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Published in:
Journal of Bacteriology

DOI:
10.1128/JB.01616-08

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Copper Acquisition Is Mediated by YcnJ and Regulated by YcnK and CsoR in Bacillus subtilis

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Received 14 November 2008/Accepted 9 January 2009

Copper is an essential cofactor for many enzymes, and at over a threshold level, it is toxic for all organisms. To understand the mechanisms underlying copper homeostasis of the gram-positive bacterium Bacillus subtilis, we have performed microarray studies under copper-limiting conditions. These studies revealed that the ycnJ gene encodes a protein that plays an important role in copper metabolism, as it shows a significant, eightfold upregulation under copper-limiting conditions and its disruption causes a growth-defective phenotype under copper deprivation as well as a reduced intracellular content of copper. Native gel shift experiments with the periplasmic N-terminal domain of the YcnJ membrane protein (135 residues) disclosed its strong affinity to Cu(II) ions in vitro. Inspection of the upstream sequence of ycnJ revealed that the ycnK gene encodes a putative transcriptional regulator, whose deletion caused an elevated expression of ycnJ, especially under conditions of copper excess. Further studies demonstrated that the recently identified copper efflux regulator CsoR also is involved in the regulation of ycnJ expression, leading to a new model for copper homeostasis in B. subtilis.

Transition metals such as copper, iron, and zinc play important roles in bacterial metabolism as essential cofactors for numerous enzymes. However, when the concentrations of these metals increase in the cell, they undergo undesirable redox reactions or bind inappropriately to the metal binding sites of several enzymes, thereby altering their specificity and finally leading to toxic effects (4, 24). Thus, the acquisition of these metals has to be strictly balanced in the cells, and hence bacteria tend to evolve in response to the bioavailability of metals to sustain the metal homeostasis.

Copper homeostasis is well studied in gram negative bacteria such as Enterococcus hirae and Escherichia coli. In E. hirae, the process occurs at the plasma membrane and includes four genes, i.e., copY, copZ, copA, and copB. copA and copB encode two integral membrane P-type ATPases that are necessary for the transport of copper into the cells under copper-limiting conditions (20). CopA, which serves to import copper, interacts with CopZ, which acts as a copper chaperone. CopZ then chaperones the metal atom to the transcriptional repressor CopY, thereby releasing the repression of copper homeostasis genes (28, 29). In E. coli, there are several sets of genes which are responsible for copper homeostatic functions. The cutRS genes form a sensor-regulatory pair which senses copper and activates the cutCFBA genes (19). CusF is a periplasmic copper binding protein, while CusB/CusA gene products are homologous to a family of proton/cation antiporter complexes (7). In a second copper efflux system, regulated by CueR, a MerR-like transcriptional activation controls two copper efflux genes, copA and cueO (19), whereas cueO encodes a multicopper oxidase. In addition, the cutABCDEF genes are also believed to be involved in copper uptake, storage, delivery, and efflux (21, 23). Copper efflux is carried out mainly by heavy metal exporters which belong primarily to the integral membrane protein family of P-type ATPases (8, 9, 22, 27, 33), whose expression is controlled mainly at the level of transcription. These P-type ATPases are functional in translocating Cu(I) across the cytoplasmic membrane. The recently discovered copper-specific repressor CsoR in Mycobacterium tuberculosis belongs to an entirely new set of copper-responsive repressors, whose homologs are widely spread in all major classes of eu-bacteria (16). CsoR from Bacillus subtilis, which is encoded upstream of the copZA operon, is 37% homologous to M. tuberculosis CsoR, and elevated copper levels in B. subtilis are sensed by CsoR, which leads to derepression of the copZA copper efflux operon (16, 26).

