

## *Bacillus subtilis* ResD Induces Expression of the Potential Regulatory Genes *yclJK* upon Oxygen Limitation

Elisabeth Härtig,<sup>1\*</sup> Hao Geng,<sup>2†</sup> Anja Hartmann,<sup>1</sup> Angela Hubacek,<sup>3</sup> Richard Münch,<sup>1</sup>  
Rick W. Ye,<sup>4</sup> Dieter Jahn,<sup>1</sup> and Michiko M. Nakano<sup>2</sup>

*Institute of Microbiology, University of Braunschweig, Braunschweig, Germany*<sup>1</sup>; *Department of Environmental and Biomolecular Systems, OGI School of Science and Engineering, Oregon Health and Science University, Beaverton,*<sup>2</sup>  
*and Woodburn High School, Woodburn,*<sup>3</sup> *Oregon; and Experimental Station E328/148B,*  
*DuPont Central Research and Development, Wilmington, Delaware*<sup>4</sup>

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**Transcription of the *yclJK* operon, which encodes a potential two-component regulatory system, is activated in response to oxygen limitation in *Bacillus subtilis*. Northern blot analysis and assays of *yclJ-lacZ* reporter gene fusion activity revealed that the anaerobic induction is dependent on another two-component signal transduction system encoded by *resDE*. ResDE was previously shown to be required for the induction of anaerobic energy metabolism. Electrophoretic mobility shift assays and DNase I footprinting experiments showed that the response regulator ResD binds specifically to the *yclJK* regulatory region upstream of the transcriptional start site. In vitro transcription experiments demonstrated that ResD is sufficient to activate *yclJ* transcription. The phosphorylation of ResD by its sensor kinase, ResE, highly stimulates its activity as a transcriptional activator. Multiple nucleotide substitutions in the ResD binding regions of the *yclJ* promoter abolished ResD binding in vitro and prevented the anaerobic induction of *yclJK* in vivo. A weight matrix for the ResD binding site was defined by a bioinformatic approach. The results obtained suggest the existence of a new branch of the complex regulatory system employed for the adaptation of *B. subtilis* to anaerobic growth conditions.**

Current knowledge about the adaptation of *Bacillus subtilis* to oxygen limitation in the environment has revealed a redox-dependent regulation of gene expression at the transcriptional level (13, 21, 22, 34). A two-component regulatory system, composed of a histidine sensor kinase (ResE) and a response regulator (ResD), has a pivotal role in the metabolic adjustment required for anaerobic growth, with nitrate as a terminal electron acceptor (23, 31). ResD and ResE are required for the transcription of genes involved in anaerobic nitrate respiration, including *fnr* (anaerobic gene regulator; Fnr), *nasDEF* (nitrite reductase operon), and *hmp* (flavo-hemoglobin) (9, 12, 19, 23). Furthermore, ResD and ResE also play an important role in another mode of anaerobic growth, i.e., fermentation (3, 18), in which they are required for the full induction of *ldh* (lactate dehydrogenase) and *lctP* (lactate permease) expression (3). The genes encoding ResA, ResB, and ResC, which constitute an operon with the genes for ResD and ResE, are thought to be involved in cytochrome *c* biosynthesis (31). A recent study showed that ResA is required for the reduction of cysteinyl residues during heme binding to apocytochrome *c* (5). ResD and ResE are also required for the transcription of their own genes, which is initiated at the *resA* operon promoter (31). Previous studies demonstrated the physical interaction of ResD with the regulatory regions of *ctaA* (35), *resA* (35), *hmp* (20), *nasD* (20), and *fnr* (20). ResD activates the transcription of *ctaA* (25), *hmp* (8, 24), *nasD* (8), and *fnr* (8) in vitro.

The ResD-ResE signal transduction system functions early in the anaerobic gene regulatory cascade. It activates the expression of other regulatory genes that also play important roles in anaerobic gene expression. One such gene encodes Fnr, which is needed for activation of the respiratory nitrate reductase operon *narGHJI* (2). Fnr also activates the transcription of another regulator gene, *arfM*. Anaerobic expression of the fermentative operons *ldh-lctP* (lactate fermentation) and *alsSD* (acetoin formation) (14) and of the heme biosynthesis genes *hemN* and *hemZ* is partly dependent on ArfM (10).

A previous DNA microarray analysis showed that the two-component regulatory genes *yclJ* (encoding a potential response regulator) and *yclK* (encoding a potential sensor kinase) are induced by oxygen limitation (34). YclK is likely one of the class IIIA family of kinases according to a classification based on sequence similarities in the vicinity of the phosphorylated histidine. The potential response regulator YclJ belongs to the OmpR family of transcriptional regulators according to alignments of sequences within the C-terminal domains of response regulators (6). A comprehensive DNA microarray analysis was undertaken to identify target genes of uncharacterized *B. subtilis* two-component regulatory systems (11). Seventeen genes were designated as being positively controlled by YclJK, while 11 appeared to be negatively controlled. For this paper, we examined how *yclJK* transcription is activated in response to oxygen limitation and determined whether the candidate target genes are regulated by YclJK.

\* Corresponding author. Mailing address: Institute of Microbiology, University of Braunschweig, Spielmannstr. 7, D-38106 Braunschweig, Germany. Phone: 49 (0)531 391-5819. Fax: 49 (0)531 391-5854. E-mail: e.haertig@tu-bs.de.

