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Multiple Pathways of Spx (YjbD) Proteolysis in *Bacillus subtilis*

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ATP-dependent proteases degrade denatured or misfolded proteins and are recruited for the controlled removal of proteins that block activation of regulatory pathways. Among the ATP-dependent proteases, those of the Clp family are particularly important for the growth and development of *Bacillus subtilis*. Proteolytic subunit ClpP, together with regulatory ATPase subunit ClpC or ClpX, is required for the normal response to stress, for development of genetic competence, and for sporulation. The *spx* (formally *yjbD*) gene was previously identified as a site of mutations that suppress defects in competence conferred by *clpP* and *clpX*. The level of Spx in wild-type cells grown in competence medium is low, and that in *clpP* mutants is high. This suggests that the Spx protein is a substrate for ClpP-containing proteases and that accumulation of Spx might be partly responsible for the observed pleiotropic phenotype resulting from the *clpP* mutation. In this study we examined, both in vivo and in vitro, which ClpP protease is responsible for degradation of Spx. Western blot analysis showed that Spx accumulated in *clpX* mutant to the same level as that observed in the *clpP* mutant. In contrast, a very low concentration of Spx was detected in a *clpC* mutant. An in vitro proteolysis experiment using purified proteins demonstrated that Spx was degraded by ClpCP but only in the presence of one of the ClpC adapter proteins, MecA or YpbH. However, ClpXP, either in the presence or in the absence of MecA and YpbH, was unable to degrade Spx. Transcription of *spx*, as measured by expression of *spx-lacZ*, was slightly increased by the *clpX* mutation. To exclude a possible effect of *clpX* and *clpP* on *spx* transcription, the *spx* gene was placed under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible P*spac* promoter. In this strain, Spx accumulated when ClpX or ClpP was absent, suggesting that ClpX and ClpP are required for degradation of Spx. Taken together, these results suggest that Spx is degraded by both ClpCP and ClpXP. The putative proteolysis by ClpXP might require another adapter protein. Spx probably is degraded by ClpCP under as yet unidentified conditions. This study suggests that the level of Spx is tightly controlled by two different ClpP proteases.

ATP-dependent proteases play an important role in many cellular processes in both prokaryotes and eukaryotes (reviewed in reference 47). They function in the cell's response to stress by eliminating damaged or misfolded proteins and degrading truncated products of aborted translation. They are also involved in degradation of specific, short-lived regulatory proteins, many of which function in developmental processes. Members of one class of ATP-dependent proteases, the Clp proteases, are composed of two subunits, a regulatory ATPase component and the peptidase, ClpP (reviewed in reference 37). While ClpP alone exhibits peptidase activity, its association with the ATPase imparts substrate specificity and is required for the degradation of polypeptides longer than six amino acids (41). The Clp ATPase hexamers bind to and unfold substrates and then translocate them to the two rings of ClpP heptamers (16, 40). Clp ATPases, in the presence or absence of ClpP, also function as molecular chaperones (reviewed in reference 12). For example, ClpX chaperone activity is necessary for the dissociation of the MuA complex following strand transfer during Mu transposition (23). In *Escherichia coli*, ClpAP degrades RepA (46), bacteriophage P1 protein (22), and the MazE protein (1), which is involved in programmed cell death. ClpXP degrades MuA transposase (23), λ O (49), and the stationary-phase σ factor RpoS (39). Proteins from the translation of truncated mRNA are targeted for the addition of 11-residue peptides to the carboxy termini, a process which is mediated by SsrA RNA (also called 10Sa RNA or tm RNA) (42). The SsrA-tagged proteins are degraded by ClpAP and ClpXP (13).

In *Bacillus subtilis*, Clp proteases include those bearing the ClpC, ClpE, and ClpX ATPase subunits (reviewed in reference 38). Unlike *E. coli clpP* and *clpX* mutants, which grow normally (39), the *B. subtilis clp* mutants are highly pleiotropic and exhibit poor growth phenotypes on certain media such as minimal medium and Luria-Bertani (LB) medium even under nonstress conditions (10, 26, 31). Immunocytochemical experiments showed that ClpCP and ClpXP are directly involved in degradation of misfolded or denatured proteins after either heat shock or treatment with puromycin (20). These treatments also induce transcription of *clpC*, *clpP*, and *clpX* (10, 11, 19, 26, 27) and lead to increased ClpC and ClpP protein levels (20). ClpC, ClpP, and ClpX are essential for growth at high temperature following heat shock induction (10, 19, 26, 27). Other common features observed in the *clpC*, *clpP*, and *clpX* mutants include a salt-sensitive and nonmotile phenotype (10, 19, 26). No obvious phenotype which is associated with the *clpE* mutation has been found so far (5). Recently, ClpXP was shown to be responsible for degradation of SsrA-tagged proteins in *B. subtilis* cells (48).

