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Regulation of the expression of the *Acidithiobacillus ferrooxidans rus* operon encoding two cytochromes *c*, a cytochrome oxidase and rusticyanin

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The regulation of the expression of the *rus* operon, proposed to encode an electron transfer chain from the outer to the inner membrane in the obligate acidophilic chemolithoautroph *Acidithiobacillus ferrooxidans*, has been studied at the RNA and protein levels. As observed by Northern hybridization, real-time PCR and reverse transcription analyses, this operon was more highly expressed in ferrous iron- than in sulfur-grown cells. Furthermore, it was shown by immunodetection that components of this respiratory chain are synthesized in ferrous iron- rather than in sulfur-growth conditions. Nonetheless, weak transcription and translation products of the *rus* operon were detected in sulfur-grown cells at the early exponential phase. The results strongly support the notion that *rus*-operon expression is induced by ferrous iron, in agreement with the involvement of the *rus*-operon-encoded products in the oxidation of ferrous iron, and that ferrous iron is used in preference to sulfur.

INTRODUCTION

The respiratory flexibility found in Bacteria and Archaea, resulting from complex multicomponent branched electron-transfer systems, allows these micro-organisms to colonize a wide range of biotopes. The genes that encode electron-transfer proteins belonging to the same respiratory system are often organized as operons, whose expression is modulated depending on the growth conditions, thus allowing rapid adaptation to environmental changes.

Acidithiobacillus ferrooxidans is a Gram-negative, acidophilic bacterium, which thrives in harsh environments and exhibits a versatile energy metabolism. Although this obligate chemolithoautotroph obtains its energy mainly from the oxidation of ferrous iron, Fe(II), or various reduced sulfur compounds (Leduc & Ferroni, 1994; Rohwerder *et al.*, 2003), it can also use hydrogen or formate in oxic conditions (Drobner *et al.*, 1990; Pronk *et al.*, 1991). Moreover, under anoxic conditions, it is able to reduce ferric iron, Fe(III), with sulfur (S^0) or hydrogen as electron donors (Das *et al.*, 1992; Ohmura *et al.*, 2002; Pronk *et al.*, 1992). The respiratory chains involved in these redox reactions have not been clearly established. Nevertheless, three operons encoding redox proteins have been characterized in *A. ferrooxidans* (Appia-Ayme *et al.*, 1999; Levicán *et al.*, 2002; P. Bruscella and others, unpublished data). One of these, the *rus* operon, encodes two *c*-type cytochromes, Cyc1 and Cyc2 (*cyc1* and *cyc2*), an ORF of unknown function (*orf*), an aa_3-type cytochrome oxidase (*coxBACD*) and rusticyanin (*rus*) (see Fig. 6) (Appia-Ayme *et al.*, 1999). Based on the operon organization, the subcellular localization of the corresponding redox proteins (Yarzábal *et al.*, 2002a, b) and interaction studies (Giudici-Orticoni *et al.*, 1999), we have proposed the following electron transfer pathway: Cyc2→Rus→Cyc1→cytochrome oxidase→O_2. Because Rus has been shown to constitute up to 5% of total soluble proteins in Fe(II)-grown cells (Cobley & Haddock, 1975; Cox & Boxer, 1978), this protein, and therefore the electron transfer chain presented above, have been proposed to be involved in Fe(II) oxidation (Appia-Ayme *et al.*, 1999).

However, Rus has also been detected in S^0-grown cells (Cox & Boxer, 1986; Espejo *et al.*, 1988; Osorio *et al.*, 1993; Bengrine *et al.*, 1995, 1997, 1998; Yarzábal *et al.*, 2001, 2003). Furthermore, the Fe(II) oxidation activity has been reported to be maintained over several generations in cells shifted from Fe(II) to S^0 medium (Margalith *et al.*, 1966; Sugio *et al.*, 1986; Espejo *et al.*, 1988; Suzuki *et al.*, 1990; Mansch & Sand, 1992). These apparently contradictory data may reflect (i) a variability among *A. ferrooxidans* strains, (ii) a role for Rus in S^0 metabolism and/or (iii) a complex

regulation of *rus*-operon expression. An argument in favour of the last hypothesis is that at least three putative promoters have been characterized for the *rus* operon (see Fig. 6), two upstream from *cyc2* and one between *coxD* and *rus* (Bengrine *et al.*, 1998; Appia-Ayme *et al.*, 1999). Therefore, to gain further insight into its regulation, the expression of the *rus* operon in the ATCC 33020 strain was studied under various growth conditions, at both the transcriptional and translational levels. Our results show that the expression of the *rus* operon is regulated at the transcriptional level by ferrous iron. They also suggest that the expression of this operon is regulated at the post-transcriptional level and that, furthermore, transient expression of the *rus* operon takes place during the early exponential phase of S^0-grown cells.

METHODS

Strains, plasmids and culture conditions. *A. ferrooxidans* ATCC 33020 was obtained from ATCC. *Escherichia coli* TG1 strain [*supE hsd*Δ*5 thi* Δ(*lac–proAB*) F′: *traD36 proAB lacI*^{q} *lacZ*Δ*M15*] was used for plasmid propagation. BL21(DE3) strain [F^- *ompT hsdS*$_B$ (λc*Its*857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene*1*)] and the plasmids pET22b and pET21, used to produce recombinant proteins, were purchased from Novagen. Phagemid Bluescript SK^+ was purchased from Stratagene. The plasmid pEC86, which constitutively expresses the *E. coli ccm* operon encoding the cytochrome *c* maturation system (Arslan *et al.*, 1998), was kindly provided by L. Thöny-Meyer. *E. coli* strains were grown in L broth (LB; Ausubel *et al.*, 1987). *A. ferrooxidans* ATCC 33020 was grown at 30 °C under oxic conditions in Fe(II) medium or S^0 medium, as previously described (Yarzábal *et al.*, 2003), and in Fe(II) + S^0 medium consisting of 1 % (w/v) elemental S^0 and 3·5 % (w/v) $FeSO_4$ in basal salts solution [1·6 g $(NH_4)_2SO_4$ l^{-1}, 1·6 g K_2HPO_4 l^{-1}, 1·6 g $MgSO_4.7H_2O$ l^{-1}], adjusted to pH 1·6 with H_2SO_4. Usually, 99 % purity minimum S^0 (Prolabo) was used. However, in some experiments, extra-pure S^0 (Fluka) was used to avoid contamination of the S^0 medium with Fe(II) traces. In both cases, no Fe(II) was detected in S^0 medium by *o*-phenanthroline analysis (Muir & Anderson, 1977).

