Direct and Indirect Regulation of the *ycnKJI* Operon Involved in Copper Uptake through Two Transcriptional Repressors, YcnK and CsoR, in *Bacillus subtilis*

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Northern blot and primer extension analyses revealed that the *ycnKJI* operon and the *ycnL* gene of *Bacillus subtilis* are transcribed from adjacent promoters that are divergently oriented. The *ycnK* and *ycnJ* genes encode a DeoR-type transcriptional regulator and a membrane protein involved in copper uptake, respectively. DNA binding experiments showed that the YcnK protein specifically binds to the *ycnK-ycnL* intergenic region, including a 16-bp direct repeat that is essential for the high binding affinity of YcnK, and that a copper-specific chelator significantly inhibits YcnK's DNA binding. *lacZ* reporter analysis showed that the *ycnK* promoter is induced by copper limitation or *ycnK* disruption. These results are consistent with YcnK functioning as a copper-responsive repressor that derepresses *ycnKJI* expression under copper limitation. On the other hand, the *ycnL* promoter was hardly induced by copper limitation, but *ycnK* disruption resulted in a slight induction of the *ycnL* promoter, suggesting that YcnK also represses *ycnL* weakly. Moreover, while the CsoR protein did not bind to the *ycnK-ycnL* intergenic region, *lacZ* reporter analysis demonstrated that *csoR* disruption induces the *ycnK* promoter only in the presence of intact *ycnK* and *copZA* genes. Since the *copZA* operon is involved in copper export and repressed by CsoR, it appears that the constitutive *copZA* expression brought by *csoR* disruption causes intracellular copper depletion, which releases the repression of the *ycnKJI* operon by YcnK.

Copper ion is an essential cofactor for many proteins involved in various physiological processes, such as respiration, oxidative stress response, and redox reactions in metabolic pathways. Two useful features of the copper ion as an active center of these proteins are that it is able to tightly bind to polar functional groups of amino acid residues and that it is interconvertible between +1 and +2 oxidation states. However, these features also make copper ion highly toxic when free copper ion increases in the cell, as it undergoes undesirable redox reactions to generate reactive oxygen species and inappropriately binds to the other metal binding sites of the proteins, thereby altering their properties. Recent studies suggest that the cytotoxicity of copper ion is primarily derived from its destabilizing effect on iron-sulfur clusters (10, 21). To avoid the potential toxicity of copper ion, organisms have evolved systems to strictly control the cytoplasmic concentration of copper ion, as well as its intracellular trafficking to target proteins (11).

In a Gram-positive soil bacterium, *Bacillus subtilis*, when copper ion is in excess in its cytoplasm, a specific efflux system encoded by the *copZA* operon is induced (13). In a previous study, it was reported that strong induction of the *copZA* operon occurs in the presence of copper ion, although it is not induced by the other metal ions tested, and that the CopZA system is required for resistance to a high level of copper ion (13), which accentuates its specialized function in copper export. The CopA protein, belonging to the integral membrane protein family of P-type ATPases, functions to translocate Cu^+ across the cytoplasmic membrane, while CopZ is an Atx1-like soluble protein playing a role in the Cu^+ transfer to the N-terminal domain of the CopA protein; this domain shows structural similarity to CopZ and transiently interacts with it when the transfer occurs (4, 30, 31). It seems that CopZ also serves as a copper chaperone with high binding affinity, so that the reactive free copper ion is kept at a very low level in the *B.*

subtilis cell, as demonstrated for the yeast cell (26) (Fig. 1). Eukaryotic Sco proteins play a copper chaperone role in addition to their major role in the assembly of the two copper ions forming a dinuclear center (Cu_2) in subunit II of cytochrome *c* oxidase (3). However, the *B. subtilis* Sco protein is unlikely to act as a copper chaperone, although it fulfills a redox role in Cu_2 assembly (3).

copZA transcription is controlled by a copper-sensing repressor, CsoR, dependent on copper availability within the cytoplasm (20, 32). The gene encoding CsoR is located immediately upstream of the *copZA* operon and aligned in the same direction as *copZA*. The CsoR protein forms a tetramer, and two molecules of the CsoR tetramers cooperatively bind with high affinity to an operator site that includes a characteristic palindrome sequence overlapping the *copZA* promoter. The DNA binding of CsoR is specifically inhibited by the addition of copper salts, and it was found that CsoR binds to one mol equivalent Cu^+ per monomer. Thus, it is presumed that *copZA* is derepressed by the release of CsoR from the operator when the cytosolic copper ion is in excess (Fig. 1).

Studies on copper resistance in *Pseudomonas syringae* strains infective to tomato revealed that the *copABCD* operon, located on a plasmid, confers copper resistance and that it is regulated by the CopRS two-component system. The genes encoding CopRS are located immediately downstream from and in the same direction as *copABCD*, but the *copRS* genes are transcribed from a distinct

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FIG 1 Regulatory system for the homeostasis of intracellular copper ion in *B. subtilis*. The coding regions of the *ycnKJI* operon, the *ycnL* gene, the *csoR* gene, and the *copZA* operon are indicated by large open arrows, and four promoters and four hairpin structures, probably functioning as ρ -independent transcription terminators, are indicated by bent arrows and stem-loops, respectively. The YcnK protein, found in this study to form a dimer (two ovals), binds to the intergenic region between *ycnK* and *ycnL* to repress the *ycnKJI* operon effectively and the *ycnL* gene weakly. When the cytosolic copper ion (Cu^{+} , small circles) is depleted, the binding affinity of YcnK is impaired, which leads to derepression of, at least, the *ycnKJI* operon. The YcnJ protein, predicted to possess nine transmembrane domains (17) (<http://bp.nuap.nagoya-u.ac.jp/sosui/>), functions to import copper ion from outside the cell. The CsoR protein forms a tetramer (four circles), and two molecules of the CsoR tetramer bind to the regulatory region of the *copZA* operon to repress its expression. When the cytosolic copper ion is in excess, CsoR is detached from the regulatory region, causing derepression of the *copZA* operon. The CopZ protein acts as a copper chaperone and transfers copper ion (Cu^{+}) to the N-terminal domain of the CopA protein. The CopA protein, predicted to possess seven transmembrane domains, functions to export excess copper ion out of the cell, coupling with ATP hydrolysis.

constitutive promoter (23). Similar genes for copper resistance (*pcoABCD*) and for its regulatory system (*pcoRS*) were found in a gene cluster (*pcoABCDRSE*) on a plasmid of an *Escherichia coli* strain that was isolated from a copper-rich habitat (27). Despite the high similarity between these two systems, the mechanisms that afford copper resistance are quite different, i.e., the copper resistance of the *E. coli* *pco* system is achieved by an energy-dependent efflux of copper, whereas the mechanism taken by the *P. syringae* *cop* system is copper sequestration outside the cytoplasm (7).

The *B. subtilis* YcnJ protein was found as a homolog of *P. syringae* CopCD; CopC and CopD are considered to be a periplasmic copper-binding protein and an inner membrane protein, and the N- and C-terminal parts of YcnJ show high homology to CopC and CopD, respectively (7–9). It was demonstrated that *copCD* expression in a *P. syringae* host, which does not have copper resistance, causes copper hypersensitivity and hyperaccumulation, suggesting that CopCD is also capable of copper uptake (8). In *B. subtilis*, disruption of *ycnJ* resulted in a growth-defective phenotype under copper limitation and a reduced intracellular copper content. Moreover, the *ycnJ* transcript increased greatly under copper limitation (9). Thus, these findings indicated that the primary role of *B. subtilis* YcnJ is associated with copper uptake (Fig. 1). The *ycnK* gene, encoding a transcriptional regulator belonging to the DeoR family, is located upstream of *ycnJ*. Dot blot analysis showed that the *ycnK* disruptant has higher levels of *ycnJ* tran-

scription than the wild-type strain, and its degree of increase under the copper excess conditions was remarkable. *ycnJ* transcription was also enhanced by *csoR* disruption (9). These findings suggest that both regulators participate in the control of *ycnJ* expression.

In this study, we show that the *ycnKJI* genes constitute an operon, whereas the *ycnL* gene, located immediately upstream of this operon in the opposite direction, is monocistronic (Fig. 2). The YcnK protein specifically bound to an operator in the intergenic region between *ycnK* and *ycnL* to repress the *ycnKJI* operon effectively and the *ycnL* gene weakly. YcnK's binding affinity was impaired by copper limitation, causing the *ycnKJI* derepression. Although the CsoR protein did not bind to the *ycnK-ycnL* intergenic region, it was suggested that *csoR* disruption indirectly affects YcnK's activity to release the repression of the *ycnKJI* operon by inducing intracellular copper depletion through constitutive *copZA* expression.

