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Bacillus subtilis CPx-type ATPases: Characterization of Cd, Zn, Co and Cu efflux systems

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Abstract

Metal ion homeostasis requires the balanced expression of metal ion uptake systems, when metals are limiting, and corresponding efflux or storage systems, when metals are in excess. CPx-type ATPases are a family of membrane-bound transporters that often function to export toxic metals from cells. The *Bacillus subtilis* genome encodes three CPx-type ATPases: *zosA, yvgW* and *yvgX*. We demonstrate that *yvgW* and *yvgX* encode CadA and CopA, respectively, and that these genes function in metal ion resistance. A *cadA* mutant was sensitive to Cd(II), Zn(II) and Co(II), but not copper. Transcription of *cadA* initiates from a single, σ^A-type promoter and was induced by Cd(II), Zn(II), and Co(II). The adjacent *copZA* operon is expressed as a bicistronic transcript from a σ^A-type promoter and is selectively induced by copper. Mutation of either *copZ*, encoding a metallochaperone, or *copA* sensitizes the cells to copper but not to other metal ions.

Introduction

Metals play many important roles in bacterial metabolism as both enzyme cofactors and structural components of proteins. Since metal ions are often limiting for growth in natural settings, bacteria have evolved numerous high affinity transporters to import these essential nutrients. However, at high concentrations many metal ions can participate in undesirable redox reactions or can bind inappropriately to macromolecules leading to toxic effects. Thus, the intracellular concentration of heavy metal ions has to be tightly controlled, even for relatively low-toxicity metals such as zinc (Nies 1999; Outten & O'Halloran 2001). Resistance to many metals is mediated by a variety of chromosomal-, transposon- and plasmid-encoded resistance systems (for reviews: Silver & Phung 1996; Nies 1999; Bruins *et al.* 2000). Efflux systems represent the largest category of resistance systems and are often highly specific for the metal(s) that they export (Nies & Silver 1995).

Metal transport often involves P-type ATPases: ubiquitous, polytopic membrane proteins that share a conserved DKTGT motif (containing the aspartate residue that is phosphorylated during the catalytic cycle) and an ATP-binding motif (GDGXNDXP) (Palmgren & Axelsen 1998). Metal transport is typically mediated by the CPx sub-family of P-type ATPases, which can be further divided into monovalent and divalent cation transporters (Axelsen & Palmgren 1998; Rensing *et al.* 1999). CPx-type ATPases that transport copper have been characterized from human (Menkes and Wilson disease protein) (Bull *et al.* 1993; Vulpe *et al.* 1993), *Saccharomyces cerevisiae* (Fu *et al.* 1995), *Enterococcus hirae* (Solioz & Odermatt 1995), *Synechococcus* (Kanamaru *et al.* 1994), *Helicobacter pylori* (Ge *et al.* 1995), and *E. coli* (Petersen & Moller 2000; Rensing *et al.* 2000). Related Cd/Zn-ATPases have been characterized from *E. coli* (Rensing *et al.* 1997, 1998), *Staphylococcus aureus* (Tsai *et al.* 1992), *Helicobacter pylori* (Herrmann *et al.* 1999) and *Pseudomonas putida* (Lee *et al.* 2001).

Bacillus subtilis encodes four P-type ATPases. The *yloB* gene encodes a type IIA P-type ATPase similar to Ca^{2+} transport systems while the other three genes (*ykvW, yvgW* and *yvgX*) encode type IB (CPx-

type) metal-associated ATPases (Palmgren & Axelsen 1998). YkvW (re-named ZosA) was recently found to be a zinc uptake system expressed as part of the peroxide stress stimulon and under the regulation of *perR* (Gaballa & Helmann 2002). The predicted YvgW and YvgX proteins are > 45% identical in sequence to an *S. aureus* Cd efflux system and an *E. hirae* Cu efflux system, respectively (Tsai *et al.* 1992; Solioz, 1995). Indeed, *yvgW* (re-named *cadA*) was found to be important for Cd(II)-resistance and is induced by Cd(II) (Solovieva & Entian 2002). While physiological studies of the *yvgX(copA)* and *yvgY(copZ)* genes have not been reported, the corresponding proteins have been the subject of detailed structural investigation. The 3D structure of the N-terminal domain of CopA has been determined in both Cu-free and Cu-bound forms (Banci *et al.* 2002). The conserved CXXC motif of CopA can bind Cu(I) and interact with the metallochaperone, CopZ (Banci *et al.* 2001, 2002).

