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

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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 *cusRS* genes form a sensor-regulatory pair which senses copper and activates the *cusCFBA* genes (19). CusF is a periplasmic copper binding protein, while *cusCBA* 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 eubacteria (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 *copABCD* is plasmid encoded and is regulated by the two-component system *copRS* (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).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Function	Source or reference
B. subtilis strains			
ATCC 21332	WT		5a
CSP100	Δ*csoR*::*erm*	Copper efflux transcriptional regulator	This study
CSP101	Δ*ycnJ*::*spc*	Copper homeostasis	This study
CSP 102	Δ*ycnK*::*erm*	Transcriptional regulation	This study
CSP 103	Δ*csoR*::*erm* Δ*ycnK*::*spc*	Transcriptional regulation	This study
E. coli strains			
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*)ϕ80*lacZ*ΔM15 Δ*lacX74 deoR nupG recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1* λ$^-$	Transformation	Invitrogen
BL21(DE3)	F$^-$ *ompT gal dcm lon hsdSB* (r_B^- m_B^-) λ(DE3)	Overexpression	Novagen
Plasmids			
pUS 19	Spc^r	Antibiotic resistance cassette Spc^r	3a
pMUTIN	Erm^r	Gene disruption vector; antibiotic resistance	31a
pET28a+	Kan^r	Expression vector	Novagen
pCSP01	pET28a+ containing N-terminal 135 codons of *ycnJ* as C-terminal His_6 tag fusion	Possible copper import	This study
pCSP02	pET28a+ containing *yvgZ* as a C-terminal His_6 tag fusion	Transcriptional regulation	This study
pCSP03	pET28a+ containing *ycnK* as a C-terminal His_6 tag fusion	Possible copper import transcriptional regulator	This study

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 21332 (wild type [WT]) was grown in Belitsky 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. Unless otherwise indicated, liquid medium was inoculated from an overnight preculture and incubated at 37°C with constant shaking at 225 rpm. All glassware were washed with 0.1 M HCl and double-distilled water before autoclaving. The antibiotics erythromycin (1 μg ml^{-1}) and lincomycin (25 μg ml^{-1}), both for testing macrolide-lincosamide-streptogramin B resistance, and spectinomycin (100 μg ml^{-1}) were used for the selection of various *B. subtilis* mutants after construction. For selection of *E. coli* Top10 strains with transformed plasmids, the antibiotic kanamycin (50 μg ml^{-1}) was used. *E. coli* strain BL-21 was used for protein overexpression. For RNA preparations, bacteria were harvested at mid-log phase.

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 21332 strain for mutant construction. Restriction enzymes, T4 DNA ligase, and calf intestinal phosphatase were used according to the manufacturer's instructions (New England Biolabs).

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 21332 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 21332 to generate the Δ*csoR*, Δ*ycnK*, Δ*ycnJ*, and 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. The erythromycin resistance cassettes used for the construction of the Δ*csoR* and Δ*ycnK* single mutants were amplified from the pMUTIN vector. To generate the Δ*ycnJ* mutant, the spectinomycin resistance cassette was amplified from the pUS19 vector, while the resistance cassettes for the Δ*csoR* Δ*ycnK* double mutant were amplified from pMUTIN and pUS19, respectively.

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 21332 as the template and cloned with NcoI and XhoI 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 the integration of the fragment and transformed into BL21 cells using electroporation. Overnight cultures were grown by inoculating an isolated colony from a plate into 5 ml LB medium containing kanamycin (50 μg ml^{-1}). The overnight cultures were used to inoculate 2 liters of LB medium containing kanamycin (50 μg ml^{-1}) with a starting optical density at 600 nm (OD_{600}) of 0.05. Inoculated cultures were grown under continuous shaking at 225 rpm at 35°C, and the temperature was decreased to 30°C after 1 hour. When the OD_{600} of the cells reached 0.5 to 0.6, IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.1 mM was added to the culture flasks and growth was continued for 4 hours. Cells were harvested by centrifugation (Sorvall RC 5B plus), and the resulting pellets were resuspended in HEPES-A buffer (50 mM HEPES, 300 mM NaCl [pH 7.5], 1 mM dithiothreitol [DTT]) and lysed with EmulsiFlex-C5 Avestin. The lysate obtained

