J. Biochem. **133**, 295–302 (2003)
DOI: 10.1093/jb/mvg040

Involvement of ClpX Protein in the Post-Transcriptional Regulation of a Competence Specific Transcription Factor, ComK Protein, of *Bacillus subtilis*

Hideaki Nanamiya[1], Emiko Shiomi[1], Mitsuo Ogura[2], Teruo Tanaka[2], Kei Asai[3] and Fujio Kawamura[*,1]

[1]Laboratory of Molecular Genetics, College of Science, Rikkyo (St. Paul's) University, Toshima-ku, Tokyo 171-8501; [2]Department of Marine Science and Technology, Tokai University, Shimizu, Shizuoka 424-0902; and [3]Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, Saitama, Saitama 338-8570

Received November 27, 2002; accepted December 24, 2002

ComK protein of *Bacillus subtilis* positively regulates the transcription of several late competence genes as well as *comK* itself. We constructed a *clpX* disrupted mutant of *B. subtilis* and studied its effect on the regulation of ComK activation. When P*spac*, which controls the *comK* gene in a multicopy plasmid, was induced by the addition of IPTG, *comK* transcripts were detected in both the *clpX* mutant and the wild type. However, the ComK protein could not be detected in the *clpX* disrupted mutant. To obtain further information, we constructed several *comK-lacZ* translational fusions covering different lengths of the *comK* gene, whose transcription is controlled by an IPTG inducible P*spac* promoter. We found that both the expression of *comK-lacZ* directed β-galactosidase and the accumulation of ComK-LacZ fused protein, derived from the fusion containing the entire *comK* open reading frame, were extremely reduced in the *clpX* mutant compared with the wild type, while the accumulation of *comK-lacZ* transcripts in the *clpX* mutant after the addition of IPTG was about half that in the *clpX*+ background. On the other hand, transcription, translation and activity of *comK-lacZ* were detected in both the *clpX* mutant and the wild type when the *comK-lacZ* fusion lacking the 3′ region of the *comK* gene was induced. These results indicate that ClpX plays an important role in the regulation of ComK at the post-transcriptional level.

Key words: *Bacillus subtilis*, Clp protease, ClpX, ComK, competence development.

Bacillus subtilis, one of the best-characterized Gram-positive soil bacteria, can develop the ability to take up exogenous DNA and to undergo homologous recombination between chromosomal and incoming single stranded DNA (*1*, *2*). In the developmental pathway of natural genetic competence, ComK protein has been identified as a key transcription factor in competence development (*3*, *4*). During the exponential-growth phase, ComK activity is inhibited by direct protein-protein interaction with MecA (*5*, *6*). The bound ComK protein is targeted for degradation by an ATP-dependent ClpC/ClpP protease (*5*, *6*). When cell density increases, ComS protein, a 46 amino acid polypeptide encoded by the *comS* gene within the *srf* operon (*7*), is fully synthesized by the activation of the ComP-ComA two component regulatory system (*8*). ComS binds to MecA, and thus liberates ComK from degradation by the ClpCP protease (*6*). ComK protein is required for full induction of the expression of the late competence genes including the *comC* gene, *comG* operon, *comF* operon and *recA* as well as *comK* itself (*3*, *4*, *9–11*). Since the late competence genes are essential for the incorporation of external DNA in macromolecular form (*1*, *12*) and the *recA* gene product is indispensable for homologous DNA recombination, activation of ComK protein is one of the key regulatory events during competence development (*3*). Moreover, the expression of *comK* is positively regulated by DegU(*13*) and Med (*14*, *15*), and negatively regulated by AbrB and CodY(*8*, *16*). These results indicate that the molecular mechanisms for ComK activation involve a complex network including transcriptional regulation and post-translational regulation.

ClpX, which functions as the regulatory subunit of the ATP- dependent Clp protease and as a molecular chaperone, belongs to a family of Clp/Hsp100 proteins that are highly conserved among eukaryotes and procaryotes (*17*, *18*). In *B. subtilis*, it has been reported that ClpX is involved in several cellular processes such as stress response, cell division, motility, competence and sporulation (*19–22*). It has recently been shown that the introduction of *clpX* disruption decreases the expression of both *srfA-lacZ* and *comK-lacZ* during competence development (*20*). Moreover, *comK* expression in the *clpX* mutant can be partially suppressed by the introduction of a *mecA* mutation (*20*). These results suggest that ClpX regulates *comK* expression by controlling the ComA-dependent activation of *srf* transcription initiation (*20*).

Our attempt to clarify the involvement of ClpX protein in competence development was started by monitoring the expression of various competence genes, *comK*, *comG* and *srfA*, as previously reported by Nakano *et al*. (*20*).

[*]To whom correspondence should be addressed. Tel/Fax: +81-3-3985-2401, E-mail: kawamura@rikkyo.ne.jp

Table 1. ***Bacillus subtilis*** **strains used in this study.**

Strain	Genotype	Source or Reference
UOT1285	*trpC2 lys-1 aprEΔ3 nprR2 nprE18*	(*23*)
RIK770	*trpC2 lys-1 aprEΔ3 nprR2 nprE18 clpX*::*neo*	This study
RIK772	*trpC2 lys-1 aprEΔ3 nprR2 nprE18* pULI7KEm	This study
RIK774	*trpC2 lys-1 aprEΔ3 nprR2 nprE18* pULI7KEm *clpX*::*neo*	This study
RIK778	*trpC2 lys-1 nprR2 nprE18 aprE*::P*spac-comK1-lacZ spc*	This study
RIK779	*trpC2 lys-1 nprR2 nprE18 aprE*::P*spac-comK2-lacZ spc*	This study
RIK784	*trpC2 lys-1 nprR2 nprE18 clpX*::*neo aprE*::P*spac-comK1-lacZ spc*	This study
RIK785	*trpC2 lys-1 nprR2 nprE18 clpX*::*neo aprE*::P*spac-comK2-lacZ spc*	This study
RIK789	*trpC2 lys-1 nprR2 nprE18 aprE*::P*spac-comK3-lacZ spc*	This study
RIK790	*trpC2 lys-1 nprR2 nprE18 aprE*::P*spac-comK4-lacZ spc*	This study
RIK793	*trpC2 lys-1 nprR2 nprE18 clpX*::*neo aprE*::P*spac-comK3-lacZ spc*	This study
RIK794	*trpC2 lys-1 nprR2 nprE18 clpX*::*neo aprE*::P*spac-comK4-lacZ spc*	This study

Although the basal expression of the *comK* and *comG* genes in the *clpX* mutant could be monitored as described (*20*), we could not obtain the same results for *srfA* expression. Our preliminary experiments led us to consider other possible roles for ClpX in the regulation of ComK expression during competence development. In this study, we analyzed the effect of *clpX* mutation on the intracellular levels of ComK protein and found that ClpX is required for the production of ComK at a post-transcriptional level.

