The ATPases CopA and CopB both contribute to copper resistance of the thermoacidophilic archaeon *Sulfolobus solfataricus*

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INTRODUCTION

Transition elements such as copper, zinc, nickel and cobalt are essential trace constituents occurring in many important cellular processes and structures. At high intracellular concentrations however, these metals become deleterious when undergoing Fenton-type redox reactions (Haber & Weiss, 1932; Goldstein et al., 1993; Liochev & Fridovich, 2002) or blocking the cellular antioxidant defence system. The highly reactive oxygen species suppress metabolic pathways by affecting iron–sulfur proteins or damaging other proteins and DNA (Imlay, 2006). Thus, metal concentrations have to be equilibrated carefully and cells need powerful mechanisms to avoid metal accumulation up to toxic levels.

The mechanisms of transition metal uptake into bacterial cells are largely unknown. Metals may enter the cytoplasm by using either non-selective pores or membrane-embedded permeases, or by means of chalkophores (Balasubramanian et al., 2011). In contrast, export of transition metals is carried out by P-type ATPases acting as specific transporters by coupling the energy of ATP hydrolysis to the ion transfer across cytoplasmic membranes (for reviews, see Møller et al., 1996; Silver & Phung, 1996; Axelsen & Palmgren, 1998; Kaplan, 2002; Nies, 2003; Rensing & Grass, 2003; Kühlblandt, 2004; Argüello et al., 2011). According to an extensive sequence comparison, they belong to subgroup IB of the P-type ATPase superfamily whose members are widely distributed in archaea, bacteria and eukaryotes (Axelsen & Palmgren, 1998). Transition metal transporters of this subgroup are also referred to as CPx-ATPases because of their transmembrane ion binding sequence motif Cys-Pro-Xxx (Solioz & Vulpe, 1996).

*S. solfataricus* is a thermoacidophilic archaeon thriving at low pH and high temperatures (Brock et al., 1972; de Rosa et al., 1975) in hot springs which are potentially rich in diverse metals. The organism has developed effective mechanisms to withstand toxic concentrations of metals (Grogan, 1989; Villafane et al., 2009). In this study, we investigated the copper resistance of *S. solfataricus* whose genome exhibits only two P-type ATPases (She et al., 2001), both of which belong to the heavy metal translocating class IB. We particularly addressed questions regarding substrate specificity and relative contributions of both P-type ATPases to the metal resistance.

**Abbreviations:** HMA, heavy-metal-associated; TMPD, NN,N,N'-tetramethyl-p-phenylenediamine.

Five supplementary figures and a supplementary table are available with the online version of this paper.
**METHODS**

**Growth of bacteria.** *Escherichia coli* strain XL1-Blue was grown in LB medium and used for cloning of plasmids for disruption of *S. solfataricus* genes. Unless indicated otherwise, *S. solfataricus* PBL2025 (Schelert et al., 2004) and its mutants were grown aerobically at 80 °C and 110 r.p.m. in Brock’s medium (Brock et al., 1972). Strains are listed in Table 1.

For metal resistance tests, solid media were prepared using Brock’s medium and Gelrite gellan gum (Shungu et al., 1983) as gelling agent. Plates poured with 0.54 % (silver) or 0.66 % (copper) Gelrite provided even surfaces at pH 3 and temperatures of up to 80 °C and hence were preferred over agar plates for our experiments.

**Measurement of growth rates.** For measurement of growth rates, *S. solfataricus* and its mutant strains were grown in a copper-depleted medium, also referred to as standard medium. This consisted of a modified basal salt medium (Brock et al., 1972) containing 1 mM (NH₄)₂SO₄, 2 mM KH₂PO₄, 25 μM CaCl₂, 50 μM MgSO₄, 5 μM FeSO₄, 9.1 μM MnCl₂, 765 mM Na₂S, 119 mM VOSO₄, 42 mM CoCl₂, 22 mM Na₂B₄O₇, 248 mM Na₂MoO₄, 1 μM KI, 0.2 % (w/v) sucrose and 0.2 % (w/v) sterilized ion-depleted (see below) casein hydrolysate (Oxoid) in quartz-distilled water, adjusted to pH 3 with 50 % (w/v) H₂SO₄. A 10-fold concentrated ion-depleted stock solution was prepared by dissolving 20 g casein hydrolysate per litre and separating by chromatography on a Serdolit CHE (Serva) column. For standard growth experiments, 50 ml ion-depleted media was supplemented with the desired concentrations of CuSO₄ in 100 ml flasks were inoculated to OD₆₀₀ 0.04, using centrifuged and washed cells from a preculture grown in Brock’s medium.

