane cytochrome Cyc2, which catalyzes the extracellular reduction of Fe^{3+} to Fe^{2+}.

However, our results do not exclude participation of the cytochrome bc_1 complex II in an alternative respiratory chain, as previously suggested occurs in the *A. ferrooxidans* type strain [15,44]. Two proteins encoded by the *petII* operon could mediate electron transport from cytochrome bc_1 complex II to an unknown terminal Fe^{3+} reductase. One is CycA2, which has been recently proposed to be included in the anaerobic electron transport pathway based on its transcript-level overexpression in *A. ferrooxidans* ATCC 23270 [15]. Nevertheless, its induction was not demonstrated in *A. ferrooxidans* CCM 4253 in this study (Fig. 2). The other is periplasmic Hip, but its gene expression only significantly increased ($P < 0.05$) at the end of the anaerobic culture (Fig. 2). In addition, Hip contains an O_2-labile [4Fe-4S] cluster with ideal properties for participation in anaerobic electron transport.

3.2.3 Regulation of anaerobic respiratory pathway

Four potential genes encoding members of the one-component regulator Fnr family, which is required for the switch from aerobic to anaerobic metabolism [48], have been found in the *A. ferrooxidans* ATCC 23270 genome sequence [49]. Transcript levels of *fnrACD* genes were increased after two days of anaerobic growth (Fig. 2), suggesting that they might participate in regulation of the transition between aerobic and anaerobic states. In contrast, *fnrB* was underexpressed during anaerobic growth (Fig. 2), and we obtained no confirmatory indications of its involvement in the switch. In facultative anaerobic bacteria, expression of

genes involved in the anaerobic respiratory pathway and related functions in the absence of O_2 are controlled by the reversible activation of Fnr [50]. In addition, under the same conditions Fnr represses some genes of the aerobic respiratory pathway [51]. In *A. ferrooxidans*, Fnr probably has quite modest effects on activation of genes involved in the anaerobic respiratory pathway because most of them also participate in the aerobic respiratory pathway. Transcript levels of *regA*, part of the two-component regulatory system RegBA, increased both at the beginning of anaerobic cultivation (when small quantities of Fe^{2+} were present), and at the end of the cultivation, when almost all of the Fe^{3+} had been reduced to Fe^{2+} (Figs. 1 and 2). The transcript levels of *regA* mirror the concentration of iron in the environment [23]. This system positively regulates *rus* operon expression [29] and could therefore enhance induction of respiratory genes as the local concentration of Fe^{2+} increases. Changes in transcript level of other genes in late stages of anaerobic growth with elevated concentrations of Fe^{2+} could also contribute to different results in end-point analyses.

It has been proposed that anaerobic sulfur respiration is one of the earliest mechanisms of microbial energy conservation [52]. It is possible that the anaerobic sulfur respiratory system was the original system in *A. ferrooxidans* from which the aerobic system (which uses O_2 as a much more efficient electron acceptor than Fe^{3+}) subsequently evolved. The ability to facultatively utilize energy under anaerobic conditions may have been retained since bacteria frequently exist under O_2-limited conditions.

4. Conclusion

Fast adaptation of Fe^{2+}-grown *A. ferrooxidans* CCM 4253 cells to anaerobic S^0 oxidation coupled with dissimilatory Fe^{3+} reduction caused notable induction of genes with functions in iron and sulfur metabolism and regulation of associated processes. In addition,

none of the genes encoding components of the iron-oxidizing system were repressed during anaerobic growth, and some were even induced. Clearly, relatively low-fold inductions of monitored genes may indicate that Fe^{2+}-grown cells use most of the respiratory components that are present in and used by cells under aerobic conditions to produce energy by anaerobic Fe^{3+} respiration. Apart from a few sulfur metabolism genes induced anaerobically, some of them that are probably also involved in both aerobic and anaerobic sulfur oxidation were expressed at basal levels in anaerobic cells, corresponding to levels observed in Fe^{2+}-grown cells. This suggests that the sulfur metabolism enzymes are temporarily expressed in Fe^{2+}-grown cells, but to a lesser extent than in S^0-grown cells. It might also explain the smooth (lag-free) adaptation of Fe^{2+}-grown *A. ferrooxidans* CCM 4253 cells to S^0 oxidation, whereas S^0-grown cells lacking a complete iron-oxidizing system only oxidize Fe^{2+} after a 4- to 7-day lag phase [23].