In contrast, in Saccharomyces cerevisiae, high-affinity copper uptake is mediated by two transmembrane transport proteins, Ctr1p and Ctr3p. Prior to uptake, Cu(II) is reduced to Cu(I) by Cu(II)/Fe(III)-specific reductases Fre1p and Fre2p (11). The CTR1, CTR3, and FRE1 genes are activated under copper starvation and repressed under copper repletion by the copper-sensing transcription factor Mac1p (6, 36). Studies undertaken to understand the copper resistance in Pseudomonas syringae strains that infect tomato revealed that the copper resistance operon pcoABCD is plasmid encoded and is regulated by the two-component system pcoRS (3, 4, 15). Similar copper resistance (pcoABCD) and regulatory (pcoRS) genes are also plasmid encoded in E. coli (14, 31, 35). In spite of high homology between these two systems, the resistance mechanisms dealing with an excess of copper inside the cell are completely different. Copper resistance in E. coli is achieved mainly by a copper efflux mechanism, whereas P. syringae performs sequestration of excess cytosolic copper (15).
Here, we explore the role of *B. subtilis YcnJ*, which is a homolog of *P. syringae* CopCD, in copper homeostasis. The *ycnJ* gene from *B. subtilis* is highly induced under copper-limiting conditions, and a Δ*ycnJ* mutant shows reduced growth under copper-limiting conditions. Uptake components for copper in *B. subtilis* have not been reported so far, and we demonstrate that YcnJ is a candidate for such a function. The ycnK gene located upstream from *ycnJ* was investigated and shown to encode a transcriptional regulator which acts, in addition to the investigated regulator CsoR, as a copper-specific repressor for *ycnJ*.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. *Bacillus subtilis* ATCC 23132 (wild type [WT]) was grown in Beltsky minimal medium (BMM) supplemented with 0.5% (wt/vol) glucose as a carbon source and with all essential nutrients required (30). Freshly prepared CuCl (0.5 mM) was used to maintain the copper excess conditions, and 0.25 mM bathocuprione disulfonate (BCS) (Sigma-Aldrich) was used as a Cu(I)-specific chelator for maintaining copper-limiting conditions.

**Mutant construction.** Deletion mutants were generated by the long flanking homology PCR method (34). In the first-round PCR, long flanking homologous PCR fragments were amplified from upstream and downstream regions of the gene to be deleted. The 3' ends of the resulting homologous PCR products were designed to be complementary to the resistance cassette and were used in the second-round PCR, generating a fusion construct which replaces the gene of interest with a resistance marker to facilitate the selection of the mutant on antibiotic plates. The primers used in generating the mutants are listed in Table 2. All PCRs were performed using Platinum Pfx DNA polymerase (Invitrogen). Chromosomal DNA of *B. subtilis* ATCC 23132 was used as a template to amplify the corresponding upstream and downstream flanking regions. The Expand long-template PCR system was used to fuse the homologous flanks with the corresponding resistance markers. PCR fusion products were used directly for the transformation of *B. subtilis* strain ATCC 23132 to generate the Δ*csoR*, Δ*ycnK*, Δ*csoR* Δ*ycnK* mutants. Transformants were selected on the respective antibiotic-containing LB plates. Chromosomal DNA was isolated from all the mutants, and the recombinations were confirmed by PCR.

**Purification of CsoR, YcnK, and the N-terminal 135 amino acids (aa) of YcnJ.** The open reading frames of genes *csoR*, *ycnK*, and *ycnJ* were amplified by PCR using chromosomal DNA isolated from *B. subtilis* strain ATCC 23132 as the template and cloned into Ncol and Xhol sites into the pET28a+ vector for overexpression. The ligated plasmids were then transformed into *E. coli* DH5α cells. The resulting plasmids were confirmed by restriction analysis. For purification, the fragment and transformed into BL21 cells using electrotransformation.

**DNA manipulations and genetic techniques.** DNA preparations and transformations were carried out as described previously (12, 25). Electroporation was used for the transformation of plasmids into *E. coli* Top10 cells. Homologous recombination was used for transforming the *B. subtilis* ATCC 23132 strain for mutant construction. Restriction enzymes, T4 DNA ligase, and calf intestinal phosphatase were used according to the manufacturer's instructions (New England Biolabs).

