Copper resistance and its regulation in the sulfate reducing bacterium *Desulfosporosinus* sp. OT

Stefano Mancini¹*, Ranjeet Kumar²*, Helge K. Abicht¹, Elisabeth Fischermeier¹ and Marc Solioz¹,²

¹Department Clinical Research, University of Bern, 3010 Bern, Switzerland
²Laboratory of Biochemistry and Molecular Biology, Tomsk State University, 634050 Tomsk, Russian Federation

*These authors contributed equally to the work

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Correspondence
Marc Solioz
marc@solioz-scientific.ch

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Abbreviations: MBD, metal binding domain; Ni-NTA, nickel-nitrilotriacetic acid; EMSA, electrophoretic mobility shift assay; IPTG, isopropyl-β-D-thiogalactoside; ROS, reactive oxygen species; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(N-morpholino)propanesulfonic acid, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodiumdodecyl sulfate; rTEV, recombinant tobacco etch virus; Ec_CopA, *Escherichia coli* CopA; Eh_CopA, *Enterococcus hirae* CopA; DOT_CopA, *Desulfosporosinus* sp. OT CopA; DOT_CopA2, *Desulfosporosinus* sp. OT CopA2.
Desulfosporosinus sp. OT is a Gram-positive, acidophilic sulfate-reducing Firmicute isolated from copper tailings sediment in the Norilsk mining-smelting area in Siberia and represents the first Desulfosporosinus species whose genome has been sequenced. Desulfosporosinus sp. OT is exceptionally copper resistant, which made it of interest to study the resistance mechanism. It possesses a copUAZ operon which is here shown to be involved in copper resistance. The copU gene encodes a CsoR-type homotetrameric repressor. By electrophoretic mobility shift assay, it was shown that CopU binds to the operator/promoter region of the copUAZ operon in the absence of copper, and is released from the DNA by Cu⁺ or Ag⁺, implying that CopU regulates the operon in a copper/silver-dependent manner.

DOT_CopA is a P1B-type ATPase related to other characterized, bacterial copper ATPases. When expressed in a copper-sensitive Escherichia coli ∆copA mutant, it restores copper resistance to wild-type levels. His-tagged DOT_CopA was expressed from a plasmid in E. coli and purified by Ni-NTA affinity chromatography. The purified enzyme is most active in the presence of Cu(I) and bacterial phospholipids. These findings indicate that the copUAZ operon confers copper resistance to Desulfosporosinus sp. OT, but do not per se explain the basis of the high copper resistance of this strain.

INTRODUCTION
Copper is a cofactor in many bacterial enzymes such as cytochrome aa₃-type terminal oxidases, Cu,Zn-superoxide dismutase required for defense against oxidative stress, plastocyanins and azurins which act as electron carriers, and periplasmic multicopper oxidases which can oxidize Cu⁺ to less toxic Cu²⁺ (Grass et al., 2001; Solioz et al., 2010; Abicht et al., 2013). It is currently believed that copper-loading of all these proteins takes place in the periplasmic space and that there is no requirement for cytoplasmic copper in bacteria, with the exception of photosynthetic organisms (Raimunda et al., 2011). While this concept still awaits more extensive scientific proof, it is clear that excess cytoplasmic copper is toxic and all bacteria are endowed with one or several copper export mechanisms as well as various cytoplasmic copper sequestration and detoxification schemes (Dupont et al., 2011; Bondarczuk and Piotrowska-Seget, 2013; Osman and Cavet, 2008).

The ability of copper to catalyze the formation of reactive oxygen species (ROS) via Fenton-type chemistry has frequently been stated to be the primary toxicity mechanism of copper. However, recent work suggests that in vivo copper toxicity is primarily due to the displacement of iron from iron-sulfur clusters, leading to the inactivation of essential enzymes (Macomber and Imlay, 2009; Macomber et al., 2007; Chillappagari et al., 2010; Azzouzi et al., 2013; Fung et al., 2013; Foster et al., 2014).
The key components of copper homeostasis in all bacteria are Cu\(^{2+}\)-ATPases, usually termed CopA, CopB, or CupA. They belong to the heavy metal-transporting P1B-type ATPases subgroup of the P-type ATPases superfamily (Lutsenko and Kaplan, 1995). Their characteristics are one to six CxxC metal binding domains at their N-termini, and conserved domains, ATP-binding domains, and intramembranous CPC or CPH motifs involved in copper transport. The X-ray crystal structure has so far only been solved for CopA of *Legionella pneumophila* (Gourdon *et al.*, 2011). It is now clear that CopA-type ATPases remove excess Cu\(^{2+}\) from the cytoplasm by pumping it across the cytoplasmic membrane.

We recently announced the draft genome of a Gram-positive, sulfate-reducing bacterium, *Desulfosporosinus* sp. OT (Abicht *et al.*, 2011). *Desulfosporosinus* bacteria were identified as key players in microbial sulfate reduction in a low-sulfate peatland. Sulfate reduction contributes to precipitation of metal sulfides and thereby to the immobilization of toxic metals (White *et al.*, 1997). In culture, *Desulfosporosinus* sp. OT exhibits unusually high copper tolerance, being able to grow in 236 mM copper under sulfate reducing conditions (Abicht *et al.*, 2011).

