CsoR regulates the copper efflux operon copZA in Bacillus subtilis

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The adaptation of Bacillus subtilis to elevated levels of copper ions requires the copper-inducible copZA operon encoding a copper chaperone and efflux ATPase. Here we identify CsoR (formerly YvgZ) as the copper-sensing repressor that regulates the copZA operon. CsoR binds with high affinity to an operator site overlapping the copZA promoter and its binding is specifically inhibited by copper salts. As previously described, the YhdQ (CueR) protein also binds to the copZA regulatory region, but genetic experiments indicate that this protein is not responsible for the copper-dependent regulation of this operon.

INTRODUCTION

Metals are essential cofactors for many enzymatic reactions within the cell. Yet, at high concentrations, many metals become toxic. Toxicity can arise from metal ions binding inappropriately to metal-binding sites in enzymes, thereby inhibiting activity, or by the generation of reactive oxygen species that can damage the genetic, enzymic and structural components of the cell (Imlay, 2002; Touati, 2000). Thus, it is vitally important for the cell to closely regulate metal concentrations within the cytosol. When limited for metal ions, many bacteria express high-affinity uptake systems. Conversely, when metals are in excess the cell will often express specific efflux systems or metal-storage proteins. The regulated expression of metal homeostasis mechanisms, including both uptake and efflux systems, is controlled by metalloregulatory proteins that sense metal availability within the cytosol.

Copper is an essential element for Bacillus subtilis aerobic respiration because it is a cofactor for haem-copper oxidases, the terminal enzymes in the respiratory pathway (Mattatall et al., 2000). The pathways responsible for the uptake of copper are not yet defined, but copper present in the cytosol appears to be tightly chaperoned. The Sco chaperone characterized in yeast, humans and bacteria (YpmQ in B. subtilis) mediates the insertion of copper into the CuA copper centre of cytochrome c oxidase (Mattatall et al., 2000). It has also recently been suggested that Sco may play a role in the insertion of copper into the CuB centres of the haem-copper oxidases, and may play a more general role as a copper chaperone for other metalloproteins (Banci et al., 2003).

When copper is present in excess, a specific efflux system encoded by the copZA operon is induced (Gaballa & Helmann, 2003). Strong induction of copZA was noted in the presence of copper salts, but not other metals tested, and the CopZA system was shown to be required for resistance to high levels of copper (Gaballa & Helmann, 2003). CopZ functions as a copper chaperone to deliver copper to CopA, a CPx-type efflux ATPase (Banci et al., 2001; Radford et al., 2003). Structural analyses have allowed visualization of B. subtilis Sco, CopZ and the N-terminal domain of CopA, thereby affording a detailed look at the molecular mechanics of copper homeostasis in this model organism (Balatri et al., 2003; Banci et al., 2001, 2003).

The regulatory proteins controlling the expression of these copper homeostasis proteins are not as well defined. Previously, we identified a MerR-type regulatory protein (YhdQ; previously renamed CueR) as a candidate regulator for the copZA operon (Gaballa et al., 2003). Here, we provide evidence that this assignment was incorrect: although YhdQ does bind to the copZA regulatory region (P<sub>copZA</sub>) in vitro, this binding may not be physiologically relevant. Our previously reported genetic studies linking yhdQ to the copper-dependent induction of copZA were incorrect due to an inadvertent error in strain construction. Here, we present evidence that YvgZ, an orthologue of the recently described copper-sensing metalloregulator CsoR from Mycobacterium tuberculosis (Liu et al., 2007), is the copper-sensing regulator of the copZA operon.

