Differential expression of two *bc*₁ complexes in the strict acidophilic chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans* suggests a model for their respective roles in iron or sulfur oxidation

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Three strains of the strict acidophilic chemolithoautotrophic *Acidithiobacillus ferrooxidans*, including the type strain ATCC 23270, contain a *petI/ABC* gene cluster that encodes the three proteins, cytochrome *c*₁, cytochrome *b* and a Rieske protein, that constitute a *bc*₁ electron-transfer complex. RT-PCR and Northern blotting show that the *petI/ABC* cluster is co-transcribed with *cycA*, encoding a cytochrome *c* belonging to the *c*₄ family, *sdrA*, encoding a putative short-chain dehydrogenase, and *hip*, encoding a high potential iron–sulfur protein, suggesting that the six genes constitute an operon, termed the *petI* operon. Previous results indicated that *A. ferrooxidans* contains a second *pet* operon, termed the *petI* operon, which contains a gene cluster that is similarly organized except that it lacks *hip*. Real-time PCR and Northern blot experiments demonstrate that *petI* is transcribed mainly in cells grown in medium containing iron, whereas *petII* is transcribed in cells grown in media containing sulfur or iron. Primer extension experiments revealed possible transcription initiation sites for the *petI* and *petII* operons. A model is presented in which *petI* is proposed to encode the *bc*₁ complex, functioning in the uphill flow of electrons from iron to NAD(P), whereas *petII* is suggested to be involved in electron transfer from sulfur (or formate) to oxygen (or ferric iron). *A. ferrooxidans* is the only organism, to date, to exhibit two functional *bc*₁ complexes.

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INTRODUCTION

The cytochrome *bc*₁ complex is a central component of the energy transduction of the biosphere and is involved in almost all respiratory (aerobic as well as anaerobic) and photosynthetic electron-transfer chains (Hunte *et al.*, 2003 and references therein). In prokaryotes, the *bc*₁ complex, while common, is not universal. The complex is composed of three subunits, a cytochrome *c*₁, a cytochrome *b* and a Rieske iron–sulfur protein, that transfer electrons from a membrane-localized quinone to a small soluble redox protein such as cytochrome *c*, plastocyanin, or a

high-potential iron–sulfur protein (HiPIP) (Menin *et al.*, 1998; Hunte *et al.*, 2003 and references therein).

Reverse electron flow from cytochrome *c* through the cytochrome *bc*₁ complex to quinone has also been reported in a variety of chemolithoautotrophic organisms. During autotrophic growth of these bacteria, the energetic substrate has to provide electrons for reduction of NAD(P) to NAD(P)H, which is required for CO₂ fixation and other anabolic processes. When the midpoint potential of the electron donor is more positive than that of the NAD(P)/NAD(P)H couple, the reduction of NAD(P) requires energy. Depending on the level at which the electrons enter the respiratory chain, they have to be transported ‘uphill’ to NAD(P) by reverse electron transport through the cytochrome *bc*₁ complex, the quinone pool and the NAD(P)H dehydrogenase (Griesbeck *et al.*, 2000). This uphill (reverse) electron transport uses the proton-motive

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force generated by hydrolysis of ATP derived from electron donor oxidation.

In the strictly acidophilic chemolithoautotrophic Gram-negative bacterium *Acidithiobacillus ferrooxidans*, a cytochrome *bc₁* complex was shown by spectroscopic techniques to function in reverse in ferrous-iron-grown cells, even in the presence of thiosulfate, while it functioned in the normal (downhill) direction in sulfur-grown cells (Brasseur *et al.*, 2002, 2004). This raised questions regarding the mechanism regulating the flow of electrons either uphill or downhill in the same complex. A candidate operon, termed *petI*, was identified in *A. ferrooxidans* ATCC 33020 and ATCC 19859 (Levicán *et al.*, 2002) that could potentially encode a *bc₁* complex, and a second distinct operon, termed *petII*, was experimentally validated in *A. ferrooxidans* ATCC 33020 (Bruscella *et al.*, 2005) and bioinformatically detected in the type strain *A. ferrooxidans* ATCC 23270 (Brasseur *et al.*, 2002), raising the possibility that one of the two distinct *bc₁* complexes might be involved in the uphill flow of electrons and the other in the downhill flow (Brasseur *et al.*, 2004). The aim of the research described in this paper was to deepen our understanding of the *petII* operon and to study the expression of both the *petI* and *petII* operons in response to ferrous iron and sulfur in order to gain further insight into the respiratory chains in which these two cytochrome *bc₁* complexes are involved and how their synthesis might be regulated.