Liquid cultures of S^0- and/or Fe(II)-adapted cells were carefully washed with the respective basal salts solution, before inoculating a fresh medium. Growth was followed by direct cell counting in a Neubauer chamber. Samples were removed at 1 day intervals from the different cultures and centrifuged at 15 000 ***g*** for 5 min. Pelleted cells were stored at −20 °C for further studies.

DNA manipulations. General DNA manipulations were performed according to standard procedures (Ausubel *et al.*, 1987). *A. ferrooxidans* genomic DNA was prepared with the NucleoSpin Tissue kit (Macherey-Nagel). YellowStar DNA polymerase (Eurogentec) was used for PCR amplifications. The nucleotide sequences of the cloned fragments were determined by GENOME express. *A. ferrooxidans* ATCC 23270 preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org.

Plasmid construction. All the plasmid constructions are summarized in Table 1. For the construction of expression plasmids to produce wild-type polypeptides fused to a hexa-histidine tag at the C-terminus, the DNA fragment corresponding to the sequence of the peptide was amplified by PCR as far as the last codon upstream from the translational termination site. For *cyc2*, *cyc1* and *orf*, the cloned fragments started upstream from the Shine–Dalgarno sequence. For *coxB*, the peptide corresponding to the periplasmic domain of CoxB was fused at its NH_2-terminus to the PelB signal sequence from the pET22 vector. In this case, the Shine–Dalgarno sequence and the translational start site were both provided by the vector. The 'upstream' oligonucleotides contained a *Bam*HI restriction site, and the 'downstream' oligonucleotides a *Xho*I site (Table 2), to allow cloning of the amplified products in the correct orientation in *Bam*HI- and *Xho*I-digested pET21 or pET22 vectors (Table 1).

For the construction of the plasmids from which RNA probes were synthesized, an internal fragment of the gene of interest was amplified by PCR (Table 2) and cloned between the T7 and the T3 promoters in the *Eco*RV site of the Bluescript SK^+ vector (Table 1).

All these constructions were checked by nucleotide sequencing.

Biochemical methods. Protein analyses were performed as previously described (Yarzábal *et al.*, 2003). The primary antibodies used in immunodetection studies were: (i) mouse anti-His (Invitrogen); (ii) rabbit anti-Rus serum, kindly provided by M. Bruschi; (iii) mouse anti-Omp40 serum, kindly provided by N. Guiliani; and (iv) rabbit anti-Cyc2–HisTag, anti-Cyc1–HisTag, anti-ORF–HisTag and anti-CoxB–HisTag sera (see below).

Table 1. Plasmids constructed in this study

See Table 2 for description of oligonucleotides.

Expression plasmid	Vector	Insertion site	Oligonucleotides
pET21*cyc2*-HisTag	pET21	*Bam*HI–*Xho*I	cyc2N-*Bam*HI; cyc2C-*Xho*I
pET21*cyc1*-HisTag	pET21	*Bam*HI–*Xho*I	*B*ass; cyc1C-*Xho*I
pET21*orf*-HisTag	pET21	*Bam*HI–*Xho*I	ORFN-*Bam*HI; ORFC-*Xho*I
pET22-*coxB*P-HisTag	pET22	*Bam*HI–*Xho*I	coxBp-*Bam*HI; coxBC-*Xho*I

Transcription plasmid	Vector	Insertion site	Oligonucleotides	Linearization site	RNA polymerase
SK-*cyc2*	Bluescript SK^+	*Eco*RV	verif2; Averif1	*Hin*dIII	T3 RNA polymerase
SK-*coxB*	Bluescript SK^+	*Eco*RV	av3; Aav4	*Hin*dIII	T3 RNA polymerase
SK-*rus*	Bluescript SK^+	*Eco*RV	RSD*Bam*HI; RusC*Xho*I	*Eco*RI	T7 RNA polymerase

Table 2. Oligonucleotides used in this study

Restriction sites are shown in italics. Nucleotides have been added between this site and the 5′ end of the oligonucleotide according to the restriction endonuclease requirement. The translational start site is underlined with a solid line, and the Shine–Dalgarno sequence is underlined with a dotted line. Position refers to EMBL nucleotide sequence AJ006456 for the *rus* operon, X95571 for the *alaS* gene and AJ278719 for the *rrs* (16S rRNA) gene. (+), Coding strand orientation; (−), non-coding strand orientation.

