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

Ann-Charlotte M. Toes, Maria H. Daleke,[†] J. Gijs Kuenen and Gerard Muyzer

Correspondence
Gerard Muyzer
g.muyzer@tudelft.nl

Department of Biotechnology, Delft University of Technology, Julianalaan 67, NL-2628 BC Delft, The Netherlands

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*.

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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*, *Ferropasma acidiarms* 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*

[†]Present address: Department of Medical Microbiology and Infection Control, VU Medical Centre, Amsterdam, The Netherlands.

Abbreviations: HME, heavy metal efflux; Q-PCR, quantitative PCR; RND, resistance, nodulation and cell division; TMH, transmembrane helix.

aeruginosa, conferring resistance to acriflavin 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 cytoplasm 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.*, 2005). 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, Legatzki *et al.* (2003) investigated the interplay between the *czc* system (RND) and two P-type ATPases in cadmium and zinc resistance of *C. metallidurans*. In this bacterium, it was shown that the *czc* 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), *Cupriavidus 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.fhcr.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 with help of the predictive tool TMHMM (Sonnhammer *et al.*, 1998; <http://www.cbs.dtu.dk/services/TMHMM/>). As the LPL regions are possibly involved in metal-binding and show relatively high sequence variability (Goldberg *et al.*, 1999), new primers specifically targeted the second loop between helices VII and VIII. Consensus primers were designed from the nucleotide sequences for *cusA* in *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* are 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 OD₅₉₅ 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 RNeasy lysis buffer (Qiagen) 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). RNeasy lysis buffer was removed by centrifugation and cells were lysed in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) containing 1 mg lysozyme 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 MgCl₂) 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 1. Primers used for amplification and quantification of *cusA*, *copA* and bacterial 16S rRNA genes

Target	Name*	Sequence (5'–3')†	Size (bp)	Reference
<i>copA</i>	<i>copA_F</i>	CGC CAC CAT GAA CAA CAT GAA RCA RAA YYT	151	This study
	<i>copA_R</i>	GTA ATG GAA GAC AGT GCC ATI GCI GC†		
<i>cusA</i>	<i>cusA_F</i>	ATG ACG AAT GGC GTG AAG G	112	This study
	<i>cusA_R</i>	GAT ACG GGT TTT GAT GGG TTG		
16S rRNA	1055F	ATG GCT GTC GTC AGC T	352	Ferris <i>et al.</i> (1996)
	1392R	ACG GGC GGT GTG TAC		

*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 μ l 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 °C and increasing by 0.5 °C 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 μ M *copA*_F primer, 0.3 μ M *copA*_R primer, 0.08 μ g

BSA μ l⁻¹, 4.5 mM MgCl₂ and DNA template were added. The PCR programme included a hot start activation step of 1 cycle at 95 °C for 15 min, followed by 30 cycles of 95 °C for 1 min and a combined annealing/extension step with fluorescence detection at 57 °C for 30 s. For *cusA* amplification both primers were added to a final concentration of 0.3 μ M. Other conditions and reagents were similar to Q-PCR of *copA*, except that the annealing temperature was 59 °C. For amplification of fragments of the 16S rRNA genes an identical reaction mixture was used. The Q-PCR programme started with 95 °C for 15 min, followed by 27 cycles of 95 °C 20 s, 55 °C 30 s and 72 °C 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 α - and γ -subdivisions of *Proteobacteria* and from the *Firmicutes*) resulted in the phylogenetic trees shown in Fig. 1 (ATPases) and Fig. 2 (RND proteins).

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.

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.

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 typhimurium*), 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. 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.