**Determination of metal resistance of wild-type *S. solfataricus.***

The sublethal (=maximum tolerated) concentration of a specific metal was defined as the concentration still allowing 10 % survival of cells. It was measured by growth in standard medium with the respective metal ion added at defined concentrations. Cell survival was quantified by reading OD₆₀₀. Metal ion resistances were 10 μM CoCl₂, 12 μM NiSO₄, 1.4 mM CuSO₄, 2 mM CdSO₄, 1 μM Pb(NO₃)₂, 2 mM ZnSO₄, 10 μM AgNO₃ and 4 μM mercury acetate.

**Construction of plasmids.** For copA disruption, the upstream flanking region of Sso2651 was amplified using the primers 5’UFR-A_KpnI/3’UFR-A_NcoI; the downstream flanking region of Sso2651 was amplified using the primers 5’DFR-A_BamHI/3’DFR-A_NotI (Table S1, available with the online version of this paper). For copB disruption, the upstream flanking region of Sso2896 was amplified using the primers 5’UFR-B_KpnI/3’UFR-B_NcoI; the downstream flanking region of Sso2896 was amplified using the primers 5’DFR-B_BamHI/3’DFR-B_NotI. The obtained fragments (400–1000 bp) were cloned in plasmid pET2268 (Albers & Driessen, 2008), containing the lacS cassette flanked by upstream (NcoI and KpnI) and downstream (BamHI and NotI) fragment insertion sites. The plasmids used are listed in Table 1.

**Transformation of *S. solfataricus* and screening for recombinants with defective copA and copB genes.** Deletion mutants were obtained as described by Albers & Driessen (2008). In brief, linearized deletion plasmids were electroproporated in PBL2025. The transformants were selected in lactose minimal medium for 12–14 days. After reinoculation in lactose minimal medium, cells were streaked on 0.1 % tryptone/Gelite plates and incubated at 76 °C for 6 days. Colonies were sprayed with X-Gal solution as described previously (Jonuscheit et al., 2003) and blue colonies were inoculated in tryptone liquid medium. Successful deletion of the target genes was determined as described below.

**PCR analysis of deletion mutants.** Analysis of *S. solfataricus* ΔcopA and ΔcopB deletion mutants was performed by PCR using the primers 5’UFR-A/3’DFR-A or 5’UFR-B/3’DFR-A yielding a product only in case of successful insertion of lacS gene into the copA or copB loci. Mutants were double-checked using the primers 5’copA/3’lacS or 5’copB/3’lacS yielding a product only in the case of non-successful deletion of copA or copB loci.

**Isolation of genomic DNA.** Genomic DNA from *S. solfataricus* was isolated from 200 μl cultures by addition of 200 μl phenol/Tris and chloroform/isoamyl alcohol (24:1) and centrifugation. DNA was precipitated from the aqueous phase by 200 mM NaCl and 30 % (v/v) ethanol and dissolved in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0 (TE buffer).

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**Table 1.** Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<tr>
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<td>XL1 blue</td>
<td><em>endA1 gyrA96(nalR)</em> thi-1 recA1 relA1 lac gbnV44 F’::Tn10 proAB+ lacF Δ(lacZ)M15 hisR17lacY1 mX28</td>
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<td>pBAD His/MycA</td>
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<td>Invitrogen</td>
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<td><em>E. coli</em> copA</td>
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<td><em>bla lacS</em> cassette containing its own promoter and terminator region</td>
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<td>pET2268 ΔcopB</td>
<td><em>copB::lacS</em></td>
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**Membrane preparation.** Cells were disrupted by using a Branson sonicator for 5 min at level 7 and 50 % pulse length. Homogenates were centrifuged twice for 15 min at 5000 g. Membranes were pelleted from supernatants for 1 h at 150 000 g and suspended in TEN-buffer [TE buffer plus 110 mM NaCl and 10 % (w/v) glycerol]. Protein concentrations were determined as described by Smith et al. (1985).