† E.H. and H.G. contributed equally to this work.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All *B. subtilis* strains used for this work are listed in Table 1. Luria-Bertani medium was used for standard cultures of *B. subtilis* and *Escherichia coli* unless otherwise indicated. For investigations of

TABLE 1. Bacterial strains used for this study

<i>B. subtilis</i> strain	Genotype	Source or reference
JH642	<i>trpC2 pheA1</i>	BGSC <sup>a</sup>
LAB2135	<i>trpC2 pheA1 ΔresDE::tet</i>	23
LAB2234	<i>trpC2 pheA1 ΔresE::spec</i>	18
THB2	<i>trpC2 pheA1 fur::spec</i>	9
BEH1	<i>trpC2 pheA1 ΔyclJ::ery</i>	This study
BEH2	<i>trpC2 pheA1 amyE::yclJ-lacZ cat</i>	This study
BEH3	<i>trpC2 pheA1 ΔyclJ::ery amyE::yclJ-lacZ cat</i>	This study
BEH4	<i>trpC2 pheA1 fur::spec amyE::yclJ-lacZ cat</i>	This study
BEH5	<i>trpC2 pheA1 ΔresDE::tet amyE::yclJ-lacZ cat</i>	This study
BEH6	<i>trpC2 pheA1 amyE::muta yclJ-lacZ cat</i>	This study
BEH7	<i>trpC2 pheA1 amyE::mutb yclJ-lacZ cat</i>	This study

<sup>a</sup> BGSC, *Bacillus* Genetic Stock Center.

the expression of various *lacZ* fusions and for preparations of RNA, the strains were grown at 37°C in minimal medium (80 mM K<sub>2</sub>HPO<sub>4</sub>, 44 mM KH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.5 mM thiamine, 40 μM CaCl<sub>2</sub>·2H<sub>2</sub>O, 68 μM FeCl<sub>2</sub>·4H<sub>2</sub>O, 5 μM MnCl<sub>2</sub>·4H<sub>2</sub>O, 12.5 μM ZnCl<sub>2</sub>, 24 μM CuCl<sub>2</sub>·2H<sub>2</sub>O, 2.5 μM CoCl<sub>2</sub>·6H<sub>2</sub>O, 2.5 μM Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 50 mM glucose, 50 mM pyruvate, 1 mM L-tryptophan, 0.8 mM L-phenylalanine), and where indicated, 10 mM nitrate or 10 mM nitrite was added. For aerobic growth, 100 ml of medium was inoculated at an optical density at 578 nm (OD<sub>578</sub>) of 0.05 with an aerobically grown overnight culture and then incubated in a 500-ml baffled flask with shaking at 250 rpm. For anaerobic fermentative growth, the bacteria were incubated in completely filled flasks with rubber stoppers and with shaking at 100 rpm in an incubation shaker to minimize aggregation of the bacteria. Inoculation was performed aerobically with an aerobically grown overnight culture with an OD<sub>578</sub> of 0.3. Anaerobic conditions were achieved after a short time through the consumption of residual oxygen by the inoculated bacteria. After 3 h in the midst of the exponential growth phase and after 6 h at the beginning of the stationary growth phase, samples for β-galactosidase assays were taken. The cells for preparations of RNA were harvested after 3 h in the midst of the exponential growth phase.

**Construction of *B. subtilis* *yclJK* mutant strain.** A 1,197-bp PCR fragment containing parts of the coding region of *yclJK* was amplified by PCR with primers EH100 5'-GGTTTTGAAGCCGAATTCGTTTCATGAC-3' (the 5' end corresponds to position 69 of the *yclJ* coding sequence) and EH101 5'-CGCTTCTC TCAGCTCTAGAATCCGCTTGAC-3' (the 5' end corresponds to position 593 of the *yclK* coding sequence, with two base changes to create an internal XbaI restriction site [underlined]). This fragment was cleaved at an internal KpnI site (positions 106 to 112 of the *yclJ* coding sequence) and at the XbaI site and ligated into vector pBluescript SK(+) II (Stratagene) digested with the same restriction enzymes. The plasmid was then cut at an internal HindIII restriction site in the *yclJK* fragment, and an erythromycin resistance gene cassette liberated from the vector pDG646 (7) by HindIII digestion was inserted. The plasmid was transformed into *B. subtilis* strain JH642 and screened for erythromycin-resistant clones. The desired double-crossover event was confirmed by PCR and Southern blot analysis. The resulting mutant strain, BEH1, carries an inactivated *yclJ* gene.

**Preparation of RNA and Northern blot analysis.** For preparations of RNA, 25 ml of cell culture was added to 25 ml of an ice-cold solution containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, and 20 mM Na<sub>3</sub>. The cells were harvested by centrifugation (5 min at 7,155 × g and 4°C). The cell pellets were resuspended in 200 μl of supernatant, immediately dropped into a Teflon disruption vessel, filled, and precooled with liquid N<sub>2</sub>. The cells were disrupted with a Mikro-Dismembrator S instrument (B. Braun Biotech International, Melsungen, Germany) for 2 min at 2,600 rpm. The resulting frozen powder was resuspended in 1 ml of prewarmed (50°C) cell lysis solution consisting of 4 M guanidine thiocyanate, 25 mM sodium acetate (pH 5.2), and 0.5% (wt/vol) *N*-laurylsarcosine. After complete cell lysis, the solution was immediately placed on ice. The RNA was extracted twice with acidic phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) and once with chloroform-isoamyl alcohol (24:1 [vol/vol]). After ethanol precipitation, the RNA pellet was resuspended in 180 μl of 20 mM phosphate buffer (pH 6.5)–1 mM EDTA and 20 μl of a solution containing 200 mM sodium acetate (pH 4.5), 180 mM MgCl<sub>2</sub>, 100 mM NaCl, and 15 U of DNase I and then was incubated for 30 min at room temperature. Twenty microliters of 250 mM EDTA, pH 7.0, was added, followed by phenol-chloroform extraction and ethanol precipitation. The resulting RNA pellet was dissolved in 50 μl of H<sub>2</sub>O. For Northern blot analysis, 10 μg of RNA was separated under denaturing conditions

in a 1% agarose–670 mM formaldehyde–morpholinepropanesulfonic acid gel, stained with ethidium bromide, and transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) by vacuum blotting. The approximate sizes of the mRNAs were estimated by the use of RNA standards (Bethesda Research Laboratories, Inc.) labeled with digoxigenin. Hybridization and detection was performed as described elsewhere (4). A digoxigenin-labeled RNA probe was synthesized in vitro with T7 RNA polymerase and a 556-bp *yclJ*-specific PCR fragment as a template, previously amplified with the following primers: EH34, 5'-TATGTACGATGACGAGATG-3'; and EH35, 5'-CTAATACGACTCACTATAGGGAGATAAAATTGATAGCCCCATAC-3'.