METHODS

Bacterial strains, plasmids and growth conditions. *Acidithiobacillus ferrooxidans* ATCC 33020, ATCC 19859 and ATCC 23270 were obtained from the American Type Culture Collection. *A. ferrooxidans* was grown at 30 °C under oxic conditions in ferrous iron or sulfur medium as described previously (Yarzabal *et al.*, 2003).

Escherichia coli strain TG1 (*supE hsdΔ5 thi Δ(lac-proAB) F': traD36 proAB lac^q lacZΔM15*) was used for phagemid propagation and was grown on LB medium (Ausubel *et al.*, 1992). The phagemid SK⁺ Bluescript was purchased from Stratagene.

Analytical methods. The ferrous iron concentrations were determined by the *o*-phenanthroline method (Muir & Anderson, 1977).

DNA manipulations. General DNA manipulations were performed according to Ausubel *et al.* (1992). Before manipulations, *A. ferrooxidans* cells were washed several times in basal salt solution corresponding to the medium in which they were grown, in order to remove ferrous iron precipitates or sulfur aggregates. Genomic DNA from *A. ferrooxidans* was prepared using the NucleoSpin Tissue kit (Macherey-Nagel), according to the manufacturer's instructions for bacterial DNA extraction. *Taq* polymerase purchased from Eppendorf was used for PCR. The oligonucleotides were obtained from Sigma-Genosys Corporation. The nucleotide sequences of the cloned fragments were determined from both strands by GENOME Express.

Plasmid construction. In order to synthesize the *hip* and *cycA1* RNA probes used for the Northern blot experiments, an internal fragment of each gene was amplified by PCR (Table 1) and cloned into the *EcoRV* restriction site of the SK⁺ Bluescript vector, between the T7 and T3 promoters.

RNA manipulations. Total RNA was extracted from 500 ml ferrous iron- or sulfur-cultures at different stages of growth (early exponential, mid exponential, late exponential or stationary phase) with the High Pure RNA isolation kit (Roche) as described previously (Guiliani *et al.*, 1997). For RT-PCR and real-time PCR, total RNA was treated twice with DNase I (Roche) and DNA contamination was checked by PCR.

Northern blotting. Formaldehyde gels were used for Northern blotting, as described by Ausubel *et al.* (1992). RNA was transferred by capillary action to positively charged nylon membranes purchased from Roche. RNA was UV cross-linked to the membrane with the Stratalinker from Stratagene. DIG-labelled *hip* and *cycA1* RNA probes were obtained by *in vitro* transcription performed on SK-*hip* or SK-*cycA1* plasmids, linearized with *EcoRI* or *HindIII* restriction enzymes, using T7 or T3 RNA polymerase and DIG-UTP from the Strip-EZ kit (Ambion). Prehybridization and hybridization steps were performed under high-stringency conditions with the DIG-labelled *hip* and *cycA1* RNA probes. Detection was performed by chemiluminescence with CSPD (Roche).

Reverse transcriptase-PCR (RT-PCR). Coupled RT-PCR experiments were performed with the Promega Access RT-PCR system. RT-PCR was carried out in two steps: (i) the reverse transcription was done on approximately 1 µg total RNA (DNA free) extracted from ferrous-iron-grown cells of *A. ferrooxidans*, with the Omniscript RT kit purchased from Qiagen; (ii) routine PCR amplification, with the oligonucleotides of interest (Table 1), was done using the cDNA obtained as matrix, as described above. For each RT-PCR experiment, three controls were used: one without template to detect potential contamination, one with genomic DNA as a positive control for PCR amplification and one with RNA not treated with reverse transcriptase to check for DNA contamination during RNA preparation.

