Expression of copA and cusA in *Shewanella* during copper stress

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Copper homeostasis is tightly regulated in all living cells as a result of the necessity and toxicity of this metal in free cationic form. In Gram-negative bacteria CPx-type ATPases (e.g. CopA in *Escherichia coli*) and heavy-metal efflux RND proteins (e.g. CusA in *E. coli*) play an important role in transport of copper across the cytoplasmic and outer membrane. We investigated the expression of CusA- and CopA-like proteins in *Shewanella oneidensis* MR1 and *Shewanella* strain MB4, a Mn(IV)-reducing isolate from a metal-polluted harbour sediment. Q-PCR analysis of total mRNA extracted from cultures grown under aerobic conditions with 25 μM copper showed significantly increased expression of *cusA* (Student’s *t*-test: MR1, *P*<0.0001; MB4, *P*=0.0006). This gene was also induced in the presence of 100 μM copper and 10 or 25 μM cadmium in both tested strains. In the absence of oxygen, with fumarate as final electron acceptor and 100 μM copper, a prolonged lag phase (5 h) was observed and general fitness decreased as evidenced by twofold lower copy numbers of 16S rRNA compared to aerobic conditions. *cusA* expression in cells grown under these conditions remained at comparable levels (MR1) or was slightly decreased (MB4), compared to aerobic copper challenges. A gene homologous to the *copA* gene of *S. oneidensis* was not detected in strain MB4. Although low *copA* copy numbers were observed in strain MR1 under conditions with 25 and 100 μM copper, *copA* was not detected in mRNA from cultures grown in the presence of 10 μM cadmium, or in the absence of added heavy metals. However, *copA* was highly induced under anaerobic conditions with 100 μM copper (*P*=0.0011). These results suggest essentially different roles for the two proteins CopA and CusA in the copper response in *S. oneidensis* MR1, similar to findings in more metal-resistant bacteria such as *Escherichia coli* and *Cupriavidus metallidurans*.

**INTRODUCTION**

Copper is required for growth in all living organisms, yet becomes toxic at high concentrations. Therefore, transport of copper ions across the cell membrane and subsequent incorporation into metallo-proteins is tightly controlled (Rae *et al.*, 1999; Nies, 1999). Two types of proteins involved in bacterial heavy metal efflux (HME) are CPx-type ATPases and HME RND proteins (from the Resistance, Nodulation and Cell Division protein family) (Paulsen *et al.*, 1996; Coombs & Barkay, 2004, 2005; Saier, 2003). CopA in *Escherichia coli* was among the first heavy-metal-transporting ATPases to be fully characterized; it showed transport of Cu(I) out of the cytoplasm at the expense of ATP hydrolysis (Rensing *et al.*, 2000). Other relevant examples include CadA and ZntA, involved in export of Zn\(^{2+}\), Cd\(^{2+}\) and Pb\(^{2+}\) (Tsai *et al.*, 2002; Banci *et al.*, 2003; Gaballa & Helmann, 2003). CopA-like transporters are quite common and have been detected in numerous micro-organisms (or their genomes), e.g. *Enterococcus hirae*, *Synechocystis* spp., *Bacillus subtilis*, *Streptococcus mutans*, *Archeoglobus fulgidus*, *Ferroplasma acidarmanus* and lower eukaryotes such as *Cryptosporidium parvum* and *Candida albicans* as well as mammals (Baker-Austin *et al.*, 2005; Coombs & Barkay, 2005; Ettema *et al.*, 2006).

Examples of HME RND proteins are found only in proteobacteria and include the CzcCBA system of the extremely metal-resistant bacterium *Cupriavidus metallidurans* (formerly known as *Ralstonia*, *Alcaligenes* and *Wautersia*), which exports divalent cobalt, zinc and cadmium, and the CusCFBA system in *E. coli*, responsible for transport of monovalent copper and silver (Goldberg *et al.*, 1999; Franke *et al.*, 2003; Nies, 2003). These proteins are distantly related to well-studied multidrug transporters such as AcrB in *E. coli* and MexB in *Pseudomonas*.
P. aeruginosa, conferring resistance to acriflavine and other antibiotics. In contrast to ATPases, metal transport by RND proteins occurs in an antiport fashion, i.e. at the expense of proton-motive force. Additionally, ATPases only span the inner membrane and transport from the periplasm to periplasm or vice versa. RND proteins, however, take up substrates from the cytoplasm (possibly also from the periplasm: Outten et al., 2001) and transport them across both membranes to the cell’s exterior. This suggests essentially different, although perhaps complementary, functions in metal extrusion.