**Construction of reporter gene fusion and site-directed mutagenesis of *yclJ* regulatory region.** A transcriptional fusion between the *E. coli lacZ* gene and the *yclJ* upstream region was constructed. A 545-bp PCR fragment spanning the region from positions –495 to +50 relative to the translational start of *yclJK* was amplified with the primers EH29 (5'-CGAGGAATTCGCATCAGACACTTT-3') and EH28 (5'-TAATGGATCCGTCATCGTACATACA-3'). Using the restriction sites for EcoRI and BamHI created by the primers (underlined), we cloned the promoter region of *yclJK* into the plasmid pDIA5322 (15), resulting in plasmid *PyclJ-lacZ*. This plasmid was transformed into *B. subtilis* strains JH642, LAB2135 (*ΔresDE*) (23), LAB2234 (*ΔresE*) (18), THB2 (*Δfur*) (9), and BEH1 (*ΔyclJ*), and transformants were screened for double-crossover integration at the *amyE* locus.

The potential ResD binding sites were changed from TATTTTTTTCATAC to TcTTggTcTCATgC (part a) and from TAGATTGTTTCATAT to TcGaggGcT-CATgT (part b), respectively (exchanged bases are shown in lowercase). Crossover PCRs were performed with the following two primers containing the desired base exchanges (in bold letters): EH88 (5'-GAAAAAATCAGGCCCTCGA TTTCTAG-3') and EH89 (5'-CTAGAAATCGAGGGCTCATGTTTTTTTC-3') for part a and EH86 (5'-TCCAAAGGCATGAGACCAAGATGAAC-3') and EH87 (5'-GTTTCATCTTGGTCTCATGCCCTTGGGA-3') for part b. Two PCR products were generated with primer pairs EH28-EH88 (180 bp) and EH29-EH89 (393 bp) for part a and with primer pairs EH28-EH86 (191 bp) and EH29-EH87 (380 bp) for part b. In a second PCR, we used the first two PCR products as templates and amplified the whole promoter with the primer pair EH28-EH29. The complete promoter fragments were cloned into the plasmid pDIA5322, as described above for the wild-type sequence, resulting in the plasmids *pmuta yclJ-lacZ* and *pmutb yclJ-lacZ*. After transformation into *B. subtilis* strain JH642, strains BEH6 and BEH7 were obtained.

**Identification of *yclJK* transcription start site.** Fifty micrograms of RNA was used for a primer extension analysis of the *yclJ* transcript. Reverse transcription was initiated from the γ-<sup>32</sup>P-end-labeled primer EH36 (5'-CGTCATCGTACATACACTAACATTATC-3') by a standard procedure (1). The sequencing reaction was performed with the same primer. The primer extension products and the sequencing reactions were analyzed in a 6% denaturing polyacrylamide gel in Tris-borate buffer. The dried gel was analyzed by use of a phosphorimager.

**Measurement of *yclJ-lacZ* expression.** For β-galactosidase assays, cells were harvested by centrifugation 3 h after inoculation. β-Galactosidase activities were determined by a standard method and are given in Miller units (16).

**Prediction of ResD binding sites.** A model of the ResD binding site was created by computing an information weight matrix with the following equation:  $R_{iw} = 2 + \log_2 f(b,l) - e(n)$  (bits per base), where  $f(b,l)$  is the frequency of each base ( $b$ ) at position  $l$  in the aligned binding sites and  $e(n)$  is a small sample size correction factor (29). By adding the weights together for various positions in a

particular binding site, we could measure the total individual information ( $R_i$ ) in bits. We employed Virtual Footprint software for pattern searches and the prediction of potential binding sites (17). This program is able to use the information weight matrix model and is connected interactively to the ProDoric database (<http://prodoric.tu-bs.de>). The sequence logo was created with Web-Logo software (<http://weblogo.berkeley.edu>).

**Electrophoretic mobility shift assays (EMSAs).** The ResD and ResE proteins were overproduced in *E. coli* carrying pXH22 or pMMN424, respectively, and were purified by chromatography as described previously (20). DNA fragments carrying the *yclJ* promoter region (−220 to +30 with respect to the *yclJ* transcription start site) were amplified by PCR with the primers oMN02-201 (5′-GCCCTTCATATTCAAAAG-3′) and oMN02-202 (5′-TATATCCTCCGGTTGT TT-3′) and with JH642 chromosomal DNA as a template. Mutant *yclJ* promoters were amplified from plasmids *pmutA yclJ-lacZ* and *pmutB yclJ-lacZ* with the same oligonucleotides. The primers were 5′ end labeled on the coding and noncoding strands with T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP. DNA fragments were produced by PCR as described above, but with one labeled and one unlabeled primer. The radiolabeled promoter fragments were separated in 6% nondenaturing polyacrylamide gels and then purified in Elutip-d columns (Schleicher & Schuell, Dassel, Germany). Unlabeled probes employed in competition experiments were produced by PCR as well and were purified in 1.2% agarose gels, followed by fragment recovery by use of a gel extraction kit (Qiagen, Hilden, Germany).

EMSAs were performed by incubating the labeled fragments (0.18 pmol or 1,000 cpm per reaction) with the indicated amounts of the ResD protein, with or without ResE, in 20  $\mu$ l of reaction buffer [50 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 50  $\mu$ g of poly(dI-dC)/ml, 50  $\mu$ g of bovine serum albumin (BSA)/ml, 0.25 mM ATP]. After incubation for 15 min at room temperature, the reaction mixtures were separated in 5% nondenaturing polyacrylamide gels in Tris-acetate buffer. The gels were dried and analyzed by use of a phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). In vitro phosphorylation assays using [ $\gamma$ - $^{32}$ P]ATP showed that ResD was phosphorylated under this conditions.

**DNase I footprinting analysis.** The *yclJ* fragment, labeled as described above (50,000 cpm per reaction), was incubated with 2 to 6  $\mu$ M ResD and/or ResE protein in the same buffer as that used for EMSAs, except that glycerol, poly(dI-dC), and BSA were omitted. The reaction was treated with 60 ng of DNase I at room temperature for 20 s for free probes and 40 s for reactions containing the protein(s). The same primers used for the labeling of DNA fragments were used for sequencing of the template DNAs with a Thermo Sequenase cycle sequencing kit (USB, Cleveland, Ohio). The sequencing reactions were run together with the footprinting reactions in 8% polyacrylamide-urea gels in Tris-borate buffer. The dried gels were analyzed by phosphorimaging.

