Respiratory gene clusters of *Metallosphaera sedula* – differential expression and transcriptional organization

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*Metallosphaera sedula* is a thermoacidophilic Crenarchaeon which is capable of leaching metals from sulfidic ores. The authors have investigated the presence and expression of genes encoding respiratory complexes in this organism when grown heterotrophically or chemolithotrophically on either sulfur or pyrite. The presence of three gene clusters, encoding two terminal oxidase complexes, the quinol oxidase SoxABCD and the SoxM oxidase supercomplex, and a gene cluster encoding a high-potential cytochrome *b* and components of a *bc*, complex analogue (*cbsBA–soxL2N* gene cluster) was established. Expression studies showed that the *soxM* gene was expressed to high levels during heterotrophic growth of *M. sedula* on yeast extract, while the *soxABCD* mRNA was most abundant in cells grown on sulfur. Reduced-minus-oxidized difference spectra of cell membranes showed cytochrome-related peaks that correspond to published spectra of *Sulfolobus*-type terminal oxidase complexes. In pyrite-grown cells, expression levels of the two monitored oxidase gene clusters were reduced by a factor of 10–12 relative to maximal expression levels, although spectra of membranes clearly contained oxidase-associated haems, suggesting the presence of additional gene clusters encoding terminal oxidases in *M. sedula*. Pyrite- and sulfur-grown cells contained high levels of the *cbsA* transcript, which encodes a membrane-bound cytochrome *b* with a possible role in iron oxidation or chemolithotrophy. The *cbsA* gene is not co-transcribed with the *soxL2N* genes, and therefore does not appear to be an integral part of this *bc*, complex analogue. The data show for the first time the differential expression of the *Sulfolobus*-type terminal oxidase gene clusters in a Crenarchaeon in response to changing growth modes.

INTRODUCTION

Sulfide forms highly insoluble minerals with a number of metals of major economic and industrial importance. Since the isolation of bacteria such as *Acidithiobacillus* (formerly *Thiobacillus*) *ferrooxidans* in the 1950s it has been established that micro-organisms greatly enhance the extraction of metals by leaching processes (reviewed by Rawlings, 2001). The predominantly accepted view of the role of micro-organisms in the solubilization of metal sulfides is that they provide protons from the oxidation of sulfur compounds and maintain iron in its oxidized form for Fe3+/H+ attack on the mineral (Sand et al., 2001). Over three decades of research on mesophilic bacteria such as *A. ferrooxidans* has led to an understanding of the organization of the electron transfer chain in this chemolithoautotroph (Brasseur et al., 2002; Ohmura et al., 2002).

In addition to the acidophilic bioleaching bacteria, a number of thermophilic Crenarchaeota are also able to oxidize sulfur compounds and sulfidic metal ores and can achieve leaching rates superior to those of mesophilic microorganisms, making them attractive for use in commercial tank leaching operations (Dew et al., 2000). However, at present, our knowledge about the physiological features of these organisms which enable this enhanced performance is rather limited.

