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CopZ from *Bacillus subtilis* interacts in vivo with a copper exporting CPx-type ATPase CopA

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Abstract

The structure of the hypothetical copper-metallochaperone CopZ from *Bacillus subtilis* and its predicted partner CopA have been studied but their respective contributions to copper export, -import, -sequestration and -supply are unknown. Δ*copA* was hypersensitive to copper and contained more copper atoms cell^{-1} than wild-type. Expression from the *copA* operator-promoter increased in elevated copper (not other metals), consistent with a role in copper export. A bacterial two-hybrid assay revealed in vivo interaction between CopZ and the N-terminal domain of CopA but not that of a related transporter, YvgW, involved in cadmium-resistance. Activity of copper-requiring cytochrome *caa*$_3$ oxidase was retained in Δ*copZ* and Δ*copA*. Δ*copZ* was only slightly copper-hypersensitive but Δ*copZ*/Δ*copA* was more sensitive than Δ*copA*, implying some action of CopZ that is independent of CopA. Significantly, Δ*copZ* contained fewer copper atoms cell^{-1} than wild-type under these conditions. CopZ makes a net contribution to copper sequestration and/or recycling exceeding any donation to CopA for export.
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Keywords: CopZ; CopA; Metallochaperone; P-type ATPase; Cytochrome oxidase; Atx1

1. Introduction

Copper is an essential cofactor for a number of enzymes with roles including electron transfer, oxidase and oxygenase activities, and detoxification of oxygen derived radicals [1]. However, copper can also be toxic in excess due to its binding to adventitious sites and promotion of oxidative damage through the catalysis of free radical formation. It has become apparent that, at least in yeast, efficient homeostatic mechanisms maintain essentially no free copper in the cell cytosol [2] whilst assisting in the delivery of copper to specific intracellular compartments and/or copper-requiring proteins [3–5]. These include copper transporters and copper metallochaperones.

In *Saccharomyces cerevisiae* the copper metallochaperone Atx1 interacts with and delivers copper to the CPx (or P_1)-type ATPase Ccc2 which imports copper into the Golgi-apparatus for insertion into copper enzymes (reviewed in [4]). Atx1-related proteins have also been identified in some prokaryotes, including Atx1 from the cyanobacterium *Synechocystis* PCC 6803 [6] and CopZ from *Enterococcus hirae* [7]. The latter (EhCopZ hereafter) influences DNA binding by the copper-responsive transcriptional repressor CopY and copper exchange between EhCopZ and EhCopY has been observed in vitro [8]. The *cop* operon includes *copY* and *copZ* along with *copA* and *copB* that encode CPx-type ATPases [7] with proposed roles in copper import and export, respectively [9]. Both EhCopA and EhCopB have been suggested as further interactive partners for EhCopZ [4,10], but it remains to be established whether or not EhCopZ interacts with these proteins in vivo.

An Atx1-like protein, designated CopZ, was recently identified in *Bacillus subtilis* (BsCopZ hereafter) and the solution structures of the apo and a copper(I)-bound form resolved [11]. The structure is similar to related proteins, including EhCopZ [12] and yeast Atx1 [13,14], with typical βαββαβ ferredoxin-like folding. The cytosolic N-terminal region of yeast Ccc2 contains two soluble domains that

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each adopt a structure similar to Atx1 [15] and possess complementary charged surfaces to Atx1 that contribute significantly to interactions between the two proteins [16]. Both proteins possess the motif M*X*C*XX*C (where *X* represents any amino acid) associated with metal binding and the formation of copper-bridged hetero-dimeric species during copper transfer [16]. A potential partner for BsCopZ was identified as the deduced CPx-type ATPase CopA (BsCopA hereafter) [17] encoded adjacent to *copZ* in the *B. subtilis* genome (Fig. 1). The N-terminal region of BsCopA ($BsCopA_N$) possesses two putative metal-binding domains and the solution structure of the second, resolved in the apo and copper(I) bound forms, reveals a high degree of similarity to BsCopZ but with complementary charged residues surrounding the M*X*C*XX*C metal-binding site that may contribute towards interactions [17].

Copper-transporting CPx-type ATPases are widespread with representatives described in bacteria, yeast, higher plants and man (reviewed in [20,21]). Other CPx-type ATPases are also known that transport different metal ions including cadmium [22], zinc and lead [23–25], cobalt [26] and silver [27]. These proteins display a high degree of specificity with respect to the metal ions transported but the determinants of metal specificity remain unresolved. Most importantly the metal ion transported or direction of transport cannot be predicted from the sequence of a CPx-type ATPase based upon the current level of understanding. However, similarity of BsCopA to known copper transporters, including CopA (49% identity) and CopB (26%) from *E. hirae* [9], PacS (45%) and CtaA (35%) from *Synechocystis* PCC 6803, and CopA (39%) from *Escherichia coli* [28] encouraged the prediction that BsCopA contributes to copper homeostasis. During the writing of this manuscript Gaballa and Helmann [29] have reported that BsCopA confers copper-resistance and is induced by elevated copper. We attribute similar phenotypes to BsCopA and also detect increased copper accumulation in mutants with disrupted *copA* supporting a role for BsCopA in copper export.