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Function</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 23132</td>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSP100</td>
<td>ΔcsoR::erm</td>
<td>Copper eflux transcriptional regulator</td>
<td>This study</td>
</tr>
<tr>
<td>CSP101</td>
<td>ΔycnJ::cpc</td>
<td>Copper homeostasis</td>
<td>This study</td>
</tr>
<tr>
<td>CSP 102</td>
<td>ΔcsoR::erm ΔycnK::cpc</td>
<td>Transcriptional regulation</td>
<td>This study</td>
</tr>
<tr>
<td>CSP 103</td>
<td>ΔcsoR::erm ΔycnK::cpc</td>
<td>Transcriptional regulation</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP10</td>
<td>F' mcrA Δ(mrr-hsdRMS-mcrBC)Δ880lacZΔM15 ΔlacX74 deoR nutG recA1 araD139 (Δara-leu)7697 galU galK rpsL (Str') endA1 k</td>
<td>Transformation</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F' ompT gal dcm lon hsdSB (rB- mB-) λ(DE3)</td>
<td>Overexpression</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUS 19</td>
<td>Spe'</td>
<td>Antibiotic resistance cassette Spe'</td>
<td>3a</td>
</tr>
<tr>
<td>pMUTIN</td>
<td>Erm'</td>
<td>Gene disruption vector; antibiotic resistance</td>
<td>31a</td>
</tr>
<tr>
<td>pET28a+</td>
<td>Kan'</td>
<td>Expression vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pCSP01</td>
<td>pET28a+ containing N-terminal 135 codons of <em>ycnJ</em> as C-terminal His6 tag fusion</td>
<td>Possible copper import</td>
<td>This study</td>
</tr>
<tr>
<td>pCSP02</td>
<td>pET28a+ containing <em>yvgZ</em> as a C-terminal His6 tag fusion</td>
<td>Transcriptional regulation</td>
<td>This study</td>
</tr>
<tr>
<td>pCSP03</td>
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<td>Possible copper import transcriptional regulator</td>
<td>This study</td>
</tr>
</tbody>
</table>

**TABLE 1. Strains and plasmids used in this study**
was clarified by centrifugation (Sorvall RC 26 plus) at 17,000 rpm for 30 min, and an Ni-nitriilotriacetic acid (NTA) column was used to purify the protein using a linear gradient with HEPES-B buffer (50 mM HEPES, 300 mM NaCl, 250 mM imidazole [pH 7.5], 1 mM DTT). The purified protein was dialyzed against HEPES-A buffer containing 100 mM NaCl. Protein purity of more than 90% was observed by Coomassie blue staining of a sodium dodecyl sulfate-polyacrylamide gel.

**Native gel electrophoresis.** Purified protein samples (~100 μM) were incubated with 200 μM Cu(I) or Cu(II) for about 5 h until the bromophenol blue ran out of the gel. Gels were stained using Coomassie blue and destained using 10% acetic acid.

**Microarray analysis.** For microarray analysis, both the WT (ATCC 21332) and the ΔcsoR mutant were grown in BMM under copper-replete conditions (growth in the presence of 0.5 mM CuCl2) and copper-depleted conditions (growth in the presence of the copper-specific chelator BCS at 0.25 mM). Cultures were harvested in mid-log phase for RNA extraction. The Macaloid/Roche method was used for the RNA extraction (13). Concentrations were measured using inductively coupled plasma mass spectrometry. The data were exported to Microsoft Excel. The expression levels were processed and normalized (Lowess method) with Micro-Prep (10, 32). The in-transformed ratios of the expression levels of mutant versus WT were subject to a t test using the Cyber-T tool (2). Three independent measurements for each condition along with a dye swap were analyzed. The results obtained were averaged, the raw data were processed to Cyber-T web interface software for the calculation of the expression ratios, and the data were exported to Microsoft Excel.