**In vitro transcription assay.** A mutant ResD protein (D57A) was overproduced in *E. coli* pMMN539. ResE and wild-type and mutant ResD proteins were chromatographically purified as described previously (8). The linear template of *yclJ* (−129 to +116) used for in vitro transcription assays was PCR amplified with the primers oHG-11 (5′-CCCCTTGCTGATAAATTAATA-3′) and oHG-12 (5′-AATTCGGCTTCAAACCTTCT-3′). The PCR products were purified with a PCR purification kit (Qiagen). In vitro transcription buffer contained 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 0.25 mM ATP, 50  $\mu$ g of BSA/ml, 10% glycerol, and 0.4 U of RNasin RNase inhibitor (Promega)/ $\mu$ l. ResD (0.35, 0.7, and 1.4  $\mu$ M), ResE (1  $\mu$ M), or both were incubated in 20  $\mu$ l of transcription buffer at room temperature for 10 min. RNA polymerase and templates were added at final concentrations of 25 and 5 nM, respectively, and the reaction mixtures were incubated for 10 min at room temperature. ATP, GTP, CTP (each at 100  $\mu$ M), UTP (25  $\mu$ M), and 5  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]UTP (800 Ci/mmol) were added to start transcription. After incubation at 37°C for 20 min, 10  $\mu$ l of stop solution (1 M ammonium acetate, 100  $\mu$ g of yeast RNA/ml, 30 mM EDTA) was added. The nucleic acids in the reaction mixture were precipitated with ethanol, and the pellet was dissolved in 3.5  $\mu$ l of loading dye solution (7 M urea, 100 mM EDTA, 5% glycerol, 0.05% bromophenol blue). The transcripts were analyzed in a urea-8% polyacrylamide gel. RNA markers were prepared according to the Decade marker system protocol (Ambion Inc., Austin, Tex.).

## RESULTS

**Analysis of *yclJK* operon structure.** In a microarray analysis, the *yclJ* and *yclK* genes for a potential two-component system were found to be induced under anaerobic conditions (34). In order to analyze the expression and organization of these

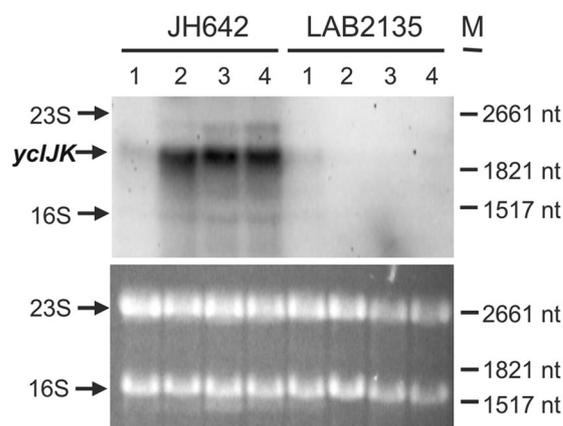


FIG. 1. Anaerobic expression of *yclJK* operon is ResDE dependent. Total RNAs were extracted from wild-type strain JH642 and the *resDE* mutant strain LAB2135 grown under the following growth conditions: aerobic (1), fermentative (2), anaerobic plus nitrate (3), and anaerobic plus nitrite (4). RNAs (10  $\mu$ g) were separated in a 1% denaturing agarose gel and analyzed by Northern blotting. A *yclJ*-specific RNA probe was used for hybridization. A single transcript of 2.1 kb was detected, which corresponds to the size of a *yclJK* transcript. Ethidium bromide staining of the gel showed that equal amounts of RNA were analyzed. The size standards are RNA molecular weight marker no. 1 (Roche Diagnostics GmbH) and the 16S and 23S rRNA species.

genes in detail, we performed a Northern blot analysis. A sequence analysis of the *yclJK* region showed that the *yclK* start codon resides eight nucleotides upstream of the *yclJ* stop codon (<http://genolist.pasteur.fr/Subtilist/>). The partial overlap between the *yclJ* and *yclK* open reading frames suggested that these genes constitute an operon. Northern analysis results listed in the BSORF *Bacillus subtilis* Genome Database (<http://bacillus.genome.ad.jp/>) indicated that *yclJ* and *yclK* are cotranscribed. We examined whether the two genes are coinduced by anaerobiosis by examining *yclJK* transcript levels on Northern blot membranes. Equal amounts of total RNAs isolated from wild-type cells cultured under aerobic and three different anaerobic conditions (fermentative, anaerobic with nitrate, and anaerobic with nitrite) were analyzed with a *yclJ*-specific RNA probe (Fig. 1). A single transcript of 2.1 kb, which corresponds to the size of the *yclJK* operon, was detected for RNAs isolated from anaerobically grown cells, whereas the transcript was barely detected in RNAs from aerobic cultures. A transcript of the same size was detected with a *yclK*-specific probe (data not shown). The *yclJK* transcript was not present in RNA prepared from a *resDE* mutant strain that was grown anaerobically. This result clearly demonstrated that the anaerobic induction of *yclJK* is dependent on *resDE*. The Northern blot analysis showed that *yclJ* and *yclK* are likely cotranscribed from a promoter residing upstream of the *yclJ* gene. The transcription start site was identified by primer extension analysis (Fig. 2). Upstream of the transcription start site a potential  $\sigma^A$ -type −10 (TATTAT) sequence was detected, but no sequence resembling a −35 region was present, suggesting the involvement of an additional activator for efficient transcription of *yclJK*.

