Insights into the iron and sulfur energetic metabolism of \textit{Acidithiobacillus ferrooxidans} by microarray transcriptome profiling

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Abstract

\textit{Acidithiobacillus ferrooxidans} is a well known acidophilic, chemolithoautotrophic, Gram negative, bacterium involved in bioleaching and acid mine drainage. In aerobic conditions, it gains energy mainly from the oxidation of ferrous iron and/or reduced sulfur compounds present in ores. After initial oxidation of the substrate, electrons from ferrous iron or sulfur enter respiratory chains and are transported through several redox proteins to oxygen. However, the oxidation of ferrous iron and reduced sulfur compounds has also to provide electrons for the reduction of NAD(P) that is subsequently required for many metabolic processes including CO\textsubscript{2} fixation. To help unravel the enzymatic pathways and the electron transfer chains involved in these processes, a genome-wide microarray transcript profiling analysis was carried out. Oligonucleotides corresponding to approximately 3000 genes of the \textit{A. ferrooxidans} type strain ATCC23270 were spotted onto glass-slides and hybridized with cDNA retrotranscribed from RNA extracted from ferrous iron and sulfur grown cells. The genes which are preferentially transcribed in ferrous iron conditions and those preferentially transcribed in sulfur conditions were analyzed. The expression of a substantial number of these genes has been validated by real-time PCR, Northern blot hybridization and/or immunodetection analysis.

Our results support and extend certain models of iron and sulfur oxidation and highlight previous observations regarding the possible presence of alternate electron pathways. Our findings also suggest ways in which iron and sulfur oxidation may be coordinately regulated. An accompanying paper (Appia-Ayme et al.) describes results pertaining to other metabolic functions.

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1. Introduction

\textit{Acidithiobacillus ferrooxidans} is one of the most studied microorganism commonly found in acid mine drainage (see references in [1,2]). One can wonder at how life is possible in such extreme environments (high proton
and metal concentration). In fact, A. ferrooxidans takes advantage of these conditions and gains energy required for growth from the aerobic oxidation of ferrous iron (FeII) and reduced sulfur compounds to ferric iron (FeIII) and sulfuric acid, respectively. The oxidation products then attack chemically the metal sulfides [3] leading to metal solubilization and ore desulfurization. Therefore, elucidating the unusual energetic metabolism of A. ferrooxidans is of central importance to understanding its bioleaching and bioremediation properties. Consequently, understanding the respiration of iron and reduced sulfur compounds is of wide interest, not only scientifically, but also from an economic and environmental point of view.

Iron-based energetic metabolism has been studied for several years leading to the proposal of a number of respiratory chain models [4–9]. The currently accepted model [9] is based on genetic [9,10] and biochemical evidence [11,12], and is further supported by the subcellular localization of the proposed redox partners [13,14]. On the other hand, the study of the energetic metabolism of reduced sulfur compounds is more complicated than for iron, because (i) it involves several enzymatic reactions, (ii) only some of them are coupled to the respiratory chain(s) and (iii) these couplings occur at different points of the respiratory chain(s). While several enzymes proposed to be involved in sulfur dissimilation have been known for a long time [15–21], it is only recently that some results were reported on the sulfur respiratory chains [22,23]. To add to the complexity of A. ferrooxidans energetic metabolism, the oxidation of ferrous iron and of reduced sulfur compounds has to provide electrons for the reduction of NAD(P) that is subsequently required for many metabolic processes, such as carbon and nitrogen fixation. It is now clear that A. ferrooxidans respiratory chains to oxygen are redundant and branched [22]. Furthermore, from the A. ferrooxidans genome sequence analysis, the existence of several electron transfer proteins has been inferred [13,22,24], but the respiratory chain to which they belong was not known for most of them.

Despite considerable effort, A. ferrooxidans remains largely recalcitrant to standard genetic techniques for its manipulation, although progress has been made for developing a conjugation system for genetic exchange with Escherichia coli [25,26]. Such deficiency seriously impedes the direct experimental investigation of the metabolism of A. ferrooxidans and gene knockouts are still difficult to obtain. Nevertheless, the study and comparison of gene expression profiles is an alternative that can circumvent this limitation. Recently, the A. ferrooxidans genome sequence has been made available by The Institute for Genomic Research. As part of the growing effort to understand A. ferrooxidans physiology its genome has been annotated and curated (Holmes et al., 2005, manuscript in preparation), making feasible genome-wide comprehensive studies through the construction and use of microarray technology.