Real-time PCR. The *rrs* gene encoding the 16S rRNA has been shown to be expressed at the same (constitutive) level under both conditions of growth examined (on ferrous iron and sulfur media) (Yarzabal *et al.*, 2004) and was used as a reference standard. Equal amounts of total RNA, extracted from ferrous iron- and sulfur-grown cells at different stages of growth, were retrotranscribed with the Superscript II reverse transcriptase (InVitro Life Technologies) at 42 °C for 50 min, followed by 15 min at 70 °C to inactivate the enzyme. Real-time PCR quantification was performed on the total cDNA obtained, using the LightCycler instrument and the LightCycler Fast Start DNA master (plus) SYBR Green I kit, with external standards, as described in Roche Molecular Biochemicals technical note no. LC 11/2000 and Yarzabal *et al.* (2004). Real-time PCR experiments were performed several times, using RNA samples from at least two independent cultures. The sequences of the oligonucleotide primers are given in Table 1.

Primer extension. Primer extension was performed with Superscript II reverse transcriptase (InVitro Life Technologies) as follows: extension at 42 °C or 50 °C for 50 min, followed by heating at 70 °C for 15 min to inactivate the enzyme. The oligonucleotides used (Table 1) were [³²P]ATP-labelled with T4 polynucleotide kinase from Biolabs. The experiments were done in duplicate, using RNA samples from independent cultures.

Nucleotide sequence accession numbers. The GenBank/EMBL database accession numbers, for the *petI* operon sequences from *A. ferrooxidans* ATCC 33020 are: AJ438314 (5' untranslated region upstream from *cycA1*), AM261982 (*cycA1* internal region), AJ318502 (intergenic region *cycA1-sdrA1*), AM261983 (*sdrA1* internal region), AJ318503 (intergenic region *sdrA1-petA1*), AJ318504 (*petA1* internal region), AJ318505 (intergenic region *petA1-petB1*), AJ318506 (*petB1* internal region), AJ413191 (intergenic region *petB1-petC1*),

