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Characterization of an Operon Encoding Two *c*-Type Cytochromes, an aa_3-Type Cytochrome Oxidase, and Rusticyanin in *Thiobacillus ferrooxidans* ATCC 33020

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Despite the importance of *Thiobacillus ferrooxidans* in bioremediation and bioleaching, little is known about the genes encoding electron transfer proteins implicated in its energetic metabolism. This paper reports the sequences of the four *cox* genes encoding the subunits of an aa_3-type cytochrome *c* oxidase. These genes are in a locus containing four other genes: *cyc2*, which encodes a high-molecular-weight cytochrome *c*; *cyc1*, which encodes a c_4-type cytochrome (c_{552}); open reading frame 1, which encodes a putative periplasmic protein of unknown function; and *rus*, which encodes rusticyanin. The results of Northern and reverse transcription-PCR analyses indicated that these eight genes are cotranscribed. Two transcriptional start sites were identified for this operon. Upstream from each of the start sites was a σ70-type promoter recognized in *Escherichia coli*. While transcription in sulfur-grown *T. ferrooxidans* cells was detected from the two promoters, transcription in ferrous-iron-grown *T. ferrooxidans* cells was detected only from the downstream promoter. The cotranscription of seven genes encoding redox proteins suggests that all these proteins are involved in the same electron transfer chain; a model taking into account the biochemistry and the genetic data is discussed.

The gram-negative eubacterium *Thiobacillus ferrooxidans* is important for industry and ecology because (i) this microorganism is able to solubilize metals from ores, such as copper, uranium, and cobalt, and to decompose recalcitrant gold-containing ores (39) and (ii) it is able to remove heavy metals from contaminated industrial effluents or soils and to desulfurize fossil fuels to avoid corrosion and atmospheric acid depositions (7, 21, 22). In addition to its industrial importance, *T. ferrooxidans* is of fundamental interest since its way of life is one of the "most primitive extant" (8): for growth, this microorganism requires only air, which provides carbon from carbon dioxide, nitrogen, and oxygen, and ores containing ferrous iron (Fe^{2+}) or reduced sulfur compounds, from which it derives its energy. Furthermore, it thrives at extremely low pHs (between 4 and 1.5). *T. ferrooxidans* is one of the most studied bioleaching microorganisms, but little is known about its physiology and, more particularly, its energy metabolism. Because its energy metabolism is responsible for its bioleaching and bioremediation abilities, any attempt to improve these properties is dependent on an understanding of the respiratory mechanisms. Although several redox proteins have been identified (49), the electron pathways from Fe^{2+} to oxygen (O_2) and from reduced sulfur compounds to O_2 are not established. Several models for the iron respiratory chain which differ with regard to the electron carriers and the side of the cytoplasmic membrane on which oxygen reduction takes place have been proposed (2, 5, 13, 17, 18, 50).

As an approach for elucidating the *T. ferrooxidans* respiratory chains, we are studying the genes encoding electron transfer proteins. We have previously cloned and sequenced the *rus*, *cyc1*, and *cyc2* genes, which encode, respectively, the rusticyanin (4) and two cytochromes *c* (2) from strain ATCC 33020. The sequences of internal fragments of the *rus* genes from strain ATCC 19859 (38) and from strain ATCC 23270 (15) and the sequence of the *iro* gene from strain Fe-1 (26) have also been reported. We have also shown that in strain ATCC 33020 the *cyc1* and *cyc2* genes are cotranscribed with at least two downstream open reading frames (ORFs) (2) and that the *rus* gene is cotranscribed with at least three upstream ORFs (4). In this paper, we demonstrate that all these genes together with the genes encoding the four subunits of a cytochrome *c* oxidase belong to the same operon.

MATERIALS AND METHODS

Strains, plasmids, growth conditions, and β-galactosidase assays. *T. ferrooxidans* ATCC 33020 was obtained from the American Type Culture Collection. *Escherichia coli* MC4100 [*araD139* Δ(*lacIPOZYA-argF*)U169 *rpsL thi*] was used when β-galactosidase activities had to be determined.

Phagemid Bluescript SK was purchased from Stratagene. Plasmid pGE593 is an operon fusion vector containing the *lacZ* gene as the reporter gene (10).

E. coli strains were grown in Luria-Bertani medium (31). Conditions for growth of *T. ferrooxidans* on iron or sulfur medium have been described previously (4).

β-Galactosidase activities were determined according to the method of Miller (31) in whole cells grown on Luria-Bertani medium containing ampicillin.

DNA manipulations. General molecular biology techniques were carried out by standard procedures (3) or as recommended by the manufacturer. *T. ferrooxidans* genomic DNA preparation has been described previously (4). Ultrapure plasmid DNA was obtained with a Wizard *plus* Spin or vacuum minipreps DNA purification system plasmid kit from Promega. Ligations were generally carried out in the presence of the restriction enzyme used to cleave the vector to prevent its recircularization.

