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

Stefano Mancini, † Ranjeet Kumar, ‡ Helge K. Abicht, † Elisabeth Fischermeier † and Marc Solioz †, 2

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

*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 shown here 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* *Δ*copA mutant, it restores copper resistance to WT levels. His-tagged DOT_CopA was expressed from a plasmid in *E. coli* and purified by Ni-NTA affinity chromatography. The purified enzyme was 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 *aa*₃-type terminal oxidases, Cu/Zn-superoxide dismutase required for defence 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 (Osman & Cavet, 2008; Dupont et al., 2011; Bondarczuk & Piotrowska-Seget, 2013).

The ability of copper to catalyse the formation of reactive oxygen species 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 et al., 2007; Macomber & Imlay, 2009; 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⁺-ATPases, usually termed CopA, CopB or CupA. They belong to the heavy-metal-transporting P1B-type ATPases subgroup of the P-type ATPase superfamily (Lutsenko & Kaplan, 1995). Their characteristics are one to six CxxC metal-binding domains (MBDs) at their N-termini, and conserved DKTGT (in the one-letter

These authors contributed equally to this work.

**Abbreviations:** EMSA, electrophoretic mobility shift assay; MBD, metal-binding domain; rTEV, recombinant tobacco etch virus.

One supplementary table and four supplementary figures are available with the online Supplementary Material.
Copper resistance in Desulfosporosinus

amino acid code, used throughout this paper) phosphorylation 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⁺ 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 other Gram-positive organisms, i.e. Lactococcus lactis and Enterococcus hirae (Soliz 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). CopA 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 Enterococcus 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, CopU, 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 otherwise. Ni-NTA affinity resins were from Qiagen and asolectin was from Associated Concentrates. Escherichia coli and Enterococcus hirae lipids were isolated as described previously (Ames, 1968).

Strains and culture conditions. Desulfosporosinus sp. OT was grown as described previously (Karnachuk et al., 2005) and DNA was isolated by alkaline lysis as described elsewhere (Aussubel et al., 1995). E. coli W3110 and W3110ΔcopA were kindly provided by Christopher Rensing (University of Copenhagen, Copenhagen, Denmark). All E. coli strains were grown aerobiically in LB media at 37 °C, unless indicated otherwise.

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, available in the online Supplementary Material). The resulting PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen), yielding pCa6. The Ec_copA gene was excised from pCa6 with NarI/XbaI and subcloned into pProExHTa, digested with the same enzymes. The resulting construct, pCB1, encoded EcCopA with an N-terminal His-tag that could be cleaved with recombinant tobacco etch virus (rTEV) protease. Desulfosporosinus sp. OT copU (GenBank accession number 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/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 (GenBank 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 protease-cleavable, N-terminal 6-His-tag. DOT_copA2 (GenBank accession number 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 protease-cleavable, N-terminal 6-His-tag. The absence of mutations was verified in all the constructs by commercial DNA sequencing.

Purification of CopU. E. coli BL21 (DE3) RIL with plasmid pOU02 was grown aerobiically at 37 °C in 300 ml LB containing 50 μg ampicillin ml⁻¹ to OD₆₀₀ 0.8. The culture was then induced with 0.1 mM IPTG and incubated for an additional 16 h at 20 °C. The cells were harvested at 7000 g for 10 min and the resultant pellet was stored at −70 °C until further use. His-tagged CopU was purified by resuspending pelleted cells at 2 ml (g wet weight)⁻¹ in lysis buffer (50 mM Na-HEPES, pH 7.5), containing 1 mM PMSF, added from 100 mM stock in DMSO. Cells were disrupted by two passages through a French press at 30 MPa. Cell debris was removed by centrifugation at 15 000 g for 30 min. The supernatant obtained was applied to a 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. 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 analysed by 15 % SDS-polyacrylamide gels (Laemmli, 1970). CopU-containing fractions were pooled and dialysed against 2 × 100 vol. 20 mM Na-HEPES, pH 7.5, 1 mM DTT, 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 aa protein with a predicted molecular mass of 13 kDa exhibited high purity (>95 %), as determined by SDS-PAGE (Fig. S1). Purified CopU was incubated with 1/20 vol. Chelex 100 (Bio-Rad) for 2 h at 4 °C to remove metal ions, followed by dialysis against 2 × 100 vol. 20 mM Na-HEPES, pH 7.5, 1 mM DTT, for 2 h each at 4 °C. The protein concentration was determined by the method of Bradford, using BSA as a standard (Bradford, 1976).