Findings of this and previous studies [8,11,12,15] suggest possible mechanisms of anaerobic S^0 oxidation coupled with dissimilatory Fe^{3+} reduction in *A. ferrooxidans* CCM 4253 (Fig. 3). The main mechanism involves the outer-membrane Cyc2 acting as a terminal Fe^{3+} reductase; rusticyanin acting as a periplasmic electron shuttle; and c_4-type cytochrome CycA1, the inner-membrane cytochrome bc_1 complex I, and the inner-membrane quinone pool providing electron transfer connections to key components of the sulfur metabolism machinery (heterodisulfide reductase, thiosulfate:quinone oxidoreductase and tetrathionate hydrolase). Additionally, an alternative mechanism involving the inner-membrane cytochrome bc_1 complex II together with c_4-type cytochrome CycA2, as previously reported [15], Hip and the unknown terminal Fe^{3+} reductase cannot be excluded. However, our conclusions differ from the model presented in the cited study on the type strain [15], which includes H_2S formation and participation of different genes. Thus, the findings indicate strain- or phenotype-dependent variations in the anaerobic respiratory pathway.

Acknowledgments

This work was supported by grant no. GP14-27075P from the Czech Science Foundation.

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Legends to figures

Fig. 1. Growth kinetics of an anaerobic *A. ferrooxidans* CCM 4253 culture during oxidation of S^0 with Fe^{3+}. The culture was generated using Fe^{2+}-grown cells as an inoculum, with an initial density of 10^8 cells per mL. Cell numbers are indicated by open squares, Fe^{2+} formation by filled triangles, and pH by open circles. The error bars represent means ± SD (n = 2).

Fig. 2. Transcriptional profiles of energy metabolism genes during anaerobic oxidation of S^0 with Fe^{3+} in growing *A. ferrooxidans* CCM 4253 cells. Gene transcripts were monitored in the culture in Fig. 1. Predicted functions and names of the proteins which are encoded by genes monitored are described in the text and summarized in Table S1 in the Supplementary material. The color scale shows the logarithm of the relative expression ($\log_2$ ratio). All expression values are listed in Table S2 in the Supplementary material.

Fig. 3. Model of anaerobic oxidation of S^0 coupled with dissimilatory Fe^{3+} reduction in *A. ferrooxidans* CCM 4253. The electron transport pathways from S^0 to Fe^{3+} are based on findings in this and previously published, corroborative studies [9-16]. Electrons derived from S^0 oxidation catalyzed by heterodisulfide reductase (Hdr) and other RISC metabolism processes mediated by tetrathionate hydrolase (Tth) and thiosulfate:quinone oxidoreductase (Tqo) are collected by the quinone pool (Q/QH_2). The cytochrome bc_1 complex I (PetA1B1C1) probably passes most of the electrons to the c_4-type cytochrome (CycA1) that may interact with rusticyanin (Rus) and transfer the electrons to the outer membrane *c*-type cytochrome (Cyc2) where Fe^{3+} is reduced outside the cells. Alternatively, the cytochrome bc_1 complex II (PetA2B2C2) may pass the electrons to the c_4-type cytochrome (CycA2), which might interact with the high potential iron-sulfur protein (Hip) and transfer the electrons to an unknown outer membrane Fe^{3+} reductase. Some of the electrons cross the quinone pool to the

NADH complex (NDH-1) and reduce NAD^+. ATP synthase phosphorylates ADP using the proton motive force generated by the electron transport chain. Proteins encoded by genes of the same operon are shown in the same color. Dashed arrows indicate electron transfer and solid arrows indicate H^+ transport. Black circles represent the copper centre, circles within bars represent the haem centre, crosses represent the [2Fe–2S] cluster, and star shapes represent the [4Fe–4S] cluster. See text for more details.