MATERIALS AND METHODS

Bacillus Subtilis Strains and Strain Construction—The *B. subtilis* strains used in this study are listed in Table 1. All strains are isogenic with UOT1285 (*trpC2 lys-1 aprEΔ3 nprR2 nprE18*) (*23*). RIK770 carrying a *clpX* disrupted gene was constructed as follows. A PCR product covering the entire *clpX* gene and its upstream region was generated using the primers CX2F (5′-CTTCAAGGATCCGCTTGCGAAAGCGGAAAACC-3′; underlined sequence represents a *Bam*HI restriction site) and CXR (5′-TGCAGATGTTTTATCTTGGC-3′). The amplified DNA fragment was cleaved with *Bam*HI/*Eco*RI and inserted into pBR322. This recombinant plasmid, named pRCX and containing a *Hin*dIII cleavage site in the *clpX* coding region, was cut with *Hin*dIII. The digest was ligated with a neomycin resistance cassette, a derivative of pBEST501 (*24*), after blunting the *Hin*dIII cleavage sites. The resulting plasmid, pRCXd, was linearized by *Pvu*II and transformed into *B. subtilis* strain UOT1285 for integration at the *clpX* site of the chromosome by double crossing over, and a neomycin resistant transformant was selected. Proper integration was confirmed by PCR amplification and by checking that the cells could not grow at 50°C and above (heat sensitive phenotype) (*19*, *21*). To exclude some unexpected mutations that existed in the *clpX* mutant, chromosomal DNA extracted from the *clpX* mutant was used to transform RIK771 carrying a *leuD*::*cat* mutation. A Leu+ transformant showing the heat-sensitive phenotype was selected and named RIK770. RIK771 was constructed as follows. Oligonucleotide primers were used to amplify the upstream (LDFF: 5′-GTGTCGATATCCGGTGTGAAATTCGGCACAG-3′, LDFR: 5′-CTAGTCTAGACAACGAAGTGTCCGTGAATG-3′) and downstream (LDBF: 5′-TTTCGGGATCCGGCTTCAAGCCTGAAAAAGC-3′, LDBR: 5′-CTTGCGTCGACCAGCAATTTCGGCTGGAACG-3′) regions of the *leuD* gene. The underlines represent *Eco*RV/*Xba*I (upstream) and *Bam*HI/*Sal*I (downstream) restriction sites, respectively. After PCR amplification, the fragments were cut with these restriction enzymes, and ligated simultaneously with pBR322 *Eco*RV/*Sal*I digests and a chloramphenicol resistance cassette derived from pCBB31 *Xba*I/*Bam*HI digests (*25*). The recombinant plasmid, pRLDC, was linearized with *Sca*I and used to transform UOT1285, and a chloramphenicol resistant transformant was selected. Proper integration was confirmed by PCR amplification and the Leu$^-$ phenotype.

pULI7KEm, a derivative of pULI7KS27 (*26*), was constructed by inserting an erythromycin resistance gene derived from pAE41 (*25*) into the kanamycin resistance gene of pULI7KS27 to avoid cross resistance between kanamycin and neomycin in the *clpX*::*neo* mutant.

To construct *comK-lacZ* translational fusions whose transcription is controlled by an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible P*spac* promoter, DNA fragments carrying different portions of the *comK* gene were obtained by PCR amplification using a common forward primer, CMKF-*Bgl*II (5′-GGAAGATCTGTATAAATTTTGCAGAAAAAGG-3′), and reverse primers, CMKR1-*Bam*HI (5′-CGCGGATCC*CC*TGTCACTTCATAAGTTCCTGC-3′), CMKR2-*Bam*HI (5′-CGCGGATCC*CC*ATACCGTTCCCCGAGCTCAC-3′), CMKR3- *Bam*HI (5′-CGCGGATCC*CC*ATGCACATGGGAAATCCAGCC-3′), and CMKR4-*Bam*HI (5′-CGCGGATCC*CC*CACTTCCGTATCGTCGAATTCAG-3′) to generate *comK1*, *comK2*, *comK3*, and *comK4* DNA fragments covering –32 to +219 (*comK1*), –32 to +576 (*comK2*), –32 to +321 (*comK3*), and –32 to +369 (*comK4*) of the *comK* gene relative to the translational start site, respectively. A *Bgl*II site and a *Bam*HI site were introduced at the 5′ and 3′ ends, respectively, and additional common sequences (CC) were introduced after the 3′ ends of each generated *comK* fragment to allow co-translation with the fused *lacZ* gene. The obtained fragments were digested with *Bgl*II and *Bam*HI and inserted into *Bam*HI-digested pAPNC213 (*27*) to generate pHEFK1, pHEFK2 pHEFK3 and pHEFK4. The resulting plasmids were sequenced to confirm the *comK* gene nucleotide sequence. These recombinant plasmids were cut with *Bam*HI and ligated with the *lacZ* gene fragment derived from pMC1871 *Bam*HI digestion (*28*). The obtained plasmids, named pHEFK1Z, pHEFK2Z, pHEFK3Z, and pHEFK4Z, respectively, were linearized

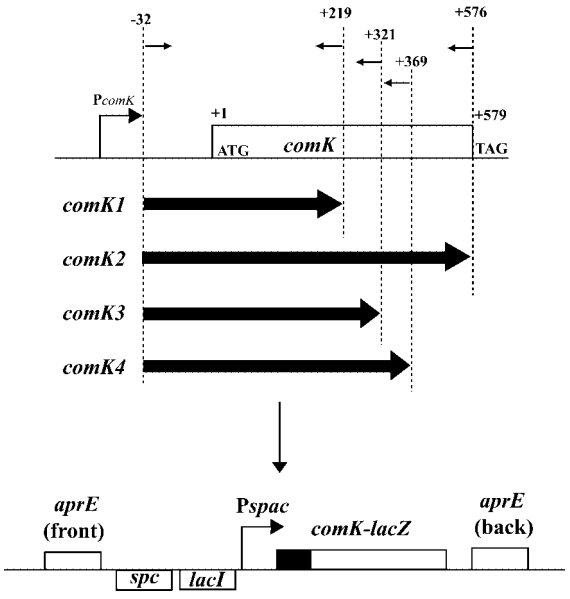

Fig. 1. **Structure of the *comK-lacZ* translational fusion.** The bold bars with arrows indicate the *comK1, comK2, comK3, and comK4* DNA fragments generated through PCR amplification using the primers shown by the arrows. +1 defines the translational start site of *comK*. These fragments were fused to the *lacZ* gene whose gene product lacked the 8 amino acid residues of the N-terminal region, and were integrated at the *aprE* site of the chromosome using pAPNC213 (*27*). The details of the construction procedures are described in "MATERIALS AND METHODS."