Cross-linking of CopU. Purified CopU (5 μg) was suspended in 50 mM Na-HEPES, pH 7.5, and cross-linked for 5–20 min with 0.2 % glutaraldehyde in a total reaction volume of 25 μl. Reactions were
stopped by the addition of 5 μl 1 M Tris/HCl, pH 8, and cross-linking 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 binding buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 100 mM KCl, 200 μM magnesium acetate, 100 μM DTT, 1 μg BSA, 5 % glycerol). Samples were separated on 10 % polyacrylamide gels prepared in a 1 : 1 mixture of binding buffer and 40 nM Tris/acetate, pH 8, 1 mM EDTA. Following electrophoresis, gels were stained for 60 min with RedSafe, 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/ms12 and ms115/ms116 were pairwise annealed at 65 °C for 10 min for EMSA. PCR products were amplified with primer pairs sm144/ms10 and ms109/sm143 (Table S1) from Desulfovoruspinus 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 for both DOT_CopA and DOT_CopA2. Either E. coli BL21(DE3)RII transformed with plasmid pOU1 or pOU3 was grown with 250 ml 0.9 % NaCl. They were finally resuspended in G-buffer by centrifugation for 10 min at 8000 g. The resultant membrane pellet was resuspended in 2.5 ml 0.7 % agar and poured on LB plates. Filter discs containing 5 μl of different repressor solutions were added to the cultures as it had a strong inhibitory effect on growth. Glucose, 100 mM MgSO4, 1 mM PMSF, 100 mM Na2SO4, 1 mM K2SO4, 1 mM β-mercaptoethanol, 20 % (v/v) glycerol, 5 μg DNase I per ml (g wet pellet)1 and 0.1 mM PMSF solved in DMSO, and extracted with dodecyl-β-maltoside. Samples were added to the cultures grown for an additional 4 h. Cells were collected by centrifugation for 15 min at 12 000 g and the supernatant was centrifuged for 45 min at 80 000 g. The resultant membrane pellet was resuspended in 2.5 ml 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−1, supplemented with 1/100 vol. protease inhibitor cocktail (100 mM N-α-p-tosyl-l-lysine-chloromethylketone, 100 mM N-p-tosyl-l-phenylalanine-chloromethylketone, 100 mM P-amnobenzamidine, 100 mM PMSF dissolved in DMSO), and extracted with dodecyl-β-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 g and the supernatant loaded onto a 1 ml Ni-NTA-agarose column, pre-equilibrated with buffer JD. Cleavage of the 6-His-tag chaperone (Fig. 1a). Downstream of copZ are two more ORFs, 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 TATAAGTA(N6)TATACTATA, which could represent the CopU-binding site (Fig. 1b). This figure also shows the oligonucleotides used to test the CopU–DNA interaction.

Primary and quaternary structure of CopU

The predicted gene product of copU exhibits extensive sequence similarity to characterized CsrR-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 & Giedroc, 2014). They revealed a flat, disc-like homotetrmeric 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 CsrR 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 & Giedroc, 2014; Chang et al., 2015). All these amino acids are conserved in CopU, suggesting a similar regulatory mechanism.

Structural modelling of CopU predicts a homotetrameric structure that is essentially identical to that of other CsrR-type repressors (Fig. S2) (Liu et al., 2007; Chang et al., 2014; Jacobs et al., 2015; Porto et al., 2015). The subunit composition of purified CopU (Fig. S1) was verified experimentally by cross-linking of the purified protein with glutaraldehyde. The subunits of multimeric proteins in suspension can readily be cross-linked with bifunctional cross-linking reagents, while monomeric proteins in
solution do not significantly cross-link under similar conditions. Cross-linking CopU with glutaraldehyde led to the formation of dimers, trimers and tetramers of the expected molecular masses of 26, 39 and 52 kDa, but not to higher-order structures (Fig. 3). This suggested that CopU was 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 (compare with Fig. 1b), was tested for interaction with CopU by EMSA. Fig. 4(a) shows that CopU formed a complex with this DNA region in the absence of copper. Cu\(^{+}\) and Ag\(^{+}\), but not Zn\(^{2+}\), Co\(^{2+}\), Cd\(^{2+}\) or Ni\(^{2+}\), dissociated the complex. Silver induction of CopU supported 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 Cu\(^{+}\); 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 duplex (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 position −96 to −273 and not containing the proposed CopU-binding site but including the inverted