Here we investigate the role of the putative copper metallochaperone BsCopZ. A bacterial two-hybrid assay shows in vivo interaction between BsCopZ and $BsCopA_N$, but not of a second CPx-type ATPase, YvgW, encoded adjacent to *copA* (Fig. 1) but with a role in cadmium-resistance [30]. Activity of copper-requiring cytochrome caa_3 oxidase at the cytoplasmic membrane is not dependent upon *copA* or *copZ* (or *yvgW*). We show that *copZ* is required for normal cellular copper content and that *copZ* alone confers some copper tolerance. This is consistent with a model in which BsCopZ mediates greater internal sequestration of copper in vivo either via accumulation of Cu(I)-BsCopZ or by trafficking to some other 'advantageous' site.

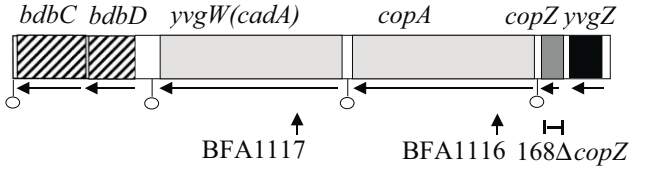

Fig. 1. Physical map of the *copZ–copA* region. The *copZ* and *copA* genes, corresponding to ORFs *yvgY* and *yvgX* respectively in the sequenced *B. subtilis* genome [18], are shown with the adjacent ORFs *yvgZ*, *yvgW*, *bcdC* and *bcdD* (shaded rectangles coincide with ORFs); the latter two encoding thiol-disulfide oxidoreductases [19]. The insertion sites of pMUTIN in BFA1116 and BFA1117 (bold arrows), the region of DNA (239 bp) deleted in 168Δ*copZ* by introduction of the kanamycin-resistance gene (horizontal line), and the positions of deduced [18] transcriptional terminators (circles) are shown.

2. Materials and methods

2.1. Bacterial strains, growth conditions and DNA manipulations

B. subtilis strains 168 or 1A1 (*trpC2*) (Bacillus Genetic Stock Center), BFA1116 and BFA1117 (http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl) were used. The latter two were generated within the framework of the *B. subtilis* European consortium and contain insertionally inactivated *yvgX* (now designated *copA*) and *yvgW*, respectively due to integration of pMUTIN [31] into the 168 genome (Fig. 1). *B. subtilis* strains were grown at 37°C in Luria–Bertani medium (LB), nutrient sporulation medium with phosphate (NSMP) [32] or on tryptose blood agar base (Oxoid) plates. *E. coli* strains JM101, JM109 (Promega) or BacterioMatch™ (Stratagene) were used. The media were supplemented with antibiotics when appropriate: for *B. subtilis*, lincomycin (25 μg ml^{-1}), erythromycin (0.3 μg ml^{-1}) or kanamycin (5 μg ml^{-1}) were used; for *E. coli*, ampicillin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) were used. DNA manipulations were performed as described by Sambrook et al. [33]. Extraction of *B. subtilis* genomic DNA and transformation of *B. subtilis* by the Groningen method was performed as described by Bron [34]. All generated plasmid constructs were checked by restriction digestion and DNA sequencing.

2.2. Generation of a copZ deletion mutant and a copA/copZ double mutant

B. subtilis 1A1 genomic DNA was used as template for PCR with primers 5′-ATGTCTAGACAACCGTTTGGAC-3′ and 5′-CCTGTGAATTCTTTCTATTTTCATCC-3′ and the amplification product, containing 423 bp from immediately upstream of *yvgY* (now designated *copZ*), was ligated into the *Xba*I/*Eco*RI site of pBluescript (SK), creating pYDS1. A second amplification product generated using primers 5′-ATGACGTCGACAAGTGATTCAAGG-3′ and 5′-GACGGTACCTGTTTCTAAAGCG-3′, containing 427 bp from immediately downstream of *copZ*, was subsequently ligated into the *Sal*I/*Kpn*I site

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of pYDS1, creating pYDS1S2. A kanamycin-resistance gene, released from pDG780 [35] on an *Eco*RV/*Sal*I fragment, was ligated into the *Eco*RV/*Sal*I site of pYDS1S2, between the *copZ* flanking sequences, creating pMKNC10. *Sca*I linearised pMKNC10 (to favour a double crossover recombination event) was used to transform *B. subtilis* 1A1 to kanamycin-resistance, and deletion of *copZ* upon integration of the kanamycin-resistance gene was confirmed by PCR. Genomic DNA from *B. subtilis* 1A1 with disrupted *copZ*, 1A1Δ*copZ*, was then used to transform *B. subtilis* 168 to kanamycin-resistance and *copZ* deletion again confirmed by PCR and the resulting strain designated 168Δ*copZ*.