Table 1. Oligonucleotides used in this study

Primer (names used in Figs 1 and 2)	Sequence	Gene
Expression plasmid construction (for <i>in vitro</i> transcription for RNA probes)		
Hip9	AgTgAgAAACTgAAggAAAC	<i>hip</i>
Hip10	gCgggCATCggCgTAAAC	<i>hip</i>
cycA1-1	gCTCCCTATCTggTCAAAAC	<i>cycA1</i>
cycA1-2	CTCgTCgTTgACCTgCCAAg	<i>cycA1</i>
Primer extension		
HipC ₄ -1 (23)	AgAgAATAgTgggATAACgg	<i>cycA2</i>
Ext HC ₄ (24)	CgCTTgTTgATAgTTATgg	<i>petII</i> 5' untranslated region
ResC ₄ -1 (25)	ggCATCgCACCCATACC	<i>cycA1</i>
II Ext RC ₄ (26)	ACCATCTATCTCCACCCC	<i>petI</i> 5' untranslated region
PCR, RT-PCR and real-time PCR*		
C _{4C} -6154 (1) ^{ab}	gCagAAACgCACCTATgTgA	<i>cycA2</i>
DH _N -6154 (2) ^{ab}	CgCCTTggTATAATCATTTTCCg	<i>sdrA2</i>
DH _C -6154 (3) ^{ab}	CTgggTCATAACggCgAgTTg	<i>sdrA2</i>
R _N -6154 (4) ^{ab}	TgCTgTCTTgCgTTTCgTC	<i>petA2</i>
R _C -6154 (5) ^{ab}	CCAAgAAAACgTCgTTgTC	<i>petA2</i>
B _N -6154 (6) ^{ab}	ggTAACCCCAACgCCgATAAAC	<i>petB2</i>
B _C -6154 (7) ^{ab}	CggAAAgATTgggggCgTTC	<i>petB2</i>
C _{1N} -6154 (8) ^{ab}	gCCATTCTgCCCACACAACA	<i>petC2</i>
C _{1C} -33020 (9) ^{ab}	CTggAAggAggTAAAgTg	<i>petC2</i>
Div-hip3 (10) ^{ab}	ACCTTACAgCACTTCggggC	<i>hip</i>
cycA2-1 (11) ^{ac}	ATggAAgCCTTACCTTTTgC	<i>cycA2</i>
cycA2-2 (12) ^{ac}	TCACATAggTgCgTTTCTgC	<i>cycA2</i>
sdrA2-1 (13) ^{ac}	CggAAAATgATTATACCAAggC	<i>sdrA2</i>
sdrA2-2 (14) ^{ac}	gTgAACTCgCTggAAATggC	<i>sdrA2</i>
ipetA1 (15) ^{ac}	TCAGACgAAACgCAAgACAg	<i>petA2</i>
ipetA2 (16) ^{ac}	gACAACgACggTTTTCTTgg	<i>petA2</i>
ipetB1 (17) ^{ac}	gTTACATTggTgTTTCTggTC	<i>petB2</i>
ipetB2 (18) ^{ac}	TgAACgCCCCCAATCTTTCC	<i>petB2</i>
ipetC1 (19) ^{ac}	gTTgTgTgggCaggAATgg	<i>petC2</i>
ipetC2 (20) ^{ac}	CCgCACgAAgCAgTTTCCC	<i>petC2</i>
Hip9 (21) ^{ac}	AgTgAgAAACTgAAggAAAC	<i>hip</i>
Hip10 (22) ^{ac}	gCgggCATCggCgTAAAC	<i>hip</i>
cycA1-1 ^c	gCTCCCTATCTggTCAAAAC	<i>cycA1</i>
cycA1-2 ^c	CTCgTCgTTgACCTgCCAAg	<i>cycA1</i>
sdrA1-1 ^c	gTTTgggTgCTgAAgTAGTg	<i>sdrA1</i>
sdrA1-2 ^c	CgTTgTCACCgTTTgTCCg	<i>sdrA1</i>
rpetA1 ^c	TgACAACAgTTACAgATTcgg	<i>petA1</i>
rpetA2 ^c	TAgCACTCATCTTCTCTgTgg	<i>petA1</i>
rpetB1 ^c	TTCCCTATTATTCTCgCAgCC	<i>petB1</i>
rpetB2 ^c	AAACCCCGCAACAATAAgC	<i>petB1</i>
rpetC1 ^c	TATCTATggTTTTgTTggTg	<i>petC1</i>
rpetC2 ^c	CTTTgTATgggTATTACTAC	<i>petC1</i>

*The superscript letters indicate the use(s) of the primer: a, PCR; b, RT-PCR; c, real-time PCR.

AJ413192 (*petC1* internal region), AJ413171 (intergenic region *petC1-resB*), AJ413193 (*resB* internal region) and AJ413194 (intergenic region *resB-resC*).

The GenBank/EMBL database accession numbers for the *petII* operon sequences from *A. ferrooxidans* ATCC 33020 are: AJ427631 (5' untranslated region upstream from *cycA2*), AM261984 (*cycA2* internal region), AJ318500 (intergenic region *cycA2-sdrA2*), AM261984 (*sdrA2*

internal region), AJ311888 (intergenic region *sdrA2-petA2*), AJ413195 (*petA2* internal region), AJ311889 (intergenic region *petA2-petB2*), AJ318501 (*petB2* internal region), AJ413196 (intergenic region *petB2-petC2*) and AJ320262 (*petC2-hip* region).

Bioinformatic techniques. Potential sigma-70-like promoters were detected using an HMM model trained on *A. ferrooxidans* sigma-70-like promoters (M. Santa Ana, J. Valdes, M. Chacon, T.

L'Heureaux, E. Jedlicki, & D. S. Holmes, unpublished results). Potential transcription factor binding sites were searched for using MAT inspector (Cartharius *et al.*, 2005) and information theory (Schneider, 1999). Potential rho-independent translational stop sites were detected according to de Hoon *et al.* (2005).