Routine PCRs were performed with Boehringer Mannheim *Taq* DNA polymerase according to the manufacturer's recommendations in a Mini-cycler (MG Research). For cloning purposes, the *Pwo* polymerase was preferred. Amplification of flanking sequences by inverse PCR was as described by Ochman et al. (36). All synthetic oligonucleotides for PCR and sequencing were purchased from Genset.

Sequences were determined with a Thermo Sequenase II dye terminator cycle sequencing premix kit from Amersham. The DNA sequences were compiled and analyzed through the Worldwide Web Netscape facilities. The predicted proteins

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence	Positions (orientation)[a]
a	5′-CGTATTCATCTATGTGCTGGTCGG-3′	3472–3495 (+)
b	5′-CACCATCCCGTCTGAAACCCG-3′	4757–4787 (−)
c	5′-TTGGCATGTCGATTTTTGGACC-3′	1–22 (+)
d	5′-ATGGTTAACATGATAAAATAACG-3′	53–75 (+)
e	5′-TGTTCGTTATTTACTTTATTGC-3′	98–119 (+)
f	5′-GTTTGTATTAAATAGAACGTGTGG-3′	269–292 (+)
g	5′-GGTTATGGTGTCATCGTCCGTTGG-3′	446–469 (+)
h	5′-CCAACGGACGATGACACCATAACC-3′	446–469 (−)
i	5′-CGCAAAGGATGGCAGTGCCCAGG-3′	535–557 (−)
j	5′-GAACATTATTGTTGGGAGAAGC-3′	810–831 (−)
k	5′-ACACGTTCTATTTAATACAAACCG-3′	267–290 (−)
l	5′-ATGGTCATAACTATAATGCTTTTA-3′	167–190 (−)
m	5′-GCTCATGCGCCCGGTCTTCCTGCC-3′	374–397 (−)
n	5′-GACCTTTAACCAATGTTGTTGCG-3′	306–328 (−)
o	5′-TGCCGTTTAATTATAGGCCG-3′	34–53 (−)
p	5′-ATGTCTCGTTGCCACAAATCAAGG-3′	3963–3985 (−)

[a] The positions refer to the nucleotide sequences submitted to the EMBL data bank. + corresponds to the coding strand, and − corresponds to the noncoding strand.

were compared with EMBL, GenBank, and SwissProt database entries by using the BLASTP program (1) with the BLOSUM62 scoring matrix or, when specified, with the BLOSUM30 matrix.

Plasmid constructions. Different DNA fragments containing the putative promoters of the *cyc* operon were amplified by PCR with oligonucleotides d to h, f to h, d to k, or c to l (Table 1 and see Fig. 1A and 3A) from *T. ferrooxidans* genomic DNA and cloned into the *Sma*I site of pGE593 to yield plasmids 1, 2, 4, and 5, respectively. The insert of plasmid 3 is the larger fragment obtained after digestion with *Ssp*I of the PCR fragment obtained with oligonucleotides d to h.

Plasmid SK/*coxB* was obtained by cloning the DNA PCR fragment amplified between oligonucleotides a and p (Table 1; see Fig. 1A) into the *Eco*RV site of the Bluescript SK vector. Screening was performed by PCR with the oligonucleotides used to amplify the DNA. In all cases, the sequence of the cloned fragment was checked.

RNA manipulations. *T. ferrooxidans* total RNA was prepared as described previously (14). RNA electrophoresis was performed with agarose-formaldehyde gels (3). RNA was transferred to a positively charged nylon membrane from Boehringer Mannheim by capillary blotting. The digoxigenin (DIG)-labeled RNA probe was obtained by in vitro transcription with T7 RNA polymerase from the *Eco*RI-linearized SK/*coxB* plasmid described above with DIG-UTP as the substrate according to the instructions of the Boehringer Mannheim DIG RNA labeling kit. Prehybridization and hybridization with a DIG-labeled probe were performed under stringent conditions according to the recommendations of Boehringer Mannheim. RNA was detected by a chemiluminescent reaction with disodium 3-(4-methoxyspuro{1,2-dioxelane-3,2′-(5′-chloro)tricyclo [3.3.1.1^{3,7}] decan}-4-yl) (CSPD) as recommended by Boehringer Mannheim.

Primer extension was performed with the Superscript II RNase H$^-$ reverse transcriptase from Gibco BRL. The k, l, m, n, and o primers used (Table 1; see Fig. 3A) were labeled with [γ-^{32}P]ATP. Coupled reverse transcription and PCR amplification (RT-PCR) was performed with the Promega Access RT-PCR system as described previously (14) with the a, b, c, d, e, f, g, h, i, and j oligonucleotides (Table 1; see Fig. 1A). For each RT-PCR experiment, three control experiments were performed: one without template to detect any contamination, one with genomic DNA as a control for PCR amplification, and one with RNA but without the reverse transcriptase to ensure that there were no DNA traces in the RNA preparation.