MATERIALS AND METHODS

Construction and cultivation of *B. subtilis* strains. The *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* strain 168 was used as the standard strain (wild type). Strains YCNKd, YCNJd, and YCNLd were constructed by integrating plasmid pMUTIN2 (35) into the *ycnK*, *ycnJ*, and *ycnL* genes of strain 168 at the 344th, 415th, and 137th bases, respectively, downstream from the translation start positions (18) (<http://bacillus.genome.jp/>).

FIG 2 Organization of the divergently oriented *ycnKJI* operon and *ycnL* gene and their promoter regions. (A) Organization of the *ycnKJI* operon and the *ycnL* gene. These genes and neighboring genes are indicated by large open arrows, and the two promoters are indicated by bent arrows and hairpin structures, respectively. aa, amino acids. (B) Promoter regions of *ycnK* and *ycnL*. The -35 and -10 sequences are underlined, and the transcription start sites (+1) and the Shine-Dalgarno (SD) sequences are enclosed in boxes. The partial coding regions of the two genes are indicated by lines, and the deleted region in the *PycnK*ΔD probe is also indicated by a line. The protected regions in the coding and noncoding strands detected on DNase I footprinting are indicated by gray bars. The 16 bp of a direct repeat sequence in the *ycnL* promoter are indicated by tandem arrows.

TABLE 1 *B. subtilis* strains used in this study

Strain	Genotype	Reference or source
168	<i>trpC2</i> (standard strain, wild type)	
YCNKd	<i>ycnK</i> ::pMUTIN2 <i>trpC2</i>	18
YCNJd	<i>ycnJ</i> ::pMUTIN2 <i>trpC2</i>	18
YCNLd	<i>ycnL</i> ::pMUTIN2 <i>trpC2</i>	18
FU1130	Δ <i>ycnK</i> :: <i>cat</i> <i>trpC2</i>	This study
FU1144	Δ <i>ycnK</i> :: <i>erm</i> <i>trpC2</i>	This study
FU1131	Δ <i>csoR</i> :: <i>cat</i> <i>trpC2</i>	This study
FU1132	Δ <i>csoR</i> :: <i>tet</i> <i>trpC2</i>	This study
FU1133	Δ <i>copZA</i> :: <i>cat</i> <i>trpC2</i>	This study
FU1145	Δ <i>copZA</i> :: <i>tet</i> <i>trpC2</i>	This study
FU1134	Δ (<i>csoR copZA</i>):: <i>cat</i> <i>trpC2</i>	This study
FU1146	Δ (<i>csoR copZA</i>):: <i>tet</i> <i>trpC2</i>	This study
FU1135	Δ <i>csoR</i> :: <i>cat</i> <i>ycnK</i> ::pMUTIN2 <i>trpC2</i>	This study
FU1136	Δ <i>copZA</i> :: <i>cat</i> <i>ycnK</i> ::pMUTIN2 <i>trpC2</i>	This study
FU1137	Δ (<i>csoR copZA</i>):: <i>cat</i> <i>ycnK</i> ::pMUTIN2 <i>trpC2</i>	This study
FU1138	Δ <i>csoR</i> :: <i>cat</i> <i>ycnJ</i> ::pMUTIN2 <i>trpC2</i>	This study
FU1139	Δ <i>copZA</i> :: <i>cat</i> <i>ycnJ</i> ::pMUTIN2 <i>trpC2</i>	This study
FU1140	Δ (<i>csoR copZA</i>):: <i>cat</i> <i>ycnJ</i> ::pMUTIN2 <i>trpC2</i>	This study
FU1141	Δ <i>ycnK</i> :: <i>cat</i> <i>ycnL</i> ::pMUTIN2 <i>trpC2</i>	This study
FU1142	Δ <i>csoR</i> :: <i>tet</i> <i>ycnL</i> ::pMUTIN2 <i>trpC2</i>	This study
FU1143	Δ <i>ycnK</i> :: <i>cat</i> Δ <i>csoR</i> :: <i>tet</i> <i>ycnL</i> ::pMUTIN2 <i>trpC2</i>	This study
FU1147	<i>amyE</i> ::[<i>cat</i> <i>PycnK</i> (-190 to 109)- <i>lacZ</i>] <i>trpC2</i>	This study
FU1148	Δ <i>ycnK</i> :: <i>erm</i> <i>amyE</i> ::[<i>cat</i> <i>PycnK</i> (-190 to 109)- <i>lacZ</i>] <i>trpC2</i>	This study
FU1149	Δ <i>ycnK</i> :: <i>erm</i> Δ <i>csoR</i> :: <i>tet</i> <i>amyE</i> ::[<i>cat</i> <i>PycnK</i> (-190 to 109)- <i>lacZ</i>] <i>trpC2</i>	This study
FU1150	Δ <i>ycnK</i> :: <i>erm</i> Δ <i>copZA</i> :: <i>tet</i> <i>amyE</i> ::[<i>cat</i> <i>PycnK</i> (-190 to 109)- <i>lacZ</i>] <i>trpC2</i>	This study
FU1151	Δ <i>ycnK</i> :: <i>erm</i> Δ (<i>csoR copZA</i>):: <i>tet</i> <i>amyE</i> ::[<i>cat</i> <i>PycnK</i> (-190 to 109)- <i>lacZ</i>] <i>trpC2</i>	This study

Strains FU1130, FU1131, FU1133, and FU1134, which carry the *ycnK*, *csoR*, *copZA*, and *csoR-copZA* deletions, respectively, were constructed as follows. Long-flanking homology PCR (38) was performed to create DNA fragments in which the chloramphenicol acetyltransferase gene (*cat*) was in the same direction as the target genes and sandwiched by the upstream and downstream flanking regions of these genes. The upstream and downstream regions were amplified by PCR with genomic DNA of strain 168 as the template and primer pairs as follows: *ycnK*UpF1/*ycnK*UpR_catup for Δ *ycnK*, *csoR*UpF1/*csoR*UpR_catup for Δ *csoR* and Δ (*csoR copZA*), and *copZ*UpF1/*copZ*UpR_catup for Δ *copZA* (the upstream regions) and *ycnK*downF_catdown/*ycnK*downR1 for Δ *ycnK*, *csoR*downF_catdown/*csoR*downR1 for Δ *csoR*, and *copZ*downF_catdown/*copZ*downR1 for Δ *copZA* and Δ (*csoR copZA*) (the downstream regions) (Table 2). The *cat* cassette was amplified by PCR with primer pair *catF*/*catR* (Table 2) and plasmid pCBB31 bearing the *cat* gene (29) as the template. The three PCR products (the upstream, *cat*, and downstream fragments) were appropriately combined by conducting cycles of denaturation, annealing, and extension in a reaction mixture containing *Ex Taq* DNA polymerase (TaKaRa-bio, Japan) and deoxynucleoside triphosphates without any primer oligonucleotide. Nested PCR with the resultant fragments as the template and primer pairs *ycnK*UpF2/*ycnK*downR2, *csoR*UpF2/*csoR*downR2, *copZ*UpF2/*copZ*downR2, and *csoR*UpF2/*copZ*downR2 (Table 2) was performed to amplify the recombinant DNA fragments, which were then used to transform strain 168 to chloramphenicol resistance (5 μ g/ml) to yield strains FU1130 (Δ *ycnK*::*cat*), FU1131 (Δ *csoR*::*cat*), FU1133 (Δ *copZA*::*cat*), and FU1134 [Δ (*csoR copZA*)::*cat*] (Table 1). The *ycnK* and *csoR* coding regions were completely deleted in the corresponding mutant strains, whereas the 3'-terminal 58 bases of the *copA* coding region remain in strains FU1133 and FU1134. Correct replacement of the target genes with *cat* was confirmed by means of PCR and DNA sequencing. Strains FU1131, FU1133, and FU1134 were transformed with plasmid pCm::Tc (33) to change chloramphenicol resistance to tetracycline resistance (10 μ g/ml), yielding strains FU1132 (Δ *csoR*::*tet*), FU1145 (Δ *copZA*::*tet*), and

