The RSP_2889 gene product of *Rhodobacter sphaeroides* is a CueR homologue controlling copper-responsive genes

Verena Peuser, Jens Glaeser and Gabriele Klug

INTRODUCTION

Copper is an essential trace element required by organisms from bacteria to humans. Due to its redox chemistry copper ions can act as cofactors for redox-active proteins (Rensing & Grass, 2003). Copper proteins are widely distributed in aerobic organisms and mainly function in electron-transfer reactions. They are involved in biological processes such as respiration, iron transport and oxidative stress protection. On the other hand copper is toxic to cells even at low concentrations since it contributes to the generation of reactive oxygen species (ROS), including the highly reactive hydroxyl radical (Bremner, 1998; Halliwell & Gutteridge, 1984). ROS are harmful to the cells due to protein and lipid oxidation and modification, and cleavage of nucleic acids (Imlay, 2003). Copper can also exhibit toxic activity by displacing other metal cofactors from their natural ligands and thereby interfering with the function of cellular proteins (Macomber & Imlay, 2009). In order to maintain appropriate copper levels in the cell the uptake of copper, its distribution within the cell and its removal need to be well balanced. Copper-resistance systems have been described in several bacteria, e.g. *Escherichia coli* (Reeve et al., 2002), *Salmonella enterica* (Kim et al., 2002) and *Escherichia coli* (Outten et al., 2000; Petersen & Møller, 2000; Stoyanov et al., 2001). Despite the importance of copper homeostasis, the underlying mechanisms are only partly understood in bacteria.

*C. sphaeroides* is a facultatively photosynthetic alpha-proteobacterium with remarkably high metabolic versatility, widely distributed in freshwater habitats. While aerobic respiration generates ATP in the presence of oxygen, anoxicogenic photosynthesis or anaerobic respiration can guarantee energy supply in the absence of oxygen. Due to changing conditions in its environment *R. sphaeroides* can be exposed to ROS-generating conditions, and different aspects of its response to ROS have been elucidated in the past (Glaeser & Klug, 2005; Glaeser et al., 2007; Zeller et al., 2005). The RSP_2889 gene product of *R. sphaeroides* was originally annotated as SoxR (Gene ID 3720629; protein ID YP_352951.1), a transcriptional regulator first identified in *E. coli*, which activates certain set of target genes in response to superoxide. However, the similarity to other SoxR proteins is limited and higher similarity is found to CueR proteins of e.g. *E. coli* with a role in copper resistance. In *E. coli* CueR senses the intracellular copper concentration and activates the copA gene at elevated copper levels. The CopA protein transports copper ions from the cytoplasm to the periplasm (Petersen & Møller, 2000). Here we show that the RSP_2889 gene product affects copper homeostasis of *R. sphaeroides*, confirming the more recent annotation as CueR. Furthermore we show that CueR binds to the RSP_2890 (Cu-translocating P-type ATPase; copA) and RSP_2891 (Cu-chaperone; copZ) promoter regions and activates these genes in response to high copper concentrations.

METHODS

**Bacterial strains, plasmids and growth conditions.** Strains and plasmids used in this study are listed in Supplementary Table S1, available with the online version of this paper.
Construction of a \textit{R. sphaeroides} cueR deletion mutant. \textit{R. sphaeroides} strain 2.4.1\textit{ΔcueR} was generated by transferring the suicide plasmid pPHU2.4.1\textit{ΔcueR}:Km into \textit{R. sphaeroides} 2.4.1, and screening for insertion of the kanamycin-resistance cassette into the chromosome by homologous recombination. Briefly, parts of the \textit{cueR} gene of \textit{R. sphaeroides} 2.4.1, together with upstream and downstream sequences, were amplified by PCR using oligonucleotides 9RSP2889downBSphI (5'-AGG CAA ATG TCT AGG CGG CCC G-3') and 9RSP2889downABam (5'-AGG CAA ATG TCT AGG CGG CCC G-3') and were added to the liquid or solid growth medium at the following concentrations: kanamycin 25 µg ml\(^{-1}\), tetracycline 2 µg ml\(^{-1}\) (for \textit{R. sphaeroides}); and ampicillin 200 µg ml\(^{-1}\), tetracycline 20 µg ml\(^{-1}\) (for \textit{E. coli}).

