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Original article

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

Jiri Kucera*, Eva Pakostova, Jan Lochman, Oldrich Janiczek, Martin Mandl

Department of Biochemistry, Faculty of Science, Masaryk University, Brno, Czech Republic

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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 involved in aerobic iron and sulfur respiration were induced during anaerobic growth of ferrous iron-grown cells. Most sulfur metabolism genes were either expressed at the basal level or their expression declined. However, transcript levels of genes assumed to be responsible for processing of elemental sulfur and other sulfur intermediates were elevated at the beginning of the growth period. In contrast, genes with predicted functions in formation of hydrogen sulfide and sulfate were significantly repressed. The main proposed mechanism involves: outer membrane protein Cyc2 (assumed to function as a terminal ferric iron reductase); periplasmic electron shuttle rusticyanin; c_4-type cytochrome CycA1; the inner membrane cytochrome bc_1 complex I; and the quinone pool providing connection to the sulfur metabolism machinery, consisting of hetero-disulfide 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 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 insight 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. (*Acidithiobacillus ferrooxidans*, *Acidithiobacillus ferrivorans*, *Acidithiobacillus ferridurans*) and thermo-tolerant *Acidiferrobacter thiooxydans*

* Corresponding author.
E-mail addresses: jiri.kucera@mail.muni.cz (J. Kucera), 150560@mail.muni.cz (E. Pakostova), lochik@mail.muni.cz (J. Lochman), janiczek@chemi.muni.cz (O. Janiczek), mandl@chemi.muni.cz (M. Mandl).

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(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 *Sulfobacillus 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].

$$8Fe^{3+} + 5Fe_2(OH)_2^{4+} + 3S^0 + 11SO_4^{2-} + 2H_2O \rightarrow 10Fe^{2+} + 8FeSO_4^0 + 6HSO_4^- + 8H^+ \quad (1)$$

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 downregulation 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, 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 the cells' growth phases. The results of this and previous studies indicate that there may exist 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 closely 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