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New Genes Encoding Subunits of a Cytochrome bc_1–Analogous Complex in the Respiratory Chain of the HyperThermoacidophilic Crenarchaeon *Sulfolobus acidocaldarius*

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The *soxL* gene from *Sulfolobus acidocaldarius* (DSM 639) encodes a Rieske iron–sulfur protein. In this study we report the identification of two open reading frames in its downstream region. The first one, named *soxN*, codes for a membrane protein bearing a resemblance to the *b*-type cytochromes of the cytochrome bc_1 and b_6f complexes. The protein is predicted to contain at least 10 transmembrane helices and features the two conserved histidine pairs coordinating the heme groups of these cytochromes. The second open reading frame, named *odsN*, encodes a soluble protein of unknown function. The genomic region displays a complex transcription pattern. Northern blot and RT-PCR analyses revealed the presence of mono- and bi-cistronic transcripts as well as a tri-cistronic transcript of *soxL* and *cbsAB*, encoding the mono-heme cytochrome $b_{558/566}$. Phylogenetic analyses of the genes of the *soxLN* pair and of other archaeal gene pairs encoding Rieske iron–sulfur proteins and *b*-type cytochromes revealed an identical branching patterns for both protein families, suggesting an evolutionary link of these genes provided by the functional interaction of the proteins. On the basis of the findings of this study and the previously studied properties of the soxL and cbsA proteins, we propose the occurrence of a novel cytochrome bc_1–analogous complex in the membranes of *Sulfolobus*, consisting of the cytochrome *b* homolog soxN, the Rieske protein soxL, the high potential cytochrome cbsA, as well as the non–redox-active subunits cbsB and odsN.

KEY WORDS: Rieske iron–sulfur protein; cytochrome *b*; bc_1 complex; b_6f complex; phylogeny; transcription analysis; *cbsAB*; *soxL*; *soxN*; *odsN*.

INTRODUCTION

It is still an open question whether enzymes homologous or analogous to the cytochrome bc_1 complex or the closely related b_6f complex can be found in the membranes of hyperthermophilic archaea. As central components of many respiratory and photosynthetic electron-transfer chains, these complexes are widely distributed among the bacteria and eukarya. They catalyze the oxidation of membrane-embedded ubi-, plasto-, or menaquinol using soluble *c*-type cytochromes or blue copper proteins as electron acceptors. The free energy of this reaction is used to generate a proton gradient across the membrane. The general outline of the reaction mechanism of these complexes, the protonmotive Q-cycle, is well understood (Mitchell, 1975). However, essential details of this process are still an issue in current research (Bartoschek *et al*., 2001; Covián and Moreno-Sánches, 2001; Lange *et al*., 2001; Zhang *et al*., 1998).

The currently known bc_1-homologous complexes can be classified into three groups: the bc_1 complexes of the mitochondria, proteobacteria, and *Aquifex*; the b_6f complexes of chloroplasts and cyanobacteria; and a third, rather heterogeneous group consisting of the enzymes from the Firmicutes (*Bacillus*,

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Heliobacillus), the Chlorobiacaea (*Chlorobium*), and the Thermus/Deniococcus group (*Deniococcus*) (Schmidt and Shaw, 2001; Schütz *et al*., 2000).

At present, there is only indirect evidence for the existence of archaeal homologs to these complexes. For example, genes encoding Rieske proteins and *b*-type cytochromes have been detected in the genomes of *Pyrobaculum aerophilum* (Henninger *et al*., 1999), *Aeropyrum pernix* (Kawarabayasi *et al*., 1999), *Sulfolobus solfataricus* (She *et al*., 2001), *S. tokodaii* (Kawarabayasi *et al*., 2001), *S. acidocaldarius* (Castresana *et al*., 1995; Schmidt *et al*., 1996), *Thermoplasma acidophilum* (Ruepp *et al*., 2000), and *Thermoplasma volcanium* (Kawashima *et al*., 1999). A protein fraction containing *b*- and *c*-type cytochromes as well as a Rieske protein has been enriched from the membranes of *Halobacterium salinarum.* The ubiquinol–cytochrome *c* reductase activity of this preparation in combination with the Antimycin- and Myxothiazol-sensitive NADH– and succinate–cytochrome *c* reductase activities of the membranes (Sreeramulu *et al*., 1998) strongly hints at the presence of a cytochrome *bc*–homologous complex in this organism. Moreover, Rieske iron–sulfur proteins—potentially indicating the presence of a bc_1/b_6f complex or a related structure—have been isolated from *Sulfolobus acidocaldarius* (Schmidt *et al*., 1995, 1996) and *Sulfolobus sp.* strain 7 (recently renamed as *Sulfolobus tokodaii* strain 7) (Iwasaki *et al*., 1995). However, the protein from the latter organism is soluble and appears to belong to a cytoplasmic redox system comparable to the Rieske-type proteins from the bacterial oxygenases (Mason and Cammack, 1992). The two Rieske proteins from *S. acidocaldarius* are membrane-bound. The soxF protein was isolated as subunit of a terminal oxidase supercomplex (Castresana *et al*. 1995; Lübben *et al*., 1994). The *soxF* gene is part of an operon together with a gene for a di-heme *b*-type cytochrome (soxG) and other subunits of the soxM terminal oxidase supercomplex (Castresana *et al*., 1995). The organization of the operon, i.e. *soxF*, followed by *soxG* is the same as in the bacterial *fbc* operons (Schütz *et al*., 2000). Since *c*- or *f*-type cytochromes are absent in *Sulfolobus*, their function is obviously adopted by other subunits, as was recently demonstrated by the isolation of the active soxM oxidase complex (Komorowski *et al*., 2002). SoxL, the second Rieske protein of *S. acidocaldarius* was the first protein of this family to be purified and characterized from a member of the archaea (Schmidt *et al*., 1995, 1996). It displays ubiquinol–cytochrome *c* reductase activity (Schmidt *et al*., 1995) as was reported for the isolated beef heart Rieske protein (DegliEsposti *et al*., 1990). A comparative analysis of the archaeal Rieske protein sequences revealed rather low similarities to the bacterial and eukaryal Rieske proteins as well as within the group of the archaeal proteins (Schmidt and Shaw, 2001).

In this study we report the identification of new genes in the downstream region of the *soxL* gene from *S. acidocaldarius*, an analysis of the transcription pattern and a comparison of this region to those of the currently available archaeal genomes.