RESULTS

Genetic organization and conservation of the *petII* operon in multiple strains of *A. ferrooxidans*

Using information derived from the fully sequenced genome of the type strain *A. ferrooxidans* ATCC 23270 (<http://www.tigr.org/>), DNA primers were designed for each gene and intergenic region of the *petII* operon. The sizes of the resulting amplified fragments suggest that the organization of the *petII* operon previously described in the type strain of *A. ferrooxidans* ATCC 23270 (Brasseur *et al.*, 2002) is conserved in *A. ferrooxidans* ATCC 33020 (Fig. 1a, b) and *A. ferrooxidans* ATCC 19859 (data not shown). The sequences of the PCR fragments obtained from the ATCC 33020 strain display higher identity to the *petII* than to the *petI* locus from ATCC 23270 (data not shown). Taken together, these results

indicate that, as in ATCC 23270, there are two *pet* loci in ATCC 33020 and ATCC 19859.

The *petII* gene cluster constitutes an operon

RT-PCR experiments were performed with convergent oligonucleotide primers designed from two adjacent genes of the ATCC 23270 *petII* locus (see Table 1 and Fig. 1) on total RNA extracted from ATCC 33020 grown with ferrous iron (Fig. 1c). RT-PCR products of the expected size were generated between each gene pair of the *petII* cluster, suggesting that it is an operon.

A candidate transcriptional start site of the *petII* operon was detected by reverse-transcriptase-mediated primer extension experiments (Fig. 2a). This was compared to the predicted start site of the *petI* operon using similar techniques (Fig. 2b and data not shown). In both cases putative sigma-70-like promoter sequences were detected by bioinformatic techniques upstream of the respective transcriptional start sites (Fig. 2c). Transcription from the *petI* promoter was detected only in iron-grown cells and transcription from the *petII* promoter in iron- and in sulfur-grown cells (Fig. 2a, b), which was in agreement with

Fig. 1. Characterization of the *petII* operon in *A. ferrooxidans* ATCC 33020. (a, b) PCR amplification of (a) intergenic fragments and (b) gene internal fragments from the *petII* locus; (c) RT-PCR amplification of the intergenic regions of the *petII* locus. The locations of the oligonucleotides used for PCR and RT-PCR experiments are shown above and below the map of the *petII* operon. The sequences of the oligonucleotides are given in Table 1. *L_R*, DNA molecular mass marker X from Roche; *L₁*, 1 kb Plus DNA ladder from Invitrogen.

Fig. 2. (a, b) Reverse-transcriptase-mediated primer-extension experiments. Reverse transcription experiments were performed with specific primers from (a) the *petII* (23 and 24) and (b) the *petI* (25 and 26) operons of *A. ferrooxidans* ATCC 33020: one internal to the first gene of the operon (23 and 25) and one located in the 5' untranslated region of the operon (24 and 26). Total RNA was extracted from early-exponential iron-grown cells (lane F), or mid-exponential sulfur-grown cells (lane S). RT products are indicated with arrows. A sequence ladder, produced with primers 24 for the *petII* operon and 26 for the *petI* operon, is shown to the left of the RT products (lanes T, G, C and A). The DNA sequences are shown on the right. Possible transcriptional start sites are indicated with an asterisk (*). (c) The 5' untranslated regions of the *petII* and *petI* operons are shown with the putative -35 and -10 regions in bold underlined lower case, and transcriptional start sites in bold upper case.

Northern blot hybridization and real-time PCR experiments described below.

Regulation of the *petI* and *petII* operons

Northern blotting and real-time PCR experiments were carried out to determine the relative levels of expression of the *petI* and *petII* operons in cells grown in either iron or sulfur medium.

Antisense RNA probes complementary to the *cycA1* and *hip* genes were hybridized to total RNA extracted from *A. ferrooxidans* ATCC 33020 grown in iron or sulfur medium. The largest transcript detected with the *cycA1* probe was 4000 nt long (Fig. 3a), which suggested that the *petI* operon includes transcripts that correspond to the full length operon, confirming results obtained from RT-PCR experiments (Levicán *et al.*, 2002). Smaller transcripts were also observed. Because the *cycA1* probe corresponds to the first gene of the *petI* operon, these data suggest that the largest transcript is processed. With the *hip* probe, the largest transcript detected was 4600 nt long (Fig. 3b), again potentially corresponding to a full-length transcript of the operon. Smaller transcripts were also observed, perhaps resulting from RNA processing or transcription from internal promoters. However, no internal promoters were

detected upstream of *hip* by reverse transcriptase-mediated primer extension experiments (data not shown), reducing the likelihood of this explanation.

