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A MecA Paralog, YpbH, Binds ClpC, Affecting both Competence and Sporulation

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ComK, the master regulator of competence, is degraded by the general stress-related protease ClpCP but must be targeted to this protease by binding to the adapter protein MecA. The genome of *Bacillus subtilis* contains a paralog of *mecA*, *ypbH*. We show in the present study that YpbH, like MecA, binds ClpC and that its elimination or overproduction affects competence and sporulation.

The development of genetic competence, the ability to bind and internalize free DNA from the environment, is tightly regulated in *Bacillus subtilis*. The competence transcription factor ComK is necessary and sufficient to activate the transcription of late competence genes, which encode components of the DNA uptake machinery, as well as many other operons (6, 25). It has been suggested that this massive reorganization of the transcriptional program defines a unique physiological condition, the K state (3). ComK is regulated both transcriptionally and posttranscriptionally. Five transcription factors, including ComK itself, control the level of *comK* transcription (6–8, 19, 21). ComK acts as a positive autoregulator, thus imposing a switch-like behavior on K-state development. These mechanisms ensure a low level of *comK* transcription during exponential growth and a postexponential burst of ComK synthesis.

Another layer of control, regulated proteolysis, also keeps the concentration of active ComK at the basal level during exponential growth. Two key players in this process are MecA and ClpC. MecA binds and targets ComK for degradation by the ClpCP protease (22, 23). ClpCP is composed of two heat shock proteins, the protease subunit ClpP and ClpC. ClpC, an ATPase belonging to the family of Clp/HSP100 proteins (18), is widespread among bacteria. The role of ClpC is complex: cells deficient in *clpC* are affected in sporulation, competence, and growth at high temperatures (4, 10, 11, 14). ClpC probably forms a hexameric structure (M. Persuh and D. Dubnau, unpublished data) and together with ClpP presumably forms a higher order hetero-oligomer similar to ClpAP or ClpXP in *Escherichia coli* (5). By binding to both ComK and ClpC, MecA adapts the general stress-related proteolytic machine for the degradation of the competence transcription factor ComK. Upon entry into the stationary phase, the small protein ComS is synthesized in response to the quorum-sensing pheromones ComX and CSF (12, 13, 20). ComS binds to MecA, causing the release of ComK and protecting it from degradation. MecA consists of two domains: its N-terminal domain recognizes ComK and ComS while its C-terminal domain binds ClpC (16). There are other phenotypes associated with *mecA* knockout or overexpression in *Bacillus*. For instance, MecA-overproducing cells are sporulation deficient (9), and the inactivation of *mecA* causes a rough colony shape (Persuh and Dubnau, unpublished). These phenotypes are not dependent on ComK, and MecA probably targets proteins other than ComK for degradation by ClpCP.

YpbH is a paralog of MecA. The search of sequence databases with *mecA* as a query revealed the presence of a gene, *ypbH*, the product of which shows high similarity to MecA. The *B. subtilis* MecA paralog shares 26% sequence identity and 52% sequence similarity with the *B. subtilis* MecA on the amino acid level. MecA consists of two domains, the N- and C-terminal domains, with a linker region between them (16). The similarity of YpbH to MecA extends over both domains, but YpbH is 24 amino acids shorter than MecA and lacks the linker region. *ypbH* is present in two other sequenced *Bacillus* species: *Bacillus anthracis* and *Bacillus halodurans*. It seems that *ypbH* is present only in the genus *Bacillus* while MecA, with a much broader distribution, is found in essentially all low-GC gram-positive bacteria (*Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Lactococcus*, and *Enterococcus*) (16).

YpbH binds to ClpC. Cells overexpressing YpbH are very deficient in sporulation, and we have observed that colonies of the overexpressing strain lyse on plates within 48 h. The introduction of a *clpC* knockout in the YpbH-overproducing strain reversed this lysis phenotype. Although *clpC* knockout cells are themselves sporulation deficient, the expression of the *spoII* genes is not affected and rapid lysis does not occur (15). These results demonstrated that YpbH needs ClpC for its effect on sporulation. The strong similarity of YpbH to MecA further implied that YpbH, like MecA, might bind to ClpC. We used surface plasmon resonance to detect this interaction. The His-tagged YpbH protein was purified as described previously for MecA (23). A *clpC*-intein construct (a gift from M. Nakano) in pTYB2 (New England Biolabs) was used for expression, and ClpC was purified according to the manufacturer's instructions. Protein concentrations were determined by using the Bio-Rad reagents with bovine serum albumin as the protein standard. His-tagged YpbH (800 response units) was noncovalently immobilized to the surface of a nitrilotriacetic acid chip by the injection of a 150 nM solution of YpbH-His in eluent buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 50 μM EDTA, and 0.005% polysorbate 20) at flow rate of 10 μl/min.