Nucleotide sequence accession number. The EMBL accession number of the 8,007-bp DNA nucleotide sequence containing the *cyc2*, *cyc1*, ORF1, *coxB*, *coxA*, *coxC*, *coxD*, and *rus* genes is AJ006456.

RESULTS AND DISCUSSION

Characterization of the cytochrome oxidase genes. The *cyc2* and *cyc1* genes encoding a high-molecular-weight cytochrome *c* and a c_4-type cytochrome are cotranscribed with at least one other gene (ORF1) encoding a putative periplasmic protein of unknown function (2). We have determined the nucleotide sequence downstream from ORF1 by chromosome walking using PCR and inverse-PCR approaches. Four putative open reading frames, each preceded by a correctly positioned putative ribosome binding site, were found on the same DNA strand between positions 3296 and 4060, 4417 and 6000, 6019 and 6570, and 6611 and 6805. The *rus* gene, which we have already characterized (4), lies immediately downstream. Downstream from ORF1, the first and second ORFs encode proteins presenting significant similarities to subunits II and I, respectively, of an aa_3-type cytochrome *c* oxidase and will be referred to herein as the *coxB* and *coxA* genes. The two ORFs downstream from the *coxA* gene are those found upstream from *rus* (ORF1 and ORF2 in reference 4) and will be referred to herein as *coxC* and *coxD* (see below). Thus, the gene order in this locus is *cyc2*-*cyc1*-ORF1-*coxB*-*coxA*-*coxC*-*coxD*-*rus*.

Analysis of the *coxB*-encoded polypeptide. The *coxB* gene encodes a putative 254-amino-acid polypeptide ($CoxB_{Tf}$) with a calculated molecular weight of 28,240. The first 51 amino acids may constitute a long but standard signal sequence. The mature protein has a higher similarity to subunit II of aa_3-type cytochrome *c* oxidases (and more particularly to those of *Synechococcus vulcanus*, *Anabaena* sp. strain PCC7120, and *Synechocystis* sp. strain PCC6803) than to subunit II of quinol oxidases. Similar to the cytochrome oxidases, the mature $CoxB_{Tf}$ has two putative N-transmembrane segments that serve as membrane anchors and a large periplasmic carboxy-terminal domain (for extensive references, see references 40 and 41). In the periplasmic domain, the aromatic amino-acid-rich region (145-WKWTFSY-151) involved in the electron transfer between subunits I and II is present (20, 45). Furthermore, the residues binding the dinuclear copper center (CuA) (H181, C222, C226, H230, and M233) (20, 25, 40), the residues stabilizing CuA (W145 and D178), and three of the four highly conserved residues interacting with cytochrome *c* (Q144, D178, and D193) (20, 27) are also present, suggesting that $CoxB_{Tf}$ belongs to a *c*-type cytochrome oxidase.

In spite of the acidic pH of the *T. ferrooxidans* periplasm, the periplasmic domain of $CoxB_{Tf}$ is well conserved. Acid-stable proteins generally contain a relatively low number of charged residues (29), but this is not the case in the $CoxB_{Tf}$ periplasmic domain (11.6% R+H+K; 8% D+E). It is noteworthy that two *T. ferrooxidans* periplasmic redox proteins, the high-potential iron sulfur protein (HiPIP) encoded by *iro* and rusticyanin, have already been noted as exceptions to this rule (29). A possibility is that these electron transfer proteins are inaccessible to the periplasm medium because they are buried in a supercomplex.

Analysis of the *coxA*-encoded polypeptide. The *coxA* gene encodes a putative 627-amino-acid polypeptide ($CoxA_{Tf}$) with a calculated molecular weight of 69,090. This protein is related to subunit I of both quinol and cytochrome *c* oxidases from archaea, eucarya, and bacteria but more particularly to subunit I of the aa_3-type cytochrome *c* oxidase from *Synechocystis* sp. strain PCC6803, *Synechococcus vulcanus*, and *Anabaena* sp. strain PCC7120. $CoxA_{Tf}$ contains the 12-transmembrane segment core common to all cytochrome oxidase subunits I (41). In this core region are the residues binding and stabilizing the low- and high-spin hemes (*a* and a_3) and the copper atom (CuB): H159, H333, H382, H383, H467, H469 (Cu and heme binding), W329, Y337 (CuB stabilization), W145, R529, and R530 (heme stabilization) (20, 40, 44). The invariant phenylalanine residue (F468) involved in electron transfer between the two hemes is also present (41). Based on the predicted topology of $CoxA_{Tf}$, all the residues binding hemes and copper are within the membrane bilayer but near the periplasm. This may explain why Kai et al. (23, 24) found that the pH optimum for *T. ferrooxidans* cytochrome oxidase was pH 3.5, a value corresponding to that of the periplasm. From the crystal structure of the *Paracoccus denitrificans* cytochrome *c* oxidase, two proton transfer pathways have been proposed: the K and the D