MATERIALS AND METHODS

Sulfolobus acidocaldarius cells were grown as previously described (Schäfer *et al*., 2001). The isolation of *Sulfolobus* DNA, Southern blot analysis, as well as the construction and the screening of genomic libraries were performed as outlined in (Schmidt *et al*., 1996). A 525-bp Pst I fragment was isolated using the 83-bp Pst I/EcoR I fragment AGCGTTCATTTATACTG TAGTTTCAGGTCTTATCCTCTTATTATACTATAACG CGGAGGC TGGCTACTCCTCAACTGAATTCC from a previously sequenced clone (Schmidt *et al*., 1996) as a probe. This clone was sequenced as previously described (Schmidt *et al*., 1996). A 2-kb EcoR I/Xba I fragment was detected in a further Southern blot analysis probed with the synthetic oligonucleotide SaciSoxN (AATTATGACGCTGGATATTATGGAAGAGTCTTAG CTTGGCACAT) derived from the previous clone. Since several attempts to clone this fragment failed for unknown reasons, we performed a PCR reaction using the products of the ligation reaction containing the genomic *Sulfolobus* DNA and the plasmid pBSII SK as template. The probe SaciSoxN and the vector-specific oligonucleotide T3 (CAATTAACCCTCACTAAA) were used as primers with the following "touch down" protocol: Initial denaturation for 4 min at 94°C; First stage – 30 s 94°C; 150 s, 72°C; 5 cycles; Second stage – 30 s, 94°C; 30 s, 65°C; 120 s, 72°C; 5 cycles; Final amplification – 30 s, 94°C; 30 s, 58°C; 120 s, 72°C; 25 cycles. The obtained 2-kb product was purified by electrophoresis on an agarose gel and directly sequenced by a commercial company.

The expression of the *odsN* gene in *E. coli* was accomplished as outlined previously (Schäfer *et al*., 2001) for the *soxL* gene.

RNA was isolated from *S. acidocaldarius* cells using the Invisorb RNA Kit II (Invitek, Berlin) according to the manufacturer's instructions.

Northern blotting was carried out as previously described (Purschke *et al*., 1997). The blots were hybridized with DNA probes generated by PCR reactions and labeled with digoxigenin-11-dUTP (Boehringen-Mannheim, Germany) according

Table I. Primer Combinations for RT-PCR Analysis

Product	First strand synthesis	Amplification
cbsA-soxL	5′-GACTCCAGGTATAATT CCTACAA (soxLrev)	5′-GGAAAGACATACTATGTTGCCTTT (cbsA) ↔ 5′-AGCCATTCCAAATATCAGTGACTT (soLnt)
cbsB-soxL	5′-TGAGGAGAAGGGATT TTCTGTTTGA (soLct-01)	5′-CAACACTAGGAATTGACCTGTATAA (cbsL) ↔ 5′-TGATATAGGGACTATTTACTGGTAAA (anti cbsL)
soxL	soLct-01	soLct-01 ↔ 5′-ATGGATTCCTCAGGAAATCCGGTAA (LntI33M)
soxL-soxN	5’-AACAAATGTTATTAAA GCTCCGGCAA (soxN ct3)	5′-TGTTGGTGTAGCAGTTTATCCAAA (soxL rtl) ↔ 5′-GTTAGTACAAGCATTATAACACCAATTA (anti soxLN)
soxN	5′-TTCCTTATGGACAGGA CAAATATTAA (soxN ct2)	5′-ATGGCTGAGCACTACGGCATTAT (soxN nt2) ↔ socN ct3
soxN-odsN	5′-TGAAGTACTCTATGGG TAGGTTT (anti orfL	5′-AGAAGAAGATCGCTGAAATACTGATAA (orfL) ↔ 5′-CAAGTATTGTCTTACCGTAGCTAA (anti orfL2)
soxL-odsN	anti orfL	anti orfL2 ↔ soxL rtl
cbsB-odsN	anti orfL	anti orfL2 ↔ cbsL
cbsB-soxN	soxN ct2	soxN ct3 ↔ cbsL

to manufacturer's instructions. The probes PsoxL and PodsN were generated using plasmids with the cloned genes as templates and the primer ATGGATTCCTCAGGAAATCCGGTAA in combination with TGAGGAGAAGGGATTTTCTGTTTGA (PsoxL), and ATGAGAATGATAAATGTAGGGTTTTACT with TTGAAGTACTCTATGGGTAGGTTTT (PodsN). The probe PsoxN was generated by a nested PCR reaction using *Sulfolobus* genomic DNA as a template. In a first reaction, the primers TTCCTTATGGACAGGACAAAT ATTAA and ATGGCTGAGCACTACGGCATTAT were used to amplify part of the soxN gene. The product of this reaction was purified by agarose gel electrophoresis and used as template for the second PCR and labeling reaction using the primers AACAAATGTTATTAAAGCTCC GGCAA and GTATGCGATAGAAGAGTGGCAAA. The probe PcbsL was generated by a simple PCR reaction, with genomic DNA as the template and the primers CAACACTAGGAATTGACCTGTATAA and TGAGGA GAAGGGATTTTCTGTTTGA.

RT-PCR analysis was performed utilizing gene-specific primers and the Superscript™ First-strand synthesis Kit (GIBCO BRL) for PCR products up to 1.3 kb or the ImProm II™ reverse transcriptase (Promega) for larger products. The primer combinations are listed in Table I. The PCR products were analyzed by agarose gel electrophoresis as previously described (Purschke *et al*., 1997). Control reactions were performed with genomic DNA (positive control) and with RNA without reverse transcription (negative control).

Phylogenetic trees and alignments were calculated using the program ClustalX (version 1.64b) (Thompson *et al*., 1997). The phylograms were drawn with the program "NJplot" (Perrière and Gouy, 1996).

RESULTS

Two open reading frames were detected downstream of the *soxL* gene of *Sulfolobus acidocaldarius* (Fig. 1). The first, named *soxN*, codes for a protein of 528 amino acid residues (1584 bp). The second named ods*N* (*o*rf *d*ownstream *s*ox*N*) codes for a protein of 99 residues (297 bp). Reevaluation of previously published data (Hettmann *et al*., 1998; Schmidt *et al*., 1996) revealed that the *cbsAB* genes are located immediately upstream of *soxL*. The previously detected open reading frame orf1 (Hettmann *et al*., 1998) was identified as the 5′ end of *soxL*. Potential transcription termination signals (T-tracks) (Reiter *et al*., 1988) are located 3 bp downstream of the stop-codon of *cbsB* (Hettmann *et al*. 1998) and 15 bp downstream of the stop-codon of *soxL*. Potential promotor elements (box A) (Hain *et al*., 1992) are located 39 bp upstream of the first methionine codon of soxL. 12 or 27 bp upstream of the first two methionine codons of *soxN*, and 15 or 21 bp upstream of the first two methionine codons of *odsN* (Fig. 1).