The results of Northern blotting experiments indicated that the *petI* and *petII* operons are differentially expressed. Using the same amount of total RNA, the hybridization signal is clearly much more intense for *cycA1* when cells are grown in iron versus sulfur medium (Fig. 3a). However, *hip* appears to be transcribed under both conditions, with slightly more transcripts in sulfur medium (Fig. 3b). This suggests that the *petI* and *petII* operons are regulated in response to the energetic substrate.

To corroborate the Northern blot results, the amount of transcripts corresponding to *petI* and *petII* was quantified at all stages of the cell growth by real-time PCR. The results are shown in Table 2. The *petI* and *petII* pattern of expression in response to the energetic substrate was similar to that detected by Northern blotting, with much higher expression of *petI* in iron- than in sulfur-grown cells and expression of *petII* in sulfur- and in iron-grown cells. In addition, several interesting points were noticed: (i) as shown for the *rus* operon (Yarzabal *et al.*, 2004), the amounts of all the *petI* transcripts decreased significantly after 3 days of growth in ferrous-iron-grown cells when complete oxidation of

Fig. 3. Northern blot hybridization analysis. Total ATCC 33020 *A. ferrooxidans* RNA from early-exponential iron-grown cells or mid-exponential sulfur-grown cells probed with DIG-labelled *cycA1* (a) or *hip* (b) RNA. Arrows show the largest transcripts. The positions and sizes of the RNA ladder (Invitrogen) are indicated on the left of each blot.

ferrous iron to ferric iron had occurred; (ii) the *petII* operon was expressed in ferrous iron-grown cells, mainly in the early exponential phase; (iii) *hip* transcripts were clearly more abundant in sulfur- than in iron-grown cells. *hip*

may have an additional regulatory mechanism, which we propose is at a post-transcriptional level because no internal promoter could be detected upstream of *hip* by primer extension (data not shown).

Table 2. Quantification of the *petI* and *petII* transcripts by real-time PCR in ferrous iron (F)- and sulfur (S)-grown cells at different stages of growth

Total RNA was extracted from ferrous-iron-grown cells at 17 h (early-exponential phase), 1 day (mid-exponential phase), 2 days (late-exponential phase) and 3 days (stationary phase). Total RNA was extracted from sulfur-grown cells at 1 day (early-exponential phase), 2, 3 and 4 days (mid-exponential phase), and 5 days (late-exponential phase). All values (except for *rrs*, encoding the 16S rRNA) are expressed as *n*-fold relative to 16S rRNA ($\times 10^6$). The data are representative of real-time PCR experiments performed on at least two independent cultures. The experiment shown used single cultures and the values are the mean of duplicate assays that almost always varied by less than 25%.

Gene	Growth conditions:								
	F				S				
	17 h	1 d	2 d	3 d	1 d	2 d	3 d	4 d	5 d
<i>rrs</i>	1	1	1	1	1	1	1	1	1
<i>sdrA1</i>	600.9	761.2	302.1	190.3	8	1.3	1.3	1.8	1.2
<i>petA1</i>	609.9	627	149.7	110.9	3.1	1.1	0.6	0.8	0.8
<i>petB1</i>	1119	1383.1	360.2	115.1	4.9	4.3	5.3	4.7	4
<i>petC1</i>	39.8	348.6	23.9	3	0.2	0.03	0.1	0.4	0.1
<i>cycA2</i>	87	553.9	14.3	41.2	134.9	94.1	54	25	36.8
<i>sdrA2</i>	154.7	573.1	16.6	85.4	439.5	144.9	89.4	26.4	54.3
<i>petA2</i>	103.9	704.3	21.8	33.3	90.6	39.3	28.9	24.6	34
<i>petB2</i>	143.5	639.2	33.8	84	179.8	110.4	61.4	52	107.7
<i>petC2</i>	84.5	331.3	11.4	32.3	75.9	42.7	22.8	29	36.4
<i>hip</i>	158.4	959.3	134.3	155.1	1806.9	1482.5	601.1	83.6	95.7

To explore possible mechanisms involved in the differential expression of the *petI* and *petII* operons depending on energy source, the 5' untranslated regions of both operons were compared using MAT inspector. No obvious conserved regulatory motifs were detected.