**Inhibition zone assays.** For these assays cultures were grown microaerobically overnight at 32 °C and then diluted to an OD_{600} of 0.2. Cultures were grown to an OD_{600} of 0.4 and 200 µl aliquots of culture were mixed with 5 ml prewarmed top agar (0.8 %, w/v, agar) and layered onto plates of malate minimal salt medium. A 0.55 cm filter disc containing 5 µl CuSO\(_4\) solution was placed on the hardened top agar. The plates were incubated for 3 days at 32 °C in the dark. After incubation the diameter of the inhibition zone was measured to determine the sensitivity of the strains to the agent. The assay was performed at least three times.

**Extraction of RNA and quantitative real-time RT-PCR.** Cell samples from growth experiments were rapidly cooled on ice and harvested by centrifugation at 10 000 g in a cooled centrifuge. Total RNA was isolated by the Total RNA Isolation Reagent (TRIR, Thermo Fisher Scientific) as described by the manufacturer. Samples were treated with 1 unit of RNase-free DNase I (Invitrogen) per 1 µg RNA to remove contaminating DNA. After DNase I treatment, the RNA was purified by standard procedures using phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol before precipitating with sodium acetate and 2-propanol. Contamination with remaining DNA was checked by PCR amplification of RNA samples using primers targeting \textit{gaaB} (RSP_0799-A, 5'-GAA CAA TTA CGC CCT CTC-3'; RSP_0799-B, 5'-CAT CAG CTG GTA GCT CTC-3') as described previously (Glaeser & Klug, 2005).

Oligonucleotides used for gene amplification are listed in Supplementary Table S2. Conditions for real-time RT-PCR were described earlier in detail (Glaeser & Klug, 2005). A final concentration of 4 ng µl\(^{-1}\) of total RNA was used in a one-step RT-PCR kit (Qiagen). For detection of double-stranded DNA, SYBR Green I (Invitrogen) was added in a final dilution of 1:50 000 to the master mix. For normalization of mRNA levels the \textit{rpoZ} gene, which encodes the \textit{rpoZ} subunit of RNA polymerase of \textit{R. sphaeroides}, was used (Gomelsky et al., 2003). Expression of target genes was calculated relative to the expression of untreated samples and relative to \textit{rpoZ} (Pfaffl, 2001). PCR efficiencies were determined experimentally using serial dilutions of RNA at a final concentration of between 8 and 0.5 ng µl\(^{-1}\) (Supplementary Table S3).

**5’RACE.** For the determination of 5’ mRNA ends using 5’ rapid amplification of cDNA ends (5’RACE), 2–4 µg total RNA isolated from wild-type cells after 20 min of copper stress was reverse transcribed into cDNA by using avian myeloblastosis virus reverse transcriptase (Promega) and gene-specific primers (2890\_RACE1 and 2891\_RACE1; see Table S2). The 5’ RACE protocol was performed as described previously (Nuss et al., 2009).