To generate mutants lacking both *copZ* and *copA*, *B. subtilis* 168Δ*copZ* was transformed to erythromycin- and lincomycin-resistance using genomic DNA from BFA1116 and inactivation of both *copZ* and *copA* confirmed by PCR.

2.3. Analyses of metal tolerance and copper accumulation

To determine the minimum inhibitory/maximum permissive concentrations of a range of metal ions, cells were grown overnight in LB medium, diluted 1:100 in fresh medium supplemented with $ZnSO_4$, $CuSO_4$, $NiSO_4$, $AgNO_3$, $CdCl_2$ or $CoSO_4$, and growth monitored after ca. 6 h by measuring the absorbance at 600 nm. Subsequent experiments quantified the effects on growth of selected (from the previous experiment) concentrations as a function of time.

To examine copper contents, overnight cultures were diluted 1:100 in LB supplemented with various concentrations of $CuSO_4$ (described in individual experiments) and grown for 4 h. Cells from the resulting cultures, of standardised optical density (A_{600}), were harvested and washed three times with 10 mM Tris–HCl (pH 7.5), 1 mM EDTA and once with Milli-Q H_2O. Pelleted cells were dried overnight at 80°C, dissolved in 70% nitric acid, and the metal content measured by atomic absorption spectrophotometry. Metal contents were determined as atoms cell^{-1} (determined here as a colony-forming unit). Parallel control experiments eliminated any metal contamination from the materials used.

2.4. Generation of bacterial two-hybrid constructs containing copA$_N$, yvgW$_N$ and copZ

B. subtilis 168 genomic DNA was used as template for PCR with primers 5′-GAATTCCATGGAACAAAAAACATTGC-3′ and 5′-GCTCGAGTCACTTGGCTAC-3′ to amplify *copZ*, primers 5′-GGATCCATGTTGAGTGAAC-3′ and 5′-GCTCGAGTTACAGTCTCGCCG-3′ to amplify codons 1–163 of *copA* (*copA*$_N$) and primers 5′-GGATCCATGAGACTAGTG-3′ and 5′-GCTCGAGTCACATATTGACCATTC-3′ to amplify codons 1–93 of *yvgW* (*yvgW*$_N$). All PCR products contained introduced restriction sites suitable for introduction into BacterioMatch™ two-hybrid vectors (Stratagene). The amplification products were ligated to pGEM-T prior to subcloning; *copZ* into the *Not*I/*Eco*RI site of pBT creating pBTCOPZ; and *copA*$_N$ and *yvgW*$_N$ into the *Bam*HI/*Eco*RI site of pTRG creating pTRGCOPA$_N$ and pTRGYVGW$_N$, respectively.

2.5. β-Galactosidase assays

These assays were performed as described previously [36]. *B. subtilis* cultures were grown overnight in LB medium, diluted 1:100 in fresh medium supplemented with maximum permissive concentrations of Zn(II), Cu(II), Ni(II), Ag(I), Cd(II) or Co(II) and grown at 37°C until OD_{595} of 0.2–0.5 prior to assay. *E. coli* cultures (for the two-hybrid assays) were used with an OD_{595} of 0.6 following 20 h growth at 30°C.

2.6. Membrane isolation and assays of cytochrome oxidase activities

For these assays cells were cultured in NSMP. Colony staining for *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene diamine (TMPD) oxidation activity was carried out as previously described [37]. Membranes were prepared [38] and cytochrome *c* oxidase activities measured as described [39] but using a membrane protein concentration of 20 μg ml^{-1} and reduced cytochrome *c* (20 μM) from *S. cerevisiae* (Sigma). Protein concentrations were determined using the BCA method [40] with bovine serum albumin standards.

