Apparent redundancy of electron transfer pathways via bc_1 complexes and terminal oxidases in the extremophilic chemolithoautotrophic *Acidithiobacillus ferrooxidans*

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Abstract

Acidithiobacillus ferrooxidans is an acidophilic chemolithoautotrophic bacterium that can grow in the presence of either the weak reductant Fe^{2+}, or reducing sulfur compounds that provide more energy for growth than Fe^{2+}. We have previously shown that the uphill electron transfer pathway between Fe^{2+} and NAD^+ involved a bc_1 complex that functions only in the reverse direction [J. Bacteriol. 182, (2000) 3602]. In the present work, we demonstrate both the existence of a bc_1 complex functioning in the forward direction, expressed when the cells are grown on sulfur, and the presence of two terminal oxidases, a *bd* and a ba_3 type oxidase expressed more in sulfur than in iron-grown cells, besides the cytochrome aa_3 that was found to be expressed only in iron-grown cells. Sulfur-grown cells exhibit a branching point for electron flow at the level of the quinol pool leading on the one hand to a *bd* type oxidase, and on the other hand to a $bc_1 \rightarrow ba_3$ pathway. We have also demonstrated the presence in the genome of transcriptionally active genes potentially encoding the subunits of a bo_3 type oxidase. A scheme for the electron transfer chains has been established that shows the existence of multiple respiratory routes to a single electron acceptor O_2. Possible reasons for these apparently redundant pathways are discussed.

Keywords: *Acidithiobacillus ferrooxidans*; Acidophile; Electron transfer chain; Iron and sulfur oxidation; bc_1 complex; Terminal oxidase

1. Introduction

Acidithiobacillus ferrooxidans is one of the principal bacteria of a consortium of microorganisms used in the industrial extraction of copper from ores and in gold beneficiation [1]. The energy required for its growth and cell maintenance involves the oxidation of a number of reducing sulfur compounds (S°, S^{2-}, $S_2O_3^{2-}$, SO_3^{2-}, $S_4O_6^{2-}$) and/or ferrous ion under acidic conditions, using O_2 as the oxidant [2–4]. However, under certain conditions, Fe^{3+} may also serve as an alternative oxidant for sulfur compounds [5–7].

When this bacterium grows on Fe^{2+}, little energy is available as the result of the oxidative reaction. However, the bacterium fixes its own CO_2, and Fe^{2+} oxidation (downhill pathway) must therefore be coupled to the reduction of $NAD^+(P)$ required for this fixation and also for other anabolic processes. As the redox midpoint potential (E_m) of the couple Fe^{3+}/Fe^{2+} (+650 mV at pH 2) is much more positive than that of the couple $NAD(P)^+/NAD(P)H$ (−305 mV at pH 6.5, the cytoplasmic pH of *A. ferrooxidans* [8]), the reduction of $NAD(P)^+$ from Fe^{2+} requires energy. It has been suggested that an uphill electron transfer, established at the expense of the energy derived from the oxidation of Fe^{2+} by oxygen, may be involved in the reduction of $NAD(P)^+$ from Fe^{2+} [9]. The downhill electron transfer chain from Fe^{2+} to O_2 is thought to involve a Fe^{2+} -cytochrome *c* oxido-reductase [10,11], rusticyanin [12–14], several *c*-type cytochromes (the 14-kDa soluble cytochrome *c* [15] and/or c_4-type cytochromes [16–18], and the outer membrane Cyc2 cytochrome [19], which is similar to the 46-kDa cytochrome *c* [18,20]), and a cytochrome *c* oxidase [21]. In addition, a 30-kDa membrane-bound *c* type cytochrome has been isolated and

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characterized from iron-grown *A. ferrooxidans* [18,20]. A bioinformatic analysis of the nearly complete genome sequence of the type strain *A. ferrooxidans* ATCC 23270 provided by The Institute for Genome Research (TIGR) has revealed the presence of 11 putative cytochromes *c*, four of which belong to the c_4 cytochrome family [22]. Despite substantial efforts, the pathway between Fe^{2+} and O_2 has not yet been completely elucidated and several different models have been proposed [17,19,23–25]. Studies on the uphill electron transfer pathway carried out by Elbehti et al. [18,26] provided evidence for the presence of a bc_1 and a NDHI complex in iron-grown *A. ferrooxidans* and their involvement in the electron transfer between reduced cytochrome *c* and NAD^+. Moreover, Brasseur et al. [27] showed that the bc_1 complex expressed in the iron-grown cells did not function in the forward direction.

Whereas considerable progress has been made in the elucidation of the electron transfer chains involved in downhill and uphill flow from Fe^{2+}, less is known about the mechanism and components of electron transfer during the oxidation of elemental sulfur (E_m of the couple $S°/HS^- = -270$ mV at pH 7) and reduced sulfur compounds (the E_m values for the couples $S_2O_3^{2-}$ (thiosulfate)/$HS^- + HSO_3^-$ and SO_4^{2-}/HSO_3^- are -400 and -520 mV, respectively). Two models for the sulfur oxidizing respiratory systems of *A. ferrooxidans* have been proposed [5,6]. In the first model, Sugio et al. [5] suggested that the electron transfer chain between sulfur compounds and oxygen consists only of a sulfur:ferric oxido-reductase and a cytochrome oxidase, the electrons produced by the oxidation of reduced sulfur compounds enter the respiratory chain at the same site as electrons derived from Fe^{2+}. Therefore, according to this model, the same terminal oxidase is involved in iron and sulfur oxidation pathways. In the second model, studying the oxidation of elemental sulfur on Fe^{2+} grown cells, Corbett and Ingledew [6] proposed that all the electrons arising from sulfur take a pathway through a bc_1 and a cytochrome oxidase complex to O_2. However, this model was based on the erroneous assumption that HQNO is a specific inhibitor of the bc_1 type complexes, whereas it is now known that it is a general inhibitor of the quinone-reacting *b* type cytochromes [28]. Moreover, we have recently shown that the bc_1 complex expressed in iron-grown cells cannot function in the forward direction and a recent bioinformatic analysis of the *A. ferrooxidans* ATCC 23270 genome demonstrated the existence of two different bc_1 complexes in *A. ferrooxidans*, encoded by two different operons [27]. We have then hypothesized that, in sulfur-grown cells, the bc_1 complex might function in the forward direction [27]. These results raised the intriguing possibility that one of the bc_1 complexes might function in the uphill or reverse electron flow from Fe^{2+} to NAD+, whereas the other bc_1 might be involved in the electron transfer in a downhill or forward direction from sulfur to oxygen. In addition, genetic evidence for the existence of an alternate route for electron transfer from sulfur versus Fe^{2+} comes from the observation that insertional inactivation of the *resB* gene, encoding a chaperone known to be involved in cytochrome *c*-type maturation, resulted in cells able to grow on sulfur but not on Fe^{2+} [29,30]. This supports the hypothesis that an alternative electron transfer pathway, not involving a *c*-type cytochrome, can be utilized during sulfur oxidation.

In order to investigate these hypotheses, we carried out biochemical and biophysical studies on electron transfer components of *A. ferrooxidans* grown in the presence of either Fe^{2+} or sulfur. Our experimental results showed the presence of a bc_1 complex functioning in the forward direction in sulfur-grown cells, and of four different terminal oxidases differentially expressed depending on the growth substrate. These results, combined with those obtained previously [26,27], have led us to propose a scheme that involves multiple, divergent electron transfer pathways from both Fe^{2+} and sulfur compounds to O_2. Possible reasons for the existence of these apparently redundant pathways will be discussed.