The hydropathy analysis indicates that soxN is a membrane protein containing at least 10 trans-membrane helices, whereas odsN displays the hydropathy profile of a soluble protein (Fig. 1). The latter conclusion was confirmed by heterologous expression of the *odsN* gene in *E. coli* (data not shown).

The soxN protein shows clear similarities to the sequences of the *b*-type cytochromes from the bc_1 and b_6f complexes (Fig. 2). The position and the distances of the hemecoordinating histidine residues are strictly conserved (Fig. 1 and 2). The classification of soxN as a member of the cytochrome *b* family is also supported by the phylogenetic analysis shown in Fig. 3. The sequence

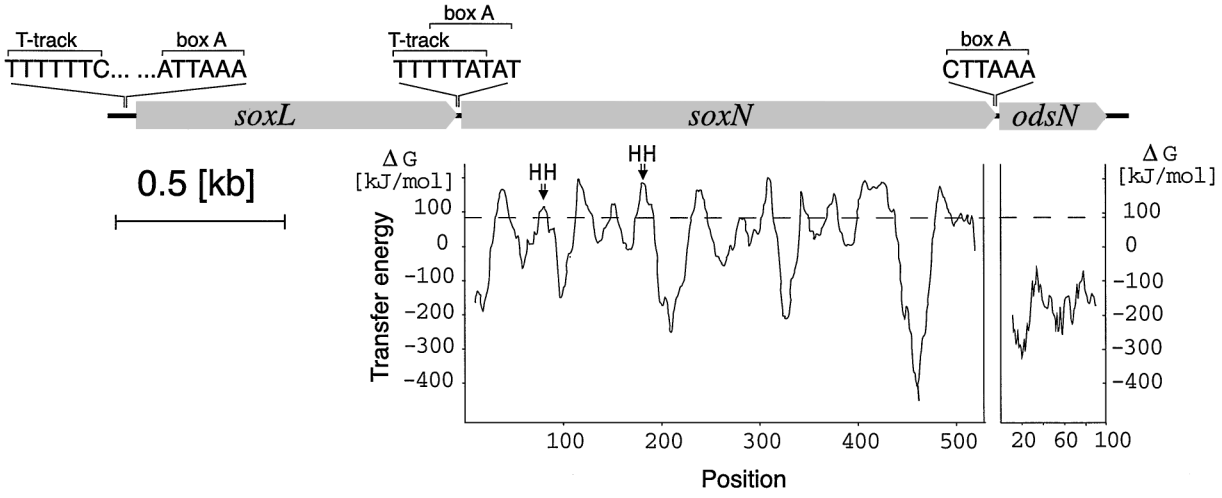

Fig. 1. Organization of the *soxLN–odsN* locus of *S. acidocaldarius.* Potential transcription regulation signals identified by sequence analysis are indicated. The lower part of the figure shows the transfer energy plots for the proteins encoded by *soxN* and *odsN*. The threshold of 86 (Kj/mol) for potential transmembrane helices is marked by a dashed line. The arrows indicate the positions of the conserved pairs of histidine residues involved in the binding of the putative heme groups of soxN. The DNA sequences were submitted to the Genbank database under the accession numbers AF202954 (*soxN*) and AF202955 (*odsN*).

segregates into a separate group together with the sequences of other putative, or confirmed (SULaci soxC, SULaci soxG) (Castresana *et al*., 1995; Schäfer *et al*., 1999), archaeal *b*-type cytochromes.

In contrast, database searches revealed no conclusive evidence regarding the classification of the odsN protein. The sequence does not contain known motifs associated with the binding of prosthetic groups. It exhibits 22% similarity to the protein encoded by the aknX gene from *Streptomyces galilaeus* (Fig. 4). However, for reasons discussed below we do not expect both proteins to be functionally related. Even within the archaeal domain, the odsN protein shows only a relatively low degree of conservation (Fig. 4 and 5). The recombinant protein did not exhibit any tendency to bind metal ions (Fe^{2+}, Co^{2+}, Cu^{2+}, or Mn^{2+}) (data not shown).

Figure 5 shows a comparison of the genomic context of the *soxL* gene of *S. acidocaldarius* relative to other archaeal genes encoding high-potential Rieske iron–sulfur proteins. The previously sequenced (Hettmann *et al*., 1998) *cbsA* gene encodes a unique high-potential, highly glycosylated membrane-bound *b*-type cytochrome. The cbsB protein has not been isolated so far. It is most certainly an integral membrane protein owing to its high percentage of hydrophobic residues (Hettmann *et al*., 1998). The occurrence of the *cbsAB* genes appears to be restricted to the Sulfolobales. No homologous genes were detected in any of the other archaeal genomes sequenced so far. Genes encoding Rieske iron–sulfur proteins and *b*-type cytochromes have been detected in several, but not all, archaeal genomes. In many cases these genes are organized in the same order as in the bacterial *fbc* operons (Fig. 5) (Schütz *et al*., 2000). Several archaeal genomes (Fig. 5) contain more than one Rieske–cytochrome *b* gene pair. The similarity between these gene pairs within the same genome is relatively low, being in the range 27–32% for the Rieske proteins and 24–30% for the *b*-type cytochromes (Figs. 5 and 6). A phylogenetic analysis reveals an identical branching pattern for both genes of each pair (Fig. 6). Thus, it appears that the occurrence of these different copies of Rieske–cytochrome *b* gene pairs is the result of ancient gene duplications and that the evolution of the genes in each pair may be linked by close functional interactions of the proteins.

Genes encoding odsN-homologous proteins are detectable immediately downstream of the *soxN*-homologous genes of *Sulfolobus solfataricus* (SSO11196) and *Aeropyrum penix* (APE1727) (Fig. 5). The corresponding open reading frames in *Sulfolobus tokodaii* (ST1668) and *Thermoplasma acidophilum* (TA1221) show only very little (ST1668), or no significant, similarity (TA1221) to the *odsN* gene.

The transcription patterns of the *soxLN* and *odsN* genes were analyzed by Northern blot analysis (Fig. 7). Hybridization with the *soxL*-specific probe PsoxL (Fig. 8) detected a major transcript of 1.05–1.15 kb, corresponding to the length of the *soxL* gene (0.945 kb) and a minor transcript of 3.2–3.4 kb (Fig. 7). The same signals were detected using the probe PcbsL that should hybridize with transcripts of *soxL* and *cbsB* (data not shown).