with *Sca*I and integrated at the *aprE* site of the chromosome of *B. subtilis* strain 168 by double crossing over recombination. Spectinomycin resistance transformants were selected as RIK775, RIK776, RIK786, and RIK787. Proper integrations were verified by PCR. Chromosomal DNAs extracted from RIK775, RIK776, RIK786, and RIK787 were used to transform UOT1285 and RIK771, to generate RIK778, RIK779, RIK789, and RIK790, and to generate RIK781, RIK782, RIK791, and RIK792. Finally, chromosomal DNA extracted from RIK770 was used to transform RIK781, RIK782, RIK791, and RIK792. Leu+ transformants showing the heat sensitive phenotype were selected and named RIK784, RIK785, RIK793, and RIK794 (Fig. 1).

Media—For competence medium, CI medium (*26*) was used. Chloramphenicol (5 μg/ml), neomycin (5 μg/ml), erythromycin (0.5 μg/ml), and spectinomycin (100 μg/ml) were added as required. Solid medium consisted of Luria broth (LB) (*29*) agar supplemented with antibiotics as required.

Western Blot Analysis—Cells were incubated in CI competence medium (*26*) at 37°C with shaking. After inoculation, cells were collected at the indicated time before or after the end of exponential growth defined as *T*0. Cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 0.2 mg of lysozyme per ml, 10 mM $MgCl_2$, 0.1 mg of DNase I per ml, 0.1 mM dithiothreitol, 2 mM PMSF) and disrupted by sonication on ice. Aliquots of cell extracts containing 20 μg of total protein were electrophoresed and transferred to polyvinilidene difluoride (PVDF) membranes (Millipore). Immunodetection procedures were carried out as described previously (*30*). Anti-LacZ antibody, purchased from Chemicon International (cat. no. AB986), and anti-ComK antibody (*15*) were used at a 1:1,000 dilution.

SI Protection Assay—Cells carrying pULI7KEm were grown in CI medium at 37°C with shaking. IPTG was added to the culture at a final concentration of 1 mM when the $OD_{600\ nm}$ reached ca. 0.2 (mid log phase) as the zero time. Cells were collected at the indicated times after the addition of IPTG and resuspended in LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.4, 1% SDS), and then total RNA was extracted using glass beads and phenol-chloroform. Aliquots of extracts containing 15 μg of total RNA and 0.1 μg of the digoxigenin UTP-labeled specific RNA probe for *comK* mRNA were mixed with hybridization buffer (80% deionized formamide, 40 mM PIPES pH 6.5, 0.4 M NaCl, and 1 mM EDTA) and hybridized for 16 h at 45°C after boiling at 90°C for 5 min. The *comK* probe was synthesized according to the manufacturer's procedures (Roche Molecular Biochemicals). The template for the *comK* probe was obtained by PCR using the primers KP.f (5′-AGTCAGAAAACAGACGCACC-3′) and KP.r (5′-TAATACGACTCACTATAGGGCGAATACCGTTCCCCGAGCTCAC-3′). The underline represents a recognition site for T7 RNA polymerase. After hybridization, samples were treated with 1,000 units of SI nuclease (TAKARA BID Inc., Shiga, Japan) as described previously (*31*) and electrophoresed through MOPS-formaldehyde denaturing 1% agarose gels (*29*). Samples were blotted onto Hybond N+ membrane (Amersham Biosciences), and detection procedures were performed according to the manufacturer's procedures (Roche Molecular Biochemicals) using NBT/BCIP as the substrate for alkaline phosphatase.

Assay of β-Galactosidase Activity—The β-galactosidase activity was determined as described previously (*26*, *30*). Cells were grown in CI medium with or without 1 mM of IPTG and 1 ml aliquots of were collected at the indicated times for the assay of β-galactosidase activity. One unit is equivalent to $1{,}000 \times A_{420}/OD_{600\ nm}$/ml/min, where A_{420} is the absorbance at 420 nm.

Northern Blot Analysis—Cells carrying a *comK-lacZ* fusion were grown in CI medium at 37°C with shaking and IPTG was added to the culture at a final concentration of 1 mM when the $OD_{600\ nm}$ reached ca. 0.20 (mid log phase) as the zero time. Cells were collected at the indicated times after the addition of IPTG and total RNA was extracted from the cells as described for the "S1 protection assay." Aliquots of extracts containing 15 μg of total RNA were electrophoresed through MOPS-formaldehyde denaturing 1% agarose gels (*29*) and blotted onto a Hybond N+ membrane by capillary transfer. After baking at 120°C for 30 min, the membrane was soaked in hybridization buffer (5× SSC, 50% deionized formamide, 0.02% SDS, 0.1% *N*-lauroylsarcosine, and 2% Blocking Reagent [Roche Molecular Biochemicals]) containing a *lacZ*-specific RNA probe at a final concentration of 100 ng/ml, and incubated at 68°C for 16 h. The *lacZ* probe was obtained according to the manufacturer's procedures (Roche Molecular Biochemicals). The template for the *lacZ* probe was obtained by PCR by using the primers, LACZP.f (5′-GATACACTTGCTGATGCGGTGCTG-3′) and LACZP.r (5′-TAATACGACTCACTATAGGGCGACCAGACCAACT-

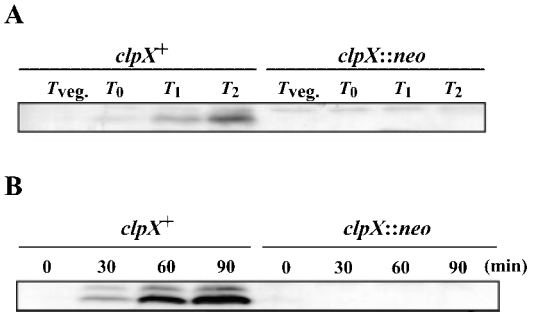

Fig. 2. **Western blot analysis of ComK protein in the *clpX* mutant.** (A) Intracellular levels of ComK protein in *clpX*+ (UOT1285) and *clpX*::*neo* (RIK770) mutant strains during competence development. Cells were incubated in CI competence medium (*26*) at 37°C with shaking and collected during the exponential growth (*T*veg.) or at the indicated time (expressed in hours) after the end of exponential growth, which is designated as *T*0. Aliquots of cell extracts containing 20 μg of total protein were electrophoresed through an SDS–15% (wt./vol.) polyacrylamide minigel. Western blot analysis was performed as described in "MATERIALS AND METHODS." (B) Intracellular levels of ComK protein in the *clpX* mutant carrying a multi-copy plasmid containing an inducible *comK* gene. *clpX*+ (RIK772) and *clpX*::*neo* (RIK774) mutant strains carrying pULI7KEm were grown in CI medium at 37°C with shaking and IPTG was added to the culture at a final concentration of 1 mM when $OD_{600\,nm}$ reached *ca.* 0.2 (mid log phase) as the zero time. Samples were collected at the indicated times after the addition of IPTG and were used for western blot analysis as described above.