A number of studies have indicated this apparent overlap in functions between different proteins in heavy metal resistance. In E. coli, the interactions between proteins from the cus (RND) and the cop (ATPase) operons were investigated under copper stress (Outten et al., 2001; Kershaw et al., 2001). Expression of copA was observed under most conditions tested, whereas cusA was only induced at higher concentrations of copper (experiments carried out under both aerobic and anaerobic conditions). An additional gene, cueO, was associated with the physiological response to copper and hypothesized to convert Cu(I) to the less toxic Cu(II) in the periplasm (under aerobic conditions). On the other hand, Legatzi et al. (2003) investigated the interplay between the ccc system (RND) and two P-type ATPases in cadmium and zinc resistance of C. metallidurans. In this bacterium, it was shown that the ccc system alone was sufficient for removal of both metal cations under moderate levels of stress, but one of the ATPases (cadA) proved essential for full cadmium tolerance. More recently, transcriptional profiling of copper-stressed P. aeruginosa indicated upregulation of two RND efflux systems and an ATPase, as well as a general fine-tuning of iron acquisition (Teitzel et al., 2006).

Although newly annotated genomes suggest a wealth of genes encoding putative HME pumps, physiological evidence is only available for a few representatives. Members of the genus Shewanella represent interesting study objects in this sense, as many representatives are known for their capacity to reduce and detoxify a number of (toxic) metals (Nealson & Myers, 1992; Guha et al., 2003; Saltikov et al., 2003; Venkateswaran et al., 1999), yet little is known about their resistance to copper. In addition, Shewanella species have been isolated from (and detected in) a wide range of aquatic habitats, including both relatively pristine and polluted environments (Ziemke et al., 1998; Ivanova et al., 2003). Enrichments for Mn(IV)-reducing bacteria from a heavy-metal-polluted harbour sediment led to isolation of a Shewanella strain designated MB4 (98 % similarity to Shewanella marisflavi based on 16S rRNA and gyrB gene). Physiological responses of isolate MB4 and Shewanella oneidensis strain MR1 to the heavy metals cobalt, cadmium, zinc and copper under varying growth conditions are described elsewhere (Toes et al., 2008). Copper tolerance of both strains in the presence of oxygen, as evidenced from lag phase and growth rate, was not significantly different from copper tolerance during anaerobic growth on lactate and fumarate. This is in contrast to results obtained for E. coli (Outten et al., 2001).

In this study we report the detection of copA and cusA transcripts in mRNA of S. oneidensis MR1 and strain MB4 in response to copper, monitored by quantitative PCR (Q-PCR). Cultures of S. oneidensis and isolate MB4 were subjected to copper challenges and cells harvested for mRNA extraction at different points during the growth cycle. Reproducibility between different cultures was tested and results used to design an optimal experimental set-up for comparison between different stress conditions. These included, in the presence of oxygen: 25 µM and 100 µM copper and 10–25 µM cadmium. In addition, the 100 µM copper trial was conducted under anaerobic growth conditions.

METHODS

Strains and cultivation conditions. Shewanella oneidensis MR1 was kindly provided by K. Nealson, University of Southern California, Los Angeles, USA. Strain MB4 was isolated from a metal-polluted marine harbour sediment under Mn(IV)-reducing conditions; it showed 97 % 16S rRNA gene similarity to S. marisflavi. A photoluminescent, Vibrio-like bacterium was isolated from the skin of North Sea fish, during a practical course (identified as 97 % similar to Vibrio alginolyticus). From the Netherlands Culture Collection of Bacteria (Utrecht) we obtained the following cultures: E. coli K-12 (MG 1655 NCCB 4007), Capriavidus necator (formerly known as Ralstonia eutropha H16, NCCB 82042) and Bacillus azotoformans (NCCB 100003).

All cultures were kept as glycerol stocks at −80 °C. Each test was carried out with cultures that had not been previously exposed to heavy metals. Each culture was transferred three times to new medium after approximately 8 h of growth, prior to the actual experiments. The growth medium used in the aerobic experiments consisted of 10-fold diluted LB broth, containing, per litre (g): 1 tryptone, 0.5 yeast extract and 5 NaCl, with extra salt added for MB4 (27.5 g total). The minimal marine medium used for anaerobic tests contained, per litre (g): 1 NH₄Cl, 0.2 MgSO₄·7H₂O, 0.1 CaCl₂·2H₂O, 0.05 K₂HPO₄·3H₂O, 27.5 NaCl (5 for strain MR1) 0.5 yeast extract and 2.38 HEPES (pH 7.5). The medium was divided into 60 ml portions in glass flasks (100 ml), closed with butyl rubber stoppers and the gas phase was flushed with argon. After sterilization, lactate (20 mM), fumarate (10 mM) and iron (20 µM) were aseptically added from anaerobic stock solutions. Flasks were incubated at room temperature in the dark. Growth was monitored by microscopy and by measuring OD₅₉₅.