Supplementary material

Legends to supplementary figures

Fig. S1. Effect of varying the CO_2 supply on an anaerobic culture of *A. ferrooxidans* CCM 4253 oxidizing S^0 with Fe^{3+}. The culture was generated using Fe^{2+}-grown cells as an inoculum, with an initial density of 10^8 cells per mL. Circles indicate cell numbers and triangles indicate Fe^{2+} formation, with 1.5-L CO_2 supply if filled, and no CO_2 supply if open. The error bars represent means ± SD (n = 3).

Fig. S2. Relationship between H^+ and Fe^{2+} concentrations during anaerobic oxidation of S^0 by Fe^{3+} in growing *A. ferrooxidans* CCM 4253 cells. The data relate to the cultures profiled in Fig. 1. The line represents a linear regression of experimental results for two independent cultures under anaerobic growing conditions.

Legends to supplementary tables

Table S1. The list of primers used in real-time reverse transcription quantitative PCR analysis.

Table S2. Transcriptional profiles of energy metabolism genes during anaerobic oxidation of S^0 with Fe^{3+} in growing *A. ferrooxidans* CCM 4253 cells (source of data shown in Fig. 2).

Response to Reviewer:

To the individual reviewer comments:

In the following answers, all reviewers' comments are in bold and our answers are in normal.

Comments:

The manuscript is much improved and I have just a few minor comments.

Page 5, line 9: Add a comma after B2.
It was corrected. (Page 5 Line 9)

Page 6, line 19: Is this better? "Ferrous grown cells were harvested by centrifugation at 15,000 × g for 10 min and then inoculated into the bioreactor at a final density of around 10E8 per mL".
The recommended sentence has been accepted. (Page 6 Line 19-21)

Page 6, line 25: mL
It was corrected. (Page 6 Line 25)

Page 8, line 23: "…results indicated that…"
It was corrected. (Page 8 Line 24)

Fig. 1: Replot the figure with an x-axis from -0.5 to 8.5. This will allow the 0 and 8 day data points to be fully visible.
Figure 1 has been revised according to the Reviewers' suggestion. Also Figures S1 and S2 contained data points which were not fully visible. Therefore we have also revised all the Figures containing graphs (including Figs S1 and S2) to unify the graphic art of the manuscript.

Fig. 2: To differentiate from the NADH complex I, rename "Complex I" and "Complex II" as "PetI bc1 complex" and "PetII bc1 complex".
We assume that the Reviewer had in mind the Figure 3. Fig. 3 has been revised according to the Reviewers' suggestion.

Page 9, line 20: Add a comma after petI.
It was corrected. (Page 9 Line 21)

Page 12: Could the low level of sat gene RNA transcripts (for sulfite oxidation) and increased tqo gene transcripts indicate that sulfite abiotically reacts with elemental sulfur to form thiosulfate (that is oxidized by Tqo)?
Based on the reviewers' valuable remark, the new sentence has been added into the manuscript. (Page 12 Line 23-25)

Page 14, Line 19: Add a comma after A. ferridurans.
It was corrected. (Page 14 Line 22)

Page 15, line 5: Has the rus operon been shown to be co-transcribed in strain CCM 4253? If not, I suggest the authors use the term "gene cluster".
It was corrected. (Page 15 Line 7)

Page 16, lines 21 and 24: Add a comma after petB2.
It was corrected. (Page 16 Line 23 and Page 17 Line 1)

Ref 21: Abbreviate the journal name.
It was corrected.