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 10^7 copies per ng total mRNA at OD₅₉₅ 0.05 to 10^3 at OD₅₉₅ 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 (OD₅₉₅ 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 μ M) 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 0.5×10^5 and 2×10^5 . *cusA* quantification indicated 20–50 copies per ng mRNA during growth with 25 μ M 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 μ M copper and with 25 μ M cadmium (10 μ M in the case of *S. oneidensis*). Furthermore, growth with 100 μ M copper was studied during anaerobic growth with fumarate as terminal electron acceptor. Fig. 5 summarizes Q-PCR results for

Fig. 4. Comparison of Q-PCR (*cusA*, *copA* and 16S rRNA) results of four biological replicates from two separate experiments with cultures of isolate MB4 (a), and of *S. oneidensis* (b) growing with or without 25 μ M copper (dosed at $t=0$ h and OD₅₉₅<0.02; RNA extracted at OD₅₉₅ 0.2). Relative copy number is the natural logarithm of the calculated gene copy number per ng total RNA. Error bars represent standard deviation between three technical replicates.

cultures of isolate MB4 (Fig. 5a) and *S. oneidensis* (Fig. 5b) harvested at OD₅₉₅ 0.2 (OD₅₉₅ 0.15 for anaerobic cultures). The data presented in Fig. 5(a) indicate that *cusA* expression increased approximately twofold between 25 and 100 μ M 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 μ M, $P=0.0059$). 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

Fig. 5. Quantification of *cusA* and 16S rRNA fragments in isolate MB4 (a), and of *cusA*, *copA* and 16S rRNA copy numbers in RNA of *S. oneidensis* (b) during aerobic growth with 25 μ M copper, 100 μ M copper or 10–25 μ M cadmium, and during anaerobic growth with fumarate and 100 μ M Cu and under control conditions (0). Copper or cadmium was present from $OD_{595} < 0.02$ and cells were harvested at OD_{595} 0.2 (aerobic) and 0.15 (anaerobic). The experiment was carried out in duplicate and error bars indicate standard deviation between three technical replicates.

presence of 100 μ M 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 (all $P < 0.0001$). 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 (Bencheikh-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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REFERENCES