TABLE 2. Primers

Primer (5′ → 3′)	Sequence[a]
Csp100-Us-FP	CCA CAT GAC GAA GCA ACT TCG TAC AG
Csp100-Us-RP	GCA AGT CAG CAC GAA CAC GAA CC GCT TTT ATG GTT TAA TGT TTT ATG TTC GTT ATG CTT TTC CAT
Csp100-Ds-FP	GTC TAT TTT TAA TAG TTA TCT ATT ATT TAA CGG GAG GAA A TAA GGG GAA CAG GCC ATT TCT GAG C
Csp100-Ds-RP	GGC TTC CCG TTT GTC ACG GTT CC
Csp101-Us-FP	GGC AAG GAG CAG GCA AAA GTG G
Csp101-Us-RP	CTC TTG CCA GTC ACG TTA CGT TAT TAG GGC CTG ACC GGC GAC TTT AAC G
Csp101-Ds-FP	CTA TAA ACT ATT TAA ATA ACA GAT TAA AAA AAT TAT AA GCA GAA GGA TGA TCC ACC ATC TGT TTC G
Csp101-Ds-RP	CGC TCA AAT AAC TCC CAA AGC GTT GC
Csp102-Us-FP	CCG ATC CTA CAA TCA CCC CAA TTG C
Csp102-Us-RP	GCA AGT CAG CAC GAA CAC GAA CC CAG CCA CTT CAG TAT GTG TTG CTG TC
Csp102-Ds-FP	CTA TAA ACT ATT TAA ATA ACA GAT TAA AAA AAT TAT AA GCA GAA GGA TGA TCC ACC ATC TGT TTC G
Csp102-Ds-RP	GAG AGC AGC AAA ACG AGT GCC G
Csp103 Δ*ycnK*-Us-FP	CCG ATC CTA CAA TCA CCC CAA TTG C
Csp103 Δ*ycnK*-Us-RP	CTC TTG CCA GTC ACG TTA CGT TAT TAG CAG CCA CTT CAG TAT GTG TTG CTG TC
Csp103 Δ*ycnK*-Ds-FP	CTA TAA ACT ATT TAA ATA ACA GAT TAA AAA AAT TAT AA CGA AAG AAA CGC TGC ACC TGC ACC
Csp103 Δ*ycnK*-Ds-RP	GAG AGC AGC AAA ACG AGT GCC G
ycnJ pET28a+FP	ATA TAT CCA TGG ACA TGA AGC GAA ACA GAT GGT GG
ycnJ pET28a+RP	TAT ATA CTC GAG TGA ATC GGC TGC TTT TTG GC
csoR pET28a+FP	ATA TAC CAT GGA AAA GCA TAA CGA ACA TAA AAC ATT AAA CCA TAA AAG C
csoR pET28a+RP	ATA TAC TCG AGT GAT TTT GTG AAC TTT TTA AAT ACG TCC AAA AGC TCA G
ycnK pET28a+FP	ATA TAC CAT GGA CAT GCT TCC G AT TAA TAG ACA GCA ACA C
ycnK pET28a+RP	TAT ATC TCG AGT TTC TTG GTG CAG GTG CAG CG

[a] Underlining indicates the flanking regions of resistance cassette and restriction sites.