3. Results

3.1. Disruption of copA causes reduced tolerance to copper and increased cellular copper content

B. subtilis copA encodes an 803-residue protein with sequence features of metal-transporting CPx-type ATPases [41,42] including two M*X*C*XX*C metal-binding motifs in the N-terminal region. Disruption of *copA* in *B. subtilis* strain BFA1116, due to integration of vector pMUTIN (Fig. 1), was confirmed using PCR (data not shown). Growth of BFA1116 and *B. subtilis* 168 (wild-type) was tested in multiple liquid cultures supplemented with a range of levels of copper, silver, zinc, cadmium, cobalt and nickel ions to determine maximum permissive concentrations (data not shown). Only resistance to copper appeared to be reduced in BFA1116, with growth inhibited above 0.2 mM copper (inset Fig. 2A). Subsequently, growth was examined as a function of time in response to selected concentrations of copper (Fig. 2A). Unlike wild-type *B. subtilis*, BFA1116 is unable to grow in LB medium containing 1.5 mM copper.

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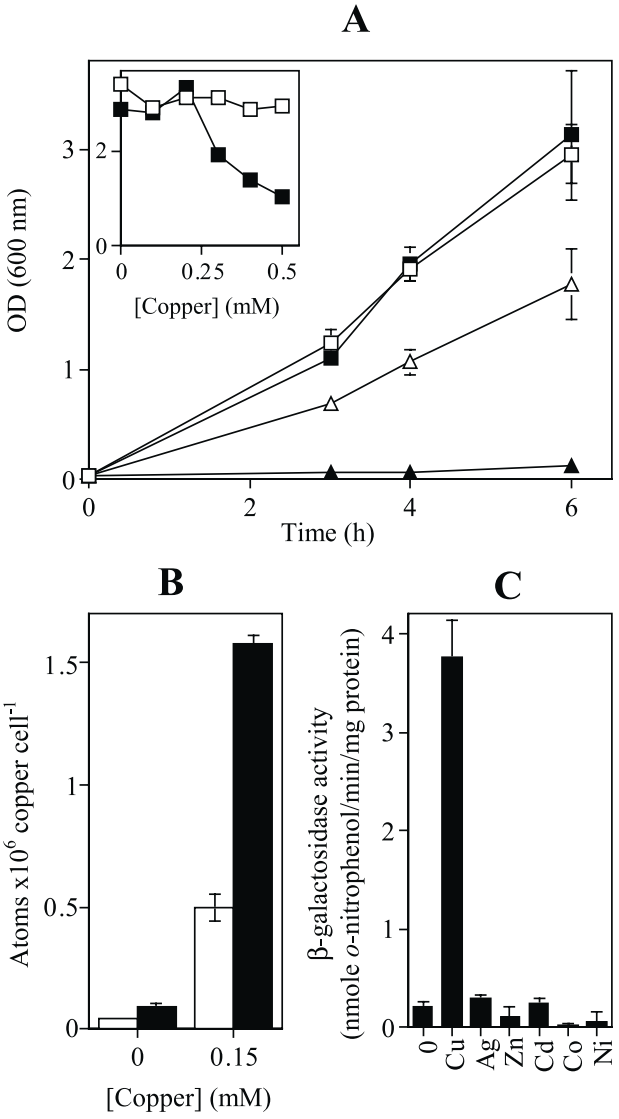

Fig. 2. Analysis of Δ*copA*. A: Growth of wild-type *B. subtilis* 168 (open symbols) and BFA1116 (closed symbols) in LB medium supplemented with 0 (squares), or 1.5 mM (triangles) Cu(II). Inset, OD_{600} cultures (*y*-axis) against added [Cu(II)] (*x*-axis) following 6 h growth. B: Copper contents of *B. subtilis* 168 (open bars) and BFA1116 (closed bars) grown in media supplemented with 0 or 0.15 mM Cu(II). Data points represent the mean values from three separate cultures with standard errors. C: β-Galactosidase activity in BFA1116 grown with no metal supplement or with maximum permissive concentrations of Cu(II) (0.2 mM), Ag(I) (0.5 μM), Zn(II) (0.1 mM), Cd(II) (0.5 μM), Co(II) (0.1 mM) or Ni(II) (0.25 mM). The data points represent the means of three separate assays with standard errors. Similar trends were obtained when the experiment was repeated on two further occasions.

The total copper content, or copper quota, of both wild-type and BFA1116 cells were determined for cultures grown in normal LB medium or following supplementation with copper at a level non-inhibitory to either strain (0.15 mM). Values were expressed as number of atoms per cell. These values increase by 11-fold in wild-type cells and 18-fold in BFA1116 following copper supplementation, with BFA1116 containing significantly more (three-fold) copper than wild-type cells (Fig. 2B). Disruption of *copA* promotes copper accumulation consistent with a role in export.