TABLE 2 Oligonucleotide primers used in this study

Primer	Sequence (5'–3') ^a
ycnKupF1	CAGAGAATGATGCTTCCCTTTGAC
ycnKupR_catup	CTAATGGGTGCTTTAGTTGAAGATCATA CACCTCTTCATTAAGATC
ycnKdownF_catdown	GAGATAATGCCGACTGTACTTAACACAA CACATACAGCGGAAGG
ycnKdownR1	CTGCGTTCGCTTTTGTCTGAATCG
ycnKupF2	CTATGTTAAAACGCTTACAGTCCC
ycnKdownR2	GCTGAAGCAAAAGCGCCAGCCC
csoRupF1	CCGCCACCGCGCTTGAACCTTCAG
csoRupR_catup	CTAATGGGTGCTTTAGTTGAAGAGCGTA CACCTCTGTTAATGATATTA
csoRdownF_catdown	GAGATAATGCCGACTGTACTTAAAGCGT TTTTTATGTGAATACCCTAC
csoRdownR1	CAATCCGATTGGCGCAAGCCGCAC
csoRupF2	GTATTGATATCTTCAAGGGATCC
csoRdownR2	GTAACGACGTGATAGCCAGCTTC
copZupF1	TCAAGCTCATGTACAACCTCAGC
copZupR_catup	CTAATGGGTGCTTTAGTTGAAGAATCAA TTCCTCCTGTTTATTCTTT
copAdownF_catdown	GAGATAATGCCGACTGTACTAATGGCAT TCAGCTCCGTTTCCG
copAdownR1	GCTCACCGATTGATAAAACAGC
copZupF2	CTGGAATAATCCCGTTTCACTTC
copAdownR2	CCTCAGGGTATTGCTGAATCAG
catF	TCTTCAACTAAAGCACCCATTAG
catR	AGTACAGTCGGCATTATCTC
PycnK_XF	ATGATCTAGACATATCGCCTGTC
PycnK_BR	GAACGCGGATCCTTGCCTGATG
ycnK_NF	GTTTCGAAATGACGGTATATAG
ycnK_NR	AGCCGAACCGGCACTGACGG
ycnJ_NF	ACCGTTTGGATACGGACAAGAC
ycnJ_NR	CTTTATACCAGAAAAGATGGAAG
ycnI_NF	GAGTCTGCTGCAGGCTCTTGGG
ycnI_NR	GCTGCCGTCTTTGTAATATTGGT
ycnL_NF	CATGCCGAACTGCGGCAAAAC
ycnL_NR	TTTGAATCACGTACTTGTGCAATC
ycnK_PE	TTTCGAAACGCGCAACCTTGCG
ycnL_PE	ATGATCTGACCATATCGCCTGTC
PycnKF	GGTTTGCCGACGTTCCGGCATG
PycnKR	CAAGCAATAGCTGCACATATGATC
PycnK_delDF	GTTTCGTTTGTGTGCTTCAATTATCA TTCCACTTACTGTCTC
PycnK_delDR	GAGACAAGTAAGTGATGATAATTGAAC AGCAACAAAACGAAAC
PcopZF	GTGGCGCTTCATTTGCTTGAGG
PcopZR	GACATGAACGGCACTGACGC
ycnKORF_FF	CTTAATGGATGGGGTGTATGCATGCTTC
ycnKORF_BR	CTCAGGATCCACCTTCCGCTGTATGTG
csoRORF_NF	GTGTACCCATGGAAGCATAACG
csoRORF_BR	CTTTCTATTTGGATCCTACCATAACC

^a Restriction enzyme sites are underlined.

FU1146 [Δ (*csoR copZA*)::tet]. Strain FU1130 was transformed with plasmid pCm::Er (33), yielding the erythromycin-resistant (0.3 μ g/ml) strain FU1144 (Δ *ycnK::erm*) (Table 1). Strains YCNKd and YCNJd were transformed with genomic DNAs of strains FU1131, FU1133, and FU1134 to obtain chloramphenicol resistance, which resulted in strains FU1135, FU1136, FU1137, FU1138, FU1139, and FU1140 (Table 1). Strain YCNLd was transformed with genomic DNAs of strains FU1130 and FU1132 to obtain resistance to chloramphenicol and/or tetracycline, which resulted in strains FU1141, FU1142, and FU1143 (Table 1).

Strain FU1147 was constructed by ectopically introducing the *ycnK*

promoter region (bases –190 to 109; base 1 is the transcription start base identified in this study) fused to the *lacZ* reporter gene [*PycnK*(–190 to 109)–*lacZ*] into the *amyE* locus of strain 168 as follows. The corresponding DNA fragment was amplified by PCR with genomic DNA of strain 168 and primer pair PycnK_XF and PycnK_BR (Table 2), followed by trimming with XbaI and BamHI digestion. It was then cloned into the pCRE-test2 vector (24) that had been treated with the same restriction enzymes. Correct construction was confirmed by DNA sequencing. The resulting plasmid was linearized by PstI digestion and then integrated into the *amyE* locus of strain 168 through double-crossover transformation to obtain chloramphenicol resistance, which resulted in strain FU1147 (Table 1). Strain FU1147 was transformed with genomic DNAs of strains FU1144, FU1132, FU1145, and FU1146 to obtain resistance to erythromycin and/or tetracycline, which resulted in strains FU1148, FU1149, FU1150, and FU1151 (Table 1).

B. subtilis cells were pregrown at 30°C overnight on tryptose blood agar base (Difco) plates supplemented with 0.18% glucose, which contained chloramphenicol (5 μ g/ml), erythromycin (0.3 μ g/ml), and/or tetracycline (10 μ g/ml) according to the drug resistance of the cells. The cells were inoculated into a minimal medium (40) supplemented with a mixture of 16 amino acids (MM+16aa medium; glutamine, histidine, tyrosine, and asparagine were omitted) (2) to give an optical density at 600 nm (OD₆₀₀) of 0.05 and then incubated at 37°C with shaking. It is noteworthy that the MM+16aa medium contains CuCl₂ at 2.5 μ M.

Northern blot analysis. For the analysis of transcripts from the *ycnK* promoter, total RNAs were extracted and purified, as previously reported (41), from the cells of strain 168 grown in MM+16aa medium without or with the Cu²⁺-specific chelator bathocuproine disulfonate (BCS) (Sigma-Aldrich) at 0.5 mM to induce copper limitation. BCS was added to one part of the cultures when its OD₆₀₀ reached 0.2, and the BCS-treated and untreated cells were harvested at an OD₆₀₀ of 0.5 (3 h after BCS addition). For the analysis of transcripts from the *ycnL* promoter, total RNAs were similarly prepared from the cells of strains 168 and FU1135, both of which were grown in MM+16aa medium and harvested at an OD₆₀₀ of 0.5.

The RNA samples (10 μ g for the detection of transcripts from the *ycnK* promoter and 50 μ g for the detection of transcripts from the *ycnL* promoter) were electrophoresed in a glyoxal gel and transferred to a Hybond-N membrane (GE Healthcare). To prepare probes for the detection of transcripts carrying *ycnK*, *ycnJ*, *ycnI*, and *ycnL*, the DNA fragments (each 300 bp) were obtained by PCR with genomic DNA of strain 168 and primer pairs ycnK_NF/ycnK_NR, ycnJ_NF/ycnJ_NR, ycnI_NF/ycnI_NR, and ycnL_NF/ycnL_NR (Table 2) and labeled using a BcaBEST labeling kit (TaKaRa-bio) and [α -³²P]dCTP (MP Biomedicals). The radiolabeled DNA probes were hybridized to the RNAs blotted on the membrane, which was then washed, as described previously (28). The autoradiograms obtained were quantified using a Typhoon 9400 variable image analyzer (GE Healthcare).

Primer extension analysis. To determine the transcription start sites from the *ycnK* and *ycnL* promoters, primer extension analysis was performed using the same RNA samples that were prepared for the Northern blot analysis: 45 μ g of total RNAs from the strain 168 cells treated and untreated with 0.5 mM BCS for the *ycnK* promoter and 50 μ g of total RNAs from strains 168 and FU1135 for the *ycnL* promoter. The RNA samples were annealed to 1 pmol of primers ycnK_PE and ycnL_PE (Table 2), respectively, which had been 5' end labeled with a Megalabel kit (TaKaRa-bio) and [γ -³²P]ATP (MP Biomedicals), and the primer extension reaction was then conducted with ThermoScript reverse transcriptase (Life Technologies) as described previously (29). Templates for the dideoxy sequencing reactions for ladder preparation, starting from the same 5'-end-labeled primers that were used for the reverse transcription, were generated by PCR with genomic DNA of strain 168 and primer pairs PycnKF/ycnK_PE and ycnL_PE/PycnKR (Table 2).

Preparation of the YcnK and CsoR proteins. The *ycnK* and *csoR* coding regions were amplified by PCR with genomic DNA of strain 168 and primer pairs ycnKORF_FF/ycnKORF_BR and csoRORF_NF/cso-

RORF_BR (Table 2), digested with FokI/BamHI and NcoI/BamHI, and then cloned into the pET-16b vector (Merck), which had been treated with NcoI and BamHI, to yield expression plasmids pET-ycnK and pET-csoR, respectively. The correct cloning of the *ycnK* and *csoR* genes was confirmed by DNA sequencing.