Here we demonstrate that in addition to Cd(II) (Solovieva & Entian 2002), *cadA* is also important for resistance to Zn(II) and Co(II) and is selectively induced at by these same metal ions. We also demonstrate that the adjacent *copZA* operon encodes proteins important for copper resistance, as predicted from the previous biochemical studies of copper binding (Banci *et al.* 2001, 2002). This operon is induced by copper, but not by other metal ions tested, in contrast with previously reported results (Solovieva & Entian 2002).

Material and methods

Media and growth conditions

B. subtilis CU1065 (Table 1) was grown on LB and metals were added from filter-sterilized stocks before the inoculation. *E. coli* DH5α was used for routine DNA cloning (Sambrook *et al.* 1989). Unless otherwise indicated, liquid media were inoculated from an overnight pre-culture and incubated at 37 °C with shaking at 200 rpm. Erythromycin (1 μg/ml) and lincomycin (25 μg/ml) (for testing macrolide-lincosamide-streptogramin B resistance), spectinomycin (100 μg/ml), kanamycin (10 μg/ml), neomycin (10 μg/ml), and chloramphenicol (5 μg/ml) were used for the selection of various *B. subtilis* strains.

DNA manipulation

Routine molecular biology procedures were done according to Sambrook *et al.* (1989). Isolation of *B. subtilis* chromosomal DNA, transformation and specialized SPβ transduction was done according to Cutting and Vander Horn (1990). Restriction enzymes, DNA ligase, Klenow fragment and DNA polymerase were used according to the manufacturer's instructions (New England Biolabs).

Construction of yvgW (cadA), yvgZ, yvgY (copZ), and yvgX(copA) transcription fusions

Promoter regions were amplified from the *B. subtilis* genome by PCR using primers 5′-GCGAAGCTTGGC TATAACAGCCTG-3′ and 5′-GCGGATCCAAACCG TCCAGAACATA-3′ for *yvgW*; primers 5′-TCCAAGC TTGATCGTATCCACTTTTCT-3′ and 5′-AATCGGA TCCTTTTCTTTCGAGCTT-3′ for *yvgZ*; primers 5′-GCGAAGCTTGAGGATCATGCCCATC-3′ and 5′-GCCGGATCCGCAATATCTTTGACTGAT-3′ for *yvgY* and primers 5′-GCGAAGCTTGTCAGCACTGC GTCAA-3′ and 5′-CGGGATCCTTTTGTTCACTCA ACATAC-3′ for *yvgX*. The resulting products were cloned as *Hin*dIII-*Bam*HI fragments (sites underlined) into pJPM122 (Slack *et al.* 1993) to generate the corresponding (promoter)-*cat-lacZ* operon fusions. The resulting plasmids were linearized with *Sca*I and transformed into ZB307A (Table 1) with selection for neomycin resistance. SPβ transducing lysates were prepared by heat induction and transduced to *B. subtilis* CU1065.

Construction of yvgW (cadA), yvgX(copA) and yvgY(copZ) mutants

Chromosomal DNA of the *yvgW* region was amplified by using the primers 5′-GCGGATCCTTTGCCTTG TTCTGAAT-3′ and 5′-GCGCATGCGGATCGCAA TTGACCCA-3′ and then digested with *Bam*HI and *Sph*I (sites underlined); the resulting fragment was cloned in pGEM3Zf(+)cat-1 (Youngman 1990). An internal *Sac*II-*Hinc*II fragment within yvgW was replaced by a gene cassette coding for spectinomycin resistance (Spc^r) that was isolated from pKF59 (Perego 1993) as a *Pvu*II-*Sac*II fragment. The resulting construct was linearized with *Sca*I and transformed into *B. subtilis* CU1065 selecting for Spc^R and the transformants were screened for loss of the plasmid borne Cm^R to ensure the double crossover event. Genomic DNA was isolated from selected transformants and the mutation was confirmed by PCR. To construct the *yvgX* mutant, an internal fragment of *yvgX* was amplified from genome DNA by PCR using as primers 5′-GGGACTGCAGCGATTCAGGAA-3′

Table 1. Bacterial strains used in this study.