Copper homeostasis has been well characterized in two other Gram-positive organisms, namely *Lactococcus lactis* and *Enterococcus hirae* (Solioz *et al.*, 2010). In *L. lactis*, the core element of copper resistance is the *copRZA* operon, which encodes the CopR copper-inducible repressor, the CopZ copper chaperone and the CopA copper export ATPase (Magnani *et al.*, 2008). Copper activates the operon by inducing the release of the CopR repressor from the *copRZA* operator-promoter (Portmann *et al.*, 2006). A second putative copper ATPase on a monocistronic operon, CopB, is also under the control of CopR; however, its role remains unclear (Magnani *et al.*, 2008). Likewise, the copper resistance determinant of *E. hirae* is the *copYZAB* operon. It is under the control of the copper-inducible repressor CopY. Of the two ATPases, CopA and CopB, only CopB appears to contribute to copper resistance. CopA, like CopB of *L. lactis*, has recently been proposed to have a role in the supply of copper to the periplasm for copper-loading of enzymes (Raimunda *et al.*, 2011).

The genome of *Desulfosporosinus* sp. OT encodes two putative copper exporting ATPases, CopA and CopA2. In this work, the determinant of copper resistance was shown to be the *copUAZ* operon, encoding a copper-responsive inducer, CupU, which regulates the operon, a copper-exporting ATPase, CopA, and a putative cytoplasmic copper chaperone, CopZ. No role in copper resistance could be defined for CopA2.
METHODS

Reagents and chemicals. All reagents were of analytical grade and were obtained from Sigma-Aldrich if not indicated differently. Ni-NTA affinity resins were from Qiagen and asolectin from Associated Concentrates, Woodside, NY. *E. coli* and *E. hirae* lipids were isolated as previously described (Ames, 1968).

Strains and culture conditions. *Desulfov sporosinus* sp. OT was grown as previously described (Karnachuk et al., 2005) and DNA was isolated by alkaline lysis as described elsewhere (Ausubel et al., 1995). *E. coli* W3110 and W3110Δ*copA* were kindly provided by Christopher Rensing, University of Copenhagen. All *E. coli* strains were grown aerobically in LB media at 37 °C, unless indicated differently.

Vector construction. The *E. coli* CopA gene (*Ec_copA*) was cloned by PCR amplification from *E. coli* W3110 genomic DNA with primers ha95 and ha96 (Table S1). The resulting PCR product was cloned into PCR Blunt II TOPO (Invitrogen, California, USA), yielding pCA6. The *Ec_copA* gene was excised from pCA6 with NarI and XbaI and subcloned into pProExHTa, digested with the same enzymes. The resulting construct, pCB1, encoded *Ec_CopA* with an N-terminal His-tag that could be cleaved with recombinant tobacco etch virus (rTEV) protease. *Desulfosporosinus* sp. OT *copU* (NCBI accession: AGAF01000118) was cloned by PCR amplification with *Pfu* DNA polymerase using primers U1 and U2. The PCR product was cloned into pCR-Blunt II-TOPO, generating pOT07, from where the *copU* gene was excised with NarI and XbaI and subcloned into pProExHTa, digested with the same enzymes. The resulting construct, pOU02, encoded CopU with an rTEV-cleavable, N-terminal 6-His-tag. *DOT_copA* (NCBI accession number: EGW37486) was similarly cloned using PCR primers ha91 and ha92 for cloning into PCR Blunt II TOPO, yielding pOT5, followed by subcloning into pProExHTa. The resulting construct, pOU1, encoded *DOT_CopA* with an rTEV-cleavable, N-terminal 6-His-tag. *DOT_copA2* (NCBI accession: AGAF01000248) was cloned by the same strategy, using PCR primers A21 and A22 to generate pOT91, from which the gene was subcloned into pProExHTa to generate pOU03, encoding DOT_CopA2 with an rTEV-cleavable, N-terminal 6-His-tag. The absence of mutations was verified in all the constructs by commercial DNA sequencing.

Purification of CopU. BL21(DE3)RIL with plasmid pOU02 was grown aerobically at 37 °C in 300 ml of LB containing 50 µg/ml of ampicillin to an OD at 600 nm of 0.8. The culture was then induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated for additional 16 h at 20 °C. The cells were harvested at 7000 x g for 10 min and the resultant pellet was stored at −70 °C until further use. His-tagged CopU was purified by re-suspending
pelleted cells in 2 ml per g of wet weight in lysis buffer (50 mM Na-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5), containing 1 mM phenylmethylsulfonyl fluoride (PMSF), added from 100 mM stock in dimethylsulfoxide. Cells were disrupted by two passages through a French press at 30 MPa. Cell debris was removed by centrifugation at 15,000 x g for 30 min. The supernatant obtained was applied to a Ni-nitrilotriacetic acid (Ni-NTA) column equilibrated with lysis buffer. The column was washed with 5 column volumes each of lysis buffer, lysis buffer with 25 mM imidazole, and lysis buffer with 50 mM imidazole. CopU was eluted with lysis buffer containing 200 mM imidazole. Eluted fractions were analyzed in 15% sodiumdodecyl sulfate (SDS) polyacrylamide gels (Laemmli, 1970).

