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Cytochromes *c* of *Acidithiobacillus ferrooxidans*

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Abstract

The chemolithoautotrophic Gram-negative bacterium *Acidithiobacillus ferrooxidans* is versatile and can grow on a number of electron donors and acceptors. In the *A. ferrooxidans* ATCC 23270 genome, computer analysis identified 11 genes encoding putative cytochromes *c*. At least eight putative cytochromes *c* were differentiated on gels in ATCC 33020 cells grown on ferrous iron or sulfur. All these cytochromes were associated with the inner or the outer membranes. Lower levels of total cytochromes *c* were observed in sulfur- than in ferrous iron-grown cells. One cytochrome *c* was specific for sulfur conditions while three were specific for iron conditions, suggesting that cytochrome *c* synthesis is modulated depending on the electron donor.

Keywords: Cytochrome *c*; Ferrous iron; Sulfur; *Acidithiobacillus ferrooxidans*

1. Introduction

Nearly all bacteria have multiple branched respiratory pathways with a diverse range of electron donors and acceptors. In most cases, the expression of the genes encoding electron transporters is tightly regulated depending on the growth conditions, providing respiratory flexibility to adapt efficiently to environmental changes. Among these electron transporters, cytochromes *c* are widely distributed in eukarya, archaea and eubacteria. In bacteria, apocytochromes *c* are translocated to the periplasm where heme C is covalently attached generally to the CXXCH signature motif [1]. The holocytochromes *c* are often associated with the inner or outer membranes but can also be found in the periplasm where they may function as mobile electron carriers [1].

Acidithiobacillus ferrooxidans exhibits an unusually high content of cytochromes *c*, which can constitute as much as 10% of the total proteins synthesized in cells grown on ferrous iron (FeII) [2]. In its natural habitat, this acidophilic chemolithoautotrophic Gram-negative bacterium gains its energy from the oxidation of FeII and sulfur present in pyrite. Several membrane and soluble *c*-type cytochromes have been identified in different strains of *A. ferrooxidans* (Table 1) and proposed to be involved in the respiratory pathway of FeII oxidation. Furthermore, the energy-dependent electron transfer pathway involved in the reduction of $NAD(P)^+$ required for CO_2 fixation in FeII-grown cells has been proposed to include a *bc*1 complex [3]. A bc_1 complex has been also suggested to participate in the electron pathway from sulfur (S^0) to oxygen [4]. Therefore, *c*-type cytochromes seem to be involved in the oxidative pathways of both FeII and S^0.

As an approach for assignment of functions to the multiple cytochromes *c* of *A. ferrooxidans*, we have characterized the pattern of cytochromes *c* which are detected in ATCC 33020 under different growth conditions and identified genes encoding putative cytochromes *c* in the ATCC 23270 genome.

2. Materials and methods

A. ferrooxidans ATCC 33020, obtained from the American Type Culture Collection, was grown at 30°C on FeII medium at pH 1.6 or on S^0 medium at pH 3.5 [5]. Total membrane, outer membrane and periplasmic fractions were obtained after EDTA–lysozyme treatment of cells as previously described [6].

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Table 1
Cytochromes *c* identified in various *A. ferrooxidans* strains