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In addition to their growth phenotype and sensitivity to stress, mutants lacking ClpC, ClpP, or ClpX show defects in developmental pathways including sporulation and competence (10, 26, 31, 32). A *clpP* null mutation confers the most severe sporulation defect, followed, in order of decreasing severity, by the *clpC* and *clpX* mutations. The sporulation phenotype of the *clpC* mutation appeared more obvious at high temperature (32), while a mutation in *clpE*, encoding another ATPase subunit, produces no sporulation phenotype (5). Since ClpP is thought only to function with Clp ATPases, it is not clear why mutations in *clpX*, *-C*, and *-E* have little or no sporulation phenotype. Perhaps the ATPases perform redundant functions during the sporulation process. That ClpP is necessary for entry into sporulation is supported by the finding that Spo0A-dependent expression of *spoIIA* and *spoIIG* operons, which encode σ^F and σ^E, respectively, was adversely affected by a *clpP* mutation (26). This result suggests that the phosphorelay leading to the phosphorylation of Spo0A is defective in the *clpP* mutant. In fact, a *spo0E* mutation partially suppresses *clpP*; that is, Spo0A-dependent gene expression is observed in a *spo0E clpP* mutant background but sporulation was not restored (33). The *clpC* mutation did not affect Spo0A-dependent expression of *spoIIA* and *spoIIE*; however, the mutation caused an impairment of σ^F-dependent gene expression (35). Apparently ClpCP is required after phosphorylation of Spo0A and before activation of σ^F. A recent study demonstrated that ClpCP increased σ^F activity by directly degrading SpoIIAB and anti-σ^F factor and by indirectly promoting polar septum formation, which is a prerequisite for the activation of σ^F (35). ClpCP is also involved in the degradation of σ^H, an RNA polymerase sigma subunit required for sporulation initiation, at high temperature and might be responsible for the degradation of σ^H after it accomplishes its task in early sporulation (32). Finally ClpX, but not ClpP, is required for σ^H-RNA polymerase activity in vitro and in vivo (25). Thus the role of the ClpXP protease in sporulation, if any, is elusive.

Although ClpC, ClpP, and ClpX are involved in competence development, the effect of *clpC* is opposite to that of *clpP* and *clpX*. Both *clpP* and *clpX* mutants are defective in competence (26, 31), while the *clpC* mutant shows a *mec* (medium-independent expression of competence) phenotype (27), indicating a negative role for ClpC. ClpC forms a complex with MecA (18) and ComK (45) in noncompetent cells; this complex sequesters transcription factor ComK in an inactive state (44). Thus, ClpC negatively regulates *comK*-dependent expression of *comK* and late competence genes. In addition, ComK is degraded by ClpCP, and the proteolysis requires adapter protein MecA (43). The small peptide ComS (7, 14), whose synthesis is activated by a quorum-sensing pathway, accumulates in stationary phase and releases ComK from the inhibitory complex (44). Liberated ComK is no longer a target of ClpCP; instead MecA and ComS are degraded by ClpCP (43). Two additional roles for ClpP in competence have been found. ClpP, together with ClpX, is required for *srf* (*comS*) expression (31). Amino acid substitutions in the carboxy-terminal domain of the α subunit of RNA polymerase suppress either *clpX* or *clpP* with respect to *comS* expression (31). Although the exact role of ClpXP in *comS* expression remains to be elucidated, a direct involvement of ClpXP in *comS* transcription was suggested. Furthermore ClpP is also needed to degrade Spx (YjbD), the accumulation of which negatively affects competence and sporulation (28). The *clpP spx* double mutant completely and partially restored competence and sporulation, respectively. In this study, we examined how the level of Spx is regulated by ClpP proteases and found that both ClpCP and ClpXP are involved in degrading Spx, probably under different conditions.

MATERIALS AND METHODS

Bacterial strains and media. All *B. subtilis* strains listed in Table 1 are derivatives of JH642. Construction of LAB2876 (*clpX*::*spc*) (25), LAB2972 (*clpP*::*erm*) (34), and ORB3834 (*spx*::*neo*) (28) was described previously. ORB3976 was constructed by transforming JH642 with chromosomal DNA prepared from QPB418 (*clpC*::*tet*) (35).