- Baker-Austin, C., Dopson, M., Wexler, M., Sawers, R. G. & Bond, P. L. (2005). Molecular insight into extreme copper resistance in the extremophilic archaeon *Ferroplasma acidarmanus* Fer1. *Microbiology* **151**, 2637–2646.
- Banci, L., Bertini, I., Ciofi-Baffoni, S., Del Conte, R. & Gonnelli, L. (2003). Understanding copper trafficking in bacteria: interaction between the copper transport protein CopZ and the N-terminal domain of the copper ATPase CopA from *Bacillus subtilis*. *Biochemistry* **42**, 1939–1949.
- Bencheikh-Latmani, R., Williams, S. M., Haucke, L., Criddle, C. S., Wu, L., Zhou, J. & Tebo, B. M. (2005). Global transcriptional profiling of *Shewanella oneidensis* MR-1 during Cr(VI) and U(VI) reduction. *Appl Environ Microbiol* **71**, 7453–7460.
- Bikandi, J., San Millán, R., Rementeria, A. & Garaizar, J. (2004). In silico analysis of complete bacterial genomes: PCR, AFLP-PCR and endonuclease restriction. *Bioinformatics* **20**, 798–799.
- Coombs, J. M. & Barkay, T. (2004). Molecular evidence for the evolution of metal homeostasis genes by lateral gene transfer in bacteria from the deep terrestrial subsurface. *Appl Environ Microbiol* **70**, 1698–1707.
- Coombs, J. M. & Barkay, T. (2005). New findings on evolution of metal homeostasis genes: evidence from comparative genome analysis of bacteria and archaea. *Appl Environ Microbiol* **71**, 7083–7091.
- Ettema, T. J. G., Brinkman, A. B., Lamers, P. P., Kornet, N. G., de Vos, W. M. & van der Oost, J. (2006). Molecular characterization of a conserved archaeal copper resistance (*cop*) gene cluster and its copper-responsive regulator in *Sulfolobus solfataricus* P2. *Microbiology* **152**, 1969–1979.
- Fan, B. & Rosen, B. P. (2002). Biochemical characterization of CopA, the *Escherichia coli* Cu(I)-translocating P-type ATPase. *J Biol Chem* **277**, 46987–46992.
- Ferris, M. J., Muyzer, G. & Ward, D. M. (1996). Denaturing gradient gel electrophoresis profiles of 16S rDNA-defined populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* **62**, 340–346.
- Fleige, S. & Pfaffl, M. W. (2006). RNA quality and the effect on the real-time qRT-PCR performance. *Mol Aspects Med* **27**, 126–139.
- Franke, S., Grass, G., Rensing, C. & Nies, D. H. (2003). Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *J Bacteriol* **185**, 3804–3812.
- Gaballa, A. & Helmann, J. D. (2003). *Bacillus subtilis* CPx-type ATPases: characterization of Cd, Zn, Co and Cu efflux systems. *Biometals* **16**, 497–505.
- Gao, H., Wang, Y., Liu, X., Yan, T., Wu, L., Alm, E., Arkin, A., Thompson, D. K. & Zhou, J. (2004). Global transcriptome analysis of the heat shock response of *Shewanella oneidensis*. *J Bacteriol* **186**, 7796–7803.
- Goldberg, M., Pribyl, T., Juhnke, S. & Nies, D. H. (1999). Energetics and topology of CzcA, a cation/proton antiporter of the resistance-modulation-cell division protein family. *J Biol Chem* **274**, 26065–26070.
- Groh, J. L., Luo, Q., Ballard, J. D. & Krumholz, L. R. (2007). Genes that enhance the ecological fitness of *Shewanella oneidensis* MR-1 in sediments reveal the value of antibiotic resistance. *Appl Environ Microbiol* **73**, 492–498.
- Guha, H., Jayachandran, K. & Maurrasse, F. (2003). Microbiological reduction of chromium(VI) in presence of pyrolusite-coated sand by *Shewanella alga* Simidu ATCC 55627 in laboratory column experiments. *Chemosphere* **52**, 175–183.
- Heidelberg, J. F., Paulsen, I. T., Nelson, K. E., Gaidos, E. J., Nelson, W. C., Read, T. D., Eisen, J. A., Seshadri, R., Ward, N. & other authors (2002). Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. *Nat Biotechnol* **20**, 1118–1122.
- Huggett, J., Dheda, K., Bustin, S. & Zumla, A. (2005). Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun* **6**, 279–284.
- Ivanova, E. P., Sawabe, T., Zhukova, N. V., Gorshkova, N. M., Nedashkovskaya, O. I., Hayashi, K., Frolova, G. M., Sergeev, A. F., Pavel, K. G. & other authors (2003). Occurrence and diversity of mesophilic *Shewanella* strains isolated from the north-west Pacific Ocean. *Syst Appl Microbiol* **26**, 293–301.
- Kershaw, C. J., Brown, N. L., Constantinidou, C., Patel, M. D. & Hobman, J. L. (2005). The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. *Microbiology* **151**, 1187–1198.
- Kolker, E., Picone, A. F., Galperin, M. Y., Romine, M. F., Higdon, R., Makarova, K. S., Kolker, N., Anderson, G. A., Qiu, X. & other authors (2005). Global profiling of *Shewanella oneidensis* MR-1: expression of hypothetical genes and improved functional annotations. *Proc Natl Acad Sci U S A* **102**, 2099–2104.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Lassmann, T. & Sonnhammer, E. L. (2005). Automatic assessment of alignment quality. *Nucleic Acids Res* **33**, 7120–7128.
- Legatzki, A., Grass, G., Anton, A., Rensing, C. & Nies, D. H. (2003). Interplay of the Czc system and two P-type ATPases in conferring metal resistance to *Ralstonia metallidurans*. *J Bacteriol* **185**, 4354–4361.
- Liang, S.-T., Xu, Y.-C., Dennis, P. & Bremer, H. (2000). mRNA composition and control of bacterial gene expression. *J Bacteriol* **182**, 3037–3044.
- Nealon, K. H. & Myers, C. R. (1992). Microbial reduction of manganese and iron: new approaches to carbon cycling. *Appl Environ Microbiol* **58**, 439–443.
- Nielsen, K. K. & Boye, M. (2005). Real-time quantitative reverse transcription-PCR analysis of expression stability of *Actinobacillus pleuropneumoniae* housekeeping genes during in vitro growth under iron-depleted conditions. *Appl Environ Microbiol* **71**, 2949–2954.
- Nies, D. H. (1999). Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* **51**, 730–750.
- Nies, D. H. (2003). Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol Rev* **27**, 313–339.
- Outten, F. W., Huffman, D. L., Hale, J. A. & O'Halloran, T. V. (2001). The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *J Biol Chem* **276**, 30670–30677.
- Paulsen, I. T., Brown, M. H. & Skurray, R. A. (1996). Proton-dependent multidrug efflux systems. *Microbiol Rev* **60**, 575–608.