channels (16, 20, 37). Although not strictly invariant, most of the residues involved in these channels are conserved in the $CoxA_{Tf}$ subunit. Five of the seven residues constituting the K channel and six of the nine residues constituting the D channel are present. Altogether, these data strongly suggest that the *cox* genes encode an aa_3-type cytochrome *c* oxidase.

In addition to the core region, $CoxA_{Tf}$ contains an extended N terminus with two hydrophobic regions that have no similarity to sequences in the protein data banks. Extra hydrophobic regions have been also described for the N termini of subunits I of the cbb_3-type cytochrome oxidases from *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* (41).

The estimated molecular masses of subunits I of the aa_3-type cytochrome oxidases purified from the *T. ferrooxidans* Fe-1, AP19-3, and OK1-50 strains (53, 53 and 55 kDa, respectively) (19, 24) do not correspond with the molecular mass deduced from the *coxA* gene sequence reported here (69 kDa). This discrepancy may be due to an aberrant migration of subunit I on sodium dodecyl sulfate gels, as was previously observed with other integral membrane proteins. Another possibility is that the cytochrome oxidase encoded by the *coxBACD* gene cluster described in this paper is distinct from the oxidases which have been purified.

Analysis of the *coxC*- and *coxD*-encoded polypeptides. *coxC* and *coxD* genes encode putative polypeptides of 183 and 64 amino acids with calculated molecular weights of 20,202 and 7,211, respectively. These proteins are integral membrane proteins, with five transmembrane helices for $CoxC_{Tf}$ and one for $CoxD_{Tf}$ (4).

By specifying alternate scoring matrices, $CoxC_{Tf}$ exhibits some similarity with the *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Synechocystis* sp. strain PCC6803, *Synechococcus vulcanus*, and *Anabaena* sp. strain PCC7120 cytochrome *c* oxidase subunits III. Although slight, this similarity is significant. Furthermore, the carboxy-terminal region is the best conserved, as has been observed for other cytochrome oxidase subunits III (40).

As a general rule, subunit III is the second most conserved subunit in the cytochrome oxidase family after subunit I. Surprisingly, $CoxC_{Tf}$ is less conserved than subunit II, suggesting that it has evolved more quickly because of an interaction with another protein partner(s) specific to *T. ferrooxidans*.

Whatever the scoring matrices used, no similarity was detected for $CoxD_{Tf}$. Because the bacterial cytochrome *c* oxidase structural genes are always in the order *coxB-coxA-coxC-coxD* if they are clustered in the same locus (11, 46) and because cytochrome *c* oxidase subunit IV is generally a small protein which has one transmembrane helix and a sequence which is not always conserved (48), we have inferred that the *coxD* gene encodes cytochrome oxidase subunit IV.

Interestingly, even though *T. ferrooxidans* belongs to the phylogenetic β subdivision of the *Proteobacteria*, $CoxA_{Tf}$, $CoxB_{Tf}$, and $CoxC_{Tf}$ amino acid sequences are most closely related to those of cyanobacteria (*Synechococcus vulcanus*, *Synechocystis* sp., and *Anabaena* sp). However, no significative similarities were detected at the nucleotide level, dismissing the hypothesis of a lateral gene transfer. On the other hand, the ancestors of the cyanobacteria were the first to introduce oxygen into an anaerobic environment and the *T. ferrooxidans* way of life has been described as one of the "most primitive extant" by Cairns-Smith et al. (8), arguing rather for a convergent evolution.

Transcription of the *coxB*, *coxA*, *coxC*, and *coxD* genes in *T. ferrooxidans*. If the genes encoding the different cytochrome oxidase subunits are clustered in the same locus, they are always cotranscribed (11, 46). To determine if the *cox* genes of *T. ferrooxidans* ATCC 33020 are also organized in an operon, we used the RT-PCR approach, which combines RNA RT and cDNA amplification (PCR). An amplification product of the expected size was obtained between oligonucleotides a and b, corresponding to the *coxB* and *coxA* genes, respectively (Fig. 1A), indicating that these two genes are cotranscribed (Fig. 1B). We have shown previously that (i) *coxB* (ORF2 in reference 2) is cotranscribed with *cyc2*, *cyc1*, and ORF1 and (ii) *coxA* (ORFA in reference 4) is cotranscribed with *coxC*, *coxD* (ORF1 and ORF2, respectively, in reference 4), and *rus*, the *rus* gene being the last gene of this operon. All these results suggest that *cyc2*, *cyc1*, ORF1, *coxB*, *coxA*, *coxC*, *coxD*, and *rus* constitute an operon.