Re: RESMIC-D-15-00403R1

Are there multiple mechanisms of anaerobic sulfur oxidation with ferric iron in *Acidithiobacillus ferrooxidans*?

by Jiri Kucera, Eva Pakostova, Jan Lochman, Oldrich Janiczek, and Martin Mandl

February 9, 2016
Research in Microbiology

Dear Prof. Fabiano,

we are very pleased that the Reviewer found improvements in our manuscript. We would like to thank the Reviewer for the valuable comments again. All the minor comments or questions have been included and answered in the revised version of the manuscript; see the attached "Responses to the reviewers" file.

Thank you for your assistance and consideration.

Yours sincerely,
Jiri Kucera, jiri.kucera@mail.muni.cz
Corresponding author

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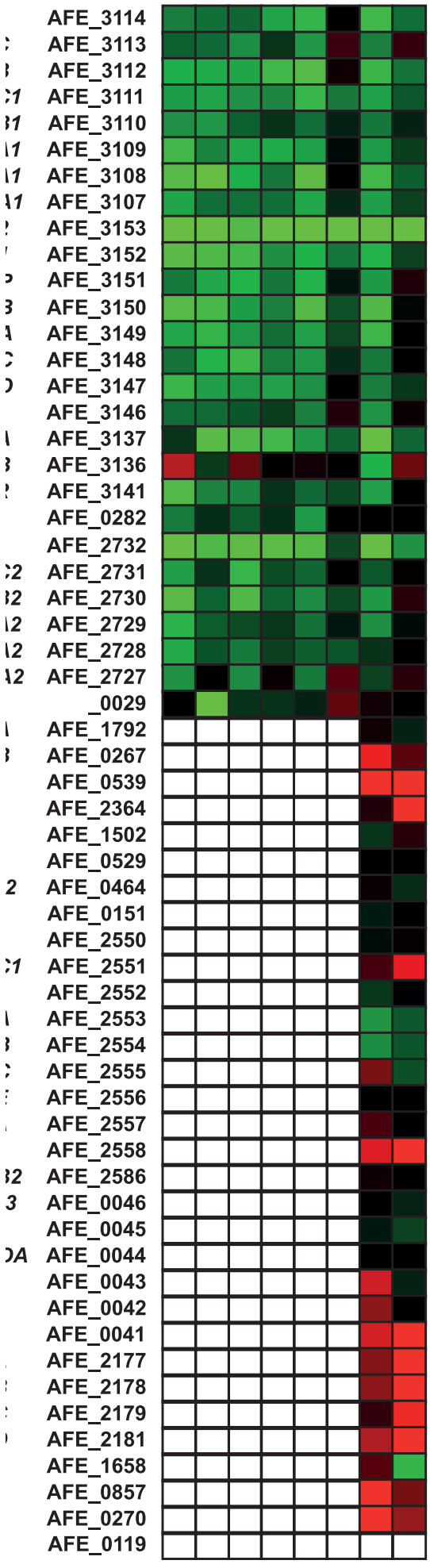
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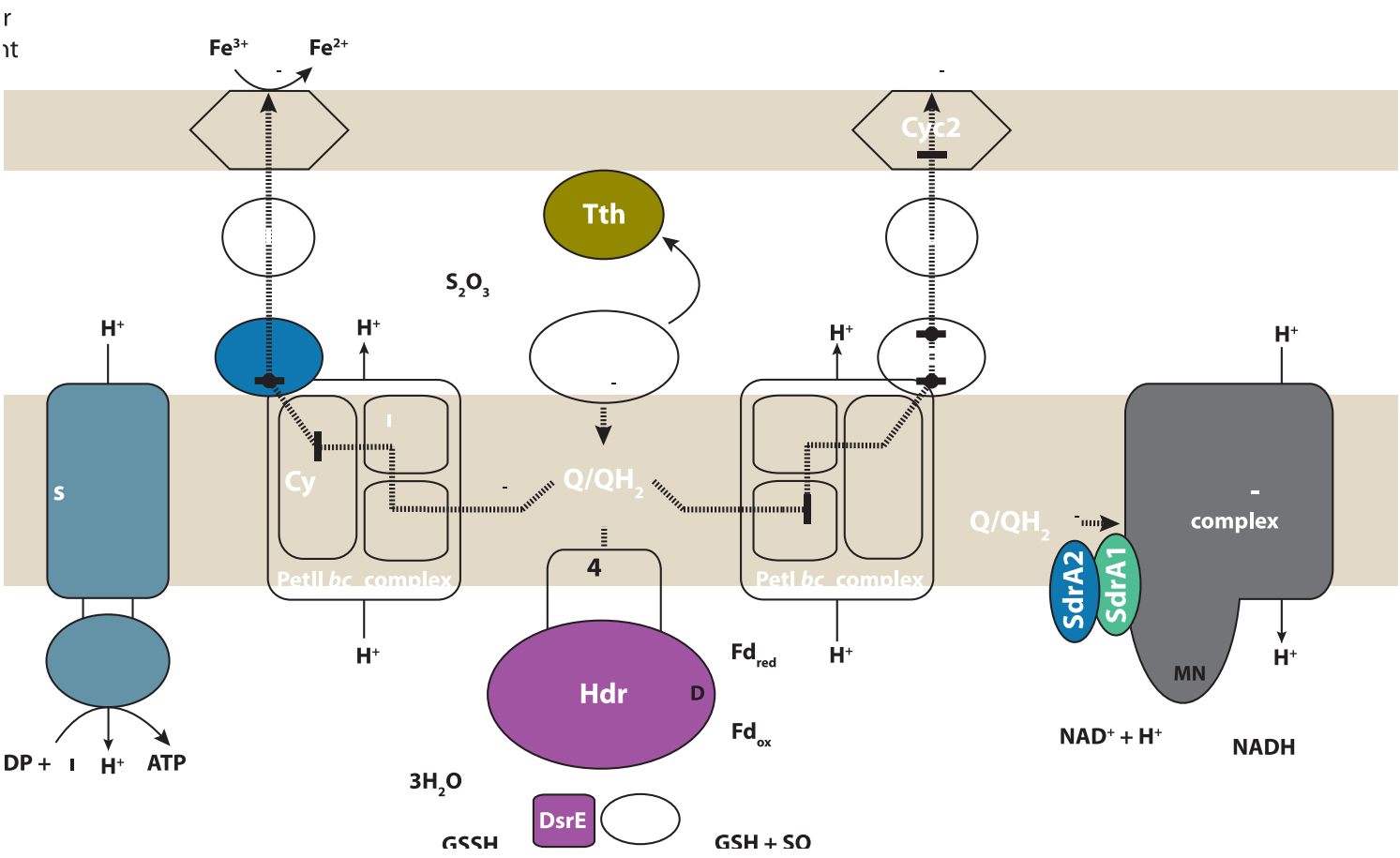
Fe3+
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PetI bc complex
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Fdred
Fdox
3H2O
DsrE
GSSH
GSH + SO
SdrA2
SdrA1
complex
MN
NAD+ + H+
NADH
ATP

Table 1. Gene transcript ratio between *petI* and *petII* operons (*petI* gene divided by *petII* gene) during anaerobic oxidation of S^0 with Fe^{3+} in growing *A. ferrooxidans* CCM 4253 cells.

	Cultivation time (d)								
Gene	0	1	2	3	4	5	6	7	8
petA	1.05	1.10	1.26	1.32	1.13	1.01	1.03	1.13	1.13
petB	0.84	1.26	0.76	0.96	0.95	0.94	0.92	1.52	0.89
petC	1.00	1.35	0.95	1.17	1.26	1.35	1.20	1.38	1.34
sdrA	1.19	1.94	1.16	1.14	1.52	0.78	1.45	1.28	1.58
cycA	1.09	1.32	0.95	1.46	1.16	1.46	1.42	1.41	1.31

Note: Presented values are based on ratios of the C_T values normalized using normalization factors from the cultures used to obtain the transcription profiles shown in Fig. 2.

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