**Estimation of copper concentrations inside cells.** Total cytoplasmic copper concentrations were measured using inductively coupled plasma mass spectrometry. B. subtilis strain ATCC 21332 and the ΔcsoR mutant were grown in BMM overnight. Fresh BMM (100 ml) either with 0.5 mM copper in excess or under copper-limiting conditions achieved by addition of 0.25 mM BCS was inoculated with overnight cultures with a starting OD600 of 0.05, and the cells were harvested in mid-log phase. Cells were centrifuged at 13,000 rpm (18,000 × g) for 5 min, and the pellets were washed three times with buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA and finally with MilliQ water. Cells were dried overnight at 85°C, and the total copper concentration was determined by breaking the cells using nitric acid.

**Dot blot analysis.** B. subtilis strains were grown under normal (BMM), copper limited (BMM plus 0.25 mM BCS), and copper-replete (BMM plus 0.5 mM copper in excess) conditions. Overnight cultures were inoculated into fresh medium to an initial OD600 of 0.05, and cells were harvested at an OD600 of 0.25. Total RNA was isolated from these cells using the RNAzol method (http://microarrays.nki.nl/download/protocols.html). RNA concentrations were measured using the nanodrop method at 260 nm/280 nm. The ratios of RNA concentration to protein concentration were above 1.65 in all samples. Denaturing gel electrophoresis was run to test the quality of RNA for 16S and 23S RNAs. Two micrograms of RNA from each sample were loaded on a 1% agarose gel, and the RNA was transferred to a nylon membrane using a dot blot apparatus and hybridized after UV crosslinking with a UTP-11-digoxigenin-labeled antisense RNA probe specific for ycnJ mRNA. The riboprobe was synthesized by in vitro transcription using T7 RNA polymerase. The T7 promoter sequence was introduced into the PCR product of

**TABLE 2. Primers**

<table>
<thead>
<tr>
<th>Primer (5′ → 3′)</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csp100-Us-FP</td>
<td>CCA CAT GAC GAA GCA ACT TCG TAC AG</td>
</tr>
<tr>
<td>Csp100-Us-RP</td>
<td>GCA AGT CAG CAC GAA CAC GAA AGT TTT TAT ATG TAA TGT TTT ATG</td>
</tr>
<tr>
<td>Csp100-Ds-FP</td>
<td>TTC GTC ATG TTG TCT GCT TAT TTT TAA TAG TAA TCT ATT ATT TAA CGG GAG GAA A TAA GGG</td>
</tr>
<tr>
<td>Csp100-Ds-RP</td>
<td>GGC TTC CGG TTT GTC AGT TTG</td>
</tr>
<tr>
<td>Csp101-Us-FP</td>
<td>GCC AAG GAG CAG GAA AAA GTG G</td>
</tr>
<tr>
<td>Csp101-Us-RP</td>
<td>CTC TTT CCA GTG TAA CTG TAG TAT TAG GC TGC ACC GGC GAC TAT TTT TGG</td>
</tr>
<tr>
<td>Csp101-Ds-FP</td>
<td>TAA ACT GTT TAA ACT ACA GAT TAA AAA AAT TAT AA GCA GAA GGA</td>
</tr>
<tr>
<td>Csp102-Us-FP</td>
<td>CGG ATC CTA CAA TCA CCC CAA TTG C</td>
</tr>
<tr>
<td>Csp102-Ds-FP</td>
<td>TAA ACT ATG GAA CCC ATG GAG CAG CAG</td>
</tr>
<tr>
<td>Csp103 ΔcsoK-U6-RP</td>
<td>TGG ACC TCC AAT TAA GAG GAG</td>
</tr>
<tr>
<td>Csp103 ΔcsoK-U6-RP</td>
<td>TGG ACC TCC AAT TAA GAG GAG</td>
</tr>
<tr>
<td>ycnK pET28a + FP</td>
<td>ATA TAT TCA TCG AAG GAA ACA TGG GAT GGT G</td>
</tr>
<tr>
<td>ycnK pET28a + RP</td>
<td>TGG ACC TCC AAT TAA GAG GAG</td>
</tr>
<tr>
<td>ycnJ pET28a + FP</td>
<td>ATA TAT TCA AAT TAA GAG GAA ACA TGG GAT GGT G</td>
</tr>
<tr>
<td>ycnJ pET28a + RP</td>
<td>ATA TAT TCA AAT TAA GAG GAA ACA TGG GAT GGT G</td>
</tr>
<tr>
<td>csoR pET28a + FP</td>
<td>TGG ACC TCC AAT TAA GAG GAG</td>
</tr>
<tr>
<td>csoR pET28a + RP</td>
<td>TGG ACC TCC AAT TAA GAG GAG</td>
</tr>
</tbody>
</table>