Molecular mass in kDa	Localization	*c* family	Heme number	Strain	Growth conditions	References
68[a]	membrane bound			ATCC 13661	FeII+O_2	[31]
60[a]	membrane bound			F-427	FeII+O_2	[29]
51[a]	membrane bound			Fe-1	FeII+O_2	(ref. in [20])
46[a]; 50.4[c]	outer membrane		1	ATCC 33020	FeII+O_2	([6,13], this study)
46[a]	membrane bound			BRGM	FeII+O_2	[12]
30[a]	membrane bound			BRGM	FeII+O_2	[12]
30[a]	membrane bound			ATCC 13661	FeII+O_2	[31]
30[a]	membrane bound			F-427	FeII+O_2	[29]
29[a]; 26.5[b]	soluble	c_4	2	BRGM	FeII+O_2	[21]
28[a]	soluble			JCM 7811	H_2+FeIII; FeII+O_2	[27]
25[a]; 21.2[c]	membrane bound	c_4	2	ATCC 33020	FeII+O_2	([13], this study)
25[a]	membrane bound			F-427	FeII+O_2	[29]
24.9[c]		c_1	1	ATCC 19859		[15]
22.3[a]	membrane bound		1	Fe-1	FeII+O_2	(ref. in [20])
22[a]	outer membrane			ATCC 33020	FeII+O_2	([6], this study)
21.9[c]		c_4	2	ATCC 19859		[15]
21[a]	membrane bound			BRGM	FeII+O_2	[12]
21[a]	membrane bound		1	ATCC 13661	FeII+O_2	[31]
20[a]; 21.2[b]	soluble	c_4	2	BRGM	FeII+O_2	[19]
14[a]	membrane bound			ATCC 13661	FeII+O_2	[31]
14[a]	soluble			BRGM	FeII+O_2	[12]
14[a]	soluble		1	Fe-1	FeII+O_2	(ref. in [20])
12.8[a]	soluble	c'	1	BRGM	FeII+O_2	[16]

[a]Molecular mass deduced from SDS–PAGE analysis.
[b]Molecular mass deduced from mass spectroscopy.
[c]Molecular mass deduced from amino acid sequence.

Protein concentrations of cell suspensions were determined with the Bio-Rad Protein Assay, after hydrolysis of the cells in 0.5 N NaOH at 100°C for 10 min. Protein concentrations of membrane and periplasmic fractions were determined according to Peterson [7].

SDS–PAGE and Western immunoblotting were carried out by standard procedures, except for the use of Tris(2-carboxyethyl)phosphine as a reducing agent in Laemmli's sample buffer. After separation of polypeptides in polyacrylamide gels, hemoproteins were detected by two methods: *o*-dianisidine staining [8] and the chemiluminescence procedure of Dorward et al. [9] slightly modified [Yarzábal, A., unpublished results].

Room-temperature optical spectra were obtained as described previously [6]. The heme C content was determined from the reduced minus oxidized difference spectra, with a millimolar extinction coefficient for the α-peak of $\Delta\varepsilon_{550-540\ nm} = 18\ mM^{-1}\ cm^{-1}$.

For the genome analysis, *A. ferrooxidans* ATCC 23270 preliminary sequence data were obtained from The Institute for Genomic Research (http://www.tigr.org). The following programs were used: Patscan (http://ir2lcb.cnrs-mrs.fr/local) for pattern detection in translated ORFs; ORF Finder [Tatusov, T. and R. Tatusov, unpublished results] (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to analyze ORFs in DNA sequences; TBLASTN (http://www.ncbi.nlm.nih.gov/Microb_blast/) and BLASTP (http://www.ncbi.nlm.nih.gov/blast/) for similarity searches; and Psort [10] (http://psort.nibb.ac.jp:8800/form.html) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) [11] to predict the cellular localization of putative proteins.

3. Results

3.1. Cytochromes c detected in A. ferrooxidans ATCC 33020

Based on room-temperature optical spectra analyses (α-band at 551 nm and γ-band at 421 nm), S^0-grown cells of *A. ferrooxidans* were shown to contain lower levels of total cytochromes *c* (0.30–0.57 μmol g^{-1} protein) than FeII-grown cells (1.2 μmol g^{-1} protein) (Fig. 1A).

To determine the number of different cytochromes *c* synthesized in exponentially growing cells, cell extracts were resolved by SDS–PAGE and cytochromes visualized by two different procedures: the *o*-dianisidine technique (Fig. 2B) and a chemiluminescent detection protocol (Fig. 2C,D). These two techniques allowed the detection of at least five heme-containing bands in S^0- (46, 27, 24.5, 22 and 14 kDa) and in FeII-grown cells (46, 30, 27, 22 and 14 kDa) (Fig. 2B,C). The 30 kDa chemiluminescent band was resolved into two bands (31 and 29.5 kDa) when the concentration of the polyacrylamide was decreased (Fig. 2D). Whether these two bands corresponded to two different cytochromes *c* or to the same, as shown with the 30kDa cytochrome of *A. ferrooxidans* BRGM strain [12],