Gene	Primer	Sequence	Position and orientation
Expression plasmid construction			
cyc2	cyc2N-*Bam*HI	5′-AA*GGATCC*GCGTATTTTGTTTATCTAATATGCC-3′	396–420 (+)
cyc2	cyc2C-*Xho*I	5′-AAA*CTCGAG*GTATGATATCCAGGCCAGCAGTTCAAGG-3′	1886–1913 (−)
cyc1	*B*ass	5′-AA*GGATCC*AATAAAGAGAACGACACGATGACC-3′	1961–1984 (+)
cyc1	cyc1C-*Xho*I	5′-AAA*CTCGAG*CAGCGATGAAAGATAAGCCGCCACATCC-3′	2590–2617 (−)
orf	ORFN-*Bam*HI	5′-AA*GGATCC*CTCAAAGGAGGTAGAGGTATGGCAGC-3′	2713–2738 (+)
orf	ORFC-*Xho*I	5′-AAA*CTCGAG*TGAAGTGTTGACCCGTCTGCAGAAAACACC-3′	3203–3232 (−)
coxB	coxBp-*Bam*HI	5′-AA*GGATCC*GGGTCTGGAACAACTTTGGGGTATCC-3′	3656–3681 (+)
coxB	coxBC-*Xho*I	5′-AAA*CTCGAG*GAAGCTATTGTTTGCCACCCAAGCC-3′	4034–4058 (−)
Transcription plasmid construction			
cyc2	verif2	5′-ATCAGCAAAGCAGGCGGCCCC-3′	1437–1457 (+)
cyc2	Averif1	5′-AAGCGGGTATTCCACCACGGC-3′	1775–1795 (−)
coxB	av3	5′-CGTATTCATCTATGTGCTGGTCGG-3′	3473–3496 (+)
coxB	Aav4	5′-ATGTCTCGTTGCCACAAATCAAGG-3′	3964–3986 (−)
rus	RSD*Bam*HI	5′-TT*GGATCC*TAAGGAGAAGGGATAACTTATG-3′	7157–7178 (+)
rus	RusC*Xho*I	5′-CCA*CTCGAG*CCTTGACAATGATTTTACCAAACATAC-3′	7711–7737 (−)
Real-time PCR			
rrs (16S)	16S-G	5′-ACACTGGGACTGAGACACGG-3′	277–296 (+)
rrs (16S)	16S-D	5′-ACCGCCTACGCACCCTTTAC-3′	534–553 (−)
alaS	alaS13	5′-GTGCCTTTCCCGAACTCACG-3′	1681–1700 (+)
alaS	alaS3	5′-TCCTCCAGCAGACTGAGTCC-3′	1770–1789 (−)
cyc2	verif1	5′-TGTTGGTGTCTATGGGGCGG-3′	1169–1188 (+)
cyc2	Ainv1	5′-ACCCCAATCGTGCTGATAGGC-3′	1338–1358 (−)
cyc1	C87	5′-CCGCCGATGCAAAGCAGC-3′	2330–2347 (+)
cyc1	cyc1C-*Xho*I	5′-AAA*CTCGAG*CAGCGATGAAAGATAAGCCGCCACATCC-3′	2590–2617 (−)
orf	ORF-G	5′-GACTACGGTGCTTGTATCC-3′	2751–2769 (+)
orf	ORF-D	5′-AGCCTGTGATTTTGGAAAG-3′	3027–3045 (−)
coxB	coxB-G	5′-CATCAACCTGGCAAATACC-3′	3620–3638 (+)
coxB	Aav4	5′-ATGTCTCGTTGCCACAAATCAAGG-3′	3964–3986 (−)
coxA	Av6	5′-ATGTTGTTGGGTGTGGTGGC-3′	4604–4623 (+)
coxA	rusAm5	5′-CACCATCCCGTCTGAAACCCG-3′	4768–4788 (−)
coxC	RuAm	5′-TCAGCCTGGTGCCGGTGAC-3′	6213–6234 (+)
coxC	RΔ5	5′-GCTGGTGATGTCATAAATCCC-3′	6371–6391 (−)
rus	RusNm	5′-GGCACGCTGGATTCCACATGGAAAGAGGCG-3′	7272–7301 (+)
rus	R6	5′-ACTTCAAAGCTCGGGAACGG-3′	7419–7438 (−)
Primer extension			
Promoter	**Primer**	**Sequence**	**Position and orientation**
PI	Ainv3	5′-ACACGTTCTATTTAATACAAACCG-3′	267–290 (−)
PII	Ainv6	5′-GCTCATGCGCCCGGTCTTCCTGCC-3′	374–397 (−)
P_{orf}	orf-inv	5′-ATACAAGCACCGTAGTCATACC-3′	2746–2767 (−)
P_{rus}	RΔ2	5′-GCCCTACAAAACCATCACCG-3′	7112–7131 (−)

Amino acid sequence determinations were carried out with an Applied Biosystems gas-phase sequenator (model 473A) at the Protein Sequencing Unit of the IBSM.

Production and purification of His-tagged recombinant proteins. The BL21(DE3) strains carrying pET22-*coxB*P-HisTag, pET21*orf*-HisTag, pET21*cyc2*-HisTag and pEC86, or

pET21*cyc1*-HisTag and pEC86, were grown at 37 °C in LB with 50 μg ampicillin ml^{-1} and 25 μg chloramphenicol ml^{-1}, when required, to an OD_{600} of 1·0. IPTG was then added to the cultures to a final concentration of 1 mM, and the cultures were grown for a further 2 h. The cells were harvested, washed once with 40 mM sodium phosphate, pH 7·2, and stored at −20 °C until use. The cell pellets from 400 ml culture were suspended in 40 ml freshly prepared buffer I [20 mM sodium phosphate, pH 7·2; 0·15 mM phenyl-methyl-sulfonyl fluoride (PMSF); 5 mM benzamidine], and passed three times through a French Press. Cyc2, Cyc1, ORF and CoxB were mainly present in inclusion bodies. Inclusion bodies, unbroken cells and cellular debris were pelleted by centrifugation at 10 000 ***g*** for 20 min, suspended in 8 ml freshly prepared buffer I containing 2 % Triton X-100, incubated on ice for 2 h, and centrifuged for 20 min at 10 000 ***g***. The pellet was suspended in 20 ml freshly prepared buffer I containing 1 % Triton X-100, and immediately centrifuged at 10 000 ***g*** for 20 min. The pellet corresponding to the inclusion bodies was kept at −20 °C. Solubilization of inclusion bodies with urea and purification of the His-tagged recombinant proteins were performed with the HisTrap kit (Amersham Pharmacia), according to the manufacturer's instructions. The fractions eluted from Ni^{2+} columns were analysed by immunodetection with antibodies directed against the HisTag and by NH_2-terminus sequencing.

Production of specific antisera. The purified His-tagged recombinant proteins were electrophoresed in 15 % acrylamide gels. The bands corresponding to the His-tagged proteins were detected by $CuCl_2$ staining (Lee *et al.*, 1987) and excised from the gel. Gel strips were crushed and used to immunize New Zealand SSC rabbits, following standard procedures.

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

RNA manipulations. *A. ferrooxidans* total RNA was prepared from 500 ml of S^0 or Fe(II) culture, as described previously (Guiliani *et al.*, 1997). The Northern blotting protocol was as described in Bengrine *et al.* (1998). The DIG-labelled RNA probes hybridizing to *rus*, *cyc2* and *coxB* mRNA were obtained from the SK-*rus*, SK-*cyc2* and SK-*coxB* plasmids described in Table 1. Following linearization with *Eco*RI or *Hin*dIII, *in vitro* transcription with T7 or T3 RNA polymerase was performed on these plasmids, using DIG-UTP as substrate, according to the instructions of the Strip-EZ kit (Ambion) (Table 1). RNA was detected by a chemiluminescent reaction with disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3·3·1·$1^{3,7}$]decan}-4-yl) phenyl phosphate (CSPD; Roche), following the manufacturer's instructions.

Primer extension experiments were performed with the Superscript II RNase H^- reverse transcriptase (InVitro Life Technologies), as previously described (Bengrine *et al.*, 1998; Appia-Ayme *et al.*, 1999), with Ainv3, Ainv6, orf-inv, RΔ2 and $P_{orf\text{-}inv}$ oligonucleotides (Table 2). The experiments were performed in duplicate, using RNA samples from independent cultures.

Real-time PCR. The relative abundances of *cyc2*, *cyc1*, *orf*, *coxB*, *coxA*, *coxC* and *rus* transcripts in Fe(II)- and S^0-grown cells were determined by real-time PCR with the LightCycler instrument and the LightCycler-FastStart DNA Master SYBR Green 1 kit (Roche), with external standards as described in Roche Molecular Biochemicals technical note no. LC 11/2000. The *alaS* gene, encoding alanyl tRNA synthetase (Guiliani *et al.*, 1997), and *rrs* (16S rRNA) genes were shown to be expressed at the same (constitutive) level under both conditions of growth (data not shown), and were therefore used as reference standards. Primer pairs are given in Table 2. The real-time PCR quantification was performed twice, using RNA samples from independent cultures.