was clarified by centrifugation (Sorvall RC 26 plus) at 17,000 rpm for 30 min, and an Ni-nitrilotriacetic 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 15 to 30 min at room temperature and loaded onto 6% continuous native polyacrylamide gels. Samples were run at 100 V and 10 mA for about 4 to 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 Δ*csoR* mutant were grown in BMM under copper-replete conditions (growth in the presence of 0.5 mM CuCl) and copper-depleted conditions (growth in the presence of the copper-specific chelator BCS at 0.25 mM). Cultures were harvested in the mid-log phase for RNA extraction. The Macaloid/Roche method was used for the RNA extraction (13). Concentrations of RNA were measured using the nanodrop method. For reverse transcription, a solution containing 18 μl annealing mix (10 to 20 μg total RNA and 2 μl random nonamers adjusted to a final volume of 18 μl with nuclease-free water) was incubated for 5 min at 70°C and 10 min at 4°C for annealing. Reverse transcription was performed by addition of this annealing mix to a solution containing 6 μl Superscript III buffer (supplied with the reverse transcriptase), 10 mM DTT, nucleotide master mix containing aminoallyl-dUTP, and 300 U Superscript III reverse transcriptase (Invitrogen). cDNA was synthesized overnight at 42°C in a total volume of 30 μl. The aminoallyl-modified cDNA was incubated with the CyDye NHS ester (Cy3 or Cy5 monoreactive dye) at room temperature in the dark for 60 to 90 min. Labeled cDNAs were purified using Nucleo Spin Extraxt II columns, and the dye incorporation was measured using the nanodrop method. Equal quantities of Cy3/Cy5-incorporated cDNAs were mixed, dried in a Speedvac, and then dissolved in 5 μl H_2O, followed by heating at 94°C for 2 min. The heated sample was subsequently mixed with 30 μl preheated hybridizing buffer (68°C), hybridized onto microarray glass slides, and incubated overnight at 68°C in a hybridization oven.

Microarray data analysis. Hybridized DNA arrays were read using a GenePix 4200AL autoloader (Axon Instruments), and the data obtained were processed with ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD) ArrayVision software. Expression levels were processed and normalized (Lowess method) with Micro-Prep (10, 32). The ln-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 Δ*csoR* 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 OD_{600} 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 OD_{600} of 0.05, and cells were harvested at an OD_{600} 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 was subsequently dotted onto a nylon membrane using a dot blot apparatus and hybridized after UV cross-linking 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 3. Microarray results

Genetic background	Ratio of expression levels[a]			
	WT (BMM−Cu)/ WT (BMM)	Δ*csoR* (BMM−Cu)/ WT (BMM−Cu)	WT (BMM+Cu)/ WT (BMM)	Δ*csoR* (BMM+Cu)/ WT (BMM+Cu)
copA	0.90	7.80	0.93	4.20
copZ	0.98	16.64	2.61	6.53
ycnJ	8.17	0.93	0.40	2.31
ycnI	6.36		0.29	
ycnK	10.9		0.34	
ycnL	1.77		0.68	

[a] Less than 1, downregulation; greater than 2, upregulation.

the *ycnJ* gene by using primers 5′-AGCTCGTCAAACGGACGAGACG-3′ and 5′-TAATACGACTCACTATAGGGGAAATCCATCAAAATGCCGACCG-3′ (the T7 promoter extension is underlined). After hybridization and washing, the filters were treated with a digoxigenin-specific antibody fragment conjugated with alkaline phosphatase (Roche) and AttoPhos (Amersham Biosciences) as an enhanced chemifluorescence substrate. The hybridization signals were detected with a Storm860 fluorescence imager, and relative signal quantification was performed with ImageQuant software.

RESULTS AND DISCUSSION

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) Δ*csoR* mutant (BMM without Cu) compared with WT (BMM without Cu), (iii) WT (BMM plus Cu) compared with WT (BMM), and (iv) Δ*csoR* 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 Δ*ycnK* mutant showed no or little difference in growth under copper-limiting conditions (Fig. 1B). In contrast, it exhibited