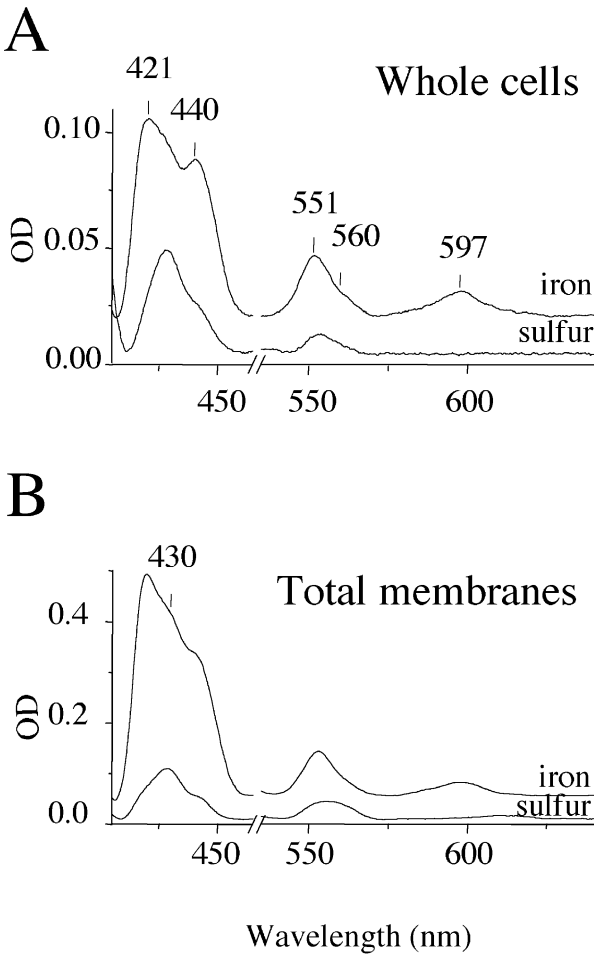

Fig. 1. Room-temperature optical spectra of whole cells and membrane fractions of *A. ferrooxidans*. Spectra were obtained as the difference between dithionite-reduced minus Na_2IrCl_6-oxidized samples. A: Spectra of whole cells grown with FeII or S^0 as electron donor. Both samples were suspended at 1.2 mg ml^{-1} in 20 mM β-alanine buffer (pH 3.5). B: Total membrane fractions obtained from cells grown on FeII or S^0 and suspended at 6.3 mg ml^{-1} in 20 mM β-alanine buffer (pH 3.5).

remains to be answered. Some of these bands were detected only by chemiluminescence (e.g. 14 kDa) while others reacted better with *o*-dianisidine (e.g. 46 kDa). With both procedures, all the bands corresponded to proteins containing a covalently bound heme C because they were detected even after heating these samples at 95°C for 5 min under reducing conditions. The apparent molecular masses of these hemoproteins are summarized in Table 2A.

In the ATCC 33020 strain, the genes encoding two cytochromes *c* have been studied by our group [13]. These genes (*cyc1* and *cyc2*) and the genes encoding an aa_3-type cytochrome oxidase (*coxBACD*) and rusticyanin (*rus*) are cotranscribed [14], and we have deduced that these redox proteins are involved in the same electron transfer chain [14]. The Cyc1 cytochrome belongs to the c_4-type cytochrome family and has been proposed to be the physiological electron donor to the terminal cytochrome oxidase [13,14]. The Cyc2 cytochrome has been located in the outer membrane and was supposed to be the primary electron acceptor [6,14]. The Cyc1 and Cyc2 molecular masses, deduced from their amino acid sequence, are 21.2 and 50.4 kDa (Table 1). To determine unambiguously to which cytochromes resolved on SDS–PAGE Cyc1 and Cyc2 corresponded, immunodetection with specific antibodies directed against Cyc1 and Cyc2 were performed. The 46kDa hemoprotein corresponded to Cyc2 (data not shown). The cytochrome Cyc1 migrated as a 25kDa protein which was unambiguously distinct from the 22 and 24.5kDa cytochromes (Figs. 2 and 3), and was detected only by immunoassay.