Primer design. Primers for amplification of copA-like genes from members of the genus Shewanella were designed using multiple protein sequence alignments. S. oneidensis MR-1 ‘CopA’-like protein (NP_717300, http://www.ncbi.nlm.nih.gov) was queried against the KEGG GENES Database (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/). Top matching sequences from several other representatives of the γ-subclass of the Proteobacteria were retrieved and used to generate a multiple sequence alignment in CLUSTAL W (Lassmann & Sonnhammer, 2005; http://www.ebi.ac.uk/Tools/clustalw2/index.html). This alignment was imported into the primer design tool CODEHOP to suggest primers (http://blocks.fhcrc.org/codehop.html, Rose et al., 2003). Secondary structures were predicted using NetPrimer (Premier Biosoft International; http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch) with
default settings. Potential primers were blasted against all bacterial genomes with the BLASTN search (for short, nearly exact matches) in order to avoid non-specific amplification. Potential primers were tested in silico in closely and distantly related bacterial genomes (Bikandi et al., 2004; http://insilico.ehu.es/). Several primer sets were tested in vitro on target and non-target DNA (see next paragraph).

A similar strategy was followed to design primers for the genes encoding CusA-like proteins of S. oneidensis (NP_720114 and NP_720469) and related sequences. However, the primers obtained resulted in formation of a non-specific, larger amplicon in PCRs with E. coli control DNA. The second attempt involved a more topological approach; the 12 transmembrane helices (TMH) and the two large periplasmic loops (LPL) were detected in the amino acid sequence approach; the 12 transmembrane helices (TMH) and the two large periplasmic loops (LPL) were detected in the amino acid sequence of S. oneidensis and its two marine relatives Shewanella baltica OS155 and Shewanella frigidimarina NCIMB 400 with Primer3 (Rozen & Skaletsky, 2000; http://frodo.wi.mit.edu/). Secondary structures and in silico specificity were analysed as described above.

**Primer specificity.** Specificity of primers was tested by PCR. All amplifications were carried out with the Taq PCR Master Mix kit (Qiagen) in a Thermocycler (BioMetra). Each reaction contained 1× Taq PCR Master Mix, forward and reverse primers with final concentrations of 1 μM each, PCR-grade water and approximately 50 ng template DNA. Sequences of forward and reverse primers copA_F and copA_R are shown in Table 1. Positive controls were DNA from S. oneidensis and V. alginolyticus, and negative controls were DNA from B. azotoformans, E. coli and C. necator. Cycling steps consisted of an initial denaturation step (95 °C for 5 min), followed by amplification [95 °C for 1 min, 59 °C for 1 min, 72 °C for 30 s (30 cycles)], and a final extension of 72 °C for 10 min. Sequences of forward and reverse primers cusA_F and cusA_R were shown in Table 1. Positive controls were genomic DNA from S. oneidensis and MB4, and negative controls were DNA from C. necator and B. azotoformans. The PCR for cusA was identical to the programme described above, except that the optimal annealing temperature was determined at 57 °C.

Universal primers 1055F and 1392R were used for amplification of bacterial 16S rRNA genes (Ferris et al., 1996). All primers were purchased from Thermo Electron. PCR products were visualized by electrophoresis on 2% (w/v) agarose gels (80 V for 30 min). The molecular marker GeneRuler 100 bp DNA Ladder (Fermentas) was used for size confirmation. After electrophoresis, gels were stained with ethidium bromide and bands were visualized with the Bio-Rad Gel Doc 1000 system under UV illumination.

**Cloning, sequencing and phylogenetic analysis.** Specificity of the primers was confirmed by sequencing. PCR products of cusA amplified from S. oneidensis and MB4 genomic DNA, and of copA amplified from S. oneidensis and V. alginolyticus, respectively, were extracted from 2% agarose gels and purified with the QIAQuick Gel Extraction kit (Qiagen). DNA sequencing was performed with the appropriate forward primers by BaseClear.