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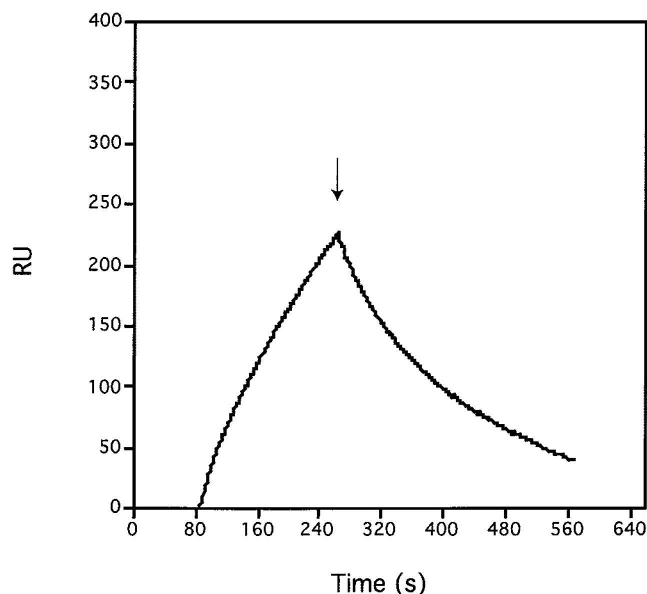

FIG. 1. Analysis of ClpC binding to YpbH by surface plasmon resonance. YpbH was bound to the surface of a chip, and 200 nM ClpC was passed over the chip surface. At the time indicated by the arrow, buffer lacking ClpC was introduced, permitting observation of the dissociation of the YpbH-ClpC complex. The binding response is measured in resonance units (RU).

To follow the binding of ClpC, a 200 nM solution of ClpC in buffer A (20 mM Tris, pH 8, 150 mM KCl, 10 mM $MgCl_2$) was passed over the chip. After each run, the chip was regenerated with 0.3 M EDTA and recharged with a 200 μM solution of $NiCl_2$. This experiment demonstrated that ClpC was able to bind YpbH (Fig. 1). In the same experiment, a ComK fusion to the maltose binding protein was passed over the chip and no binding to YpbH was detected (data not shown), indicating the specificity of the YpbH-ClpC interaction. Like MecA, YpbH was able to stimulate the ATPase activity of ClpC in vitro (K. Turgay, personal communication), confirming its interaction with ClpC.