CopU-containing fractions were pooled and dialyzed against 2 x 100 volumes of 20 mM Na-HEPES pH 7.5, 1 mM dithiothreitol, for 2 h each. The His-tag of CopU was removed by cleavage with 1/10 the amount of rTEV protease (purified in-house) overnight at room temperature in the same buffer, followed by passage through a Ni-NTA column to remove uncleaved His-CopU and the His-tagged rTEV protease. The resulting 115 amino acid protein with a predicted molecular weight of 13 kDa exhibited high purity (> 95%), as determined by SDS polyacrylamide gel electrophoresis (Fig. S1). Purified CopU was incubated with 1/20 volume of Chelex 100 (Bio-Rad, Berkeley, USA) for 2 h at 4 °C to remove metal ions, followed by dialysis against 2 x 100 volumes of 20 mM Na-HEPES pH, 1mM dithiothreitol, for 2 h each at 4 °C. The protein concentration was determined by the method of Bradford, using bovine serum albumin as a standard (Bradford, 1976).

Crosslinking of CopU. Purified CopU (5 µg) was suspended in 50 mM Na-HEPES pH 7.5 and crosslinked for 5 to 20 min with 0.2% glutaraldehyde in a total reaction volume of 25 µl. Reactions were stopped by the addition of 5 µl of 1 M Tris-Cl pH 8 and crosslinking was evaluated by electrophoresis on a 10% SDS-polyacrylamide gel, followed by staining with Coomassie blue.

Electrophoretic mobility shift assays (EMSA). Purified CopU and DNA were incubated at molar ratios of 240:1 and incubated for 1 h at room temperature in a total volume of 20 µl of binding buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA, 100 mM KCl, 200 µM Mg-acetate, 100 µM dithiothreitol, 1 µg bovine serum albumin, 5% glycerol). Samples were separated on 10% polyacrylamide gels prepared in a 1:1 mixture of binding buffer and 40 mM Tris-acetate pH 8, 1 mM EDTA. Following electrophoresis, gels were stained for 60 min with RedSafe (Sigma-Aldrich, St. Louis, MO, USA), diluted 1:20'000 in 40 mM Tris-acetate pH 8, and photographed under blue LED illumination with a Bio-Rad ChemiDoc imaging system. Primers ms111/ms112 and ms115/ms116 were pairwise annealed at 65 °C for 10 min for
EMSA. PCR products were amplified with primer pairs sm144/ms110 and ms109/sm143 (Table S1) from Desulfosporosinus sp. OT DNA with Pfu DNA polymerase under standard conditions. Identical EMSA results were obtained with His-tagged CopU and CopU without the His-tag.

**Purification of DOT_CopA and DOT_CopA2.** The same procedure was used both, for DOT_CopA and DOT_CopA. E. coli BL21(DE3)RIL transformed with either plasmid pOU1 or pOU3 was grown aerobically in 1 l (2 x 0.5 l in baffled 1 l Erlenmeyer flasks) of 1% Trypticase-peptone, 1% yeast extract, 0.5% NaCl, at 37 °C to an OD at 600 nm of 0.5–0.7. Expression was then induced with 0.1 mM IPTG and the cultures grown for an additional 4 h.

Cells were collected by centrifugation for 10 min at 8000 x g at room temperature and washed with 250 ml of 0.9% NaCl. They were finally resuspended in 10 ml per g of wet pellet of G-buffer (20 mM Tris-SO₄ pH 7.5, 5 mM MgSO₄, 25 mM Na₂SO₄, 25 mM K₂SO₄, 1 mM β-mercaptoethanol, 20% (v/v) glycerol, 5 μg/ml DNaseI, 1 mM PMSF) and passed through a French press twice at 30 MPa. Unbroken cells were removed by centrifugation for 15 min at 12,000 x g and the supernatant was centrifuged for 45 min at 90,000 x g. The resultant membrane pellet was resuspended in 2.5 ml of G-buffer and stored frozen at -70°C. For ATPase purification, the membranes were suspended in G-buffer at a protein concentration of 24 mg/ml, supplemented with 1/100 volume of a protease inhibitor cocktail (100 mM N-α-p-tosyl-L-lysine-chloromethylketone, 100 mM N-p-tosyl-L-phenylalanine-chloromethylketone, 100 mM p-aminobenzamidine, 100 mM phenylmethylsulfonyl fluoride dissolved in dimethylsulfoxide), and extracted with dodecyl-β-D-maltoside at a detergent/protein ratio of 8 (w/w) with stirring on ice for 1 h. Undissolved membranes were sedimented for 45 min at 90,000 x g and the supernatant loaded onto a 1 ml Ni-NTA-agarose column, pre-equilibrated with buffer JD (20 mM Tris-SO₄ pH 7.5, 5 mM MgSO₄, 1 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.05% dodecyl-β-D-maltoside). Weakly bound proteins were washed from the column with buffer JD buffer containing 10 mM of imidazole. Elution of the ATPase was accomplished with a 30 ml linear gradient of 10–250 mM imidazole in buffer JD. The eluate was desalted on a PD10 column (GE Healthcare Life Sciences) equilibrated with buffer JD. Cleavage of the 6 His-tag was accomplished as described above for CopU. Purified ATPase was stored at -70 °C. The yield was typically 1 mg of ATPase.