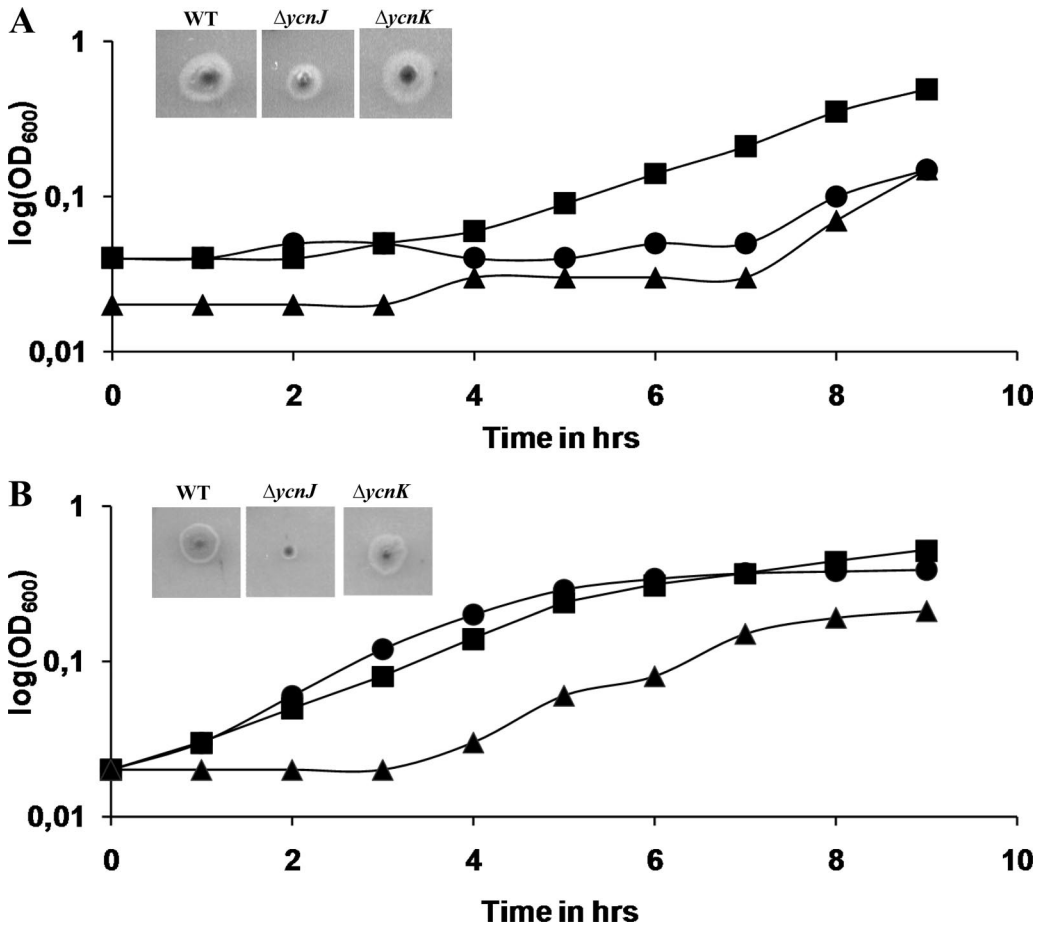

FIG. 1. (A) Analysis of copper-dependent growth in BMM with 1 mM copper in excess. Growth curves of the WT (●), the Δ*ycnJ* mutant (▲), and the Δ*ycnK* mutant (■) are shown. The insets show the phenotypes of the WT and the Δ*ycnJ* and Δ*ycnK* mutants on BMM with 1 mM copper in excess. (B) Analysis of copper-dependent growth in BMM with 0.5 mM BCS. Growth curves of the WT (●), the *ycnJ* mutant (▲), and the Δ*ycnK* mutant (■) are shown. The insets show the phenotypes of the WT and the Δ*ycnJ* and Δ*ycnK* mutants on BMM agar with 0.5 mM BCS.