RESULTS AND DISCUSSION

Expression of the *rus* operon is dependent on the energetic substrate

To determine the relative abundance of products encoded by the *rus* operon during growth, Fe(II) or S^0 cells were used to inoculate Fe(II), S^0 and/or Fe(II) + S^0 medium. Aliquots were removed every day and cells pelleted. Time-course analysis of protein levels was monitored by immunodetection (Figs 1 and 2, and data not shown).

No change in electron donor (Fe(II)- or S^0-adapted cells)

When exponentially Fe(II)-grown cells were inoculated into Fe(II) medium, Cyc1, CoxB and Rus were detected throughout the growth cycle, and their levels remained almost unchanged after complete oxidation of Fe(II), that is after 2–3 days of culture (Fig. 1a), and even after 16 days (data not shown). Because the Fe(II) medium used was buffered to slow down the precipitation of ferric salts, the pH of the medium did not vary significantly (1·6–1·8 after 4 days of growth). As expected, growth stopped when no more Fe(II) was available, though the proteins tested were still present, suggesting that they are very stable. When exponentially S^0-grown cells (3–4 days) were inoculated into S^0 medium, Rus was detected only during the early exponential phase, though its concentration was significantly lower than the level detected in Fe(II)-grown cells (Fig. 1a). Indeed, semi-quantitative analysis from Western blot experiments showed that the highest amount of Rus in S^0-grown cells (i.e. cells from day 1 in S⟶S cultures in Fig. 1a) never exceeded 20 % of the amount observed in Fe(II)-grown cells. Cyc1 and CoxB were also detected during the exponential phase in S^0-grown cells, but higher exposure times were needed to visualize them clearly (data not shown). The three proteins disappeared towards the late-exponential phase. The pH of the medium decreased gradually from 3·5 to 1·0, due to H_2SO_4 production during S^0 oxidation. The levels of an outer-membrane protein used as reference, Omp40, whose synthesis is independent of the oxidizable substrate (Osorio *et al.*, 1993), showed no significant variation under any of the conditions tested (data not shown). These results show that *rus*-operon expression depends on the electron donor available.

Cells switched from Fe(II) to S^0 and vice versa (non-adapted cells)

When switching exponentially growing cells from Fe(II) to S^0 medium, cell growth was observed after a 1 day lag (Fig. 1b). Cyc1 and CoxB, already present in the inoculum, disappeared completely after 2 days of growth. The Rus level decreased more slowly as growth proceeded (Fig. 1b). When exponentially growing cells were transferred from S^0 to Fe(II) medium, after a 1 day lag we observed simultaneous cell growth, Fe(II) oxidation, and a rapid increase in Cyc1, CoxB and Rus levels, relative to the levels observed in the

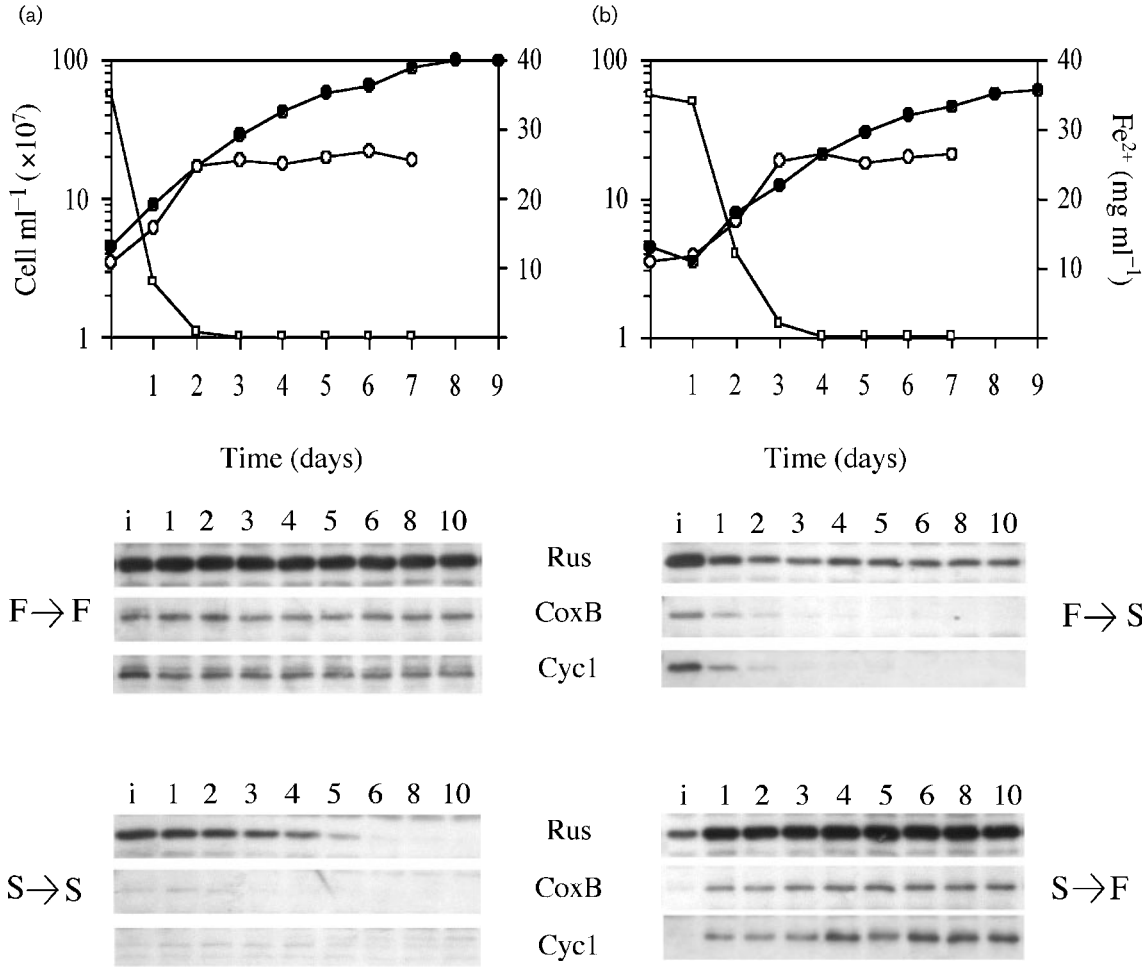

Fig. 1. Effect of Fe(II)- and S^0-dependent growth on *rus*-operon product levels in *A. ferrooxidans* ATCC 33020. (a) Cells were grown on iron (○; F→F) or sulfur medium (●; S→S) for several generations. Ferrous iron concentrations are shown (□). (b) Cells were switched from iron to sulfur medium (●; F→S) and vice versa (○; S→F). Ferrous iron concentrations are shown (□). Samples were removed every 24 h to measure cell densities. The samples were subjected to SDS-PAGE and transferred to a PVDF membrane. Western immunoblots were performed with antisera raised against rusticyanin (Rus), the periplasmic domain of subunit II of cytochrome oxidase (CoxB), and the periplasmic membrane-bound cytochrome c_4 (Cyc1). The same results were obtained with antibodies directed against the product of the *orf* gene (data not shown). i, Inoculum.

inoculum (Fig. 1b). Again, the levels of Omp40 did not show any significant variation (data not shown). These results suggest a regulation of *rus*-operon expression in response to the electron donor.