Strain	Characteristics	Source or reference
B. subtilis strains		
CU1065	W168 *attSPβ trpC2*	(Vander Horn & Zahler 1992)
ZB307A	W168 SP*βc2*Δ2::Tn*917*::pSK10Δ6 (MLS^R)	(Zuber & Losick 1987)
HB8112	CU1065 *copA::cm*	This work
HB8113	CU1065 *cadA::spc*	This work
HB8121	ZB307A *SPβc2Δ2::T*n917::*φ*(*cadA′-cat-lacZ*) (MLS^r Neo^r)	This work
HB8122	ZB307A *SPβc2Δ2::T*n917::*φ*(*copA′-cat-lacZ*) (MLS^r Neo^r)	This work
HB8123	ZB307A *SPβc2Δ2::T*n917::*φ*(*copZ′-cat-lacZ*) (MLS^r Neo^r)	This work
HB8124	ZB307A *SPβc2Δ2::T*n917::*φ*(*yvgZ′-cat-lacZ*) (MLS^r Neo^r)	This work
HB8125	CU1065 *SPβc2Δ2::T*n917::*φ*(*cadA′-cat-lacZ*) (MLS^r Neo^r)	This work
HB8126	CU1065 *SPβc2Δ2::T*n917::*φ*(*yvgZ′-cat-lacZ*) (MLS^r Neo^r)	This work
HB8127	CU1065 *SPβc2Δ2::T*n917::*φ*(*copZ′-cat-lacZ*) (MLS^r Neo^r)	This work
HB8128	CU1065 *SPβc2Δ2::T*n917::*φ*(*copA′-cat-lacZ*) (MLS^r Neo^r)	This work
HB8174	CU1065 *copZ::em*	This work
E. coli strains		
DH5α	*φ*80 Δ(*lacZ*)M15 Δ (*argF-lac*)*U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1*	(Sambrook *et al.* 1989)

and 5′-CTCCGTCGACAGGAATTCGT-3′ and cloned in cloned in pGEM3Zf(+)cat-1 (Youngman 1990) as *Pst*I-*Sal*I (sites underlined in the primers). The resulting plasmid was transformed to *B. subtilis* CU1065 selecting for Cm^r. The genomic DNA was isolated from selected transformants and the mutation was confirmed by PCR. To construct the *yvgY* mutant, an internal fragment of the *yvgY* was amplified by PCR using the primers 5′-GCGGAATTCAAAGCAGTAGAAACAAGCGT-3′ and 5′-GCCGGATCCGTGACACCGGGCATC-3′ and cloned as *Eco*RI-*Bam*HI fragment in pMUTIN vector (Vagner *et al.* 1998). This plasmid was used because it allows the expression of the downstream gene, *yvgX*, under the control of the IPTG-induced P_{spac} promoter. The resulting construct was transformed to *B. subtilis* CU1065 selecting for MLS^r. Genomic DNA was isolated from selected transformants and the mutation was confirmed by PCR.

β-galactosidase assays

Overnight cultures were diluted 1:100 in LB liquid media containing different concentration of metal ions (as indicated) and grown to mid-log phase. Cells were collected and assayed for β-galactosidase as described (Miller 1972; Chen *et al.* 1993).

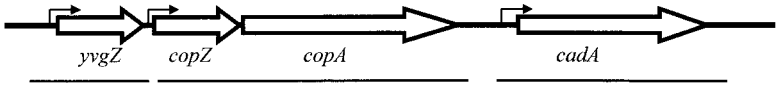
Northern and dot blot

Total RNA was isolated from cells grown with or without 0.5 mM $CuSO_4$ at the mid-exponential growth phase Ten μg of RNA was analyzed using denaturing agarose gel electrophoresis, the RNA was transferred to a nylon membrane, and Northern blotting was done using the NothernMax Kit (Ambion, USA) according to the manufacturer's instructions. For Dot blot, 5 μl of different RNA dilutions were spotted on a nylon membrane and the dot blotting was done using the NothernMax Kit (Ambion, USA) according to the manufacturer's instructions.

Primer extension assays

Total RNA was isolated from cells grown under different conditions using the RNeasy RNA isolation kit (Qiagen). For primer extension analysis, 100 μg of total RNA was precipitated with 4 pmol of end-labeled reverse primer and the reverse transcripts generated as described (Huang *et al.* 1997; Huang & Helmann 1998). Reverse transcripts were analyzed using 8 M urea/6% PAGE. The PCR product was sequenced using the same primer to index the reverse transcripts.