To gain further insight into the physiological role of the known redox proteins and to help identify yet unknown or missing components involved in iron and sulfur respiratory chains to oxygen, we have undertaken a global transcriptomic approach. In the present work, we have compared the expression profiles of the A. ferrooxidans cells grown on iron with those grown on sulfur, focusing our interpretations on the energy metabolism functions.

2. Materials and methods

2.1. Strain and culture conditions

A. ferrooxidans ATCC 23270 was obtained from the American Type Culture Collection. A. ferrooxidans was grown at 30°C under oxic conditions (200rpm) in (i) FeII-medium consisting of 62mM FeSO4–7H2O in 9K basal salts solution ((NH4)2SO4: 0.4g/l; K2HPO4: 0.4g/l; MgSO4–7H2O: 0.4g/l; H2SO4 adjusted to pH 1.6 with H2SO4; (ii) S0-medium consisting of 1% (w/v) elemental sulfur in 9K basal salts solution ((NH4)2SO4: 0.4g/l; K2HPO4: 0.4g/l; MgSO4–7H2O adjusted to pH 3.5 with H2SO4.

2.2. Materials

The TRIZOL® reagent was from Invitrogen. The High Pure RNA isolation kit was from Roche Applied Biosystem. The 50-mer oligonucleotides were synthesized, and HPCR® (High Purity Salt Free) purified by MWG Biotech. The slides (UltraGAPS), the spotting buffer and clean-up reagents (ChipShot™ Universal Printing Kit), the labelling reagents (Cy®3 and Cy®5-labeled nucleotides were from Amersham Biosciences. LightCycler Fast Start DNA master (plus) SYBR Green I PCR kit was from Roche Applied Biosystems.

2.3. Oligonucleotide design

For each of the 3037 putative ORFs predicted in the genomic sequence of A. ferrooxidans ATCC 23270 (TIGR release September 2003; 2.98 Mb), an internal 50-mer oligonucleotide was designed using the Oligoarray
software (Version 1.0). Oligonucleotide design considerations include narrow melting temperature (92 ± 7°C), and avoidance of self-annealing structures. To exclude redundant oligonucleotides and minimize cross-hybridization, a Blast search (using e^{-10} as cutoff) was carried out. A special set of control oligonucleotides (negative, positive, tiled and antisense sequences) was included to evaluate probe specificity and adjust the hybridization conditions. 50-mer oligonucleotides corresponding to the CDS of the gfp gene from Aequorea sp., the rad9 gene form Saccharomyces cerevisiae and the idi2 gene from human were used as negative controls.

Unmodified 50-mer oligonucleotides have been shown to enable high signal intensity and high specificity [27]. Therefore, unmodified oligonucleotides, synthesized by MWG Biotech at a 10nm scale and at 100μM were used.

2.4. Oligonucleotides spotting

The unmodified 50-mer oligonucleotides were diluted to 50μM in Corning Pronto!™ Universal Spotting Solution for spotting purposes. They were spotted in duplicate on Corning UltraGAPS slides, coated with Gamma Amino Propyl Silane, with an 8 pin Affimetrix 427 Arrayer at the Marseille-Nice Genopole (France). They were subsequently immobilized by UV cross-linking with the UV Stratalinker 2400 (Stratagene) and stored in a dessicator cabinet until use.

2.5. RNA preparation

A. ferrooxidans mid logarithmic cultures grown in the presence of FeII (62 mM FeSO₄) or S⁰ (0.5% S⁰) as energy source (30°C, 200rpm) were harvested by centrifugation at 4°C and immediately processed for RNA extraction. Cells were washed in 9K buffer (pH adjusted) to remove iron traces or sulfur particles before further treatment. Total RNA was extracted using a modified acid–phenol extraction method [28], including a preliminary TRIZOL® reagent (Invitrogen) extraction step. This RNA was used directly for cDNA synthesis and labelling. For real-time PCR, RNAs were additionally purified with the High Pure RNA isolation kit (Roche Applied Biosystem) and treated twice with DNase I (Roche Applied Biosystem). The lack of DNA contamination was checked by PCR on each RNA sample.