Underlining indicates the flanking regions of resistance cassette and restriction sites.
Microarray analyses reveal ycnJ as a putative copper uptake determinant. In order to gain insights into the transcriptional response under various copper conditions, microarray experiments were performed with the following combinations: (i) WT (BMM without Cu) compared with WT (BMM), (ii) ΔcsoR mutant (BMM without Cu) compared with WT (BMM without Cu), (iii) WT (BMM plus Cu) compared with WT (BMM), and (iv) ΔcsoR mutant (BMM plus Cu) compared with WT (BMM plus Cu). The essential results from these experiments are summarized in Table 3. B. subtilis WT cultures grown under copper-limiting conditions (without Cu) show a significant increase in the upregulation of the copper-responsive genes ycnI, ycnJ, ycnK, and ycnL. In contrast, in the WT the same genes are significantly downregulated in copper-replete medium (plus Cu). Thus, we speculated that these genes play a possible role in copper acquisition. To investigate this hypothesis, deletion mutants were constructed and checked for their ability to grow under different copper availability conditions. The ΔycnK mutant showed no or little difference in growth under copper-limiting conditions (Fig. 1B). In contrast, it exhibited...
enhanced growth under copper excess conditions (Fig. 1A). On the other hand, the ∆ycnJ mutant exhibited a growth-defective phenotype under copper-limiting conditions (Fig. 1B), suggesting an important role for YcnJ in copper import. In case of the ∆ycnL mutant, no effect on growth under both conditions was observed (data not shown).

The CopC and CopD gene products of *P. syringae* exhibit a high sequence identity (28%) with YcnJ. The N-terminal region of YcnJ (1–135 aa) is homologous to the periplasmic protein CopC, a copper binding protein which is involved in copper uptake in *P. syringae* (1, 5). The putative copper binding amino acid residues (His-24, Glu-51, Asp-108, and His-110) identified within the N-terminal region of YcnJ are highly homologous to the Cu(II) binding sites in CopC (1). The C-terminal region of YcnJ (401 aa) represents a transmembrane domain that is homologous to the inner membrane copper transport protein CopD of *P. syringae* (1, 5). Alignments of the N- and C-terminal regions of YcnJ with CopC and CopD are shown (Fig. 2). For the genes *ycnK* and *ycnL*, encoding a putative transcriptional regulator and a putative signal permease, respectively, several metal binding sites (Cys-x-x-Cys and Cys-x-Cys) were identified. YcnK was identified as a putative transcriptional regulator with a conserved helix-turn-helix motif in its N-terminal region and a C-terminal region with a sensor function. The sensor function is defined by a NosL superfamily motif which indicates specific binding of Cu(I) (17). Apart from these genes, the copper-inducible *copZA* operon, encoding a copper chaperone and an efflux ATPase, was found to be strongly upregulated in the ∆csoR mutant, suggesting a considerable role of CsoR in copper efflux as recently described (26).