GGTAATGGTAGCG-3′). The underline represents a recognition site for T7 RNA polymerase. Detection procedures were performed according to the manufacturer's procedures (Roche Molecular Biochemicals) using NBT/BCIP as the substrate for alkaline phosphatase.

RESULTS

Intracellular Level of ComK Protein in the clpX Mutant—To obtain further information about the function of ClpX protein during competence development, we constructed a *clpX* disrupted mutant as described in "MATERIALS AND METHODS." This *clpX*::*neo* mutant does not develop competence at all as reported by Nakano *et al*. (*20*); however, it shows genetic instability and forms various types of colonies with different morphologies when cells are grown on LB plates containing 5 μg/ml of neomycin. To exclude the possibility that some suppressor mutations might occur in the *clpX* mutant, we constructed a strain RIK771 carrying a disrupted mutation of the *leuD* gene, which lies about 3 kbp upstream of the *clpX* gene, and chromosomal DNA extracted from the *clpX*::*neo* mutant was used to transform RIK771 at a final concentration of *ca*. 0.01 μg/ml. Among Leu+ transformants, several colonies that did not grow at 50°C and over (*19*, *21*) were selected. Most of these transformants were expected to carry the *clpX*::*neo* mutation from the genetic linkage between *leuD* and *clpX*. In fact, the *clpX* mutation was confirmed in these transformants by PCR amplification. A representative transformant was used for this analysis without using neomycin.

It has been reported that the expression of the *comK* gene is severely inhibited in the *clpX* mutant during competence development (*20*). We also examined *comK-lacZ* and *comG-lacZ* expression in the *clpX* mutant during competence development and obtained results similar to those reported by Nakano *et al*. (*20*) who showed that these expressions are at almost a basal level in the *clpX* mutant compared with the wild type (data not shown). These results indicate that *comK* can not be activated in the *clpX* mutant and thus suggest the possibility that the ComK protein does not accumulate in the *clpX* mutant. To examine the intracellular levels of ComK protein during competence development, we carried out western blot analysis of ComK protein in the *clpX* mutant using a polyclonal anti-ComK antibody. As shown in Fig. 2A, ComK protein could hardly be detected in the *clpX* background during competence development. Since the expression of *comK* requires ComK itself (*11*, *32*), it is very difficult to determine whether *comK* gene expression is affected at either the transcription level or the post-transcription level in the *clpX* mutant. To answer this question, we introduced a multi-copy plasmid, pULI7KEm, in the *clpX* mutant. pULI7KEm, a derivative of pULI7KS27 (*26*), carries a *comK* gene whose transcription is controlled by an IPTG (Isopropyl-β-D-thiogalactopyranoside) inducible P*spac* promoter, and thus the expression of cloned *comK* gene in the plasmid can be induced independently of ComK itself. Although the intracellular level of ComK protein is drastically increased after the addition of 1 mM IPTG to the wild type strain during early competence development, ComK protein was not observed in the *clpX* mutant even when the transcription of *comK* was induced independently of ComK itself (Fig. 2B). To examine the possibility that the P*spac* promoter switches on rapidly following IPTG addition in the *clpX* mutant, we carried out an S1 protection assay using a *comK* specific RNA probe. The *comK* transcripts in the *clpX* mutant were detected with a slight difference compared with those in the wild type (Fig. 3). Although the intracellular levels of *comK* mRNA in the *clpX* mutant were slightly lower than those in the wild type, these results clearly indicate that ClpX is involved in the regulation of ComK protein at the post-transcriptional level during competence development.

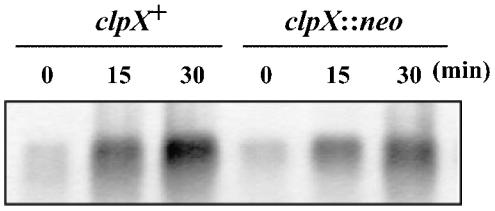

Fig. 3. **S1 protection assay for *comK* mRNA.** *clpX*+ (RIK772) and *clpX*::*neo* (RIK774) mutant strains carrying pULI7KEm were grown in CI medium at 37°C with shaking and IPTG was added to the culture at a final concentration of 1 mM when OD600nm reached *ca.* 0.2 as the zero time. Cells were collected at the indicated times after the addition of IPTG. The SI protection assay was performed as described in "MATERIALS AND METHODS."

To examine whether *comK* mRNA in the *clpX* mutant is degraded at a higher rate than that in the wild type strain, an S1 protection assay was carried out to determine the relative stability of *comK* mRNA in the wild