The oxidation of ferrous iron and inorganic sulfur molecules is an aerobic process that is linked to the respiratory chain. Over the last decade or more there has been considerable progress towards an understanding of the molecular properties of the oxidases and associated electron transfer proteins in Crenarchaeota, especially *Sulfolobus acidocaldarius* (Pereira et al., 2004; Schafer et al., 1999; Schmidt, 2004). Biochemical and spectroscopic characterization of respiratory complexes has been complemented by molecular genetic studies, and the latter have been greatly facilitated by the availability of sequenced genomes for *Sulfolobus solfataricus* and *Sulfolobus tokodaii* (Kawarabayasi...
et al., 2001; She et al., 2001). The current view of aerobic respiratory electron transfer in *S. acidocaldarius* is that it involves at least two oxidases. The SoxABCD complex functions as a quinol oxidase and contains a haem *aa*<sub>3</sub>-Cu<sub>B</sub> centre that is the site of oxygen reduction. The second complex in *S. acidocaldarius* is the SoxM supercomplex. It contains a subunit (SoxM) that has a haem *bb*<sub>3</sub>-Cu<sub>B</sub> centre and a second subunit (SoxH) that is also found in enzymes of the superfamily of cytochrome oxidases (Komorowski et al., 2002). The SoxH subunit contains a binding motif for a binuclear Cu<sub>A</sub> centre that is absent in the analogous SoxA subunit of the quinol-oxidizing SoxABCD oxidase. SoxHM does not oxidize quinol directly. Instead, it is thought that quinol oxidation occurs via the SoxFG component, which is analogous to the cytochrome *bc*<sub>1</sub> complex of a bacterial respiratory chain. It has been proposed that electron transfer from the SoxFG components to the SoxHM oxidase components is mediated by a small copper protein, sulfocyanin (SoxE), which appears to be anchored to the cell membrane via a single N-terminal transmembrane helix (Komorowski & Schafer, 2001). A third type of terminal oxidase found in species of the Sulfolobales is another quinol oxidase (Dox-type) that has so far been found exclusively in *Acidianus* species (Giuffre et al., 1997; Puschke et al., 1997). Recently, a second analogue of bacterial cytochrome *bc*<sub>1</sub> complexes (referred to as the Cbs/SoxL2N complex) has been identified in *S. acidocaldarius* (Hiller et al., 2003). It is composed of a SoxC-like cytochrome *b* (SoxN), a Rieske protein (SoxL2) and a high-potential *b*-type cytochrome (CbsA). The Cbs/SoxL2N complex clearly represents a third quinol-oxidizing species, although it is not established how this complex interacts with the respiratory oxidases.

A major task in describing chemolithotrophy in Crenarchaeota is to understand how ferrous iron- and sulfur-oxidizing pathways are connected to the aerobic respiratory chain. Unfortunately, none of the species described above for which we have genomic information is able to grow efficiently on pyrite or sulfur. In order to address this deficiency we have investigated the expression of respiratory gene clusters in *Metallosphaera sedula*, a Crenarchaeon related to the aforementioned *Sulfolobus* species (Huber et al., 1989). This thermophilic archaon is able to grow chemoeauto- and chemomixotrophically using pyrite or sulfur as energy source and can also grow heterotrophically using yeast extract. Thus, *M. sedula* represents an excellent model organism for basic research into bioreaching processes. In the present paper we report the identification of genetic loci encoding components of respiratory complexes from *M. sedula* as well as the expression of relevant genes under different growth conditions.

**METHODS**

**Microorganisms, plasmids, media and growth conditions.** *Escherichia coli* strain DH5α (Invitrogen) was routinely cultivated on LB medium; when necessary the medium was supplemented with antibiotics (Sambrook et al., 1989). *M. sedula* DSMZ 5348<sup>T</sup> (Huber et al., 1989) was cultivated in DSMZ medium 88 supplemented with one of the following: (1) 0·1 % yeast extract (YE), (2) 0·02 % YE and 0·05 % elemental sulfur (S), (3) 0·02 % YE and 2 % pyritic ore (size fraction ~<200 μm, BHP Billiton, Randburg, South Africa) (PYR). The pH of the medium was adjusted to pH 2·9 for growth on pyrite and yeast extract and to pH 3·0 for growth on sulfur using dilute sulfuric acid. pH values were stable for 72 h in pyrite and yeast extract cultures, in sulfur-containing culture the pH dropped to 1·5–2 over 72 h. Cultures were inoculated with 5 % of a preculture grown on the same medium and incubated at 65 °C, 120 r.p.m. in a shaking waterbath (Julabo SW-22). Cells were cultivated in either 100 ml (250 ml flask) or 200 ml (500 ml flask) medium for the time period indicated. For strain maintenance, the cells were transferred every 7 days.

**Growth curves.** Growth of *M. sedula* in 100 ml YE and S medium was monitored by following the increase in OD<sub>600</sub> over a period of 80 h. In addition, growth of S and PYR cultures was monitored by whole-cell protein determination (duration of experiment 102 h, culture volume 100 ml). Relationships between protein concentration and OD<sub>600</sub> were computed based on the S medium data. Aliquots (2 ml) of culture were harvested in a microcentrifuge and the cell pellets extracted with ice-cold acetone/methanol (7:2) solution at −20 °C overnight. Protein pellets were collected by spinning the extracted samples at 4 °C for 30 min and air-dried. The protein pellets were then resuspended in 100 μl distilled water and used for a microscale Lowry protein assay (range 0–25 μg protein ml<sup>−1</sup> at an appropriate dilution (Lowry et al., 1951). In all cultures growth rate began to decline after ~60–65 h.