A strain which carries the *spx* coding sequence downstream of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible P*spac* promoter was constructed as follows. Primers oMN01-173 (5′-CGAGGAAGCTTAGATGTTCA TCCTACTA-3′) and oMN01-174 (5′-TACCAGCAGGTCGACAAATAAAAG AAGG-3′) were used to amplify the *spx* gene by using JH642 chromosomal DNA as the template. The PCR product was digested with *Hin*dIII and *Sal*I and ligated with pDR66 digested with the same enzymes. After the *spx* sequence was verified, the resultant plasmid, pMMN497, was used to transform JH642 to generate ORB4075. ORB4075 was generated by integration of pMMN497 at the *amyE* locus by double-crossover recombination and screening for the Amy$^-$ (amylase-negative) phenotype. ORB4078 was constructed by transforming OEB4075 with chromosomal DNA prepared from LAB3834 (*spx*::*neo*). The insertion of the *neo* marker into *spx* located at the original locus and not into P*spac-spx* located at *amyE* was confirmed by testing the genetic linkage between the chloramphenicol resistance (Cm^r; derived from pMMN497) and neomycin resistance (Neo^r) markers. ORB4079, ORB4080, and ORB4081 were constructed by transforming ORB4078 with LAB2876 (*clpX*::*spc*), LAB2972 (*clpP*::*erm*), and ORB3976 (*clpC*::*tet*) DNA, respectively.

B. subtilis cells were grown in rich media, 2× yeast extract-tryptone (YT) (29) and LB medium, Difco sporulation medium (DSM) (29), and one-step competence medium (CM) (8). Antibiotics were added to the following concentrations: chloramphenicol, 5 μg/ml; erythromycin plus lincomycin, 1 and 25 μg/ml, respectively; tetracycline, 12.5 μg/ml; spectinomycin, 75 μg/ml; neomycin, 5 μg/ml; ampicillin, 25 μg/ml.

Purification of proteins. For production of proteins used in this study, the IMPACT system (New England BioLabs) (4), which utilizes the inducible self-cleaving intein tag, was used. Overproduction and purification of Spx (28) and ClpX (25) were previously described.

The *clpP* gene was amplified by PCR using primers oclpPex1 (5′-GGAGGA GCCATATGAATTTAAT-3′) and oclpPex2 (5′-ATTAGGAAGAGCCCTTTT TGTCTTCTGTGTGA-3′) and chromosomal DNA prepared from JH642 as the template. The PCR product digested with *Nde*I and *Sap*I was cloned into pTYB1 (New England BioLabs), which had undergone digestion with the same enzymes. The resultant plasmid was digested with *Sap*I, treated with T4 DNA polymerase in the presence of deoxynucleoside triphosphate to fill in the single-stranded end, and self-ligated to generate pClpP.

The *clpC* gene was amplified by PCR using primers oClpCex1 (5′-GGATGA ATCCATATGATGTTTGGAAGATTT-3′) and oClpCex2 (5′-TCCCCCGGG ATTCGTTTTAGCAGTCGTTTT-3′) and JH642 chromosomal DNA as the template. The amplified fragment, after being digested with *Nde*I and *Sma*I, was cloned into pTYB2 (New England BioLabs) digested with the same enzymes to generate pClpC.

The *mecA* gene was amplified by PCR using JH642 chromosomal DNA and two primers, oMN01-156 (5′-GGAAGGTTGGCATATGGAAATTG-3′) and oMN01-157 (5′-GACTTAGCTCTTCCGCATGATGCAAAGTGTTTT-3′). The amplified fragment, after being digested with *Nde*I and *Sap*I, was inserted into pTYB1 (digested with the same enzymes) to construct plasmid pSN3.

The *ypbH* gene was amplified by PCR using JH642 chromosomal DNA and two primers, oMN01-164 (5′-GAAGATCATATGCGGCTTGAGCGT-3′) and oMN01-165 (5′-GCCGCCGCTCTTCCGCATGAAAAATGAGTTTGTA-3′). The amplified fragment was inserted into pTYB1 by using the same enzymes as those used for cloning the *mecA* gene to generate pSN5.

The sequences of all the genes cloned above were verified by DNA sequencing. The *clpC* gene in pClpC has a mutation in codon 795; however, the amino acid (Asp) was not affected by the change, GAT to GAC. *E. coli* ER2566 (New England BioLabs) carrying pClpP, pClpC, or pSN3 was used to produce ClpP,