YpbH plays a role in sporulation and competence. To explore the role of YpbH, we constructed a deletion-insertion mutant of *ypbH* as well as a *ypbH* multicopy construct in the vector pUB110 and examined the associated phenotypes. The phenotype of the *ypbH* knockout was not due to polarity since the downstream gene, *gudB*, codes for an inactive protein (2). To make the *ypbH* knockout, the regions 200 bp upstream and 500 bp downstream from *ypbH* were amplified with the primers *ypbH-Eco*RIF1 (5′-CGG AAT TCT GTT GCG GCA GCG G-3′) and *ypbH-Bam*HIR1 (5′-CGG GAT CCA CGT CTT TTG TCG GGC-3′). The resulting 2.1-kb fragment was cut with *Eco*RI and *Bam*HI and cloned into pUCCm18. The recombinant plasmid was cut with *Spe*I and *Bgl*II, which removes nearly all of *ypbH*, and a spectinomycin cassette was cloned between the sites. The resulting plasmid was transformed into *B. subtilis* to make strain BD3349, and transformants were checked for chloramphenicol sensitivity (the plasmid contained a chloramphenicol marker which is lost upon the double crossover). The construct in the chromosome was verified with a PCR. To make the overproducing strain BD3350, the *ypbH* gene was amplified with the primers *ypbH-Bam*HIF1 (5′-GCG GAT CCT GCA TTG TGC TTG TCT TAC-3′) and *ypbH-Eco*RIR1 (5′-CGG AAT TCG GCT GCC ATT TGA G-3′). The PCR fragment was then cut with *Bam*HI and *Eco*RI and cloned between the corresponding sites in pUB110 to produce pMB1. The growth of *B. subtilis* strains in competence medium and transformation were carried out as described previously (1). The morphology of colonies lacking or overexpressing *ypbH* suggested the involvement of *ypbH* in sporulation. Measurements of sporulation frequency, carried out as described previously (17), confirmed this impression. A *ypbH* knockout formed 20-fold fewer spores than the wild type while a strain overexpressing *ypbH* on a multicopy plasmid completely lacked the ability to sporulate (a sporulation frequency of less than 10^{-8}). We tested the same mutant strains for the expression of a *spoIIE-lacZ* reporter and found that the *ypbH* strain showed reduced *spoIIE-lacZ* activity while the overexpressing strain was completely deficient in this activity (data not shown). The effect of YpbH overproduction was not due to titration of ClpC, since ClpC deficiency does not affect the expression of *spoIIE* (15). The sporulation deficiency of the YpbH overproducer was not due to the titration of a regulatory molecule by the *ypbH* promoter, since a multicopy plasmid with a partially deleted YpbH coding region had no sporulation phenotype. Since active Spo0A is required for *spoIIE* transcription (26), YpbH may affect the transcription of *spo0A* or the phosphorylation of its gene product. We tested the competence phenotype of the *ypbH* mutant strain by measuring the expression of ComK-dependent genes. β-Galactosidase assays were carried out as described previously (16). *comG-lacZ* transcription was delayed in the strain lacking *ypbH* and increased in the overexpressing strain (Fig. 2A and B). Similar results were obtained with other late competence genes (*comF*, *comE*, and *comC*) (data not shown). The results shown in Fig. 2 demonstrate that in the YpbH-overproducing strain, the mechanisms that regulate the timing of competence gene expression are still functional. The effect of YpbH overproduction is to increase competence gene expression at all time points but particularly during exponential growth. In this respect, the effect of YpbH overproduction is what would be expected from a decrease in MecA. Western blot analysis suggests that this is the case (data not shown). Since ComK is needed for the transcription of late competence genes, we measured *comK-lacZ* expression and found it was elevated in a strain overproducing YpbH, particularly before T_0 (0 h after the end of log-phase growth) (Fig. 2C), suggesting that the effect of YpbH overproduction on late competence gene expression is due to the increased synthesis of ComK.

The transcription of *ypbH* is growth-stage regulated. We constructed a transcriptional *ypbH-lacZ* fusion and measured its activity in Luria-Bertani (LB), sporulation, and competence media. The vector pMutin2 (24) was used to make a transcriptional fusion to *ypbH.* pMB1 was cut with *Spe*I, the ends were filled in, and the fragment was cut with *Bgl*II. This treatment generated an N-terminal fragment of *ypbH*. pMutin2 was cut with *Hin*dIII, the ends were filled in, and the fragment was cut with *Bam*HI. The N-terminal *ypbH* fragment was cloned into the vector fragment. The resulting plasmid, pMB3, was used to transform *B. subtilis* to make strain BD3351. Proper insertion in the chromosome was checked by PCR. The results are shown in Fig. 3. The transcription of *ypbH* increased at the end