**Cloning of *M. sedula* oxidase gene clusters.** Standard methods were used throughout (Ausubel et al., 1987; Sambrook et al., 1989). DNA from *M. sedula* was isolated using the DNAzol reagent (Invitrogen). Degenerate primers targeting *soxC*/*soxN*-like genes (SOSOXCF2, TggTggCCWAgRAAYTT; SOSOXCF1, TDTWCAGT-CAAYTTDTATgg; SOSOXCR2, gAgAgAACCttggNggATA), the *soxM* (SOSOXMF1, CTTgTTCTTyygggHCAYCC; SOSOXMR, ACCATgTA-RTgKAARTgHCC) and *soxG* genes (SOSOXGF, AWtgTTAgAD-AgAKTAgg; SOSOXGR, ACYAADgATCMACRRCC) were designed using sequences available in GenBank and taking the codon usage into account (45 mol% GC for *M. sedula*). *M. sedula* genomic DNA fragments in the size range 4–50 kbp were readily obtained using genomic DNA as template, an annealing temperature of 48 °C and an extension time of 3 min 30 s. The products were cloned into pGemTeasy, resulting in constructs pMssoxC1 and pMssoxM1. The plasmid inserts were sequenced and used to generate DIG-DUTP (Roche)-labelled PCR products (probe sizes 600 and 500 bp, respectively) for use in Southern blot experiments. PCR-labeling was performed according to the manufacturer’s instructions. Southern blot hybridizations were performed at 68 °C for 16–18 h followed by chemiluminescence detection using CDP-Star (Roche). Two positive DNA fragments, a ~4.5 kbp *HindIII* fragment and a ~4·5 kbp *EcoRV* fragment hybridizing with the *soxM* and the *soxCSoxN* probe, respectively, were chosen for cloning. Partial genomic libraries of *M. sedula* genomic DNA fragments in the size range 4·5–6·5 kbp (*HindIII*) and 3·5–5·5 kbp (*EcoRV*) were constructed in pBluescript and ~200 clones screened by Southern blotting of digested plasmids using the *soxM* and the *soxCSoxN* gene probes. A clone strongly hybridizing to the *soxCSoxN* probe and one clone that weakly hybridized to the *soxM* probe could be isolated. They were designated pMSE91 and pMSH182 and the inserts sequenced completely, showing that they contained a *cbsBA*-soxL2N and a partial *soxL1*- *soxABC* gene cluster, respectively. This result was unexpected, and it is assumed that the similarity of the *soxB* and *soxM* genes (48 % identity at the protein level) may have given rise to the positive
hybridization result leading to identification of pMSH182. In fact, there is a potential soxM probe-binding site located in the soxB gene, as shown by sequence alignments (see Fig. 2). Multiple binding sites exist for the soxC/soxN gene probe in the M. sedula soxN gene, one of which is located within the soxN fragment encoded by pMSE91. It is assumed that this fragment did not show up in the original Southern blot analyses because the probe interaction is not strong enough to give a detectable signal when only a few copies of the fragment are present, as would be the case when blotting genomic DNA digests. The soxN probe sequence itself is not contiguous with the partial soxN gene sequence obtained (see Fig. 5). To obtain further sequence for the soxM gene cluster, a size-fractioned HindIII digest of M. sedula genomic DNA was ligated overnight and used in an inverse PCR reaction. Primers for this PCR were designed based on the sequence obtained from pMSsoxM. The inverse PCR product was partially sequenced.

Preparation of cell-free extracts. M. sedula cells were harvested after 48 h incubation by centrifugation for 20 min at 4000 g. To remove solids, S and PYR medium samples were filtered through Whatman no. 1 paper prior to centrifugation. The resulting pellets were either stored at −20 °C until further use or immediately resuspended in 50 mM MES, pH 6.0. Pefabloc protease inhibitor (1 mM) (Roche) was added to the samples followed by two passages through a French Pressure cell (SIM Aminco) at 1000 p.s.i. Cell debris was removed by low-speed centrifugation, and the resulting supernatant (crude extract) subjected to ultracentrifugation at 4 °C, 140,000 g for 90 min. Cell membranes were resuspended in 50 mM MES, pH 6.0, using a homogenizer and used for optical difference spectroscopy at once.