Cells grown in the presence of both Fe(II) and S^0

When *A. ferroxidans* cells were grown in the presence of both energetic substrates for several generations, i.e. in Fe(II) + S^0 medium, Fe(II) was oxidized rapidly, and the pH increased concomitantly (Fig. 2a). This pH increase is likely to be due to proton consumption when Fe(II) is oxidized, suggesting that Fe(II) is used immediately in preference to S^0. Cyc1, Rus and CoxB levels increased during Fe(II) oxidation, reaching a maximum at day 3, and then decreased after complete oxidation of Fe(II) (day 4), as growth proceeded, to become undetectable in late-exponential-phase cells (day 8) (Fig. 2b, and data not shown). This decrease, presumably due to dilution of the proteins during subsequent cell growth and division, coincided with a drop in pH, from 1·8 to 1·0 (Fig. 2a), which is probably due to H_2SO_4 production during S^0 oxidation and to ferric hydroxide formation, which leads to proton liberation. In conclusion, the *rus* operon is expressed as long as Fe(II) is present in the medium, even in the presence of S^0, indicating that *rus*-operon expression is not repressed by S^0 itself in the presence of Fe(II). However, repression of *rus*-operon expression by sulfur oxidation product(s), as soon as S^0 is metabolized, cannot be excluded.

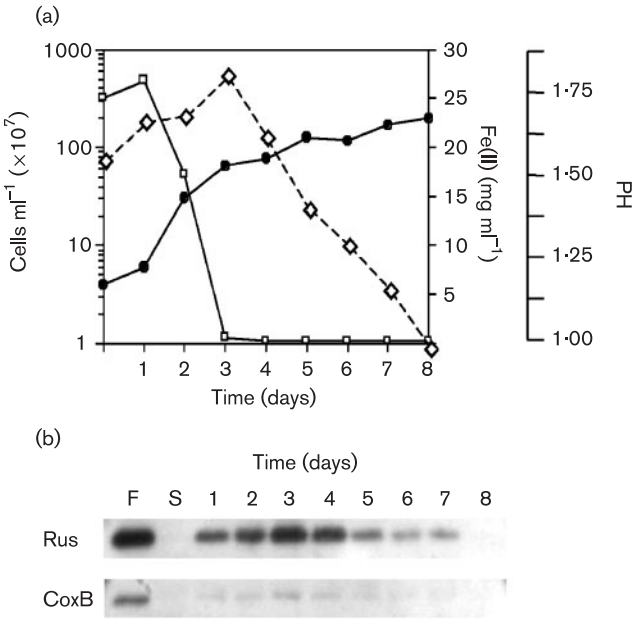

Fig. 2. Expression of the *rus* operon in *A. ferrooxidans* ATCC 33020 cells grown on medium containing both ferrous iron and sulfur. (a) Cell density (●), ferrous iron concentration (□) and pH (◇) were monitored in daily harvested samples. (b) Western immunodetection of rusticyanin (Rus) and the periplasmic domain of subunit II of cytochrome oxidase (CoxB). Samples of iron- (F) and sulfur-grown cells (S) were included as controls. The same results were obtained with antibodies directed against the products of the *orf* and *cyc1* genes (data not shown).

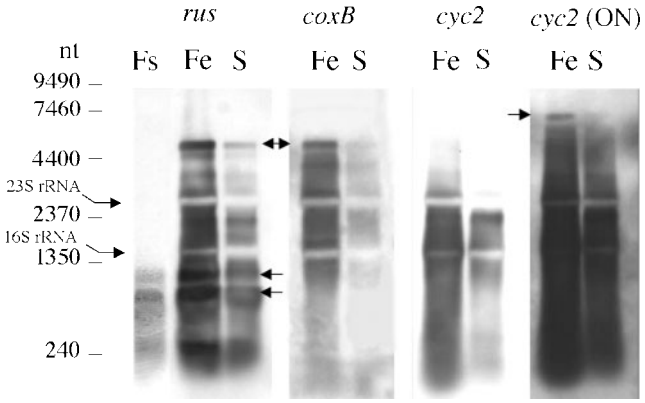

Fig. 3. Northern analysis. Total *A. ferrooxidans* RNA was extracted from ATCC 33020 cells grown on iron medium for 1 day (Fe) or 3 days (Fs), and on sulfur for 4 days (S). RNA (10 μg) was subjected to agarose-formaldehyde gel electrophoresis, transferred to a positively charged nylon membrane and hybridized to DIG-UTP-labelled antisense-RNA probes complementary to *rus*, *coxB* and *cyc2* transcripts. Arrows show major transcripts (5100, 1300 and 850 nt), and the largest transcripts detected (7500 nt), following overnight exposure (ON). rRNA, which appeared as white bands, is indicated with bent arrows.

Transcription of the *rus* operon is dependent on the energetic substrate

The data presented above show that the level of the *rus*-operon products depends primarily on the electron donor available. To determine whether the protein levels reflected the corresponding mRNA levels, antisense RNA probes complementary to *cyc2*, *coxB* and *rus* were hybridized to total RNA extracted from Fe(II)- and S^0-grown cells in mid-exponential phase (see Fig. 1a), as described in Methods. Comparison of the transcript profiles revealed that more transcripts are detected in Fe(II) than in S^0 exponentially growing cells, whatever the probes used (Fig. 3). Since the patterns obtained were reproducible in at least three independent experiments, these differences probably reflect regulation at the transcriptional level in response to the energetic substrate. Furthermore, we noticed that in Fe(II) medium, *rus* transcripts are more abundant in cells after 1 day than after 3 days of growth (Fig. 3, lanes Fs and Fe), that is before and after complete oxidation of Fe(II) to Fe(III) (see Fig. 1a). Similar results were obtained with the *cyc1* probe (data not shown). These results explain the data obtained previously (Bengrine *et al.*, 1995; Yarzábal *et al.*, 2001), in which the level of *rus* transcripts was apparently higher in S^0- than in Fe(II)-grown cells, since the RNA was extracted from a Fe(II) culture at day 3, when all the Fe(II) was oxidized. The possibility of *rus*-operon transcripts being degraded by Fe(III) during the extraction procedure was rejected, because: (i) in this experiment, Fe(III) was already present at day 1 (Fig. 1a); (ii) no obvious RNA degradation was observed in the ethidium bromide-stained gel (data not shown); and (iii), when RNA samples were prepared from S^0-grown cells in the presence or absence of the filter-sterilized supernatant of a five-day Fe(II)-grown culture, the *rus* transcripts presented the same pattern (data not shown). Therefore, the most likely explanation is the induction of *rus* operon transcription by Fe(II).