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

Strain	Relevant genotype or properties	Source and/or reference
B. subtilis strains		
JH642	*trpC2 pheA1*	J. A. Hoch
QPB418	*clpC*::*tet*	35
LAB2876	*trpC2 pheA1 clpX*::*spc*	25
LAB2972	*trpC2 pheA1 clpP*::*erm*	34
ORB3673	*trpC2 pheA1 clpX*::*spc yjbFG*::pPS34	28
ORB3834	*trpC2 pheA1 yjbD*::*neo*	28
ORB3976	*trpC2 pheA1 clpC*::*tet*	This study
ORB4059	*trpC2 pheA1 yjbD-lacZ cat*	This study
ORB4065	*trpC2 pheA1 clpX*::*spc yjbD-lacZ cat*	This study
ORB4075	*trpC2 pheA1 amyE*::P*spac-yjbD cat*	This study
ORB4078	*trpC2 pheA1 yjbD*::*neo amyE*::P*spac-yjbD*	This study
ORB4079	*trpC2 pheA1 yjbD*::*neo clpX*::*spc amyE*::P*spac-yjbD*	This study
ORB4080	*trpC2 pheA1 yjbD*::*neo clpP*::*erm amyE*::P*spac-yjbD*	This study
ORB4081	*trpC2 pheA1 yjbD*::*neo clpC*::*tet amyE*::P*spac-yjbD*	This study
Plasmids		
pDR66	Plasmid allowing IPTG-dependent gene expression	A. Grossman, 15
pLysS	Plasmid to produce T7 lysozyme	Stratagene
pPS34	Integrative plasmid	P. Serror and A. L. Sonenshein, unpublished
pTKlac	Plasmid for construction of *lacZ* transcriptional fusion	17
pTYB1	Cloning vector for IMPACT T7 system	New England BioLabs
pTYB2	Cloning vector for IMPACT T7 system	New England BioLabs
pClpC	pTYB2 with *clpC*	This study
pClpP	pTYB1 with *clpP*	This study
pMMN464	pPS34 carrying *oppB* to *yjbF*	This study
pMMN470	pTYB4 with *yjbD*	28
pMMN497	pDR66 with *yjbD* under control of P*spac*	This study
pSN3	pTYB1 with *mecA*	This study
pSN5	pTYB1 with *ypbH*	This study
pSN16	pTKlac carrying *yjbD-lacZ*	This study

ClpC, and MecA, respectively. *E. coli* BL21(DE3) carrying pLysS (Stratagene) and pSN5 was used for the production of YpbH. The proteins were purified by using a chitin column as recommended by the manufacturer. Purified MecA and YpbH produced by this construct have no extra amino acids. ClpP and ClpC have extra amino acids at their carboxy ends (Gly-Ser-Ser-Tyr for ClpP and Pro-Gly for ClpC).

Purification of the Spx protein in the presence of $CuSO_4$. *E. coli* ER2566 carrying pMMN470 was cultured in 2× YT at 37°C. When the optical density at 600 nm reached 0.4, $CuSO_4$ was added to the culture at the final concentration of 500 μM. The procedure for further purification was described previously (28).

In vitro degradation assay. An in vitro degradation assay was carried out as described previously (43) with slight modification. ClpP (4 μM) and Spx (4 μM) were incubated at 37°C for 0 or 30 min in the presence or absence of MecA (2.5 μM), YpbH (2.5 μM), ClpX (2.5 μM), and ClpC (2.5 μM) in a final volume of 50 μl of reaction buffer (25 mM MOPS [morpholinepropanesulfonic acid]-KOH [pH 7.0], 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 2 mM phosphoenol pyruvate, 0.93 μM pyruvate kinase [Sigma]). Phosphoenol pyruvate and pyruvate kinase were used to regenerate ATP. After the incubation, 10-μl portions of the samples were transferred to 5 μl of stop solution. The proteins were analyzed on a 15% polyacrylamide–sodium dodecyl sulfate (SDS) gel, followed by staining with Coomassie blue.

Western blot analysis. Cells were cultured in CM and harvested at T_2 (2 h after the onset of the stationary phase). Cells were broken by passage through a French press, and whole-cell extracts were used in Western analysis or cell debris was first removed by centrifugation (17,000 × *g* for 15 min) to generate a cleared extract. The protein concentration of each sample was measured with a Bio-Rad protein assay kit. Thirty micrograms of each protein was applied to a 15% polyacrylamide–SDS gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. Immunodetection was performed with the anti-Spx antibody (28) followed by incubation with the secondary antibody conjugated to alkaline phosphatase.

Construction of a transcriptional *spx-lacZ* fusion. *B. subtilis* strain ORB3673, which has plasmid pPS34 inserted into the *yjbFG* region, was constructed in a previous study (28). Chromosomal DNA prepared from ORB3673 was digested with *Apa*I and self-ligated. The ligation mixture was used to transform *E. coli* DH5α with selection for ampicillin resistance (Amp^r). Plasmid pMMN464, isolated from the Amp^r transformant, carries 9.3 kbp of *B. subtilis* chromosomal DNA containing the 3′ ends of *oppB*, *oppCDE*, *yjbBCDE*, and *mecA* and the 5′ end of *yjbF*. pMMN464 was cut with *Bgl*II, and the resulting 4.6-kbp fragment was recovered from a low-melting-point agarose gel. The fragment was then digested with *Bcl*I and *Hae*III sequentially, which produces a 1.4-kbp fragment containing the 3′ end of *yjbB*, the entire *yjbC* gene, and the 5′ end of *spx* (*yjbD*). The fragment was inserted into promoter-probe vector pTK*lac* (17), which was digested with *Sma*I and *Bam*HI to generate pSN16. JH642 was transformed with pSN16, and a Cm^r transformant was selected as ORB4059. ORB4065 was isolated by transforming ORB4059 with chromosomal DNA prepared from LAB2876 (*clpX*::*spc*) with selection for Cm^r and Spc^r.