Analytical methods. Electronic absorption spectra were recorded at 25 °C on a split-beam UV-3000 spectrophotometer (Hitachi). Membrane fractions were analysed as prepared (oxidized) and after reduction by addition of solid dithionite.

RNA isolation. M. sedula cultures (100 ml) were harvested 24, 48 and 72 h after inoculation. Cultures were chilled to 4 °C immediately and harvested by centrifugation (4 °C, 10 min, 4000 g). RNA was isolated from cell pellets using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA pellets were stored at −80 °C until further use. RNA yields were assessed using a spectrophotometer, and the integrity of the samples was checked by gel electrophoresis using a 10 mM BES buffer system (50 × buffer: 500 mM BES, 5 mM EDTA, pH 6.7) and DMSO/glyoxal as the denaturing agents. For Northern blot analysis, RNA was transferred from such gels to a positively charged nylon membrane (Hybond-N+, Amersham Biosciences) by capillary blotting (Sambrook et al., 1989). The membranes were subjected to UV cross-linking and hybridized for 18 h at 50 °C in DIG Easyhyb solution (Roche) using DIG-DUTP (Roche) labelled dsDNA probes (see above), followed by chemiluminescent signal detection using CPD-Star (Roche).

RT-PCR and real-time RT-PCR. RNA for use in RT-PCR experiments was DNase treated using Qiagen RNaseasy columns and the RNase-free DNase set (Qiagen). To detect DNA contaminations, 50–100 ng RNA were used as a template in a ‘non-RT’ PCR reaction, and only samples that resulted in no amplification after 35 PCR cycles were considered DNA-free. RNase inhibitor (RNasin, Promega) was included in all RT reactions. RT-PCR was conducted using a Qiagen One-Step RT-PCR kit; for real-time RT-PCR, 1 μg RNA was reverse transcribed using random hexamer primers (Invitrogen, 0.2–2 μg per reaction) and the Omniscript RT Kit (Qiagen). The single-stranded cDNA was diluted to appropriate concentrations before being used in real-time PCR experiments. Primers for use in such experiments were designed to produce amplicons of 201 bp using the Primer Express software (Applied Biosystems). The primers used were: MS16SF, TAGTCCTCCGGCCT-TAGCGATG; MS16SR, CCCGCCAATTCCCTTAAGTTTCT; MSSOXMF, TCATTTTTCGCCGCACTG; MSSOXMRQ, CTACAAA-CCTCCAGAACTGACA; MSSOXQF, TCGGCGTCTCATTGCTGTC; MSSOXQR, GAGGACAGTGGCGGAATGTCG; MSQoxL2F, GGAGGAGTTGGGTTACCCGT; MSQoxL2R, GGCTACTGGC-TCTCCCCAAGT; MSQcsbAF, CCTGCATCCTTTAGGTAAGC; MSQcsbR, TGGTATTACCCTGACAGGGCTG; MSQoxBF, TCCT-CGGAGCGCCAGACATG; MSQoxBR, GCTTACGGCAAGGCCG-CAGATA. Real-time PCR was performed on an Applied Biosystems 7700 sequence detection system using 25 μl total reaction volume, 5 μl cDNA, 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems) and an appropriate amount of each primer (100–400 nM) per reaction. Preliminary experiments showed that all primer pairs gave rise to unique amplification products. During real-time PCR experiments, the formation of PCR products and PCR artefacts (e.g. primer dimers) was routinely monitored by melting-curve analysis. At least three independent reactions were performed for each data point; values for 16S RNA gene expression were collected for each RNA sample and used for normalization of the expression values. PCR efficiencies were determined for each reaction, as suggested by Liu & Saint (2002), and averaged for related samples present on the same 96-well plate. Normalized expression values were then determined from the ratios of target gene expression to reference gene expression using means of the CT values obtained for each experiment, similar to the method of Pfaffl (2001). These values were corrected for differences in the template concentration used. The relative standard deviation of the normalized expression values was assessed on the basis of the observed variation in CT values.