E. coli strain BL21(DE3) (Merck), transformed with pET-ycnK, was grown in Luria-Bertani medium (28) supplemented with ampicillin (50 µg/ml) at 37°C to an OD₆₀₀ of 0.4. After isopropyl-β-D-thiogalactopyranoside had been added to a final concentration of 1 mM, the cells were cultivated at 30°C for another 3 h. The cells harvested from 1 liter of the culture were disrupted by sonication in buffer A (20 mM Tris-Cl buffer [pH 8.0] containing 10% [vol/vol] glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol [DTT]). After centrifugation (17,000 × *g* for 4°C at 20 min) and filtration (0.45 µm), the supernatant was subjected to ammonium sulfate precipitation. The fraction precipitated at 30 to 50% saturation was recovered and dialyzed against buffer A and then applied to a heparin Sepharose 6FF column (GE Healthcare) equilibrated with buffer A. The column was washed with buffer A and eluted with a linear gradient of 0 to 1 M NaCl. The YcnK fraction was collected and concentrated by ultrafiltration (Ultracel-10k; Millipore) and then subjected to gel filtration (Sephacryl S-200HR; GE Healthcare) with buffer A containing 0.1 M NaCl at 1.5 ml/min to determine the molecular mass of the native form of YcnK. Low- and high-molecular-weight (LMW and HMW) gel filtration calibration kits (GE Healthcare) were used for the column calibration. The purity of the YcnK protein was evaluated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with Coomassie brilliant blue (CBB) staining. The protein concentrations were determined by the Bradford method with bovine serum albumin as the standard (6).

E. coli strain BL21(DE3) bearing pET-csoR or vector pET-16b was cultivated to prepare crude lysates as described above. The crude lysates were then dialyzed against buffer A. The content of the CsoR protein in the lysate was evaluated by SDS-PAGE.

DNase I footprinting analysis. DNase I footprinting analysis was performed as described previously (12). The DNA probe for the footprinting (*PycnK* probe) was prepared by PCR with genomic DNA of strain 168 and primer pair PycnKF/PycnKR (Table 2). Prior to PCR amplification, the 5' terminus of only one of the primers was labeled with [γ-³²P]ATP using a Megalabel kit. The *PycnK* probe (0.04 pmol), labeled at the 5' end, was mixed with the YcnK protein to obtain a DNA-protein complex, which was then partially digested with DNase I (TaKaRa-bio) in 50 µl of a reaction mixture and subjected to urea-PAGE with sequencing ladders prepared using genomic DNA of strain 168 and the primer pair PycnKF/PycnKR.

Gel retardation analysis. Gel retardation analysis was performed essentially as described previously (39). The *PycnK* probe that was used for the DNase I footprinting was labeled by PCR with genomic DNA of strain 168 and the primer pair PycnKF/PycnKR in the presence of [α-³²P]dCTP (MP Biomedicals). To create a *PycnKΔD* probe, a derivative of the *PycnK* probe from which the internal 16-bp direct repeat was deleted, recombinant PCR (16) was performed with the internal overlapping primer pair of PycnK_delDF/PycnK_delDR (Table 2) together with the outer primer pair PycnKF/PycnKR and genomic DNA of strain 168. The radiolabeled probe (0.02 pmol) was mixed and incubated at 30°C for 10 min with various amounts of the YcnK protein in a 25-µl binding mixture, and then the mixture was subjected to PAGE. To evaluate the effect of the copper ion concentration on the DNA binding affinity of YcnK, copper-limiting and copper excess conditions were produced by adding BCS and CuCl₂ into the binding mixture at 1 mM and 0.1 mM, respectively. It is noteworthy that the binding mixture contained 5 mM DTT to reduce Cu²⁺ to Cu⁺ (4), which corresponds to its cytosolic state. Although no copper salt was added to the original binding mixture, it probably contained a small amount of copper ion that had bound tightly to the YcnK protein during purification or slightly contaminated the reagent solutions.

The binding ability of CsoR to the *PycnK* probe was similarly tested

using the crude lysate containing the CsoR protein, as well as the vector control. To confirm that the active CsoR protein was obtained, a *PcopZ* probe, corresponding to the *copZ* promoter region, was created by PCR with genomic DNA of strain 168 and primer pair PcopZF/PcopZR (Table 2).

lacZ reporter analysis. *B. subtilis* strains carrying the promoter-*lacZ* fusion were grown in 50 ml of MM+16aa medium at 37°C with shaking. When the OD₆₀₀ reached 0.2, BCS and CuCl₂ solutions were added to cultures to give final concentrations of 0.25 mM and 1 mM, respectively, to induce copper-limiting and copper excess conditions. Then, 1-ml aliquots of the culture were withdrawn at intervals of 1 h, and the β-galactosidase (β-Gal) activity in crude cell extracts was spectrophotometrically measured using *o*-nitrophenyl-β-D-galactopyranoside (Wako Pure Chemical Industries, Japan) as a substrate, according to the procedure described previously (2). To reduce the effect of BCS and copper ion on β-Gal, the harvested cells were washed with a 100 mM phosphate buffer (pH 7.5) before lysozyme treatment.

RESULTS

Northern blot analysis of transcripts, including *ycnK*, *ycnJ*, *ycnI*, and *ycnL*. The *ycnJ* and *ycnK* genes encode a membrane protein for copper uptake and a DeoR family transcriptional regulator for *ycnJ* expression, respectively (9). Although the actual functions of the products of the other genes remain unclear, homology search predicts that the *ycnI* and *ycnL* genes encode a membrane protein and a reductase or disulfide isomerase, respectively. The *ycnK*, *ycnJ*, and *ycnI* genes are located successively in the same direction on the *B. subtilis* chromosome, while the *ycnL* gene is located immediately upstream of *ycnK* in the opposite direction (Fig. 2A). Their arrangement, as well as the direction of their flanking genes, suggested that the *ycnK*, *ycnJ*, and *ycnI* genes constitute an operon. To confirm this, Northern blot analysis was performed using specific DNA probes and total RNAs that were prepared from cells of strain 168 grown in MM+16aa medium and in medium containing 0.5 mM BCS to induce copper limitation (Fig. 3A). A 2.9-kb transcript was detected when the probes specific to *ycnK*, *ycnJ*, and *ycnI* were used, indicating that these genes constitute an operon. Moreover, the band intensities increased when the RNA of the cells exposed to BCS was used (Fig. 3A, lane 2), indicating that the transcription of the *ycnKJI* operon is inducible by copper depletion.

The transcriptional unit from the *ycnL* promoter was determined similarly, using the *ycnL*-specific probe and total RNAs from strains 168 (wild type) and FU1135 (*ycnK* and *csoR* double disruptant). A specific band of 0.5-kb transcript was observed when either RNA sample was used, indicating that the *ycnL* gene is monocistronic (Fig. 3B). As no clear difference was observed between the band intensities of the two total RNAs, *ycnL* repression by YcnK and/or CsoR was not verified by this experiment; slightly less total RNA from strain FU1135 might have been applied, judging from the band intensities of the 16S rRNA (Fig. 3B).

Determination of the transcription start sites of the *ycnK* and *ycnL* promoters. To determine the transcription start sites of the *ycnK* and *ycnL* promoters, we performed primer extension analysis using the same RNA samples that were used for the Northern blot analysis. As shown in Fig. 4 (left), the specific band corresponding to runoff cDNA of the *ycnKJI* transcript was detected with both RNA samples, but the band intensity of the BCS-treated sample was much higher than that of the untreated one, supporting the idea that *ycnKJI* transcription is induced by copper depletion.

FIG 3 Northern blot analysis of transcripts of *ycnK*, *ycnJ*, *ycnI*, and *ycnL*. (A) RNA samples were prepared from cells of strain 168 (wild type) grown in MM+16aa medium (lane 1) and from those treated with 0.5 mM BCS, a Cu^{+} -specific chelator (lane 2), and Northern blotting was performed, as described in Materials and Methods. The blotted membranes were stained once with methylene blue to ensure equal amounts of the RNA samples; the bands corresponding to 16S rRNA are shown underneath. ^{32}P -labeled DNA probes specific to *ycnK*, *ycnJ*, and *ycnI* were used. The positions of the RNA size marker are indicated by arrows on the left. The detected bands of transcripts are indicated by arrows on the right. (B) RNA samples were prepared from strains 168 (lane 1) and FU1135 (*ycnK* and *csor* double disruptant) (lane 2), both of which were grown in MM+16aa medium. Northern blotting was performed as for the experiments whose results are shown in panel A but using the ^{32}P -labeled *ycnL*-specific probe and 5-fold larger amounts of the RNA samples (50 μg) than were used in those experiments. The bands corresponding to 16S rRNA stained with methylene blue are shown underneath. The detected bands of transcripts are indicated by an arrow on the right. The faint broad bands of around 3 kb in size probably resulted from nonspecific hybridization of the *ycnL* probe to 23S rRNA, as reported previously (29).

tion. As for the *ycnL* promoter, the specific band of runoff cDNA was faintly detected only when the RNA sample of strain FU1135 was used (Fig. 4, right), suggesting that *ycnL* transcription is repressed by YcnK and/or CsoR.