METHODS

Bacterial strains, media and growth conditions. Bacterial strains used in this study are listed in Table 1. Escherichia coli DH5x was used for routine DNA cloning (Sambrook et al., 1989). Liquid media were inoculated from overnight pre-culture and incubated at 37 °C with shaking at 225 r.p.m. Erythromycin (1 μg ml<sup>-1</sup>) and lincomycin (25 μg ml<sup>-1</sup>) [for MLS (macrolide-lincosamide-streptogramin B) resistance], spectinomycin (100 μg ml<sup>-1</sup>), kanamycin (10 μg ml<sup>-1</sup>),
neomycin (10 μg ml⁻¹) and chloramphenicol (5 μg ml⁻¹) were used for the selection of various B. subtilis strains. To determine growth in the presence of copper, strains were cultivated overnight in LB, diluted 1:100 in 5 ml LB, and grown to mid-exponential phase (OD₆₀₀ ~0.4). Three microtitre plates of cultures were inoculated into 197 μL LB containing CuSO₄ from 0 to 6 mM in a 100-well microtitre plate and growth was monitored after overnight growth with shaking at 37 °C using a BioScreen C plate reader.

**DNA manipulations.** Routine molecular biology procedures were performed according to Sambrook et al. (1989). Transformation and specialized SPβ transduction were performed as described by Cutting & Vander Horn (1990). Restriction enzymes, DNA ligase and T4 PNK were all used according to the manufacturer’s instructions (New England Biolabs).

**Strain construction.** Null mutants were generated by allelic replacement via a modified long flanking homology PCR protocol (Wach, 1996; Gaballa et al., 2003). The resulting PCR products were purified and introduced by transformation into B. subtilis wild-type strain CU1065 or appropriate mutant strain with appropriate antibiotic selection. Mutants generated in this study are listed in Table 1.

**β-Galactosidase assay.** The PₐcopZA-cat-lacZ operon fusion, carried on the SPβ prophage, was introduced by specialized transduction. Overnight cultures were diluted 1:100 in LB liquid medium with or without 0.5 mM CuSO₄ and grown to mid-exponential phase. Cells were collected and the expression of β-galactosidase was measured (modified from Miller, 1972).

**Purification of CsoR.** The yvgZ (csoR) gene was PCR amplified and inserted into the Ncol and the BamHI sites of the overexpression vector pET16b (Novagen). This was then introduced into E. coli DH5α by transformation. The sequence of the resulting plasmid (pGS001) was verified by DNA sequencing (Cornell Life Sciences Core Laboratories Center) and was introduced into E. coli BL21 (DE3)(pLysS). A single colony was grown overnight in 5 ml LB containing ampicillin (100 μg ml⁻¹). The overnight culture was used to inoculate 1 litre of LB containing ampicillin (100 μg ml⁻¹). Cells were incubated with vigorous shaking until an OD₆₀₀ of 0.4 (Milton Roy Spectronic 21) was reached, at which point IPTG was added to a final concentration of 4 mM, and the cells were allowed to grow an additional 2 h. Cells were recovered by centrifugation, resuspended in buffer A (20 mM Tris/HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 %, v/v, glycerol), and sonicated. The extract was clarified by centrifugation and then purified by sequential application to heparin-Sepharose, mono-Q ion-exchange and Superdex-200 size-exclusion columns. Purified protein was stored in Buffer A at −80 °C for later use. CsoR purity was determined to be >95 % by SDS-PAGE with Coomassie staining. YhdQ had been purified previously during studies (Gaballa et al., 2003).

**Electrophoretic mobility shift assays (EMSAs).** PCR fragments containing the copZA promoter (246 bp) and the control non-specific yoeB promoter (106 bp) were amplified and labelled with T4 polynucleotide kinase (PNK) and [γ-³²P]ATP. EMSA reactions were carried out in 10 μL EMSA buffer (20 mM Tris/HCl pH 8.0, 50 μg BSA ml⁻¹, 50 mM NaCl, 1 mM DTT, 5 μg salmon sperm DNA ml⁻¹, 5 %, v/v, glycerol). Increasing concentrations of CsoR and YhdQ were incubated for 10 min at room temperature with the labelled promoters in both the presence and absence of 10 μM CuSO₄ (as indicated). DTT was added to the reaction to reduce Cu(II) to Cu(I) (Banci et al., 2001). The copZA promoter was digested with BstI and the control non-specific yoeB promoter with EcoRI where indicated. All samples were loaded onto a 6 % polyacrylamide gel and electrophoresed for 1 h at 90 V in 45 mM Tris/borate buffer (without EDTA), pH 8.0. The gel was dried and imaged on a Storm 840 PhosphorImager scanner (Molecular Dynamics) after overnight exposure of a PhosphorImager screen.