3.2. *Copper induces* copA *expression*

The pMUTIN vector used to disrupt *copA* contains a *lacZ* reporter gene such that, upon integration into the chromosome, transcription of the target gene can be monitored [43]. Expression of *copA* was therefore examined in BFA1116. Following exposure of cells to biologically significant levels of various metal ions, induction of β-galactosidase activity was only observed in cells exposed to copper (Fig. 2C).

3.3. *CopZ interacts with* $CopA_N$, *but not* $YvgW_N$, *in a bacterial two-hybrid assay*

B. subtilis copZ (Fig. 1) encodes a 69-amino acid protein with significant similarity to copper metallochaperones. A likely candidate partner for BsCopZ is $BsCopA_N$ [17]. It is now possible to analyse protein–protein interactions within a bacterial (*E. coli*) cell using the BacterioMatch™ two-hybrid system (Stratagene), and we have used this method previously [6] to reveal interactions between cyanobacterial Atx1 and the N-terminal regions of CtaA and PacS from *Synechocystis* PCC 6803. Greatly elevated β-galactosidase activity was detected in cells in which BsCopZ and $BsCopA_N$ (which included residues preceding the first predicted trans-membrane α-helix of BsCopA) were used as target and bait within this system compared with cells in which one or both partners was/were absent (Fig. 3A). BsCopA can therefore act as an interactive partner for BsCopZ.

Adjacent to *copA* in the *B. subtilis* genome is a second gene, *yvgW*, for a deduced metal-transporting CPx-type ATPase (Fig. 1). In contrast to BsCopA, YvgW contains only a single M*X*C*XX*C motif in its N-terminal region and was shown [30] to have a role in cadmium, but not copper, resistance. We investigated whether or not BsCopZ could also interact with $YvgW_N$ (the N-terminal region of YvgW). Fig. 3B shows no detectable increase in β-galactosidase activity when BsCopZ and $YvgW_N$ (which included residues preceding the first predicted trans-membrane α-helix of YvgW) were used within the bacterial two-hybrid system compared with cells in which one or both partners was/were absent.

3.4. *Deletion of* copZ *causes a slight reduction in copper tolerance*

The demonstrated in vivo interaction between BsCopZ and $BsCopA_N$ (Fig. 3A) suggests that BsCopZ may also have a role in copper homeostasis. To test this, a *copZ*-deficient mutant of *B. subtilis* strain 1A1 was obtained following chromosomal integration of pMKNC10, which contains sequences from immediately upstream and down-

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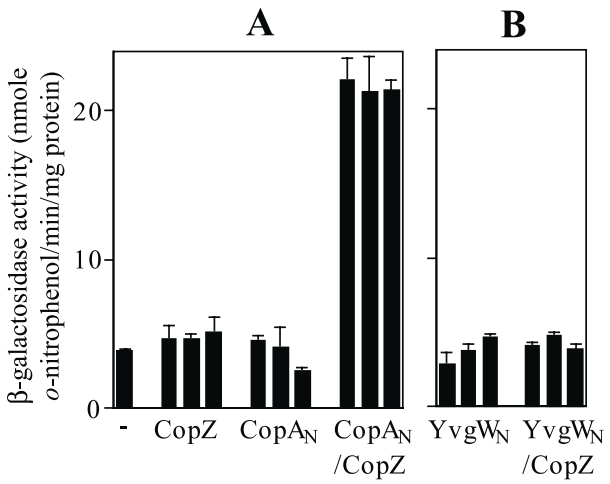

Fig. 3. In a bacterial two-hybrid assay BsCopZ interacts with the amino-terminal domain BsCopA$_N$, but not with YvgW$_N$. A: β-Galactosidase activity in *E. coli* (BacterioMatch®, Stratagene) containing: the control plasmids pBT and pTRG (-), pBTCOPZ and pTRG (CopZ), pBT and pTRGCOPA$_N$ (CopA$_N$) or pBTCOPZ and pBTCOPA$_N$ (CopA$_N$/CopZ). B: As panel A but using cells containing pBT and pTRGYVGW$_N$ (YvgW$_N$), or pBTCOPZ and pBTYVGW$_N$ (YvgW$_N$/CopZ). The data for three independent transformants are shown for cells containing translational fusions of *copA$_N$*, *yvgW$_N$* and/or *copZ* within pBT and pTRG. Data points represent the means of three separate assays for each transformant, with standard errors. Similar trends were obtained when the experiment was repeated on two further occasions.