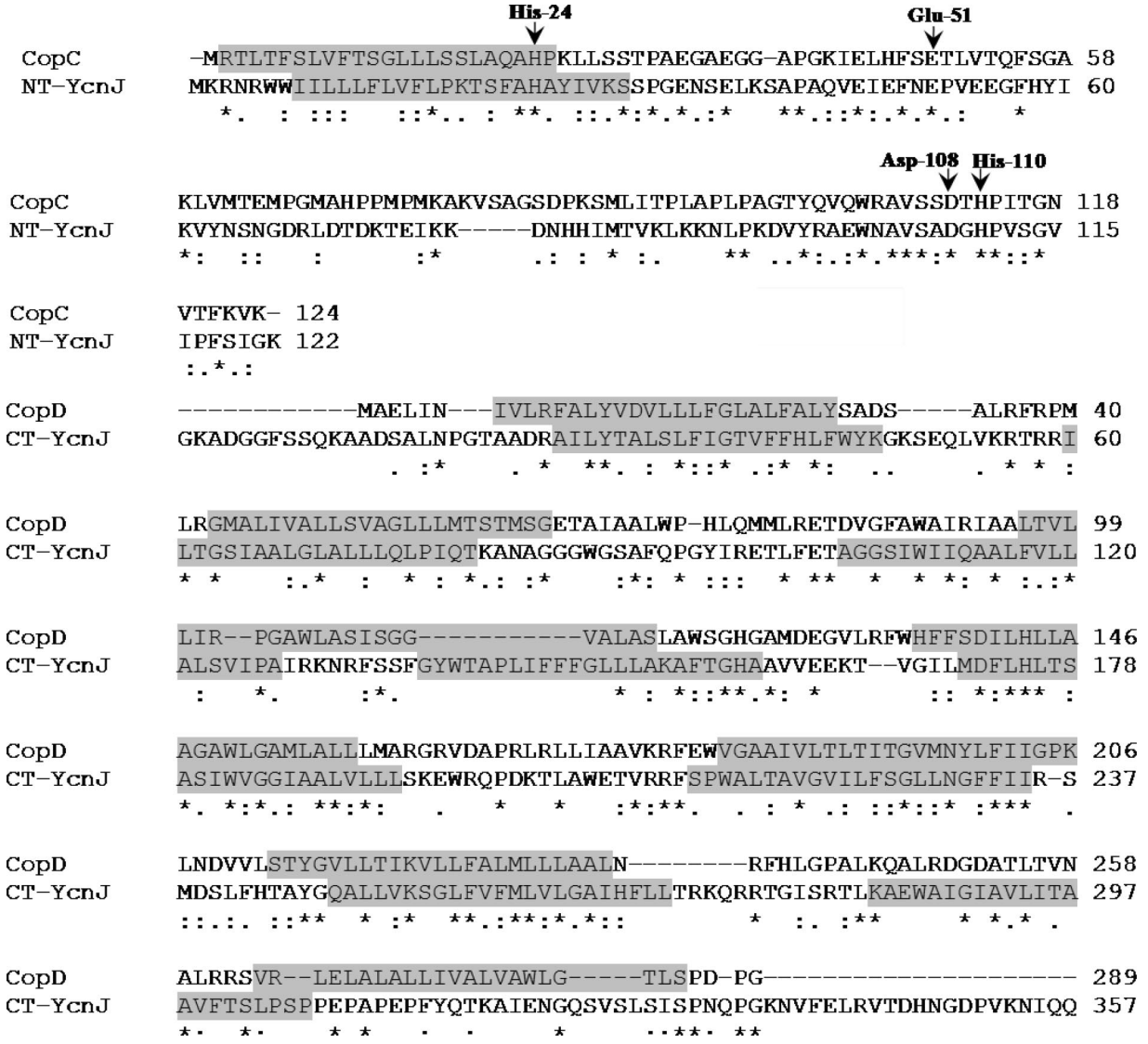

FIG. 2. Alignment of amino acid sequences of *P. syringae* CopC and CopD and *B. subtilis* YcnJ (ClustalW). NT, N terminal; CT, C terminal. Arrows indicate conserved copper binding residues. Highlighted amino acids represent the predicted transmembrane domains.

enhanced growth under copper excess conditions (Fig. 1A). On the other hand, the Δ*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 Δ*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 (~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 Δ*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

