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

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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. Pseudomonas putida (Adaikkalam & Swarup, 2002), Rhizobium leguminosarum (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.

Rhodobacter 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, anoxygenic 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 a 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.
E. coli strains were cultivated in Luria–Bertani medium at 37 °C with shaking (180 r.p.m.) or on solid growth medium, which contained 1.6% (w/v) agar. R. sphaeroides strains were grown at 32 °C in a malate minimal salt medium (Drews, 1983). Microaerobic cultures were grown in Erlenmeyer flasks up to a culture volume of 80% with shaking at 140 r.p.m. For aerobic growth during copper-stress experiments cultures were gassed with air. When required, antibiotics were added to the liquid or solid growth medium at the following concentrations: kanamycin 25 µg ml⁻¹, tetracycline 2 µg ml⁻¹ (for R. sphaeroides); and ampicillin 200 µg ml⁻¹, tetracycline 20 µg ml⁻¹ (for E. coli).

**Construction of a R. sphaeroides cueR deletion mutant.** R. sphaeroides strain 2.4.1 cueR was generated by transferring the suicide plasmid pPHU2.4.1cueR⁺; Km into R. sphaeroides 2.4.1, and screening for insertion of the kanamycin-resistance cassette into the chromosome by homologous recombination. Briefly, parts of the cueR gene of R. sphaeroides 2.4.1, together with upstream and downstream sequences, were amplified by PCR using oligonucleotides RSP2889upBBam (5'-GAA CGC ATG CCC GCT GAC GCC C-3') and RSP2889downBSphI (5'-CGC GGC CCT CTA-3'). The amplified PCR fragments were cloned into the EcoRI-BamHI and BamHI-SphI sites of the suicide plasmid pPHU281 generating the plasmid pPHU2.4.1cueR⁺. A 1.3 kb BamHI fragment containing the kanamycin-resistance cassette from pUC4K was inserted into the BamHI site of pPHU2.4.1cueR⁺ to generate pPHU2.4.1cueR⁺; Km. This plasmid was transferred into E. coli strain S17-1 and biparentally conjugated into R. sphaeroides 2.4.1 wild-type. Conjugants were selected on malate minimal salt agar plates containing 15 µg ml⁻¹ of kanamycin, 10 µg ml⁻¹ tetracycline and 25 µg ml⁻¹ of kanamycin. Southern blot analysis of chromosomal DNA was carried out to confirm the double-crossover event of the kanamycin-resistance cassette into the R. sphaeroides chromosome (data not shown).

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'-GTC CGT TTA ACG CCA CGC AG-3'); RSP_2891, RSP2891up_fwd (5'-GTT AAC GTG GGG TCC GGG-3') and RSP2891up_rev (5'-GTC CGT TAA TAC GCG AGC 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 (Qiagen), and isolated from the vector by using the restriction enzyme Hincl. 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 [α-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, [α-32P]ATP-labelled DNA probe (5000 c.p.m.), salmon sperm DNA (1 μg), and 7.5 μl of a 2 x 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 1x TB buffer (89 mM Tris/HCl pH 8.0, 6.7 mM sodium acetate, 1 mM EDTA) 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 Cue-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...
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

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**Fig. 2.** 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.** 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 mutant strain (Fig. 6). Since metal homeostasis is linked to oxidative stress (Touati, 2000) the sensitivity of the 2.4.1 ΔcueR mutant and of the isogenic wild-type strain 2.4.1 to CuSO₄. Strain 2.4.1 ΔcueR 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 2.4.1 ΔcueR(pRKcueR), an even higher sensitivity to CuSO₄ was observed compared to the wild-type and the mutant strain (Fig. 6).

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

Inhibition zone assays were performed to compare the sensitivity of the 2.4.1 ΔcueR mutant and of the isogenic wild-type strain 2.4.1 to CuSO₄. Strain 2.4.1 ΔcueR 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 2.4.1 ΔcueR(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 2.4.1 ΔcueR 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
increased levels of H$_2$O$_2$ 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 Streptagged version of the CueR homologue RSP_2889 specifically binds to a DNA fragment carrying the upstream
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.

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