2.6. Labelled cDNA synthesis and hybridization

The ChipShot™ Labelling Clean-Up System (Promega-Corning) was used to generate fluorescently labeled cDNA via direct incorporation of Cy®3 and Cy®5-labeled nucleotides from Amersham Biosciences. The reverse transcription reaction was performed in the presence of 5μg total RNA and random hexamers (Protocols: www.promega.com). Two independent cDNA preparations were labeled once with each dye (reverse dye labeling) to account for sampling differences, biases in dye coupling or emission efficiency of Cy®-dyes. Labelled cDNA was purified from contaminating fluorescent dNTPs and degraded RNA using the ChipShot Labelling Clean-Up System (Promega-Corning). Dye incorporation efficiency was determined by absorbance readings at 260, 550 and 650nm and the frequency of incorporation (FOI, pmol of dye incorporated per ng of cDNA) was calculated according to Promega’s instructions. Optimally labelled samples were combined, vacuum-dried and resuspended to a final volume of 40–50μL in Corning Pronto! Universal Hybridization Buffer.

The Pronto!™ Universal Hybridization kit was used according to Promega’s instructions. Briefly, the combined denatured target cDNA samples (95°C for 5 min) were hybridized to the spotted slides for 14h at 42°C in the Corning hybridization chamber. Following hybridization, slides were washed in serial dilutions of Corning Wash Buffers as recommended by the manufacturer and dried by centrifugation at 1200×g for 2 min.

2.7. Image acquisition and data analysis

Microarrays were scanned for the Cy®3 and Cy®5 fluorescent signals using the ScanArray 4000 Microarray Analysis System (PerkinElmer Life Sciences, Inc.) at a resolution of 10μm per pixel. Scanning parameters (laser power: 85–95 and photomultiplier voltage: 90–95) were adjusted so that overall intensities in both fluorescent channels were balanced and few spots were saturated (to maximize the dynamic range). Scans stored as 16-bit TIFF (Tagged Information File Format) image files and then analyzed with the image quantification software package GenePix Pro 4.0 (Axon Instruments, Inc.). Addressing information, raw signal intensity readings and quality parameters for each spot were recorded. Low-quality spots (spots smaller than 60μm or bigger than 160μm in diameter, sub-circular in shape and/or exhibiting uneven fluorescence distribution) were flagged and filtered out. Genes with median signal intensities less than two standard deviations above background in both channels were considered as not detected and array elements that approached the maximum value (65.535 per pixel for a 16-bit scanner) were considered as saturated and excluded from further
statistical analysis. Local background was subtracted from the recorded spot intensities (median values). Negative values (i.e. local background intensities higher than spot signal) were considered as no data. Median spot intensities were Log transformed (Log2) and normalized to account for any difference in total intensity between the scanned images due to pin effects (Pin group location normalization), dye and slide effects (Scale normalization) and intensity-dependent effects (Lowees normalization) using the R package (Version 1.8.1; URL http://www.R-project.org.). The processed FeII and S0 signal intensities for each spot were used for calculating the expression ratio FeII/S0. Eight ratio values per gene, resulting from direct and reverse labelling and replicate experiments, were used for further statistical analysis of the data. The statistical significance of differential expression in FeII or S0 grown cells, was assessed using R package applying to the data set, the Student t-test [28].