stream of the *copZ* coding region separated by a kanamycin-resistance gene. PCR analyses confirmed integration via a double homologous recombination event at the *copZ* locus (data not shown) and the resulting strain was designated 1A1Δ*copZ*. A *copZ* deletion mutant of *B. subtilis* strain 168 was subsequently generated using genomic DNA from 1A1Δ*copZ* to transform *B. subtilis* 168 to kanamycin-resistance. Deletion of *copZ* was again confirmed by PCR and the resulting strain designated 168Δ*copZ*. Growth of *B. subtilis* 168 (wild-type) and 168Δ*copZ* was tested in multiple liquid cultures supplemented with a range of levels of copper. Resistance to copper appeared to be slightly reduced in 168Δ*copZ* compared to wild-type cells, with a greater inhibition of growth observed in medium containing ≥ 1 mM copper (inset of Fig. 4A). Subsequently, growth was examined as a function of time in LB medium with or without 1.5 mM copper added (Fig. 4A). Growth of 168Δ*copZ* was significantly more inhibited than growth of wild-type cells in medium containing 1.5 mM copper, revealing a contribution of *copZ* to copper tolerance.

3.5. copZ *enhances cellular copper content*

BsCopZ binds copper(I) in vitro [11,44]. Two naive models are that *copZ*-mediated resistance to elevated exogenous copper results from (i) enhanced export via donation to BsCopA or (ii) enhanced intracellular sequestration either directly by BsCopZ or by donation to other 'non-adventitious' copper sites. To test this the copper quotas of *B. subtilis* 168 (wild-type) and 168Δ*copZ* were examined following growth in normal LB medium or in LB medium supplemented with a level of copper non-inhibitory to either strain (0.4 mM). Values increased by 40-fold and 22-fold for wild-type and 168Δ*copZ*, respectively, as exogenous copper levels increased (Fig. 4B). Most notably, cells containing functional *copZ* had significantly, 2.6-fold, more cellular copper than 168Δ*copZ* cells. The reduced copper content in 168Δ*copZ* implies that BsCopZ binds and sequesters copper in vivo, thereby increasing endogenous copper levels.

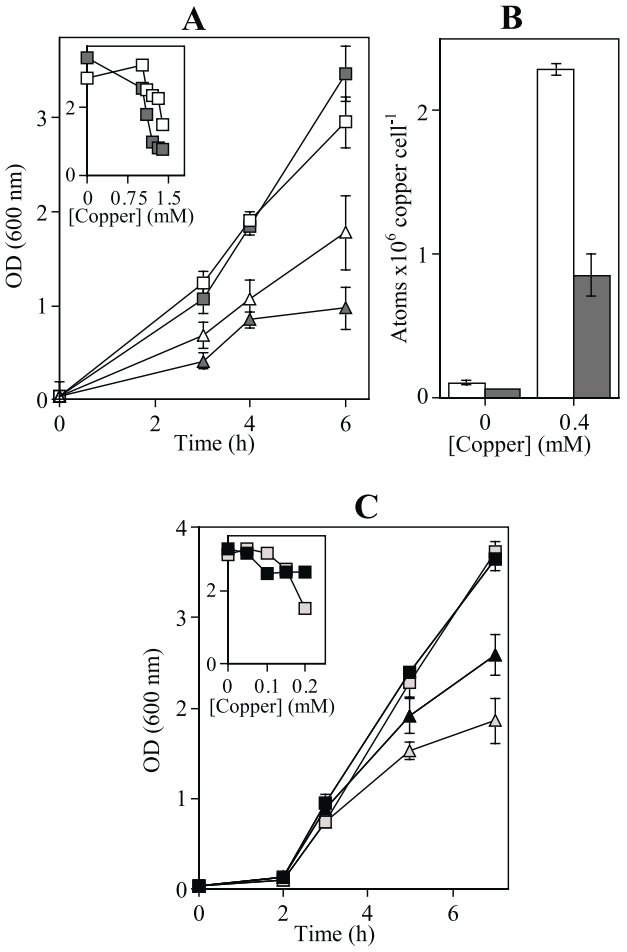

Fig. 4. Mutants deficient in *copZ* have reduced copper content and a slight reduction in copper tolerance. A: Growth of wild-type *B. subtilis* 168 (open symbols) and 168Δ*copZ* (dark-grey symbols) in LB medium supplemented with 0 (squares) or 1.5 mM (triangles) Cu(II). Inset, OD$_{600}$ cultures (*y*-axis) against added [Cu(II)] (*x*-axis) following 6 h growth. B: Copper contents of *B. subtilis* 168 (open bars) and 168Δ*copZ* (dark-grey bars) grown in media supplemented with 0 or 0.4 mM Cu(II). C: Growth of BFA1116 (closed symbols) and 168Δ*copZ*/Δ*copA* (light-grey symbols) in LB medium supplemented with 0 (squares) or 0.2 mM (triangles) Cu(II). Inset, OD$_{600}$ cultures (*y*-axis) against added [Cu(II)] (*x*-axis) following 7 h growth. Data points represent the mean values from three separate cultures with standard errors. Similar trends were obtained when the experiments were repeated on two further occasions.