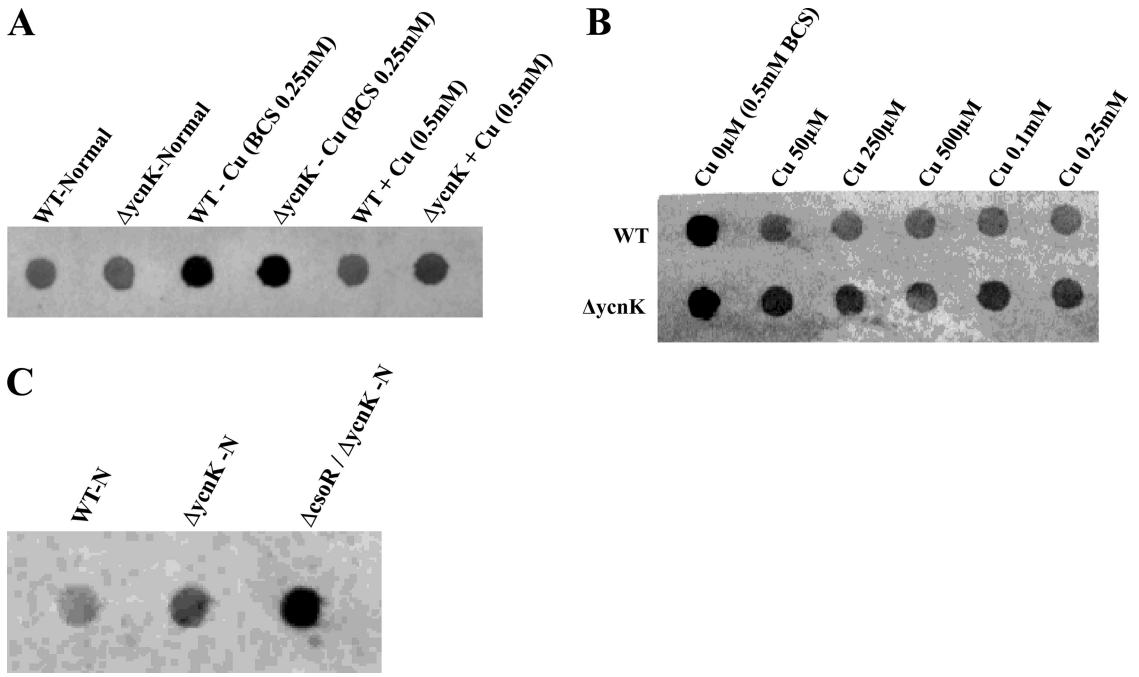

FIG. 3. Transcriptional analysis of *ycnK*- and *csoR*-dependent *ycnJ* expression. Two micrograms of total RNA isolated from the WT and mutants under normal, 0.25 mM BCS, and 0.5 mM copper excess conditions was blotted onto a nylon membrane by dot blotting and hybridized with a digoxigenin-UTP-labeled antisense riboprobe specific for the *ycnJ* transcript. Hybridization signals were detected by using a digoxigenin-specific antibody fragment conjugated with alkaline phosphatase and AttoPhos as chemifluorescence substrate. (A) *ycnK*-dependent expression of *ycnJ* with different copper concentrations. (B) *ycnK*-dependent expression of *ycnJ* under increasing copper concentrations. (C) Expression of *ycnJ* in the WT and the Δ*ycnK* and Δ*csoR* Δ*ycnK* mutants under normal conditions.

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 semiquantitatively 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 Δ*ycnK* 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 Δ*csoR* 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 Δ*csoR* and Δ*ycnK* backgrounds were combined in a Δ*csoR* Δ*ycnK* double mutant which was tested for *ycnJ* expression, an additional elevation of *ycnJ* expression compared to that in the Δ*ycnK* 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 Δ*ycnK* mutant contains approximately double the amount of cytoplasmic copper (511.1 ppb) in the WT (278,87 ppb) and the Δ*ycnJ* 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 Δ*ycnJ* 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 those grown without the addition of BCS (1.84 ppb). This might be a possible consequence of *ycnJ* upregulation under copper-limiting conditions, where the Δ*ycnJ* mutant under normal conditions holds only 1.2 ppb (Fig. 3A). The total copper content measured in the Δ*ycnJ* mutant when

