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 enzymic 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 cytoplasm. 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 Cu_A 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 Cu_B centres of the haem-copper oxidases, and may play a more general role as a copper chaperone for other metalloproteins (Banci *et al.*, 2007).

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*_{copZA}) *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* DH5 α 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 μ g ml⁻¹) and lincomycin (25 μ g ml⁻¹) [for MLS (macrolide-lincosamide-streptomycin B) resistance], spectinomycin (100 μ g ml⁻¹), kanamycin (10 μ g ml⁻¹),

Abbreviation: EMSA, electrophoretic mobility shift assay.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid		Relevant characteristic(s)	Source or reference
<i>B. subtilis</i>			
CU1065	W168 <i>att</i> SP β <i>trpC2</i>		Vander Horn & Zahler (1992)
HB7301	CU1065 <i>yhdQ::kan</i>		This study
HB7350	CU1065 <i>csor::spc</i>		This study
HB7351	CU1065 <i>yhdQ::kan csor::spc</i>		This study
HB7352	CU1065 <i>csor::spc</i> SP β (P _{copZA} - <i>cat-lacZ</i>) (MLS ^R Neo ^R)		This study
HB7353	CU1065 <i>yhdQ::kan csor::spc</i> SP β (P _{copZA} - <i>cat-lacZ</i>) (MLS ^R Neo ^R)		This study
HB7354	CU1065 <i>csor::spc amyE::csor-cat</i>		This study
HB7355	CU1065 <i>yhdQ::kan csor::spc amyE::csor-cat</i>		This study
HB7356	CU1065 <i>csor::spc amyE::csor-cat</i> SP β (P _{copZA} - <i>cat-lacZ</i>) (MLS ^R Neo ^R)		This study
HB7357	CU1065 <i>yhdQ::kan csor::spc amyE::csor-cat</i> SP β (P _{copZA} - <i>cat-lacZ</i>) (MLS ^R Neo ^R)		This study
HB7358	CU1065 SP β (P _{copZA} - <i>cat-lacZ</i>) (MLS ^R Neo ^R)		This study
HB7359	CU1065 <i>yhdQ::kan</i> SP β (P _{copZA} - <i>cat-lacZ</i>) (MLS ^R Neo ^R)		This study
<i>E. coli</i>			
DH5 α	ϕ 80 Δ (<i>lacZ</i>)M15 Δ (<i>argF-lac</i>)U169 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>deoR thi-1 supE44</i>		Sambrook <i>et al.</i> (1989)
BL21 (DE3)(pLysS)	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)/pLysS		Novagen
HE7307	BL21 pGS001		This study
HE8248	BL21 with pET16X containing <i>yhdQ</i> cloned into the <i>NcoI</i> and <i>BamHI</i> sites		Gaballa <i>et al.</i> (2003)
Plasmid			
pGS001	pET16b containing <i>csor</i> cloned into the <i>NcoI</i> and <i>BamHI</i> sites		This study

neomycin (10 μ g ml⁻¹) and chloramphenicol (5 μ g 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 microlitres of cells were inoculated into 197 μ l 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 SP β 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 P_{copZA}-*cat-lacZ* operon fusion, carried on the SP β 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 *NcoI* and the *BamHI* sites of the overexpression vector pET16b (Novagen). This was then introduced into *E. coli* DH5 α 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 μ g ml⁻¹). The overnight culture was used to inoculate 1 litre of LB containing ampicillin (100 μ g 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 during previous 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 μ l EMSA buffer (20 mM Tris/HCl pH 8.0, 50 μ g BSA ml⁻¹, 50 mM NaCl, 1 mM DTT, 5 μ g 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 μ M 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 *BtsI* and the control non-specific *ytiA* 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 P_{copZA} 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 μ l 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 μ l DNase I reaction mixture (0.06 units DNase I μ l⁻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 μ l -20 °C absolute ethanol, 50 μ l 3 M sodium acetate and 5 μ l 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 μ l formamide loading buffer. Samples were incubated at 90 °C for 3 min before loading. The G+A ladder was generated by adding 1 μ l labelled promoter to 3 μ l formamide loading buffer with 1 % formic acid added; the reaction was incubated at 90 °C for 20 min. Then 3 μ l G+A ladder and 7.5 μ l of the DNase I footprinting reactions were loaded onto a 6 % polyacrylamide sequencing gel with 6 M urea pre-run in 0.5 $\times$ 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 σ^A promoter sequence with an overlapping GC-rich pseudo-inverted repeat (Fig. 1). As noted previously, P_{copZA} 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 P_{copZA} reporter fusion relative to levels in a wild-type background. Together with biochemical studies that demonstrated binding of YhdQ to P_{copZA}

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 P_{copZA} 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 P_{copZA} (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

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 *copZ* 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.

Fig. 2. Expression of P_{copZA} is affected in a *csoR* null background. (a) Expression of P_{copZA} -cat-lacZ promoter fusion in different null backgrounds with (grey bars) and without (white bars) $CuSO_4$ added to a final concentration of 0.5 mM was measured using β -galactosidase assays. Dbl, $\Delta csoR$ $\Delta yhdQ$ double mutant; $\Delta csoR$ (+csoR), *csoR* null mutant complemented ectopically with *csoR*. Results presented are the means $\pm$ SD of three experiments where the total $n=7$. (b) Growth yield for wild-type (CU1065; white bars) and its isogenic *csoR* null mutant (grey bars) after overnight growth in medium either lacking (0) or supplemented with $CuSO_4$ at the indicated concentration.

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_{copZA} -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

Fig. 3. EMSAs. (a) CsoR EMSA was carried out with increasing amounts of protein and a constant amount of both specific *copZA* promoter and non-specific *yoeB* promoter; 10 μ M $CuSO_4$ in the presence of 1 mM DTT was added to the last lane to test the effects of copper on DNA-binding activity. (b) YhdQ EMSA with increasing amounts of protein and a constant amount of both specific *copZA* promoter and non-specific copper uninduced *yoeB* promoter. (c) YhdQ EMSA carried out with both specific *copZA* promoter and non-specific *ytiA* promoter. Both promoters were digested with either *BtsI* or *EcoRI* to generate a promoter specific fragment (upper band in '0' lane) and a non-specific fragment (lower band in '0' lane).

regulation. These results suggest that CsoR functions as a repressor for P_{copZA} . In contrast, induction of P_{copZA} 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 *BtsI* (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

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.

protects 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_{copZA} 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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