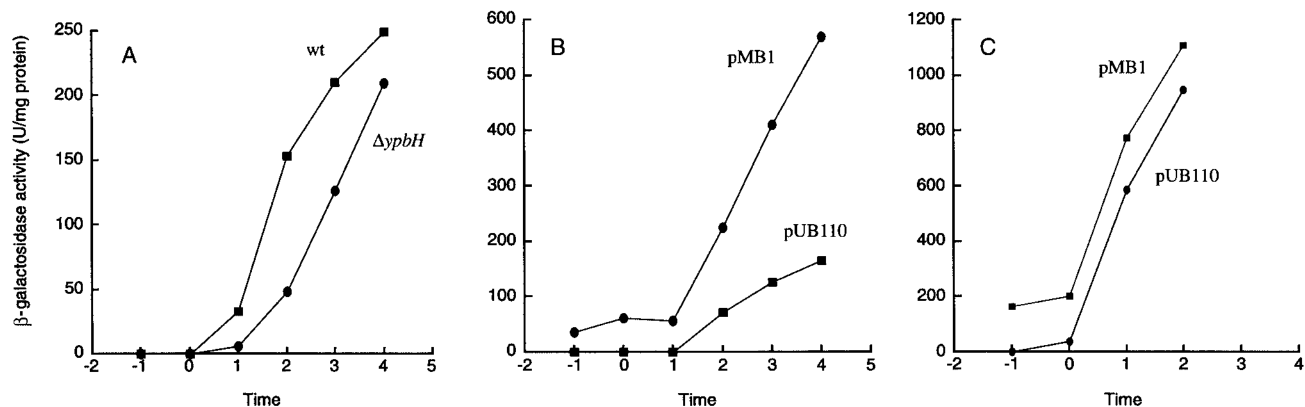

FIG. 2. The effect of *ypbH* knockout on *comG-lacZ* expression (A) and of *ypbH* overproduction on *comG-lacZ* (B) and *comK-lacZ* (C) expression. The expression of β-galactosidase from a *lacZ* fusion to the promoters of *comG* or *comK* was measured as a function of the growth stage. In panel B, the strains carried either the vector pUB110 or the *ypbH* plasmid, pMB1. The time scale refers to hours before and after the transition from the exponential to the stationary growth phase (T_0). wt, wild type.

of exponential growth in both LB and competence medium and reached approximately the same level in both. In contrast, *lacZ* activity slowly decreased after T_0 in sporulation medium and was at least fourfold lower than in LB or competence medium. These results are consistent with the effects of YpbH on competence and sporulation and suggest that this molecule may play a regulatory role in vivo. In an additional experiment we found that the overproduction of YpbH had no effect on *ypbH-lacZ* expression (data not shown).

The effect of YpbH on late competence gene transcription can be explained by the modulation of *comK* transcription (Fig. 2C). There is a striking parallel between MecA and YpbH regarding their effects on sporulation. Both of these proteins eliminate sporulation when overproduced, and in both cases this effect is dependent on ClpC (9, 16), indicating that YpbH-ClpC hetero-oligomers are likely the functional regulatory complexes inside the cell. These observations and the patterns of *ypbH* transcription in competence and sporulation media suggest that YpbH is a pleiotropic regulator affecting both competence and sporulation.

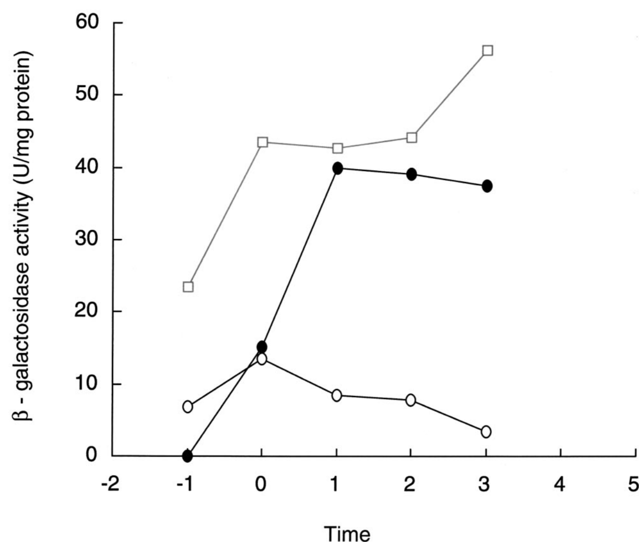

FIG. 3. Expression of *ypbH-lacZ* in competence (□), LB (●), and sporulation (○) medium. The expression of β-galactosidase from a *lacZ* fusion to the promoter of *ypbH* (BD3351) was measured as a function of the growth stage. The time scale refers to hours before and after the transition from the exponential to the stationary growth phase (T_0).