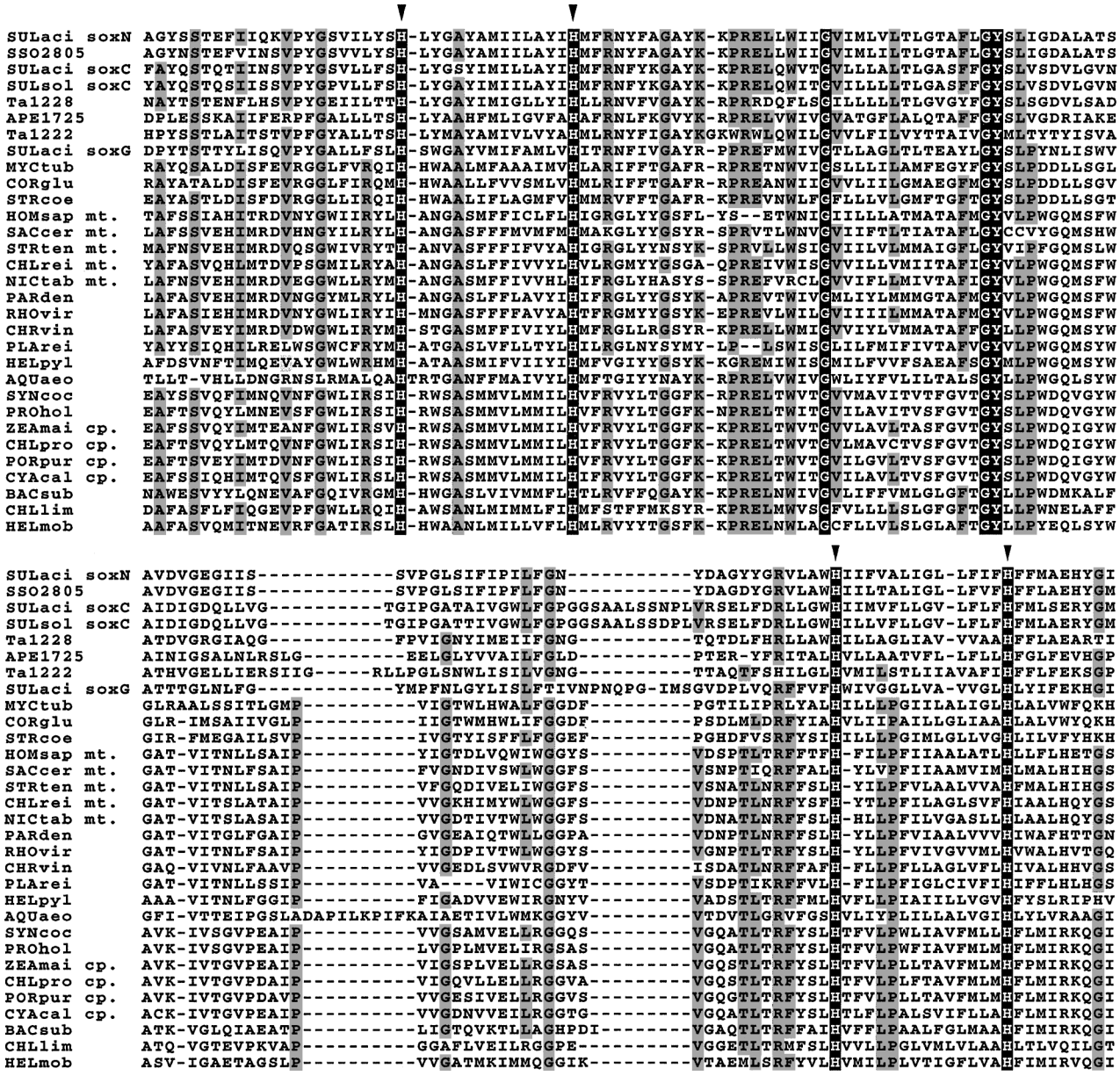

Fig. 2. Partial alignment of the heme-binding regions of selected *b*-type cytochromes. Residues conserved in all sequences are printed inverse. Shading indicates residues that are conserved in the majority of the sequences. Arrows mark the ligands of the heme groups. Abbreviations – SULaci: *Sulfolobus acidocaldarius*; SULsol: *Sulfolobus solfataricus*; MYCtub: *Mycobacterium tuberculosis*; CORglu: *Corynebacterium glutamicum*; STRcoe: *Streptomyces coelicolor*; HOMsap: *Homo sapiens*; SACcer: *Saccharomyces cerevisiae*; STRten: *Strobilurus tenacellus*; CHLrei: *Chlamydomonas reinhardtii*; NICtab: *Nicotiana tabacum*; PARden: *Paracoccus denitrificans*; RHOvir: *Rhodopseudomonas viridis*; CHRvin: *Allochromatium vinosum*; PLArei: *Plasmodium reichenowi*; HELpyl: *Helicobacter pylori*; AQUaeo: *Aquifex aeolicus*; SYNcoc: *Synechococcus sp.* PCC7942; PROhol: *Prochlorothrix hollandica*; ZEAmai: *Zea mais*; CHLpro: *Chlorella protothecoides*; PORpur: *Porphyra purpurea*; CYAcal: *Cyanidium caldarium*; BACsub: *Bacillus subtilis*; CHLlim: *Chlorobium limicola*; HELmob: *Heliobacillus mobilis*; mt.: mitochondria; cp.: chloroplast. SSO2805, Ta1228, APE1725, and Ta1222: Open reading frames as identified in the legend to Fig. 5.

Hybridization with the probes PsoxN or PodsN (Fig. 8) detected a single transcript of 1.95–2.1 kb, corresponding to the size expected for a common transcript of *soxN* and *odsN* (1.899 kb). Thus, the two genes appear to be transcribed into a common mRNA, whereas *soxL* seems to be predominantly transcribed into a mono-cistronic mRNA. Since the 3.2–3.4-kb transcript was detectable with the probes PsoxL and PcbsL, but not with PsoxN or PodsN,