2.8. Real-time PCR

The relative abundances of a set of differentially expressed genes and a set of invariant genes, according to the microarray results obtained, were determined in FeII- and S0-grown cells by real-time PCR. Specific primers for the genes of interest amplifying average products of 300bp with about 50GC % and about 55°C Tm were designed. Equal amounts of A. ferrooxidans DNAseI-treated total RNA were retro-transcribed from these primers with the Superscript II™ RNase H−(Invitro Life Technologies) at 42°C for 50min, and then treated at 70°C for 15min to inactivate the enzyme. The real-time PCR quantifications were realized at the transcriptome platform of the I.B.S.M. (Marseille, France) on the total cDNA obtained, using the LightCycler instrument and the LightCycler Fast Start DNA master (plus) SYBR Green I PCR kit (Roche Applied Biosystems) with external standards as described in Roche Molecular Biochemicals technical note no. LC 11/2000. The real-time PCR experiments were performed twice, with both independent total RNA and cDNA preparations by the comparative threshold cycle method. The calculated threshold cycle (Ct) for each gene was normalized to Ct of the rrs gene, coding for the 16S rRNA which expression is invariant under FeII- and S0-growth conditions [10].

3. Results and discussion

3.1. A. ferrooxidans microarrays analysis

The genomic expression profiles of A. ferrooxidans ATCC 23270 growing on FeII or S0 media were determined and compared with one another. To identify the genes differentially affected by the energy source, the following statistical criteria were employed: (i) genes with P values >0.01 were considered as not regulated regardless of their differential expression level; (ii) genes with a P value of <0.01 were considered to have significant differential expression and thus regarded as up-regulated either in FeII or in S0

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Fig. 1. Differentially expressed genes in A. ferrooxidans microarrays. (A) Odds of being differentially expressed [B-statistic versus M (Log2 ratio FeII/S0)]. (B) Fold-change cut-off determination by hybridization of identical cDNA samples labelled with Cy®3 and Cy®5.
conditions (Fig. 1A). The fold-change cut-off to be used for the identification of the genes exhibiting differential expression between treatments was assessed by hybridization of identical cDNA samples (total RNA extracted from FeII-grown cells) labeled with Cy®3 or Cy®5 dyes. The pattern of hybridization revealed a linear correlation of the data and a fold change in gene expression level ($M$: Log2 ratio FeII/S0) no larger than 1.5 (Fig. 1B). Similar cut-off values have been reported as being biologically significant elsewhere. Therefore, genes with a positive Log2 FeII/S0 ratio ($M$) were considered to be more expressed in FeII- than in S0 and vice-versa, while the genes with a Log2 FeII/S0 ratio ($M$) close to zero were considered to be similarly expressed in both conditions.

On the basis of the criteria described above, the expression level of more than 200 genes of *A. ferrooxidans* was affected by the presence of either FeII or S0 as energy source in the growth media (Fig. 2). The comparison of the transcriptional profiles of FeII-grown cells versus S0-grown cell indicated that about 120 genes were induced (Max=up to 38 fold) in FeII conditions and about 90 were specifically induced (Max=up to 10 fold) in S0 conditions.

### 3.2. Energy source (FeII or S0) expression profile

The genes expressed differentially according to the energy source can be divided into seven functional categories: energy metabolism, central intermediary metabolism, regulatory functions, protein translation, amino acid biosynthesis, cell wall and envelope constituents, and miscellaneous (unknown function). In this paper, we will analyze the genes encoding proteins involved in the energetic metabolism while results pertaining to the other metabolic functions, and more particularly to the carbon metabolism, are described in the accompanying paper (Appia-Ayme et al.).

Table 1 summarizes the data obtained on genes encoding electron transfer proteins we have previously suggested to be involved in FeII or S0 energetic metabolism ([9,22,30,31], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in preparation).

<table>
<thead>
<tr>
<th>Up-regulated genes</th>
<th>$P$ value</th>
<th>$B$-statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>265</td>
<td>211</td>
</tr>
<tr>
<td>FeII</td>
<td>145</td>
<td>120</td>
</tr>
<tr>
<td>S $\frac{1}{2}$</td>
<td>120</td>
<td>91</td>
</tr>
</tbody>
</table>

3.2.1. Characterization of FeII-induced genes encoding electron transporters

In FeII conditions, the *petI* operon encoding one of the bc$_1$ complexes ([24,30,31], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in preparation), was highly expressed (Table 1 and Fig. 3) in agreement with Northern hybridization and real time PCR experiments ([31], Bruscella, Appia-Ayme, Levican, Ratouchniak Holmes, Jedlicki and Bonnefoy, manuscript in preparation).