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REFERENCES

1. **Albano, M., J. Hahn, and D. Dubnau.** 1987. Expression of competence genes in *Bacillus subtilis*. J. Bacteriol. **169:**3110–3117.
2. **Belitsky, B. R., and A. L. Sonenshein.** 1998. Role and regulation of *Bacillus subtilis* glutamate dehydrogenase genes. J. Bacteriol. **180:**6298–6305.
3. **Berka, R. M., J. Hahn, M. Albano, I. Draskovic, M. Persuh, X. Cui, A. Sloma, W. Widner, and D. Dubnau.** Microarray analysis of the Bacillus subtilis K-state; genome-wide expression changes dependent on ComK. Mol. Microbiol., in press.
4. **Dubnau, D., and M. Roggiani.** 1990. Growth medium-independent genetic competence mutants of *Bacillus subtilis*. J. Bacteriol. **172:**4048–4055.
5. **Gottesman, S., S. Wickner, and M. R. Maurizi.** 1997. Protein quality control: triage by chaperones and proteases. Genes Dev. **11:**815–823.
6. **Hahn, J., A. Luttinger, and D. Dubnau.** 1996. Regulatory inputs for the synthesis of ComK, the competence transcription factor of *Bacillus subtilis*. Mol. Microbiol. **21:**763–775.
7. **Hahn, J., M. Roggiani, and D. Dubnau.** 1995. The major role of Spo0A in genetic competence is to downregulate *abrB*, an essential competence gene. J. Bacteriol. **177:**3601–3605.
8. **Hamoen, L. W., A. F. Van Werkhoven, G. Venema, and D. Dubnau.** 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.
9. **Kong, L., K. J. Siranosian, A. D. Grossman, and D. Dubnau.** 1993. Sequence and properties of *mecA*, a negative regulator of genetic competence in *Bacillus subtilis*. Mol. Microbiol. **9:**365–373.
10. **Krüger, E., T. Msadek, and M. Hecker.** 1996. Alternate promoters direct stress-induced transcription of the *Bacillus subtilis clpC* operon. Mol. Microbiol. **20:**713–723.
11. **Krüger, E., T. Msadek, S. Ohlmeier, and M. Hecker.** 1997. The *Bacillus subtilis clpC* operon encodes DNA repair and competence proteins. Microbiology **143:**1309–1316.
12. **Lazazzera, B. A., J. M. Solomon, and A. D. Grossman.** 1997. An exported peptide functions intracellularly to contribute to cell density signaling in *B. subtilis*. Cell **89:**917–925.
13. **Magnuson, R., J. Solomon, and A. D. Grossman.** 1994. Biochemical and genetic characterization of a competence pheromone. Cell **77:**207–216.

14. **Msadek, T., F. Kunst, and G. Rapoport.** 1994. MecB of *Bacillus subtilis* is a pleiotropic regulator of the ClpC ATPase family, controlling competence gene expression and survival at high temperature. Proc. Natl. Acad. Sci. USA **91:**5788–5792.
15. **Pan, Q., D. A. Garsin, and R. Losick.** 2001. Self-reinforcing activation of a cell-specific transcription factor by proteolysis of an antisigma factor in *B. subtilis*. Mol. Cell **8:**873–883.
16. **Persuh, M., K. Turgay, I. Mandic-Mulec, and D. Dubnau.** 1999. The N- and C-terminal domains of MecA recognize different partners in the competence molecular switch. Mol. Microbiol. **33:**886–894.
17. **Roggiani, M., J. Hahn, and D. Dubnau.** 1990. Suppression of early competence mutations in *Bacillus subtilis* by *mec* mutations. J. Bacteriol. **172:**4056–4063.
18. **Schirmer, E. C., J. R. Glover, M. A. Singer, and S. Lindquist.** 1996. HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem. Sci. **21:**289–296.
19. **Serror, P., and A. L. Sonenshein.** 1996. CodY is required for nutritional repression of *Bacillus subtilis* genetic competence. J. Bacteriol. **178:**5910–5915.
20. **Solomon, J. M., B. A. Lazazzera, and A. D. Grossman.** 1996. Purification and characterization of an extracellular peptide factor that affects two developmental pathways in *Bacillus subtilis*. Genes Dev. **10:**2014–2024.
21. **Tran, T. H., P. Tortosa, M. Albano, and D. Dubnau.** 2002. Rok (YkuW) regulates genetic competence in *Bacillus subtilis* by directly repressing *comK*. Mol. Microbiol. **43:**15–26.
22. **Turgay, K., J. Hahn, J. Burghoorn, and D. Dubnau.** 1998. Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. EMBO J. **17:**6730–6738.
23. **Turgay, K., L. W. Hamoen, G. Venema, and D. Dubnau.** 1997. Biochemical characterization of a molecular switch involving the heat shock protein ClpC, which controls the activity of ComK, the competence transcription factor of Bacillus subtilis. Genes Dev. **11:**119–128.
24. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.
25. **van Sinderen, D., A. Luttinger, L. Kong, D. Dubnau, G. Venema, and L. Hamoen.** 1995. *comK* encodes the competence transcription factor, the key regulatory protein for competence development in *Bacillus subtilis*. Mol. Microbiol. **15:**455–462.
26. **York, K., T. J. Kenney, S. Satola, C. P. Moran, Jr., H. Poth, and P. Youngman.** 1992. Spo0A controls the σ^A-dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. J. Bacteriol. **174:**2648–2658.