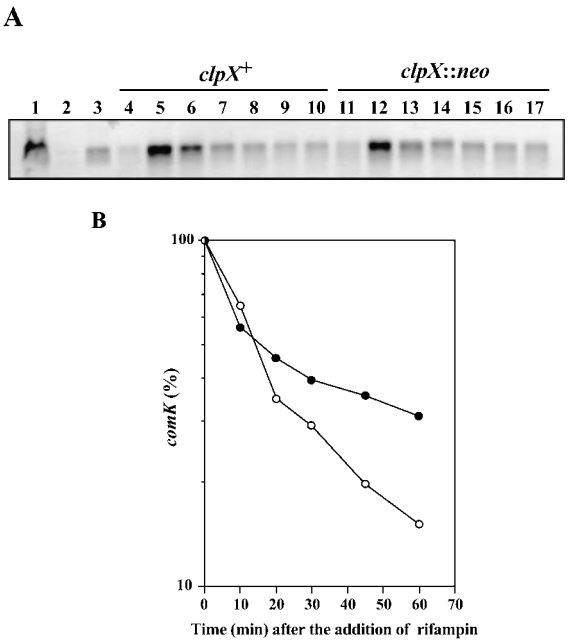

Fig. 4. **Relative stability of *comK* mRNA in the *clpX* mutant.** (A) S1 protection assay for the half-lives of *comK* mRNA in *clpX*+ (RIK772, lanes 4–10) and *clpX*::*neo* (RIK774, lanes 11–17) mutant strains. Strains carrying pULI7KEm were grown in CI medium at 37°C with shaking, and IPTG was added to the culture at a final concentration of 1 mM when $OD_{600\ nm}$ reached ca. 0.2 (lanes 4 and 11). After further incubation for 30 min, rifampin was added to the culture at a final concentration of 10 μg/ml to inhibit transcription (lanes 5 and 12). Samples were collected 10 min (lanes 6 and 13), 20 min (lanes 7 and 14), 30 min (lanes 8 and 15), 45 min (lanes 9 and 16), and 60 min (lanes 10 and 17) after the addition of rifampin and subjected to the S1 protection assay as described in the legend to Fig. 3. As controls, equal concentrations of *comK* probe were mixed with 15 μg of *Escherichia coli* tRNA (Sigma), and samples were loaded after incubation at 37°C for 60 min with (lane 2) or without (lane 1) S1 nuclease. To visualize the position of *comK* mRNA on the image more easily, diluted (1:50) *comK* probe was loaded on lane 3. (B) Relative stability of *comK* mRNA in *clpX*+ and *clpX*::*neo* (mutant strains. Quantification of the relative amounts of *comK* mRNA was done using NIH Image software. The ordinate shows the percentage *comK* mRNA remaining. Open circles, *clpX*+ (RIK772); closed circles, *clpX*::*neo* (RIK774).

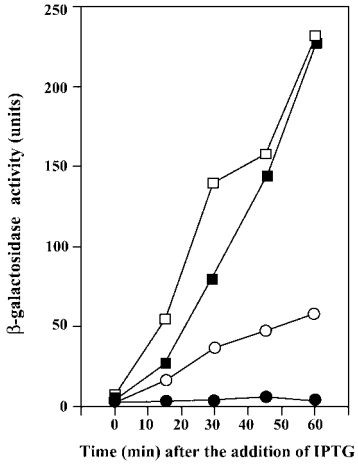

Fig. 5. **Expression of *comK-lacZ* in the *clpX* mutant.** Strains carrying *comK-lacZ* fusions were incubated in CI medium at 37°C with shaking, and IPTG was added to the culture at a final concentration of 1mM when $OD_{600\ nm}$ reached *ca.* 0.2 as the zero time. Cells were collected at the indicated times after the addition of IPTG. Symbols: open squares, RIK778 (*comK1-lacZ clpX*+); closed squares, RIK784 (*comK1-lacZ clpX*::*neo*); open circles, RIK779 (*comK2-lacZ clpX*+); closed circles, RIK785 (*comK2-lacZ clpX*::*neo*).

type and *clpX* mutant strains using a strain carrying pULI7KEm. As shown in Fig. 4A, similar band images were observed in both *clpX*+ and *clpX*– cells after the addition of rifampin, although the induction of *comK* in the *clpX* mutant seemed to be lower than that in the wild type. Our densitometry analysis using NIH Image software showed that the half-life of *comK* turnover was about 15 min in both strains (Fig. 4B). However, *comK* transcripts in the *clpX* mutant seemed to be less stable than those in the wild type before 15 min and more stable after 15 min. These profiles were consistent with several individual examinations (data not shown). From these results, a slightly more rapid degradation rate of *comK* transcripts in the *clpX* mutant at the initial periods after the addition of rifampin might be the cause for the slightly lower expression of *comK*. Although we can not at present fully explain why the decay curve of *comK* transcripts in the *clpX* mutant is strikingly biphasic, these results imply that ClpX is required for the accumulation of ComK protein by involvement in translational events and/or post-translational events.

Effect of the clpX Mutation on the Expression of comK-lacZ Translational Fusion—It has been shown that the proteolysis of ComK is regulated by the MecA-ClpCP protease complex (*5*, *6*). The possibility was considered that the ComK protein might be rapidly degraded in the *clpX* mutant due to the overexpression of proteins that comprise the MecA-ClpCP complex. We, therefore, examined the intracellular levels of ClpC and ClpP proteins in the *clpX* mutant during competence development by Western blot analysis, and found no significant difference from that in the wild type (data not shown). These results, together with the fact that intracellular levels of MecA protein in the *clpX* mutant are almost the same as those in the wild type (*6*), indicate that a *clpX* mutation has no effect on the accumulation of the ClpCP-MecA complex.

Our results described above suggest another possibility that ComK protein synthesis could be inhibited in the *clpX* mutant. Therefore, we constructed two *comK-lacZ* translational fusions at the *aprE* site of the chromosome (Fig. 1) to examine whether ClpX affects ComK synthesis. The transcription of these fusions was controlled by an IPTG inducible P*spac* promoter, and thus the expression of the fused *comK-lacZ* gene could be induced independently of ComK itself. By using this system, we monitored *comK-lacZ* directed β-galactosidase activity in the *clpX*+ and *clpX* mutant strains. As shown in Fig. 5, the expression of *comK1-lacZ* (containing –32 to +219 of the *comK* gene) directed β-galactosidase activity in the *clpX* mutant was highly induced, although its level was slightly lower than that in the wild type after the addition of IPTG. On the other hand, the expression of *comK2-lacZ* (containing –32 to +576 of the *comK* gene) directed β-galactosidase activity after the addition of 1 mM IPTG was almost completely abolished in the *clpX*