GenBank accession numbers and computer-based analyses. DNA sequences have been deposited with GenBank under accession numbers AY452058, AY452059, AY452060 and AY452061. Analysis of the available Sulfolobus genomes was undertaken using the BLAST algorithm (Altschul et al., 1997). Assembly of DNA sequences and determination of codon usages was carried out using the 2D-ANGIS and Web-ANGIS programs (ANGIS). Analysis of putative gene products was conducted using SignalP, DAS, TMPred and TMHMM (Cserzo et al., 1997; Krogh et al., 2001; Moller et al., 2001; Nielsen et al., 1997a, b) programs accessible through the Expasy-molecular biology server.

RESULTS

Differential expression of respiratory complexes in M. sedula cells grown heterotrophically or chemolithoheterotrophically

Initial evidence for a differential expression of respiratory complexes in M. sedula came from optical spectroscopy of cell membrane fractions. Reduced-minus-oxidized difference spectra (Fig. 1) of membranes from cell material grown on yeast extract, sulfur- or pyrite-containing medium clearly showed the presence of cytochromes, but differed in regard to the content and proportion of the different haem species present. The spectrum of membranes isolated from YE-grown cells showed a strong absorption in the x-band region that was characterized by a peak at 591 nm with a shoulder at around 608 nm (Fig. 1a). It was reminiscent of published spectra of the SoxM supercomplex purified at near-neutral pH (Lubben et al., 1994a; Lubben, 1995), but without the absorption peak at 562 nm. The spectrum of membranes from cells grown on
sulfur exhibited a prominent absorption peak at 587 nm with a shoulder at 604 nm and an additional absorption band at 565 nm (Fig. 1b), and clearly resembled spectra obtained from the purified SoxABCD complex of *S. acidocaldarius* (Lubben et al., 1994b; Lubben, 1995; Schafer et al., 1994). In contrast, two distinctive absorption peaks were observed in membranes for pyrite-grown cells (Fig. 1c). A broad absorption band was centred at 591 nm with a shoulder at 603 nm. In addition, a peak at 573 nm and a broad absorption band centred at about 690 nm were observed. A clear relationship of this spectrum to published spectra of *Sulfolobus* oxidase complexes was not obvious.

### Cloning of the terminal oxidase genes of *M. sedula*

There is currently no genome sequence for *M. sedula*, and genes encoding terminal oxidases from this organism have not been reported previously. Hence, sequences of genes encoding respiratory complexes in related archaea (*S. acidocaldarius*, *S. solfataricus*, *S. tokodaii*) were used to design degenerate primers for amplification of partial SoxM- and SoxC/SoxN-like genes. PCR products of the expected size were readily obtained using *M. sedula* genomic DNA, and the amplicons were cloned into pGemTeasy and sequenced (plasmids pMSsoxM1, pMSsoxC1). Gene probes for Southern blotting against *M. sedula* genomic DNA were amplified from these plasmids. The Southern blot using the SoxC/SoxN gene probe clearly shows that multiple copies of related genes exist in the *M. sedula* genome. The SoxC and SoxN genes are closely related (~60% similarity at the protein level), and the cloned fragment of a SoxC/SoxN-like gene from *M. sedula* is probably part of a SoxN-like gene.