**Complementation of E. coli.** The E. coli W3110ΔcopA strain was transformed with either the empty vector or constructs pCB01, pOU1 or pOU3, expressing Ec_CopA, DOT_CopA, or DOT_CopA2, respectively. Cultures were grown in LB media containing 50 μg/ml of ampicillin and no copper, 1.5 or 3 mM CuSO₄ and the optical density was determined at 600
nm after 48 h of aerobic growth at 37 °C. No IPTG was added to the cultures, since it had a strong inhibitory effect on growth, presumably due to excessive expression of the ATPases compared to wild-type levels. For complementation studies on solid media, 100 µl of exponential-phase cultures were suspended in 2.5 ml of 0.7% agar and poured on LB plates. Filter disks containing 5 µl of 1 M CuSO₄ were deposited on the plates and after 24 h of incubation at 37 °C, the plates were photographed with a digital camera.

**ATPase activity measurements.** Enzyme activity was measured at 37 °C in a volume of 1 ml 40 mM Na-3-(N-morpholino)propanesulfonic acid (MOPS), pH 6.5, 150 mM NaCl, 20 mM NH₄SO₄, 20 mM L-cysteine, 5 mM KCl, 5 mM MgCl₂, 2 mg/ml dodecylmaltoside, 100 µM CuSO₄, and supplemented with different lipids as indicated under Results. The reaction was started by the addition of 1 mM Na-ATP and was followed by measuring Pi-release by the method of Lanzetta et al. (Lanzetta et al., 1979).

**RESULTS**

**Structure of the copUAZ operon**

*Desulfosporosinus* sp. OT contains a predicted *copUAZ* operon, encoding a copper-responsive repressor, CopU, a P1B-type ATPase, DOT_CopA, and a CopZ copper chaperone (Fig. 1a). Downstream of *copZ* are two more open reading frames, *orf1* and *orf2*, encoding proteins of unknown function. Whether *orf1* and *orf2* are part of the *cop*-operon currently remains unclear and the operon will henceforth only be referred to as the *copUAZ* operon. The *copUAZ-orf1-orf2* gene cluster is braced by terminators with predicted stabilities of -11.4 and -16.9 kcal. Upstream of *copU* are predicted -10 and -35 regions, and a dyad symmetry with sequence TATAGTATA(N₆)TATACTATA, which could represent the CopU binding site (Fig. 1b). Also shown in this Figure are the oligonucleotides used to test CopU-DNA interaction.

**Primary and quaternary structure of CopU.** The predicted gene product of *copU* exhibits extensive sequence similarity to characterized CsoR-type repressors (Fig. 2). This type of repressor has been characterized in great detail in recent years and a number of structures have been solved (Higgins and Giedroc, 2014). They reveal a flat, disc-like homotetrameric structure; two such tetramers form a "sandwich" complex with the operator DNA region in the absence of copper to suppress transcription (Chang et al., 2015). In CsoR of *Mycobacterium tuberculosis*, Cu(I) binds to C36 on one subunit and H61 and C65 on the adjacent subunit (Liu et al., 2007). Furthermore, Y35 and E81 were shown to be involved in a hydrogen bonding network between the subunits (Higgins and Giedroc, 2014; Chang et al.,
2015). All these amino acids are conserved in CopU, suggesting a similar regulatory mechanism.

Structural modeling of CopU predicts a homotetrameric structure that is essentially identical to that of other CsoR-type repressors (Fig. S2) (Porto et al., 2015; Jacobs et al., 2015; Chang et al., 2014; Liu et al., 2007). The subunit composition of purified CopU (Fig. S1) was verified experimentally by crosslinking of the purified protein with glutaraldehyde. The subunits of multimeric proteins in suspension can readily be crosslinked with bifunctional crosslinking reagents, while monomeric proteins in solution do not significantly crosslink under similar conditions. Crosslinking CopU with glutaraldehyde led to the formation of dimers, trimers, and tetramers of the expected molecular weights of 26, 39, and 52 kDa, but not to higher-order structures (Fig. 3). This suggests that CopU is indeed a homotetramer.

**CopU-DNA interaction**

Sequence analysis of the promoter/operator region revealed a 9-bp inverted repeat at position -22 to -45 that likely constitutes a repressor binding site. An 80 bp (ms115/ms116) double stranded oligonucleotide, encompassing this region (cf. Fig. 1b), was tested for interaction with CopU by EMSA. Fig. 4a shows that CopU forms a complex with this DNA region in the absence of copper. Cu\(^+\) and Ag\(^+\), but not Zn\(^{2+}\), Co\(^{2+}\), Cd\(^{2+}\), or Ni\(^{2+}\), dissociate the complex. Silver induction of CopU supports Cu\(^+\) rather than Cu\(^{2+}\) as inducer: Ag\(^+\) is chemically similar to Cu\(^+\) but not Cu\(^{2+}\) (Outten et al., 2001; Migocka et al., 2015) and Ag\(^+\) is a known mimetic of copper; induction by Ag\(^+\) has been shown for many copper-responsive repressors (Odermatt et al., 1993; Rensing et al., 2000; Liu et al., 2007; Magnani et al., 2008). Titration of the CopU-DNA interaction with Cu\(^+\) showed a gradual dissociation of the complex, with half-maximal release occurring at 10 µM Cu\(^+\) (Fig. 4b).