In order to quantify cusA and copA during different experiments, the PCR amplicons obtained were cloned in E. coli, and resulting plasmids used as molecular standards in Q-PCR. PCR fragments amplified from S. oneidensis genomic DNA were purified as described above, cloned into pCR 2.1-TOPO TA cloning vectors and transformed into chemically competent TOP10 E. coli cells according to the manufacturer's manual (Invitrogen). Plasmids with cusA and copA inserts were extracted using the QIAprep Spin Miniprep kit (Qiagen). Quality check and concentration measurements were performed with NanoDrop (Thermo Fischer Scientific), and the plasmids stored in aliquots at −20 °C until use.

Protein and nucleotide sequences were compared to sequences in different nucleotide and protein databases, as described under primer design. Selected sequences were used to generate alignments in CLUSTAL W (Lassmann & Sonnhammer, 2005), which were imported into MEGA3 (Kumar et al., 2004). Final neighbour-joining trees were created with Poisson and Kimura correction, for proteins and nucleotides, respectively.

**RNA extraction and quantification.** Cell numbers were determined by measuring OD 565 prior to harvesting cells. Samples (1–3 ml) of culture were taken aseptically and centrifuged at 1000 r.p.m. for 25 min; the cell pellets were resuspended in RNAlater (Ambion) and used directly or stored at −20 °C for 2 days before further treatment. Care was taken to start RNA extraction with approximately equal amounts of bacterial cells, and with cell numbers below the maximal loading capacity (7.5 × 10^8) of the columns used in the RNeasy Mini kit (Qiagen). RNAlater was removed by centrifugation and cells were lysed in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) containing 1 mg lysosome ml\(^{-1}\) for 5 min at room temperature. RNA was stabilized in RLT buffer containing β-mercaptoethanol (from the extraction kit) and subsequently purified on columns. DNase treatment was performed in a waterbath (10 min, 37 °C) with 1–2 μg RNA in DNase buffer (50 mM Tris/HCl, pH 8, 5 mM MgCl\(_2\)) with DNase I (0.5 Kunitz units). Then 2 μl 140 mM EDTA was added followed by incubation at 65 °C for 5 min, and inactivated enzymes were removed. This protocol showed less RNA loss compared to on-column DNA digestion. Total extracted RNA was quantified by Nanodrop measurements before and after DNase treatment, which

<table>
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<tr>
<th>Target</th>
<th>Name*</th>
<th>Sequence (5′–3′)†</th>
<th>Size (bp)</th>
<th>Reference</th>
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<tbody>
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<td>cusA</td>
<td>cusA_F</td>
<td>CGC CAC CAT GAA CAA CAT GAA RCA RAA YYT</td>
<td>151</td>
<td>This study</td>
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<td>151</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>copA_R</td>
<td>GTA ATG GAA GAC AGT GCC ATI GCI GC†</td>
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<tr>
<td>16S rRNA</td>
<td>1055F</td>
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<td>352</td>
<td>Ferris et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>1392R</td>
<td>ACG GGC GGT GTG TAC</td>
<td></td>
<td></td>
</tr>
</tbody>
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*F, forward primer; R, reverse primer.
†R, A or G; Y, C or T; I, inosine.
enabled normalization of the amount of input RNA in the cDNA synthesis reaction. Only samples with good quality, i.e. \( A_{260}/A_{280} \) ratio ~2.0 (Fleige & Pfaffl, 2006), were considered acceptable for further processing, with the Bio-Rad iScript cDNA synthesis kit. Control PCRs with 16S rRNA primers were carried out to check for DNA contamination, and cDNA dilutions of unknown samples were amplified to confirm equal target concentrations.

**Q-PCR and statistical analyses.** Amplification and detection of \( czcA \) and \( copA \) genes and bacterial 16S rRNA genes were performed with the iCycler detection system (Bio-Rad). Approximately 50 ng cDNA was added to each reaction, based on the amount of total extracted RNA. For 16S rRNA amplification 5 ng target was needed. Data analysis was performed with the iCycler iQ Software. All reactions were performed in 25 µl volumes and utilized the QuantiTect SYBR Green kit (Qiagen). All PCR runs included triplicates of unknown samples, no-template controls, and standard DNA in 10-fold dilution series. After amplification, melting curve analysis was performed to confirm amplification specificity. This was carried out in 80 cycles with 10 s per cycle, starting at 55 ℃ and increasing by 0.5 ℃ per cycle. Q-PCR products were also analysed by gel electrophoresis (2 %, w/v, agarose) to check the amplicon size and detect primer-dimers. Threshold cycle values and the log starting quantities for standards in real-time PCR assays were used to obtain standard curves from which target concentrations in unknown samples could be calculated. Detection limits and valid template DNA input ranges were determined by performing real-time PCR runs with dilution series of standards and samples prior to quantification runs. The unpaired Student’s \( t \)-test was used to determine whether two datasets were significantly different (\( P < 0.05 \)).