A



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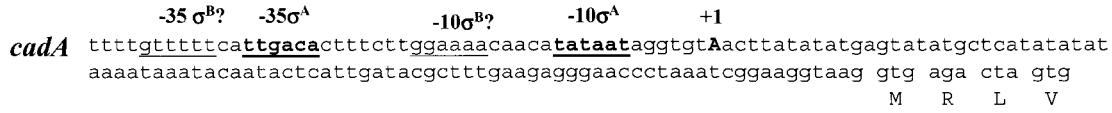

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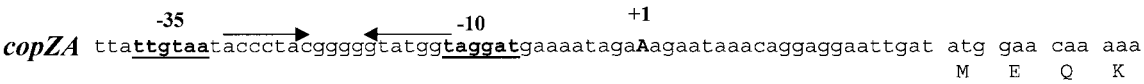

Fig. 1. The *yvgZ copZA cadA* region of *B. subtilis* A. Open reading frames are indicated by open arrows, promoter sites by bent arrows, and transcription products indicating operon structure by thin solid lines. Original gene names are: *copZ (yvgY), copA (yvgX)*, and *cadA (yvgW)*. B. Promoter region of *cadA* with σ^A-dependent (bold underlined) and previously proposed σ^B-dependent–10 and –35 regions shown. The A residue corresponding to the transcription start site (bold) is indicated by +1. C. Promoter region of the *copZA* operon showing the −35 and −10 region (bold underlined) with the A residue corresponding to the transcription start site indicated. A possible binding site for a MerR-type regulator is shown with arrows to indicate the inverted repeats.

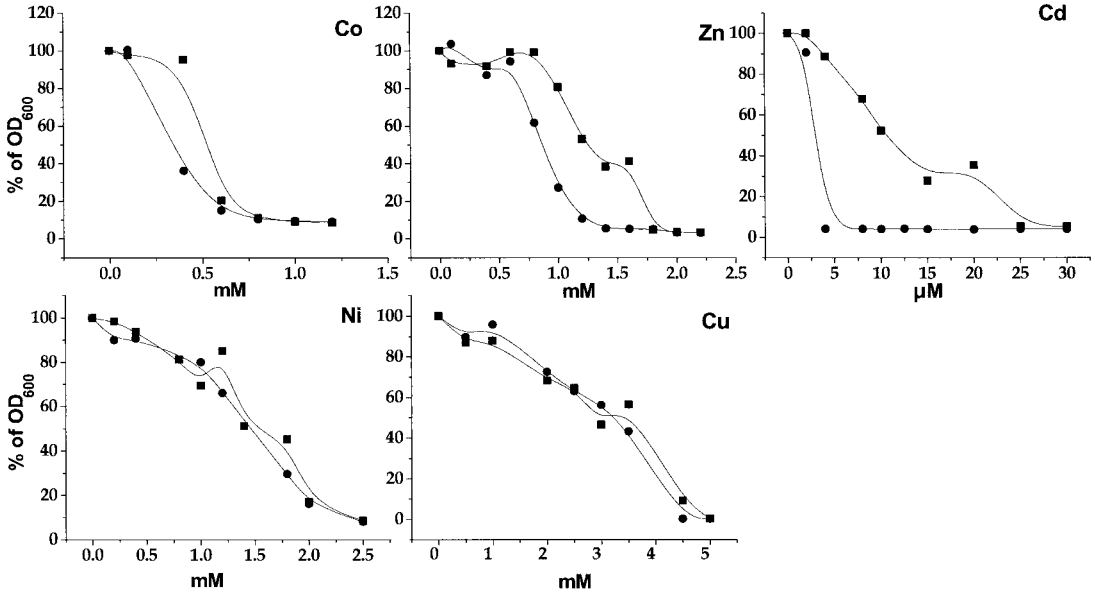

Fig. 2. Effects of metal ions on growth yield of the wild type (■) and *cadA* (●) mutant strains. Strains were grown overnight in LB with divalent metal ions added as indicated (note that Cd(II) levels are in micromolar) and the % of the OD_{600} of the control culture was calculated.

Results and discussion

B. subtilis encodes three CPx-type ATPases predicted to function in metal ion transport: *zosA, yvgW(cadA)* and *yvgX(copA)*. We have previously demonstrated that ZosA is a zinc uptake system under the control of peroxide regulon repressor, *perR* (Gaballa & Helmann 2002). The latter two genes are adjacent and co-directional in the chromosome, raising the possibility that they might be coordinately regulated (Figure 1A). Recent studies suggest that *yvgW* (*cadA*) plays a role in Cd(II) resistance (Solovieva & Entian 2002), but other metal ions were apparently not tested. Biochemical and structural studies indicate that the YvgY (CopZ) and YvgX (CopA) proteins interact with Cu(I) ions (Banci *et al.* 2001; 2002). Studies in *Enterococcus hirae* have demonstrated that CopA and CopB are transport proteins specific for copper while CopZ is a