To confirm this hypothesis, the transcription of the *cox* genes was studied by Northern hybridization with total RNAs from ferrous-iron-grown cells. The RNA probe was chosen to hybridize to the *coxB* transcript. As predicted, one major transcript of approximately 7.4 kb was detected (Fig. 2). The size of this transcript is in agreement with *cyc2-cyc1*-ORF1-*coxB-coxA-coxC-coxD-rus* cotranscription. Larger minor transcripts were also observed. These minor transcripts can be due either to mRNA processing, to transcription initiation from several promoters (see below), or to the presence of more than one cytochrome *c* oxidase in *T. ferrooxidans*. The last hypothesis is supported by the facts that (i) different cytochrome oxidases have been detected in several *T. ferrooxidans* strains (9, 17, 28) and (ii) cytochrome *c* oxidases, in which subunit I has a lower apparent molecular weight than that predicted for $CoxA_{Tf}$, have been previously purified in *T. ferrooxidans* (19, 24).

Characterization of the operon promoter(s). Because no ORF has been detected in the 450 bp upstream from *cyc2* (2), this gene is likely the first cistron of the operon. To determine approximately where the transcription of this operon is initiated, RT-PCR experiments were performed with a set of oligonucleotides hybridizing in the 5′ untranslated region of *cyc2* (c, d, e, and f) and convergent oligonucleotides hybridizing at the beginning of *cyc2* (h and i) (Fig. 1A) on total RNA extracted from sulfur- or Fe^{2+}-grown cells. With RNAs extracted from sulfur-grown cells, cDNAs of the expected sizes were obtained between all the different pairs of oligonucleotides except with oligonucleotide c (Fig. 1C), suggesting that the mRNA starts between oligonucleotides c and d, which are between positions 1 and 75. With RNA extracted from Fe^{2+}-grown cells, however, no amplification product was obtained with the different pairs of oligonucleotides except a faint band with oligonucleotide f (Fig. 1D), suggesting that under Fe^{2+} growth conditions, the mRNA starts in the region where oligonucleotide f hybridizes. Because an amplification product was obtained with two *cyc2* internal oligonucleotides, g and j (Fig. 1D), the inhibition of avian myeloblastosis virus reverse transcriptase or *Tfl* polymerase in RNA preparation from Fe^{2+}-grown cells is excluded. From these RT-PCR experiments, we conclude that *cyc2* is the first gene of the operon and that this operon is transcribed from at least two promoters, the upstream promoter being nonfunctional under Fe^{2+} growth conditions. According to these results, the eight genes of the operon are transcribed in sulfur- as well as in ferrous-iron-grown ATCC 33020 cells, confirming that rusticyanin is synthesized when thiosulfate, sulfur, or ferrous iron is present in the growth medium (4) and suggesting that the proteins encoded by the operon play a role not only in ferrous-iron- but also in sulfur-grown cells.

The 5′ ends of these transcripts were determined more precisely by primer extension analysis with RNA samples prepared from *T. ferrooxidans* cells grown with ferrous iron or sulfur as the energy source. No signal was obtained with oligo-