With both heme detection techniques, the intensity of each band reflected the relative level of the hemoprotein in the sample, as observed with a dilution series of horse-heart cytochrome *c* (data not shown). We observed that the bands were much less intense in S^0- than in FeII-grown cells (Fig. 2B,C), indicating thus a lower cytochrome *c* content. Furthermore, some cytochromes were

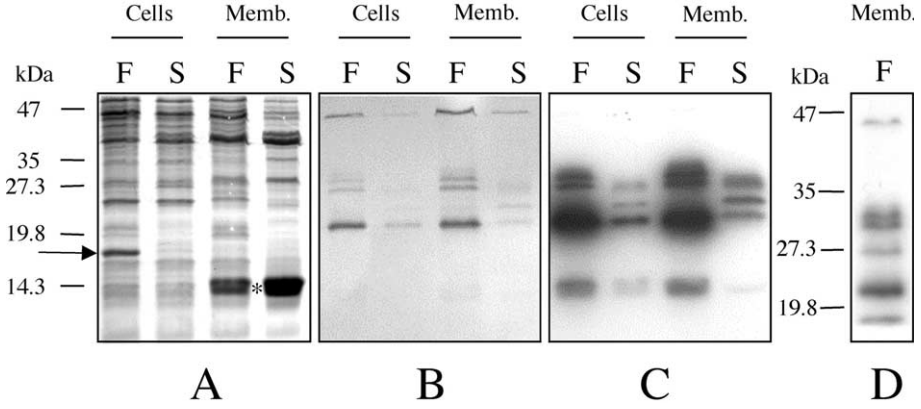

Fig. 2. Cytochromes *c* detected in *A. ferrooxidans* ATCC 33020. SDS–15% polyacrylamide (A–C) or –12% polyacrylamide (D) gels were stained for total proteins with Coomassie blue (A), and hemoproteins by *o*-dianisidine staining (B) or by chemiluminescence (C,D). Whole cells (Cells) and total membrane (Memb.) fractions were prepared from *A. ferrooxidans* cells grown in FeII (F) or S^0 (S) medium. Each lane was loaded with 10 μg of protein. The position of rusticyanin is indicated by an arrow on the left side. Asterisk indicates the position of lysozyme used to prepare the total membrane fractions. The position of the molecular mass markers is given on the left side.

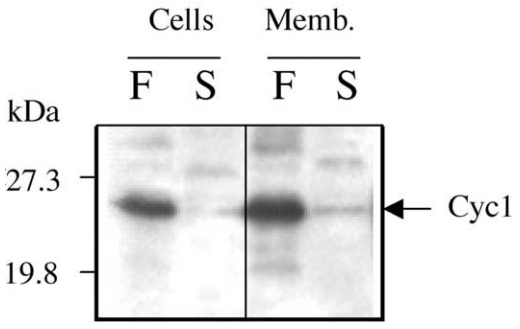

Fig. 3. Detection of Cyc1 cytochrome c_4 in *A. ferrooxidans* grown on FeII or S^0 medium. Western blots of whole cells (Cells) and total membrane fractions (Memb.) from exponentially growing cells on FeII (F) and S^0 (S) were probed with antibodies directed against Cyc1. Each lane was loaded with 1 μg of protein. The position of the molecular mass markers is given on the left side.

detected in only one of the growth conditions examined. The 29.5kDa, 31kDa and Cyc1 cytochromes, all present in FeII-grown cells, were almost absent in S^0-grown cells (Figs. 2 and 3). Instead, a 24.5kDa cytochrome, absent in FeII-grown cells, was observed in S^0-grown cells (Fig. 2B,C).

3.2. Subcellular localization of the c-type cytochromes detected in FeII- and in S^0-grown cells

The total membrane fractions from S^0- and FeII-grown cells contained about 0.3 and 0.9 μmol cytochromes *c* g^{-1} protein respectively, as determined by room-temperature optical spectra analyses (Fig. 1B).