2. Materials and methods

2.1. Bacterial growth and isolation of membranes

A. ferrooxidans ATCC 19859 was grown aerobically at 30 °C on Fe^{2+} medium at pH 1.6 or on S° medium at pH 3.5 as described in Ref. [31]. Total membranes were obtained after EDTA-lysozyme treatment of cells as described previously [19]. Protein concentration of cells suspensions and membrane fractions were determined as in [22]. All the measurements were carried out on cells or membranes suspended in a 20 mM β-alanine–H_2SO_4 buffer pH 3.5.

2.2. Optical and EPR spectroscopy

Low temperature optical absorption spectra were obtained as described previously [32]. The heme content was determined from the reduced minus oxidized difference spectrum using the following extinction coefficients for the α peak: 18 mM^{-1} cm^{-1} (hemes c), 24 mM^{-1} cm^{-1} (hemes b), 21 mM^{-1} cm^{-1} (hemes a_3), 18.8 mM^{-1} cm^{-1} (heme d). EPR spectra were recorded as in [18].

2.3. Activities measurement

Fe^{2+} oxidase, thiosulfate oxidase and cytochrome *c* oxidase activities of the whole cells were measured polarographically with a Clark electrode (Gilson oxygraph). Cytochrome *c* oxidase activity was started by adding 16 mM ascorbate and 0.1 mM cytochrome *c* (horse heart cytochrome *c*). Inhibitors of the bc_1 complex were added from an ethanolic solution at the final concentration indicated in the legend of the figures. Thiosulfate, sulfide and sulfite quinone reductase activities were measured with a dual wavelength Aminco DW2A spectrophotometer in a

stirred-reaction cuvette, by following quinone reduction (DB) at 280/325 nm with saturating amounts of substrate.

2.4. Expression of the bo_3 and bd terminal oxidases

RNA and DNA were prepared from cells grown in Fe^{2+} or sulfur medium as described previously [30]. PCR and RT-PCR were carried out as described in Ref. [30]. Primers used for the PCR amplification of genes and for the RT-PCR amplification of RNA are as follows: *cydB* (1) forward: ATTCTGGTTCCTGATTCTGGG and (2) reverse: AGCACGCCGCAGAGAATCGC; between *cydA* (3) forward: AAGCTGGCGGCCATGGAAGC and *cydB* (4) reverse: CACACCTGGTTACCGTCCCA; *cyoB* (5) forward: CTTGCCTGGTCTCAGATTCC and (6) reverse: GGTGCATAACCGAACCAGCC and between *cyoA* (7) forward: GACCACGACATTCTTCATTCC and *cyoB* (6) reverse: GGTGCATAACCGAACCAGCC. The positions of the respective primers are shown in Fig. 2.

3. Results

3.1. A. ferrooxidans grown under aerobic conditions expresses different terminal oxidases, depending on the substrate used

Cells and membranes of *A. ferrooxidans* grown on iron or on sulfur were examined for their content of various cytochromes. Low temperature (liquid nitrogen) optical difference spectra between dithionite reduced and oxidized cells and membranes are shown in Fig. 1A and B, respectively. With iron as substrate, cells showed Soret peaks at 418, 428, 440 with a shoulder at about 445 nm. In the α region, peaks were detected at 551 nm with a shoulder at 561 nm, and at 597 nm (Fig. 1A). In spectra derived from membrane preparations, the maximum at 551 nm was shifted to 548 nm and an additional shoulder at 553 nm was observed. This is most likely due to the loss of periplasmic or loosely membrane-associated cytochromes *c*, which leads to a decrease of the absorption band at 551 nm, allowing the peak at 548 and 553 nm to be distinguished. Slight absorptions were also observed at 613 and 630 nm and are more pronounced in membrane spectra (Fig. 1B). These results indicate the presence of various cytochromes *c* (418, 548, 551, 553 nm), cytochromes *b* (428, 561 nm) and of three terminal oxidases: a prevailing cytochrome aa_3 (440 with shoulder at 445 nm, 597 nm), a small amount of cytochrome *d* (630 nm) of a *bd* oxidase and of a cytochrome absorbing at 613 nm, which is probably of a ba_3 type (see Discussion).

When sulfur was used as a growth substrate, cells showed Soret peak at 428 nm with shoulders at about 418 and 440 nm. In the α region, peaks are visible at 551 nm with a shoulder at 561 nm (548, 554 and 561 nm in the membrane preparation) and at 613 and 630 nm (more obvious in membrane spectrum). A slight and broad absorption was also observed at 595 nm whereas the band at 597 nm observed in

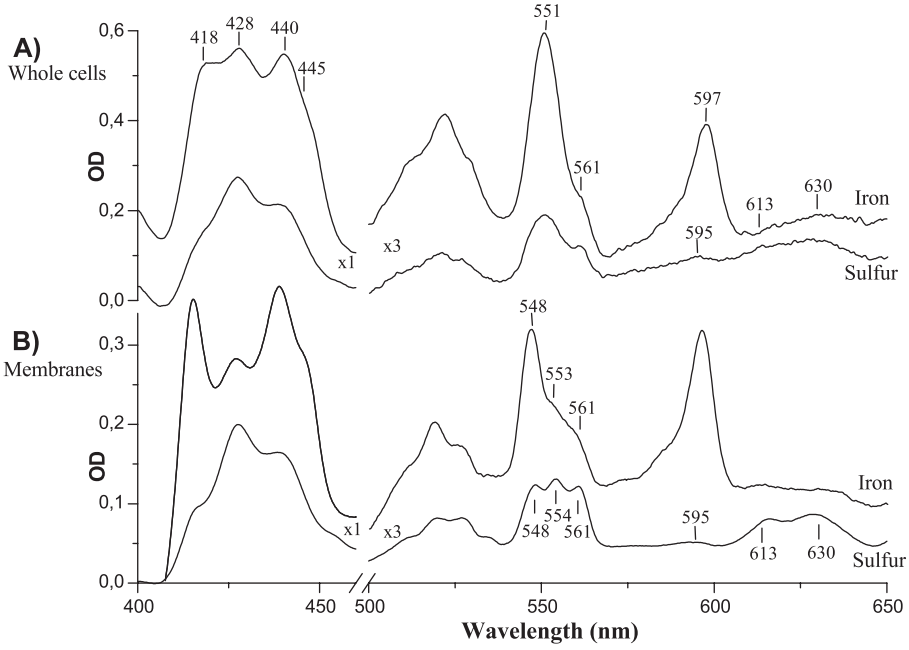

Fig. 1. Low temperature optical spectra of cells and membranes obtained from *A. ferrooxidans* grown on iron or sulfur. Spectra were recorded at liquid nitrogen temperature as the difference between dithionite-reduced minus Na_2IrCl_6-oxidized sample. (A) Spectra of whole cells grown with iron or sulfur as substrate. Both samples were suspended at 4.2 mg/ml in 20 mM β-alanine–H_2SO_4, pH 3.5. (B) Total membrane fraction obtained from cells grown on iron or sulfur and suspended at 3.8 mg/ml in 20 mM β-alanine–H_2SO_4, pH 3.5. Note that the β and α regions (after the *x*-axis break) are amplified three times (×3) in comparison with the Soret region for a better visualization of the cytochromes.