DISCUSSION

Two differentially expressed operons encoding *bc₁* complexes in *A. ferrooxidans*

Bioinformatic analysis, PCR experiments and DNA sequence analysis indicate that the genomes of three strains of *A. ferrooxidans*, ATCC 19859, ATCC 33030 and the type strain ATCC 23270, contain two operons, *petIABC* and *petIIABC*, encoding two complete cytochrome *bc₁* complexes. Both are cotranscribed with *cycA* and *sdrA*, which encode a *c₄*-type cytochrome and a short-chain dehydrogenase respectively.

Data from primer extension, Northern blotting and real-time PCR experiments (Figs 2 and 3, Table 2) and preliminary transcriptome analysis (Quatrini *et al.*, 2006) demonstrate that *petI* and *petII* are differentially transcribed depending on the growth conditions. The *petI* operon is principally transcribed only when ferrous iron is provided in the growth medium as an energy and electron source. In contrast, the *petII* operon is transcribed in sulfur- or iron-grown cells. Transient transcription is observed from the *petII* operon when cells are first placed in ferrous iron medium but at later stages of cell growth significantly less expression is detectable (Table 2), a situation that is reminiscent of the transient expression of the *rus* operon

(Yarzabal *et al.*, 2003, 2004). Although no explanation for this low amount of transient expression has been experimentally validated, it has been speculated that, in the case of the *rus* operon, it could be a response to an increase in particular nutrients to allow quick adaptation to the environment, perhaps mediated by a Fis-like protein (Yarzabal *et al.*, 2004). However, no obvious Fis-binding DNA motif, which is particularly degenerate (Hengen *et al.*, 1997), was detected in the proposed regulatory region of *petII*.

Given that *petI* is induced in ferrous iron medium, a computational search was carried out for known iron-regulated transcription factor binding sites in the region upstream of *cycA1* where a sigma-70-like promoter is predicted (Fig. 2c). No obvious Fur-binding site (Lavrrar & McIntosh, 2003) could be detected, although the *A. ferrooxidans* genome is known to encode Fur and has been shown experimentally to have Fur-binding sites upstream of known iron-regulated genes (Quatrini *et al.*, 2005). Computational analysis also failed to detect significant similarity in the *A. ferrooxidans* genome with the two-component system PmrA–PmrB (Wosten *et al.*, 2000), and no *cis*-acting iron-responsive operators (IROs) of the RirA regulatory system (Todd *et al.*, 2005) could be detected upstream of either the *petI* or the *petII* operons, leaving open, for the moment, the question of how these operons are differentially regulated.

Proposed role for the redox proteins encoded by the *petI* and *petII* operons

A. ferrooxidans is the first organism described so far to have two complete and functional *bc₁* complexes, raising the

Fig. 4. Proposed model of ferrous iron [Fe(II)] and sulfur (S⁰) energetic metabolism of *A. ferrooxidans*. The transcriptional units and the corresponding redox proteins are represented with the same background patterns. Transcriptional start sites and putative rho-independent transcriptional termination sites are represented by bent arrows and stem-loops, respectively.