In our study, all the cytochromes remained associated with the total membrane fractions (Figs. 2B,C and Fig. 3). Heme-stained bands were virtually undetectable in the periplasmic fractions obtained after treatment of FeII- and S^0-grown cells with EDTA and lysozyme, even after concentration by ultrafiltration (data not shown).

Room-temperature optical spectra of the outer membrane fraction also showed lower amounts of total *c*-type cytochromes in S^0- than in FeII-grown cells (data not shown). Cyc2 as well as the 22kDa cytochromes were both detected in the outer membrane fractions, as previously demonstrated [6].

3.3. Identification of putative cytochromes c from analysis of the A. ferrooxidans ATCC 23270 genome sequence

As the *A. ferrooxidans* ATCC 23270 genome sequence was being completed (but not annotated) by The Institute for Genomic Research during this study, we carried out a search of genes encoding putative heme binding proteins in the genome sequence translated in all six reading frames to predict the total number of *c*-type cytochromes that may be synthesized in this microorganism. Using the Patscan program with the consensus classical heme C binding site CXXCH [1], 70 such candidate proteins were detected. Each of these amino acid sequences was compared with TBLASTN to eubacteria complete genomes and with BLASTP to the non-redundant protein data base at the National Center for Biotechnology Information. In this search, 33 proteins did not match any known amino acid sequence, two were similar to previously identified hypothetical proteins, 24 exhibited similarities to proteins with known function but are not cytochromes, and 11 exhibited similarities to known cytochromes *c*. The nucleotide sequences corresponding to the 33 putative unknown proteins were further analyzed with ORF Finder. In an overlapping but different frame, 23 of them encoded proteins of the data base that were not cytochromes. According to Psort and SignalP programs, the 10 remaining putative proteins had no recognizable signal sequences and thus, were not further considered as putative cytochromes *c*. Therefore, based on the ATCC 23270 genome analysis, we have identified 11 putative cytochromes *c* (Table 2B). All of them were mono- or di-hemic cytochromes.

Two putative cytochromes c_1 were detected (25 571 and 24 417 Da), including one encoded by an orthologue of the ATCC 19859 *petC* gene [15]. It has to be pointed out that, so far, *A. ferrooxidans* is the only organism characterized to contain two c_1-type cytochromes. Four cytochromes belonged to the c_4-type cytochrome family (24 925, 23 783, 21 171 and 20 768 Da), one of them being encoded by an orthologue of the *A. ferrooxidans* ATCC 33020 *cyc1*

Table 2A
Cytochromes *c* detected in *A. ferrooxidans* ATCC 33020 cells

Apparent molecular mass (kDa)	Detection method			Electron donor	
	o-dianisidine	chemiluminescence	immunoassay	FeII	S^0
46 (Cyc2)	++	ε	+	++	+
31	–?	++	nd	++	–
29.5	+?	++	nd	++	–
27	+	++	nd	++	+
25 (Cyc1)	–	–	+	+	ε
24.5	ε	+	nd	–	++
22	+++	+++	nd	++++	++
14	–	++	nd	++	+

–: Undetectable; ε: barely detectable; +: detectable; nd: not determined.

Table 2B
Putative cytochromes *c* identified from the *A. ferrooxidans* ATCC 23270 genome sequence analysis

Theoretical molecular mass of putative holocytochromes (Da)[a]	Similarities to known cytochromes
49 915	Cyc2 of *A. ferrooxidans* ATCC 33020 [23]
49 884[b]	Cyc2 of *A. ferrooxidans* ATCC 33020 [23]
25 571[b]	cytochrome c_1 of *A. ferrooxidans* ATCC 19859 [24]
24 925[b]	cytochrome c_4 of *A. ferrooxidans* ATCC 19859 [24]
24 417	cytochrome c_1 of *A. ferrooxidans* ATCC 19859 [24]
23 783	cytochrome c_4 of *A. ferrooxidans* ATCC 19859 [24]
21 171[b]	Cyc1 (c_4) of *A. ferrooxidans* ATCC 33020 [23]
20 768	Cyc1 (c_4) of *A. ferrooxidans* ATCC 33020 [23]
15 889	cytochrome c_{552} of *H. thermophilus* [26]
13 081	cytochrome c_{552} of *H. thermophilus* [26]
13 063	brown *c′*-type cytochrome [25]