**Regulation of ycnJ by YcnK and CsoR.** The initial transcriptome studies were followed by a closer examination of *ycnJ* regulation with respect to different copper concentrations and metal binding sites.
possibly involved transcription factors. As a first candidate, we addressed the putative transcriptional regulator-encoding gene ycnK, which is located directly upstream from ycnJ. A ycnK deletion mutant was constructed and grown under different copper conditions along with the WT. Total RNA was isolated at mid-log phase, and ycnJ gene expression was estimated semi-quantitatively by use of dot blots (Fig. 3). As expected, transcription of ycnJ in the WT was elevated under copper-limiting conditions. In comparison to the WT, the ycnK mutant showed an upregulation of ycnJ expression, especially under copper excess conditions (Fig. 3A). Further in this context, the effect of increasing copper concentrations on ycnJ gene expression was tested, and the ΔycnK mutant was found to upregulate ycnJ expression during copper excess approximately twofold compared to the WT (Fig. 3B). This points to a function of YcnK in which it acts as a negative regulator of ycnJ. Since this function is present mainly under conditions of high copper concentrations, YcnK is predicted to use copper as a corepressor. In addition, enhanced growth of the ycnK mutant under copper excess conditions was observed (Fig. 1A). Thus, as long as toxic copper concentrations are compensated for by copper efflux detoxification systems such as CopZA, induction of the predicted copper uptake system YcnJ is not detrimental for cell growth without carbon and energy source limitation.

As a second putative candidate for ycnJ regulation, we examined the recently described copper efflux regulator CsoR (16, 26), according to the finding of ycnJ upregulation in the ΔcsoR background during the microarray studies. Since absence of the CsoR repressor leads to strong derepression of the copZA copper efflux system (26), effects of CsoR on ycnJ expression might be regarded as rather indirect via modulating the intracellular copper concentration and, in the course of that, also YcnK activity. However, when the ΔcsoR and ΔycnK backgrounds were combined in a ΔcsoR ΔycnK double mutant which was tested for ycnJ expression, an additional elevation of ycnJ expression compared to that in the ΔycnK mutant was observed (Fig. 3C), suggesting also a direct participation of CsoR in ycnJ regulation.

**Estimation of intracellular copper content.** To further verify the predicted roles of ycnJ and ycnK as a copper uptake mediator and corresponding regulator, respectively, total intracellular copper contents were measured by using inductively coupled plasma mass spectrometry analysis. These studies revealed that the ΔycnK mutant contains approximately double the amount of cytoplasmic copper (511.1 ppb) in the WT (278.87 ppb) and the ΔycnK mutant (205.0 ppb). These high values are a result of copper accumulation under copper excess conditions. In agreement with the dot blot results, these findings suggest that YcnK may act as a negative transcriptional regulator of copper uptake that is active in the presence of copper. In addition, the entire copper contents within the WT and ΔycnJ cells under normal and copper-limiting conditions were determined. The results revealed that the amount of copper in WT cells grown in the presence of the copper-specific chelator BCS is approximately twofold higher (3.58 ppb) than that in cells grown without the addition of BCS (1.84 ppb). This might be a possible consequence of ycnJ upregulation under copper-limiting conditions, where the ΔycnJ mutant under normal conditions holds only 1.2 ppb (Fig. 3A).

The total copper content measured in the ΔycnJ mutant when
grown in the presence of BCS was found to be 1.57 ppb and thus more than twofold less than that in the WT under these conditions. Together with the growth experiments, this supports the supposed role for YcnJ in copper acquisition under copper-limiting conditions.