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3.6. copZ *and* copA *are additive with respect to copper tolerance*

To test for additivity with respect to copper tolerance, a double mutant with both *copA* and *copZ* disrupted was generated by transforming 168Δ*copZ* to erythromycin and lincomycin-resistance using genomic DNA from BFA1116 (Δ*copA*). Disruption of *copA* by pMUTIN and retention of the *copZ* deletion were confirmed using PCR (data not shown) and the resulting strain designated 168Δ*copZ*/Δ*copA*. Copper-resistance appeared to be slightly reduced in 168Δ*copZ*/Δ*copA* compared to BFA1116 (inset Fig. 4C). Supplementation of the medium with 0.2 mM copper caused a greater reduction in the growth of 168Δ*copZ*/Δ*copA* compared to that of BFA1116 (Fig. 4C). While the interaction between BsCopZ and $BsCopA_N$ provides support for BsCopZ acting in conjunction with BsCopA, BsCopZ alone also provides some copper-resistance.

3.7. *Cytochrome* caa_3 *oxidase activity is unaffected in mutants with disrupted* copA, copZ *or* yvgW

Disruption of Atx1 and the copper transporters CtaA (cellular import) and PacS (thylakoid import) in *Synechocystis* PCC 6803 results in phenotypes associated with impaired copper supply to plastocyanin and cytochrome *c* oxidase at the thylakoid compartment [6,45]. *B. subtilis* of course lacks an internal thylakoid compartment and contains a copper-requiring caa_3-type cytochrome oxidase at the cytoplasmic membrane [39]. To examine whether or not the related proteins in *B. subtilis* have a role in the supply of copper to this enzyme, cytochrome caa_3 oxidase activity was examined in 168Δ*copZ* and BFA1116 (Δ*copA*). Due to the location of *yvgW*, adjacent to *copA*, activity was also examined in BFA1117 in which *yvgW* is disrupted (confirmed by PCR, data not shown). Staining of colonies with the cytochrome caa_3-specific substrate TMPD, showed that the mutants have the same positive TMPD-oxidation activity as wild-type cells (data not shown) revealing that cytochrome caa_3 oxidase activity was retained. The level of cytochrome oxidase activity in isolated membranes was subsequently determined and activity was found to be similar in membranes isolated from BFA1116, 168Δ*copZ*, BFA1117 and wild-type cells (Table 1). These values correlate well with the level of activity (0.15 μmol min^{-1} mg $protein^{-1}$) previously reported for *B. subtilis* strain 3G18 [39]. In contrast, Δ*ctaCD* mutants which lack two of the structural genes for cytochrome caa_3 oxidase [39], have only 3% of the cytochrome oxidase activity detected in wild-type cells (data not shown).

Table 1

Strain	Cytochrome oxidase activity (μmol min^{-1} mg $protein^{-1}$)
B. subtilis 168	0.168 (±0.011)
BFA1116	0.196 (±0.012)
168Δ*copZ*	0.215 (±0.022)
BFA1117	0.229 (±0.027)

Cytochrome oxidase activity (normalised for cytochrome *c* concentration) in membrane preparations of cells grown in NSMP. Data values are the mean (with standard errors) of at least three separate assays performed using membranes from two separate preparations.

4. Discussion

Evidence that BsCopA is involved in copper export includes: (i) disruption of *copA* caused increased cytosolic copper levels (Fig. 2B), (ii) disruption of *copA* caused a reduction (five-fold) in tolerance to elevated copper while normal tolerance to other metals was retained (Fig. 2A), and (iii) *copA* expression was substantially increased by elevated copper but not by other metals (silver, zinc, cadmium, nickel and cobalt) at maximum permissive concentrations (Fig. 2C). The observed in vivo interaction between BsCopZ and $BsCopA_N$ (Fig. 3A) suggests that BsCopZ may also contribute to copper export, while the absence of any detectable interaction between BsCopZ and $YvgW_N$ (Fig. 3B) illustrates the specificity of BsCopZ towards the copper transporter. From the structures of BsCopZ and BsCopA, a mechanism of copper transfer and adduct formation similar to that described [16] for eukaryotic Atx1 and Ccc2 has been proposed [17].