**Immunoblotting.** Antisera to purified CopA of *S. solfataricus* and *E. coli* (unpublished data) were raised commercially (Seqlab). Rabbit antisera to CopB of *S. solfataricus* were produced by immunization with the recombinant fragment CopB-A or CopB-B (Deigweiler et al., 2004). CopB-A antisera exhibits cross-reactivity to CopA of *S. solfataricus* and hence was used to display comparative expression profiles of both ATPases.

**Cytochrome oxidase assay and redox-difference spectroscopy.** The specific oxidase activity was measured spectrophotically at 546 nm at room temperature in a buffer containing 20 mM 2-(N-morpholino)ethanesulfonic acid adjusted to pH 6.0 with KOH, 10 mM potassium sulfate, 1 mM EDTA [buffer A] and 0.1 mM of the artificial substrate *N*,*N*,*N*,*N*′-tetramethyl-p-phenylenediamine (TMPD). The reaction was started by addition of 10 μl cytoplasmic membrane fraction, and inhibited by 0.2 mM potassium cyanide. Reduced-minus-oxidized absorbance difference spectra in the visible region were recorded with a Jasco V-650 spectrophotometer at final protein concentrations of 1.5 mg ml⁻¹ in buffer A, supplemented with 0.1 % (w/v) β-β-dodecylmaltoside. Spectra of samples oxidized with 1 mM ferricyanide were subtracted from those obtained in the presence of 10 mM sodium dithionite.

**Cloning of copA and copB in *E. coli* expression vectors.** copA and copB were amplified from *S. solfataricus* genomic DNA using primer pairs 5′-copA_NcoI/copB_BamHII and 5′-copB_NcoI/3′-copA_BamHII (Table S1). *E. coli* copA was amplified from genomic DNA using primers 5′-EcoA_NcoI/3′-EcoA_BamHII. The resulting PCR products were cloned into pBADHis/MycA (Invitrogen) yielding plasmids pBAD-Sso_copA, pBAD-Sso_copB and pBAD-Eco_copA.

**Heterologous complementation of *E. coli* copA mutants.** Plasmids pBADHis/MycA, pBAD-Sso_copA, pBAD-Sso_copB and pBAD-Eco_copA were transformed into the copper-sensitive *E. coli* strain DC194 (Rensing et al., 2000), resulting in strains DC194-pBAD, DC194-Sso_copA, DC194-Sso_copB and DC194-Eco_copA. Starter cultures were grown overnight at 37 °C in LB medium containing ampicillin (100 μg ml⁻¹), chloramphenicol (20 μg ml⁻¹) and kanamycin (50 μg ml⁻¹). For the complementation experiments, cultures were diluted 1 : 1000 in the same medium supplemented with 0.0004 % l-arabinose and 3 mM CuSO₄. Growth was measured by recording OD₆₀₀ after approximately 12 h.

**Sequence alignment and phylogenetic tree.** Multiple sequence alignments were done using the computer program CLUSTAL W (Larkin et al., 2007). The PHYLIP suite (Felsenstein, 1989), version 3.69, was used to calculate and to graphically display the phylogenetic tree of PIB-ATPases from distance matrix analysis. Bootstrapping based on 1000 samples was used to determine the phylogenetic branching order.

**RESULTS AND DISCUSSION**

**Gene organization of copA and copB**

The structural gene encoding CopA (Sso2651) is in a typical operon structure together with a gene encoding the small copper binding protein CopT which bears the metal coordinating ligands within the so-called TRASH domain (Ettema et al., 2003) (Fig. 1). It is associated with a transcriptional unit containing the copper-responsive regulator gene copR (Villafane et al., 2011). Both copT and copA have been shown to be transcribed in response to increased copper concentrations (Ettema et al., 2006; Villafane et al., 2009). The copB gene (Sso2896) is organized in a different region (Fig. 1). Next to the translation initiation site of the presumed monocistronic copB, the two genes copY (encoding a regulator-type protein) and copZ [encoding a small copper chaperone of the heavy-metal-associated (HMA) group] are arranged in opposite orientation to copB. Notably, two pairs of insertion sequence elements are located at positions adjacent to the copYZ/copB cluster (Fig. 1).