Assay of β-galactosidase activity. Cells were grown in CM, DSM, or 2× YT, and samples were withdrawn at 30-min or 1-h time intervals during growth. β-Galactosidase activity in each sample was determined as described previously (29) and is presented as Miller units.

RESULTS

Spx accumulates in *clpP* and *clpX* mutants but not in a *clpC* mutant. We have previously shown that Spx concentration was low in wild-type cells grown in CM but markedly higher in a *clpP* mutant (28). This suggests that Spx is a substrate of ClpP protease(s). In *B. subtilis* ATPase subunits ClpC, ClpX, and ClpE are known or thought to function with catalytic subunit ClpP (reviewed in reference 38). To identify which regulatory subunit is involved in degradation of Spx, we first examined the amount of Spx in a *clpX* mutant. The result showed that Spx accumulated in the *clpX* mutant to a level similar to that observed in a *clpP* strain (Fig. 1A). In contrast, Spx concentration in a *clpC* mutant was as low as that in the wild type (Fig. 1B). As was the case for the *clpC* mutant, no increase in Spx concentration was observed in either the *mecA* or *ypbH* mu-

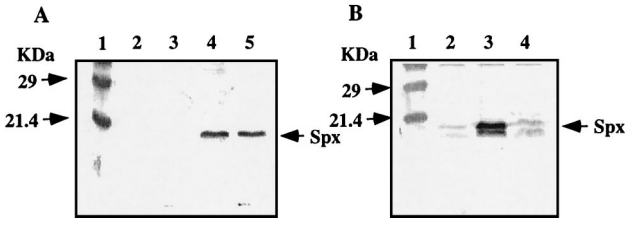

FIG. 1. Effect of mutations on Spx level as measured by Western blot analysis using anti-Spx antiserum. Samples were taken at T_2 (2 h after the onset of the stationary phase) from cells grown in CM. (A) Lane 1, molecular mass markers; lane 2, JH642 (wild type); lane 3, ORB3834 (*spx*::*neo*); lane 4, LAB2972 (*clpP*::*erm*); lane 5, LAB2876 (*clpX*::*spc*). (B) Lane 1, molecular mass markers; lane 2, JH642; lane 3, LAB2972; lane 4, ORB3976 (*clpC*::*tet*).

tant. This result suggested that degradation of Spx is dependent primarily on ClpXP.

Spx is degraded in vitro by ClpCP but not ClpXP. Although the result described above suggests that Spx is a substrate of ClpXP, alternative interpretations, such as the possibility that a regulator of *spx* expression is a target of ClpXP protease, could not be excluded. To investigate whether Spx is degraded by ClpXP, in vitro proteolysis experiments using purified proteins were carried out. We have previously found that Spx binds to copper (30), and Fig. 2 shows the data from experiments using copper-bound Spx, prepared as described in Materials and Methods. However, a similar pattern of proteolysis was observed by using the copper-free Spx protein (data not shown). We also examined the effect of ClpCP protease on Spx degradation, since we observed that Spx could form a complex with the ClpCP adapter, MecA, and could contribute to ComK inhibition (30). Degradation of Spx was observed in the presence of ClpP, ClpC, and MecA (Fig. 2, lanes 3 and 4). MecA, to a lesser extent, was also degraded by ClpCP, as previously reported (43). In the absence of MecA (lanes 1 and 2) or ATP (data not shown), Spx was stable after incubation for 30 min. This indicates that MecA acts as the adapter for ClpCP-catalyzed proteolysis of Spx, as well as for MecA-dependent ComK and ComS proteolysis by ClpCP (43). Under these same conditions, we observe that ComK is degraded by MecA-dependent, ClpCP-catalyzed proteolysis (data not shown), as was shown previously. YpbH is similar in amino acid sequence to MecA and is a possible MecA paralog. Therefore we examined whether YpbH can act as the adapter protein for ClpC. The result showed that Spx was degraded by ClpCP in the presence of YpbH (lanes 5 and 6), indicating that YpbH acts as the adapter for Spx proteolysis by ClpCP. We could not discern whether YpbH itself was degraded by ClpCP because YpbH and ClpP comigrated during SDS-polyacrylamide gel electrophoresis. Surprisingly, Spx was not degraded in vitro in the presence of ClpX, ClpP, and ATP (lanes 7 and 8). Addition of MecA and YpbH did not promote ClpXP-dependent proteolysis (data not shown).