**Examination of *yclJ-lacZ* expression in various regulatory mutant strains.** To gain further insights into the regulation of *yclJK* transcription, we fused the promoter region of *yclJ* to a

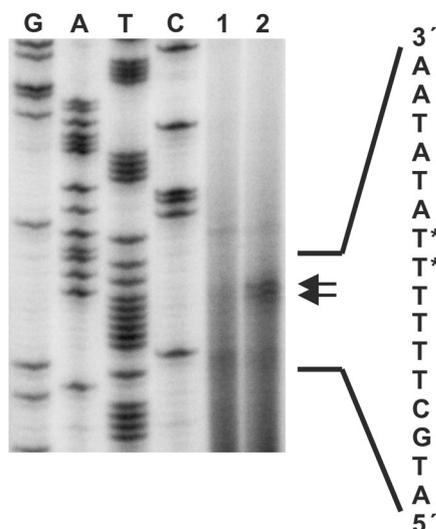


FIG. 2. Determination of transcription start site of *yclJK* by primer extension analysis. The total RNA was isolated and analyzed from JH642 cells grown aerobically (1) and under fermentative conditions (2). The same primer used for the primer extension analysis was used for sequencing reactions (lanes G, A, T, and C). Arrows indicate the primer extension products and asterisks mark the 5' end of the *yclJK* mRNA in the sequence.

promoterless *E. coli lacZ* gene. The expression of *yclJ-lacZ* in wild-type cells was induced 8- to 10-fold under all employed anaerobic conditions compared to aerobic conditions (Fig. 3). In accordance with the results of the Northern blot analysis, the introduction of the *resDE* mutation completely abolished the anaerobic induction of *yclJK*. Only a residual expression of

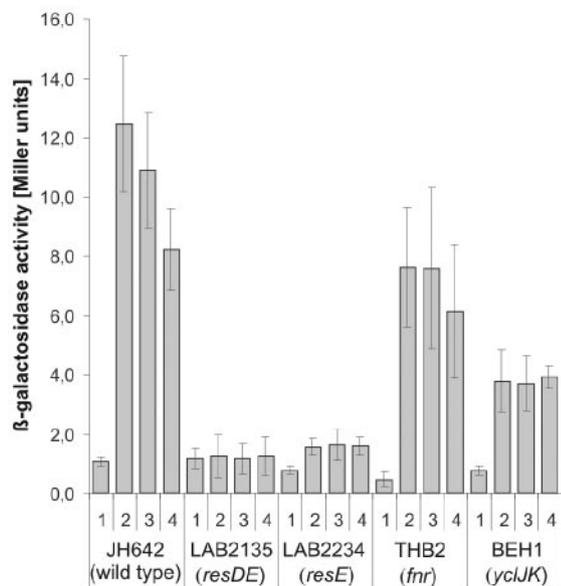


FIG. 3. Expression of *yclJ-lacZ* in various regulatory mutant strains.  $\beta$ -Galactosidase activities were measured in the JH642 wild-type strain and *resDE*, *resE*, *fnr*, and *yclJ* mutant strains after 3 h of culture under the following growth conditions: aerobic (1), fermentative (2), anaerobic plus nitrate (3), and anaerobic plus nitrite (4). Error bars indicate standard deviations ( $n \geq 3$ ).

*yclJ-lacZ* was found in a *resE* mutant strain, indicating that the phosphorylation of ResD by ResE is required for the anaerobic induction of *yclJK* (Fig. 3). In contrast to the effect observed for the *resDE* mutant, mutations in *fnr* had no significant effect on *yclJ-lacZ* expression. In agreement with this finding, no obvious Fnr binding site was detected in the *yclJK* promoter region. The expression level of *yclJ-lacZ* in an *arfM* mutant strain was similar to the wild-type level as well (data not shown). These results indicate that ResDE does not exert its positive role through Fnr or ArfM and that it may play a more direct role in *yclJK* activation. The  $\beta$ -galactosidase activity in a *yclJ* mutant was reduced by half compared to that in the wild type, suggesting that *yclJK* expression is under moderate auto-regulatory control. Nevertheless, ResD is the essential regulator of *yclJK* expression. YclJK is able to increase *yclJK* expression to some extent, but only in cooperation with ResD. Therefore, a *resD* mutation abolishes the expression of *yclJK*.

**ResD binds to the *yclJ* promoter.** We examined by EMSA the possibility that ResD directly binds to the *yclJ* promoter. A 250-bp DNA fragment carrying the *yclJ* promoter (positions -220 to +30) was end labeled with [ $\gamma$ - $^{32}$ P]ATP and incubated with increasing amounts of purified recombinant ResD in the presence or absence of ResE, followed by electrophoresis in order to resolve DNA-protein complexes (Fig. 4A). The results indicated that ResD forms a stable complex with the *yclJ* promoter. However, the phosphorylation of ResD via ResE only slightly stimulated DNA binding.

To localize the ResD binding site in the *yclJ* promoter, we performed DNase I footprinting analysis (Fig. 5). A strongly protected area between positions -92 and -73 relative to the transcription start site was observed on both strands, and ResD-P protected the same regions with a similar affinity as unphosphorylated ResD.

**Phosphorylation of ResD by ResE is needed for maximal transcriptional activation of *yclJK*.** Since the binding of ResD to the *yclJ* promoter was not stimulated by phosphorylation, we investigated whether phosphorylation is mainly required for transcriptional activation by using an in vitro transcription assay. In the absence of ResD and ResE, the transcript of *yclJ* was barely detected (Fig. 6, lane 1). Increasing amounts of unphosphorylated ResD only slightly stimulated in vitro transcription (Fig. 6, lanes 2 to 4). However, in vitro transcription was significantly stimulated when both ResD and ResE were present (Fig. 6, lanes 5 to 7). This indicated that the phosphorylation of ResD is required for full transcriptional activation. It was shown previously that the aspartate residue at position 57 of ResD is the phosphorylation site of the response regulator. A mutant ResD protein carrying an amino acid exchange at position 57 from aspartate to alanine (D57A) can no longer be phosphorylated by ResE (8). To further determine the effect of ResD phosphorylation on *yclJK* transcription in vitro, we tested the mutant ResD D57A protein with the in vitro transcription assay. Transcription was stimulated by the mutant ResD D57A protein to a small extent, similar to that by unphosphorylated wild-type ResD (Fig. 6, compare lanes 2 to 4 with lanes 8 to 10). Moreover, the level of the *yclJK* transcripts was not further increased by ResD D57A in the presence of ResE (Fig. 6, lanes 11 to 13). These results clearly demonstrated that unphosphorylated ResD, although it binds to the promoter region, is not sufficient to activate the maximal transcription of *yclJK*. The