**Assignment of CopA and CopB to the PIB-ATPase group**

In microbes, translocation of heavy metals across cytoplasmic membranes is primarily related to membrane-associated transport ATPases of the P-type (Solioz & Vulpe, 1996; Palmgren & Axelsen, 1998). The number of genes encoding these transporters in micro-organisms varies between one, as in *Sulfolobus acidocaldarius* (Chen

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**Fig. 1.** Organization of cop genes. copR and copY (red arrows) are putative regulator genes; copT and copZ (light blue arrows) encode small copper-binding proteins probably acting as copper chaperones; copA and copB (grey arrows) are structural genes for copper ATPases. Pairs of insertion sequence elements (IS elements; dark blue arrows) are flanking the genes related to the transcriptional units of copB. In the single mutant strains ΔcopA and ΔcopB, the respective genes are replaced by lacS (green arrows) of *S. solfataricus.*
et al., 2005), and nine, as in Haloarcula marismortui (Baliga et al., 2004). This variability could be a response to environmental challenges by diverse heavy metals such as copper, zinc, cobalt or cadmium. As mentioned above, the genome of Sulfolobus solfataricus (She et al., 2001) has two loci encoding P-type ATPases. Fig. S1 shows a sequence alignment of both Sulfolobus solfataricus ATPases, with the Pβ-ATPases from E. coli and Archaeoglobus fulgidus being well-characterized copper translocators. Sequence features clearly demonstrate the characteristic P-type domain arrangement in the order of A-, P- and N-domains. In addition, the N-terminal HMA-domains displaying a CXXC metal binding motif allow assignment of CopA and CopB of Sulfolobus solfataricus to subclass type IB according to Palmgren’s nomenclature (Axelsen & Palmgren, 1998). CopA exhibits a canonic CPC sequence motif within the putative hydrophobic region 6, and it is recognized as contributing to the transmembrane metal binding site. On the other hand, CopB holds the very rare sequence YPC at this position. According to a BLAST search, this feature prevails in only 18 out of more than 1000 known Pβ-type ATPases, indicating its unusually rare occurrence in metal transport.

Phylogenetic relationship of CopA and CopB

The relationships of the Sulfolobus solfataricus ATPases can be studied by comparing a larger series of sequences. Pairwise distances are calculated from the multiple sequence alignment, and a phylogenetic tree of the Pβ-ATPases was constructed by distance matrix analysis (Fig. 2, Fig. S2 and Table S2). The unrooted tree divides the ATPases in two major subgroups named CopA-1 and CopA-2/FixI according to a recent analysis (González-Guerrero et al., 2010). In another publication (Coombs & Barkay, 2005), the assignment to CopA-1 and CopA-2 is contrary to that outlined by González-Guerrero et al. (2010). The CopA-2 subgroup contains ATPases such as FixI that are described as being assembly factors of metalloproteins such as cytochrome oxidases (Kahn et al., 1989; Preisig et al., 1996; Koch et al., 2000; Hassani et al., 2010). Recent investigations have demonstrated that CopA-2 of Pseudomonas aeruginosa is an ATP-driven copper export system operating at low rates (González-Guerrero et al., 2010). The CopA-2 lineage and other branches are not shown in detail in Fig. 2, because both ATPases of Sulfolobus solfataricus belong to the other subgroup CopA-1, which comprises ATPases in charge of copper homeostasis. CopA-1-type ATPases are assigned as resistance factors exporting copper at high rates (Argiello et al., 2007; Osman & Cavet, 2008), whereas a few ATPases have been previously considered to be copper importers due to their phenotypic characteristics (Odermatt et al., 1993; Tottey et al., 2001; Lewison et al., 2009). These apparently conflicting views have been lately reconciled by the statement that “all Cu⁺-ATPases drive cytoplasmic Cu⁺-efflux, albeit at quite different transport rates” (Raimunda et al., 2011).