A 32 bp DNA douplex (oligonucleotide dimer ms111/ms112), encompassing the copUAZ promoter region from position -22 to -45, was apparently too short to form a DNA-CopU complex (not shown, see discussion). A PCR product generated with primers sm144 and ms110, covering nucleotides -96 to -273 and not containing the proposed CopU binding site but including the inverted repeat of the predicted upstream terminator, did also not show CopU-DNA interaction (Fig. 4c). On the other hand, a PCR product of similar size, generated with primers ms109 and sm143, but encompassing nucleotides -145 to +30 and containing the proposed CopU binding site, exhibited copper-dependent CopU binding.

A homotetrameric DNA binding protein must necessarily bind to an inverted repeat sequence for symmetry reasons. There can thus be little doubt that CopU binds to the inverted repeat TATAGTATAggggggTATACTATA, indicated in Fig. 1b. Taken together, these
results show that CopU binds to the promoter region of the copUAZ operon in a copper-dependent manner and suggest that CopU regulates the expression of the downstream genes, thereby regulating intracellular copper homeostasis in Desulfosporosinus sp. OT.

**Primary structure of the DOT_CopA ATPase**

In the annotated genome of Desulfosporosinus sp. OT, two genes were predicted to code for copper ATPases: DOT_copA, which is part of the copUAZ-operon, and DOT_copA2, which is located elsewhere in the genome (Abicht et al., 2011). Both genes code for proteins which strongly resemble the experimentally characterized Ec_CopA of Escherichia coli and Eh_CopA of Enterococcus hirae and possess the conserved elements typical of copper ATPases (Fig. S3) (Solioz et al., 1994).

A signature feature of heavy metal ATPases is a conserved proline residue in membrane helix six, usually in the context CPC. This site has been shown to directly be involved in transport of Cu\(^+\) ions across the membrane (Mandal et al., 2004). The amino acids surrounding the conserved proline confer metal specificity to the ATPase. The universal DKTGT motif encompasses the aspartic acid residue which is phosphorylated during catalysis. Other conserved features typical of P1B-ATPases are also conserved in DOT_CopA and DOT_CopA2. These are the TEGS motif, the HP-motif, the TGDN motif, and the GDGINDAPAL motif (see ref. (Smith et al., 2014) for recent review). The presence of these motifs in DOT_CopA and DOT_CopA2 suggests that both enzymes are heavy metal ion translocating ATPases of P1B-type.

**Complementation of E. coli with DOT_CopA and DOT_CopA2**

To functionally characterize DOT_CopA and DOT_CopA2, the respective genes were cloned in the pProExHTa expression vector and transformed into the copper sensitive E. coli W3110ΔcopA strain, which is devoid of the single transport system, CopA, which can expel cytoplasmic copper (Rensing et al., 2000). Complementation studies were performed both in liquid (Fig. 5a) and on solid media (Fig. 5b) in the presence and absence of copper. As expected, E. coli CopA used as a positive control, complemented the copper sensitive phenotype of W3110ΔcopA in liquid culture as well as on solid media. DOT_CopA also complemented the copper-sensitive phenotype under both conditions, indicating that this enzyme functions as a copper-exporting ATPase in E. coli. However, DOT_CopA2 was unable to restore the copper sensitive phenotype of the host strain, suggesting that only DOT_CopA, but not DOT_CopA2, functions in copper resistance of Desulfosporosinus sp. OT.

**In vitro activity of purified DOT_CopA**
To confirm the function of DOT_CopA as a copper ATPase, the enzyme was purified to greater than 90% purity by Ni-NTA affinity chromatography (Fig. S4). ATPase activity by DOT_CopA was highest in the presence of 100 μM Cu^{+}, 20 mM L-cysteine, and 1 mg/ml of phospholipids. Different lipid preparations were tested for their effect on ATPase activity.

Asolectin, a commercial crude soy bean phospholipid preparation, was previously found to optimally stimulate different ATPases (Portmann and Solioz, 2005; Wunderli-Ye and Solioz, 2001; Wyler-Duda and Solioz, 1996; Apell and Solioz, 1990). With asolectin, the specific ATPase activity of DOT_CopA was 12 ± 5 nmol/min/mg, depending on the preparation. In the presence of E. coli or E. hirae phospholipids in lieu of asolectin, the activity was 1.5 to 2.5 times and 2.5 to 3.5 times higher, respectively (Fig. 6). No enzymatic activity could be measured for DOT_CopA2 (not shown). Taken together, the structural features of DOT_CopA, its regulation by the copper-responsive CopU repressor, the complementation of a copper-sensitive E. coli phenotype by DOT_CopA, and the in vitro ATPase activity strongly suggest that DOT_CopA functions as a copper exporting ATPase in Desulfosporosinus sp. OT.