Table 1
Cytochrome content of *A. ferrooxidans* cells grown on iron or sulfur

Cytochromes	Growth substrate	
	Iron	Sulfur
c (550 nm)	1.55	0.56
b (560 nm)	0.31	0.25
aa_3 (597 nm)	0.86	ε
ba_3 (613 nm)	0.06	0.13
d (630 nm)	0.11	0.19

Cells were suspended in 20 mM β-alanine–H_2SO_4, pH 3.5. The concentration of cytochromes is expressed in nmol/mg protein.

iron-grown cells was not detected. These results are consistent with the presence of cytochromes *c* (418, 548, 554 nm), cytochromes *b* (428, 561 nm), the heme a_3 (438 and 613 nm) of a probably ba_3 cytochrome *c* type oxidase (see Discussion) and the heme d (630 nm) of the *bd* type oxidase. This latter oxidase, which absorbs at 630 nm in the α region, exhibits a slight absorption at 438 nm (it is known that the absorption in the γ region of heme *d* is small, at best equal to that of the α region [33,34]). The slight and broad absorption observed at 595 nm is due to heme b 595 of cytochrome *bd* oxidase. In sulfur-grown cells, cytochrome aa_3 is almost not detected (Table 1, Fig. 1). This agrees with the experiments of Yarzabal et al. which show that in sulfur-grown cells of *A. ferrooxidans* ATCC 33020 strain, this cytochrome oxidase is poorly detected and the corresponding genes are weakly transcribed (personal communication). It should be noted that cytochromes aa_3 and ba_3 belong to the heme/copper terminal oxidase superfamily.

3.2. Demonstration of the presence and transcription of genes potentially encoding bd and bo_3 terminal oxidases

A bioinformatic analysis of the partial genome of *A. ferrooxidans* ATCC 23270 from The Institute of Genome Research revealed the presence of potential genes encoding an aa_3 cytochrome *c* type oxidase (*cox1*, *2*, *3*, *4*), and a bo_3 quinol type oxidase (*cyoA, B, C, D, E*) both belonging to the heme/copper terminal oxidase superfamily, and of a third oxidase of the *bd* type (*cydA, B*). In each case, these genes appear to be organized in operons. We have not found the presence of an oxidase of the ba_3 type in the sequenced genome of *A. ferrooxidans* ATCC 23270 strain.

Because of the possibility that the genes coding for the cytochrome bo_3 oxidase were not present in the *A. ferrooxidans* ATCC 19859 strain under investigation in this paper, and in order to confirm the presence of the genes coding for the *bd* type oxidase, we carried out PCR and RT-PCR experiments using DNA and RNA, respectively, isolated from *A. ferrooxidans* ATCC 19859 (Fig. 2B). The presence of *cydA*, *cydB*, *cyoA* and *cyoB* in the genome was verified (Fig. 2B, lanes 2, 3, 4 and 7). In addition, RNA expression was detected for *cydB* and *cyoB* for cells grown in Fe^{2+} (Fig. 2B, lanes 5 and 8) or sulfur medium (Fig. 2B, lanes 6 and 9). However, since RT-PCR is not a quantita-

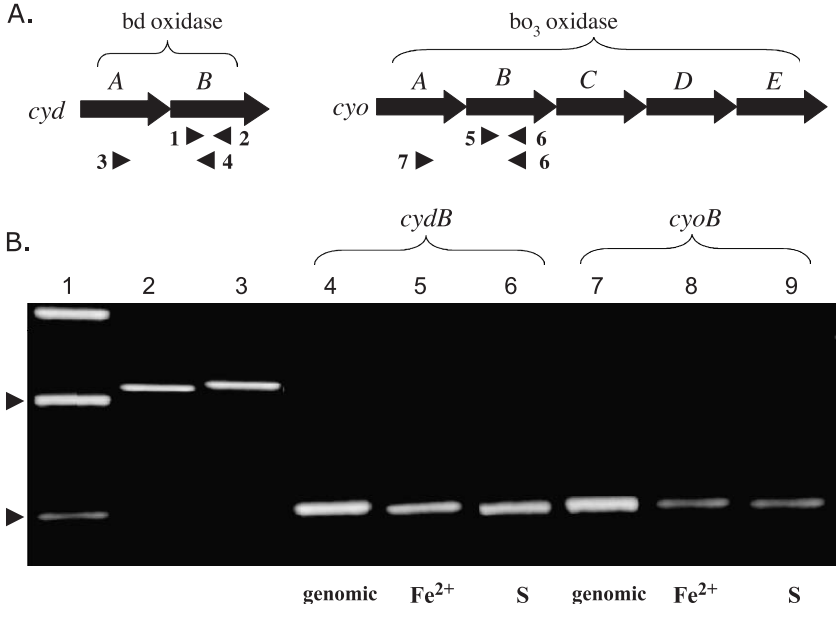

Fig. 2. (A) Bioinformatic identification of the candidate genes and their organization that potentially encode *bd* and bo_3 type terminal oxidases derived from a bioinformatic examination of the genome of *A. ferrooxidans* ATCC 23270. Small arrowheads indicate the primers used in part B below. The numbers refer to the identification of primers described in Materials and methods. (B) Experimental demonstration of the presence of the proposed *bd* and bo_3 encoding genes in *A. ferrooxidans* 19859 and their transcription in cells grown in either Fe^{2+} or sulfur as indicated. Lane 1 = DNA molecular weight standard where the arrows mark DNA bands of 1000 and 500 bp. Lanes 2 and 3 are PCR products of the amplification of genomic *cydA/cydB* (1024 bp) using primers 3 and 4 and genomic *cyoA/cyoB* (1114 bp) using primers 7 and 6, respectively, demonstrating the presence and juxtaposition of the respective gene pairs in the genome. Lanes 5, 6, 8 and 9 show RT-PCR products of total RNA of cells grown in Fe^{2+} (lane 5 = *cydB*, lane 8 = *cyoB*) or sulfur (lane 6 = *cydB*, lane 9 = *cyoB*). Lanes 4 and 7 show PCR products of the genomic DNA using primers 1 and 2 for *cydB* (lane 4) and primers 5 and 6 for *cyoB* (lane 7).

Table 2
Fe^{2+} oxidase, thiosulfate-oxidase, cytochrome *c* oxidase and quinol oxidase activities of *A. ferrooxidans* cells grown on iron or sulfur

Growth substrate	Fe^{2+} oxidase	Thiosulfate oxidase	Cytochrome *c* oxidase	Quinol oxidase
Iron	850	95	40	70
Sulfur	150	810	11	140

Cells were suspended in 20 mM β-alanine–H_2SO_4, pH 3.5. The Fe^{2+} oxidase, thiosulfate oxidase and cytochrome *c* oxidase activities are expressed in nmol O_2/min/mg. The use of a non-physiological cytochrome *c* (horse heart cytochrome *c*) accounts for the low cytochrome *c* oxidase activity. The quinol oxidase activity is expressed in nmol quinol oxidized/min/mg.

tive technique, we cannot, at present, evaluate whether these genes are differentially expressed in the two growth conditions.

3.3. The Fe^{2+} and thiosulfate oxidase activities of *A. ferrooxidans* grown on Fe^{2+} or on sulfur: action of KCN

The Fe^{2+} oxidase and thiosulfate oxidase activities of *A. ferrooxidans* cells grown either on iron or on sulfur were measured at pH 3.5 (Table 2). The Fe^{2+} oxidase activity was found to be six fold higher in Fe^{2+} than in sulfur-grown cells whereas the thiosulfate-oxidase activity was found to be eight fold higher in sulfur than in Fe^{2+} grown cells.

The Fe^{2+} oxidase activity of the cells grown on Fe^{2+} and of that grown on sulfur was found to be inhibited by low concentrations of KCN [I_{50} at about 25 and 35 μM, respectively (I_{50} = concentration of inhibitor required to inhibit 50% of the activity)] (Fig. 3) corresponding to the inhibition of a cytochrome *c* oxidase, principally of the aa_3 type in the case of iron-grown cells, and of the ba_3 type in the case of sulfur-grown cells, as suggested by the spectroscopic data.