Based on the locations of the transcription start sites of the *ycnK* and *ycnL* promoters, we predicted the -35 and -10 sequences of the promoters, with 17-bp spacers, to be TTGTCT and TACGCT for *ycnK* and ATGATA and TTGAAC for *ycnL*, respec-

FIG 4 Primer extension analysis to determine the transcription start sites from the *ycnK* and *ycnL* promoters. To determine the transcription start site from the *ycnK* promoter (left), RNA samples from cells of strain 168 grown in MM+16aa medium (lane 1) and from those treated with 0.5 mM BCS (lane 2) were used for the reverse transcription reaction to generate the runoff cDNA. To determine the transcription start site from the *ycnL* promoter (right), RNA samples of strains 168 (lane 1) and FU1135 (lane 2) grown in MM+16aa medium were used. Lanes G, A, T, and C contain the products of the dideoxy sequencing reactions with the same primer that was used for reverse transcription. Each runoff cDNA band is indicated by an arrow on the right. The partial nucleotide sequences of the coding strands corresponding to the ladders are shown, with the -35 and -10 sequences and the initiating codon underlined and the transcription start sites (+1) and the SD sequence enclosed in boxes.

tively (Fig. 2B and 4), both of which are probably recognized by σ^A RNA polymerase (15).

Preparation of the YcnK protein. To prepare YcnK protein for *in vitro* experiments, the *ycnK* gene was cloned in vector pET-16b, and recombinant YcnK was overproduced in *E. coli* BL21(DE3) cells. As described in Materials and Methods, the YcnK protein was partially purified by ammonium sulfate precipitation followed by heparin-affinity column chromatography and gel filtration; in this way, 55% purity was achieved, as judged by measurement of the band intensity on a gel of SDS-PAGE stained with CBB using ImageJ (<http://rsbweb.nih.gov/ij/>) (data not shown). Upon gel filtration with protein standards to obtain the molecular mass, the YcnK protein exhibited a molecular mass of 43.9 kDa (data not shown). The molecular mass of the YcnK monomer is calculated to be 21.4 kDa, and thus, it was estimated that YcnK forms a dimer.

Identification of the binding site of YcnK in the intergenic region between *ycnK* and *ycnL*. DNase I footprinting analysis was performed to identify the YcnK binding site in the region between the *ycnK* and *ycnL* coding sequences. When the YcnK protein was mixed with the *PycnK* probe (bases –174 to 244; base 1 is the transcription start base for the *ycnK* promoter), followed by DNase I digestion and electrophoresis, a region protected against DNase I was detected immediately upstream of the –35 sequence of the *ycnK* promoter (bases –78 to –38 of the coding strand and bases –98 to –43 of the noncoding strand) and also covering the *ycnL* promoter region (bases –40 to 16 of the coding strand and bases –45 to –5 of the noncoding strand; base 1 is the transcription start base for the *ycnL* promoter) (Fig. 5, lanes 2 and 3). A 16-bp direct repeat sequence (CACATTTTCACATTTT; bases –65 to –50 for the *ycnK* promoter) which is probably necessary for YcnK recognition was observed in the protected region (Fig. 2B).

Quantitative evaluation of the dependence of the DNA binding of YcnK and CsoR on the copper ion concentration *in vitro*. To characterize the DNA binding of YcnK, we performed gel retardation analysis using the YcnK protein that was partially purified. The YcnK concentration was calculated as a dimer from the molecular mass of the YcnK monomer (21,434 Da) and its content in total protein (55%) as estimated by SDS-PAGE.

First, to confirm whether the 16-bp direct repeat is essential for the specific YcnK binding, the *PycnK* probe (418 bp) that was used for DNase I footprinting and a *PycnK* derivative (*PycnK* Δ D probe; 402 bp) that lacks the direct repeat were used as DNA probes for gel retardation with various concentrations of the YcnK protein (Fig. 6). The YcnK protein specifically bound to the *PycnK* probe, which resulted in retarded bands on a PAGE gel depending on the YcnK concentration. The apparent dissociation constant (K_d) was estimated to be 8 nM. On the other hand, no specific band retardation was detected with the *PycnK* Δ D probe, clearly showing the importance of this direct repeat for the specific YcnK binding.

To quantitatively evaluate the effect of copper ion on the DNA binding affinity of YcnK, CuCl_2 and BCS were added to the binding mixture at 0.1 mM and 1 mM to create copper excess and copper-limiting conditions, respectively (Fig. 6). It should be noted that Cu^{2+} was reduced to Cu^+ by using 5 mM DTT (4), as it is assumed that the cytosolic copper ion is monovalent. In the presence of 0.1 mM CuCl_2 , the YcnK protein bound to the *PycnK* probe as effectively as it did under the control conditions, giving a K_d value of 8 nM. This suggests that maximal DNA binding ability

FIG 5 DNase I footprinting analysis to identify the YcnK binding site in the intergenic region between *ycnK* and *ycnL*. A DNA probe including the *ycnK-ycnL* intergenic region (*PycnK* probe), 5' end labeled at either the coding or noncoding strand, was prepared. The 5'-end-labeled probe (0.8 nM) was incubated in the reaction mixture with the YcnK protein (50 nM [lanes 2] and 25 nM [lanes 3]) as a dimer and without the YcnK protein (lanes 1 and 4). After partial digestion with DNase I, the resulting mixtures were subjected to urea-PAGE. Lanes G, A, T, and C contain the products of the dideoxy sequencing reactions with the corresponding 5'-end-labeled primers. Nucleotide sequences protected by YcnK are indicated on the right of each panel; the 16-bp direct repeat is indicated by tandem arrows.

of YcnK was obtained without additional copper ion, probably because the copper ion that had tightly bound to the YcnK protein during purification or a trace amount of copper contamination in the mixture was enough for its full activation. When BCS was added at 1 mM, the DNA binding affinity of YcnK was distinctly decreased, providing a K_d value of 64 nM, which indicates that copper limitation impaired the DNA binding activity of YcnK. Moreover, this inhibition was canceled by adding CuCl_2 at 0.1 mM, whereby the K_d value was restored to 8 nM. As two molecules of BCS stoichiometrically bind to one Cu^+ ion, it seems that the inhibitory effect of 1 mM BCS was lost with the addition of 0.1 mM CuCl_2 before all of the BCS molecules formed a Cu^+ complex. This might be ascribed to the property of YcnK of binding to Cu^+ with an affinity much higher than that of BCS, as previously reported in the case of the CopZ protein (46). The copper-dependent property of YcnK *in vitro* shown here is consistent with its physiological role in that the repression of the *ycnK* promoter forced by YcnK binding to the *ycnK* regulatory region is released by copper depletion in the *B. subtilis* cell.

We also examined whether CsoR binds directly to the *PycnK* probe by a similar gel retardation analysis using the crude lysate, including the CsoR protein (16% of total protein), from which copper ion had been significantly removed by dialysis. The binding ability of the CsoR protein was tested by gel retardation using a *PcopZ* probe (301 bp), corresponding to the *copZ* promoter region (bases –173 to 128; base 1 is the transcription start base for the *copZ* promoter), to which CsoR binding was reported (13, 32).

FIG 6 Gel retardation analysis to evaluate the *in vitro* binding affinity of YcnK to the intergenic region between *ycnK* and *ycnL* under various copper ion concentrations. The *PycnK* probe corresponding to the *ycnK-ycnL* intergenic region and a *PycnK* derivative lacking the 16-bp direct repeat (*PycnKΔD*) were ³²P labeled, incubated at 0.8 nM with various concentrations of the YcnK protein, and then subjected to PAGE. The YcnK protein solution was diluted stepwise 2-fold, and an aliquot of each dilution was added to the binding mixture to obtain the concentrations used (as a dimer). CuCl₂ and BCS were added to the mixture at 0.1 mM and 1 mM to create copper excess and copper-limiting conditions, respectively. The “no protein” lanes contained no YcnK. The horizontal bars indicate the lanes for which the K_d was determined. The experiments were repeated at least two times, and representative results are shown.

The CsoR protein in the lysate bound specifically to the *PcopZ* probe, yielding retarded bands depending on the protein amount, and sufficient retardation was achieved when the crude lysate was added at 15 μg/ml of total protein in the binding mixture. While the addition of BCS into the mixture at 1 mM did not alter the retarded band pattern, the retarded band of CsoR binding to the *PcopZ* probe was completely lost by the addition of CuCl₂ at 0.1 mM, indicating that the DNA binding of CsoR was sensitively inhibited by the copper ion (data not shown). After the CsoR protein in the lysate was ensured to be active enough by this experiment, the ability of CsoR to bind to the *PycnK* probe was examined. However, no band retardation was observed under any condition (control, copper excess, or copper limitation), which was the same result as that obtained with the vector control (data not shown). Thus, we considered that CsoR does not bind directly to the *ycnK* regulatory region, although the previous study showed that *csoR* disruption causes a substantial increase in *ycnJ* transcription (9).