Following identification of suitably sized restriction fragments (Fig. 2), clones containing partial respiratory-complex gene clusters were obtained either by screening of partial genomic libraries or by inverse PCR. Three partial gene clusters, encoding parts of a SoxABCD, a SoxM and a cbs–soxL2N cluster (Fig. 3), could be identified. Plasmid pMSH182 contains a 3391 bp HindIII fragment that encodes homologues of the SoxAB genes (quinol oxidase subunits) and the SoxL1 gene (encodes a Rieske Fe-S protein) (Fig. 3), with the SoxB and SoxL1 genes being incomplete coding regions. The SoxAB genes (separated by 34 bp) are transcribed divergently from the SoxL1 gene, which is located 112 bp upstream of SoxA. Putative promoter elements (Hain et al., 1992) could be identified 42 and 60 bp, respectively, upstream of SoxA. Putative promoter elements were transcribed divergently from the SoxL1 gene. The SoxA gene encodes a 155 aa protein that is most closely related to the SoxA proteins of *S. tokodaii* (NP_375982.1/St0135, 61% identity) and *S. solfataricus* (NP_343986.1, 57% identity). The partial SoxB gene so far encodes 512 aa of a protein (closest relatives: NP_375983.1, 62% identity and NP_343985.1, 63% identity) with at least 12 transmembrane domains and a Cox1 domain (Pfam 00115, COG0843). No transcription termination signal (Reiter et al., 1988) was detected between the SoxA and SoxB genes. The partial gene encoding...
a Rieske Fe-S protein, soxL1 (closest relatives: NP_375981.1, 69% identity and NP_343988.1, 74% identity), may be functionally linked to the soxABCD gene cluster, as it is located upstream of soxA in the three mentioned Sulfolobus species and M. sedula. The partial SoxL1 protein has 351 aa, one membrane-spanning region and a QcrA/Rieske Fe-S protein signature (COG0723).

Using inverse PCR, a partial soxM gene could be sequenced from a circularized HindIII DNA fragment. This partial gene encodes 643 amino acids (predicted full-length protein ~815 aa) which share 66% and 64% identity with soxM proteins from S. solfataricus and S. tokodaii, respectively. It has been predicted to contain at least 13 membrane-spanning regions.

In addition to the two gene clusters that encode terminal oxidases or an oxidase/bc1 supercomplex, a homologue of the cbsAB–soxL2N gene cluster from S. acidocaldarius was also isolated. In M. sedula, the order of the cbs genes has been reversed, and the cluster encodes cbsBA–soxL2N. The 3247 bp EcoRV fragment in pMSE91 contains partial sequences for cbsB and soxN and complete coding regions for cbsA and soxL2. Both complete coding regions were preceded by putative promoter elements: CTTAATA for soxL2 and TTATTTA for cbsA, located 35 and 34 bp upstream of the genes, respectively. Putative transcription termination signals were detected downstream of cbsA. Again, all of these genes are most closely related to the corresponding genes from S. solfataricus and S. tokodaii (70–73% identity for soxL2 and cbsA at the protein level).

The cbsA gene encodes a polypeptide with 464 aa and two predicted transmembrane helices, located at the N- and the C-terminus. The N-terminal transmembrane helix is likely to be an export signal (predicted signal cleavage site: aa 32–33) that is not present in the mature protein. Fourteen predicted N-glycosylation sites and a high content of serine and threonine (7–4% and 11–3% of all amino acids, respectively, in the processed CbsA protein) may indicate that the protein is a glycoprotein similar to cbsA from S. acidocaldarius (Hettmann et al., 1998).

Like its SoxL1 homologue, the SoxL2 gene product contains a QcrA/Rieske Fe-S signature (COG0723) and one membrane-spanning region. While both SoxL1 and SoxL2 are closely related (60–70% identity over 236–321 aa) in the Sulfolobus species, the SoxF Rieske Fe-S protein found in the SoxM supercomplex has only 46% similarity (206 aa alignment) to these proteins, possibly indicating a different function of the protein. This is in agreement with biochemical data showing a differing redox behaviour for soxF (Schmidt, 2004).

Expression of genes encoding respiratory complexes under different growth conditions

In order to investigate respiratory-complex gene expression under different growth conditions, M. sedula cells were grown on YE or sulfur or pyrite. Growth was followed by optical density for sulfur and YE cultures and by protein determination for sulfur and pyrite cultures. The final OD$_{600}$ reached in YE-grown cells was ~0.65 (corresponding to...
Expression of the soxABCD gene clusters