For \( copA \) amplification the master mix was diluted with PCR-grade water, and 0.6 µM \( copA \)_F primer, 0.3 µM \( copA \)_R primer, 0.08 µg BSA µl\(^{-1}\), 4.5 mM MgCl\(_2\) and DNA template were added. The PCR programme included a hot start activation step of 1 cycle at 95 ℃ for 15 min, followed by 30 cycles of 95 ℃ for 1 min and a combined annealing/extension step with fluorescence detection at 57 ℃ for 30 s. For \( cusA \) amplification both primers were added to a final concentration of 0.3 µM. Other conditions and reagents were similar to Q-PCR of \( copA \), except that the annealing temperature was 59 ℃. For amplification of fragments of the 16S rRNA genes an identical reaction mixture was used. The Q-PCR programme started with 95 ℃ for 15 min, followed by 27 cycles of 95 ℃ 20 s, 55 ℃ 30 s and 72 ℃ 1 min. Fluorescence was measured in the extension step.

## RESULTS

### Primer design for \( cusA \) and \( copA \) in Shewanella

Analyses of the annotated genome of \( S. oneidensis \) revealed several sequences with high similarity to genes encoding proteins involved in copper transport in other bacteria, i.e. CPx-type ATPases involved in active uptake or extrusion (SO_1689 and SO_2359) as well as HME RND proteins driven by proton-motive force (SO_0520, SO_4598 and SO_A0153). Comparative analysis of amino acid sequences with other \( Shewanella \) type strains and with bacterial reference sequences (from the \( \alpha \)- and \( \gamma \)-subdivisions of Proteobacteria and from the Firmicutes) resulted in the phylogenetic trees shown in Fig. 1 (ATPases) and Fig. 2 (RND proteins).

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**Fig. 1.** Neighbour-joining tree of bacterial CPx-type ATPases containing heavy-metal-associated domains, rooted with the potassium-associated pump KdpB from \( E. coli \) (750 aa). Except for sequences preceded by an underlined protein name (e.g. \( CopA \)), physiological evidence for transporter function is lacking. The box with the dashed line indicates target sequences for primer design. Sequence accession numbers are given in parentheses. Evolutionary distances were computed using Poisson correction; the scale bar represents 0.1 changes in amino acids.
Two putative copper CPx-type ATPases were found, located in different regions of the chromosome. One of them, encoding protein NP_717949, was phylogenetically most closely related to known copper importers (Fig. 1, designated cluster copA1). The other sequence (NP_717300) showed higher similarity to proteins in cluster copA2, involved in either copper export or import, such as CopA from Enterococcus hirae and Staphylococcus aureus (Solioz & Stoyanov, 2003; Coombs & Barkay, 2005). The box with the dashed line indicates the target sequences for primer design (Table 1). Regarding HME RND proteins (Fig. 2), three potential sequences were detected, two of which are encoded on the chromosome (NP_720114 and NP_716156) and one on MR1’s megaplasmid (NP_720469). Two sequences from S. oneidensis showed resemblance to CusA (E. coli) and SilA (Salmonella typhymurium), transporting monovalent Cu and/or Ag. The third protein was related to sequences in one of the clusters without immediate representatives, or knowledge of metal specificity (HME RND II). None of the sequences showed significant similarity with Czca-like proteins, although other Shewanella type strains were represented in this cluster. The designed primers targeted the copper determinants encircled by the dashed line.

The novel primer sets created for this study are listed in Table 1. Up to three degeneracies were incorporated into the oligonucleotides, in order to target potentially similar genes in isolate MB4. The primers were also tested on C. necator, E. coli, B. azotoformans and a Vibrio-like isolate (of which only the last gave a positive signal and only for the copA primer set). Fig. 3(a) shows a phylogenetic tree of the short nucleotide fragments generated with copA primers; PCR amplification of S. oneidensis and the Vibrio-like

![Fig. 2. Neighbour-joining tree of bacterial HME RND proteins, rooted with the multi-drug exporter acrB from E. coli (1050 aa). Except for sequences preceded by an underlined protein name (e.g. CusA), physiological evidence for transporter function is lacking. The box with the dashed line indicates target sequences for primer design. Sequence accession numbers are in parentheses. Evolutionary distances were computed using Poisson correction; the scale bar represents 0.1 changes in amino acids.](http://mic.sgmjournals.org)

![Fig. 3. Pruned neighbour-joining tree of nucleotide sequences obtained with novel primer sets copA (a, 130 bp) and cusA (b, 110 bp) on S. oneidensis and test strains. Sequences in bold type were determined in this study. The scale bar represents 0.05 nucleotide changes. Bootstrap values (100 replicates) of 50% and higher are given.](http://mic.sgmjournals.org)
isolate provided the expected products, but for MB4 no PCR product was detected. Amplification with the cusA primers (Fig. 3b) did give a positive signal both with isolate MB4 and for S. oneidensis.