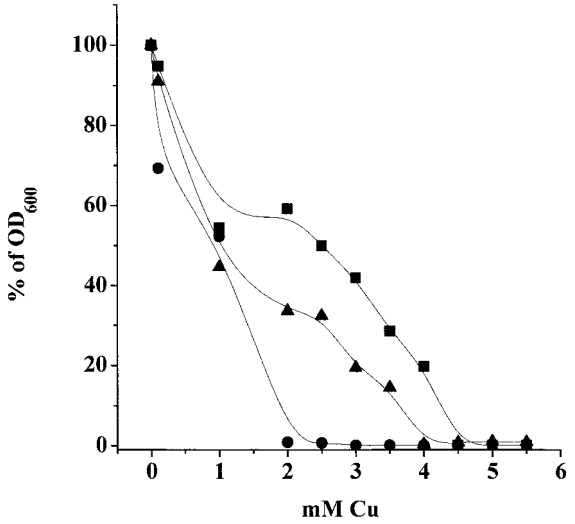

Fig. 3. Effect of divalent copper on the growth of the wild type (■), *copA* (●) and *copZ* (▲) mutants. The OD_{600} was measured after overnight growth in LB and the % of the OD_{600} of the control culture (no added metal) was calculated.

metallochaperone that specifically interacts with both CopA and the copper-responsive repressor protein, CopY (Cobine *et al.* 2002; Solioz 2002). Similarly, it has been speculated that CopA and CopZ are likely to interact in *B. subtilis* (Banci *et al.* 2002). Here, we investigate the roles of these genes in metal ion resistance, identify the promoter elements driving *copZA* and *cadA* expression, and characterize their regulation in response to metal ions.

Roles of cadA, copA, and copZ in metal ion resistance

Mutants in *cadA, copA* and *copZ* were generated by allelic replacement and tested for sensitivity to various metal ions including divalent Ni, Cd, Co, Cu and Zn. The *cadA* mutant was extremely sensitive to Cd(II), as previously shown (Solovieva & Entian 2002), and moderately sensitive to Zn(II) and Co(II) (Figure 2). No significant difference was detected in the resistance of the wild type and *cadA* mutants to Ni(II) or Cu(II). A role for CadA in modulating sensitivity to Co(II) has been suggested in *Helicobacter pylori* where it was observed that overexpression of CadA rendered the strain more resistant to Co(II) (Herrmann *et al.* 1999). Indeed, transport of Cd, Zn and Co might be a common theme for CadA-type transporters (Herrmann *et al.* 1999).

Mutation of either the *copA* or *copZ* gene led to an increased sensitivity to copper (Figure 3), but did not affect sensitivity to Cd, Ni, Co or Zn (Table 2).

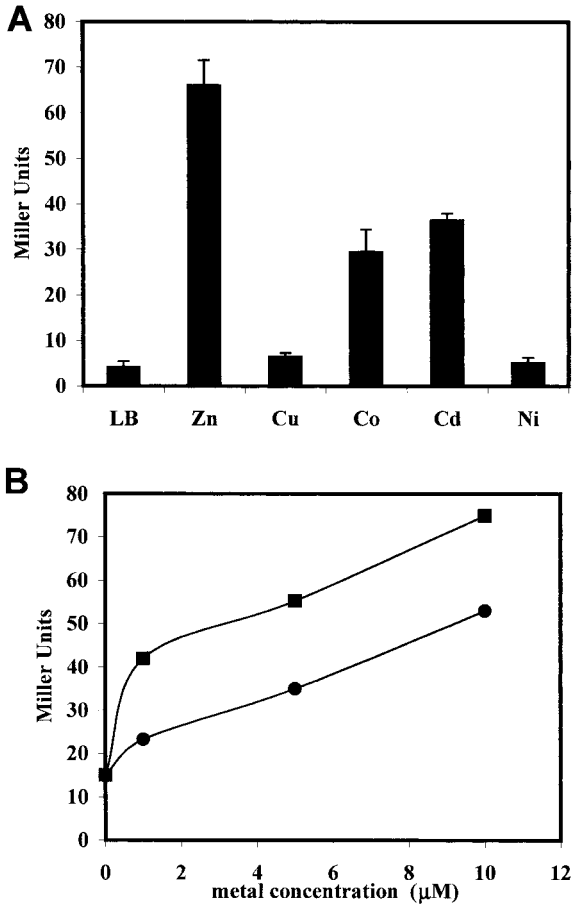

Fig. 4. Effects of metal ions on the regulation of *cadA* as determined using a P_{cadA}-*cat-lacZ* transcriptional fusion. A. Cells were grown to mid-logarithmic phase in LB medium with 0.5 mM of Zn, Cu, Co or Ni or 5 μM Cd and β-galactosidase levels were determined. B. Concentration dependence of Cd (■) and Zn (●) induction of the P_{cadA}-*cat-lacZ* transcriptional fusion.