**Expression and isolation of the \textit{R. sphaeroides} CueR protein.** The plasmid pPHU2.4.1\textit{ΔcueR} was ligated into the over-expression vector pASK-IBA3 (IBA) to generate pASK2.4.1\textit{ΔcueR}, which was transformed into \textit{E. coli} JM109. Afterwards the plasmid containing \textit{cueR} was digested with \textit{Bsal} and the purified \textit{cueR} fragment was ligated into the over-expression vector pASK-IBA3 (IBA) to generate pASK2.4.1\textit{ΔcueR}, which was transformed into \textit{E. coli} JM109. The correct construct was transformed into \textit{E. coli} BL21(DE3) for overexpression of Strep-tagged CueR. For this purpose BL21(DE3/pASK2.4.1\textit{ΔcueR}) was grown in 50 ml Luria–Bertani medium at 37 °C to an OD_{600} of 0.5–0.6 and then was shifted to 20 °C. The cells were induced with 200 ng anhydrotracyclamycin ml\(^{-1}\) for 15 h at 20 °C. Following harvest, cells were resuspended in ice-cold washing buffer (100 mM Tris pH 8.0, 150 mM NaCl) and disrupted by brief sonication. The lysate was centrifuged at 13 000 r.p.m. and 4 °C for 15 min. The clear supernatant was loaded onto Strep-Tactin Sepharose (IBA) and incubated at 4 °C for 1 h. Proteins were washed with washing buffer and were eluted with desthiobiotin (2.5 mM), a specific competitor which displaces the Strep-tag. Aliquots of the fractions were analysed on 15 % SDS-polyacrylamide gels, and fractions containing CueR protein were used for the experiments described below.
Electrophoretic mobility shift assays (EMSAs). Binding of the recombinant CueR protein to the upstream regions of RSP_2890 and RSP_2891 was determined by an EMSA. As control a DNA fragment containing the katE promoter region was used. The following oligonucleotides were used to generate DNA fragments containing the respective promoter region by PCR: RSP_2890, RSP2890up_fwd (5’-GTT AAC GCA CAG CCC GGA TGG C-3’) and RSP2890up_rev (5’-GCT CGT TTA ACC CCA CGC AG-3’); RSP_2891, RSP2891up_fwd (5’-GTT AAC GTC GCG TGG TCC GGG-3’) and RSP2891up_rev (5’-GTC CGT TAA ACC GAG CCC -3’). The PCR fragments with a length of 169 and 224 bp for RSP_2890 and RSP_2891, respectively, were cloned into pDrive cloning vector (Qagen), and isolated from the vector by using the restriction enzyme HinClI. In the case of katE the plasmid pkatEup, which contains a 352 bp fragment of the upstream region of katE (Zeller & Klug, 2004), was used. The fragment was isolated from the pDrive cloning vector by using the restriction enzymes BamHI and PstI. The restricted DNA fragments were then radioactively end-labelled with [γ-32P]ATP using T4 polynucleotide kinase (Fermentas).

Binding reactions were carried out in a final volume of 15 μl and contained an appropriate amount of protein, [γ-32P]ATP-labelled DNA probe (5000 c.p.m.), salmon sperm DNA (1 μg), and 7.5 μl of a 2× binding buffer (50 mM Tris/HCl pH 8.0; 100 mM KCl; 12.5 mM MgCl2; 20 %, v/v, glycerol; 1 mM DTT). Binding incubations were carried out for 20 min at room temperature before the samples were loaded onto a 4 % polyacrylamide gel in 1× TB buffer (89 mM Tris/HCl pH 8.0; 100 mM KCl; 12.5 mM MgCl2; 50 mM HCl PH 6.8, 89 mM boric acid) and run at 180 V for 2 h at room temperature.

RESULTS

The RSP_2889 gene product shows similarity to bacterial CueR proteins

After the genome sequence of R. sphaeroides 2.4.1 was completed, the RSP_2889 gene product was initially annotated as SoxR. SoxR is an activator of genes with a role in oxidative stress defence in E. coli and other bacteria (Greenberg et al., 1990). However, the R. sphaeroides RSP_2889 gene product shows only 24 % sequence identity to SoxR of E. coli. Notably, two of the four cysteines that are involved in coordination of the Fe–S centre are missing. However, the R. sphaeroides RSP_2889 gene product shows 47 % identity to the CueR protein of E. coli. Especially the N-terminal helix–turn–helix motif is highly conserved among different bacterial CueR proteins as well as the two cysteines near the C-terminus, which are involved in copper coordination. On the R. sphaeroides chromosome the cueR gene is located next to the genes for a putative Cu-transporting ATPase (RSP_2890; copA), a putative Cu-chaperone (RSP_2891; copZ) and a hypothetical protein (RSP_2892) (Fig. 1a). Unlike the situation in E. coli cueR is positioned directly downstream of copA in R. sphaeroides. In E. coli cueR and copA are divergently transcribed and separated by two open reading frames which seem to have no role in copper homeostasis (Petersen & Møller, 2000) (Fig. 1b). A Cu-chaperone has not been identified in E. coli up to now.