Although the tree topology is only weakly supported by low bootstrapping values at the deep branching points, the Sulfolobus solfataricus ATPases (Fig. 2, shaded in purple) are grouped separately from the proven export ATPases such as Archaeoglobus CopA, Enterococcus hirae CopB or E. coli CopA. Another cluster, the YPC-group (shaded in pink), splits off from the same precursor as the Sulfolobus group in a stable branching order. Members of the latter family carry the unusual YPC instead of the CPX signature in transmembrane helix 6. All CopA-ATPases of the Sulfolobales hold the classical CPC motif, whereas CopB of Sulfolobus solfataricus and of another strain of Sulfolobus islandicus have YPC instead (hatched shading). Three lines of evidence let us speculate about CopB-ATPases being taken up preferably from an organism belonging to the parallel YPC-group by lateral gene transfer: (i) these genes occur exclusively in these two strains but not in the other Sulfolobus species; (ii) these genes are uniquely embedded into the divergently oriented gene cluster flanked by insertion sequence elements (Fig. 1); and (iii) the copB gene has a low GC-content (30.7 %) compared with the copA gene (37.7 %) and the GC-content of the entire Sulfolobus solfataricus genome (35.8 %). One reason for the assumed gene transfer could be an unknown biological function of CopB which might be related to the unusual YPC sequence pattern. On the other hand, the copB cassette could be an evolutionary remnant of a presently needless function that is barely retained due to a slow evolutionary clock.

Construction of copA and copB deletion mutants

For independent disruption of copA and copB, their coding DNA sequences have been entirely exchanged for the endogenous lacS gene encoding β-galactosidase in the lacS⁻ strain Sulfolobus solfataricus PBL2025 via allelic replacement (Albers & Driessen, 2008). Mutants carrying a disruption in either copA or copB were identified on Gelrite plates after incubation at 76 °C by blue/white screening based on the X-Gal hydrolysis activity of the incorporated lacS gene product. The correct genotypes of these strains were verified by PCR and sequencing of isolated genomic DNA (Fig. S3). In addition to the single mutants ΔcopA and ΔcopB, the ΔcopA/ΔcopB double mutant would be highly desirable for our studies. However, its construction is difficult to perform at present with respect to the limited availability of screening or selection markers working in Sulfolobus solfataricus.

Phenotypes of copA and copB deletion mutants by growth analysis

Based on the sequence similarity of CopA and CopB to the other members of the Pβ-ATPase family, we predicted that both are copper-specific transporters. Initial growth experiments had been carried out using Gelrite plates with copper sulfate- or silver nitrate-soaked filters. With copper, no inhibition (wild-type) or a small, diffuse zone of inhibition (ΔcopB mutant) was visible, indicating relatively high resistance (Fig. 3e). In contrast, no significant growth
was observed for the ΔcopA mutant, demonstrating pronounced copper sensitivity. In the case of silver (Fig. 3f), both ΔcopA and ΔcopB mutants produced broad cell-free surroundings suggesting that CopA and CopB contribute to silver detoxification at comparable extents.

However, due to the insolubility of silver chloride and lack of a fully synthetic culture medium for *S. solfataricus*, our quantification of metal resistance in liquid media was limited to copper. For this purpose, we used depleted media containing residual copper at a concentration of

*Fig. 2.* Unrooted phylogenetic tree of type PIB-ATPases showing the subgroup of CopA-1-ATPases involved in copper homeostasis. Sequences of other PIB-ATPases, including the subgroup of CopA-2-ATPases involved in the biosynthesis of metal proteins are cut away for clarity (for a more comprehensive graph and further details, see Fig. S2 and Table S2). The tree was calculated and displayed by using CLUSTAL W and the PHYLIP suite; intermediate horizontal branch lengths assign sequence diversity, and the stability of tree topology is denoted by bootstrapping values (ranging from 0 to 100). Archaeal and eubacterial ATPases, which have the unusual YPC sequence pattern instead of the most frequently occurring CPC pattern in the putative transmembrane helix 6, form a cluster (shaded in pink). The PIB-ATPases of different *Sulfolobus* species form another cluster (shaded in purple) related to it. Herein, solely the CopB ATPases of *S. solfataricus* and of *S. islandicus* carry the YPC pattern (hatched shading).
substantially lower than 1 µM, as determined by a chemical assay (Brenner & Harris, 1995) (note that a fivefold concentrated sample of our depleted medium was still below the reported detection limit of 1 µM Cu⁺⁺ of this method), to which CuSO₄ was added at defined concentrations. Fig. 3(a and b) show the growth curves of wild-type, ΔcopA and ΔcopB mutants. Wild-type cells reach stationary phase after about 45 h in 2 µM CuSO₄ (Fig. 3a) or 1400 µM CuSO₄ (Fig. 3b). The ΔcopA mutant showed dramatically reduced growth at 2 µM (Fig. 3a), whereas for the ΔcopB deletion strain, 1400 µM CuSO₄ is needed to slow the onset and beginning of stationary phase at lower levels (Fig. 3b). The high toxicity of copper to the ΔcopA mutant is obvious by the I₅₀ (inhibitor concentration at which growth, measured by final OD, was reduced to 50% of that of the uninhibited culture) of less than 2 µM (Fig. 3c). These data are clearly compatible with the notion that CopA is an export ATPase with high affinity to the copper