[a]The theoretical molecular mass of the holocytochrome was calculated after removing the putative signal sequence predicted by Psort and SignalP from the precursor apocytochrome and adding 600 Da per heme.
[b]Cytochrome encoded by an orthologue gene in the indicated strain.

gene [13], and another by an orthologue of the *A. ferrooxidans* ATCC 19859 *cycA* gene [15]. To our knowledge, *A. ferrooxidans* is the first microorganism found to contain genes encoding multiple c_4-type cytochromes. Two putative high-molecular-mass cytochromes *c* were also found (49 915 and 49 884 Da), one of which was encoded by an orthologue of the ATCC 33020 *cyc2* gene [13]. These two cytochromes are atypical cytochromes *c* that exhibit no similarities to any known protein sequence in data banks. Additionally, one putative mature cytochrome *c* deduced from the genome analysis (13 063 Da) had an N-terminal amino acid sequence which matched that of the 13 kDa brown *c′*-type cytochrome characterized by Cavazza and Bruschi [16]. Finally, the last two proteins (15 889 and 13 081 Da) exhibited significant similarities to the cytochrome c_{552} from *Hydrogenobacter thermophilus* [17]. The precursor of the 15 889Da cytochrome exhibits a characteristic lipoprotein signal peptide and the corresponding mature cytochrome has no aspartate residue at the second position of its amino terminal sequence, suggesting that this cytochrome is anchored to the outer membrane via a lipid moiety [18].

4. Discussion

A. ferrooxidans ATCC 23270 and ATCC 33020 were shown in this paper to contain several cytochromes *c*. Computer analysis of the genome sequence of *A. ferrooxidans* ATCC 23270 revealed 11 ORFs with both the classical heme C binding motif, CXXCH, and a signal sequence for membrane translocation. Four of the *c*-type cytochromes deduced from ATCC 23270 genome analysis correspond to cytochromes *c* previously characterized in various strains of *A. ferrooxidans*: the high-molecular-mass cytochrome Cyc2 [6,12–14] and the c_4-type cytochrome Cyc1 ([13,14,19,20] and references therein) encoded by the *rus* operon, the c_4-type cytochrome [15,21] and the cytochrome c_1 [15] encoded by the *petI* operon, and the small *c′*-type cytochrome [16]. Interestingly, most cytochrome genes identified are duplicated, with paralogue genes encoding the two high-molecular-mass cytochromes (49 915 and 49 884 Da), the two putative cytochromes c_1 (25 571 and 24 417 Da) and two sets of c_4-type cytochromes (24 925 and 23 783 Da; 21 171 and 20 768 Da).

From FeII- and S^0-grown cells of *A. ferrooxidans* ATCC 33020, a total of eight putative cytochromes *c* were resolved by SDS–PAGE and identified by heme staining or immunoblotting. Several hypotheses can be proposed to explain the difference between the number of cytochromes identified by ATCC 23270 genome analysis and the number of cytochromes detected in ATCC 33020 cells. (i) First, ATCC 23270 and ATCC 33020 genomes could encode a different number of *c*-type cytochromes. However, all the ATCC 23270 genes encoding a putative cytochrome *c* seem to have an orthologue in the ATCC 33020 genome, as shown by PCR experiments ([13], Bruscella, P. and Bonnefoy, V., unpublished results). (ii) A more likely explanation is that some cytochromes could be synthesized under growth conditions different than the ones described in this paper. *A. ferrooxidans* is known to be versatile: its aerobic growth can be supported by the oxidation not only of FeII or of a variety of inorganic sulfur compounds [22] but also by the oxidation of molecular hydrogen [23] or formic acid [24]. Furthermore, *A. ferrooxidans* can grow anaerobically on the oxidation of sulfur [25,26] or hydrogen [27] by FeIII. Indeed, a soluble 28kDa cytochrome *c* was recently shown to be in higher amounts in cells grown anaerobically using FeIII as an electron acceptor and H_2 as an electron donor than in cells grown aerobically with FeII [27]. (iii) Another possibility is that some cytochromes were not detected by the techniques we used. In our hands, the intensity of heme staining, which depends on its peroxidase activity, varies from one hemoprotein to another, and from one technique to another (for example, the 14 kDa and the 46 kDa). More striking, Cyc1 cytochrome, known to be in relatively high amounts in *A. ferrooxidans* cells grown on iron [19], was