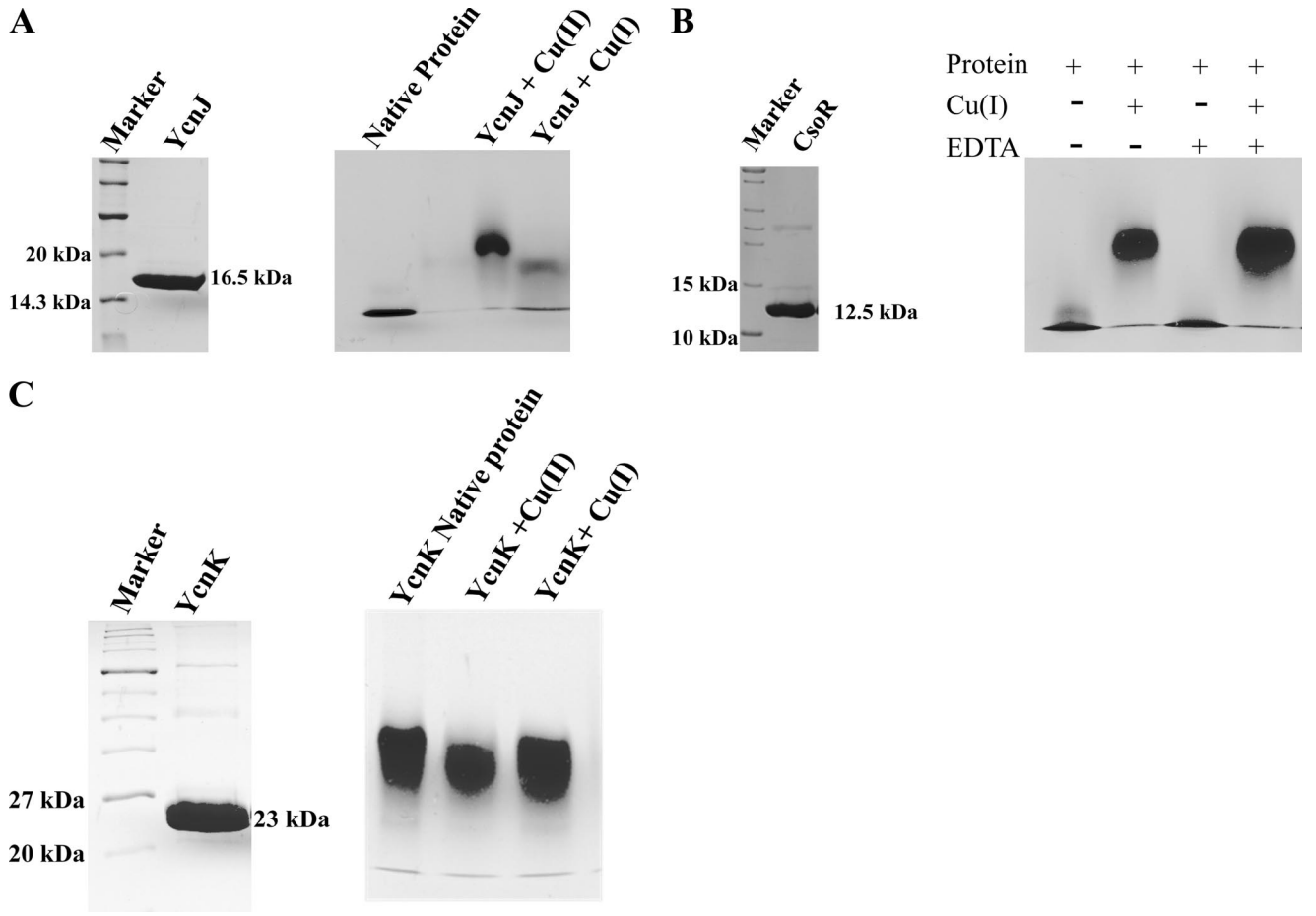

FIG. 4. Copper-induced oligomerization. (A) The N-terminal part (135 aa) of recombinant YcnJ protein, purified using Ni-NTA chromatography, was loaded on denaturing 17% sodium dodecyl sulfate gel along with markers. The molecular mass of the protein was observed to be 16.5 kDa, including the His_6 tag 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 $CuSO_4$ (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 His_6 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 His_6 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.

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 0.1 mM CuCl and 0.1 mM DTT. DTT was added to minimize the conversion of Cu(I) to Cu(II). A gel shift of the CsoR protein sample was observed in the presence of 0.1 mM CuCl and 0.1 mM DTT (Fig. 4B). Binding was also tested in the presence of the strong metal chelator EDTA. While incubation of a protein sample with 0.5 mM EDTA alone resulted in no shift,

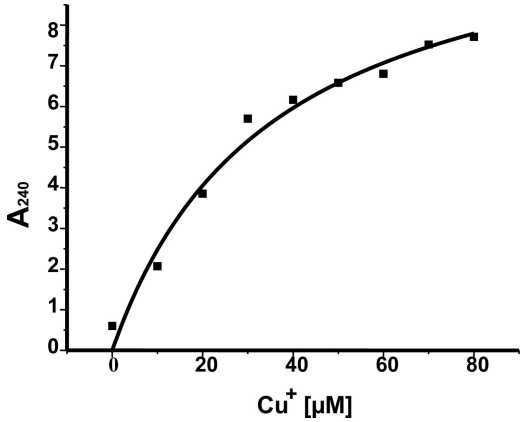

FIG. 5. Estimation of cysteine thiolate bond formation. Purified CsoR protein (80 μM) was incubated with different copper concentrations ranging from 10 μM to 80 μM, and the corresponding cysteine thiolate-copper complex formation was measured at 240 nm. The values obtained were plotted on a graph, and the $Cu_{0.5}$ was determined.