Fig. 3. Sensitivity of *S. solfataricus* wild-type, ΔcopA and ΔcopB mutants to copper and silver. (a, b) Time courses of cell growth measured in copper-depleted medium, supplemented with CuSO₄ at the indicated concentrations, by measurement of OD₆₀₀ against uninoculated medium. The respective control curves without added Cu are shown in Fig. S5. ○, 2 µM CuSO₄, ΔcopA (a) or 1400 µM CuSO₄, ΔcopB (b); ■, wild-type. (c, d) Toxicity of copper, shown in cells exposed to various concentrations of CuSO₄, measured by the final OD₆₀₀ reached after 48 h of growth. ○, ΔcopA (c) or ΔcopB (d); ■, wild-type. (e, f) Growth of *S. solfataricus* on solid media in the presence of metal ions. Gelrite plates with 20 mM CuSO₄-soaked (e) or 6 mM AgNO₃-soaked (f) filters (dark circles). WT, Wild-type.
ion and functions as a copper and silver resistance factor. CopB, although being strongly involved in silver resistance, has a less evident role in regard to copper. The latter has to be added at very high concentrations to elicit the ΔcopB phenotype, showing a 700-fold higher copper resistance than ΔcopA (Fig. 3d). Moreover, the ΔcopB mutant (I50~1400 μM) was slightly, but reproducibly, more sensitive to copper when compared with the wild-type (I50~1600 μM). This suggests that CopB can be assigned as an export pump, which becomes relevant at high copper concentrations.

Expression of copA and copB

The expression level of integral membrane proteins CopA and CopB in *S. solfataricus* can be semiquantitatively demonstrated by immunoblots of cytoplasmic membranes developed with polyclonal antisera. We used heterologously expressed CopA and CopB as molecular size markers (Fig. 4a, lanes 8 and 9). As expected, CopB is lacking in the ΔcopB mutant (Fig. 4a, lane 6), but is present in wild-type and ΔcopA mutant membranes of cells grown in copper-deficient medium (Fig. 4a, lanes 2 and 4). The CopB levels were significantly increased in the presence of copper (Fig. 4a, lanes 3 and 5). It is worth noting that 0.5 μM CuSO₄ already suffices to induce CopB accumulation (Fig. 4a, lane 5). CopA was undetectable in copper-depleted medium (Fig. 4a, lanes 2 and 6) because of its weak immunological cross-reactivity at low concentrations. However, its accumulation was sufficient to show up in blots after stimulation of wild-type and ΔcopB mutant cells with copper (Fig. 4a, lanes 3 and 7). Copper induction of CopA biosynthesis is well supported by transcriptional data obtained for the wild-type (Ettema et al., 2006). Increased expression in the presence of silver could only be demonstrated for CopB (Fig. 4b, lanes 3 and 5). Combined, these data underline the classification of CopA as a copper and silver export ATPase and support the notion that tolerance of *S. solfataricus* to these metals is controlled by both P₁β-ATPases.

To further clarify the role of CopB, membranes of wild-type cells exposed to various transition metals at sublethal concentrations have been analysed by immunoblotting. Compared with cells grown in standard (i.e. copper-depleted) growth medium, the band intensities in Fig. 4(c) showed a clear increase of CopB levels in the presence of copper, but the other tested metals had no effect. It is thus probable that external copper specifically induces the build-up of CopB-ATPase.