Table 2. Minimal inhibitory concentrations for the wild type and each mutant to the different metals.

Strain/metal	Cd	Zn	Co	Ni	Cu
Wild type	25 μM	1.8 mM	0.8 mM	1.8 mM	4.5 mM
cadA	4 μM	1.2 mM	0.6 mM	1.8 mM	4.5 mM
copA	25 μM	1.8 mM	0.8 mM	1.8 mM	2 mM
copZ	25 μM	1.8 mM	0.8 mM	1.8 mM	3 mM

Compared to the *copA* mutant, the *copZ* mutant had a relatively modest growth defect in the presence of copper (Figure 3). These studies were done using a *copZ* mutant constructed using pMUTIN to allow the expression of the downstream *copA* gene under the control of an IPTG-induced promoter. It is possible that elevated expression of CopA under these conditions partially compensated for the lack of CopZ. Table 2 summarizes the minimal inhibitory concentrations (MICs) for the wild type and each mutant to the tested metals.

Regulation of the cadA gene

To investigate metalloregulation of *cadA*, we constructed a P_{cadA}-*cat-lacZ* transcriptional fusion and integrated this fusion ectopically into the SPβ prophage. Expression driven by the *cadA* promoter region was found to be induced by Zn(II), Cd(II) or Co(II), but not by Cu or Ni (Figure 4A). Thus, there is a good correlation between the ion specificity of the CadA efflux pump as shown by the growth of the mutant in the presence of different metal ions (Figure 2) and the ability of metal ions to induce expression of CadA (Figure 4A). When measured at low concentration of inducer, *cadA* was more strongly induced by Cd(II) than Zn(II) (Figure 4B). However, Zn was used in further experiments since it allows high cell yield compared to Cd, which drastically affects the cell growth even at very low concentrations.

The *cadA* gene has been previously found to be induced by both heat shock and ethanol stress (Helmann *et al.* 2001; Petersohn *et al.* 2001), suggesting that it might be part of the σ^B-controlled general stress response. Indeed, the DNA region upstream of *cadA* contains candidate promoter elements for recognition by both σ^A and σ^B (Figure 1B). Primer extension analysis, using RNA extracted from cells grown in the presence or absence of Zn(II), indicates that transcription starts with an A residue 91 bases upstream of the translation start site (Figure 5A; Figure 1B), corresponding to a σ^A-type promoter element. This same transcript is detected in a *sigB* mutant and there is no start site corresponding to the previously proposed σ^B promoter (Helmann *et al.* 2001). Furthermore, no σ^B-dependent start could be detected even in cells treated with 4% ethanol for 10 min, a condition that induces the *sigB* regulon (data not shown). Dot blot analyses using RNA isolated from wild type and *sigB* mutant cells treated with 4% ethanol showed that *cadA* is induced by ethanol (albeit much less than zinc) in a σ^B-independent fashion (Figure 6), consistent with previous studies (Petersohn *et al.* 2001). This indicates that *cadA* is part of the ethanol stimulon but not part of the *sigB* regulon.

The identification of CadA as a determinant of Zn(II) resistance brings to four the total number of loci affecting zinc homeostasis in *B. subtilis*. Two zinc uptake systems (encoded by the *yciABC* and *ycdHIyceA* operons) are repressed in response to zinc by the Zur protein (Gaballa & Helmann 1998). A third zinc uptake system, encoded by the *zosA* (formerly *ykvW*) locus, is a CPx-type ATPase that functions to import zinc and is regulated by the peroxide-sensing transcription factor PerR (Gaballa & Helmann 2002). ZosA appears to be the major pathway of zinc uptake under high zinc conditions: a *zosA* mutation can partially suppress the zinc sensitivity of the *cadA* mutant (Gaballa & Helmann 2002). Consistent with the Zn(II) selectivity of the ZosA protein, the *cadA* and *cadA-zosA* mutant strains are equally sensitive to both Cd(II) and Co(II) (data not shown).

CadA homologs in other systems are either under the control of MerR-like activator proteins (Silver & Walderhaug 1992; Brocklehurst *et al.* 1999; Rutherford *et al.* 1999; Binet & Poole 2000; Lee *et al.* 2001) or members of the CadC/ArsR/SmtB family of metalloregulators (Busenlehner *et al.* 2002; Sun *et al.*, 2002). Recent results suggest that the *cadC* gene in *B. subtilis* is regulated by an SmtB-like protein encoded by the *yozA* gene (our unpublished results).