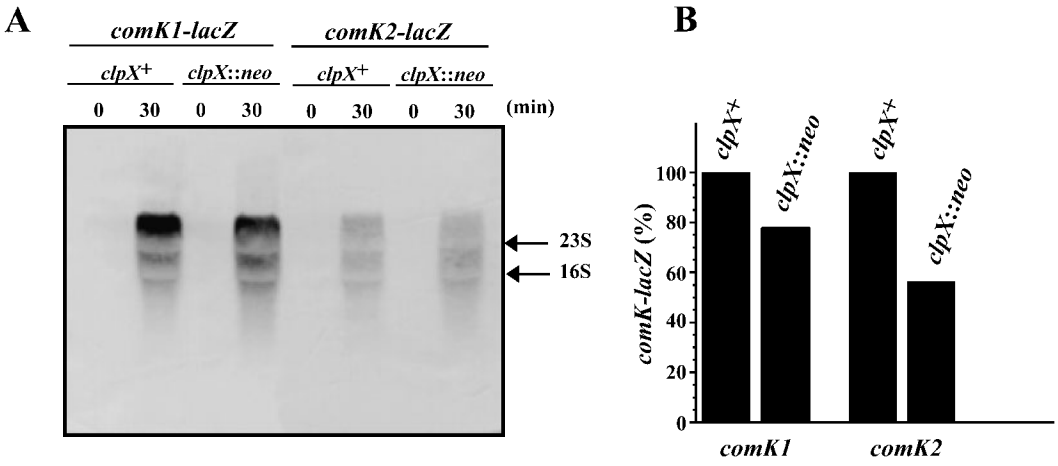

Fig. 6. **Effect of the *clpX* mutation on the accumulation of *comK-lacZ* transcripts and ComK-LacZ fusion proteins.** (A) Northern blot analysis of *comK-lacZ* fusions. Cells were grown in CI medium at 37°C with shaking, and IPTG was added to the culture at a final concentration of 1 mM when $OD_{600\ nm}$ reached *ca.* 0.2 as the zero time. Cells were then collected at 0 and 30 min after the addition of IPTG. Experimental procedures using a *lacZ*-specific probe were carried out as described in "MATERIALS AND METHODS." The arrows indicate the position of 23S rRNA and 16S rRNA. (B) Relative amounts of *comK-lacZ* transcripts. Quantification of the relative amounts of *comK-lacZ* transcripts was done using NIH Image software. The values presented are percentages of the level of *comK-lacZ* transcripts after the addition of IPTG, where the signal intensity of *comK-lacZ* transcripts observed in the wild type was defined as 100%.

mutant whereas its expression in the wild type strain was significantly induced.

To examine further the effect of the *clpX* mutation on the expression of *comK-lacZ* fusions, northern blot analysis of *comK-lacZ* transcripts was performed on the *clpX*+ and *clpX* mutant strains. As shown in Fig. 6A, the *comK1-lacZ* transcripts could be highly induced in both the *clpX*+ and *clpX* mutant strains. Our densitometry analysis showed that the relative amount of *comK1-lacZ* transcript after the addition of IPTG in the *clpX* mutant was 77.8% of that in the wild type (Fig. 6B). These results agree with those obtained in the experiments on the expression of *comK1-lacZ* activity shown in Fig. 5. On the other hand, the *comK2-lacZ* transcripts in the *clpX* mutant after the addition of IPTG was accumulated to be about half of that in the *clpX*+ background (Fig. 6, A and B). These results strongly suggest that the accumulation of the ComK2-LacZ fusion protein is affected in the *clpX* mutant, since little *comK2-lacZ* directed β-galactosidase activity was found in the *clpX* mutant.

In order to clarify this assumption, we next examined the intracellular levels of ComK-LacZ fusion protein by western blot analysis using anti-LacZ antibody. As shown in Fig. 7A, an accumulation of the ComK1-LacZ fusion protein, covering 73 amino acids (73AA) of the ComK protein, in the *clpX* mutant was detected, but at a slightly lower level than that in the wild type after the addition of IPTG. Our densitometry analysis showed that the relative amount of the ComK1-LacZ fusion protein after the addition of IPTG in the *clpX* mutant was 53.1% of that in the wild type (Fig. 7B). This result is in good agreement with the fact that *comK1-lacZ* directed β-galactosidase activity in the wild type 30 min after the addition of IPTG was about two-fold higher than that in the *clpX* mutant

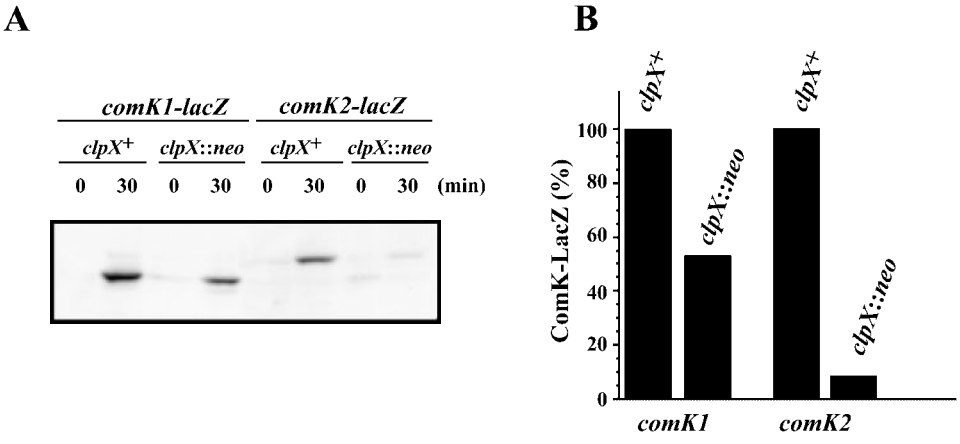

Fig. 7. **Effect of the *clpX* mutation on the accumulation of ComK-LacZ fusion proteins.** (A) Western blot analysis of ComK-LacZ fusion protein. Cell samplings were carried out as described in the legend to Fig. 6. Aliquots of extracts containing 20 μg of total protein were electrophoresed through an SDS–8% (w/v) polyacrylamide minigel. Experimental procedures using anti-LacZ antibody were performed as described in "MATERIALS AND METHODS." (B) Relative amounts of ComK-LacZ fusion proteins. Quantification of the relative amounts of ComK-LacZ fusion proteins was made using NIH Image software. The values presented are percentages of the level of ComK-LacZ fusion proteins, where the signal intensity of ComK-LacZ fusion proteins observed in the wild type was defined as 100%.

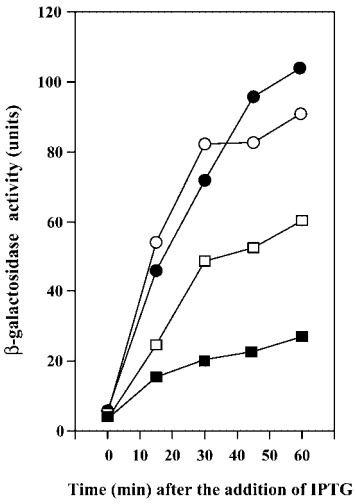

Fig. 8. **Identification of the *comK* gene region affecting ComK accumulation in the ClpX mutant.** Strains carrying *comK-lacZ* fusions were incubated in CI medium at 37°C with shaking, and IPTG was added to the culture at a final concentration of 1 mM when $OD_{600\ nm}$ reached *ca.* 0.2 as the zero time. Cells were collected at the indicated times after the addition of IPTG. Symbols: open circles, RIK789 (*comK3-lacZ clpX*+); closed circles, RIK793 (*comK3-lacZ clpX*::*neo*); open squares, RIK790 (*comK4-lacZ clpX*+); closed squares, RIK794 (*comK4-lacZ clpX*::*neo*).