only detected by immunoassay. When produced in *Escherichia coli*, Cyc1 can be visualized by *o*-dianisidine staining only when the samples were not heated prior to SDS–PAGE [Lund, K. and Bonnefoy, V., unpublished results]. Thus, it is possible that the Cyc1 hemes have lost their peroxidase activity during sample preparation. This could also happen to other cytochromes, which therefore would not be detected. (iv) Another explanation is that cytochromes of nearly identical molecular mass will not be adequately resolved on one-dimensional SDS–PAGE. This could be indeed the case since several putative cytochromes deduced from ATCC 23270 genome analysis have approximately the same apparent molecular mass (Table 2B). (v) Finally, some cytochromes may migrate aberrantly on SDS–PAGE as noticed with Cyc1 and Cyc2 (Table 2A,B). Because of the different points discussed above, it was difficult to determine to which ATCC 23270 putative cytochromes *c* the eight hemoproteins detected in ATCC 33020 corresponded. Cyc1 and Cyc2 were however identified unambiguously by immunodetection. Furthermore, the 22kDa outer membrane cytochrome (this paper and [6]) may correspond to the 15 589Da cytochrome which was predicted from its sequence to be located in the outer membrane (see above). For the other cytochromes, immunodetection or sequence of the amino terminus or internal peptides will be necessary.

The total cytochrome *c* content was lower in S^0- than in FeII-grown *A. ferrooxidans* cells. An *A. ferrooxidans* mutant, unable to oxidize FeII but still retaining sulfur-oxidation activity, has been proposed to be affected in cytochrome *c* biogenesis [28]. Since the only cytochrome *c* maturation system detected on the genome of the ATCC 23270 *A. ferrooxidans* strain is the Res system, it was tempting to suggest that the oxidation of sulfur does not require *c*-type cytochromes. Several points argue against such a hypothesis, however. First of all, S^0-grown *A. ferrooxidans* cells contain holocytochromes *c* ([29] and this paper). So far, cytochromes *c* are components of all the S^0-oxidation pathways described in the literature [30]. Furthermore, a bc_1 complex, and therefore a cytochrome c_1, has been postulated to be involved in the S^0-oxidizing respiratory system [4]. Because much more energy is available from S^0 than from FeII, a more likely explanation for the differences observed in the total content of cytochromes *c* in S^0- and FeII-grown cells is that the oxidation of FeII may require higher concentrations of electron-transport chain components, such as cytochromes *c*, to catalyze the rapid oxidation of FeII, as previously suggested by Ingledew [22]. Noteworthy, when *A. ferrooxidans* cells were switched from FeII to S^0, the levels of some cytochromes *c* tended to decrease drastically (14 kDa and Cyc2 cytochromes), even to nearly undetectable levels in some cases (31kDa, 29.5kDa and Cyc1 cytochromes). When S^0-adapted cells were transferred to FeII medium, these cytochromes *c* were synthesized de novo. It is therefore tempting to propose that these cytochromes participate in the iron-respiratory chains. In S^0- but not in FeII-grown cells, a 24.5kDa cytochrome was detected suggesting that this cytochrome is involved specifically in the electron transfer between S^0 and O_2.

The results presented in this paper show that cytochrome *c* synthesis in *A. ferrooxidans* is modulated according to the electron donor (FeII or S^0). The ability of *A. ferrooxidans* to grow on a number of electron donors and acceptors may depend on the synthesis of specific enzymes for substrate oxidation and, possibly, on the existence of different, redundant and perhaps interconnected respiratory pathways. These respiratory pathways would be expressed alternatively according to the environmental conditions, a hypothesis we are currently investigating in *A. ferrooxidans*.

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