Organization and regulation of copper efflux genes

The *copZA* operon is located 77 bases downstream of *yvgZ*, which encodes an unknown protein. Northern blot analyses, using probes in the *yvgZ* and *copA* genes, reveal a 2.75 kb, copper-induced transcript that hybridizes with *copA* but not *yvgZ* (Figure 5B). This corresponds in size to the predicted RNA for the *copZA* operon (Figure 1A). The 1.3 kb transcript detected with the *yvgZ* probe likely corresponds to a monocistronic mRNA for *yvgZ*. Primer extension analysis identified a copper-induced transcript starting with an A residue 24 bases upstream of the *copZ* translation start site (Figure 5C). This site corresponds to initiation from a predicted σ^A-dependent promoter (Figure 1C).

In *E. coli*, a CopA efflux system is regulated by CueR, a copper-sensing MerR homolog that binds to an inverted repeat sequence in the spacer region of the

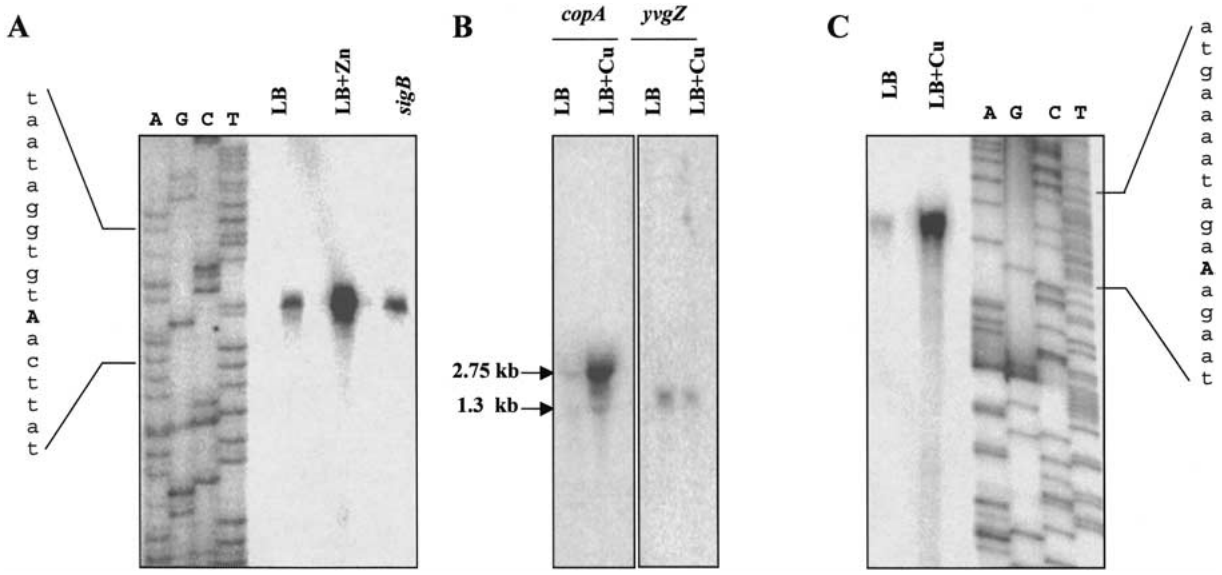

Fig. 5. Characterization of RNA products of the *copA cadA* chromosomal region. A. Primer extension mapping of the transcription start site of *cadA*. The primer extension product was generated using RNA isolated cells grown with or without 0.3 mM Zn and *sigB* mutant cells. The sequence ladder was generated using PCR cycle sequencing using the same primer as in the primer extension reaction. B. Northern blot of the RNA extracted from mid-logarithmic growing cells in the presence or absence of 0.5 mM Cu using an internal fragment of *yvgX* (*copA*, left) or *yvgZ* (right) as a probe. C. Primer extension mapping of the transcription start site of the *copZA* operon. The primer extension product was generated using RNA isolated from cells grown with or without 0.5 mM Cu. The sequence ladder was generated using PCR cycle sequencing using the same primer as in the primer extension reaction.

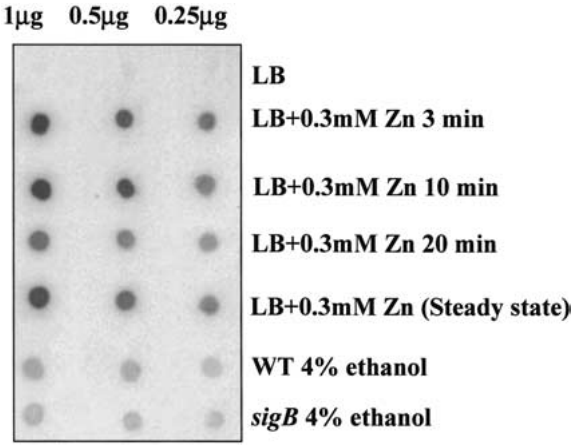

Fig. 6. Dot blot analysis of the RNA extracted from wild type cells grown in different conditions using an internal fragment of *cadA* as a probe. RNA from wild-type cells in the absence of Zn(II) treatment does not give a measurable signal with the *cadA* probe in this experiment (top row). Levels of *cadA* message are greatly increased within 3 min after treatment with zinc (rows 2–4), in cells grown in the presence of elevated zinc (row 5), or after treatment with ethanol (rows 6–7).