In the case of sulfur-grown cells, the curve corresponding to the inhibition by KCN of the thiosulfate oxidase activity appears to be biphasic with a first inhibition of about 35% of the total activity at a low concentration of KCN [I_{50} at about 35 μM]. The maximum inhibition (about 80%) was obtained at a high concentration of KCN (about 5 mM), indicating the presence of other types of terminal oxidases (Fig. 3). Optical spectra show the presence of a ba_3 (which is of the *c* type oxidase, see Discussion) and a *bd* quinol oxidase. The *bd* quinol oxidases that do not have the heme-copper binuclear center are known to be sensitive to high concentrations of KCN (for example in *Escherichia coli*, the I_{50} value was found to be 2 mM [35]). Thus, the second part of the inhibition curve can be assigned to the inhibition of the *bd* oxidase and therefore the first part of the curve should correspond to the inhibition of the ba_3 type oxidase.

The thiosulfate oxidase activity of cells grown on Fe^{2+} was not inhibited by low concentrations of KCN. The inhibition of this activity starts at a concentration of KCN above 2 mM, corresponding to the beginning of the second phase of inhibition for the cells grown on sulfur (Fig. 3). These results suggest that, in iron-grown cells, the majority of the electrons arising from thiosulfate do not take a pathway towards the aa_3 type oxidases, but instead towards the *bd* quinol oxidase. We substantiate this hypothesis in Section 3.5.

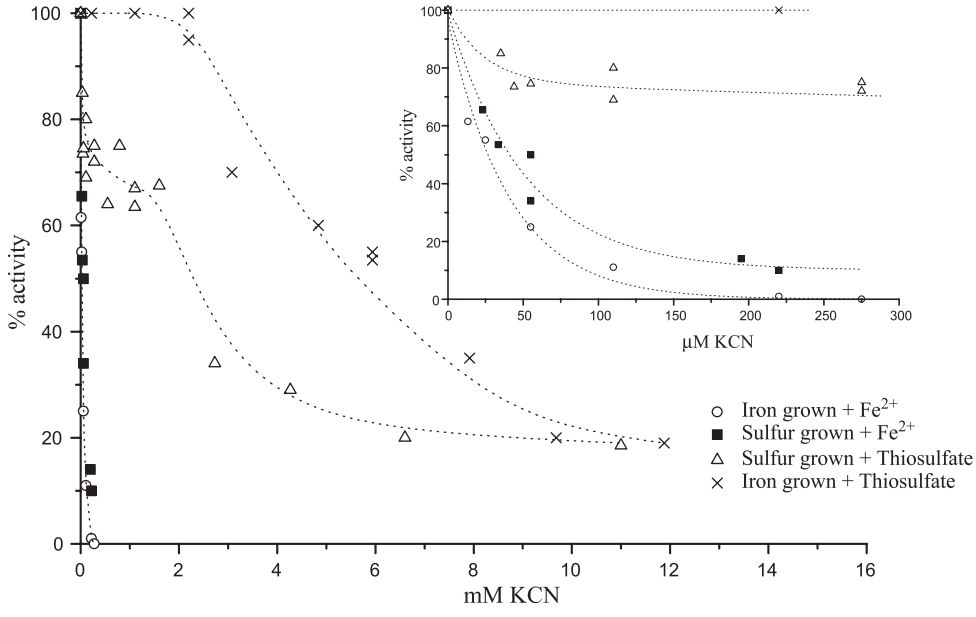

Fig. 3. Action of KCN on the Fe^{2+}-O_2 and thiosulfate-O_2 activities of *A. ferrooxidans* cells grown on iron or on sulfur. The Fe^{2+}-O_2 activity was measured on iron (○) or sulfur (■) grown cells. The thiosulfate-O_2 activity was measured on iron (×) or sulfur (△) grown cells. The rate of O_2 consumption was measured with a Clark O_2 electrode and was set to 100% in the absence of KCN. The 100% activity was 1020 (○), 150 (■), 920 (△) and 120 nmol O_2/min/mg (×). Samples were suspended in 20 mM β-alanine–H_2SO_4, pH 3.5. KCN was added 2 min before each measurement. The inset shows the same data with a micromolar scale in order to improve the visualization of the inhibition curves of the KCN-sensitive aa_3 and ba_3 oxidases in comparison with the less sensitive *bd* quinol oxidase.

Table 3
Activities and their percentage inhibition by various inhibitors of thiosulfate-quinone reductase (TQR) and sulfide-quinone reductase (SQR) in *A. ferrooxidans* cells grown either on iron or sulfur

Growth substrate	TQR activity							SQR activity	
	Without inhibitor	%Inhibition						Without inhibitor	%Inhibition HQNO
		KCN	Ant.	Myx.	Stig.	HQNO	Rot.		
Iron	18	0	0	0	0	100	0	18	100
Sulfur	425	0	0	0	0	100	0	478	100

The cells were suspended in 20 mM β-alanine–H_2SO_4, pH 3.5. Activities are expressed in nmol quinone Q_1 reduced/mn/mg protein. Inhibitors were used at the following concentrations: KCN 2 mM, antimycin (Ant.) 15 μM, myxothiazol (Myx.) 38 μM, stigmatellin (Stig.) 20 μM, HQNO 16 μM, rotenone (Rot.) 20 μM.

3.4. The quinone-reductase activities of *A. ferrooxidans* grown on Fe^{2+} or on sulfur: action of various inhibitors

A. ferrooxidans cells grown on Fe^{2+} or on sulfur catalyze the electron transfer from sulfide and thiosulfate to exogenous added quinones (Table 3). Both sulfide and thiosulfate-quinone reductase activities (SQR and TQR activities, respectively) were found to be more than 20 times higher when the cells were grown on sulfur than on Fe^{2+} and, in both cases, they were found to be inhibited by the quinone analogue HQNO. In contrast, they were not inhibited by either specific inhibitors of the bc_1 and NDHI complexes nor by KCN, even at high concentration (Table 3). When grown on sulfur, the cells also exhibit a sulfite quinone reductase activity about 10 times lower than the SQR and TQR activities and which is inhibited by HQNO (result not shown).

3.5. The bc_1 complex of the *A. ferrooxidans* cells grown on Fe^{2+} or on sulfur

When the cells were grown on Fe^{2+}, the iron and thiosulfate oxidase activities were not inhibited by the known bc_1 complex inhibitors antimycin A, myxothiazol and stigmatellin, even at high concentrations (Table 4). The thiosulfate oxidase activity was found to be inhibited by KCN only at high concentration (Fig. 3), and by the nonspecific inhibitor HQNO, which inhibits nearly all the quinol oxidase and quinone reductase enzymes (Table 4). These results show that, in iron-grown cells, the bc_1 complex does not intervene in the electron transfer, neither between iron and O_2 nor between thiosulfate and O_2. This means that the hydroquinol formed at the thiosulfate quinone reductase step (see Table 3) cannot give electrons to the bc_1 complex, but only to a quinol type oxidase that is of a *bd* type, according to our spectroscopic data. This strongly suggests that the bc_1 complex expressed in Fe^{2+} grown cells does not function in the forward direction, consistent with our previous results [27].