**Copper-induced oligomerization of CsoR and YcnJ.** We cloned and overexpressed CsoR and the cytosolic domain (N-terminal 135 aa) of YcnJ to examine possible formation of oligomeric complexes in the presence of copper by using native gel electrophoresis. Polyacrylamide gels (6%) were loaded with assay mixtures containing protein samples that were incubated with and without metal preincubation as a control (first lane) or after incubation with either 0.2 mM CuCl or 0.2 mM CuSO4 (right panel). (B) CsoR recombinant protein, purified using Ni-NTA chromatography, was loaded on a denaturing 17% gel along with markers, and the molecular mass of the protein was observed to be 12.5 kDa, including the His6 tag (left panel). Copper binding studies were performed in a 6% native gel, in which lanes were loaded with 80 μg purified protein incubated without or with 200 μM Cu(I) and/or 100 μM EDTA as indicated (right panel). (C) YcnK recombinant protein, purified using Ni-NTA chromatography, was loaded on a denaturing 17% gel along with markers, and the molecular mass of the protein was observed to be 23 kDa, including the His6 tag (left panel). Metal binding studies with purified proteins were performed in 6% native gels. Lane 1 is loaded with 100 μg purified protein. Protein samples incubated with 200 μM Cu(I) and 200 μM Cu(II) are loaded in lanes 2 and 3.

![Image](https://example.com/image1)

**FIG. 4.** Copper-induced oligomerization. (A) The N-terminal part (135 aa) of recombinant YcnJ protein, purified using Ni-NTA chromatography, was loaded on denaturing 17% sodium dodecyl sulfate gel along with markers. The molecular mass of the protein was observed to be 16.5 kDa, including the His6 tag and two additional amino acids before the start codon (left panel). A native 6% polyacrylamide gel was loaded with 15 μg of purified protein either without metal preincubation as a control (first lane) or after incubation with either 0.2 mM CuCl or 0.2 mM CuSO4 (right panel). (B) CsoR recombinant protein, purified using Ni-NTA chromatography, was loaded on a denaturing 17% gel along with markers, and the molecular mass of the protein was observed to be 12.5 kDa, including the His6 tag (left panel). Copper binding studies were performed in a 6% native gel, in which lanes were loaded with 80 μg purified protein incubated without or with 200 μM Cu(I) and/or 100 μM EDTA as indicated (right panel). (C) YcnK recombinant protein, purified using Ni-NTA chromatography, was loaded on a denaturing 17% gel along with markers, and the molecular mass of the protein was observed to be 23 kDa, including the His6 tag (left panel). Metal binding studies with purified proteins were performed in 6% native gels. Lane 1 is loaded with 100 μg purified protein. Protein samples incubated with 200 μM Cu(I) and 200 μM Cu(II) are loaded in lanes 2 and 3.

**FIG. 5.** Estimation of cysteine thiolate bond formation. Purified CsoR protein (80 μM) was incubated with different copper concentrations ranging from 10 μM to 80 μM, and the corresponding cysteine thiolate-copper complex formation was measured at 240 nm. The values obtained were plotted on a graph, and the Cu0.5 was determined.
the shift was not abolished in the presence of EDTA and copper (Fig. 4B), indicating that CsoR has higher affinity to Cu(I) than EDTA. A similar observation has been made previously in binding competition experiments with CsoR and BCS (16). To define the half-maximal copper concentration (Cu0.5), it was necessary to saturate the CsoR protein; different copper concentrations of between 10 and 80 μM were added to the assay mixture, keeping the protein concentration constant at 80 μM. The formation of cysteine thiolate-copper complex was measured at 240 nm (Fig. 5), and the calculated Cu0.5 was found to be at 35.5 μM, suggesting a Cu(I)-CsoR complex stoichiometry of 1:1.