DISCUSSION

Desulfosporosinus OT is a sulfate reducing bacterium that can withstand high ambient copper concentrations (Abicht et al., 2011). To learn about the basis of copper resistance by this organism, the putative copper homeostatic genes/operons copUAZ and copA2 were cloned and expressed in E. coli and purified for functional analysis. The copUAZ operon, but not DOT_copA2, could be shown to have a function in copper resistance.

The fist gene of the copUAZ operon, copU, encodes a CsoR-type copper-responsive repressor, closely related to CsoR of Geobacillus thermodenitrificans (Chang et al., 2015). Interestingly, the 25 N-terminal residues of CopU, which are not seen in the structural model shown in Fig. S2, feature a CxxC motif; this motif is a ubiquitous Cu(I) binding site in copper chaperones and the N-termini of copper ATPases (Boal and Rosenzweig, 2009). In GenBank, this motif is only found in CsoR-type proteins of sulfate reducing bacteria closely related to the one under study here. The Cu(I)-bound structure of CsoR from Geobacillus thermodenitrificans reveals that the N-terminus is folded over the Cu(I) binding sites (Chang et al., 2014). The N-terminal CxxC motif of CopU could conceivably participate in copper binding or serve as a docking point for copper chaperones. Why this CxxC feature is only found in acidophilic sulfate reducing bacteria remains open to speculation.
CsoR-type repressors are all-helical, homotetrameric disc-like structures. In the absence of copper, two CsoR tetramers clutch the operator region to prevent transcription (Chaplin et al., 2015). For induction of transcription by copper, four Cu⁺ per homotetramer bind cooperatively to a C and a H residue on one subunit and a C residue of the adjacent subunit (Jacobs et al., 2015). This allosterically reduces or inhibits DNA binding, allowing transcription of the downstream genes to proceed (Chang et al., 2015).

The putative DNA binding site of CopU, TATAGTATAGGGGGGTATACTATA, encompasses a 9-bp inverted repeat (underlined), separated by 6 G-residues. This surmised CopU DNA binding site differs from those of other CsoR-type repressors. CsoR of G. thermodenitrificans binds to the minimal motif TACCCCTTCGGGTA (Chang et al., 2015), while the 'CsoR box' of Corynebacterium glutamicum features the sequence ATACCCCCTAGGGGTAT and Bacillus subtilis CsoR binds to TACCCCTACGGGGGTATGGTA (Teramoto et al., 2012; Smaldone and Helmann, 2007). So the DNA binding sites for CsoR-type repressors, including some not specifically mentioned here, appear to be diverse. The observation that a 32 bp DNA fragment did not support CopU binding in vitro suggests that the DNA region occupied by CopU is larger, or could even indicate that four CopU tetramers bind to the operator/promoter region. Indeed, the observed changes in electrophoretic mobility in EMSA experiments appeared very large, although this is inherently difficult to quantify. The promoter region of DOT_CopA2, for which we could not identify a role in copper resistance, does not exhibit any sequence features that would suggest a CopU binding site.

DOT_CopA, which apparently functions as a copper exporter, features three N-terminal CxxC consensus copper binding motifs, one being located in a 26-amino acid domain not present in the other ATPases shown in Fig. S3 or other bacterial copper ATPases. An additional 21-amino acid insertion is present 44 amino acids further downstream of the CxxC-containing insertion. CxxC motifs have been shown to be modular structural elements at the N-termini of copper ATPases. Each module, or metal binding domain (MBD), encompasses around 70 amino acids that are folded in thioredoxin-like fold and can coordinate one Cu⁺ by means of the CxxC motif (Arguello and Gonzalez-Guerrero, 2008; Lutsenko et al., 2007). Bacterial copper ATPases generally feature one (Eh_CopA) or two (Ec_CopA, 2) MBDs, while eukaryotic copper ATPases may possess up to six MBDs. Structure predictions suggest that all three CxxC motifs of DOT_CopA exhibit the typical MBD-fold. For bacterial copper ATPases, the MBDs have been shown to be dispensable, at least under laboratory conditions (Arguello et al., 2007; Fan et al., 2001). It is notable that both, CopU as well as DOT_CopA,
feature additional CxxC motifs not present in analogous proteins of non-acidophilic organisms. *Desulfosporosinus* sp. OT can be cultivated in up to 236 mM copper under sulfate reducing conditions. It could be speculated that the additional CxxC motifs are an adaption to high copper concentrations and/or the acidic environments these bacteria normally live in. However, it must be considered that growth under sulfate reducing conditions leads to the release of hydrogen sulfide, which precipitates heavy metal ions in the environment as insoluble metal sulfides. This can dramatically lower the free, or bioavailable copper concentration. Given the genomic analysis and the structure and function of the *copUAZ* operon characterized here, it appears likely that *Desulfosporosinus* sp. OT is not endowed with an exceptional copper resistance system, but relies on metal sulfide precipitation as a major mechanism to lower the concentration of toxic heavy metals in the environment.