CueR of R. sphaeroides regulates expression of RSP_2890–92 in response to copper in a concentration-dependent manner

In order to elucidate the function of the RSP_2889 gene product, strain 2.4.1ΔcueR, which has this gene deleted from the chromosome, was constructed as described in Methods. Due to its position next to RSP_2890 (copA), RSP_2891 (copZ) and RSP_2892 (Fig. 1a) the CueR homologue RSP_2889 of R. sphaeroides could function as

Fig. 1. Organization of the R. sphaeroides RSP_2890 region (a) and the E. coli cueR region (b). Genes are represented by large arrows. DNA fragments used for gel-shift experiments are marked by small arrows. In R. sphaeroides, RSP_2890 (CueR homologue) is located on the minus strand as well as RSP_2890 (putative Cu-translocating ATPase; copA), whereas RSP_2891 (putative Cu-chaperone; copZ) and RSP_2892 (hypothetical protein) are located on the plus strand. In E. coli, cueR (Cu-efflux regulator) and copA (Cu-translocating ATPase) are divergently transcribed and separated by two open reading frames which seem to have no role in copper homeostasis. A Cu-chaperone has not been identified in E. coli up to now.
a regulator of these genes. The relative expression of cueR was increased about 10-fold in R. sphaeroides wild-type after 20 min of copper stress (1 μM CuSO₄) as determined by real-time RT-PCR (data not shown). Furthermore, the relative expression of RSP_2890–2892 was quantified in response to copper and the effect of CueR on this regulation was analysed. The expression of all three genes was significantly induced in a concentration-dependent manner after addition of CuSO₄ (final concentration 0.5, 1 and 5 μM) to exponentially growing wild-type cultures (Fig. 2a–c). The relative increase in expression of RSP_2890 (copA) and RSP_2892 was even higher than that of RSP_2891 (copZ). To determine whether the CueR protein of R. sphaeroides is involved in the regulation of the Cu-dependent expression of RSP_2890–92, the expression levels of these genes were also quantified in the cueR deletion strain after addition of 1 μM CuSO₄. The expression of all three genes was dependent on CueR (Fig. 3a–c; black bars). These data imply that the cueR gene product is important for regulation of copper-responsive genes in R. sphaeroides. In the complemented strain ΔcueR (pRKcueR) the expression reached wild-type-like levels in the case of RSP_2891 (copZ) (Fig. 3b; grey bars). The expression level of RSP_2890 (copA) and RSP_2892 was higher than in the mutant strain but much lower than in the wild-type (Fig. 3a, c; grey bars). Additionally, it was confirmed by real-time RT-PCR that the level of cueR transcript is increased about fivefold in the complemented mutant compared to the wild-type.

Because CueR belongs to a protein superfamily together with ZntR and MerR it was tested if RSP_2890–92 are also

![Fig. 2.](http://mic.sgmjournals.org) Relative expression of putative copper-responsive genes in R. sphaeroides wild-type after exposure to CuSO₄. Real-time RT-PCR was used to investigate the relative expression of RSP_2890 (copA) (a), RSP_2891 (copZ) (b) and RSP_2892 (c) in R. sphaeroides wild-type 7 and 20 min after incubation with 0.5 μM (grey bars), 1 μM (white bars) and 5 μM (striped bars) CuSO₄. Values were normalized to rpoZ and to the control at time point 0. The data represent the means ± SD of three independent experiments.