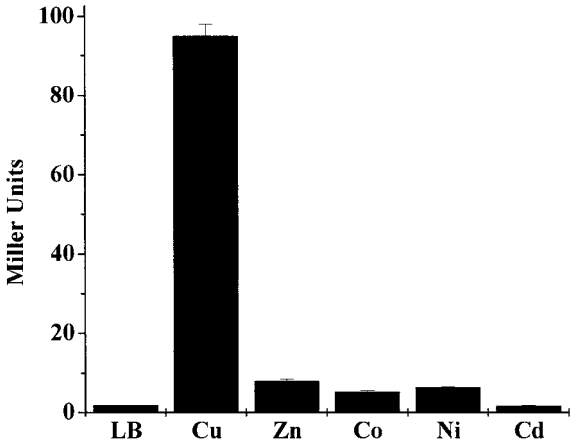

Fig. 7. Effects of metal ions on the regulation of *copZA* as determined using a P_{copZ}-*cat*-*lacZ* transcriptional fusion. Cells were grown to mid-logarithmic phase in LB medium with 0.5 mM of Zn, Cu, Co or Ni or 5 μM Cd and β-galactosidase was determined.

promoter (Outten *et al.* 2000; Stoyanov *et al.* 2001). Inspection of the *copZ* promoter reveals an inverted repeat sequence similar to that recognized by *E. coli* CueR (Figure 1C). This suggests the possible involvement of a MerR homolog in the regulation of the *copZA* operon. Indeed, preliminary results suggest that *yhdQ* encodes the suspected CueR-like regulator (our unpublished results).

To further investigate the selectivity and sensitivity of transcriptional induction, we generated *lacZ*

reporter fusions using the upstream regions of the *yvgZ, copZ* and *copA* genes. Expression from the cloned *copZ* promoter fragment was selectively induced solely by copper with maximal expression achieved in medium containing above 0.5 mM Cu(II) (Figure 7). In contrast, the *yvgZ-lacZ* fusion was constitutive ($\sim$ 25 Miller units during logarithmic growth) and was not affected by addition of metal ions including Cu(II), Cd(II), Co(II), Ni(II) or Zn(II) (data not shown). There was no detectable expression from a reporter fusion generated using a fragment containing $\sim$ 300 bp of DNA upstream of *copA* and integrated ectopically at SPβ, consistent with the hypothesis that *copA* is expressed as an operon with *copZ.*

Solovieva and Entian (Solovieva & Entian 2002) reported that Cd induced a 0.25 kb transcript corresponds to *copZ (yvgY)* and suggested that CopZ might act as positive regulator for CadA. However, we failed to detect transcripts of this size in our studies and our Northern blot and promoter cloning experiments support the idea that *copZ* and *copA* are coregulated as part of an operon. Moreover, we found that a P*copZ-cat-lacZ* fusion was induced by Cu, but not Cd (Figure 7), whereas Solovieva and Entian (2002) reported a strong transcriptional response to Cd for expression of the 0.25 kb RNA. Given our results from Northern analysis and promoter cloning experiments, the origin of the 0.25 kb RNA detected in the previous study is not clear. Since structural studies support the idea that yvgY encodes a metallochaperone (CopZ) (Banci *et al.* 2002), it seems unlikely that this gene encodes a transcription activator as proposed (Solovieva & Entian 2002).

Conclusion

Our results demonstrate that CadA functions in Cd resistance, as previously reported (Solovieva & Entian 2002), and also plays a role in resistance to Zn and Co. CadA was induced by the same metal ions that it presumably transports. A second CPx-type ATPase, CopA, has been proposed to function with the CopZ metallochaperone as a copper efflux system based on biochemical studies of Cu(I)-binding (Banci *et al.* 2001, 2002), but their physiological roles had not been reported. We demonstrate that this system is indeed an important determinant of Cu resistance. Moreover, we find that the *copZA* operon is expressed from a σ^{A}-type promoter and expression is selectively induced by Cu.

Acknowledgments

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