question of why this is necessary. Genetic evidence has been presented for the existence of a cytochrome-containing complex functioning exclusively during iron oxidation (Cabrejos *et al.*, 1999; Levicán *et al.*, 2002) and it has been reported that a *bc₁* complex functions only in reverse in iron-grown cells (Elbehti *et al.*, 2000), even in the presence of an appropriate substrate (Brasseur *et al.*, 2002). On the other hand the existence of a *bc₁* complex has been proposed to be involved in the aerobic and anaerobic oxidation of sulfur and formate processes (Corbett & Ingledew, 1987; Pronk *et al.*, 1991) and a *bc₁* complex has been shown recently to function in direct mode in sulfur-grown cells (Brasseur *et al.*, 2004). This raises the possibility that two operationally independent *bc₁* complexes are needed to cope with iron and sulfur oxidation, respectively: one for uphill flow during iron oxidation and the other for downhill flow during sulfur oxidation. A corollary of this is that neither of the two complexes can switch the direction of electron flow. Whether this imposition comes from intrinsic mechanistic differences in the sequence and structure of the two *bc₁* complexes that specify unidirectional flow of electrons or from the action of additional, as yet unknown, structural or regulatory components that could channel electrons to the correct *bc₁* complex, remains to be determined.

Our hypothesis is that the *bc₁* complex encoded by *petI* is the one functioning in reverse and transfers the electrons from ferrous iron to NAD(P), while the *bc₁* complex encoded by *petII* is the one functioning directly, transferring electrons from sulfur to oxygen and possibly involved in the aerobic and anaerobic oxidation of sulfur and formate described by Pronk *et al.* (1991).

Together with the three subunits of the *bc₁* complex, the *petI* and *petII* operons encode other redox proteins. Because these genes are in the same transcriptional unit, these proteins are probably involved in the same electron-transfer chain. The *sdrA1* gene has been predicted to encode a short chain dehydrogenase (Levicán *et al.*, 2002) but its function remains unknown. The cytochrome *c₄* encoded by the *cycA1* gene has been proposed to belong to the electron-transfer chain between ferrous iron and oxygen, and more precisely to receive the electrons directly from ferrous iron and to transfer them to rusticyanin (Giudici-Orticoni *et al.*, 2000). However, according to the data presented here, this cytochrome *c₄* is more likely to be involved in the reverse electron pathway between ferrous iron and NAD(P) because *cycA1* belongs to the *petI* operon, and because the *rus* operon contains another cytochrome *c₄* encoding gene (*cycI*) that has been suggested (Appia-Ayme *et al.*, 1999) to assume the role postulated by Giudici-Orticoni *et al.* (2000). In the reverse electron pathway the *bc₁* complex receives the electrons from a cytochrome *c* and transfers them to the quinol pool. We suggest that this cytochrome *c* is the cytochrome *c₄* encoded by the *cycA1* gene (Fig. 4). Oxygen and NAD(P) reduction have been proposed to be coupled to balance the reducing equivalent from ferrous iron between the two pathways: the exergonic one, through the *aa₃*-type

oxidase towards oxygen, and the endergonic one, through a *bc₁* complex toward NAD(P). As previously reported (Brasseur *et al.*, 2004), we propose that the branching point is at the level of rusticyanin, which can give electrons to two different cytochromes *c₄*: *CycA1* encoded by the *petI* operon or *CycI* encoded by the *rus* operon. In the former case, electrons are transferred to NAD(P), while in the latter case, they are transferred to oxygen (Fig. 4).

When functioning in direct mode, the *bc₁* complex receives electrons from the quinol pool and transfers them either to a membrane-bound cytochrome *c*, and/or to a soluble redox protein such a cytochrome *c*, or a high potential iron-sulfur protein (HiPIP) that subsequently passes the electrons to the terminal oxidase where oxygen reduction takes place (Hunte *et al.*, 2003 and references therein; Bonora *et al.*, 1999; Pereira *et al.*, 1999). Since a membrane-bound cytochrome *c* (*CycA2*) and a soluble HiPIP are encoded by the *petII* operon, we suggest that these two redox proteins transfer electrons between integral membrane complexes: *bc₁* and a terminal reductase, in particular a quinol oxidase (Brasseur *et al.*, 2004). An interesting possibility is that the electrons are transferred either to the cytochrome *CycA2* or to the HiPIP Hip depending on the environmental conditions (Fig. 4). Since *hip* is transcribed more in sulfur- than in ferrous iron-growth conditions it is proposed that Hip is involved in the electron transfer from sulfur.

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