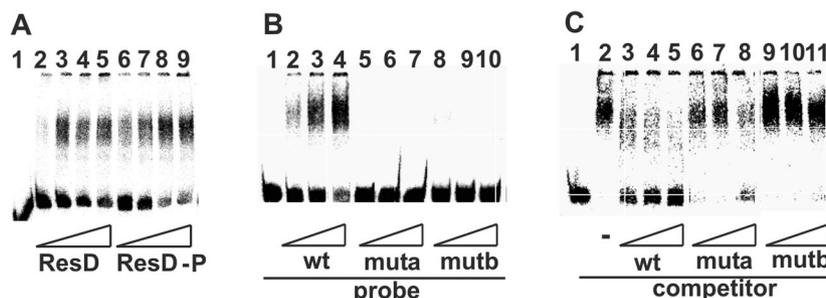


FIG. 4. EMSAs to detect binding of ResD to *ycfJ* promoter. (A) An end-labeled DNA fragment containing the *ycfJ* promoter was incubated with ResD in the presence (ResD-P) or absence (ResD) of 0.5  $\mu$ M ResE. The amounts of ResD used were 0.06  $\mu$ M (lanes 2 and 6), 0.13  $\mu$ M (lanes 3 and 7), 0.25  $\mu$ M (lanes 4 and 8), and 0.5  $\mu$ M (lanes 5 and 9). Lane 1, probe only. (B) ResD does not bind to *ycfJ* promoters carrying mutations in the putative ResD box. The same DNA fragments containing the wild-type and mutant promoters were used to examine interactions with ResD phosphorylated with 0.5  $\mu$ M ResE. The ResD concentrations used were 0.13  $\mu$ M (lanes 2, 5, and 8), 0.25  $\mu$ M (lanes 3, 6, and 9), and 0.5  $\mu$ M (lanes 4, 7, and 10). Lane 1, probe only. (C) Competition experiment with cold DNA fragment containing the wild-type and mutant promoters. The labeled *ycfJ* promoter fragment was incubated with 0.5  $\mu$ M ResD and ResE (lane 2). Increasing amounts of cold DNA, i.e., 2.5 nM (lanes 3, 6, and 9), 5 nM (lanes 4, 7, and 10), and 10 nM (lanes 5, 8, and 11), were included as competitor DNA in the reaction mixtures. Lane 1, probe only. The image shows the effect of the mutations in the putative ResD box on binding by ResD. Probes *muta* and *mutb* indicate the *ycfJ* promoter carrying the binding site a and binding site b mutations, respectively.

low level of transcription that was activated by unphosphorylated ResD and the D57A mutant was likely due to the phosphorylation-independent activation of ResD as previously described (8). These results, together with the results of the *ycfJ-lacZ* expression analysis with the *resE* mutant strain, showed that the phosphorylation of ResD by ResE is required for full anaerobic induction of *ycfJK*.

**New definition of ResD binding sites by a bioinformatic approach.** We used an extended information weight matrix model to find a better definition of ResD binding sites. The

DNA binding regions of ResD defined by footprinting analyses are part of the Prodigic database (17). They were used to create a weight matrix model, which is presented as a sequence logo (Fig. 7A) (30). The newly defined sequence is 21 bp long, with two stretches of conserved residues, specifically TTGT (positions 4 to 7) and TTTT (positions 13 to 16). The information content of these sequences is higher than 1, indicating the occurrence of major groove contacts with the DNA helix (30). The spacing of 10 between the conserved regions corresponds to one turn of the DNA helix and is typical for DNA binding motifs. The formerly proposed consensus recognition sequence for ResD, TTTGTGAAT (20, 35), corresponds to the first part of the newly defined binding sequence. In the *ycfJ*

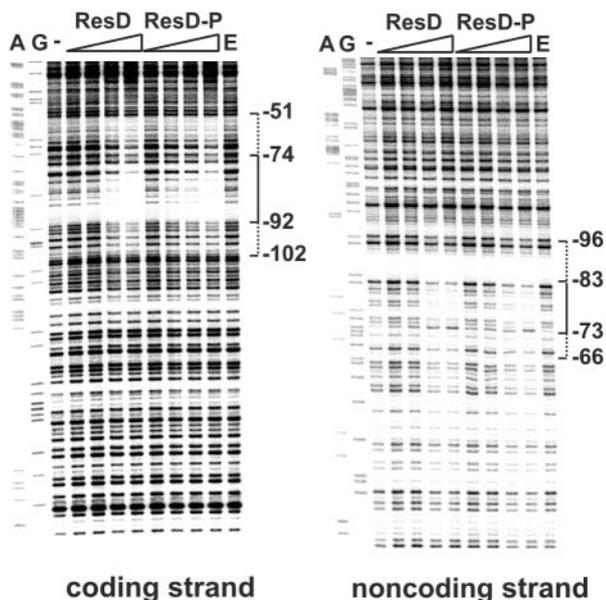


FIG. 5. DNase I footprinting experiment with ResD and the *ycfJ* promoter. Increasing concentrations of ResD and/or ResE (2, 4, 8, and 16  $\mu$ M) were incubated with  $^{32}$ P-end-labeled coding and noncoding strands of the *ycfJ* promoter. G and A sequencing ladders are included to localize the binding sites. The vertical brackets indicate protected regions. Dotted brackets show weakly protected regions. Positions relative to the transcription start site are shown.

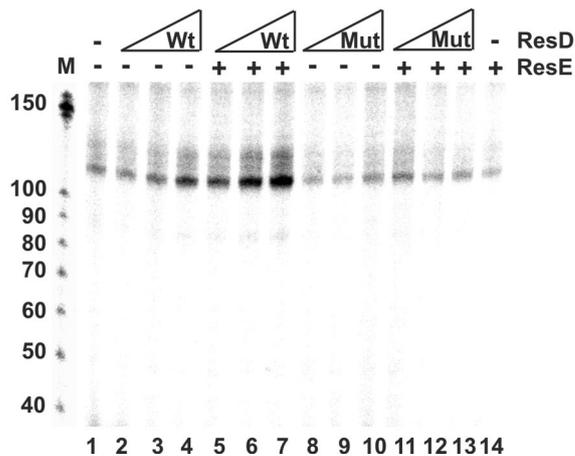


FIG. 6. In vitro transcription analysis of *ycfJ* promoter. Transcription was carried out with 25 nM purified RNA polymerase and 5 nM templates without ResD and ResE (lane 1), with increasing amounts of wild-type ResD (lanes 2 to 4), with wild-type ResD and ResE (lanes 5 to 7), with the D57A mutant ResD (lanes 8 to 10), with the D57A mutant ResD and ResE (lanes 11 to 13), and with ResE only (lane 14). The amounts of ResD used were 0.35, 0.7, and 1.4  $\mu$ M, and the amount of ResE used was 1  $\mu$ M. An RNA size marker (in nucleotides) is shown (M).