The *rus* operon, encoding electron transfer proteins involved in the respiratory chain between FeII and oxygen [9], was shown by a number of approaches to be more expressed in FeII than in S0 conditions in the strains ATCC 33020 [10,13,32,33] ATCC 19859 [22,34], or Torma [35], except in NASF-1 strain where the transcriptional level of *coxB* was similar in both conditions [23]. The spots corresponding to the genes of this operon were saturated in iron so expression ratios could not be determined. However, differential expression in iron is in agreement with previous results. Optimized image acquisition and data analysis conditions will have to be found for this case.

Surprisingly, while higher sulfide quinone reductase activity was observed in S0 than in FeII conditions [22], two out of the three putative operons encoding sulfide quinone reductase activity were induced in S0.
Table 1
Microarray expression ratios between FeII and S0 grown cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene function</th>
<th>M value</th>
<th>P value</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>rus operon</td>
<td>Outer membrane cytochrome c precursor</td>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyc2</td>
<td>Cytochrome c552 dimer precursor</td>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cycI</td>
<td>Cytochrome c oxidase II</td>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coxB</td>
<td>aa3 Cytochrome c oxidase I</td>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coxA</td>
<td>aa3 Cytochrome c oxidase III</td>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coxC</td>
<td>aa3 Cytochrome c oxidase IV</td>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rus</td>
<td>Rusticyanin</td>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cycA</td>
<td>Cytochrome b ubiquinol oxidase</td>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cycB</td>
<td>Cytochrome d ubiquinol oxidase I (bd)</td>
<td></td>
<td>0.128</td>
<td>ND</td>
</tr>
<tr>
<td>cycD</td>
<td>Cytochrome d ubiquinol oxidase II (bd)</td>
<td></td>
<td>0.065</td>
<td>ND</td>
</tr>
<tr>
<td>cyoBACD encoding bo3 ubiquinol oxidase</td>
<td>Cytochrome o ubiquinol oxidase subunit II</td>
<td>−1.97</td>
<td>0.008</td>
<td>NL</td>
</tr>
<tr>
<td>cyoB</td>
<td>Cytochrome o ubiquinol oxidase subunit I</td>
<td>−2.69</td>
<td>0.008</td>
<td>NL</td>
</tr>
<tr>
<td>cyoA</td>
<td>Cytochrome o ubiquinol oxidase subunit II</td>
<td>−2.49</td>
<td>0.020</td>
<td>ND</td>
</tr>
<tr>
<td>cyoC</td>
<td>Cytochrome o ubiquinol oxidase subunit III</td>
<td>−1.45</td>
<td>0.113</td>
<td>ND</td>
</tr>
<tr>
<td>cyoD</td>
<td>Cytochrome o ubiquinol oxidase subunit IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>doxI operon encoding thiosulfate quinol reductase</td>
<td>Thiosulfate quinol oxido-reductase</td>
<td>1.49</td>
<td>0.056</td>
<td>ND</td>
</tr>
<tr>
<td>doxDA1</td>
<td>Thiosulfate quinol oxido-reductase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>doxII operon encoding thiosulfate quinol reductase</td>
<td>Thiosulfate quinol oxido-reductase</td>
<td>−1.41</td>
<td>0.029</td>
<td>ND</td>
</tr>
<tr>
<td>doxDA2</td>
<td>Thiosulfate quinol oxido-reductase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rod</td>
<td>Rhodanese-related sulfur transferase</td>
<td>−0.95</td>
<td>0.061</td>
<td>ND</td>
</tr>
<tr>
<td>petII operon</td>
<td>Cytochrome b ubiquinol-acceptor oxidase</td>
<td>3.41</td>
<td>0.000</td>
<td>DF</td>
</tr>
</tbody>
</table>