BsCopZ binds copper(I) in vitro [11,44] and increased copper accumulation in wild-type cells compared to 168Δ*copZ*, at non-inhibitory copper levels (Fig. 4B), supports the assertion that BsCopZ binds copper in vivo. Cyanobacterial Atx1 interacts with the cellular copper importer CtaA but can also acquire copper from other locations and an attractive proposition [6] is that Atx1 contributes to recycling endogenous copper. No specific copper import proteins have so far been described for *B. subtilis*. However, it is tempting to speculate that BsCopZ contributes to endogenous copper levels by effectively scavenging copper from importers or weak cytosolic sites, such as degraded metallo-proteins or adventitious copper-binding sites, and sequestering copper either directly or by donation to advantageous copper sites. The latter could include copper requiring apo-proteins or a specific copper sequestering macromolecule.

Cyanobacterial Atx1 and PacS supply copper for cytochrome caa_3 oxidase at the thylakoid compartment and activity of this enzyme is reduced in Δ*atx1* or Δ*pacS* mutants [6]. We investigated whether or not BsCopZ and BsCopA have an analogous role in *B. subtilis*, although with BsCopA transporting copper ions across the cytoplasmic membrane rather than into the thylakoid compartment. Cytochrome caa_3 oxidase activity was retained in Δ*copA* and Δ*copZ* (and Δ*yvgW*) mutants, with similar levels of activity being detected in membranes from wild-type and the mutant cells (Table 1). Our data therefore do not support a role for BsCopA and BsCopZ in the supply

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of copper to cytochrome *caa*$_3$ oxidase. Cytochrome *c* oxidase activity in yeast requires the action of Sco1, at the inner mitochondrial membrane, in addition to the copper metallochaperone Cox17 [46]. Sco1 is proposed to accept copper(I) from Cox17 for subsequent insertion into the Cu_A site of cytochrome *caa*$_3$ oxidase [47]. A homologue of Sco1, YpmQ, has been identified in *B. subtilis* and shown to be required for the activity of cytochrome *caa*$_3$ oxidase, but not of menaquinol oxidase with only a Cu_B site [48]. It remains to be established whether or not a protein functionally analogous (but different in sequence) to yeast Cox17 therefore delivers copper to YpmQ in *B. subtilis* for subsequent incorporation into cytochrome *caa*$_3$ oxidase.

BsCopZ confers some copper-resistance (Fig. 4A). In vivo interaction with $BsCopA_N$ (Fig. 3A) is suggestive of copper donation from BsCopZ to $BsCopA_N$ for export. However, additivity with respect to copper tolerance (Fig. 4C) supports, at least some, independent action of BsCopZ and our data (Fig. 4B) are consistent with a role (direct or indirect) in intracellular sequestration (Fig. 5). While it is apparent that *copZ* causes some additional accumulation of copper it is proposed that this is less than the total amount of copper sequestered, BsCopZ thereby causing some reduction in the 'available toxic pool' of copper. At non-inhibitory copper levels BsCopZ may encounter, and donate copper to, apo-proteins at a higher frequency than to apo-$BsCopA_N$. However, at higher copper concentrations the probability of encountering holo-proteins will be greater and interactions with apo-$BsCopA_N$ may be enhanced by the increased expression (Fig. 2C) of the transporter under these conditions. With

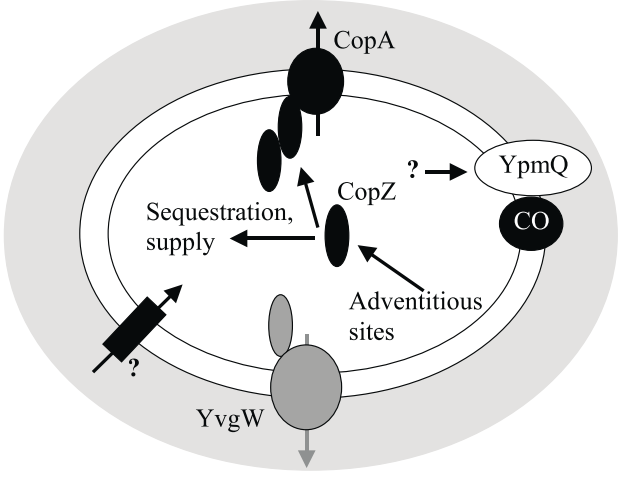

Fig. 5. The role of CopZ in *B. subtilis*. In the model black arrows represent the pathways for copper, ovals represent metal-binding domains and CO represents cytochrome *caa*$_3$ oxidase. The data shown in (B) imply that, at 0.4 mM exogenous copper, BsCopZ makes a greater contribution to sequestration/storage and/or recycling of endogenous copper than to BsCopA-mediated export. Copper-sensitivity of 168Δ*copZ* implies that removal of copper from adventitious sites and/or donation to BsCopA for export provides some resistance.

the emergence of a class of copper-metallochaperones that interact with CPx-type ATPases it is now necessary to establish what, if any, the effect is of a metallochaperone on copper transport.

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