- Qiu, X., Sundin, G. W., Wu, L., Zhou, J. & Tiedje, J. M. (2005).** Comparative analysis of differentially expressed genes in *Shewanella oneidensis* MR-1 following exposure to UVC, UVB, and UVA radiation. *J Bacteriol* **187**, 3556–3564.
- Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C. & O'Halloran, T. V. (1999).** Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* **284**, 805–808.
- Rensing, C. & Grass, G. (2003).** *Escherichia coli* mechanisms of copper homeostasis in a changing environment. *FEMS Microbiol Rev* **27**, 197–213.
- Rensing, C., Fan, B., Sharma, R., Mitra, B. & Rosen, B. P. (2000).** *copA*: an *Escherichia coli* Cu(I)-translocating p-type ATPase. *Proc Natl Acad Sci U S A* **97**, 652–656.
- Rose, T. M., Henikoff, J. G. & Henikoff, S. (2003).** CODEHOP (CONsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. *Nucleic Acids Res* **31**, 3763–3766.
- Rozen, S. & Skaletsky, H. (2000).** Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**, 365–386.
- Saier, M. H. (2003).** Tracing pathways of transport protein evolution. *Mol Microbiol* **48**, 1145–1156.
- Saltikov, C. W., Cifuentes, A., Venkateswaran, K. & Newman, D. K. (2003).** The *ars* detoxification system is advantageous but not required for As(V) respiration by the genetically tractable *Shewanella* species strain ANA-3. *Appl Environ Microbiol* **69**, 2800–2809.
- Schmittgen, T. D. & Zakrajsek, B. A. (2000).** Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods* **46**, 69–81.
- Sharkey, F. H., Banat, I. M. & Marchant, R. (2004).** Detection and quantification of gene expression in environmental bacteriology. *Appl Environ Microbiol* **70**, 3795–3806.
- Solioz, M. & Stoyanov, J. V. (2003).** Copper homeostasis in *Enterococcus hirae*. *FEMS Microbiol Rev* **27**, 183–195.
- Sonnhammer, E. L., von Heijne, G. & Krogh, A. (1998).** A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc Int Conf Intell Syst Mol Biol* **6**, 175–182.
- Teitzel, G. M., Geddie, A., De Long, S. K., Krisits, M. J., Whiteley, M. & Parsek, M. R. (2006).** Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 7242–7256.
- Toes, A.-C. M., Geelhoed, J. S., Kuenen, J. G. & Muyzer, G. (2008).** Characterization of heavy metal resistance of Fe(III)- and Mn(IV)-reducing *Shewanella* isolates from marine sediments. *Geomicrobiol J* (in press).
- Tsai, K.-J., Lin, Y.-F., Wong, M. D., Yang, H. H., Fu, H. L. & Rosen, B. P. (2002).** Membrane topology of the pl258 *cadA* Cd(II)/Pb(II)/Zn(II)-translocating p-type ATPase. *J Bioenerg Biomembr* **34**, 147–156.
- Vandecasteele, S. J., Peetermans, W. E., Merckx, R. & van Eldere, J. (2001).** Quantification of expression of *Staphylococcus epidermidis* housekeeping genes with Taqman quantitative PCR during in vitro growth and under different conditions. *J Bacteriol* **183**, 7094–7101.
- Venkateswaran, K., Moser, D., Dollhopf, M. E., Lies, D. P., Saffarini, D. A., MacGregor, B. J., Ringelberg, D. B., White, D. C., Nishijima, M. & other authors (1999).** Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *Int J Syst Bacteriol* **49**, 705–724.
- Ziemke, F., Hofle, M., Lalucat, J. & Rossello-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

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