(Fig. 5). However, the accumulation of ComK2-LacZ fusion protein, which covers the entire ComK protein (192AA), in the *clpX* mutant was much lower than that in the wild type after the addition of IPTG (Fig. 7A). The relative amount of the ComK2-LacZ fusion protein in the *clpX* mutant was 8.7% that in the wild type (Fig. 7B). These results, together with those shown in Fig. 5 and Fig. 6, suggest that the accumulation of the ComK protein is severely inhibited in the *clpX* mutant when the transcripts contain 357 nucleotides, covering between +220 and +576 of the *comK* coding sequence.

To examine further the requirement of the *comK* gene region for the accumulation of ComK protein in the *clpX* mutant, we constructed *comK3-* and *comK4-lacZ* fusions, which contain –32 to +321 (*comK3*) and –32 to +369 (*comK4*) of the *comK* gene, respectively. As shown in Fig. 8, the expression of *comK3-lacZ* directed β-galactosidase activity was highly induced in both *clpX*+ and *clpX* mutant strains after the addition of IPTG. On the other hand, the expression of *comK4-lacZ* directed β-galactosidase activity in the wild type was about two- or three-fold higher than that in the *clpX* mutant 30 min after the addition of 1 mM IPTG. These results suggest that the 48 nucleotides covering +322 and +369 of the *comK* coding sequence, affect the accumulation of the ComK protein in the *clpX* mutant.

DISCUSSION

It has been shown recently that many genes or operons, whose dependenies on ComK were previously unknown, can be identified using the DNA microarray technique (*33*, *34*). These newly identified ComK-regulated genes are not only required for DNA uptake, but also are involved in DNA repair, the uptake and utilization of nutritional sources, and the formation of cell shape (*33*, *34*). *B. subtilis* cells can develop the ability to transform when cells enter the stationary phase in a specific competent medium; however only 5–10% of the cells become competent. Moreover, it has been reported that ComK is expressed in about 10% of cells, and ComK-expressing cells are arrested with respect to growth and cell division (*35*). From these results, it has been proposed that ComK regulates the pathways for adapting to the growth-arrested state, the so-called K-state (*34*). Therefore, it seems reasonable that the expression and activation of ComK is strictly controlled by multiple regulatory mechanisms as reported previously (for review, see Refs. *33* and *34*).

In this study, we constructed *comK-lacZ* translational fusions to examine the effect of the *clpX* mutation on the accumulation of ComK protein. We found that the expression, as monitored by β-galactosidase activity and the accumulation of the ComK2-LacZ fusion protein, of *comK2-lacZ*, which contains the whole *comK* sequence from –32 to +576, was markedly decreased in the *clpX* mutant cells as compared with wild-type cells. However, the IPTG-induced level of the *comK2-lacZ* transcript in the *clpX* mutant was half that in the wild-type strain. On the contrary, the transcription, translation and activity of *comK-lacZ* were all detected in both the *clpX* mutant and the wild type when the *comK-lacZ* fusion lacking the 357 bp of the 3′region of the *comK* gene was induced. These results suggest that the region covering +220 to +576 of the *comK* gene is required for the ClpX dependence of ComK accumulation. These results also provide the first evidence that ClpX is required for the production of ComK at the post-transcriptional level.

At least three possibilities were considered for the post-transcriptional regulation of *comK* in the *clpX* mutant. First, the initiation of ComK translation might be severely blocked. Second, the translation elongation cycle might be blocked in the *clpX* mutant. Finally, the newly synthesized ComK protein might be rapidly degraded in the *clpX* mutant. Although we do not have any direct evidence to clarify these possibilities at present, we showed that the region covering +322 and +369 of the *comK* coding sequence is required for the down-regulation of the accumulation of ComK protein in the absence of ClpX. This region would act as an important signal for the post transcriptional regulation of *comK* in the *clpX* background.

Our results provide evidence for the involvement of the ClpX protein in the post-transcriptional regulation of ComK protein synthesis. Further studies are, however, clearly needed to elucidate the mechanisms of the post-transcriptional regulation of *comK* by ClpX during competence development.

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant provided by Takano Life Science Research Foundation. H.N. was supported by a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists. We are grateful to Roy H. Doi and Abraham L. Sonenshein for helpful discussion and critical reading of the manuscript.