![Fig. 3.](http://mic.sgmjournals.org) Relative expression of putative copper-responsive genes after exposure to 1 μM CuSO₄. Real-time RT-PCR was used to investigate the relative expression of RSP_2890 (copA) (a), RSP_2891 (copZ) (b) and RSP_2892 (c) in R. sphaeroides wild-type (white bars), cueR mutant (black bars) and complemented mutant (grey bars) after 7 and 20 min incubation with 1 μM CuSO₄. Values were normalized to rpoZ and to the control at time point 0. The data represent the means ± SD of three independent experiments.
responsive to zinc and mercury. All three genes of the locus showed hardly altered expression levels after 20 min exposure to 50 μM or 250 μM ZnSO₄ (see Supplementary Fig. S1A–C). cueR expression was not changed under these conditions (data not shown). In the case of mercury the relative expression of copA, copZ and RSP_2892 was increased but not as strongly as under copper-stress conditions (Fig. S1A–C). The relative expression of cueR was increased about threefold in the R. sphaeroides wild-type after 20 min of 1 μM HgCl₂ compared to about 10-fold after 20 min of 1 μM CuSO₄ as determined by real-time RT-PCR (data not shown).

**CueR binds to the upstream regions of copA and copZ**

Promoters activated by MerR homologues, like CueR, typically have a 19 or 20 bp spacer region between the −35 and −10 recognition element of RNA polymerase (Brown et al., 2003). The genome of R. sphaeroides 2.4.1 was searched for putative CueR-target promoters with a consensus matrix based on the published CueR target copA promoter sequence of E. coli (Stoyanov et al., 2001). By applying the regulon analysis tool PRODORIC (Münch et al., 2003; Münch et al., 2005) only RSP_2890 (copA) and RSP_2891 (copZ) were detected as containing a possible CueR target promoter. Furthermore it has to be noted that the promoter regions of both genes (Fig. 4) contain an inverted repeat element similar to that of the CueR-regulated copA promoter from E. coli (Stoyanov et al., 2001). This supports the idea that copA and copZ may be regulated by the CueR homologue RSP_2889. To confirm a similar function of the RSP_2889 gene product of R. sphaeroides as for the transcriptional regulator CueR of other bacteria, the protein was expressed in a Strep-tagged version in E. coli. Size-exclusion chromatography revealed that the purified protein elutes as a dimer when comparing the elution volume of the protein to elution volumes of molecular mass standards (Fig. S2). The positions of the DNA fragments used for gel-shift experiments are marked in Fig. 1(a). As shown in Fig. 5(a) CueR binds with high affinity to a DNA fragment that carries the upstream region of copA, and binding was observed both with and without CuSO₄ addition in the presence of salmon sperm DNA as unspecific competitor. The DNA fragment did not show retardation in the gel when only BSA was added (Fig. 5a, lane 9). The mobility shift was reduced by adding unlabelled copA DNA fragment as competitor DNA (Fig. 5a lane 5). In the case of the upstream region of copZ the gel shift was less pronounced and a higher protein amount was needed to observe a gel shift (Fig. 5b). No binding of CueR was observed in the case of a DNA fragment containing the upstream region of katE, which demonstrates that the binding of CueR to copA and copZ is specific (data not shown).

To confirm that transcription initiates at the putative target sequences for the CueR-dependent genes copA and copZ, the 5′ ends of the mRNA were mapped by 5′RACE. The 5′RACE was performed with RNA extracts from wild-type cultures that were exposed to copper stress. cDNA synthesis was performed with gene-specific primers in the same reaction (Table S2). For both genes, specific products were PCR amplified (Fig. S3). 5′ ends were determined by sequencing the amplified DNA fragments copA and copZ after cloning them into pDrive cloning vector (Fig. S4). Putative −10 and −35 CueR target sequences were found for both genes upstream of the 5′ end of the respective PCR products (Fig. 4).

**Effects of copper on growth of strain 2.4.1ΔcueR lacking the cueR gene**

Inhibition zone assays were performed to compare the sensitivity of the 2.4.1ΔcueR mutant and of the isogenic wild-type strain 2.4.1 to CuSO₄. Strain 2.4.1ΔcueR was as sensitive to this chemical as the wild-type (Fig. 6) or the wild-type carrying plasmid pRK415 without insert (data not shown). When the cueR gene was expressed in trans in strain 2.4.1ΔcueR(pRKcueR), an even higher sensitivity to CuSO₄ was observed compared to the wild-type and the mutant strain (Fig. 6).