Spx accumulates less in *clpX* and *clpP* cells grown in DSM than in cells grown in CM or 2× YT. Although Spx was degraded by ClpCP in vitro, Spx did not accumulate in the *clpC* mutant grown in CM. However, the *clpC* mutation might affect the concentration of Spx under different conditions. First we examined whether Spx accumulates in *clpC* cells cultured in DSM. The result showed that the level of Spx in the *clpC* strain was as low as that in the wild type (data not shown). In addition, Spx accumulates in the *clpX* and *clpP* mutants, but at a level much lower than those in the same strains grown in CM (Fig. 3A) or 2× YT (data not shown). The band with a molecular weight lower than that of Spx, which may be a degradation product of Spx, was sometimes detected (as shown in lanes 2 and 3).

The *clpX* mutation has no significant effect on *spx* transcription. That Spx is abundant in the *clpX* mutant whereas ClpXP was unable to degrade Spx in vitro might be explained if *clpX* negatively regulates production of Spx at the level of *spx* transcription. To test this possibility, a transcriptional *spx-lacZ* fusion was constructed as described in Materials and Methods and its expression in wild-type and *clpX* cells was examined. β-Galactosidase activity from *spx-lacZ* was expressed constitutively in wild-type cells during exponential and stationary phases of growth in CM. Transcription of *spx* in the *clpX* mutant was slightly affected by the *clpX* mutation (Fig. 4A). However, the increase in the *spx* transcription in the *clpX* mutant was two- to threefold at the most, which cannot fully explain the large difference in Spx level between the wild type and the *clpX* mutant.

We also examined whether the expression of *spx* is medium dependent. Levels of expression of *spx* when cells were grown in CM and DSM were similar, and the expression is higher in stationary-phase cultures grown in 2× YT (Fig. 4B). This result suggests that the smaller amount of Spx in the *clpX* and *clpP* mutant cells grown in DSM than in those grown in CM is attributable to posttranscriptional regulation.

ClpXP is responsible for low levels of Spx in P*spac-spx* cells. The results described above suggested that higher concentrations of Spx in the *clpX* and *clpP* mutants are probably due to loss of proteolysis. To confirm this, *spx* was placed under the

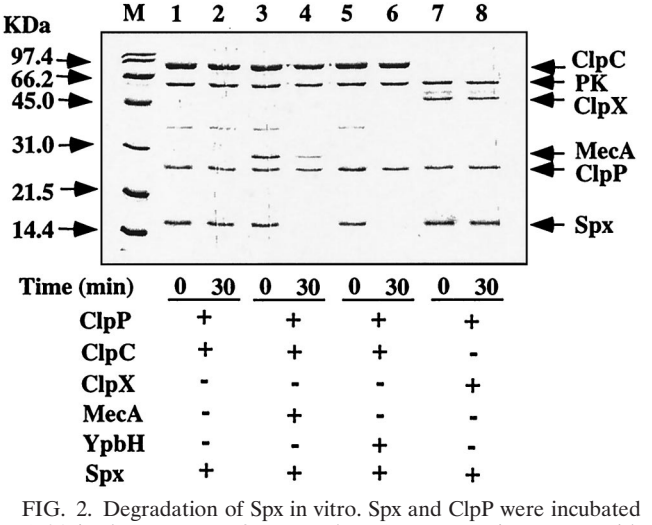

FIG. 2. Degradation of Spx in vitro. Spx and ClpP were incubated at 37°C in the presence of ATP and an ATP-generating system with (+) or without (−) ClpC, ClpX, MecA, and YpbH as described in Materials and Methods. Samples were taken at the times indicated and were analyzed by SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. Lane M, molecular mass markers. PK, pyruvate kinase, used as an ATP-regenerating system.