Several bands were detected, whatever the RNA probe used, as previously reported with a *rus* probe (Bengrine *et al.*, 1995, 1998; Yarzábal *et al.*, 2003). The largest transcript observed, 7500 nt long (Fig. 3), was detected only with the *cyc2* probe (see Fig. 6). Transcripts of 5100 nt were the largest detected with *rus* and *coxB* probes (Fig. 3). Two major bands of 850 and 1300 nt were detected with a *rus* probe only (Fig. 3). These results, which were reproducible in several independent experiments, suggest the presence of internal promoter(s) and/or the processing by endo- and exoribonucleases of *rus*-operon transcripts (see the model presented below).

To confirm that transcription of the *rus* operon is dependent on the energetic substrate, we employed real-time PCR to quantify the amount of transcript corresponding to each gene of the *rus* operon. The results are shown in Table 3. We found that, in contrast with the two genes used as reference

Table 3. Quantification of *rus* transcripts by RT-PCR

All values (except *rrs*) are expressed as *n*-fold relative to 16S rRNA ($\times 10^4$). Values shown are the mean of three independent experiments ± SD. Total RNA was extracted from mid-exponential-phase cells, after 1 day for Fe(II) and 4 days for S^0 cultures.

Gene	Growth condition		Ratio of Fe(II) to S^0
	Fe(II)	S^0	
rrs	1	1	1
cyc2	28 ± 1·76	5·7 ± 0·2	4·9
orf	77 ± 1·7	8·7 ± 0·3	8·85
cyc1	9·4 ± 0·6	0·4 ± 0·04	23·5
coxB	170 ± 3·2	9·7 ± 0·9	17·5
coxA	71 ± 1·8	7·5 ± 0·4	9·46
coxC	54 ± 1·6	9·5 ± 0·7	5·68
rus	270 ± 9·8	11 ± 0·2	24·5

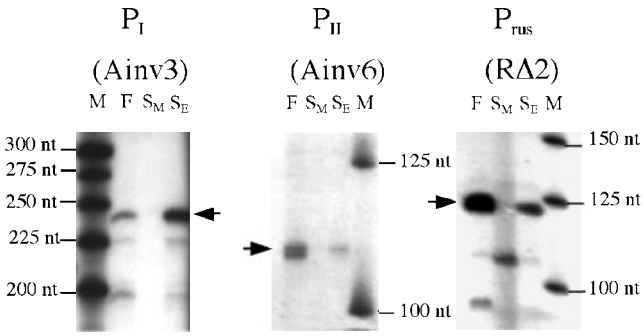

Fig. 4. Reverse transcriptase-mediated primer-extension experiments. Reverse transcription (RT) reactions were performed using the primers indicated, and total RNA was extracted from mid-exponential iron-growing cells (F) and from early exponential (S_E) or mid-exponential (S_M) sulfur-growing cells. RT products were resolved by urea-acrylamide gel electrophoresis and detected by autoradiography. Lanes M, [γ^{32}P]ATP-labelled 25 bp DNA ladder (Promega). RT products corresponding to transcription from the indicated promoters are shown with an arrow.

standards, *alaS* and *rrs*, which did not show any significant differences (data not shown), all the transcripts of the *rus* operon were more abundant in Fe(II)- than in S^0-grown cells by 4·9-fold (*cyc2*) to 24·5-fold (*rus*) (Table 3). In comparison with the other transcripts from the *rus* operon, *rus* transcripts were the most abundant, a result that matches the abundance of rusticyanin in whole-cell extracts (Cobley & Haddock, 1975; Cox & Boxer, 1978).

We have shown previously that the *rus* operon is transcribed from at least three promoters (see Fig. 6): PI and PII upstream from *cyc2* (Appia-Ayme *et al.*, 1999), and P_{rus} upstream from *rus* (Bengrine *et al.*, 1998). Transcription from these promoters was further studied by primer extension experiments with RNA extracted from mid-exponentially growing Fe(II) and S^0 cells. The results obtained clearly show that transcription from the three promoters depends on the electron donor present in the medium. Indeed, transcription from PI, PII and P_{rus} was higher in Fe(II) than in S^0 mid-exponentially growing cells (Fig. 4, lanes F and S_M).

Altogether, these results agree with those obtained at the protein level in that they demonstrate higher *rus*-operon expression in Fe(II)- than S^0-grown cells. Therefore, *rus*-operon expression is not constitutive, but likely regulated at the transcriptional level, depending on the energy source, as previously proposed for the *rus* gene alone in the ATCC 19859 strain (Pulgar *et al.*, 1993). Moreover, these data suggest that *rus*-operon products are involved in Fe(II) oxidation rather than in S^0 oxidation.

We propose that the expression of the *rus* operon is positively regulated by Fe(II), based on the following arguments: (i) the expression of the operon is higher in Fe(II)- than in S^0-grown cells (Fig. 3 and Table 3); (ii) a rapid increase in the concentrations of the proteins encoded by the *rus* operon was observed when switching cells from S^0 to Fe(II) medium (Fig. 1b); (iii) a drastic decrease of *rus*-operon protein levels was observed when cells were transferred from Fe(II) to S^0 medium (Fig. 1b); (iv) when both substrates were present, the *rus*-operon-encoded proteins were detected in large amounts only when Fe(II) was still available, and decreased rapidly once it was completely oxidized (Fig. 2), indicating that the *rus* operon is not repressed by S^0 itself. Furthermore, the levels of the transcripts from the *rus* operon decreased drastically when Fe(II) was no longer available (Figs 3 and 4). Note that, in the presence of both Fe(II) and S^0, Fe(II) is immediately oxidized, and that the pH decrease due to H_2SO_4 production occurs after complete Fe(II) oxidation (Fig. 2), suggesting that Fe(II) is apparently preferred to S^0 as an electron donor.