Indeed, efficient metal sulfide precipitation from acid mine drainage by sulfate reducing bacteria has been demonstrated in experimental systems (Webb et al., 1998). Taken together, the present work suggests that the *copUAZ* operon constitutes the major copper resistance determinant of *Desulfosporosinus* sp. OT. The only other putative copper ATPase encoded by the genome, DOT_CopA2, could not complement a copper-sensitive *E. coli* strain and did not exhibit copper-stimulated ATPase *in vitro*. DOT_CopA is under the control of a CsoR-type, copper-responsive repressor, CopU. This repressor features an N-terminal CxxC motif, not present in other characterized CsoR-type repressors, an aspect that deserves further investigation.

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**FIGURE LEGENDS**

**Fig. 1. CopUAZ operon and promoter structure.** (a) CopUAZ operon, showing the gene arrangement and predicted proteins with the number of amino acids (open arrows). The black boxes indicate predicted terminators (term) of calculated stability -11.4 kcal and -16.9 kcal. The scale gives distances in base pairs (bp). (b) Promoter/operator region of the copUAZ operon. The open arrows indicate the position of a predicted terminator (term), the arrows the location of the putative CopU binding dyad, and the dashed double-lines the primers used for EMSA. The boxes delineate the "-10" and "-35" regions. The ribosome binding site is underlined and Met- indicates the first amino acid of CopU. The numbers below the sequence give the nucleotide positions relative to the start of translation.

**Fig. 2. Alignment of CsoR-type repressors.** The alignment shows, with locus tags in parenthesis: Bsu_CsoR, Bacillus subtilis CsoR (BSU33520); DOT_CopU, Desulfovibrio sp. OT CopU (WP_009622713), Gth_CsoR, Geobacillus thermodenitrificans CsoR (GTNG_1533); Mtu_CsoR, Mycobacterium tuberculosis CsoR (RV0967); Mtu_RicR, Mycobacterium tuberculosis RicR (MT0200); Sli_CsoR, Streptomyces lividans (SCO4136). The conserved copper-binding Cys and His residues are boxed, with position numbers corresponding to Mtu_CsoR. Also boxed are Tyr-35 and Glu-81, which participate in the hydrogen bonding network between subunits. The CxxC motif of DOT_CopU is underlined.

**Fig. 3. Cross-linking of CopU.** Aliquots of 5 µg of purified CopU with the his-tag removed were crosslinked with 0.2% glutaraldehyde for the times indicated in the Figure, followed by SDS polyacrylamide gel electrophoresis and staining with Coomassie blue. Std, molecular weight standards of the sizes indicated in kDa on the left side of the gel. The scale on the right indicates monomers (1), dimers (2), timers (3) and tetramers (4).

**Fig. 4. EMSA with CopU.** (a) The oligonucleotide dimer ms115/ms116(1 pmol), covering bp -85 to -5 of the copUAZ promoter and containing the putative CopU binding dyad, was interacted with purified CopU (240 pmol) without metal ions (Ctrl) or 10 µM of the metal ions indicated in the Figure. (b) EMSA in the absence (-) or presence of increasing concentrations of Cu⁺ as indicated in the Figure. Ctrl, no CopU added. (c) EMSA with 1 pmol of PCR product generated with primers sm144/sm110, covering bp -96 to -273, not containing the CopU binding dyad, and ms109/sm143, covering bp -145 to +30 and containing the CopU binding dyad of the copUAZ promoter. Ctrl, DNA alone; -, plus 240 pmol CopU; Cu⁺, plus 240 pmol CopU and 10 µM Cu⁺. The arrows indicate the migration of free DNA and the asterisks that of the DNA-CopU complex. Other details are given under Methods.
**Fig. 5. Complementation of *E. coli*.** (a) The growth response to copper in LB media was compared between *E. coli* wild-type (●), the copper-sensitive *E. coli* ∆copA mutant (○), and *E. coli* ∆copA, complemented with either a control vector (□), or a vector expressing Ec_CopA (■), DOT_CopA (▲), or DOT_CopA2 (Δ). Cultures were challenged with the indicated CuSO₄ concentrations and grown aerobically for 48 h at 37 °C, followed by measurement of the absorption at 600 nm. The Figure is representative of three independent replicates. (b) Filter disks soaked with 5 μl of 1 M CuSO₄ were deposited on bacterial lawns of either wild-type *E. coli* (Wild-type), or an *E. coli* ∆copA mutant (∆copA), which was untransformed (−) or transformed with an empty control vector (Vector), or with plasmids harboring the ATPase genes indicated in the Figure. Following incubation for 24 h at 37 °C, the plates were photographed.