**DNase I footprinting.** Oligonucleotide primers labelled with T4 PNK and [γ-³²P]ATP were used to generate a 246 bp PcopZA fragment. PCR with the labelled forward or reverse primer (and a second,
unlabelled primer) was used to generate labelled fragments. Footprinting was carried out in 50 μl EMSA buffer. CsoR was added in increasing amounts to the top- or bottom-strand end-labelled PCR product and incubated at room temperature for 20 min. After this binding incubation, 53 μl DNase I reaction mixture (0.06 units DNase I μl⁻¹, 5 mM CaCl₂, 10 mM MgCl₂) was added to digest the labelled DNA. Digestion was performed at room temperature for 2 min and stopped by precipitation of the DNA with 645 μl −20 °C absolute ethanol, 50 μl 3 M sodium acetate and 5 μl 1 mg yeast carrier RNA ml⁻¹ at −20 °C for 20 min. DNA was collected by centrifugation, washed with cold 70 % ethanol, and the dried pellets were dissolved in 7.5 μl formamide loading buffer. Samples were incubated at 90 °C for 3 min before loading. The G+A ladder was generated by adding 1 μl labelled promoter to 3 μl formamide loading buffer with 1 % formic acid added; the reaction was incubated at 90 °C for 20 min. Then 3 μl G+A ladder and 7.5 μl of the DNase I footprinting reactions were loaded onto a 6 % polyacrylamide sequencing gel with 6 M urea pre-run in 0.5× TBE electrophoresis buffer at 1500 V for 40 min. The gel was run for 1 h at 1500 V, dried, and imaged on a Storm 840 PhosphorImager scanner (Molecular Dynamics) after overnight exposure of a PhosphorImager screen.

RESULTS AND DISCUSSION

CsoR negatively regulates the copZA operon

The copZA operon is situated downstream of a candidate σ²⁸ promoter sequence with an overlapping GC-rich pseudo-inverted repeat (Fig. 1). As noted previously, PcopZA has features similar to promoters regulated by MerR family transcription factors, including a longer than average spacer sequence and an inverted repeat element in the spacer region similar to known MerR-binding sites. These observations led us to investigate the role of MerR-like proteins as candidate regulators for copZA. Previously, we reported that a disruption of yhdQ resulted in reduced expression levels of a PcopZA reporter fusion relative to levels in a wild-type background. Together with biochemical studies that demonstrated binding of YhdQ to PcopZA we concluded that this protein mediated copper induction of copZA and we proposed to rename yhdQ as cueR (Gaballa et al., 2003). This assignment was supported by the limited similarity (17 % identity) between B. subtilis YhdQ and E. coli CueR (Outten et al., 2000; Stoyanov et al., 2001). However, we demonstrate here that yhdQ is not involved in the copper-dependent regulation of copper efflux functions, and we will henceforth refer to this gene as yhdQ (as presently annotated in the SubtiList database; Moszer et al., 1995) rather than cueR.

In the course of follow-up studies to determine the structural features of YhdQ required for copper sensing, we were unable to reproduce the previously observed defects in induction of the PcopZA reporter fusion in strains lacking YhdQ. Analysis of the original strains revealed an error in strain construction: in the course of introducing reporter fusions by phage transduction, the wrong promoter fusion had been introduced into the yhdQ null background. In our newly constructed strains, a yhdQ null mutation did not affect copper inducibility of PcopZA (Fig. 2a).