REFERENCES

1. Dubnau, D. (1999) DNA uptake in Bacteria. *Annu. Rev. Microbiol.* **53**, 217–244
2. Solomon, J.M. and Grossman, A.D. (1996) Who's competent and when: Regulation of natural genetic competence in bacteria. *Trends Genet.* **12**, 150–155
3. van Sinderen, D., Luttinger, A., Kong, L., Dubnau, D., Venema, G., and Hamoen, L. (1995) *comK* encodes the competence transcription factor (CTF), the key regulatory protein for competence development in *Bacillus subtilis*. *Mol. Microbiol.* **15**, 455–462
4. Hahn, J., Luttinger, A., and Dubnau, D. (1996) Regulatory inputs for the synthesis of ComK, the competence transcription factor of *Bacillus subtilis*. *Mol. Microbiol.* **21**, 763–775
5. Turgay, K., Hamoen, L.W., Venema, G., and Dubnau. D. (1997) Biochemical characterization of a molecular switch involving the heat shock protein ClpC, controls the activity of ComK, the competence transcription factor of *Bacillus subtilis*. *Genes Dev.* **11**, 119–128
6. Turgay, K., Hahn, J., Burghoorm, J., and Dubnau, D. (1998) Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *EMBO J.* **17**, 6730–6738
7. Hamoen, L.W., Eshuis, H., Jongbloed, J., Venema, G., and van Sinderen, D. (1995) A small gene, designated *comS*, located within the coding region of the fourth amino acid-activation domain of *srfA*, is required for competence development in *Bacillus subtilis*. *Mol. Microbiol.* **15**, 55–63
8. Grossman, A.D. (1995) Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. *Annu. Rev. Genet.* **29**, 477–508
9. Dubnau, D. (1993) Genetic exchange and homologous recombination in *Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry*, *Physiology*, *and Molecular Genetics* (Sonenshein, A.L., Hoch, J.A., and Losick, R., eds.) pp. 555–584, American Society for Microbiology, Washington, DC
10. Haijema, B.J., van Sinderen, D., Winterling, K., Kooistra, J., Venema, G., and Hamoen, L.W. (1996) Regulated expression of the *dinR* and *recA* genes during competence development and SOS induction in *Bacillus subtilis*. *Mol. Microbiol.* **22**, 75–85
11. Hamoen, L.W., van Werkhoven, A.F., Bijlsma, J.J.E., Dubnau, D., and Venema, G. (1998) The competence transcription factor of *Bacillus subtilis* recognizes short A/T -rich sequences arranged in a unique, flexible pattern along the DNA helix. *Genes Dev.* **12**, 1539–1550
12. Dubnau, D. (1997) Binding and transport of transforming DNA by *Bacillus subtilis*: the role of type-IV pilin-like proteins—a review. *Gene* **192**, 191–198
13. Hamoen, L.W., Van Werkhoven, A.F., Venema, G., and Dubnau, D. (2000) The pleiotropic response regulator DegU functions as a priming protein in competence development in *Bacillus subtilis*. *Proc. Natl Acad. Sci. USA* **97**, 9246–9251
14. Ogura, M., Ohshiro, Y., Hirao, S., and Tanaka, T. (1997) A new *Bacillus subtilis* gene *med*, encodes a positive regulator of *comK*. *J. Bacteriol*. **179**, 6244–6253
15. Ogura, M., Hashimoto, H., and Tanaka, T. (2002) Med, a cell-surface localized protein regulating a competence transcription factor gene, *comK*, in *Bacillus subtilis. Biosci. Biotechnol. Biochem.* **66**, 892–896
16. Serror, P. and Sonenshein, A.L. (1996) CodY is required for nutritional repression of *Bacillus subtilis* genetic competence. *J. Bacteriol.* **178**, 5910–5915
17. Gottesman, S., Wickner, S., and Maurizi, M.R. (1997) Protein quality control: triage by chaperones and proteases. *Genes Dev.* **11**, 815–823
18. Wawrzynow, A., Banecki, B., and Zylicz, M. (1996) The Clp ATPases define a novel class of molecular chaperones. *Mol. Microbiol.* **21**, 895–899
19. Msadek, T., Dartois, V., Kunst, F., Herbaud, M.-H., Denizot, F., and Rapoport, G. (1998) ClpP of *Bacillus subtilis* is required for competence development, motility, degradative enzyme synthesis, growth at high temperature and sporulation. *Mol. Microbiol.* **27**, 899–914
20. Nakano, M.M., Zhu, Y., Liu, J., Reyes, D.Y., Yoshikawa, H., and Zuber, P. (2000) Mutations conferring amino acid residue substitutions in the carboxy-terminal domain of RNA polymerase α can suppress *clpX* and *clpP* with respect to developmentally regulated transcription in *Bacillus subtilis*. *Mol. Microbiol.* **37**, 869–884
21. Gérth, U., Krüger, E., Derré, I., Msadek, T., and Hecker, M. (1998) Stress induction of the *Bacillus subtilis clpP* gene encoding a homologue of the proteolytic component of the Clp protease and the involvement of ClpP and ClpX in stress tolerance. *Mol. Microbiol.* **28**, 787–802
22. Liu, J., Cosby, W.M., and Zuber, P. (1999) Role of Lon and ClpX in the post-transcriptional regulation of a sigma subunit of RNA polymerase required for cellular differentiation in *Bacillus subtilis*. *Mol. Microbiol.* **33**, 415–428
23. Yamashita, S., Yoshikawa, H., Kawamura, F., Takahashi, H., Yamamoto, T., Kobayashi, Y., and Saito, H. (1986) The effect of *spo0* mutations on the expression of *spo0A*- and *spo0F-lacZ* fusions. *Mol. Gen. Genet.* **205**, 28–33
24. Itaya, M., Kondo, K., and Tanaka, T. (1989) A neomycin resistance gene cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. *Nucleic Acids Res.* **17**, 4410
25. Yamada, K. (1989) Ph. D. thesis. Hiroshima University, Hiroshima, Japan
26. Ashikaga, S., Nanamiya, H., Ohashi, Y., and Kawamura, F. (2000) Natural genetic competence in *Bacillus subtilis* Natto OK2. *J. Bacteriol*. **182**, 2411–2415
27. Morimoto, T., Loh, P.C., Hirai, T., Asai, K., Kobayashi, K., Moriya, S., and Ogasawara, N. (2002) Six GTP-binding proteins of the Era/Obg family are essential for cell growth in *Bacillus subtilis*. *Microbiology* **148**, 3539–3552
28. Shapira, S.K., Chou, J., Richaud, F.V., and Casadaban, M.J. (1983) New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to *lacZ* gene sequences encoding an enzymatically active carboxy-terminal portion of beta-galactosidase. *Gene* **25**, 71–82
29. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Nanamiya, H., Ohashi, Y., Asai, K., Moriya, S., Ogasawara, N., Fujita, M., Sadaie, Y., and Kawamura, F. (1998) ClpC regulates the fate of a sporulation initiation sigma factor, σ^H protein, in *Bacillus subtilis* at elevated temperatures. *Mol. Microbiol.* **29**, 505–513
31. Chibazakura, T., Kawamura, F., and Takahashi, H. (1991) Differential regulation of *spo0A* transcription in *Bacillus subtilis*: glucose represses promoter switching at the initiation of sporulation. *J. Bacteriol*. **173**, 2625–2632
32. van Sinderen, D. and Venema, G. (1994) *comK* acts as an autoregulatory control switch in the signal transduction route to competence in *Bacillus subtilis*. *J. Bacteriol*. **176**, 5762–5770
33. Ogura, M., Yamaguchi, H., Kobayashi, K., Ogasawara, N., Fujita, Y., and Tanaka, T. (2002) Whole-genome analysis of genes regulated by the *Bacillus subtilis* competence transcription factor ComK. *J. Bacteriol*. **184**, 2344–2351
34. Berka, R.M., Hahn, J., Albano, M., Draskovic, I., Persuh, M., Cui, X., Sloma, A., Widner, W., and Dubnau, D. (2002) Microarray analysis of the *Bacillus subtilis* K-state: genome-wide expression changes dependent on ComK. *Mol. Microbiol.* **43**, 1331–1345
35. Haijema, B.-J., Hahn, J., Haynes, J., and Dubnau, D. (2001) A ComGA-dependent checkpoint limits growth during the escape from competence. *Mol. Microbiol.* **40**, 52–64