Expression of copA and cusA during growth under aerobic and anaerobic conditions

In order to investigate whether these copper determinants showed increased expression under short-term copper stress, aerobic toxicity experiments were conducted. At different stages of growth, samples were taken for RNA extraction, and subsequent quantification of copA, cusA and 16S fragments was conducted by Q-PCR (data not shown). Increased expression of the genes of interest in both S. oneidensis and isolate MB4 was detected in response to copper. Induction was dependent on copper dosage and time of sampling; copA and cusA rose above the detection level within 2 h after dosage, but the gradual decrease observed in the amount of 16S mRNA from $10^5$ copies per ng total mRNA at OD$_{595}$ 0.05 to $10^5$ at OD$_{595}$ 0.7 also indicated strong reduction of general fitness. In order to circumvent variability in growth phase and related mRNA production, further experiments were conducted with copper added directly at the time of inoculation (t=0 h) and sampling at early exponential phase (OD$_{595}$ 0.2). Additionally, these tests aimed to determine the reproducibility between measurements of four biological replicates, cultivated and analysed on two separate occasions.

Fig. 4(a) shows the results of Q-PCR of replicates, cultivated and analysed on two separate occasions. reproducibility between measurements of four biological in the presence of copper (25 μM) was conducted by Q-PCR (data not shown). Increased expression of the genes of interest in both S. oneidensis and isolate MB4 was detected in response to copper. Induction was dependent on copper dosage and time of sampling; copA and cusA rose above the detection level within 2 h after dosage, but the gradual decrease observed in the amount of 16S mRNA from $10^5$ copies per ng total mRNA at OD$_{595}$ 0.05 to $10^5$ at OD$_{595}$ 0.7 also indicated strong reduction of general fitness. In order to circumvent variability in growth phase and related mRNA production, further experiments were conducted with copper added directly at the time of inoculation (t=0 h) and sampling at early exponential phase (OD$_{595}$ 0.2). Additionally, these tests aimed to determine the reproducibility between measurements of four biological replicates, cultivated and analysed on two separate occasions.

Fig. 4(a) shows the results of Q-PCR of cusA and 16S mRNA of four biological replicates of strain MB4 growing in the presence of copper (25 μM) and control cultures. Similar experiments for cusA, copA and 16S rRNA gene quantification in S. oneidensis MR1 were conducted (Fig. 4b). Relative copy number is defined as the natural logarithm of the calculated gene copies, corrected for input mRNA (ng). Variation between biological replicates carried out on different occasions was relatively high for the genes investigated, 2–12%. For S. oneidensis the number of 16S rRNA copies per ng total mRNA ranged between $0.5 \times 10^5$ and $2 \times 10^5$. cusA quantification indicated 20–50 copies per ng mRNA during growth with 25 μM copper, and even lower copA copy numbers (0.5–5). The correlation between the presence of copper and expression of presumed copper determinant cusA was significant in MB4 and MR1 (unpaired Student’s t-test: P<0.0006 and P=0.0001).

copA expression in MR1 in the presence of copper was not significantly different from control conditions under the tested conditions (P=0.067).

Final experiments were carried out, with the conditions described above, to determine differences in expression levels of cusA and copA under different degrees of metal stress during aerobic growth, i.e. with 25 and 100 μM copper and with 25 μM cadmium (10 μM in the case of S. oneidensis). Furthermore, growth with 100 μM copper was studied during anaerobic growth with fumarate as terminal electron acceptor. Fig. 5 summarizes Q-PCR results for cultures of isolate MB4 (Fig. 5a) and S. oneidensis (Fig. 5b) harvested at OD$_{595}$ 0.2 (OD$_{595}$ 0.15 for anaerobic cultures). The data presented in Fig. 5(a) indicate that cusA expression increased approximately twofold between 25 and 100 μM copper. Additionally, it was shown that cusA expression was significantly induced by copper under anaerobic conditions (P=0.038) and by cadmium under aerobic conditions (P=0.0013). From the data presented in Fig. 5(b), it is evident that cusA was detectable in MR1 under all conditions tested and that copy numbers were significantly higher in cultures grown in the presence of either copper or cadmium (aerobic, all P<0.0001; anaerobic 100 μM, P=0.0059). In contrast, copA was only significantly induced under anaerobic conditions (P<0.001). Quantification of rRNA suggested that copy numbers were similar throughout the experiment (in the range of $10^5$ copies ng$^{-1}$), with the exception of samples from cultures grown under anaerobic conditions in the
presence of 100 μM copper; both MB4 and MR1 produced significantly lower amounts of 16S rRNA, suggesting lower general fitness of the sampled cells.