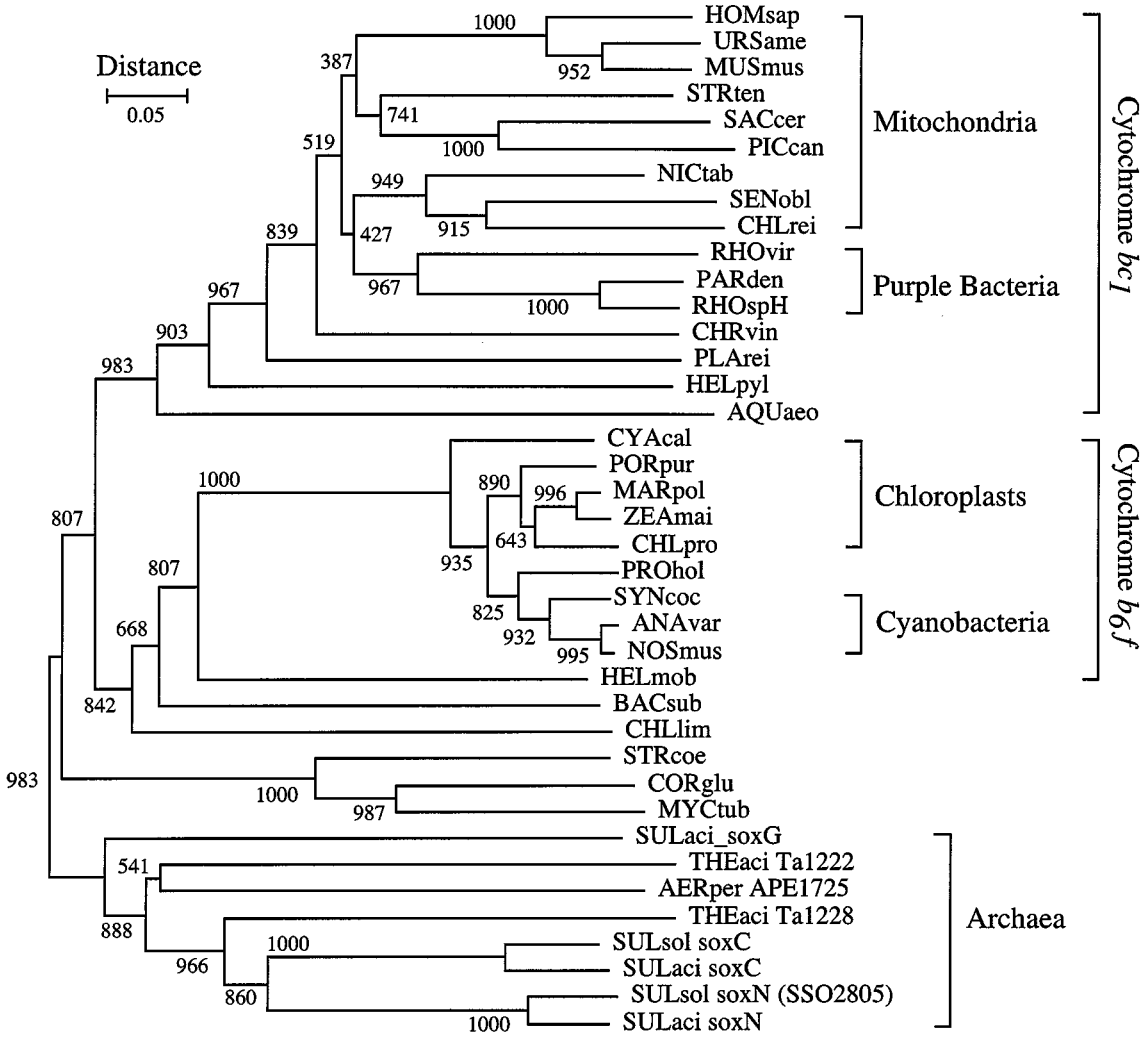

Fig. 3. Phylogram of the *b*-type cytochromes. The tree was calculated with the following settings – Pairwise alignment matrix: Blosum series; multiple alignment matrix: PAM series; delay divergent sequences: 10; hydrophilic residues: GPSNDHEK; gap separation distance: 12. All other parameter were as preset by the program. Abbreviation – URSame: *Ursus americanus*; MUSmus: *Mus musculus*; PICcan: *Pichia canadensis*; SENobl: *Scenedesmus obliquus*; RHOsph: *Rhodobacter sphaeroides*; MARpol: *Marchantia polymorpha*; ANA var: *Anabena variabilis*; NOSmus: *Nostoc muscorum* PCC 7906; THEaci: *Thermoplasma acidophilus*; AERper: *Aeropyrum pernix*. All other abbreviations as explained in the legend to Fig. 2.

it appears to be a common transcript of *cbsA*, *cbsB* and *soxL* (3.372 kb). The results from the Northern blot analysis were tested by RT-PCR experiments (Fig. 8). These experiments confirmed the transcription of the individual genes (*soxL*, *soxN*, and *odsN*) as well as the presence of common transcripts of *soxN* and *odsN* and of *cbsAB* and *soxL*. Additionally, the results of the RT-PCR experiments also suggest the presence of a common transcript of *soxL* and *soxN* (soxL–soxN in Fig. 8). However, since we were not able to detect a corresponding signal (2.558 kb) in any of the Northern blot experiments, this transcript either occurs at a rather low abundance, or is rapidly processed. Common transcripts of the *cbsB–soxL–soxN* genes (3.563 kb), or the *soxL–soxN–odsN* genes (2.875 kb) were not detectable by RT-PCR.

DISCUSSION

The presented data demonstrate that the *cbsAB–soxLN–odsnN* loci in the genomes of *Sulfolobus acidocaldarius* (DSM 639) and *S. solfataricus* P2 are similar with respect to their organization as well as their sequences, thus resolving previous contradictions.

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                 . . . .10 . . . .20 . . . .30 . . . .40 . . . .50 . . . .60 . . . .70 . . . .80
SULaci odsN   1:.......................MRMINVGFYYKIKKGHEKE.FEEKFLEIVKILKTT....NSGLIEAKLYRSVEDPT
SSO11196      1:.......................MINVGFYYKVKRGFEKD.FENKFKEVLSYLRAN....ADGFVDAKLYKNVDEPS
ST1668        1:MLKLINPTWLIFYNILIKQLRKGCFWLISVGFYYRVKRGFEGE.FERKFGEVVSFLSS.....FKGFRGARLYRSVDDPS
AEP1727       1:...MGCEMVEARLLDALYLAALAVGWLASIGGFIEFRRSLLGGGFVCKADAKGWINCRSAYVIPQAFIAGRIHLSELAPI
Ta1221        1:.......................MINVGLYYRVKKGHEEE.FERTFNSVMAMIKSS....GMGIKEVRLYRDVNDPQ
CLOace        1:.......................MVKVVAKNFIKEEKIED.VLKVAKELVEITVAK....DKGCIKYEMYQDLKDKT
STRgal aknX   1:................MTDHEPGTEGADAVTFVNTFTVHAEPEVFEKEFARTSEFMAR.....QPGFVRHTLCRHAERPG
BACsub        1:.......................MVREAAMLHIKEGLEQE.FEDAFRQAAPIISG.....MKGYITHSLSKCMEETH