When the cells were grown on sulfur, the iron oxidase activity was found to be insensitive to the bc_1 complexes inhibitors (and NDH1 inhibitors, results not shown) even at high concentration, whereas the thiosulfate oxidase activity was found to be about 50% inhibited by myxothiazol and stigmatellin at low concentration (I_{50} about 1 μM) (Fig. 4 and Table 4). At high concentration of these inhibitors, the maximum of inhibition is still around 50% (Fig. 4). Note that antimycin A partially inhibits this activity at higher concentration (10 μM), probably indicating a less favorable penetration of this inhibitor into the cells. HQNO completely inhibited this activity (Table 4). The electron transfer pathway, insensitive to myxothiazol and stigmatellin, was found to be sensitive to high concentration of KCN (2 mM) (result not shown). These results show that, for the sulfur-grown cells, a part of the electrons arising from the substrate thiosulfate took a pathway through a bc_1 type complex functioning in the forward direction, whereas the other part of the electrons took a pathway through a *bd* quinol type oxidase sensitive to HQNO and to high concentration of KCN. This is the first demonstration of the existence of a bc_1 complex functioning in the forward direction in sulfur-grown cells and of the presence of a branching point at the level of the quinone pool in *A. ferrooxidans*.

The EPR spectra recorded on membranes show that the g_y signal centered at $g = 1.895$ is two to three times higher

Table 4
Inhibition of the Fe^{2+}oxidase and thiosulfate oxidase activities by various inhibitors for *A. ferrooxidans* cells grown either on iron or sulfur

Growth substrate	Fe^{2+}oxidase activity, %inhibition			Thiosulfate oxidase activity, %inhibition			
Iron	Ant.[a]	Myx.[a]	Stig.[a]	Ant.[a]	Myx.[a]	Stig.[a]	HQNO
	< 10	0	< 10	< 10	0	< 10	100
Sulfur	Ant.[a]	Myx.[a]	Stig.[a]	Ant.[b]	Myx.[c]	Stig.[c]	HQNO
	< 10	0	< 10	27	50	50	100

The cells were suspended in 20 mM β-alanine–H_2SO_4, pH 3.5. The concentration of the inhibitors was as follows—[a]high concentration: antimycin (Ant.) 100 μM, stigmatellin (Stig.) 80 μM, myxothiazol (Myx.) 205 μM; [b]Ant. 10 μM; [c]low concentration: Stig. and Myx. 2 μM (see Fig. 4), HQNO 10 μM.

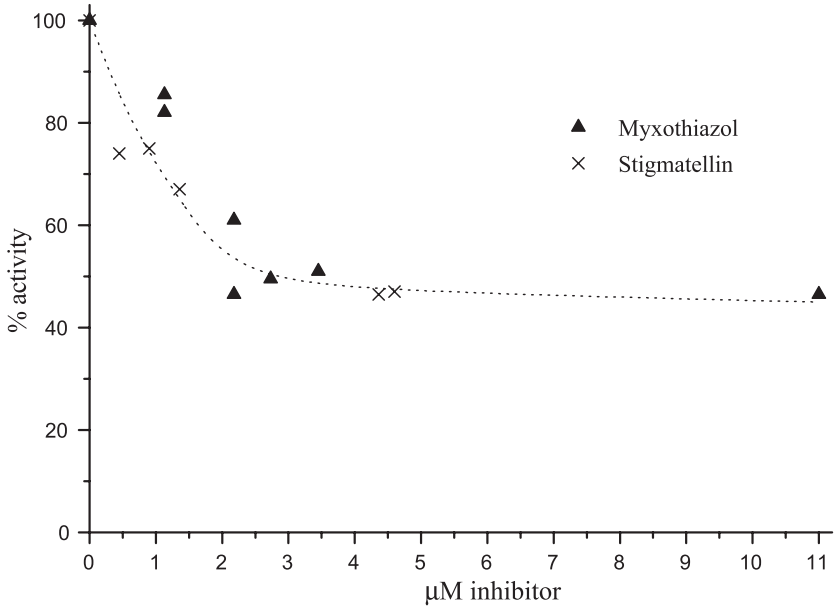

Fig. 4. Inhibition of the thiosulfate-O_2 activity by the bc_1 complex inhibitors myxothiazol (▲) and stigmatellin (×) in *A. ferrooxidans* cells grown on sulfur. The rates of O_2 consumption were measured with a Clark O_2 electrode and was set to 100% in the absence of inhibitor (1050 nmol O_2/min/mg). Samples were suspended in 20 mM β-alanine–H_2SO_4, pH 3.5. Inhibitor was added 2 min before each measurement.

in iron- than in sulfur-grown cells (Fig. 5). This signal is found in iron sulfur proteins of the bc_1 complexes and also in the Rieske type [2Fe–2S] centers, which are present in the soluble dioxygenases [36], and in arsenite oxidases [37]. A search in the complete genome of *A. ferrooxidans* ATCC 23270 for the presence of Rieske-type proteins, with the two conserved motifs CXHXG and CXXH, containing the two cysteines and histidines ligands for the [2Fe–2S] cluster, revealed, in addition to the two genes that encode the two known Rieske proteins associated with the two *pet* operons coding for the two bc_1 complexes, the presence of another gene that encodes for a soluble dioxygenase. This indicates that the EPR $g_y = 1.895$ signal, obtained with membrane fractions, is only due to the presence of the Rieske proteins from the bc_1 complexes and suggests that there is two to three times more total bc_1 complex expressed in iron- than in sulfur-grown cells. However, it is not possible to discriminate between the two Rieske proteins of the two bc_1 complexes because they have certainly the same g_y signal.

The results presented here suggest that, for sulfur-grown cells, there is a branching point at the level of the quinone pool, where some of the electrons can be shunted through a bc_1 complex to a cytochrome *c* oxidase of the ba_3 type,

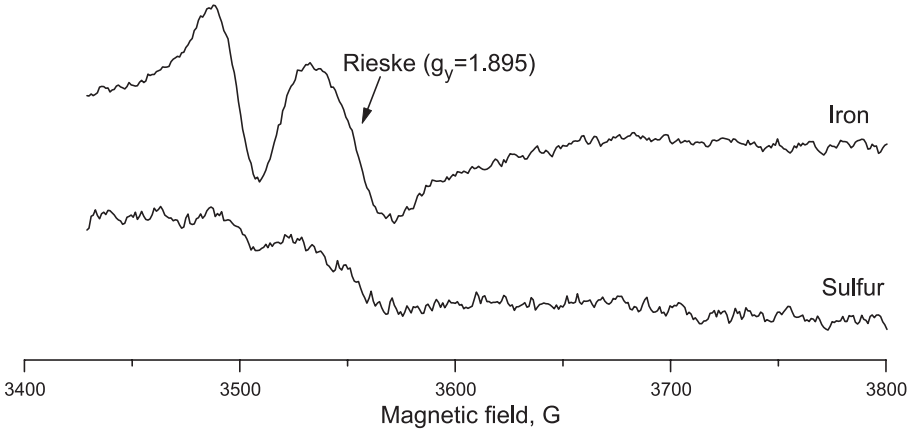

Fig. 5. EPR spectra of the Rieske protein of the bc_1 complexes. Spectra were recorded on membranes extracted from *A. ferrooxidans* grown on iron or sulfur. Membranes were suspended at 15.6 mg/ml in 20 mM β-alanine–H_2SO_4, pH 3.5. Samples were reduced by addition of dithionite. The Rieske EPR g_y signal is indicated at $g = 1.895$. EPR conditions were as follows: temperature, 15 K; microwave frequency, 9.42 GHz; microwave power, 6.3 mW; modulation frequency, 100 kHz; and modulation amplitude, 1.6 mT.

whereas the remaining electrons are transferred directly to a *bd* quinol oxidase.

4. Discussion

4.1. The iron and sulfur oxidation systems and the quinone reductase activities in A. ferrooxidans

A. ferrooxidans expresses both Fe^{2+} and thiosulfate oxidase activities in iron as well in sulfur-grown cells (Table 2). However, the Fe^{2+} oxidase activity is higher in iron- than in sulfur-grown cells, whereas the thiosulfate oxidase activity is higher in sulfur than in iron-grown cells. This indicates that both systems are regulated depending on the electron donor present in the medium. The mechanisms involved in this regulation remain to be determined.