Table 1 (continued)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene function</th>
<th>M value</th>
<th>P value</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>petII operon</td>
<td>Ubiquinol-cytochrome c reductase, cytochrome b</td>
<td>−1.42</td>
<td>0.013</td>
<td>NL</td>
</tr>
<tr>
<td>petB2</td>
<td>Ubiquinol-cytochrome c reductase, cytochrome c1</td>
<td>0.09</td>
<td>0.780</td>
<td>ND</td>
</tr>
<tr>
<td>petC2</td>
<td>Ubiquinol-cytochrome c reductase, cytochrome c1</td>
<td>−1.04</td>
<td>0.031</td>
<td>ND</td>
</tr>
<tr>
<td>hip</td>
<td>High redox potential iron–sulfur protein (HiPIP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sqrl operon encoding sulfide quinone reductase</td>
<td>Sulfide quinone reductase</td>
<td>2.16</td>
<td>0.000</td>
<td>DF</td>
</tr>
<tr>
<td>sqrl</td>
<td>Sulfide quinone reductase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sqriI operon encoding sulfide quinone reductase</td>
<td>Sulfide quinone reductase</td>
<td>2.47</td>
<td>0.000</td>
<td>DF</td>
</tr>
<tr>
<td>sqriI</td>
<td>Sulfide quinone reductase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sqriII operon encoding sulfide quinone reductase</td>
<td>Sulfide quinone reductase</td>
<td>0.59</td>
<td>0.491</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not differential (P>0.01), NL: near cut-off limit: (are significant according to one of the test criteria, e.g. P≤0.01, therefore indefinite). In bold: P≤0.01. DF: Differential FeII up (P<0.01 and M>0).

The expression of the sqri gene (sqr3), included in a different gene context, does not seem to be differentially expressed in either conditions, contrary to what was observed in *A. ferrooxidans* NASF-1, where the transcriptional level of *sqr3* was found to be three fold higher in S0- than in FeII-grown cells [23]. Further experiments are therefore required to explain these discrepancies.

### 3.2.2. Characterization of S0-induced genes encoding electron transporters

Several genes of the cyd, cyo, doxII and petII operons presented differences in expression that were very near the statistic significance cut-off limit established in the present analysis (Table 1) and were thus considered as differentially expressed. Therefore, these operons seem to be more expressed in S0 than in FeII conditions, in agreement with previous results. We have indeed shown by Northern hybridization and real time PCR that the petII operon is slightly expressed in S0 than in FeII conditions in the ATCC 33020 strain ([31], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in...
preparation). The same holds true for the rod gene encoding a rhodanese-like proteins [20] and belonging to the putative doxII operon. Furthermore, higher amounts of quinol oxidases and higher activities of quinol oxidase and thiosulfate quinol reductase have been observed in ATCC 19859 cells grown in S0 compared to those grown in FeII [22]. However, in the NASF-1 strain, the transcription of the cydA gene encoding subunit I of the alternate bd quinol oxidase was similar in both conditions [23].

### 3.3. Validation of microarrays results using real-time PCR

To confirm the expression patterns observed with the microarrays, some genes were selected for further analysis by real-time PCR. The rrs gene coding for the 16S rRNA has been shown to be expressed at the same (constitutive) level under both conditions of growth (FeII- and S0-medium) [10] and was used as the reference standard. Raw fold changes in expression are shown in Table 2. The expression ratios obtained by real-time-PCR of most of these genes correlated well with those obtained with microarrays (Table 2). However, as described previously in other cases, the transcript levels values obtained by real-time PCR were higher than with the microarrays, in accordance with the higher sensitivity of the former technique. Despite of these (minor) quantitative differences, the results obtained by real-time PCR were in good agreement with those obtained from microarrays and support the general consistency between the two methods for measuring relative expression levels.

To differentiate the expression of the two identical sqrI and sqrII operons, 20-mers oligonucleotides were designed in the very beginning or very end of the gene clusters where the sequences differ. Real-time PCR with these oligonucleotides indicated that sqrII is 10.53 fold more expressed in FeII than in S0 conditions, while sqrI is almost evenly expressed in both conditions (Table 2). Thus, differential regulation of paralogue genes which sequences are highly similar could be distinguished by real-time PCR with adequate designed oligonucleotides.

For the genes of the petII, cyd, cyo and doxII operons where B and/or P values were near the cut-off limit, real-time PCR experiments clearly showed higher expression in S0 than in FeII conditions (Table 2).

In the case of the rrs operon, which corresponding spots were saturated on microarrays, higher expression in FeII- than in S0-grown cells was obtained by real-time PCR [10,32,36] (Table 2).