                 . . . .90 . . . 100 . . . 110 . . . 120 . . . 130 . . . 140 . . . 150 . .
SULaci odsN  52:EYLMYTEWKDLDSFRN........FILSEGYKNTVSYGKTILDGKP.......THRVLQELNT...........
SSO11196     50:EYLIYSVWRDLDSFKK........FVSSSAYKNTVNYGKSIIESKP.......IHRILQEIND...........
ST1668       75:EYLIYSEWDDLESYKN........FINSTAYRETVEYGKSIIEGRP.......THKVFQQINT...........
AEP1727      78:YFTATLATAVLGVLLDIDLLAKLSYLLAAGGAASVPYLVYLEVRVAKAICLWCTIMHLSIILAVASATAKILGG
Ta1221       50:QYMIFTEWESLEKFKD........FIASRPFKETTEYGKSILEEMP.......KNRIFMNETSI..........
CLOace       50:ILTMIEEWESMECLQK........HMQSEHFKKLVPMMGEYSKKQG.......ETNIYTKVV............
STRgal aknX  60:QYVNVAEWRDLASFRA........AVSHDDFRPHAGALRALSESRPELYLVRLRREGAPGLDGPASEGEEI...
BACsub       49:KYLLLVEWETLEDHTEG.......FRGSSEYQEWKALLHRFYTPFP.......TVEHFQDV.............
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Fig. 4. Alignment of proteins bearing a resemblance to odsN. Abbreviations – CLOace: Uncharacterized conserved protein from *Clostridium acetobutylicum* (accession NP_349984); BACsub: hypothetical protein yczJ from *Bacillus subtilis* (accession O31484); STRgal aknX: uncharacterized gene from a gene cluster involved in aclacinomycin biosynthesis from *Streptomyces galilaeus* (accession AAF70105), SSO2805, Ta1228, APE1725, and Ta1222: Open reading frames as identified in the legend of Fig. 5.

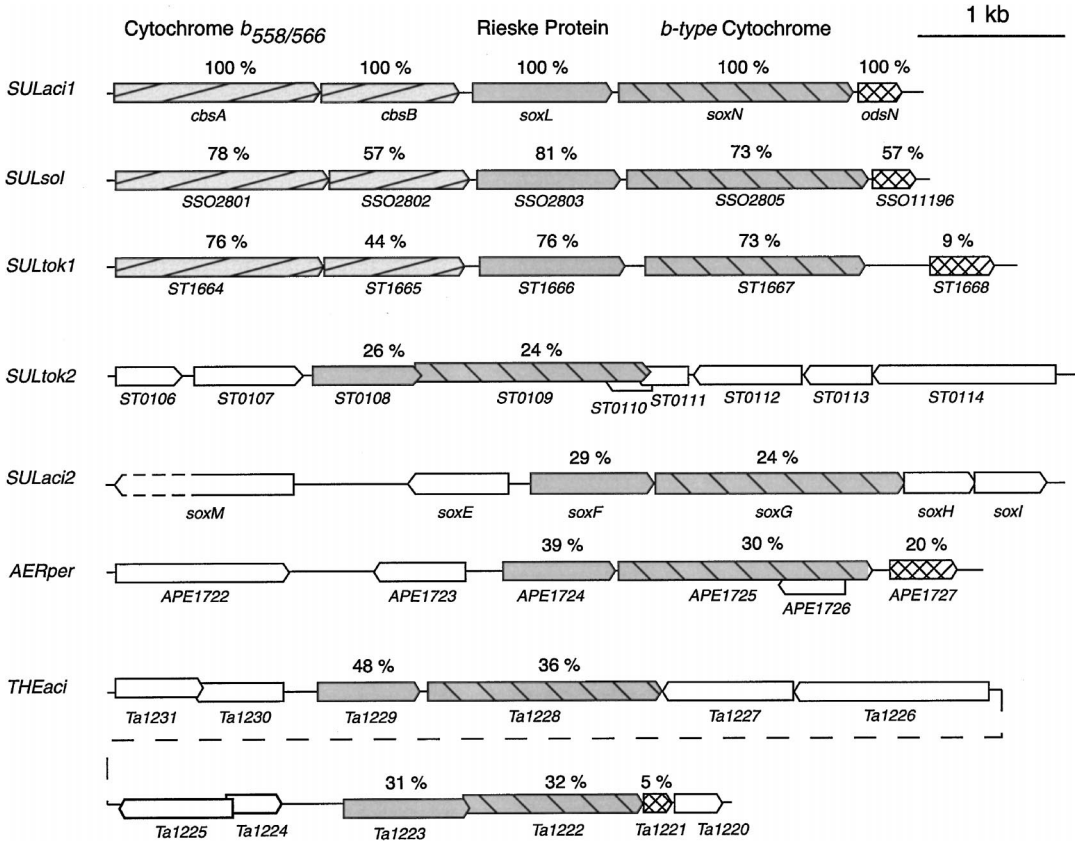

Fig. 5. Comparison of the physical organization of the *cbsAB–soxLN–odsN* locus of *S. acidocaldarius* with other archaeal loci encoding Rieske iron–sulfur proteins and *b*-type cytochromes. The numbers indicate the similarity to the *cbsAB–soxLN–odsN* genes derived from a Phylip distance matrix calculated using an identity matrix for the pairwise alignments and the Blosum series for the multiple alignments. Abbreviations – SULacil: *S. acidocaldarius cbsAB–soxLN–odsN* locus; SULsol: *cbsAB–soxLN–odsN* locus from the genome of *S. solfataricus* (accession NC_002754); SULtokl: *cbsAB–soxLN–odsN* locus from the genome of *S. tokodaii* (Kawarabayasi *et al*., 2001) (accession AP000987); SULtok2: second locus from the genome of *S. tokodaii* encoding a Rieske iron–sulfur protein and a *b*-type cytochrome (Kawarabayasi *et al*., 2001) (accession AP000981); SULaci2: gene cluster encoding the soxM oxidase from *S. acidocaldarius* (Castresana *et al*., 1995); AERper: locus from the genome of *Aeropyrum pernix* encoding a Rieske iron–sulfur protein and a *b*-type cytochrome (Kawarabayasi *et al*., 1999) (accession AP000062); THEaci: locus from the genome of *Thermoplasma acidophilum* (Ruepp *et al*., 2000) (accession AL139299).