Concurrent with this discovery, a report appeared describing a new family of copper-sensing regulatory proteins designated CsoR (Liu et al., 2007). The prototype for this family of metalloregulatory proteins is the M. tuberculosis CsoR protein, which functions as a Cu(I)-selective repressor protein and regulates expression of copper efflux systems. Giedroc and colleagues noted that apparent CsoR orthologues are present in many bacteria and they speculated that these might also play a role in regulating copper homeostasis (Liu et al., 2007). The predicted CsoR orthologue in B. subtilis is encoded by the yvgZ gene, which is located immediately upstream of the copZA operon (Fig. 1). YvgZ of B. subtilis shares 33 % amino acid identity with M. tuberculosis CsoR, consistent with a similar functional role. More importantly, the

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**Fig. 1.** Schematic representation of the csoR–copZA locus. csoR (yvgZ) is located immediately upstream of the copZA operon and is transcribed from its own promoter. The yhdQ (cueR) gene is distant from the copZA operon. The promoter region between csoR and copZA has been expanded to illustrate the sequence alignment of B. subtilis (B.su), B. amyloliquefaciens (B.am), B. licheniformis (B.li), and M. tuberculosis (M.tb). Conserved −35 and −10 promoter elements are underlined. Bold letters represent the conserved GC-rich pseudo-inverted repeat. The identified +1 sites of transcription (Gaballa & Helmann, 2003; Liu et al., 2007) are designated by capital letters.
ligand-coordinating residues shown to be essential for copper-sensing in *M. tuberculosis* CsoR are strictly conserved in *B. subtilis* (C45, H70 and C74). Other conserved residues include Y44 and E90, which have been implicated in the allosteric modulation of the DNA-binding domain in response to bound copper (Liu et al., 2007). Previous studies had demonstrated that *yvgZ* is not co-transcribed with *copZA*, nor is it copper regulated (Gaballa & Helmann, 2003). We demonstrate that *yvgZ* is required for the copper-dependent regulation of the *copZA* efflux operon. Hence, we rename this gene *csoR*, to be consistent with the *M. tuberculosis* nomenclature (Liu et al., 2007).

To determine whether CsoR regulates the *copZA* operon and to investigate the role of YhdQ, a series of allelic replacement mutations were constructed (Table 1) and tested for their effect on copper induction of a P<sub>copZA</sub>-cat-lacZ promoter fusion (Fig. 2a). In the csoR null mutant the *copZA* promoter was expressed constitutively (Fig. 2a). Complementation of the mutant strain with an ectopically integrated copy of the csoR gene restored wild-type regulation. These results suggest that CsoR functions as a repressor for P<sub>copZA</sub>. In contrast, induction of P<sub>copZA</sub> upon exposure to copper was unaffected in a yhdQ null mutant. Since YhdQ had been previously shown to bind the *copZA* regulatory region (Gaballa et al., 2003), we considered the possibility that both proteins might exert a regulatory influence over *copZA* expression. However, the csoR yhdQ double mutant was indistinguishable from the csoR single mutant (Fig. 2a).

Since a csoR null mutant expresses copper efflux functions constitutively, we hypothesized that this strain should have
a higher tolerance for copper shock. This expectation is supported by the observation that the null mutant has a slightly enhanced ability to grow relative to wild-type when diluted into medium containing high levels of copper (Fig. 2b); this is most apparent at concentrations between 5 and 6 mM copper. This effect is rather subtle, consistent with the fact that the copZA operon is probably induced even in the wild-type strain under these conditions. In contrast, a copA null mutant is unable to grow in medium containing 2 mM copper (Gaballa & Helmann, 2003).