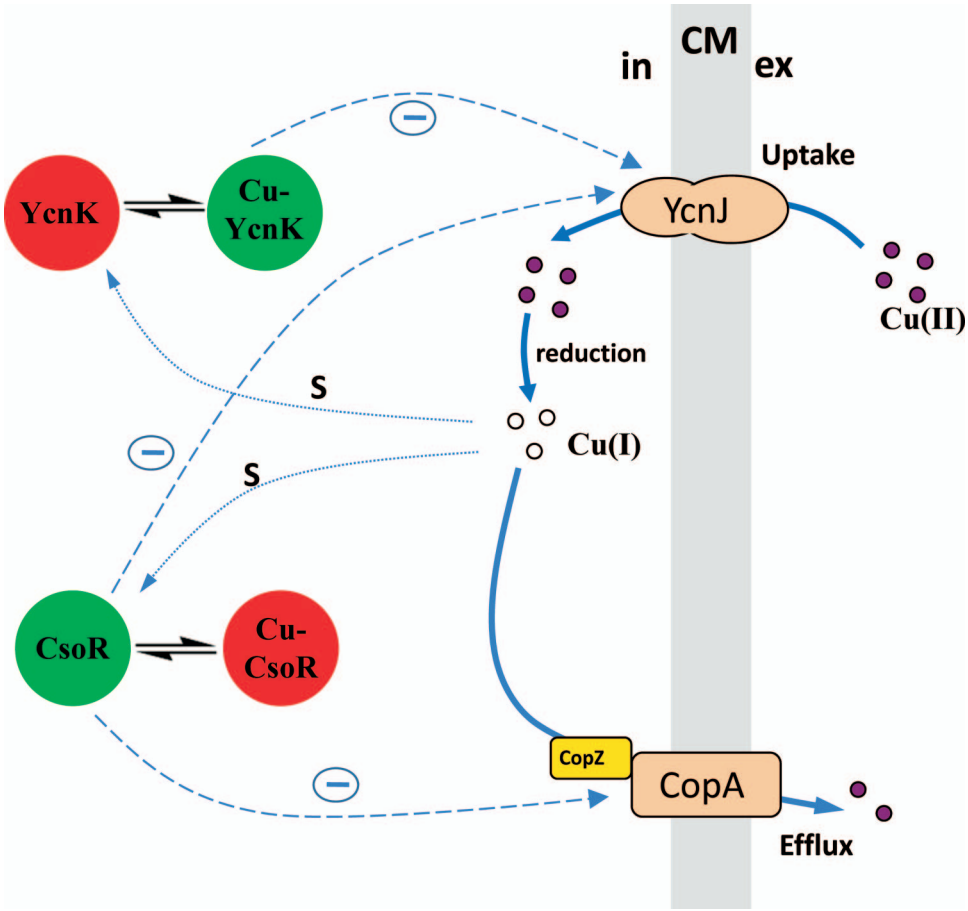

FIG. 6. Current model of copper homeostasis in *B. subtilis*, including functional components for copper uptake and efflux as well as their cognate repressors. Depending on their association with copper, the active state of transcriptional repressors YcnK and CsoR is shown in green, and the inactive state of transcriptional repressors is shown in red. The negative regulation of components (−) is indicated with dashed arrows. Copper sensing (s) is indicated with dotted arrows. Cu, copper; CM, cytoplasmic membrane; in, intracellular; ex, extracellular.

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 ($Cu_{0.5}$), 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 $Cu_{0.5}$ 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. The sequence homology studies reveal that YcnJ is highly homologous at its N terminus to CopC and at its C terminus to CopD from *P. syringae.* 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* Δ*ycnK* 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.

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