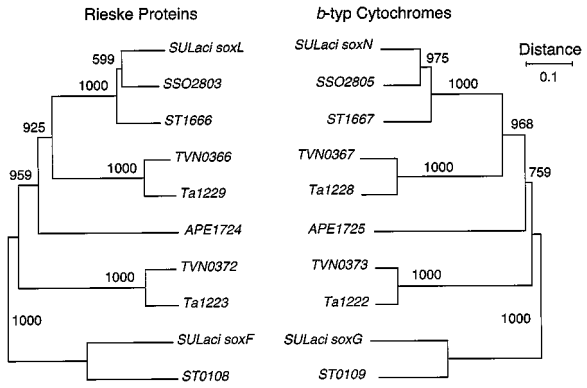

Fig. 6. Comparison of the phylograms of the archaeal Rieske iron–sulfur proteins and *b*-type cytochromes. TVN: Thermoplasma volcanium (Kawashima *et al*., 1999) (accession NC_002689), all other abbreviations as specified for Fig. 5. The alignments were calculated using an identity matrix for the pairwise alignments and the Blosum series for the multiple alignments. The protein gap penalty was set to 15. The threshold for delaying divergent sequences was set to 0. All other parameters were as preset by the program. Positions containing gaps in any of the sequences were not included for calculation of the trees.

Sequence comparisons unambiguously identified the soxN protein as a homolog of the *b*-type cytochromes from the cytochrome bc_1 and b_6f complexes (Figs. 1, 2, and 3). In contrast, the odsN protein displays only minimal similarities to sequences in the databases. With exception of aknX, an uncharacterized gene from a gene cluster involved in aklavinone–aclacinomycin biosynthesis in *Streptomyces* (Genbank accession: AB008466), all other identified sequences are classified as hypothetical proteins. However, since the other genes of this cluster have not been detected in *Sulfolobus*, and odsN shows no significant similarities to bacterial homologs of aknX, we consider this similarity to be coincidental.

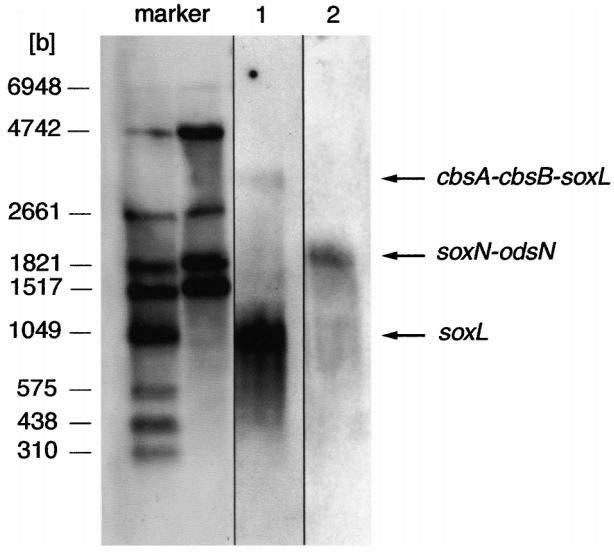

Fig. 7. Northern blot analysis of *S. acidocaldarius* RNA. Four micrograms of RNA were loaded on each lane. Lane 1 was hybridized with probe PsoxL, lane 2 with probe PsoxN specific for transcripts of the *soxL* and the *soxN* genes.

The *cbsAB–soxLN–odsnN* locus of *S. acidocaldarius* displays a complex transcription pattern (Fig. 7 and 8). The identified transcripts indicate that the potential promoter elements detected upstream of *soxL* and *soxN* as well as the transcription termination signal downstream of *soxL* (Fig. 1) are functional, whereas the potential box A detected upstream of *odsN* is not part of a functional promoter. Nevertheless, the presence of polycistronic transcripts of *soxL* indicates that the termination signal cannot be 100% effective. Furthermore, the current data do not exclude the possibility that *soxL* is exclusively transcribed into a poly-cistronic mRNA. However, this would imply an efficient processing of the primary transcripts as well as the complete degradation of all processed RNA sequences not derived from *soxL*. We consider the 3.2–3.4-kb transcript detected in this study to be identical with the previously reported (Hettmann *et al*., 1998) bicistronic *cbsAB* transcript, whose length was previously underestimated.

The *cbsAB* and *soxLN* genes are also present in the genomes of *S. solfataricus* P2 and *S. tokodaii*. *OdsN* is clearly recognizable in *S. solfataricus* P2 and *Aeropyrum pernix*, whereas the open reading frame downstream from the *soxN* homolog of *S. tokodaii* (ST1668) displays only a very low similarity to the *S. acidocaldarius* gene (Fig. 5). The overall comparison of the studied genomic region in all three *Sulfolobus* species reveals that the genes encoding cofactor-containing proteins (*cbsA*, *soxL*, and *soxN*) display a significantly higher degree of conservation than the genes encoding proteins not predicted to bind a prosthetic group (*cbsB* and *odsN*). Thus, it appears that the requirements associated with the binding of the prosthetic group exert a conserving influence on the sequences of these proteins.

Even though the occurrence of archaeal genes encoding Rieske proteins and cytochrome *b* homologs is well documented (Fig. 5, Schmidt and Shaw, 2001; Schütz *et al*., 2000), little is known about the function of these proteins. Since in many cases the genes are organized in Rieske–cytochrome *b* pairs as in the bacterial *fbc* operons, it could be speculated that these genes code for subunits of archaeal analogs of the bacterial cytochrome bc_1 complexes. Up to now, this assumption has been substantiated only for the *soxF* (Rieske protein) and *soxG* (*b*-type cytochrome) genes from *S. acidocaldarius* (compare Fig. 5 SoxF and soxG are subunits of the quinol oxidase soxM,