**Does CueR affect the oxidative stress resistance of R. sphaeroides?**

Since metal homeostasis is linked to oxidative stress (Touati, 2000) the sensitivity of the 2.4.1ΔcueR mutant to H₂O₂ and paraquat was analysed by inhibition zone assays and growth experiments (data not shown). The mutant strain was as sensitive as the wild-type to both reagents. In an additional approach, katE expression was quantified in the mutant and in the wild-type since katE responds to

---

**Fig. 4.** Determination of 5′ ends of the copA and copZ mRNA by 5′RACE. Determined 5′ ends are indicated and the putative −35 and −10 regions are printed in bold letters. The putative translational start is indicated by an asterisk. The dyad symmetrical sequence is marked with arrows.
increased levels of H₂O₂ or superoxide in the cell (Zeller & Klug, 2004). Indeed, the relative katE expression was strongly increased after 7 min of paraquat stress in both strains (data not shown). Interestingly, the basal level of katE was about two times higher in the 2.4.1ΔcueR mutant compared to the wild-type.

**DISCUSSION**

In this study, it was shown that the RSP_2889 gene product of the alpha-proteobacterium *R. sphaeroides*, which was originally annotated as SoxR, is closely related to CueR from *E. coli* and other proteobacteria as revealed by BLAST analysis (e.g. *Agrobacterium tumefaciens*: 54% sequence identity). Interestingly, RSP_2889 is localized next to genes for a putative Cu-transporting ATPase (RSP_2890; copA), a putative Cu-chaperone (RSP_2891; copZ) and a hypothetical protein (RSP_2892). The composition of this locus is unique among bacteria. Nevertheless it can be assumed that the gene product of RSP_2889 is a CueR rather than a SoxR protein because of its adjacency to putative copper-responsive genes.

In *E. coli* CueR regulates target genes that harbour promoter sequences with an unusual 19 or 20 bp spacer region between the 2₃₅ and 2₁₀ recognition element of RNA polymerase. Stoyanov et al. (2001) showed that this characteristic spacer region contains a sequence of dyad symmetry to which the CueR protein binds and seems to mediate a distortion of DNA, which favours formation of the open complex of RNA polymerase. The dyad sequence has 7 bp of perfect symmetry separated by 7 bp of non-symmetrical sequence (Stoyanov et al., 2001). In *R. sphaeroides* the neighbouring gene of cueR, RSP_2890, also contains a 19 bp spacer region with a dyad symmetrical sequence similar to copA of *E. coli* but with less perfect symmetry. BLAST analysis reveals a high similarity of the gene product of RSP_2890 to CopA of *E. coli* (40% sequence identity) and *A. tumefaciens* (58% sequence identity). In the case of RSP_2891 only an 18 bp spacer region was found but with a similar sequence of dyad symmetry as found for RSP_2890. The gene product of RSP_2891 is thought to be a Cu-chaperone similar to CopZ of *A. tumefaciens* (Nawapan et al., 2009) and *Bacillus subtilis* (Banci et al., 2001). For RSP_2892 no sequence similarity could be found to proteins of bacilli and proteobacteria. Notably copZ (RSP_2891) and RSP_2892 are on the same transcript as determined by RT-PCR (data not shown). We were able to demonstrate that a Strep-tagged version of the CueR homologue RSP_2889 specifically binds to a DNA fragment carrying the upstream

---

**Fig. 5.** Binding of purified CueR to the promoters of *copA* (a) and *copZ* (b) as determined by EMSAs. All reactions contained the same amount of ³²P end-labelled DNA fragment (5000 c.p.m. per lane) including the promoter sequence. (a) Binding of CueR to the promoter region of *copA* (169 bp). Lanes 1 and 2 contain no CueR; lanes 3–5 contain 0.3 μg CueR; lanes 6–8 contain 0.4 μg CueR; lane 9 contains 0.4 μg BSA. Reactions contained 50 μM CuSO₄ as indicated. Lanes 5 and 8 contain 80 ng unlabelled DNA fragment *copA* as competitor. (b) Binding of CueR to the promoter region of *copZ* (224 bp). Lane 1 contains no CueR; lane 2 contains 0.4 μg CueR; lane 3 contains 0.6 μg CueR; lane 4 contains 0.8 μg CueR. To all four reactions 50 μM CuSO₄ was added. All reactions contained 1 μg salmon sperm DNA as unspecific competitor. The asterisks and arrows show the location of free and CueR-bound ³²P end-labelled DNA fragments, respectively.