It was found that expression of the soxABCD quinol oxidase gene cluster, as indicated by soxB transcript levels, was highest in M. sedula cells grown on sulfur (Fig. 4b), while expression in cells grown on yeast extract (Fig. 4a) or pyrite (Fig. 4c) was lower by factors of 10–12. While soxB expression increased over time in YE-grown cells, it appeared to be steady in pyrite- and sulfur-grown cells. In contrast, the soxM gene was most highly expressed in cells grown on YE (Fig. 4a), while levels were reduced by a factor of 1:5–4 in sulfur-grown cells (Fig. 4b) and a factor of 10 in pyrite-grown cells (Fig. 4c). Under all three growth conditions monitored, the expression of the soxM gene increased over time. In sulfur-grown cells, levels of soxB transcript exceeded those of the soxM transcript by about 50 %. During growth with pyrite, expression of both the soxB and the soxM genes was very low (Fig. 4c). The fact that soxM was most highly expressed during heterotrophic growth was confirmed by Northern blot analysis, in which a transcript of 2.9 kb (corresponding to the estimated size of the soxM gene) was detected using a soxM gene probe. The signals obtained for RNA from YE-grown cells were clearly the strongest observed (data not shown).

Expression of the cbsBA–SoxL2N gene cluster

While expression patterns and levels for soxL2 and soxN differed markedly depending on the growth condition investigated, the similarity of values obtained for soxL2 and soxN under any one growth condition was striking. In YE-grown cells (Fig. 4a), soxL2 and soxN expression increased markedly over time, while in pyrite- and sulfur-grown samples expression diminished slightly with time (Fig. 4b, c). Again, gene expression for pyrite-grown cells was lowest (reduced by a factor of 8–10 relative to sulfur-grown cells), while gene expression during growth on sulfur equalled 50–60 % of the levels found for the soxB gene.

In contrast, expression of the cbsA gene exceeded the levels observed for the soxL2 and soxN genes under all three growth conditions, although cbsA expression appeared to follow a similar expression pattern. In pyrite-grown cell material, the levels of this transcript exceeded those of the soxL2N transcript by a factor of ~25. For growth on sulfur, cbsA levels tripled with respect to those of soxL2N. It was also notable that the pattern of expression of cbsA and

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**Fig. 4.** Relative normalized gene expression data for *M. sedula* respiratory gene clusters. The data were obtained from real-time RT-PCR experiments and normalized against the expression of the 16S rRNA gene in the same sample. (a) RNA from YE-grown cells; (b) RNA from sulfur-grown cells; (c) RNA from pyrite-grown cells. The three data points shown represent gene expression in samples prepared after 24, 48 and 72 h. Experimental errors are given as relative standard deviation.
soxL2N during the growth cycle appeared to be different for heterotrophically and lithotrophically grown cells. In the case of the heterotrophically grown cells, there was an increase in transcript levels with time, and it was only in the 72 h cultures that high levels of expression were observed. This contrasted with the situation for sulfur- and pyrite-grown cells, where the transcription of the above genes was high from the beginning of growth. The observed contrast between transcript levels for soxN is supported by Northern blot experiments, in which RNA (isolated after 48 h of incubation) from sulfur-grown cells gave the strongest signal, and the signal for pyrite-grown cells was at the sensitivity threshold (Fig. 5c).

Transcriptional organization of the cbsBA–soxL2N locus

In view of the overall similar expression patterns found for cbsA, soxL2 and soxN and the strikingly different levels of expression observed for these genes, one-step RT-PCR experiments were carried out to establish the transcriptional units present in this gene locus. While all primer combinations used readily gave rise to PCR products of the expected sizes when using genomic DNA as template (Fig. 5a, b), only the soxL2–soxN combination gave rise to an RT-PCR product when using 100 ng Dnase-treated RNA (isolated from cultures grown on either YE, sulfur or pyrite for 48 h) as the template. The combinations that included a cbsA gene primer and a reverse primer for the soxL2 gene did not give rise to an RT-PCR product, while RT-PCR using a primer pair targeting the cbsA gene alone established that all samples contained cbsA transcripts. Hence, cbsA seems to be located on a separate transcriptional unit, while soxL2 and soxN are co-transcribed. This result is supported by Northern blot experiments carried out using a soxN gene probe, in which two transcripts with sizes of ~2·6 kb and ~2·85–2·9 kb were detected (Fig. 5c). This would correspond well to a transcript of soxL2N (~2·6 kb) and a putative transcript including an odsN-type gene (~2·6 kb ± 0·3 kb), which is found upstream of the soxN gene in S. acidocaldarius. It is also possible, however, that the weakly hybridizing 2·6 kb transcript arises from a different gene locus present on the M. sedula genome, which was also detected in Southern blot experiments using the soxN gene probe. A transcript of soxL2N including the cbsA gene would have an expected size of at least 4·1 kb.