### 4. Conclusion

Global changes in transcript abundance, reflecting the primary response of *A. ferrooxidans* to energy source changes are in agreement with previous findings for most genes. The differential expression of the *rs*, petI, petII, cyd, cyo, doxII operons encoding electron transfer proteins, is consistent with existing data ([9,10,20,22,31–33,36], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in preparation); [3]: [data obtained from unsaturated spots].

<table>
<thead>
<tr>
<th>Gene downstream from doxDA2</th>
<th>Fold</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not differentially expressed</td>
<td>1.97</td>
<td>1.97</td>
</tr>
</tbody>
</table>

Underlined: higher expression in FeII; dotted underlined: higher expression in S0. Real-time PCR data from [10,32,33]; [31], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in preparation); [3]: [data obtained from unsaturated spots].
real-time PCR data supported the FeII and S^0 respiratory chain model we have proposed (Fig. 3) ([9, 22, 23, 29–31, 33], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in preparation). In this model, the outer membrane cytochrome c, Cyc2, transfers electrons from FeII to the periplasmic rusticyanin, which passes them to the membrane-bound cytochrome c_4, Cyc1, and from there to the cytochrome oxidase CoxBACD where oxygen is reduced to water. All these redox proteins are encoded by the rus operon [9]. FeII oxidation and NAD(P) reduction has been proposed to be coupled to explain the balance of the electron flow from FeII between two pathways: the exergonic one, through the aa_3 type oxidase towards oxygen encoded by the rus operon, and the endergonic one, through a bc_1 complex toward NAD (P) [24, 37]. In the reverse electron (endergonic) pathway the bc_1 complex receives electrons from a cytochrome c and transfers them to the quinol pool. We have proposed that this bc_1 complex and this

Fig. 3. Model proposed for iron and sulfur energetic metabolism of A. ferrooxidans. The transcriptional units and the corresponding redox proteins are presented in the same color. TQR: thiosulfate quinol reductase; SQR: sulfide quinone reductase; Hip: High Potential Iron–sulfur protein.
cytochrome $c$ are encoded by the petI operon ([30,31], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in preparation) and that the rusticyanin can give electrons to two different cytochromes $c_4$: CycA2 encoded by the petI operon or Cyc1 encoded by the Rus operon ([31], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in preparation). In the former case, electrons are transferred to the endergonic pathway to oxygen while in the later case, they are transferred to the exergonic pathway to NAD(P). In *A. ferrooxidans*, the cytochrome $bc_1$ complex has been shown to function in reverse in FeII grown cells, even in the presence of thiosulfate, while it functions in the forward direction in S$^0$ grown cells [22,24]. According to our model, the $bc_1$ complex encoded by petII is the one functioning in reverse and transfers the electrons from ferrous iron to NAD(P), while the $bc_1$ complex encoded by petII is the one functioning in the forward direction and transfers electrons from sulfur to oxygen ([31], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in preparation). When functioning in the forward direction, the $bc_1$ complex receives electrons from the quinol pool and transfers them either to a membrane-bound cytochrome $c$, and/or to a soluble reduct protein such as a cytochrome $c$, or a high potential iron–sulfur protein (HiPIP) which then gives the electron to the terminal oxidase where oxygen reduction takes place [38–40]. Since a membrane-bound cytochrome $c$ (CycA2) and a soluble HiPIP are encoded by the petII operon, we have suggested that these two reduct proteins transfer electrons between the two integral membrane complexes: the $bc_1$ and the terminal oxidase ([31], Bruscella, Appia-Ayme, Levican, Ratouchniak, Holmes, Jedlicki and Bonnefoy, manuscript in preparation).

We propose to continue our microarray investigations, not only providing a deeper analysis of the existing comparison between transcription in FeII versus S$^0$ grown cells but also extending the work to investigate transcription in cells grown in different concentrations of FeII and at different pHs. Pertinent aspects of our working models derived from the combination of bioinformatic and microarray analysis will be subjected to confirmation by real-time PCR and, where feasible, by mutant construction and analysis, genetic complementation and biochemical experiments.

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**References**