In numerous chemolithoautotrophic bacteria, electrons arising from reduced sulfur compounds form an energy source, which allows ATP synthesis and reduction of NAD^+ to NADH. Two pathways for the oxidation of sulfide to sulfate have been discussed, for which the initial step is either a sulfide-flavocytochrome *c* reductase or a sulfide-quinone reductase (SQR). In this work, we have shown the existence of both sulfide and thiosulfate quinone oxido-reductase activities (SQR and TQR, respectively) in *A. ferrooxidans* (strain ATCC 19859) and a search on the data bases showed the presence of a sulfur quinone reductase enzyme in the genome of *A. ferrooxidans* ATCC 23270 [38]. However, it is not known whether the thiosulfate quinone reductase activity corresponds to a unique enzyme or to a series of various enzymatic reactions. Although the SQR and TQR activities are present in the two growth conditions, it is noteworthy that both activities are about 20 times more expressed in sulfur than in iron-grown cells, indicating that the expression of these enzymes is regulated depending on the growth substrate (Table 3).

4.2. The terminal oxidases of A. ferrooxidans

As has been observed in many bacteria, *A. ferrooxidans* contains more than one terminal oxidase. With a growing number of bacterial genome sequences available, it is becoming increasingly clear that such redundancy is quite common. *Bacillus subtilis* strain 168 is known to contain three terminal oxidases and a fourth has been predicted from the genome sequence [39]. In *Azorhizobium caulinodans*, the presence of at least four terminal oxidases has been demonstrated, and studies on mutated strains suggested the presence of a fifth terminal oxidase [40]. In this work, we have shown that *A. ferrooxidans* ATCC 19859 strain contains four types of terminal oxidases including an aa_3 cytochrome *c* oxidase, a quinol oxidase of the *bd* type, a bo_3 quinol type oxidase and another cytochrome *c* oxidase probably of the ba_3 type. The degree of expression of the aa_3, ba_3 and *bd* oxidases was found to be dependent on the growth conditions (iron or sulfur as substrate). However, although expression of the bo_3 oxidase genes was detected in both growth conditions by RT-PCR, we were unable to determine the relative levels of expression of the genes in the two conditions because RT-PCR is not quantitative. In addition, the spectroscopic characteristics of the bo_3 oxidase are not specific. Indeed, the absorbance of hemes o is identical to that of hemes b, and thus would be superimposed with the other *b* type cytochromes from the bc_1, *bd* and bo_3 complexes. Thus, it is not possible at the present time to gain functional information about this oxidase. However, as we know that it is a quinol oxidase, it should intervene just after the quinone pool, like the *bd* oxidase (Fig. 6).

Cytochrome aa_3 was found to be the predominant oxidase in iron-grown cells and was present at barely detectable levels in sulfur-grown cells. In iron-grown cells, the two other oxidases were expressed at a lower degree, about 8 and 14 times less for cytochrome *bd* and cytochrome ba_3 with regards to cytochrome aa_3, respectively (Fig. 1 and Table 1). The reduced minus oxidized optical absorption spectra of the cells and of the membranes showed peaks at 597 nm for the α region and at 440 nm with a shoulder at 445 nm for the γ region. As usually the aa_3 type oxidases exhibit an absorption peak at 603–605 nm in the α region, it was initially thought that the oxidase found in *A. ferrooxidans*, which exhibited a peak at 597 nm, was not of the aa_3 type, but of an a_1 type. However, we have recently shown that the oxidase of another strain of *A. ferrooxidans* (BRGM strain), which absorbs at 597 nm, is of the aa_3 type (Ref. [41] and manuscript in preparation). This result can be explained in the light of the findings of Riistama et al. [42] who have shown that replacing Arg-54 by Gln or Met in subunit I of *Paracoccus denitrificans* cytochrome oxidase blue-shifts the absorption peak of cytochrome *a* from 605 (peak of the wild type) to 597 and 589 nm in the mutants, respectively. A diminution (R84Q) or a loss (R54M) of the hydrogen bonding to the formyl group of heme a is thought to be the major reason for the blue shift in the spectrum. A multiple alignment of the cytochrome oxidase subunit I from two strains of *A. ferrooxidans* (ATCC 23270 [TIGR] and ATCC 33020 [23]) with that of *P. denitrificans* shows that Arg is not present in the corresponding position (results not shown). This can explain why cytochrome oxidase of *A. ferrooxidans* is of the aa_3 type despite an absorption peak at 597 nm.

The expression of the *bd* quinol oxidase is 1.7 times higher in sulfur than in iron-grown cells, and the quinol oxidase activity is two times higher in sulfur than in iron-grown cells (Tables 1 and 2). In the case of sulfur-grown cells, cytochrome *bd* is the predominant oxidase, the ba_3 oxydase is expressed 1.5 times less than cytochrome *bd*, and the expression of cytochrome aa_3 is barely detectable. The cytochrome *bd* is unrelated to the heme-copper binuclear center oxidase superfamily, which includes the aa_3 and ba_3 cytochrome *c* oxidases. This *bd* enzyme is widely distrib-