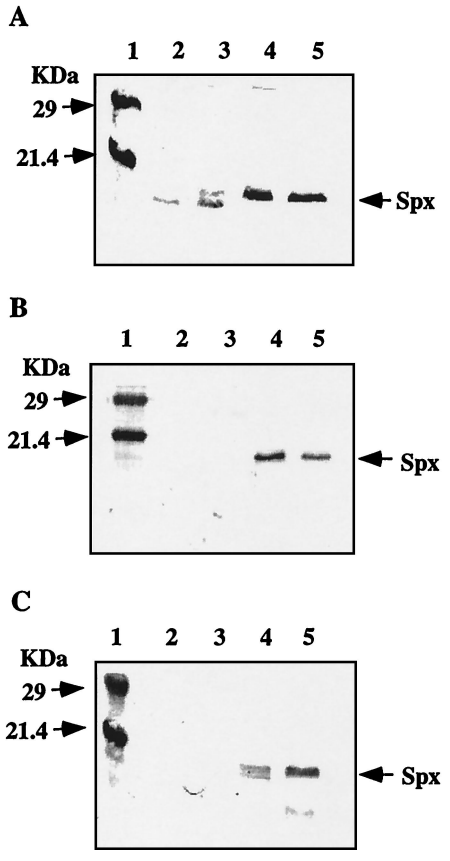

FIG. 3. Western blot analysis using anti-Spx antiserum. (A) Effect of culture media on Spx level as measured by Western blot analysis. Samples were taken at T_2 from cells grown in DSM and in CM. Lane 1, molecular mass markers; lane 2, LAB2876 (*clpX*::*spc*) grown in DSM; lane 3, LAB2972 (*clpP*::*erm*) grown in DSM; lane 4, LAB2876 grown in CM; lane 5, LAB2972 grown in CM. (B) Cells carrying P*spac-spx* were grown in CM in the presence of 1 mM IPTG. Samples were taken at T_2. Lane 1, molecular mass markers; lane 2, ORB4078 (wild type); lane 3, ORB4081 (*clpC*::*tet*); lane 4, ORB4079 (*clpX*::*spc*); lane 5, ORB4080 (*clpP*::*erm*). (C) The wild type and the *clpX* mutant carrying P*spac-spx* were grown in DSM or CM in the presence of 1 mM IPTG. Samples were taken at T_2. Lane 1, molecular mass markers; lane 2, ORB4078 in DSM; lane 3, ORB4078 in CM; lane 4, ORB4079 in DSM; lane 5, ORB4079 in CM.

control of IPTG-inducible promoter P*spac* (50) and the effect of the *clpX*, *clpP*, and *clpC* mutations on the amount of Spx was examined. In these strains, P*spac-spx* was integrated at the *amyE* locus and the original *spx* gene was mutated by insertion of a Neo^r gene cassette. When P*spac-spx* strains with or without the *clp* mutations were grown in CM in the presence of 1 mM IPTG, Spx proteins were hardly detected in the wild type and the *clpC* mutant (Fig. 3B). In contrast, the amounts of Spx in the *clpX* and *clpP* mutant strains were dramatically increased. From these results we concluded that ClpXP is required for Spx degradation. The Spx level in the P*spac-spx clpX* mutant was higher when cells were grown in CM than when they were grown in DSM (Fig. 3C). However, the Spx concentration was significantly elevated even in the DSM cultures in the absence of *clpX*. This result suggests that both ClpP and another protease(s) are responsible for the degradation of Spx in *B. subtilis* cells grown in DSM.

DISCUSSION

Spx is highly conserved in gram-positive bacteria and was identified as the site of *clpP* suppressor mutations in *Lactococcus lactis* (9) and of *clpX* (and *clpP*) mutations in *B. subtilis* (28). The accumulated Spx in *B. subtilis* is harmful to cell growth on certain media and to developmental pathways such as genetic competence and sporulation. This study indicated that the concentration of Spx is controlled by multiple pathways of ClpP-dependent proteolysis.

Western blot analysis in a previous paper (28) and in this study indicated that Spx markedly accumulated in the *clpP* and *clpX* mutants grown in CM or 2× YT. Using the P*spac-spx* construct, we demonstrated that the amount of Spx is regulated either by ClpXP-dependent proteolysis or, less likely, by an effect on *spx* translation that is indirectly influenced by ClpXP. The poor growth of the *clpX* and *clpP* mutants on minimal media (such as CM) and rich media (such as LB medium and 2× YT) is caused by the increased amount of Spx under these growth conditions. In contrast, the *clpX* and *clpP* mutants grow relatively well on DSM, where Spx accumulates to considerably lower levels. The growth of *clpP spx* and *clpX spx* mutants on CM and LB medium is similar to that of the wild type. Furthermore the *clpX* and *clpP* mutants carrying the P*spac-spx* construct grew well on CM and LB medium in the absence of IPTG; however, growth was severely reduced in the presence of IPTG (data not shown). The growth of the P*spac-spx clpC* strain was not affected by IPTG. Taken together, these results are consistent with the hypothesis that accumulation of Spx is unfavorable to normal cell growth. It also indicates that the level of Spx in the *clpP* strain is not high enough to affect growth in DSM but is sufficient to inhibit sporulation because the *spx* mutation in the *clpP* background only partially restored sporulation (28). Our results also suggested that both ClpP and another protease(s) are involved in the degradation of Spx in DSM cultures.

Although Western blot analysis clearly showed that both *clpX* and *clpP* are responsible for maintaining low Spx concentrations, Spx is not degraded by ClpXP in vitro. In fact, at present, there is no experimental evidence demonstrating that *B. subtilis* ClpXP is proteolytically active in vitro, although in vivo studies have suggested various proteins as substrates.