**CsoR specifically binds P_{copZA} in the absence, but not the presence, of copper ions**

EMSA was used to determine if CsoR is acting as a copper-sensing repressor. CsoR bound with high affinity ($K_d \sim 50$ nM) to $P_{copZA}$, but not to the non-specific control fragment ($yoeB$), and this binding was eliminated in the presence of 10 μM CuSO$_4$ and 1 mM DTT (Fig. 3a). Under these conditions, DTT is known to reduce Cu(II) to Cu(I) (Banci *et al.*, 2001), the presumed inducer by analogy with *M. tuberculosis* CsoR (Liu *et al.*, 2007). In parallel EMSA reactions with YhdQ, significant binding was detected to $P_{copZA}$, as previously reported (Gaballa *et al.*, 2003). Moreover, this binding appeared to be of higher affinity than that for the non-specific control fragment ($yoeB$), suggesting that there is some specificity for the copZA promoter DNA fragment. However, the formation of several different mobility complexes (Fig. 3b) suggests that there may be multiple YhdQ oligomers bound to this DNA fragment. To further investigate the DNA-binding properties of YhdQ, EMSAs were conducted with two additional control DNA fragments: the copZA promoter region digested with *BstI* (to generate two fragments, one of which lacks the proposed specific binding site) and the non-copper-regulated $ytiA$ promoter digested with EcoRI. Both fragments produced a ladder of shifted complexes (Fig. 3c). Taken together, these results suggest that YhdQ binds to DNA in a relatively non-specific manner while CsoR binds specifically to the $P_{copZ}$ region with high affinity.

**CsoR binds to an inverted repeat overlapping the promoter of copZA**

Using DNase I footprinting, CsoR was found to protect a 25 nt region of the top strand, overlapping the copZA promoter. In agreement with the EMSA experiments, the protection of the promoter region is lifted once copper is supplied to the reaction (Fig. 4). This result is consistent with the role of CsoR as a repressor. Furthermore, CsoR

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**Fig. 4.** The CsoR-binding site overlaps the copZA promoter. (a) DNase I footprinting of the top strand of $P_{copZA}$ with increasing amounts of CsoR. The last lane contains 10 μM CuSO$_4$ in the presence of 1 mM DTT. The G+A ladder was run as a standard; it was calibrated to a dideoxy chain-termination sequencing reaction (data not shown). (b) Schematic of the *B. subtilis* copZA promoter. The $-10$ and $-35$ elements are underlined, the GC-rich pseudo-inverted repeat is in bold, and the protected region as determined by the DNase I footprinting is boxed.
proteins the same general DNA region on the bottom strand over the same range of protein concentrations (data not shown). It is interesting to note that this operator region in *B. subtilis* and its close relatives shares similarity with the *M. tuberculosis* csoR binding site (Fig. 1). This GC-rich pseudo-inverted repeat probably mediates CsoR binding and regulation.

**Conclusions**

In this study, we have identified the regulator of the copZA operon as CsoR (formerly YvgZ). We had previously assigned this role to YhdQ (CueR), a MerR homologue, proposed to function as an activator of copper efflux (Gaballa et al., 2003). This assignment was supported by the observed binding of YhdQ to the copZA regulatory region, the loss of *P*<sub>copZA</sub> induction in a strain carrying a *yhdQ* mutation, and weak similarity in protein sequence (17% identity) between *B. subtilis* YhdQ and the CueR regulator of copper efflux gene expression in *E. coli*. However, due to an error in strain construction, the previous genetic experiments were incorrect and we now report that there is no effect on copper-dependent regulation at the copZA promoter in a *yhdQ* mutant strain. Consistent with our previous findings (Gaballa et al., 2003), YhdQ does bind with high affinity to the copZA promoter region, but it also binds to several other promoter regions, suggesting that this binding is relatively non-specific and unlikely to be physiologically relevant. Our current results support a model for CsoR as the sole repressor of the copZA operon in *B. subtilis* that is responsible for the previously characterized, copper-specific induction of this efflux system (Gaballa & Helmann, 2003).

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**REFERENCES**


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