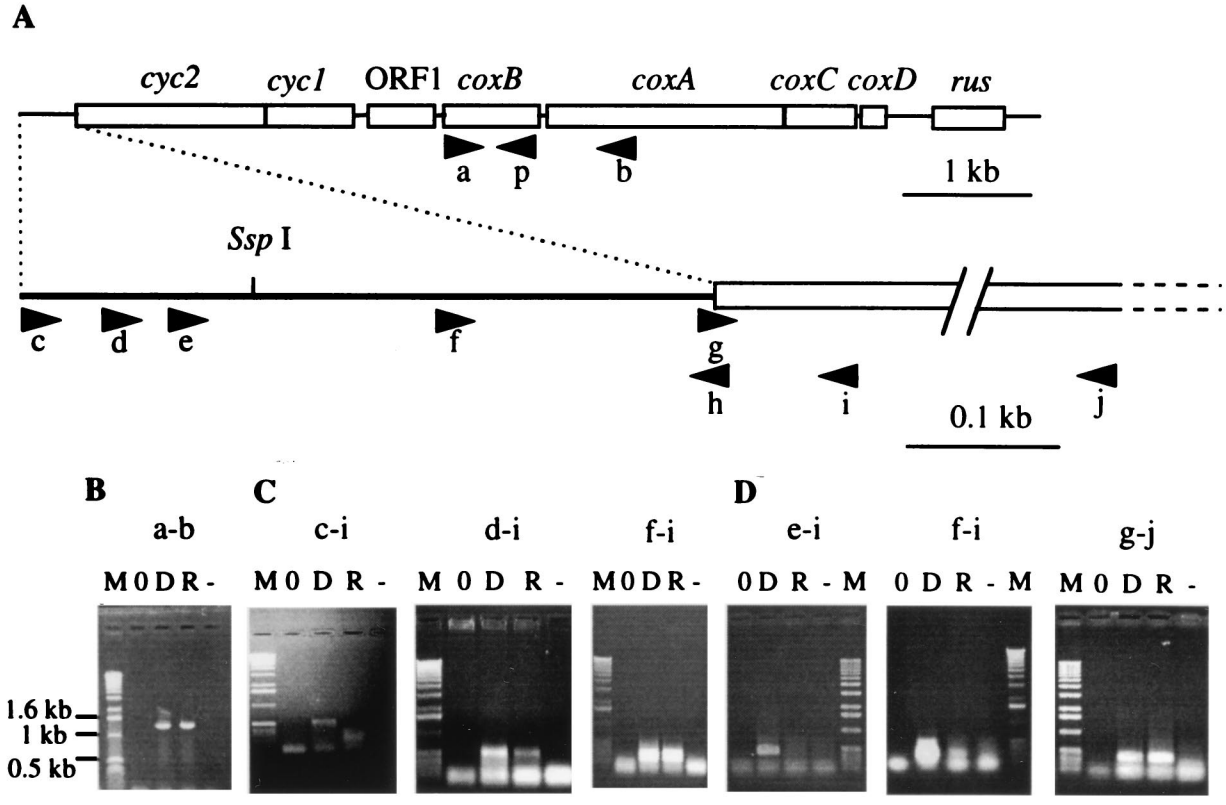

FIG. 1. RT-PCR on *T. ferrooxidans* total RNA. (A) Localizations of the oligonucleotides used for RT-PCR experiments; (B) RT-PCR experiments with oligonucleotides a and b on no template (lane 0), *T. ferrooxidans* genomic DNA (lane D), total RNA from *T. ferrooxidans* cells (lane R), total RNA from *T. ferrooxidans* but without reverse transcriptase (lane −); (C) RT-PCR experiments with oligonucleotides c and i, d and i, and f and i on RNA extracted from sulfur-grown cells (lanes R and −); (D) RT-PCR experiments with oligonucleotides e and i, f and i, and g and j on RNA extracted from ferrous-iron-grown cells (lanes R and −). Lanes M, 1-kb molecular weight ladder from Boehringer Mannheim.

nucleotide o (data not shown), indicating that the transcription of the *cyc* operon initiates downstream from this oligonucleotide (position 22). A band was obtained with oligonucleotides k and l (Fig. 3) from sulfur- but not from Fe^{2+}-grown cells. This signal corresponds to a G (position 51) located 398 bp upstream from the translational initiation site of *cyc2*. From Fe^{2+}- as well as from sulfur-grown cells, a weak band was obtained with oligonucleotide m (Fig. 3, lanes S and F). This signal corresponds to a G (position 289) located 161 bp upstream from the *cyc2* translational initiation site. This signal was confirmed with oligonucleotide n, even though other bands of unknown origin were observed with this oligonucleotide without any template (Fig. 3B, lanes 0). Correctly positioned upstream from the operon transcriptional initiation sites identified are two *E. coli* σ70-type promoters: TTGGAC(17 bp)T ATAAT for the upstream promoter and TTGCAA(17 bp)TA AATA for the downstream promoter.

To determine if these promoters are functional in *E. coli*, different regions of the 5′ untranslated region of the operon have been cloned in the operon fusion vector pGE593 (see Materials and Methods). β-Galactosidase activities of strain MC4100 (Δ*lac*) carrying the resulting plasmids were determined. The results unambiguously show that a sequence functioning as a promoter in *E. coli* is present between the *Ssp*I site and oligonucleotide k (positions 159 and 290) and that there is a second such sequence between oligonucleotides c and l (positions 1 to 190) (data not shown). Primer extension experiments with RNA samples prepared from *E. coli* MC4100 carrying plasmids 1, 2, and 3 (Fig. 3B, oligonucleotides m and n) and plasmid 5 (data not shown) have confirmed these results and have shown that the same transcriptional start sites are used in both *T. ferrooxidans* and *E. coli*.