Lack of heterologous functional complementation of an *E. coli* copA mutant

In order to prove the biological functions of the *S. solfataricus* ATPases, the cloned copA and copB genes were expressed in the *E. coli* strain DC194 (Rensing et al., 2000) which is deficient in the P₁β-type copper export ATPase copA. Growth levels of the transformed *E. coli* strains in the presence of sublethal copper concentrations are shown in Fig. 5. Strain DC194 transformed by a complementation plasmid containing *E. coli* copA served as reference and restored growth at 3 mM CuSO₄. Cells transformed with vectors carrying the *S. solfataricus* genes copA and copB yielded reproducible, but only slightly better growth than the (mock-transformed) control, which at best indicated a partial complementation. The increase in temperature resulted in a somewhat higher growth of the copA- and copB-transformed strains (Fig. 5). The absent complementation at 37 °C is not due to deficient CopA and CopB expression, as proven by immunostaining (Fig. 5, insert). In contrast with many P₁β-ATPases (Rensing et al., 2000; Lewinson et al., 2009), both *S. solfataricus* ATPases were incapable of compensating for the copper transport defect of the *E. coli* copA mutant. The small increase in growth rate at elevated temperatures may indicate weakly developing complementation. The lack of full restoration may be explained by reduced catalytic activities of the thermostable proteins at the relatively low-temperature living conditions of *E. coli*. However, this does not hold for all thermophiles, because the P₁β-ATPase of *Pyrococcus furiosus* has been reported to achieve functional complementation at 37 °C (Lewinson et al., 2009). Although *Pyrococcus* can grow at 100 °C (Fiala & Stetter, 1986), the different natural di- and tetra-ether compositions of *S. solfataricus* and *Pyrococcus* membranes may prescribe specific lipid requests for CopA and CopB to attain functional complementation in *E. coli* membranes.

Sensitivity of cop mutants towards reactive oxygen species (ROS)

It has been reported that *copA-2* mutants of *P. aeruginosa* are much more sensitive to ROS than wild-type or *copA-1* mutants (González-Guerrero et al., 2010). In order to assign a possible role for the CopA and CopB ATPases, we investigated the ROS sensitivity of *S. solfataricus*. We used Paraquat to generate ROS in our experiments. It is evident by the growth characteristics in Fig. S4 that *S. solfataricus* is highly sensitive to low concentrations of this compound, giving rise to 50 % survival at about 750 nM. This notable sensitivity is caused by its chemical reactivity leading to increased ROS formation at high growth temperatures. However, the ROS sensitivity of both ΔcopA and ΔcopB mutants is virtually imperceptible from the *S. solfataricus* wild-type strain. In contrast, wild-type *P. aeruginosa* and ΔcopA-1 strains exhibit significantly higher resistance to ROS than the *copA-2* mutants (González-Guerrero et al., 2010). It may be speculated that the high sensitivity to ROS is related to impaired biosynthesis of detoxifying Cu–Zn superoxide dismutase, which is located in the periplasm of Gram-negative bacteria (Kroll et al., 1995). However, enzymes of the Cu–Zn class did not appear to be involved in ROS metabolism of *S. solfataricus*, whereas an Fe superoxide dismutase has been isolated from culture fluids (Cannio et al., 2000).
Fig. 4. (a, b) Western blots of cytoplasmic membranes (100 μg each) from *S. solfataricus* cells, developed with polyclonal antiserum raised to the actuator domain of CopB (named CopB-A), which is cross-reactive with CopA. (a) Accumulation of CopA or CopB in wild-type and Δcop mutants in the presence of copper. (b) CopB accumulation in the presence of AgNO₃. The strain and amount of CuSO₄ or AgNO₃ added to each sample are indicated above each lane. Lanes: 1, molecular size marker; 8, CopA size marker; 9, CopB size marker. (c) CopB accumulation in wild-type *S. solfataricus* triggered by the presence of various metal ions during growth, shown by Western blotting with antiserum against the CopB-B domain, which does not cross-react with CopA. Membranes were isolated from cells grown in standard medium (lane 2) or in the presence of metal concentrations that have been previously determined to be sublethal to *S. solfataricus* (see Methods), namely 1 mM Cu²⁺ (lane 3), 10 μM Ni²⁺ (4), 8 μM Co²⁺ (5), 0.8 mM Pb²⁺ (6), 1.5 mM Cd²⁺ (7), 1.5 mM Zn²⁺ (8), 3 μM Hg²⁺ (9). Molecular size markers (lane 1) and purified CopB (lane 10) were added as references.
Probing the role of CopA and CopB in the biosynthesis of cytochrome oxidase