**Fig. 6. In vitro ATPase activity of purified DOT_ATPase with different lipids.** ATPase activity of purified CopA in the presence of 1 mg/ml of different lipids was determined by measuring the release of Pi from ATP. The following lipids were tested: asolectin (○), *E. coli* phospholipids (●), and *E. hirae* phospholipids (▲). Other details of the experiment are described under Methods. The Figure shows one of three independent experiments.
Fig. 1

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ms115/ms116

ms111/ms112

- ms109/sm143

- ms109/sm143

- sm144/ms110

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Fig. 2

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Gth_CsoR  1> MTHPSQ----------EHVLHGTKPRKEEIEINMRLKRIEGQVRGIGQ
Mtu_CsoR  1> MSK--------------ELTAKKRAALNRLKTVRGLDGV
Mtu_RicR  1> MT--------------AAGYTQQKDNAKLRRVEGQVRGIA
Sli_CsoR  17> GAVNQTVRQAEIDGTDIVTDHDRGVHGYHKQAEHKLRRIEGQIRGLQ

Bsu_CsoR  37> NMVENDRYCVDILVQISAVQAAMKNVALHLLEHAEHCVADAIKSGDGE-
DOT_CopU  51> GMIERHVYCDVLNQIASAQCSALDGARLRLEKMKSCYKEQLQAGD-E-
Gth_CsoR  42> KMVEDRNYCIDILVQISAIQAALRQVMQLEHHRHAEHCVAKAIRESGE-
Mtu_CsoR  28> RMLESDAYCVDVMKQISAVQLSRLNRNMLHLETCFSTAVLDGQ-
Mtu_RicR  30> RMIEEDMNYCIDVLTQISAVTSLRVALNLLAEHLRSCTRAVAEGGPGA
Sli_CsoR  51> RMVDENYCIDILTQASSTKALQSFALQLEEHRRFCAADAALKGGTEI

Bsu_CsoR  86> -QAISELIDVFKKFTKS
DOT_CopU  99> -QVVDELKLFIRFMR-IR
Gth_CsoR  91> -QSLRELMVIKQF-IR
Mtu_CsoR  77> -AAILELIDAVKFTPALTGPHARLGGAAVGESATEEPMFDASM
Mtu_RicR  80> DGLAAEASAAIALVRS
Sli_CsoR  101> DAKVLEDATKAIGRLLRT

Fig. 3
Fig. 4

(a) Ctrl Cu⁺ Zn²⁺ Co²⁺ Cd²⁺ Ni²⁺ Ag⁺

(b) Ctrl - 2.5 5 7.5 10 25 mM Cu⁺

(c) sm144/ms110 ms109/sm143
Ctrl - Cu⁺ Ctrl - Cu⁺
Fig. 5

(a) Absorption [600 nm] vs. CuSO₄ [mM]

(b) Wild-type vs. ΔcopA

- Vector
- Ec_copA
- DOT_copA
- DOT_copA2

Fig. 6

Pi [nmol] vs. Time [min]
Supplementary Material
Copper resistance and its regulation in the sulfate reducing bacterium Desulfosporosinus sp. OT
Stefano Mancini1*, Ranjeet Kumar2*, Helge K. Abicht1, Elisabeth Fischermeier1 and Marc Solioz1,2
1Department Clinical Research, University of Bern, 3010 Bern, Switzerland
2Laboratory of Biochemistry and Molecular Biology, Tomsk State University, 634050 Tomsk, Russian Federation
*These authors contributed equally to the work

Table S1. Primer sequences and use

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Underlined nucleotides correspond to engineered NarI and XbaI enzyme recognition sites.
**Fig. S1. Purification of CopU.** *Lane 1*, 20 µg of crude cytoplasmic extract of uninduced BL21(DE3)RIL containing the CopU-expressing plasmid pOU2; *lane 2*, 20 µg of crude cytoplasmic extract 4 h after induction with 0.1 mM IPTG containing the overexpressed 6His-CopU; *lane 3*, 2 µg of purified CopU (predicted Mw = 12.8 kDa), indicated by the arrow; *lane 4*, molecular weight markers, with the sizes in kDa indicated on the right. Proteins were resolved on a 15 % tricine-SDS polyacrylamide gel and stained with Coomassie blue.
Fig. S2. Structural model of CopU. The structure of CopU was modeled with SWISS-MODEL (Biasini et al., 2014). Each color represents a monomer of the homotetrameric structure. The modeled structure starts at H26 (N') and ends at the C-terminal residues (C). The brown spheres represent Cu(I) atoms which are predicted to be bound to H84 and C89 of one subunit and C59 of an adjacent subunit. The first 25 amino acids of CopU are missing in the model.
Fig. S3. Sequence alignment of ATPases. Protein sequences were aligned with ClustalW. Conserved amino acids are displayed in inverse type and conservative replacements by a light- or dark-grey background. Eh_CopA (accession AAA61835); DOT_CopA (accession WP_009622712); Ec_CopA (accession BAE76263); DOT_CopA2 (accession number EGW36630). Conserved domains commonly found in P1B-ATPases are boxed. See main text for additional explanations.

Fig. S4. Purification of DOT_CopA. Proteins were resolved on a 10 % glycine-SDS polyacrylamide gel and stained with Coomassie blue. Lane 1: Molecular weight marker with the sizes in kDa indicated on the left side of the Figure; lane 2: 15 µg solubilized membranes; lane 3: flow through of the Ni-NTA column; lane 4: wash with buffer JD; lane 5, elution with buffer JD + 200 mM imidazole; lane 6: 2 µg of purified DOT_CopA.