Experiments performed with the recombinant periplasmic domain of YcnJ (N-terminal 135 aa) exhibited oligomerization specifically in the presence of Cu(II), which resulted in a clear shift of the preincubated protein under these conditions (Fig. 4A). The shift observed upon incubation with Cu(I) was rather weak and might also result from partial oxidation of Cu(I) to Cu(II). However, these findings suggest that YcnJ recruits into an oligomeric state if copper sensing and/or transport into the cell is mediated. The specific response of the predicted periplasmic N-terminal domain of YcnJ to Cu(II) further supports its role in uptake of extracellular oxidized copper, whereas copper-responsive regulators such as CsoR are specific for the intracellular copper in its reduced state.

In the case of YcnK, which was also recombinantly expressed and tested for copper-induced oligomerization, no such oligomerization in the presence of copper was observed (Fig. 4C). However, since oligomerization may also take place in the absence of copper and might additionally become further stabilized but not altered by addition of the metal, as already suggested for M. tuberculosis CsoR (16), there is yet no indication that YcnK does not oligomerize or does not bind copper at all.

Conclusions. Transcriptome studies were performed to identify genes involved in copper homeostasis in B. subtilis. So far, copper transport in B. subtilis is known only as a function of efflux, which is mediated by the CsoR-dependent CopZA system and the nonspecific cation diffusion facilitator CzcD, which is repressed by the ArsR homolog CzrA (18, 26). Components and mechanisms of copper uptake have not yet been identified. In our approach, we have identified genes that were upregulated by copper deprivation and downregulated under copper excess. The ycnJ gene was especially highly upregulated under copper-limiting conditions. Unlike P. syringae, in which copper uptake is assisted by two distinct proteins (CopC and CopD), B. subtilis YcnJ is orga-
nized in a single polypeptide chain that facilitates the direct transfer of copper ions across the membrane. Studies undertaken with *P. syringae* demonstrate either that the CopC protein could interact with CopA and perhaps the outer membrane protein CopB to perform copper sequestration or that CopC along with CopD may function in copper uptake. Disruption of the *ycnJ* gene in *B. subtilis* resulted in a growth-defective phenotype under copper-limiting conditions and a reduced intracellular copper content. Thus, these findings indicate that the primary role of YcnJ in *B. subtilis* is associated with copper uptake. A putative resistance function as observed for CopC in *P. syringae* cannot be excluded. However, since this resistance was described for the periplasmic compartment, this might be a rather secondary function in *B. subtilis*. In an attempt to find the possible transcriptional factors that regulate the expression of *ycnJ*, mutants with a deletion of the unknown transcriptional regulator gene *ycnK* in combination with a deletion of the recently identified copper-sensing repressor gene *csoR* were constructed and the *ycnJ* expression was quantified using dot blots. The Δ*ycnK* mutant showed elevated expression of *ycnJ* compared to the WT, especially under copper excess conditions. Expression was further elevated in the background of the Δ*csoR ΔycnK* double mutant, suggesting that both regulators participate in *ycnJ* expression control. The current model for copper homeostasis for *B. subtilis* (Fig. 6) shows the novel components presented here and indicates the regulatory connection between copper uptake and efflux systems. As far as this interplay has been investigated, it points out the demands on a system that is developed to maintain the essential accurate levels of copper inside the cell and, at the same time, to avoid passage over the critical threshold of copper toxification in order to allow proper physiological function.

ACKNOWLEDGMENTS

We gratefully acknowledge the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie for financial support.

REFERENCES


Bacterial copper uptake is essential for growth, and the regulation of copper transport is complex and involves multiple proteins and transcriptional factors. The YcnJ protein is involved in copper homeostasis, and its expression is regulated by the copper-sensing repressor CsoR. The study also highlights the role of YcnJ in reduced intracellular copper content, which is associated with copper uptake. The findings support the idea that YcnJ is involved in copper sequestration or that the primary role of YcnJ is associated with copper uptake. The study further suggests that both regulators, YcnJ and YcnK, participate in the regulation of copper homeostasis. The model presented for copper homeostasis in *B. subtilis* indicates that copper uptake and efflux are interconnected, with the regulation of copper levels being crucial for cellular function and survival.