The transcription of the genes of the operon appears to be complex because at least three promoters have been characterized: two upstream from *cyc2* (this paper) and one between *coxD* and *rus* (4). The function of the internal promoter may be

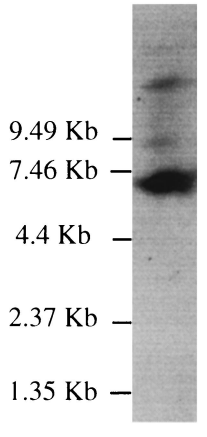

FIG. 2. Northern blot of total RNA from ferrous-iron-grown *T. ferrooxidans* cells (1.1 μg) probed with DIG-UTP-labeled *coxB* RNA. The positions and the sizes of the RNA ladder from Gibco BRL are indicated on the left.

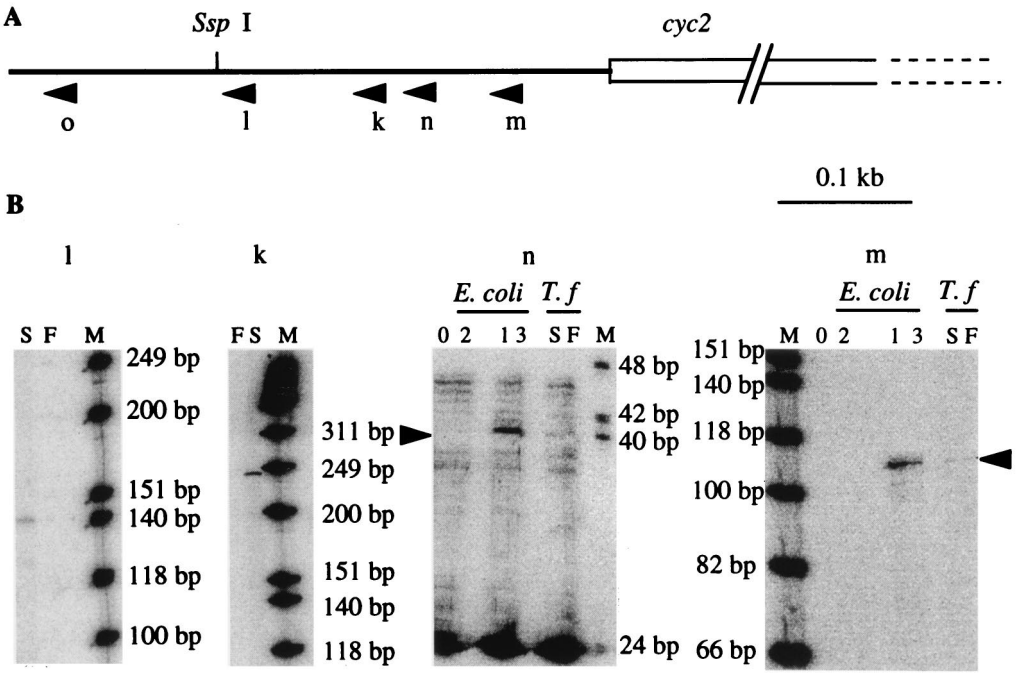

FIG. 3. Primer extension experiments. (A) Localizations of the oligonucleotides used. (B) RT experiments with the k, l, m, and n oligonucleotides on no template (lanes 0); *E. coli* carrying plasmid 1, 2, or 3 (lanes 1 to 3, respectively); total RNA from ferrous-iron (lanes F)- or sulfur (lanes S)-grown *T. ferrooxidans* (*T.f*) cells. Lanes M, [γ-^{32}P]ATP-labeled ϕX174 *Hin*fI markers (from Promega).

to uncouple expression of the *rus* gene from that of the other genes of the operon under certain growth conditions. Two of these promoters are active only under sulfur growth conditions: the most upstream promoter from *cyc2* (this paper) and the internal promoter (4). The requirement for multiple promoters suggests that the expression of the different genes of the operon must be tightly regulated depending on the growth conditions to allow the cells to adapt quickly to the environmental changes. Further work will focus on (i) looking for environmental signals involved in the transcriptional regulation of this operon and (ii) determining how these signals are transmitted to the transcriptional machinery.