***In vivo* effect of copper availability on the *ycnK* promoter controlled by YcnK and CsoR.** To investigate whether the *in vivo* repression of the *ycnK* promoter by YcnK is released by copper depletion and whether the effect of CsoR on the *ycnK* promoter is dependent on the copper ion concentration *in vivo*, we conducted a *lacZ* reporter analysis using *B. subtilis* strains carrying the *lacZ* reporter fused to the *ycnK* promoter with and without *ycnK* and *csoR* disruptions (Table 1) and grown under various conditions of copper availability. The integration of plasmid pMUTIN2 causes disruption of the target gene and *lacZ* fusion to the promoter upstream of the target gene (35). Strain YCNJd (*ycnJ*::pMUTIN2) was used to monitor the *ycnK* promoter in the presence of both YcnK and CsoR (Fig. 7, upper left). When strain YCNJd was grown in the MM+16aa medium (control), which contains 2.5 μM CuCl₂, the β-Gal activity slightly increased in the late logarithmic phase and peaked in the stationary phase (5.3 nmol/min per OD unit). Under the copper-limiting condition induced by 0.25 mM BCS, the β-Gal activity of strain YCNJd was drastically elevated shortly after the addition of BCS and reached a maximum of 45.3 nmol/min per OD unit at the end of the logarithmic phase. In contrast, the excess copper ion brought by the addition of CuCl₂ at 1 mM kept the β-Gal activity at a very low level (0.9 nmol/min per OD unit at the maximum), suggesting that YcnK fully repressed the *ycnK* promoter throughout cultivation under this condition. On the other hand, when strain YCNKd (*ycnK*::pMUTIN2) was used to monitor the *ycnK* promoter in the absence of YcnK, the β-Gal activities were much higher than those obtained with strain YCNJd under the control and copper excess conditions (23.7 and 12.9 nmol/min per OD unit at the maxima for YCNKd under the respective conditions; 4.5- and 14.3-fold higher than those for YCNJd), whereas the difference between the β-Gal activities of strains YCNKd and YCNJd under the copper-limiting condition was not as remarkable (62.7 nmol/min per OD unit at the maximum for YCNKd; 1.4-fold higher than the maximum for YCNJd) (Fig. 7, upper center). Comparison of these results showed that the repression of the *ycnK* promoter by YcnK is strengthened by an increase in the intracellular copper ion and that it is significantly weakened under the copper-limiting condition, thus leading to derepression.

The *ycnK* promoter activity in the absence of CsoR was monitored using strain FU1138 (*ΔcsoR*::*cat ycnJ*::pMUTIN2) (Fig. 7, lower left). The β-Gal activities of strains FU1138 and YCNJd were at comparable levels when the strains were grown under copper excess and copper-limiting conditions (0.6 and 45.8 nmol/min per OD unit at the maxima for FU1138 under the respective conditions). However, strain FU1138 exhibited β-Gal activity that was distinctly higher than that of strain YCNJd under the control growth condition (24.5 nmol/min per OD unit at the maximum for FU1138; 4.6-fold higher than the maximum for YCNJd). Although the *csoR* gene and the *copZA* operon are closely aligned in the same direction, they are transcribed separately (Fig. 1) (13). Thus, a *csoR* disruption causes constitutive expression of the *copZA* operon, resulting in excessive discharge of the intracellular copper ion via the overproduced CopZA protein. Copper depletion probably occurred in the FU1138 cells grown under the control condition, which was sensed by YcnK, derepressing the *ycnK* promoter. We consider that a sufficient amount of copper ion was retained by the addition of CuCl₂ at 1 mM even in the FU1138 cells overproducing CopZA and that the addition of BCS at 0.25 mM fully released the *ycnK* repression by YcnK.

FIG 7 *lacZ* reporter analysis to monitor the *ycnK* promoter in the presence and the absence of YcnK and CsoR under various copper availability conditions. Strains YCNJd (*ycnJ*::pMUTIN2), YCNKd (*ycnK*::pMUTIN2), FU1138 (Δ *csoR*::*cat ycnJ*::pMUTIN2), and FU1135 (Δ *csoR*::*cat ycnK*::pMUTIN2) were used to monitor the *ycnK* promoter activity in the presence of both YcnK and CsoR (upper left), in the absence of YcnK (upper center), in the absence of CsoR (lower left), and in the absence of both YcnK and CsoR (lower center), respectively. The *ycnK* gene was disrupted by plasmid integration in strains YCNKd and FU1135. Strains FU1148 (Δ *ycnK*::*erm amyE*::*PycnK-lacZ*) and FU1149 (Δ *ycnK*::*erm ΔcsoR*::*tet amyE*::*PycnK-lacZ*) were used to monitor the ectopically introduced *ycnK* promoter in strains with the *ycnK* null mutation (upper right) and the *ycnK* and *csoR* null mutations (lower right). Cells of each strain were cultivated in MM+16aa medium, and when the OD₆₀₀ reached 0.2, the BCS and CuCl₂ solutions were added to the cultures (indicated by an arrow) at concentrations of 0.25 mM and 1 mM, respectively, to induce copper limitation (circles) and copper repletion (triangles). The results of no addition of these reagents are indicated by squares (control condition). The open and filled symbols indicate the OD₆₀₀ values and β-Gal activities, respectively. The *lacZ* reporter experiments were repeated at least two times, and representative results are shown.

Strain FU1135 (Δ *csoR*::*cat ycnK*::pMUTIN2) was constructed to monitor the *ycnK* promoter in the absence of both YcnK and CsoR (Fig. 7, lower center). Strain FU1135 showed patterns of β-Gal activities similar to those observed in strain YCNKd under any growth condition (21.5, 12.7, and 62.9 nmol/min per OD unit at the maxima for FU1135 under the control, copper excess, and copper-limiting conditions); i.e., the introduction of *csoR* disruption into YCNKd had no effect on the *ycnK* promoter activity, suggesting that the effect of *csoR* disruption was exerted via YcnK, as described above. Interestingly, the β-Gal activity of strain FU1135, lacking both YcnK and CsoR, still showed a response to copper availability; higher activity was obtained by copper limitation and the activity was lower under the copper excess condition.

In strains YCNKd and FU1135, *ycnK* disruption was accomplished by plasmid integration (*ycnK*::pMUTIN2), while the *csoR* gene was completely replaced with the *cat* cassette for its disruption (Δ *csoR*::*cat*) in strains FU1138 and FU1135 (Table 1). Consequently, strains YCNKd and FU1135 produce a truncated form of the YcnK protein with the deletion of the C-terminal 76 residues and the alternative adduct of 5 residues (¹²⁰RSPAC¹²⁴) (data not shown). We suspected that this truncated YcnK is partially functional, resulting in copper availability-dependent variation of the *ycnK* promoter activity in strain FU1135. To clarify this, we constructed strain FU1148 [Δ *ycnK*::*erm amyE*::*PycnK*(−190 to 109)-*lacZ*] and strain FU1149 [Δ *ycnK*::*erm ΔcsoR*::*tet amyE*::*PycnK*(−190 to 109)-*lacZ*], which carry the *ycnK* promoter (bases −190 to 109; base 1 is the transcription start base)-*lacZ* fusion introduced into the *amyE* locus in the background of the *ycnK* and/or *csoR* null mutation(s) (Table 1). Prior to the *lacZ* reporter analysis of these strains, the *ycnK* promoter activity of the ectopically introduced *lacZ* fusion in the presence of both YcnK and

CsoR was measured using strain FU1147 [*amyE*::*PycnK*(−190 to 109)-*lacZ*] (Table 1). The β-Gal activity of strain FU1147 showed responses to copper availability similar to those of strain YCNJd, except that the peak activities of strain FU1147 were somewhat lower than those observed in strain YCNJd (2.9, 0.6, and 17.2 nmol/min per OD unit at the maxima for FU1147 under control, copper excess, and copper-limiting conditions). When strains FU1148 and FU1149 were analyzed, their β-Gal activities were also lower than those of strains YCNKd and FU1135, but they varied similarly depending on copper availability (12.0, 5.0, and 28.8 nmol/min per OD unit at the maxima for FU1148 and 11.8, 4.2, and 22.6 nmol/min per OD unit at the maxima for FU1149 under control, copper excess, and copper-limiting conditions) (Fig. 7, right). Thus, we conclude that the truncated YcnK protein does not contribute to the copper-dependent variation of the β-Gal activity of strain FU1135.