Transient expression of the *rus* operon in S^0-growing cells

As shown in Fig. 1, Cyc1, CoxB and Rus were always detected during the early exponential phase on S^0-grown cells, even after many subcultures on S^0, suggesting a *de novo* synthesis of these proteins. To test this hypothesis, cells from S^0 cultures (day 10 in Fig. 1a), in which Cyc1, CoxB and Rus could not be detected by Western blot analysis, were used to inoculate fresh S^0 medium. Aliquots were removed every day and analysed by immunodetection, as described above. After 1 day of growth, CoxB, Rus and Cyc1 were clearly detected by immunoassay, although their levels were lower than in Fe(II)-grown cells (Fig. 5, and data not shown). The same results were obtained using extra-pure S^0 (Fluka; data not shown). This demonstrates unambiguously a *de novo* synthesis of these proteins in S^0-grown cells. As noted previously (see Fig. 1a), Cyc1, CoxB and Rus levels rapidly

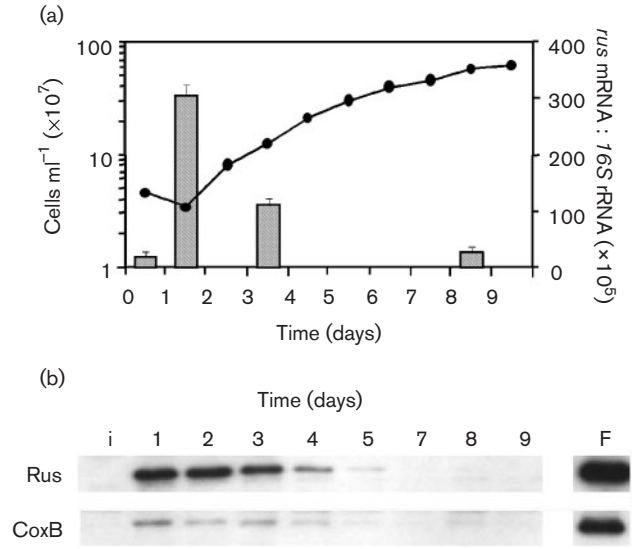

Fig. 5. *De novo* expression of the *rus* operon in *A. ferrooxidans* ATCC 33020 cells grown on sulfur. (a) Adapted cells from stationary cultures were grown on sulfur medium (●). Transcription of the *rus* gene was monitored by real-time PCR (bars). (b) Samples were harvested from sulfur cultures and monitored by Western immunodetection for the presence of rusticyanin (Rus) and the periplasmic domain of subunit II of cytochrome oxidase (CoxB). Samples from iron-grown cells (F) were included as controls. i, Inoculum.

decreased towards the end of the exponential phase, to become undetectable in the stationary phase, a pattern consistent with dilution of the proteins through growth and multiple cell divisions in the absence of any further synthesis.

To determine whether this *de novo* synthesis reflected transcription of the *rus* operon, real-time PCR experiments were performed. As can be seen in Fig. 5a, *rus* transcripts were detected 24 h after inoculation, and then decreased rapidly as growth proceeded. The same results were obtained with *cyc2* (data not shown). These results strongly suggest a transient early-exponential-phase expression of the *rus* operon in S^0-grown cells in the absence of any detectable Fe(II).

Transcription from PI, PII and P_{rus} promoters was studied further by reverse-transcriptase-mediated primer-extension experiments with RNA extracted from one-day (early exponential phase) and three-day (mid-exponential phase) S^0-grown-cells. Transcription from PI, PII and P_{rus} promoters in S^0-grown cells was not detected in mid-exponential S^0-growing cells (Fig. 4, lanes S_M), as already mentioned. However, in contrast to PII and P_{rus} transcription, transcription from PI was higher in early exponential S^0-growing cells than in Fe(II)-growing cells (Fig. 4, lanes F and S_E). Thus, the transient expression of the *rus* operon during the early exponential phase of S^0-grown cells, in the absence of any detectable Fe(II), is mainly due to transcription from PI.

As mentioned above, there are contradictory reports in the literature concerning the presence of rusticyanin and iron-oxidizing activity in S^0- or thiosulfate-grown cells: in some studies, neither were detected, while in others, both were detected (Landesman *et al.*, 1966; Margalith *et al.*, 1966; Cox & Boxer, 1986; Hazeu *et al.*, 1986; Jedlicki *et al.*, 1986; Kulpa *et al.*, 1986a, b; Sugio *et al.*, 1986, 1988; Espejo & Romero, 1987; Espejo *et al.*, 1988; Suzuki *et al.*, 1990; Mansch & Sand, 1992; Osorio *et al.*, 1993; Bengrine *et al.*, 1995, 1997, 1998; Yarzábal *et al.*, 2003). Suzuki *et al.* (1990) proposed that such conflicting results were due to strain differences. We have shown here that the *rus* operon, clearly involved in Fe(II) oxidation, is in fact transiently expressed in S^0-grown ATCC 33020 cells. Indeed, transcription of the *rus* operon and the *de novo* synthesis of the corresponding products occurred at the beginning of growth in S^0 medium (Figs 1a, 4 and 5). These results could explain the contradictory reports found in the literature: if samples were taken from exponentially growing cells, expression would be detected, but would be absent or undetectable in late-exponential or in stationary-phase samples.

Interestingly, Sugio *et al.* (1988) noticed that the iron-oxidizing activity of S^0-growing cells increased during early exponential phase and decreased later. These authors proposed that this decrease was due to the acidification of the medium. This hypothesis could also explain the *rus*-operon expression pattern under S^0-growth conditions. Indeed, the synthesis of several proteins of *A. ferrooxidans* has been reported to be pH dependent (Amaro *et al.*, 1991). *De novo* expression of the *rus* operon was observed when stationary-phase bacteria cultured in S^0 medium (pH 1) were shifted to fresh S^0 medium (pH 3·5). Furthermore, when both Fe(II) and S^0 were present in the medium, Rus and CoxB levels first increased and then decreased with the pH (Fig. 2). Therefore, the *rus*-operon expression observed in S^0-grown cells could be the consequence of a pH upshift. However, when cells of *A. ferrooxidans* grown at pH 1·5 are shifted to pH 3·5, no significant changes are observed in SDS-PAGE profiles, except the increase of a 36 kDa outer-membrane protein (Amaro *et al.*, 1991). An alternative hypothesis could explain the transient expression of the *rus* operon in S^0-grown cells. Indeed, the rapid increase in *rus*-operon transcription, after inoculation into fresh medium, followed by a steep decrease (Fig. 5), is reminiscent of the transcription pattern of genes regulated by a nutritional upshift (Nilsson *et al.*, 1990, 1992). The histone-like Fis protein of *E. coli* has been proposed to communicate the nutritional quality of the environment to various cellular processes (Ball *et al.*, 1992), including electron transfer (Green *et al.*, 1996; Wackwitz *et al.*, 1999). It is therefore possible that *rus*-operon expression in S^0-growing cells is nutrient-upshift dependent, and is mediated, directly or indirectly, by a Fis-like protein. Interestingly, an orthologous *fis* gene is present in the *A. ferroxidans* ATCC 23270