DISCUSSION

Over a decade of characterization of the biochemical properties of purified cytochrome oxidases from Archaea together with the availability of genome sequences from two Sulfolobus species has shown that there are at least three types of such enzymes associated with the Sulfolobales (Giuffre et al., 1997; Purschke et al., 1997; Schafer et al., 1999, 2001).

In this paper we have shown that M. sedula possesses at least two terminal oxidases, the SoxM complex and the
SoxABCD quinol oxidase, as well as a CbsBA-SoxL2N cytochrome bc1-complex analogue.

The main focus of our study was to determine whether there was differential expression of these respiratory complexes in M. sedula associated with different growth modes (Fig. 4). Measurements of the levels of transcripts for genes encoding the two oxidase complexes indicate that the soxM gene is most highly expressed during heterotrophic growth of M. sedula, while the soxABCD gene cluster was most highly expressed during growth on sulfur. Both findings are consistent with the optical spectra of the corresponding membrane fractions, suggesting that the transcript level reflected the formation of respiratory complexes within the membrane. The fact that neither of the monitored oxidase gene clusters was expressed to high levels in cells growing on pyrite was very surprising, in particular since optical spectra of membranes from pyrite-grown cells clearly showed the presence of oxidase-related haems. One possible explanation is that the diversity of respiratory enzymes in M. sedula is greater than can be easily gauged in the absence of a genome sequence. The presence of multiple copies of soxC/soxN-related genes in M. sedula is suggested by Southern blot analysis (Fig. 2). Multiple gene clusters encoding terminal oxidases are present in other Sulfolobales, for example the genome sequence of S. tokodaii indicated here provide a good basis for further research into mechanisms of chemolithotrophy in M. sedula.

In contrast, the independently transcribed cbsA gene was present at high levels in cells grown on both sulfur and pyrite throughout the experiments, while levels in YE-grown cells only reached high levels in the 72 h sample. The high levels of expression were particularly striking in pyrite-grown cells, as all other monitored genes were only expressed at very low level in these cells. The cbsA gene encodes an unusual glycosylated membrane-bound cytochrome b566/588 that has been purified from S. acidocaldarius. It has been proposed to function in electron transfer in the ‘pseudoperiplasmic space’ of the organism (Hettmann et al., 1998; Schoepp-Cothenet et al., 2001), and the amount of CbsA present in the cells varied in response to oxygen tension and nitrogen sources present in the medium (Hettmann et al., 1998; Schafer et al., 1994). These observations may explain why the expression of cbsA was enhanced in M. sedula cells grown for 72 h on YE, since these cultures would be denser, and therefore the oxygen tension in the culture would be lower than for the previous data points. Since the S. acidocaldarius type strain used for the CbsA induction studies is not capable of chemolithotrophic growth, there are no comparable data relating to growth on sulfur- or pyrite-containing media. However, our results suggest that the cbsA gene product is important during chemolithotrophic growth of M. sedula, and it may in fact be related to a yellow-coloured, acid- and protease-resistant haemoprotein (cytochrome b572) isolated from membranes of M. sedula cultures grown on ferrous-iron-containing media (Blake et al., 1993). The additional cytochrome peak at 573 nm observed in the spectra of pyrite-grown cells of M. sedula might be related to both this acid-resistant protein and to the high expression of the cbsA gene found under these conditions.

It is clear from our results that terminal oxidase complexes in the archaeon M. sedula are differentially expressed in relation to the prevailing growth mode, a property which, to our knowledge, has not been previously shown to exist in thermoacidophilic Crenarchaeota. The high expression levels of the cbsA gene during chemolithotrophic growth suggest a central role for the resulting protein in mediating electron transfer from as yet uncharacterized iron- and possibly sulfur-oxidizing enzymes on the outside of the cell to the respiratory oxidases during chemolithotrophic growth in M. sedula. At present, the respiratory pathway which involves CbsA is not defined, and the data presented here provide a good basis for further research into mechanisms of chemolithotrophy in M. sedula.

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