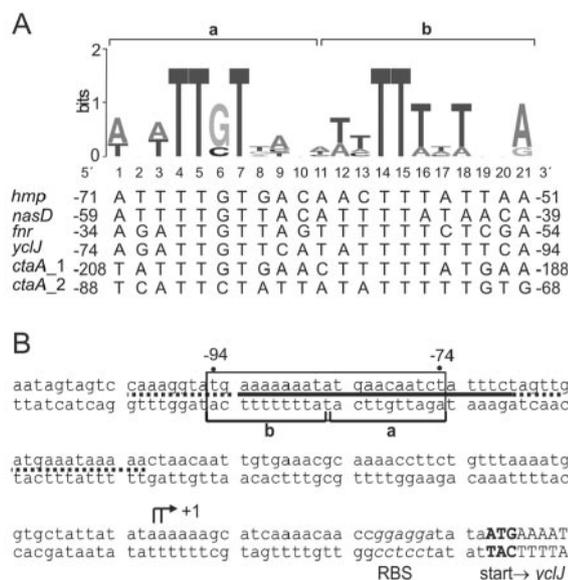


FIG. 7. New definition of ResD binding site and location in the *yclJ* promoter. (A) Sequence logo of the ResD binding site based on the information weight matrix model. The height of each stack of letters is the sequence conservation, measured in bits of information according to the equation given in Materials and Methods. The height of each letter within a stack is proportional to its frequency at that position in the binding site. The letters are sorted, with the most frequent on top. (B) ResD-dependent promoter of *yclJ*. The transcription start site obtained from primer extension analysis is marked "+1." Potential ResD binding sites, a and b, are boxed, and their positions with respect to the transcriptional start sites are given. The solid line marks the protected region from the footprinting experiments; the dashed lines mark weakly protected regions. RBS, ribosome-binding site.

promoter, the binding motif was detected on the opposite strand at positions -74 to -94 (Fig. 7B) and corresponds to the protected region from the footprinting analysis (Fig. 5).

**Mutagenesis studies of *yclJ* promoter.** Although ResD binds directly to several promoters, no detailed mutagenesis study of the ResD binding sequence is available. Based on the new computer-aided model of the ResD binding site, we exchanged three highly conserved T and two A residues in each binding part. The two mutated promoters were fused to *lacZ* and tested for  $\beta$ -galactosidase activity as described above. Both series of mutations almost completely abolished the anaerobic induction of *yclJ-lacZ* (Fig. 8), indicating that the mutated regions bear one or more nucleotide substitutions that impair ResD-dependent activation. These results suggest that the mutationally altered sequence contains the site of the ResD-DNA interaction. Confirmation of the new consensus awaits more detailed mutational analysis. To examine whether ResD binds to the mutant promoters, we performed EMSAs with the *yclJ* promoter containing the mutated a and b ResD binding sites. A concentration of 0.5  $\mu$ M ResD was sufficient for almost complete binding to the wild-type *yclJ* promoter fragment, but this concentration of ResD resulted in little, if any, binding to the mutant *yclJ* promoters (Fig. 4B). A competition experiment using excess cold DNA showed that the wild-type DNA fragment, at concentrations as low as 2.5 nM, showed significant competition with the labeled probe. In contrast, a 10 nM con-

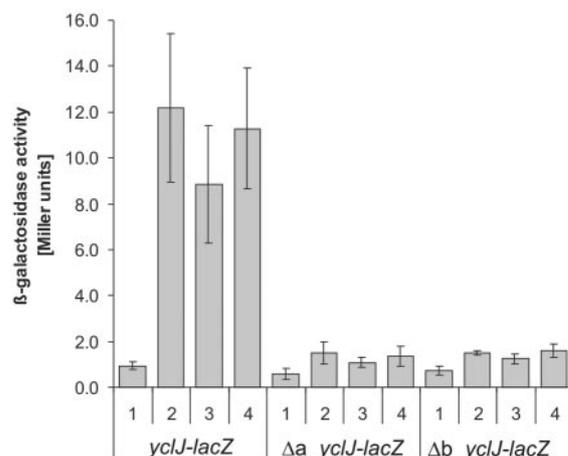


FIG. 8. Mutations in binding sites a and b prevent expression of *yclJ-lacZ*.  $\beta$ -Galactosidase activities were measured from *yclJ-lacZ* wild-type and mutant promoter constructs in the JH642 wild-type strain after 3 h of culture under the following growth conditions: aerobic (1), fermentative (2), anaerobic plus nitrate (3), and anaerobic plus nitrite (4). Error bars indicate standard deviations ( $n \geq 3$ ).

centration of the mutant *yclJ* promoters failed to compete for ResD binding to the *yclJ* promoter (Fig. 4C).

**The YclJK regulon.** To investigate the potential participation of YclJK in anaerobic growth processes, we analyzed the phenotype caused by an *yclJ* mutation. The deletion of *yclJ* had no obvious influence on aerobic or anaerobic growth. Moreover, the processes of sporulation and competence were not influenced by the *yclJ* mutation (data not shown). Subsequently, we analyzed the expression of several genes which are known to play an important role in the anaerobic metabolism of *B. subtilis*. The expression of *narG* (nitrate reductase), *nasD* (nitrite reductase), *arfM* (modulator of anaerobic respiration and fermentation), *hmp* (flavo-hemoglobin), *ldh* (L-lactate dehydrogenase), and *alsS* (alpha-acetolactate synthase) was studied by using reporter gene fusions in the *yclJ* mutant strain during aerobic and anaerobic growth. However, the *yclJ* mutation had no significant effect on the expression of any of these genes (data not shown).