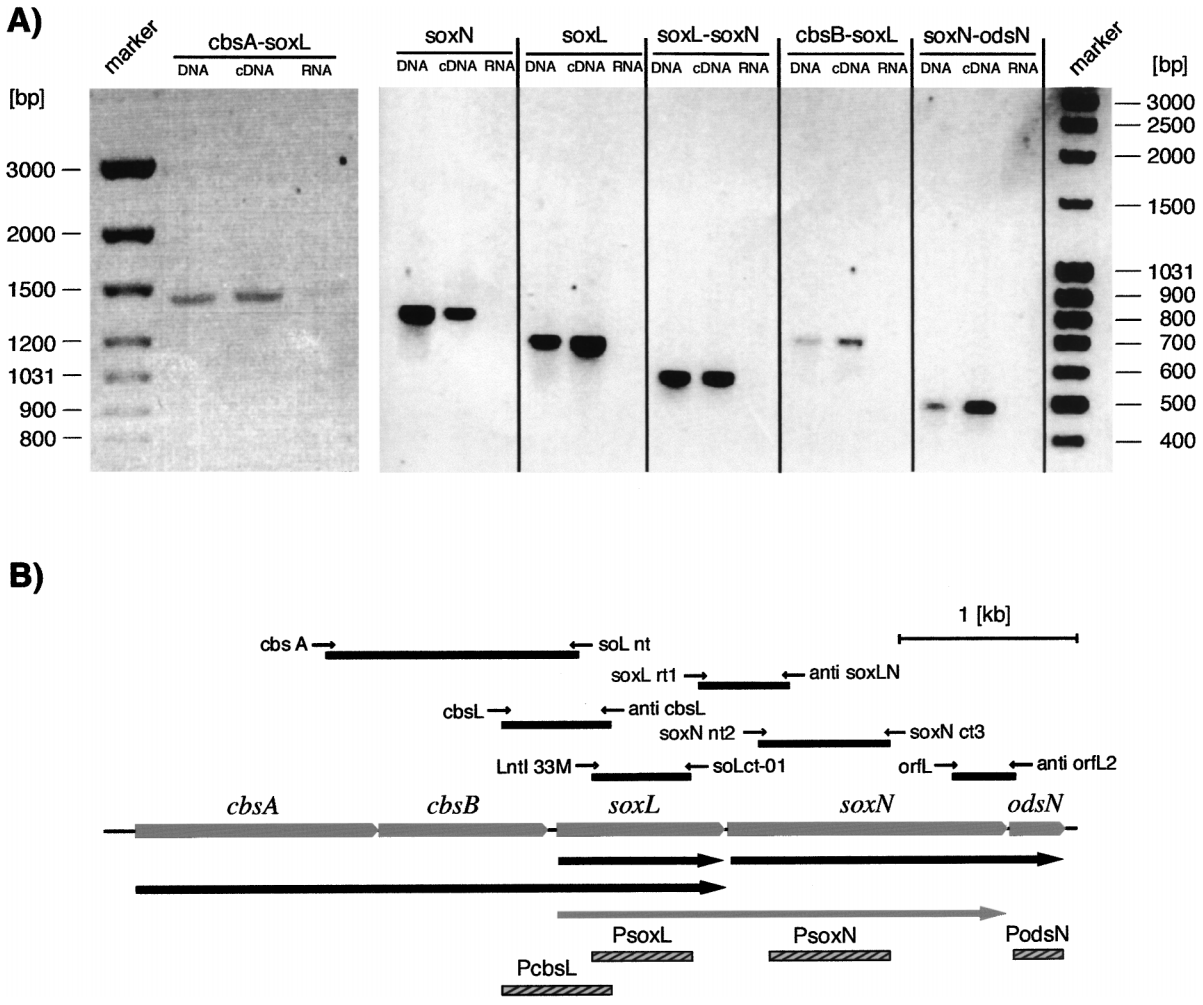

Fig. 8. (A) RT-PCR analysis of mRNAs from the *cbsAB–soxLN–odsN* locus. The amplification products were electrophoretically separated on agarose gels and visualized by ethidium bromide fluorescence. Positive and negative controls were genomic DNA (DNA) and RNA without reverse transcription (RNA). (B) Schematic representation of the RT-PCR products and the probes used for Northern blot analysis. The small arrows indicate the primers used in the individual amplification reactions. The mRNA transcripts detected by RT-PCR and Northern blot analyses are indicated by solid, black arrows. The gray arrow indicates a low abundance transcript detected only by RT-PCR.

which was recently isolated and characterized in the active state (Komorowski *et al*., 2002). The activity and the inhibitor sensitivity of this terminal oxidase supercomplex suggest an assembly of a cytochrome *bc*–homologous module and a cytochrome *c* oxidase module. Carried forward to the genomic region analyzed in the current study, this implies that the soxL and soxN proteins form the core of a second cytochrome *bc*–homologous complex in the membranes of *S. acidocaldarius.* This assumption would be in line with the previously observed quinol oxidase activity of the isolated soxL protein (Schmidt *et al*., 1995). One major question associated with this conclusion concerns the identity of the cytochrome *c*–equivalent within this hypothetical soxLN complex. The finding that *cbsAB* and *soxL* are transcribed into a single mRNA suggests that this function may be fulfilled by cbsA (cytochrome $b_{558/566}$) (Hettmann *et al*., 1998). The high redox potential of +400 mV at pH 7.0, as well as the localization of the globular, heme-containing domain on the outside of the *Sulfolobus* membranes (Hettmann *et al*., 1998; Schäfer *et al*., 2001) supports this hypothesis. The observation that the transcription pattern of the genes significantly differs from the simple organization of the *fbc* operons is not necessarily inconsistent with this model. In fact, the organization of the genes in two main transcription units, one containing the gene encoding the high-potential cytochrome and the Rieske protein, the other comprising the gene coding for the *b*-type cytochrome resembles the

situation present in the cyanobacterium *Nostoc* (Malkin *et al*., 1988). The occurrence of an additional monocistronic mRNA of *soxL* may reflect an adaptation to the growth of *Sulfolobus* in a highly acidic medium. It is conceivable that the turnover of the soxL protein containing an acid-labile iron–sulfur cluster would be significantly higher than that of the other subunits.

In conclusion, we propose the occurrence of a novel cytochrome *bc*–analogous complex in the membranes of *Sulfolobus.* The redox-active subunits are the *b*-type cytochrome soxN, the Rieske iron–sulfur protein soxL, and the high-potential cytochrome cbsA ($b_{558/566}$) adopting the function of cytochrome c_1 or f in the known complexes. CbsB and odsN are likely to be additional, non–redox-active subunits. On the basis of previous results (Schmidt *et al*., 1995) as well as on homology, this complex is predicted to function as a quinol-acceptor oxidoreductase. The nature of the electron acceptor for the cbsAB–soxLN–odsN complex remains an open question. However, the terminal electron acceptor has to be either the soxM, or the soxABCD oxidase, since these are the only terminal oxidases detected in the genomes of *S. solfataricus* and *S. tokodaii*. Because of its high activity with the single electron donor TMPD (N,N,N',N'-tetramethyl-1,4-phenylenediamine) (Gleißner *et al*., 1997), which is rather untypical for an authentic quinol oxidase, and its insensitivity to quinol oxidase inhibitors, we consider soxABCD as the most likely candidate. The electron transfer between the cbsAB–soxLN–odsN complex and soxABCD may be accomplished by a reorientation of the globular domain of cbsA, as was previously demonstrated (Schoepp-Cothenet *et al*., 2001). Thus, cbsAB–soxLN–odsN together with soxABCD may form a second respiratory supercomplex in the membranes of *Sulfolobus.*

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