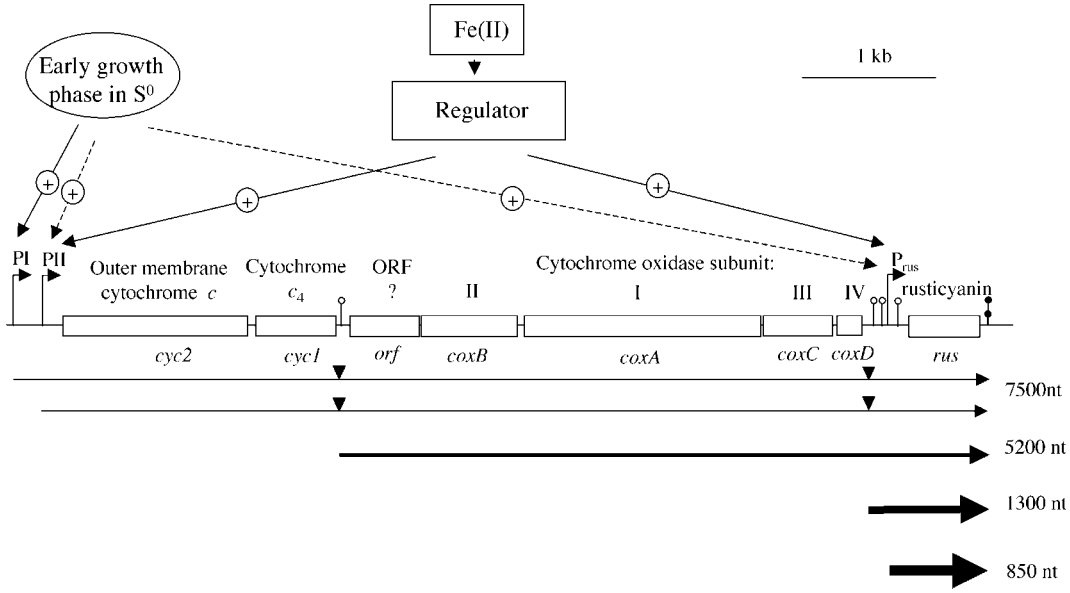

Fig. 6. Model of the transcriptional and post-transcriptional regulation of the *rus* operon. Each gene is shown by an open box. The positions of the promoters (PI, PII and P_{rus}) are indicated as bent arrows. Transcripts detected in RNA blots are shown by horizontal arrows, with thickness indicating their relative abundance. Putative RNase E cleavage sites are represented as vertical arrowheads. Inverted repeats are shown as open stem–loops, while the putative transcriptional Rho-independent termination sites are represented as a closed stem–loop.

genome. We cannot exclude, however, repression of *rus*-operon expression during growth on S^0 by accumulation of an oxidized S^0 compound.

Model for *rus*-operon regulation

In this paper, we have demonstrated that the regulation of *A. ferroxidans* ATCC 33020 *rus*-operon expression at the transcriptional level depends on the electron donor present in the medium. Furthermore, a transient expression during the early-exponential phase of S^0-grown cells suggests a nutritional- or a pH-upshift-dependent regulation. Transcription from the PI promoter seems to depend on the growth phase or pH, while transcription from PII and P_{rus} seems to be regulated mainly by the presence of Fe(II).

In addition, the *rus* operon seems to be subject to post-transcriptional mRNA processing. The largest transcript, 7500 nt, observed with the *cyc2* probe (the first gene of the *rus* operon, see Fig. 6), corresponds to a transcript covering the whole operon. The 5100 nt transcript detected with the *coxB* and *rus* probes may be due to the processing of larger transcripts or may arise from an internal promoter. Assuming that this 5100 nt transcript stops at the putative *rus* transcriptional termination site (Bengrine *et al.*, 1998), it would start in the 110 bp intergenic region between *cyc1* and *orf*. In agreement with this hypothesis, transcripts starting approximately 87 nt from the *orf* translation initiation site were detected by primer extension with the P_{orf} oligonucleotide (data not shown). Although no obvious −35 and −10 regions were detected upstream from that position, there is a putative RNase E recognition site (Fritsch *et al.*, 1995), followed by a potential stem–loop structure ($\Delta G = -38{\cdot}9$ kJ mol^{-1}). Therefore, we propose that the 5100 nt transcript originates from RNase E cleavage of larger transcripts at this site. The two major transcripts, 1300 and 850 nt, were detected only with the *rus* probe. The 850 nt *rus* transcript could correspond to transcripts starting from the internal promoter P_{rus}, previously detected between *coxD* and *rus* (Bengrine *et al.*, 1998). Since a putative RNase E recognition site (Fritsch *et al.*, 1995), followed by two potential stem–loop structures ($\Delta G = -45{\cdot}2$ and $-118{\cdot}5$ kJ mol^{-1}), lies upstream from P_{rus}, the 1300 nt *rus* transcripts may also arise from the processing of larger transcripts at this site. The model of *rus*-operon regulation we propose is summarized in Fig. 6.

It seems clear from the data presented in this paper that the *rus* operon is involved in Fe(II) rather than in S^0 respiration. Therefore, why is the *rus* operon expressed at the onset of exponential growth in S^0-grown cells? One hypothesis, proposed by Sugio *et al.* (1985), is that the iron-oxidation pathway allows the recycling of the Fe(III) that is required for S^0 oxidation (the ferric ion-reducing system, FIR). However, since the iron-oxidation system is expressed only at the early exponential phase, it is clearly not necessary for subsequent growth in S^0 medium. An alternative hypothesis is that the bacterium induces the expression of both the S^0- and the Fe(II)-oxidizing system as soon as it detects either one of these substrates in the medium, the natural substrate for *A. ferrooxidans* being metal sulfides, such as pyrite (FeS_2).

Furthermore, this hypothesis is in agreement with the model proposed by Rohwerder *et al.* (2003), according to which metal sulfides are leached by Fe(III) and/or by protons, produced by Fe(II) and S^0 oxidation, respectively. We would expect this hypothesis also to hold true for the S^0-oxidizing system, in which case we would expect the expression of this system in Fe(II)-grown cells to be early-exponential-phase dependent. Similar experiments with the S^0-oxidizing system await the detailed elucidation of the genes and gene products of that system.

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