As mentioned above, ATPases of the CopA-2 subclass have been demonstrated to be involved in the biosynthesis of metal proteins by copper export (González-Guerrero et al., 2010). In order to probe the S. solfataricus CopA- and CopB-ATPases with respect to haem-copper cytochrome oxidase production, membranes of all strains have been subjected to cytochrome analysis by reduced-minus-oxidized absorbance difference spectroscopy (Fig. 6). In copper-depleted medium, wild-type cells produced the typical cytochrome profile of \(\alpha\)-bands observed in the closely related S. acidocaldarius strain, at low but sufficient levels to sustain normal aerobic growth rates. It consists of the complex III analogon apocytochrome \(b\) bound to haem \(A_S\) which absorbs at 585 nm (SoxC and SoxG), and of the two haem-copper terminal oxidases of the SoxM-type cytochrome \(bb_3\) and the SoxB-type \(aa_3\), which absorb at 562 and 604 nm (Lübben et al., 1992, 1994; Komorowski et al., 2002). Interestingly, both \(\Delta copA\) and \(\Delta copB\) mutant membrane spectra displayed higher oxidase peaks than the wild-type, particularly pronounced by the increase of cytochrome \(aa_3\) at 604 nm (Fig. 6). Membranes of the wild-type and \(\Delta copB\) mutant from cells supplemented with 400 \(\mu\)M CuSO\(_4\) were analysed as a control, demonstrating the potential of cytochrome synthesis under conditions of copper repletion. Whereas the terminal cytochrome oxidase levels at 562 and 604 nm were only slightly enhanced compared with the copper-limiting case, the apocytochrome/haem \(A_S\) peak at 585 nm was strongly raised.

The \(bb_3\) - and \(aa_3\)-type cytochrome oxidase activities of membranes, measured by the fully cyanide-sensitive electron transfer from the pseudo-substrate TMPD (Lübben et al., 1994; Gleissner et al., 1997) further supported the above-mentioned spectroscopic observations. As shown in Table 2,
the cytochrome oxidase activities of the ΔcopA and ΔcopB mutant membranes are higher than the wild-type, but significantly smaller than after copper repletion. In relation to the bb3- and aa3-type cytochrome levels, our catalytic data suggest that several copper-involving steps are required to attain full oxidase activity which are rather speculative.

Our observations demonstrated that it is quite unlikely that any of the Pib-ATPases play an essential role in cytochrome oxidase biosynthesis, because loss-of-function phenotypes lacking cytochrome oxidase would be expected from disruption of either copA or copB genes. The copA and copB double mutant would of course fully prove this statement. Nevertheless, both single cop disruptions led to higher cytochrome oxidase levels at limiting copper concentration. Although the mechanism of CuA or CuB incorporation in S. solfataricus is unknown, our data underline the importance of internal copper to this process. Deletion of either copper-ATPase would reduce the export activity and in turn raise the cytoplasmic level of copper. Higher internal concentrations could increase the copper loading of assembly factors that may act from the outside, as postulated for the membrane-anchored mitochondrial proteins Sco1/Sco2 and Cox11 (Horng et al., 2004; Carr et al., 2005; Banci et al., 2011). Consistent with this interpretation is the increase in cytochrome levels observed in S. solfataricus after addition of CuSO4, because external copper is similarly assumed to result in higher internal levels. Alternatively, the putative assembly factors may also be copper-loaded directly from the outside.

Taken together, the increase in cytochrome oxidase absorbance and activity in the absence of either copper-translocating ATPase strongly discredits any crucial role in oxidase assembly by delivering copper to the active site in S. solfataricus.

**Conclusion**

The ATPases CopA and CopB of S. solfataricus both belong to the CopA-1 group of Pib-ATPases. They are synthesized at low levels under standard growth conditions, i.e. under copper limitation, and the expression levels increase after addition of copper. Both enzymes impart copper and silver tolerance. CopA is a very efficient copper export pump, because a disruption in its encoding gene led to severely increased copper sensitivity. The biological role of CopB is less clear. Its gene may have been acquired by lateral gene transfer, as with only one known exception its gene does not exist in other relatives of S. solfataricus. With respect to copper, the phenotype of ΔcopB differed slightly from the wild-type. Apparently, like a booster module, CopB supports the cellular resistance machinery by expanding the copper tolerance range by about 0.2 mM. In the search for a possible alternative role of CopB, for example as a copper-exporting assembly factor, we could rule out its direct involvement in the biosynthesis of Cu-proteins. The copper/silver ATPase CopB may either have a physiological assignment yet to be discovered or be a leftover from an earlier function lost during evolution.

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