**DISCUSSION**

**Copper efflux pumps encoded in the genome of *S. oneidensis* MR1**

Although generally a high degree of variability is found among CopA sequences, there are a number of strongly conserved motifs. The two putative copper CPx-type ATPases encoded in the genome of *S. oneidensis* (Fig. 1) contain the amino acid motifs characteristic of ATP-binding (GDGIN), phosphorylation (DKTG) and phosphatase domains (TGE), common for all P-type ATPases (Rensing *et al.*, 2000). In contrast to hard-metal ATPases, which contain ten transmembrane helices (TMHs), copper-transporting proteins consist of eight helices. In addition, SxHP in the periplasmic loop and the metal-binding motif CPCAL in the sixth TMH indicate heavy-metal-transporting ATPases (Coombs & Barkay, 2005). This Cys-Pro-Cys sequence is essential for binding of Cu(I) as shown by site-directed mutagenesis of CopA in *E. coli* (Fan & Rosen, 2002). Despite an extensive genomic survey of CopA-like sequences, Coombs & Barkay (2005) found no conserved motifs distinguishing proteins from the CopA1 and CopA2 clusters. However, the vast majority of genes from the CopA1 cluster were located adjacent to genes encoding the subunits of copper-haem oxidases, indicating a role in the supply of monovalent cations for metal-dependent enzymes. Likewise, in *S. oneidensis* the gene encoding NP_717949 (SO_2359) was located between genes encoding a cytochrome c maturation protein and cytochrome c oxidase subunits (http://www.tigr.com). Primary sequence differences between the two ATPases from the *S. oneidensis* genome were found in the N-terminal region. The targeted CopA-like protein (NP_717300, SO_1689) contained one CxxC motif, whereas the ATPase in the copA2 cluster contained three repeats. According to Rensing *et al.* (2000), these sequences encode cytosolic metal-binding motifs and may have regulatory functions.

Less structural information is available for HME RND proteins (Paulsen *et al.*, 1996). The roughly determined structure of CzcA in *C. metallidurans* as described by Goldberg *et al.* (1999) is a protein with 12 TMHs and two large periplasmic loops and fits the model of a two-channel pump. The highly conserved 'DDE' motif in one of these channels (TMH IV), also found in multi-drug efflux pumps such as ArcB, was essential for CzcA function in vivo and for proton/cation antiport in vitro. In RND systems involved in transport of monovalent copper and silver (CusA-like cluster in Fig. 2), a shorter DE motif is found. The DE motif was also present in all sequences from cluster HME RND II. Three methionine residues, at positions 573, 623 and 672, were shown to be essential for copper resistance by CusA in *E. coli* (Franke *et al.*, 2003), and these were also detected in all sequences from the CusA-like cluster (not in HME RND II).

The *cus* determinant in *E. coli* encodes the CusCFBA complex, including a small ORF encoding CusF, a copper-binding periplasmic protein (Rensing & Grass, 2003; Franke *et al.*, 2003). In *S. oneidensis*, the two putative CusA sequences were indeed found downstream of a membrane fusion protein (‘CusB’), but no ‘CusF’- or ‘CusC’-like sequences were detected. Instead a small hypothetical protein (86 aa), containing two putative
copper-binding histidine repeats and a copper-transporting ATPase domain were found. It is likely that the plasmid-borne cusAB determinant (SO_A0153) is a recent duplication from the one in the genome (SO_4598), as the genes are organized in a similar way, share high sequence similarity and many transposons were identified in MR1’s plasmid (Heidelberg et al., 2002; Kolker et al., 2005). The outer-membrane protein (‘CusC’) may be encoded on a different location in the chromosome as was shown for acrAB and tolC. One putative outer-membrane protein was detected upstream of the third HME RND sequence in cluster II (SO_0518) and may be recruited by the CusA-like protein systems.