Effect of *copZA* disruption on *ycnK* promoter activity. It was suggested that *csoR* disruption causes the derepression of the *copZA* operon, by which the intracellular copper ion is depleted, and in turn, YcnK sensing of this depletion derepresses the *ycnK* promoter. To verify the involvement of CopZA in this indirect regulation by CsoR, we constructed *B. subtilis* strains carrying a *copZA* disruption in addition to the *ycnK* promoter-*lacZ* fusion with and without *ycnK* and *csoR* disruptions (Table 1) and conducted a *lacZ* reporter analysis similar to that described above. Strain FU1139 (Δ *copZA*::*cat ycnJ*::pMUTIN2) was used to monitor the *ycnK* promoter in the presence of both YcnK and CsoR in the Δ *copZA* background (Fig. 8, upper left). The β-Gal activities of strain FU1139 were significantly low under any growth condition compared with those of strain YCNJd under the corresponding condition, although the β-Gal activities of strain FU1139 varied in

FIG 8 *lacZ* reporter analysis to evaluate the effect of *copZA* disruption on the *ycnK* promoter in the presence and absence of YcnK and CsoR under various copper availability conditions. Strains FU1139 ($\Delta copZA::cat ycnK::pMUTIN2$), FU1136 ($\Delta copZA::cat ycnK::pMUTIN2$), FU1140 [$\Delta(csoR copZA)::cat ycnK::pMUTIN2$], and FU1137 [$\Delta(csoR copZA)::cat ycnK::pMUTIN2$] were used to monitor the *ycnK* promoter activity in the presence of both YcnK and CsoR (upper left), in the absence of YcnK (upper center), in the absence of CsoR (lower left), and in the absence of both YcnK and CsoR (lower center), respectively, in the $\Delta copZA$ background. The *ycnK* gene was disrupted by plasmid integration in strains FU1136 and FU1137. Strains FU1150 ($\Delta ycnK::erm \Delta copZA::tet amyE::PycnK-lacZ$) and FU1151 [$\Delta ycnK::erm \Delta(csoR copZA)::tet amyE::PycnK-lacZ$] were used to monitor the ectopically introduced *ycnK* promoter in strains with the *ycnK* and *copZA* null mutations (upper right) and the *ycnK*, *csoR*, and *copZA* null mutations (lower right). Cells of each strain were cultivated in MM+16aa medium, and when the OD₆₀₀ reached 0.2, the BCS and CuCl₂ solutions were added to the cultures (indicated by an arrow) at concentrations of 0.25 mM and 1 mM, respectively, to induce copper limitation (circles) and copper repletion (triangles). The results of no addition of these reagents are indicated by squares (control condition). The open and filled symbols indicate the OD₆₀₀ values and β -Gal activities, respectively. The *lacZ* reporter experiments were repeated at least two times, and representative results are shown.

response to copper availability (1.1, 0.2, and 3.2 nmol/min per OD unit at the maxima for FU1139 under the control, copper excess, and copper-limiting conditions). Strain FU1140 [$\Delta(csoR copZA)::cat ycnK::pMUTIN2$], which was constructed to monitor the *ycnK* promoter in the absence of CsoR and CopZA, showed almost the same pattern of β -Gal activities as strain FU1139 did (1.0, 0.2, and 2.9 nmol/min per OD unit at the maxima for FU1140 under the control, copper excess, and copper-limiting conditions) (Fig. 8, lower left), indicating that the *csoR* disruption in the $\Delta copZA$ background did not enhance the β -Gal activity, unlike the case of strain FU1138 ($\Delta csoR::cat copZA^+$) (Fig. 7, lower left). These results are in good agreement with our hypothesis that CsoR is indirectly involved in the regulation of the *ycnK* promoter through *copZA* derepression.

We further examined the *ycnK* promoter activity in the absence of YcnK and CopZA using strain FU1136 ($\Delta copZA::cat ycnK::pMUTIN2$) and found that the β -Gal activities of strain FU1136 grown under the control and copper-limiting conditions were comparable to those of strain YCNkd grown under the corresponding conditions, but the β -Gal synthesis of strain FU1136 was thoroughly repressed by the excess copper ion (22.7, 2.0, and 63.7 nmol/min per OD unit at the maxima for FU1136 under the control, copper excess, and copper-limiting conditions) (Fig. 7 and 8, upper center). When strain FU1137 [$\Delta(csoR copZA)::cat ycnK::pMUTIN2$] was used to monitor the *ycnK* promoter in the absence of YcnK, CsoR, and CopZA, the pattern of the β -Gal activities was similar to that of strain FU1136 (19.9, 2.5, and 53.4 nmol/min per OD unit at the maxima for FU1137 under the control, copper excess, and copper-limiting conditions) (Fig. 8, lower center).

Strain FU1150 [$\Delta ycnK::erm \Delta copZA::tet amyE::PycnK(-190$ to 109)-*lacZ*] and strain FU1151 [$\Delta ycnK::erm \Delta(csoR copZA)::tet$

amyE::PycnK(-190 to 109)-lacZ] were constructed to monitor the ectopically introduced *ycnK* promoter with the *ycnK* and *copZA* null mutations and the *ycnK*, *csoR*, and *copZA* null mutations, respectively (Table 1). Although their peak β -Gal activities were lower than those observed in strains FU1136 and FU1137, their responses to copper availability were similar to those of strains FU1136 and FU1137 (Fig. 8, right).

Based on these results in the $\Delta copZA$ genetic background, we deduce that the intracellular copper ion level was elevated in the *copZA* disruptants even when they were grown under the copper-limiting condition, being sensed by YcnK to effectively repress the *ycnK* promoter. Under the copper excess condition, the accumulation of copper ion in the *copZA* disruptants would be more severe than that in the *copZA*⁺ strains, affecting β -Gal synthesis even in the strains carrying the *ycnK* disruption. The cell growth of the *copZA* disruptants was specifically inhibited by the excess copper ion, which might be related to the low levels of β -Gal synthesis in these strains under copper excess and also highlights the physiological importance of CopZA for resistance against a high concentration of copper ion.

Contributions of YcnK and CsoR to *ycnL* promoter activity under various conditions of copper availability. As the YcnK binding region determined by the DNase I footprinting overlaps the *ycnL* promoter region, YcnK was expected to repress the *ycnL* promoter as well as the *ycnK* promoter. Moreover, it was found that CsoR indirectly affects the *ycnK* promoter via YcnK and CopZA. Thus, we examined whether YcnK and CsoR also repress the *ycnL* promoter, being dependent on the intracellular concentration of copper ion, by means of *lacZ* reporter analysis using *B. subtilis* strains carrying the *ycnL* promoter-*lacZ* fusion with and without *ycnK* and *csoR* disruptions (Table 1) that had been grown

FIG 9 *lacZ* reporter analysis to monitor the *ycnL* promoter in the presence and the absence of YcnK and CsoR under various copper availability conditions. Strains YCNLd (*ycnL*::pMUTIN2), FU1141 (Δ *ycnK*::*cat ycnL*::pMUTIN2), FU1142 (Δ *csoR*::*tet ycnL*::pMUTIN2), and FU1143 (Δ *ycnK*::*cat* Δ *csoR*::*tet ycnL*::pMUTIN2) were used to monitor the *ycnL* promoter activity in the presence of both YcnK and CsoR (upper left), in the absence of YcnK (upper right), in the absence of CsoR (lower left), and in the absence of both YcnK and CsoR (lower right), respectively. Cells of each strain were cultivated in MM+16aa medium, and when the OD₆₀₀ reached 0.2, the BCS and CuCl₂ solutions were added to the cultures (indicated by an arrow) at concentrations of 0.25 mM and 1 mM, respectively, to induce copper limitation (circles) and copper repletion (triangles). The results of no addition of these reagents are indicated by squares (control condition). The open and filled symbols indicate the OD₆₀₀ values and β -Gal activities, respectively. The *lacZ* reporter experiments were repeated at least two times, and representative results are shown.

under various copper availabilities. When strains YCNLd (*ycnL*::pMUTIN2) and FU1142 (Δ *csoR*::*tet ycnL*::pMUTIN2) were used to monitor the *ycnL* promoter in the presence of both YcnK and CsoR and in the absence of CsoR, they showed low β -Gal activities under either growth condition of copper availability (Fig. 9, left). In contrast, the β -Gal activities of strains FU1141 (Δ *ycnK*::*cat ycnL*::pMUTIN2) and FU1143 (Δ *ycnK*::*cat* Δ *csoR*::*tet ycnL*::pMUTIN2), which were used to monitor the *ycnL* promoter in the absence of YcnK and in the absence of both YcnK and CsoR, respectively, were slightly higher than those observed in strains YCNLd and FU1142, although the β -Gal activities of strains FU1141 and FU1143 were comparable regardless of the copper availability (Fig. 9, right). These results indicate that YcnK represses the *ycnL* promoter weakly but that CsoR is not involved in its regulation and that the repressive effect of YcnK on the *ycnL* promoter is not significantly affected by copper ion in the medium. As the *cat* cassette substituted for *ycnK* is oriented opposite to the *ycnL* promoter-*lacZ* fusion in strains FU1141 and FU1143, the promoter of the *cat* cassette is unlikely to strengthen the β -Gal synthesis.