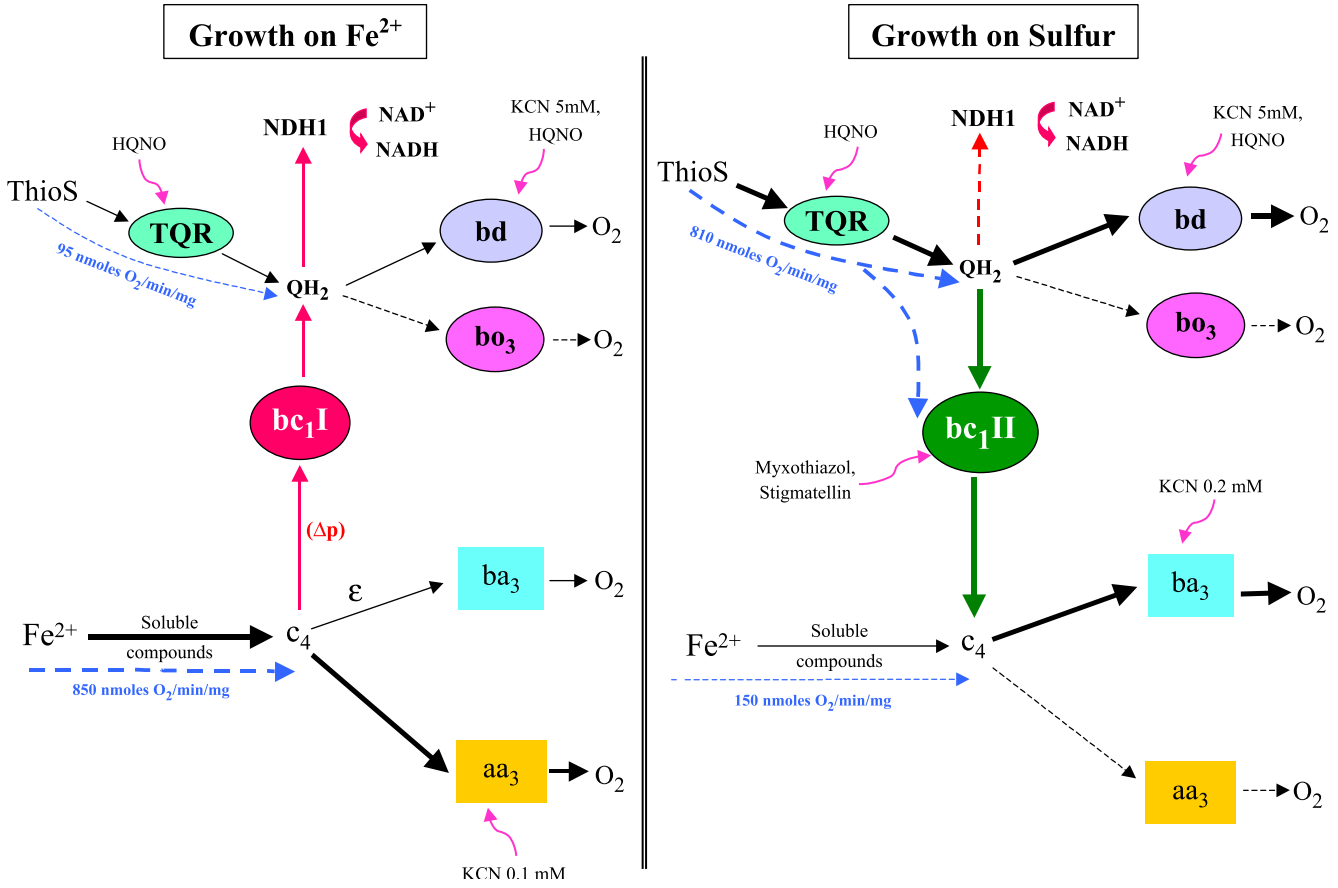

Fig. 6. Model for the electron transfer pathways in *A. ferrooxidans* grown on iron or sulfur. Uphill electron transfer steps at the level of the bc_1 complex (I) and NDHI [26] are colored in red when the cells are grown with the weak reductant Fe^{2+}. Forward functioning ($QH_2 \rightarrow$ cyt *c*) of bc_1 complex (II) is colored in green when the cells are grown with the stronger reductant sulfur. The thickness of the arrows (plain or dashed) corresponds to the flux of electrons through the corresponding steps. Blue dashed arrows indicate the values of the respiration rates. Note that, in this figure, TQR probably does not correspond to a unique enzyme but more likely to a chain of enzymes that oxidizes thiosulfate and reduces the quinone pool. Soluble compounds correspond to the small soluble and periplasmic proteins (cyt *c*, rusticyanin). The bo_3 oxidase should intervene just after the quinone pool (see Discussion). In the case of the growth on sulfur, the Q pool is reduced by the sulfur compounds and NADH could also be produced by a reverse functioning of NDHI (not demonstrated). Curvilinear red arrows indicate action of inhibitors.

uted in bacteria, particularly in gram-negative heterotrophs. It has also been found in the gram-positive bacteria *Bacillus stearothermophilus* [43]. In *Azotobacter vinelandii*, high aeration of the culture induces an increase in the cytochrome *bd* content, and an oxidase of the *bo* type is induced under oxygen limiting conditions [44]. In *E. coli*, cytochrome *d* is induced during oxygen limited growth and cytochrome *bo* prevails at higher oxygen concentration [45]. Thus, in *E. coli*, it has been suggested that cytochrome *bd* serves to protect anaerobic processes from inhibition by oxygen, whereas in *A. vinelandii* it serves to protect oxygen labile nitrogenase during aero-tolerant nitrogen fixation. In this organism, high expression of cytochrome *bd* at elevated oxygen level is also observed in the absence of diazotrophy [44]. This is what we observed in our strain, which synthesizes cytochrome *bd* in high aeration cultures under non-nitrogen fixing conditions. *A. ferrooxidans* is an aerobic N_2-reducing bacterium, which contains a nitrogenase [46], and we suggest that, in this bacterium, rapid consumption of oxygen by an active oxidase is necessary to maintain a low intracellular oxygen concentration compatible with nitrogenase function. Our results suggest that cytochrome *bd* plays this role in *A. ferrooxidans*.

Kamimura et al. [47] have purified an ubiquinol oxidase of the *bd* type from the NASF 1 *A. ferrooxidans* strain. They have shown that the purified enzyme was inhibited by antimycin and myxothiazol at concentrations of 10 and 16 μM, respectively, that correspond to 337 and 540 mol inhibitor/mol enzyme, respectively (calculation from Fig. 3 and Table 3 in their article). These concentrations are high with regards to that necessary to inhibit the bc_1 complex (1 mol inhibitor/mol enzyme). In membranes of *E. coli*, the cytochrome *bd* was found to be sensitive to antimycin A at a concentration of 50 μM (500 mol inhibitor/mol enzyme). At 200 μM of stigmatellin (2000 mol stigmatellin/mol enzyme), only a slight inhibition (14%) was observed and no inhibition with myxothiazol at 125 μM (1250 mol myxothiazol/mol enzyme)

[48]. Similarly in our strain, the iron-grown cells exhibited a thiosulfate oxidase activity inhibited by high concentrations of KCN but not by stigmatellin and myxothiazol even at high concentration (see Fig. 3 and Table 4). These results suggest that the *bd* oxidase of the *A. ferrooxidans* ATCC 19859 strain is not inhibited by myxothiazol and stigmatellin and is inhibited by high concentration of KCN.

Optical spectra of cells grown in either iron or sulfur medium show peaks at 613 nm in the α region (440 nm in the γ region), with a greater expression in sulfur-grown cells (Fig. 1). These absorption maxima are indicative of the heme a_3 of a ba_3 type cytochrome oxidase [49], suggesting that the 613-nm oxidase is of the ba_3 type. However, further characterisation of this oxidase will be necessary to support this suggestion. We have shown that sulfur-grown cells of *A. ferrooxidans* expressed a significant cytochrome *c* oxidase activity that is about one fourth of that detected in iron-grown cells (Table 2). This means that a cytochrome *c* oxidase must be used in sulfur-grown cells. We have shown that in these growth conditions, *A. ferrooxidans* expressed three terminal oxidases, *bd* and bo_3 type quinol oxidases and a ba_3 oxidase. The ba_3 oxidases can be either of the quinol type [50] or of the cytochrome *c* type [51–53]. Our results suggest that in ATCC 19859 strain, the only one possible candidate for the cytochrome *c* oxidase activity is the ba_3 oxidase, which thus would be of the *c* type. The ba_3 oxidases belong to the heme-copper superfamily and in *Thermus thermophilus*, this oxidase was found to pump protons [50]. Until now, the ba_3 cytochrome *c* type oxidases have been found in organisms that grow in extreme environmental conditions such as high temperature and low oxygen concentration or haloalkaliphilic medium [51–54]. *A. ferrooxidans* would be the first acidophilic, gram-negative eubacterium known to contain a cytochrome *c* oxidase of the ba_3 type.