**Q-PCR of copA and cusA in Shewanella strains**

A Q-PCR method was developed that allowed for detection of copA and cusA fragments in mRNA extracts of *S. oneidensis* and strain MB4. The 16S rRNA was also quantified as an indicator of physiological fitness. Although quantification of a reference gene or an internal standard may be one of the preferred methods for normalization of Q-PCR data (Sharkey et al., 2004), it is a laborious task to establish that the reference gene itself is not regulated by the test condition. For instance, at least 200 protein-encoding genes of varying function are up- or downregulated during the general stress response in *S. oneidensis* MR1 (Qiu et al., 2005), and similar effects may be expected in response to metal toxicity. Therefore, in this study results were normalized by applying equal amounts of total RNA in cDNA synthesis and subsequent Q-PCR (Schmittgen & Zakrajsek, 2000; Huggett et al., 2005). Total RNA consists primarily of rRNA (80%), and only 2–5% comprises protein-encoding mRNA. Results of Liang et al. (2000) suggest (i) that the number of available ribosomes is constant and independent of growth rate and (ii) that efficiency of translation of mRNA is influenced by the amount of bulk rRNA, due to competition for available ribosomes. Therefore, care was taken to harvest cells at similar optical densities, ensuring comparable growth rates and rRNA production. Q-PCR results of four individually treated replicates of cultures harvested at OD₅₉₅ 0.2 (early exponential phase) indicated moderate reproducibility between biological replicates (2–12% deviation). This relatively high variation is not uncommon when compared to other studies using Q-PCR to assess in vivo gene expression (Vandecasteele et al., 2001; Nielsen & Boye, 2005). Reproducibility between technical replicates was high (0.1–0.8%).

Under aerobic growth conditions, the correlation between the presence of copper (25 μM) and expression of the presumed copper determinant cusA was significant in both MB4 and MR1 (unpaired Student’s t-test: \(P<0.0006\) and \(P=0.0001\)). copA expression in MR1 in the presence of copper was not significantly different from controls under the tested conditions \(P=0.067\). Further aerobic tests indicated that cusA expression in MB4 increased approximately twofold between 25 and 100 μM copper, and was also significantly induced by cadmium \((P=0.0013)\). cusA was detectable in MR1 under all conditions tested and copy numbers were significantly higher in cultures grown in the presence of either copper or cadmium \((P<0.0001)\). When anaerobic growth conditions were applied with fumarate as terminal electron acceptor, cusA expression in the presence of 100 μM copper was lower than under aerobic conditions, but still significantly higher than controls (MB4, \(P=0.038\); MR1, \(P=0.0059\)). Interestingly, copA was only significantly induced under anaerobic conditions \((P<0.001)\) in MR1.

A number of recent studies have specifically focused on *S. oneidensis* under different types of stress. Under UVA irradiation, multi-drug-transporting RND systems were strongly induced, in addition to both presumed cusAB operons in strain MR1 (7.8- to 14-fold) (Qiu et al., 2005). This may indicate that HME RND pumps function in detoxification of radiation products or are derepressed as part of the general stress response. Transcriptome analysis of heat-shock-response genes indicated no changes in the genes under study (Gao et al., 2004). Under Cr(VI)-reducing conditions, however, genes SO0518–SO0520 were upregulated, suggesting that the sequences in cluster HME RND II may have a function in Cr(III) detoxification (Benchekh-Latmani et al., 2005). A study by Groh et al. (2007) showed that genes encoding RND proteins involved in antibiotic resistance are important determinants in the ecological fitness of *S. oneidensis* MR1. To add to this emerging picture, our results suggest that expression of copA and cusA in *S. oneidensis* mRNA extracts significantly increased during growth with copper. Furthermore, expression of cusA was observed under cadmium stress. copA in particular appeared to play an important role in copper detoxification during anaerobic growth with copper. These findings are in contrast to results obtained for *E. coli*, which indicated that the CopA protein was primarily expressed under aerobic conditions, whereas CusA was essential for full anaerobic copper tolerance (Outten et al., 2001; Kershaw et al., 2005). However, the operon encoding CopA in *E. coli* also encodes CueO, a periplasmic oxygen-dependent oxidase. Perhaps the absence of an oxygen-dependent multicopper oxidase such as CueO in *S. oneidensis* MR1 may help explain why copper resistance does not markedly change when comparing aerobic and anaerobic conditions (Toes et al., 2008). Increased copA expression in MR1 under anaerobic conditions results in active transport of Cu(I) from the cytoplasm to the periplasm, implying that an additional copper transporter may be involved to remove excess copper from the periplasm. As substantial expression levels of cusA were observed under these conditions, it is not unlikely that these HME RND protein systems are also capable of transporting metal cations from the periplasm, as suggested previously for CzcCB2A in *C. metallidurans* (Goldberg et al., 1999) and for CusCFBA in *E. coli* (Outten et al., 2001; Kershaw et al., 2005).
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