Electron transfer pathway between ferrous iron and molecular oxygen in *T. ferrooxidans* ATCC 33020. In most cases, the cytochrome oxidase subunits are encoded within an operon with the order *coxB-coxA-coxC-coxD* (11, 46). In some cases, other genes required in the biogenesis of the oxidase (biosynthesis of heme A or heme O) (11, 46) or encoding other redox proteins belong to this operon. This is the case for cbb_3-type cytochrome *c* oxidases (46) and for *Sulfolobus acidocaldarius* terminal oxidase complexes (41, 42). The cytochrome oxidase subunit I gene of the cbb_3-type oxidases is cotranscribed with the genes encoding a mono- and a bihemic membrane-bound cytochrome *c* and a small membrane protein of unknown function (46). In *Sulfolobus acidocaldarius*, the genes encoding subunits I and II of one cytochrome oxidase are cotranscribed with the genes encoding an *a*-type cytochrome and a small hydrophobic subunit (41, 42). In a second cytochrome oxidase, the gene encoding subunit II and the gene encoding a subunit I-subunit III fusion protein are cotranscribed with the genes encoding sulfocyanin, an iron-sulfur protein, and a cytochrome *b* (41, 42). In these three cases, the redox proteins encoded by the operon constitute a respiratory chain organized as a supercomplex. Because the *T. ferrooxidans* aa_3-type cytochrome *c* oxidase genes are cotranscribed with the genes encoding two cytochromes *c* (*cyc2* and *cyc1*) and rusticyanin (*rus*), we infer that all these proteins belong to the same electron transfer chain and constitute a respiratory supercomplex. We have previously proposed the following electron pathway (1): $Fe^{2+} \rightarrow X \rightarrow$ rusticyanin $\rightarrow$ cytochrome $c_4 \rightarrow$ cytochrome oxidase $\rightarrow O_2$, in which the carrier which transfers electrons from ferrous iron to rusticyanin (X) is unknown. The HiPIP encoded by the *iro* gene has been proposed to receive the electrons from ferrous iron and thus to be the first electron carrier in the respiratory chain (13, 50). However, several observations appear to disagree with this hypothesis: (i) HiPIPs are generally found in photosynthetic bacteria, where they are assumed to transfer electrons between two integral transmembrane complexes, from the bc_1 complex to the photosynthetic reaction center or to the terminal oxidase (6, 30); (ii) no HiPIP protein has been detected in the Tf-3 and F424 *T. ferrooxidans* strains (9, 17, 28); and (iii) the *iro* gene is monocistronic in strain Fe-1 (26), which suggests that the HiPIP is not synthesized concomitantly with the electron carriers encoded by the *cyc2*, *cyc1*, *coxBACD*, and *rus* genes. The HiPIP would rather be a nonobligatory carrier in ferrous-iron oxidation or an intermediate carrier between the bc_1 complex and the terminal oxidase. We propose that the high-molecular-weight cytochrome would be a better candidate for the role of the first electron acceptor because (i) the *cyc2* gene encoding this cytochrome is the first gene of the operon, (ii) this cytochrome is indeed translocated to the periplasm because it has a signal sequence that is cleaved (1), (iii) this cytochrome is likely to be acid stable because it has a low number of charged amino acids, and (iv) from its amino-acid sequence, the Psort program (35) predicts that this cytochrome *c* is an outer membrane protein. Some organisms, such as *Desulfovibrio gigas* (47), *Geobacter sulfurreducens* (12, 43), and *Shewanella putrefaciens* (32–34), have outer membrane cytochromes *c*. Interestingly, *Geobacter sulfurreducens* and *Shewanella putrefaciens* are able to reduce ferric iron and this

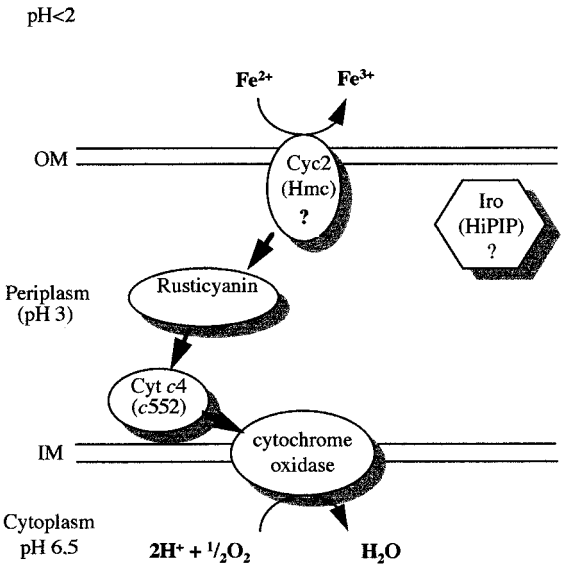

FIG. 4. Proposed electron transfer pathway between ferrous iron and oxygen in *T. ferrooxidans* ATCC 33020. OM, outer membrane; IM, inner membrane; Cyt *c*4, cytochrome c_4; Hmc, high-molecular-weight cytochrome *c*.

ferric iron reductase activity is associated with an outer membrane cytochrome *c* which makes direct contact with the solid substrate.

Based on genetic and biochemical evidence, we propose the pathway shown in Fig. 4 for electron transfer between ferrous iron and oxygen in *T. ferrooxidans* ATCC 33020; the high-molecular-weight cytochrome *c* encoded by the *cyc2* gene transfers electrons from ferrous iron to rusticyanin, which passes them to the cytochrome c_4 and from there to the cytochrome oxidase. To confirm this model, subcellular localization of the high-molecular-weight cytochrome *c* will be determined and the interaction between the cytochrome c_4 and the cytochrome oxidase subunit II will be studied.

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