**Fig. 6.** Sensitivity of the 2.4.1ΔcueR mutant to CuSO₄: inhibition of growth of 2.4.1 wild-type (white bars), cueR deletion mutant (black bars) and complemented mutant (grey bars). Each bar represents the mean ± SD of at least three independent experiments.
region of copA. The binding was not dependent on the addition of CuSO₄, and specific competitor DNA could reduce the gel shift. For the binding of CueR to the upstream region of copZ more protein was needed, indicating that the binding is weaker. The 5' ends of both genes were mapped to an appropriate distance from the putative −35 and −10 region. Thus, it can be concluded that they indeed represent transcriptional start sites. Gel filtration showed that purified CueR elutes as a dimer, which is typical for transcriptional regulators, but it did not co-purify with copper or other metals already bound as measured by ICP-MS.

Furthermore, a copper-dependent induction of RSP_2890–92 was observed in the wild-type 2.4.1, which is dependent on cueR. The relative expression of cueR was also induced upon copper stress. The expression of the whole gene locus was also increased under mercury stress conditions but to a lesser extent compared to copper stress. The response to zinc was even lower. Thus, we conclude that RSP_2889 is a CueR homologue that regulates putative copper-responsive genes by binding to its specific target sequence. The complemented strain showed wild-type-like levels of copZ mRNA. This was however not the case for copA and RSP_2892. Although the complemented strain exhibited higher levels of copA and RSP_2892 mRNA than the mutant, expression was clearly lower than in the wild-type. Notably the basal level of the cueR transcript is higher in the complemented mutant compared to wild-type. Thus, it can be assumed that balanced cueR levels are important for proper regulation of RSP_2890–92.

When testing the tolerance of the 2.4.1ΔcueR mutant to CuSO₄ no changed sensitivity was observed compared to the wild-type. R. sphaeroides harbours more than one heavy metal translocating ATPase, which may function as backup systems if the cue system is not working properly. Up to now no other copper resistance systems have been annotated in R. sphaeroides which are similar for example to the cus system of E. coli and which could function as backup systems. It is conceivable that an inactivation of copA (RSP_2890) leads to a more severe phenotype than the deletion of the transcriptional regulator cueR (RSP_2889), because other studies have shown a higher copper sensitivity of mutants lacking copA compared to mutants lacking cueR (Petersen & Möller, 2000). The observed higher sensitivity of the complemented mutant strain may be due to the fact that pRK415 is a low-copy-number plasmid. The complemented strain harbours more than one copy of cueR and apparently the level of cueR transcript is important for proper regulation. Furthermore the disruption of cueR did not lead to a changed phenotype under oxidative stress, but to a higher basal level of katE that may protect cells from oxidative damage.

This study has revealed that the gene product of RSP_2889, previously annotated as SoxR, is a homologue of CueR and proved that it is involved in copper homeostasis in R. sphaeroides. The unchanged sensitivity of the cueR deletion strain to copper indicates that further factors are involved in the regulation of copper homeostasis and that periplasmic or even outer-membrane proteins may be involved in the export of copper in R. sphaeroides.

ACKNOWLEDGEMENTS

We thank Tanja Zeller and Mobarak Mraheil for initial cloning steps, Sebastian Metz for useful help with the DNA gel shifts, Tom Rische for performing size-exclusion chromatography and Sascha Setzer and Heike Weller for ICP-MS analysis. This work was partly supported by the Deutsche Forschungsgemeinschaft (KI563/25-1) and by a fellowship from Justus Liebig University Giessen to V.P.

REFERENCES


Edited by: D. H. Nies