---

**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 and −16.9 kcal. The scale gives distances in base pairs. (b) Promoter/operator region of the copUAZ operon. The open arrows indicate the position of a predicted terminator (term), the arrows indicate the location of the putative CopU-binding dyad and the dashed double lines indicate 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.
repeat of the predicted upstream terminator, also did not show any CopU–DNA interaction (Fig. 4c). However, a PCR product of similar size, generated with primers ms109 and sm143, but encompassing position +2145 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. 1(b). 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 *E. 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 6, usually in the context CPC. This site has been shown to be directly involved in the 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-type 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 (for a recent review, see Smith et al., 2014). The presence of these motifs in DOT_CopA and DOT_CopA2 suggests that both enzymes are heavy-metal ion-translocating ATPases of the 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* W3110 \( \Delta \)copA strain that 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 W3110 \( \Delta \)copA 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 >90 % purity by Ni-NTA affinity chromatography (Fig. S4). ATPase activity containing the CopU-binding dyad of the copUAZ promoter. Ctrl, DNA alone; –, plus 240 pmol CopU; Cu\(^{+}\), plus 240 pmol CopU and 10 \( \mu \)M Cu\(^{+}\). The arrows indicate the migration of free DNA and the asterisks indicate the migration of the DNA–CopU complex. Other details are given in Methods.
by DOT_CopA was highest in the presence of 100 μM Cu²⁺, 20 mM L-cysteine and 1 mg phospholipids ml⁻¹. 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 (Apell & Solioz, 1990; Wyler-Duda & Solioz, 1996; Wunderli-Ye & Solioz, 2001; Portmann & Solioz, 2005). With asolectin, the specific ATPase activity of DOT_CopA was 12 ± 5 nmol min⁻¹ mg⁻¹, depending on the preparation. In the presence of E. coli or Enterococcus hirae phospholipids in lieu of asolectin, the activity was 1.5–2.5 and 2.5–3.5 times higher, respectively (Fig. 6). No enzymic 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 suggested that DOT_CopA functions as a copper-exporting ATPase in Desulfosporosinus sp. OT.

**Fig. 5.** Complementation of E. coli. (a) The growth response to copper in LB media was compared between E. coli WT (●), the copper-sensitive E. coli ΔcopA mutant (○) and E. coli ΔcopA complemented with either a control vector (□), or a vector expressing Ec_CopA (■), DOT_CopA (▲) or DOT_CopA2 (▲). Cultures were challenged with the indicated CuSO₄ concentrations and grown aerobically for 48 h at 37 °C, followed by measurement of the OD₆₀₀. The figure is representative of three independent replicates. (b) Filter discs soaked with 5 μl 1 M CuSO₄ were deposited on bacterial lawns of either WT E. coli or an E. coli ΔcopA mutant (ΔcopA), which was untransformed (−) or transformed with an empty control vector, or with plasmids harbouring the ATPase genes indicated. Following incubation for 24 h at 37 °C, the plates were photographed.

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

**DISCUSSION**

Desulfosporosinus OT is a sulfate-reducing bacterium that can withstand high ambient copper concentrations (Abicht et al., 2011). To determine 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 first 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 & Rosenzweig, 2009). In GenBank, this motif is only found in CsoR-type proteins of sulfate-reducing bacteria closely related to that under study here. The Cu(I)-bound structure of CsoR from G. 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...
Copper resistance in Desulfosporosinus

(Chaplin et al., 2015). For induction of transcription by copper, four Cu\(^+\) per homotetramer bind cooperatively to a C and an 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, TATAGTATAG-
GGGGTATATA, encompasses a 9 bp inverted repeat (underlined), separated by six G residues. This surmised CsoR of 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 ATACCCCTAGGGGTAT and Bacillus subtilis CsoR binds to TACCTACGGGGGTATGGTA (Smaldone & Helmann, 2007; Teramoto et al., 2012). Therefore, 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 aa domain not present in the other ATPases shown in Fig. S3 or other bacterial copper ATPases. An additional 21 aa insertion is present 44 aa 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 MBD, encompasses ~70 aa that are folded in thioredoxin-like fold and can coordinate one Cu\(^+\) by means of the CxxC motif (Lutsenko et al., 2007; Argüello & González-Guerrero, 2008). Bacterial copper ATPases generally feature one (Eh_CopA) or two (Ec_CopA) 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 (Fan et al., 2001; Argüello et al., 2007). It is notable that 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 adaptation to high copper concentrations and/or the acidic environments in which these bacteria normally live. 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, i.e. 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.

ACKNOWLEDGEMENTS

We thank Thomas Weber for expert technical assistance. This work was supported by a Russian Federation Government grant to leading scientists (contract number 14.Z50.31.0011).

REFERENCES


http://mic.microbiologyresearch.org
CopA N-terminal Cys(X)2Cys motifs are not required for Cu(I)-mediated allosteric switching in a copper-sensing operon repressor (CsoR). J Biol Chem 289, 19204–19217.