DISCUSSION

In this study, we revealed the organization of the *ycnKJI* operon and the *ycnL* gene, which are divergently oriented and adjacent to their promoter regions (Fig. 2). YcnK, encoded in the *ycnKJI* operon, specifically binds to the intergenic region, including the 16-bp direct repeat (CACATTTTCACATTTT), to repress the *ycnKJI* operon effectively and the *ycnL* gene weakly. This direct repeat was found to be indispensable for the specific binding of YcnK by gel retardation analysis using DNA probes carrying and lacking the direct repeat (Fig. 6). According to its primary structure, YcnK is classified in the DeoR family of bacterial transcriptional regulators (44). To date, many members of the DeoR family from various Gram-positive and Gram-negative bacteria have

been characterized and were found to share several common features. They contain a highly conserved region near the N terminus that includes a helix-turn-helix DNA binding motif. YcnK also possesses this N-terminal region forming the DNA binding domain, which shows high similarity to those of other members previously characterized, such as *E. coli* DeoR (25), *E. coli* GlpR (19), *E. coli* UlaR (14), *Lactococcus lactis* FruR (5), and *B. subtilis* IolR (42). The C-terminal region of the DeoR family members is assumed to be responsible for oligomerization and effector binding. Most of the members so far reported act as repressors involved in sugar and nucleoside metabolism, e.g., the metabolic systems for glycerol-3-phosphate (GlpR), L-ascorbate (UlaR), fructose (FruR), myo-inositol (IolR), and deoxyribonucleoside (DeoR). Their effectors are usually phosphorylated intermediates in the metabolic pathways that they control, e.g., glycerol-3-phosphate (GlpR), L-ascorbate-6-phosphate (UlaR), fructose-1-phosphate (FruR), 2-deoxy-5-keto-D-gluconate-6-phosphate (IolR), and deoxyribose-5-phosphate (DeoR) (5, 14, 19, 25, 43). These DeoR members possess a conserved region near their C terminus that is structurally related to *E. coli* D-ribose-5-phosphate isomerase, implying that this C-terminal region functions as the effector sensor (1). Distinct from these members, the C-terminal region of YcnK does not retain such a conserved sugar-phosphate recognition region but resembles the C-terminal regions of the NosL proteins, which are considered to act as copper chaperones involved in the metallo-center assembly of nitrous oxide reductase. It was reported that a monomeric form of NosL specifically and stoichiometrically binds to one Cu⁺ ion, which is coordinated with a Cys residue, and one Met and one His are thought to serve as the other ligands (22). However, the conserved Cys, Met, and His residues were not found in the NosL C-terminal regions to which the C-terminal region of YcnK shows similarity. Thus, we speculate that

the C-terminal region of the YcnK subunit encompasses one copper ion (probably monovalent) with a structural arrangement analogous to that of NosL, but the mode of coordination to copper ion is different in YcnK and NosL. Nevertheless, we also assume that Cys residue(s) play critical roles in the copper ion binding of YcnK, as observed in many metal ion binding proteins, including NosL, CsoR, and CopZ (11, 20, 22, 31, 46). According to our ortholog clustering search, the *ycnKJI* operon is conserved in a narrow subgroup of the *Bacillus* genus, comprising *B. subtilis*, *B. amyloliquefaciens*, *B. atrophaeus*, and *B. licheniformis* (34) (<http://mbgd.genome.ad.jp/>). A comparison of the amino acid sequences of these YcnK homologs revealed that three Cys-rich motifs are highly conserved in their central parts (⁶⁹CSYCLKP⁷⁵, ⁹²EQLCCAHA¹⁰⁰, and ¹⁴²CCQPQAIPF¹⁵⁰ for *B. subtilis* YcnK). These Cys-rich motifs might be involved in the copper ion binding of YcnK.

In general, the DeoR family members bind to several operator sites with widely varying configurations within the regulatory regions of the target genes. Also, oligomerization of the subunits is considered important for cooperative binding to the multiple operator sites, which enhances strict regulation to their target genes. *E. coli* DeoR, forming an octamer, binds simultaneously to widely spaced operators with palindrome sequences, which results in the DNA looping around the transcription start site, causing efficient repression of the target genes (25, 36). Similar DNA looping has been reported in the repression by *E. coli* UlaR, which binds to its operator sites as a tetramer (14). In contrast, the *E. coli* GlpR tetramer cooperatively binds to two adjacent operators with palindrome sequences, which is effective for strict repression (19, 45). As for YcnK, we demonstrated that the native YcnK is a dimer and binds to the region that includes the 16-bp direct repeat between *ycnK* and *ycnL*. Because no other direct repeat was identified in either the intergenic region or the coding regions of *ycnK* and *ycnL*, we assume that the binding of one molecule of the YcnK dimer to the 16-bp direct repeat is sufficient for causing effective and weak repressions of *ycnKJI* and *ycnL*, respectively, without forming a higher-order architecture of the protein-DNA complex such as those described above. Direct repeat sequences are generally acceptable for the dimeric binding of transcriptional regulators, as are palindrome sequences (37). Besides YcnK, some other DeoR family members, such as *B. subtilis* IolR and *L. lactis* FruR, are reported to recognize specific direct repeats (5, 42).

The *in vitro* gel retardation analysis showed that the YcnK protein is fully active in the presence of copper ion and that the DNA binding affinity of YcnK is distinctly impaired by copper limitation (Fig. 6). The results of the *in vivo* reporter analysis are almost consistent with the *in vitro* property of YcnK. The *ycnK* promoter activity was significantly elevated by *ycnK* disruption under any growth condition tested, but the ratio of elevation varied with the level of copper availability; the highest ratio (14.3-fold) was obtained under the copper excess condition, while the lowest one was obtained under the copper-limiting condition (1.4-fold) (Fig. 7, upper left and upper center). This indicates that the repressive effect of YcnK is enhanced with increased copper ion due to the high binding affinity of YcnK to the *ycnK* regulatory region in the presence of copper ion. As shown in the reporter analysis in Fig. 7 and 8, *csoR* disruption caused the *ycnK* promoter to be substantially elevated only when the reporter *B. subtilis* strain possessing YcnK and CopZA was grown under the control condition. We assume that *csoR* disruption indirectly enhances the *ycnK* promoter via CopZA

and YcnK, in which case YcnK senses the depletion of copper ion caused by overproduced CopZA. This assumption agrees with the *in vitro* observation that CsoR did not bind directly to the *ycnK* regulatory region (data not shown).

The reporter analysis showed that, even with the double disruption of *ycnK* and *csoR*, the *ycnK* promoter activity changed depending on the copper availability; higher β -Gal activity under copper limitation and lower activity under copper repletion were obtained (Fig. 7 and 8, center and right). Excess copper ion might affect the β -Gal synthesis by directly inhibiting the basal components of transcription and/or protein synthesis, which appears to result in the decrease in the β -Gal activity of the *ycnK* disruptants under the copper excess condition. Alternatively, in addition to the repression by YcnK, there might be another regulatory system that moderately represses *ycnKJI* expression in the presence of sufficient copper ion. This assumption could be supported by the observation that the *ycnK* promoter activity of the *ycnK* disruptants was at low levels before the middle logarithmic phase under the control and copper excess conditions. In either case, considering that the *in vitro* DNA binding affinity of YcnK is sensitive to copper limitation and that the degree of derepression of the *ycnK* promoter that is brought by *ycnK* disruption becomes prominent at a higher copper ion level, we conclude that YcnK functions as a copper-responsive transcriptional repressor.

In the *B. subtilis* strains constructed for the reporter analysis, the plasmid integrations (*ycnK*::pMUTIN2 and *ycnJ*::pMUTIN2) and the *ycnK* disruptions by its replacement with drug resistance cassettes (Δ *ycnK*::*cat* and Δ *ycnK*::*erm*) interrupt the expression of *ycnJ*, encoding a copper transporter, as well as *ycnI* expression. Nonetheless, these strains did not show severe growth defects even under the copper-limiting condition used in this study (0.25 mM BCS) (Fig. 7). This implies that some low-level copper acquisition system might be present in addition to YcnJ.

The reporter analysis of the *ycnL* promoter indicated that YcnK slightly represses the *ycnL* promoter, whereas CsoR does not. Moreover, unlike the case of the *ycnKJI* operon, the *ycnL* repression by YcnK was not significantly released by copper limitation (Fig. 9). We assume that the repressive effect of YcnK on *ycnL* might be so weak that the *ycnL* derepression by copper limitation was not detected in this experiment. Otherwise, the *ycnL* disruption caused by plasmid integration (*ycnL*::pMUTIN2) might inactivate a possible function of YcnL involved in copper trafficking. It is also possible that another transcriptional regulator that responds to some signal other than copper ion participates in the *ycnL* regulation cooperatively with YcnK. The physiological condition that induces *ycnL* expression should be further explored, as well as the functional role of the YcnL protein. Because the YcnL protein shows similarity to a reductase or disulfide isomerase, it might be involved in adaptation to some oxidative stress caused by the copper ion incorporated via the YcnJ protein, and similar but not identical oxidative stress might be a major signal for *ycnL* induction.

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