A previous DNA microarray study identified genes that are possibly regulated by YclJK (11). Candidate genes activated by YclJK were *gerKB*, *pyrR*, *dhbA*, *dhbB*, *fhuD*, and several genes of unknown function. Those repressed by YclJK included *acoA*, *acoB*, *acoC*, *acoL*, *atpA*, *atpD*, *atpE*, *qoxB*, and *qoxC*. Since *dhbA* and *dhbB*, which are involved in siderophore 2,3-dihydroxybenzoate biosynthesis (26), were shown to be induced by oxygen limitation (34), we tested whether these genes are regulated by YclJK. The expression of a *dhbA-lacZ* reporter gene fusion (27) was examined in the wild type and the *yclJ* mutant strain during aerobic and anaerobic growth. As expected, *dhbA-lacZ* expression was higher upon anaerobic cultivation. However, the higher level of anaerobic expression was not dependent on YclJ (data not shown). The *yclI* gene was also reported to be activated by YclJK (11). Since *yclI* is transcribed divergently from the *yclJK* operon, one might expect that its expression would be affected by YclJK. We constructed an *yclI-lacZ* reporter gene fusion and compared the  $\beta$ -galactosidase activities of wild-type cells and the *yclJ* mutant strain

cultivated under aerobic and anaerobic conditions. The expression of *yclI-lacZ* was two- to threefold higher under anaerobic conditions than under aerobic conditions. However, there was no significant difference in observed expression between the wild-type and *yclJ* mutant strains.

The *qoxB* and *qoxC* genes, which were identified as being negatively affected by YclJK, encode subunits of cytochrome *aa3* quinol oxidase. They are transcribed from the *qoxA* promoter (28). An examination of a *qoxA-lacZ* reporter gene fusion revealed that YclJK does not affect expression of the *qox* operon.

The discrepancy between the results of our *lacZ* reporter gene experiments and the DNA microarray analysis described by Kobayashi and coworkers was most likely caused by the different experimental conditions employed. For their DNA microarray analysis, Kobayashi et al. overproduced the response regulator YclJ in the absence of the sensor kinase YclK under aerobic conditions. This strategy is applicable to certain classes of two-component regulatory systems, such as CitST and DesKR. However, it is not applicable to others. For example, the overproduction of ResD in the absence of ResE does not result in activation of the ResDE regulon under anaerobic conditions (M. M. Nakano, unpublished result).

In order to identify genes of the YclJK regulon, we isolated RNAs from the wild type and a *yclJK* mutant grown under a variety of anaerobic conditions and then performed various DNA microarray analyses. Since *yclJK* is almost exclusively expressed under anaerobic conditions, the stimulus for the YclJK system might be present only under anaerobic conditions. As expected from the initial *lacZ* fusion analysis described above, the expression of the genes identified as YclJK targets in the previous microarray experiment (11) was not significantly affected by the *yclJ* mutation in our microarray experiment (data not shown). Moreover, in six independent microarray tests, we reproducibly detected only minor changes of less than twofold in the transcriptional profiles when the wild type and the *yclJ* mutant were compared. The only exception was the expression of the *yclJK* operon itself, which was found to be repressed by a factor of about 30 in the *yclJ* mutant, which is in agreement with the results for *yclI-lacZ* reporter gene expression. Besides *yclJK* itself, no target genes of YclJK have been identified.

## DISCUSSION

The ResD-ResE signal transduction system plays an important role in the anaerobic metabolism of *B. subtilis* and probably other low-GC-content gram-positive bacteria (22). A recent genome sequence analysis has shown that ResD orthologs exist in other gram-positive bacteria, such as *Bacillus anthracis*, *Bacillus stearothermophilus*, *Bacillus halodurans*, *Listeria monocytogenes*, and *Staphylococcus aureus* (NCBI Microbial Genome BLAST databases). The *resD* and *resE* orthologs of *S. aureus*, *sraAB* (or *srhSR*), participate in the regulation of energy metabolism in response to variations in oxygen tension (32) and in the control of virulence factor expression (33). An *srhSR* mutant exhibited reduced survival in animal hosts (33). In this paper, we have shown that ResDE regulates the anaerobic induction of the potential two-component *yclJK* system. Northern blot, reporter gene fusion, and in vitro transcription exper-

iments showed that ResD upregulates *yclJK* transcription upon oxygen limitation. EMSA and DNase I footprinting experiments demonstrated a ResD interaction with the *yclI* promoter region. Although the phosphorylation of ResD only slightly enhanced binding to the *yclI* promoter, in vitro transcription experiments showed that the phosphorylation of ResD greatly stimulates *yclI* transcription. It is likely that the phosphorylation of ResD affects the interaction of ResD with RNA polymerase at the *yclI* promoter.

We proposed a putative ResD binding site by using a bioinformatic approach. ResD likely binds as a dimer to the 21-bp sequence of the ResD box consisting of two tandemly arranged 10-bp half-sites (Fig. 7A). In the 21-bp sequence, positions 4, 5, 7, 14, and 15 were the most conserved. Position 6 is usually a G (but is a C in one of the ResD binding sites in *ctaA*), and positions 16 and 18 are occupied by T, except for an A in *nasD*. Stretches of sequences from positions 1 to 3 (ATT) and positions 9 to 11 (ACA) are identical only in *hmp* and *nasD*. Among known members of the ResDE regulon, the oxygen-dependent induction of *hmp* and *nasD* is the highest. It remains to be examined whether these sequences are important for the high-affinity binding of ResD to these promoters.

The physiological role of YclJK remains unsolved. Which signal is transmitted by the YclJK regulatory system and whether its involvement in gene regulation is related to anaerobiosis are still unknown. The *yclJ* mutant showed no obvious growth defect when it was tested under various aerobic and anaerobic conditions. The *yclJ* mutant was able to grow well under anaerobic conditions that facilitated either nitrate respiration or fermentation (data not shown). Mutant phenotypes of *yclJ* are not observed in studies listed in the BSORF *Bacillus subtilis* Genome Database. We also examined the effect of the *yclJ* mutation on the expression of genes that were predicted to be regulated by YclJK according to a previously reported DNA microarray analysis (11), and we examined DNA arrays ourselves. Our results with promoter-*lacZ* fusions showed that none of the genes tested was significantly regulated by *yclJK* under the growth conditions used. It is possible that oxygen limitation, which activates *yclJK* transcription, is not sufficient to activate the YclJK signal transduction system. Further studies are needed to identify the signal that is transmitted by YclJK, the genes that are regulated by YclJK, and the physiological role of YclJK in *B. subtilis*.

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