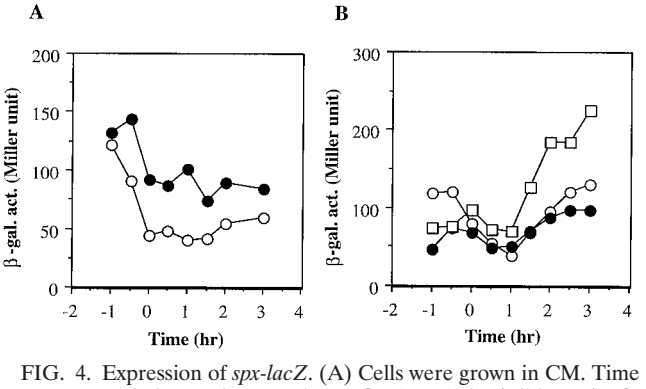

FIG. 4. Expression of *spx-lacZ*. (A) Cells were grown in CM. Time zero, onset of the stationary phase. ○, ORB4059 (wild type); ●, ORB4065 (*clpX*::*spc*). (B) ORB4059 (wild type) was grown in CM (○), DSM (●), or 2× YT (□). β-gal. act., β-galactosidase activity.

Comparison of proteins by two-dimensional gel electrophoresis showed that several proteins, such as GroEL, PpiB, PyrK, SucD, YhfP, YqkF, YugJ, and YvyD, were more abundant in *clpP* and *clpX* mutants than in the wild-type cells, suggesting that some of them might be the substrates for ClpXP (10). CtsR, the negative regulator of class III heat-inducible genes such as *clpP*, *clpE*, and *clpC*, is degraded by ClpXP under nonstress conditions (6). Finally evidence that SsrA-tagged proteins in *B. subtilis* are degraded by ClpXP was reported (48). We assume that the degradation of Spx by ClpXP requires another protein, which may work as an adapter or a stimulator. In *E. coli*, ribosome-associated protein SspB, by binding to SsrA-tagged proteins, enhances proteolysis by ClpXP but not by ClpAP (24). In the degradation of the stationary-phase sigma subunit of *E. coli*, RpoS, RssB acts as an adapter for ClpXP-dependent RpoS proteolysis (3, 51). We are currently searching for a protein that may be involved in ClpXP-dependent proteolysis of Spx in *B. subtilis.*

The in vivo studies clearly indicated that ClpXP, not ClpCP, is responsible for maintaining low intracellular levels of Spx. In contrast, in vitro proteolysis experiments using purified proteins revealed that ClpCP, but not ClpXP, degraded Spx. The degradation of Spx by ClpCP requires adapter protein MecA or YpbH, a putative paralog of MecA. Although several ClpCP target proteins were suggested by in vivo studies (see the introduction), only two cases have been analyzed in vitro. ComK and ComS are the best-studied targets of ClpCP proteolysis in *B. subtilis* (43). The degradation of ComK and ComS by ClpCP also requires MecA. The other substrate of ClpCP is CtsR. As mentioned above, CtsR is degraded by ClpXP under nonstress conditions; however, it becomes a target of ClpCP upon heat shock (21). In vitro proteolysis of CtsR by ClpCP was observed in the absence of MecA. It has yet to be determined how MecA presents ComK, ComS, and Spx to ClpCP and how CtsR is directly recognized by ClpCP. There is a similar example from studies of ClpXP proteolysis in *E. coli*. The *E. coli* stationary sigma factor, σ^s (39), and λ O (49) are substrates for ClpXP. Whereas the proteolysis of σ^s requires response regulator RssB both in vivo and in vitro, the ClpXP-dependent proteolysis of λ O does not require RssB (51). Interestingly, RssB can also act like an anti-σ^s factor when the cellular RssB/σ^s ratio is elevated and proteolysis is reduced (3). This is analogous to the ternary complex formed by MecA, ClpC, and ComK, which sequesters ComK. We have recently shown that Spx interacts with MecA, thus forming the quaternary complex and enhancing ComK binding to ClpC-MecA (30).

The contradictory in vitro and in vivo results of studies aimed at determining the role of ClpCP in Spx proteolysis are puzzling. One possibility is that the result of the in vitro proteolysis is an artifact. However, we think that this is unlikely because the proteolysis requires either MecA or YpbH. Another plausible explanation is that an unidentified protein is negatively involved in ClpCP-dependent proteolysis of Spx in vivo. The protein could be an inhibitor that specifically binds to Spx and protects it from proteolysis by ClpCP or could be the putative adapter for ClpXP that binds to Spx. This interaction could render Spx resistant to proteolysis by ClpCP.

Although the increased amount of Spx is detrimental to cells, Spx is likely needed under certain conditions. The *spx* gene resides downstream of *yjbC*, and these two genes probably constitute an operon (2). The transcription of the *yjbC* and *spx* genes was induced by heat, salt, and ethyl alcohol stress (36). A *yjbC* mutant and, to a lesser extent, an *spx* mutant exhibited a salt-sensitive phenotype (36). It is possible that Spx is produced under conditions of extreme stress as one way to suppress developmental processes and allow the cell to devote its energy to dealing with the cellular damage caused by harsh conditions. It is apparent from our studies and those of others that Spx is involved, positively and negatively, in various cellular functions in gram-positive bacteria.

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