4.3. The bc_1 complexes of A. ferrooxidans

When the cells are grown on iron, the Fe^{2+} and thiosulfate oxidase activities are not inhibited by the bc_1 complex inhibitors, suggesting that the bc_1 complex cannot function in the forward direction, even with a thermodynamically appropriate electron source such as thiosulfate (Table 4). These results are consistent with what we have already observed in another strain of *A. ferrooxidans* [27]. On the other hand, we demonstrated here for the first time that in sulfur-grown cells, the thiosulfate oxidase activity is partially inhibited by low concentration of myxothiazol and stigmatellin (about 50%) and KCN (about 35%) (Figs. 4 and 3, respectively). This indicates: (1) the presence of a bc_1 complex functioning in the forward direction, (2) that only a part of the electrons arising from thiosulfate takes a pathway through a bc_1 complex, (3) the presence of a cytochrome *c* oxidase, sensitive to low concentrations of KCN, which seems to be of the ba_3 type. The other part of the electron transfer, which was inhibited by high concentration of KCN and by HQNO, takes a pathway through a quinol oxidase identified as a *bd* type oxidase. Corbett and Ingledew [6] had suggested the involvement of a bc_1 complex in the electron transfer between sulfur and Fe^{3+} and between sulfur and O_2, in the case of sulfur-grown cells. In their model, all the electrons arising from sulfur take this pathway. However, their conclusions were based on the observation that the reduction of Fe^{3+} by sulfur was inhibited by high concentrations of HQNO (with an I_{50} higher than 350 moles of HQNO/mole of cytochrome *b*) whereas it is now known that inhibition of the bc_1 complex by HQNO is usually achieved at low HQNO concentration, and that this compound inhibits most of the quinone reacting enzymes [28]. In agreement with this contention, our results show that *A. ferrooxidans* exhibits sulfide and thiosulfate quinone reductase activities totally inhibited by HQNO (Table 3). Thus, the results of Corbett and Ingledew could be reinterpreted as an inhibition of a sulfur quinone reductase activity which would be the common step of the electron transfer pathway between, on the one hand, sulfur and Fe^{3+}, and on the other hand, sulfur and O_2. We have shown here that when the cells were grown on sulfur, a bc_1 complex functioning in the forward direction is expressed, whereas when the cells were grown on iron, the bc_1 complex expressed cannot function in the forward direction. We have also demonstrated that, in another strain of *A. ferrooxidans*, the bc_1 complex expressed when the cells were grown on iron functions only in the reverse direction, allowing the electrons to reduced NAD^+ to NADH [26]. Is the bc_1 complex expressed in iron the same as that expressed in sulfur-grown cells? Since the bc_1 complex expressed in the iron-grown cells cannot function in the forward direction whereas that expressed in sulfur-grown cells functions in the forward direction, these two bc_1 complexes have to be different. Consistent with this hypothesis is the observation that two bc_1 complexes exist in *A. ferrooxidans* ([27,30], and Bruscella, Levican, Holmes, Jedlicki and Bonnefoy, unpublished results).

4.4. Tentative scheme for the aerobic electron transfer chains in A. ferrooxidans

The experimental data presented in this paper have been summarized in a scheme presented in Fig. 6. This scheme does not show the subcellular localization of the various components.

We have seen that sulfur-grown cells exhibit a Fe^{2+} oxidase activity inhibited by low concentrations of KCN and not inhibited by the bc_1 complexes inhibitors. These results suggest that the bc_1 complex does not intervene in the electron transfer pathway between Fe^{2+} and O_2 and that this pathway proceeds through a cytochrome *c* terminal oxidase. In addition, in sulfur-grown cells, besides the quinol *bd* and bo_3 type oxidases, a ba_3 type oxidase is

expressed at significant levels, whereas cytochrome aa_3 is almost not detected. Thus, in this growth condition, the only candidate for the terminal cytochrome *c* oxidase is the ba_3 type oxidase. Based on these results, we propose that, in sulfur-grown cells, the majority of electrons arising from Fe^{2+} takes a pathway essentially through a ba_3 type cytochrome *c* oxidase (Fig. 6). In this growth condition, experimental results show that about 50% of the thiosulfate oxidase activity is inhibited by low concentrations of the bc_1 complex inhibitors, meaning that about half of the electrons arising from this sulfur compound takes a pathway through the bc_1 complex. Experimental results also show that about 35% of the thiosulfate oxidase activity is inhibited by low concentrations of KCN, indicating that a part of the electrons arising from thiosulfate takes a pathway through a cytochrome oxidase which is a ba_3 cytochrome *c* type oxidase, as suggested by the spectroscopic data. In addition, we have shown that the thiosulfate oxidase activity insensitive to the bc_1 complex inhibitors is sensitive to HQNO and to high concentrations of KCN. This indicates that a part of the electrons arising from thiosulfate takes a pathway through a quinol oxidase, which can be a *bd* and/or a bo_3 oxidase, as suggested by the experimental data. We have suggested that in *A. ferrooxidans* cytochrome *bd* serves to protect oxygen labile nitrogenase during aero-tolerant nitrogen fixation. A possible hypothesis concerning the presence of the two quinol oxidases in *A. ferrooxidans* is that cytochrome *bd* prevails in high oxygen concentrations, whereas cytochrome bo_3 prevails under oxygen limiting conditions. Experiments are in progress to obtain a better understanding of the respective role of these two oxidases.

Taken as a whole, our results show that electrons arising from thiosulfate take two pathways, the first one through a *bd* or a bo_3 type quinol oxidase and the second one through a bc_1 complex functioning in the forward direction (bc_1 II) and a ba_3 type cytochrome *c* oxidase. The thiosulfate quinol reductase activity is common to these two pathways and the reduced Q pool is the branching point between them (Fig. 6).

When grown on iron, electrons arising from Fe^{2+} take two pathways, the first one (exergonic) through the aa_3 type oxidase (and possibly, but to a less extent, through a ba_3 cytochrome *c* oxidase) and the second (endergonic) through a bc_1 type complex functioning in the reverse direction (bc_1 I, Fig. 6) [26]. Cytochrome c_4 of the *petI* operon (coding for the bc_1 complex proposed to function in the reverse direction [30]) and cytochrome c_4 of the *rus* operon (coding for the cytochrome oxidase aa_3 and rusticyanin [23]) are different. These observations suggest than in iron-grown cells, the electrons arising from Fe^{2+} take the two pathways described above, through two different cytochromes c_4 (not indicated in the figure). On the other hand, electrons arising from sulfur compounds (sulfide and thiosulfate) take a pathway to O_2 through a sulfide (or a thiosulfate) quinone reductase and a *bd* or a bo_3 type oxidase (Fig. 6).

4.5. Why does A. ferrooxidans have apparently redundant pathways for electrons to a single electron acceptor dioxygen?

Our results have shown that *A. ferrooxidans* contains two bc_1 complexes (this work and Ref. [27]) and four types of oxidase (this work). Whereas the existence of numerous terminal oxidases in bacteria has been previously substantiated, *A. ferrooxidans* is the only organism known up to now to contain two complete classical bc_1 complexes. Why this peculiarity? We can hypothesize that it is not possible for one bc_1 complex to be thermodynamically competent both for the forward and reverse direction of electron flow. Indeed, the values of the redox potential of the reverse functioning bc_1 complex (bc_1 I) render its forward functioning very difficult [27]. In addition, in natural environments the bacterium oxidizes sulfur compounds to SO_4^{2-} and Fe^{2+} to Fe^{3+}. It has been suggested that in the presence of both substrates, ferrous ions are first oxidized to ferric ions, followed by sulfur oxidation, and both substrates are then used at the same time by the bacteria population [55]. In these conditions, it is comprehensible that even if the bc_1 complex expressed in iron-grown cells was capable of functioning in the forward direction, it cannot function simultaneously in both directions, necessitating the presence of a second bc_1 complex.

We have mentioned above a possible reason for the presence of *bd* and bo_3 oxidases in *A. ferrooxidans*: these oxidases could allow the O_2-labile nitrogenase to function either under conditions of air saturating medium (presence of cytochrome *bd*) or in microaerophilic conditions (cytochrome bo_3). More surprising is the expression of a ba_3 type oxidase in sulfur-grown cells instead of the classical aa_3 oxidase predominantly expressed in iron-grown cells. A possible hypothesis would be that in natural environments, when the two substrates are used at the same time, it would be structurally and/or kinetically easier for electrons arising from the sulfur compounds through the bc_1 complex (bc_1 II) to reduce another oxidase rather than the aa_3 oxidase already used by electrons arising from iron. Studies carried out on cells grown on a mixture of Fe^{2+} and sulfur compounds are in progress to try to answer these questions.

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