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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*

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Development of genetic competence in *Bacillus subtilis* is controlled by the competence-specific transcription factor ComK. ComK activates transcription of itself and several other genes required for competence. The activity of ComK is controlled by other genes including *mecA*, *clpC*, and *comS*. We have used purified ComK, MecA, ClpC, and synthetic ComS to study their interactions and have demonstrated the following mechanism for ComK regulation. ClpC, in the presence of ATP, forms a ternary complex with MecA and ComK, which prevents ComK from binding to its specific DNA target. This complex dissociates when ComS is added, liberating active ComK. ClpC and MecA function as a molecular switch, in which MecA confers molecular recognition, connecting ClpC to ComK and to ComS.

Competent *Bacillus subtilis* cells are able to bind and internalize exogenous transforming DNA. Competence is a physiological state distinct from that of vegetatively growing or sporulating cells, and is the endpoint of a complex developmental process. A minor subpopulation (never exceeding 20%) of the cells in a given culture develop competence under specific growth and nutritional conditions. The regulation of this developmental process is embedded in a complex signal transduction network, linking it to other processes that occur at the onset of the postexponential growth phase, like sporulation and the synthesis of degradative enzymes or secondary metabolites (for review, see Dubnau 1993; Grossman 1995).

The regulation of competence development has been shown to work via diverse mechanisms, involving quorum-sensing pheromones, protein phosphorylation, and protein–DNA and protein–protein interactions. The crucial step in this regulatory pathway is the control of the activity of ComK, the competence transcription factor (Hahn et al. 1994; van Sinderen and Venema 1994; van Sinderen et al. 1995). ComK is necessary and sufficient

for the transcription of the late competence genes, which encode a set of proteins responsible for the binding, processing, and transport of transforming DNA. The transcription of *comK* is activated by ComK itself (van Sinderen and Venema 1994) and also requires the transcriptional activators AbrB, SinR, and DegU (Hahn et al. 1994, 1996; van Sinderen and Venema 1994).

In addition, the synthesis of ComK requires ComS, a small protein of 46 amino acid residues, encoded by an open reading frame embedded in the *srfA* operon (D'Souza et al. 1994; Hamoen et al. 1995). *srfA* transcription is induced toward the end of exponential growth by the action of two quorum-sensing pheromones, ComX (Magnuson et al. 1994) and CSF (Solomon et al. 1995). ComX acts via the ComP and ComA signal transduction proteins. ComP is a histidine protein kinase that is presumed to phosphorylate itself and then donate its phosphate to ComA, thereby activating the latter protein, which then functions positively as a transcription factor for *srfA* (Nakano and Zuber 1993; Roggiani and Dubnau 1993). CSF probably acts to increase the level of ComA phosphorylation by inhibiting a specific protein–phosphate phosphatase (Solomon et al. 1996). Converging pathways thus serve to regulate the intracellular level of ComA–PO$_4$ in response to population density (Solomon et al. 1995).

The activity of ComK is negatively controlled by a pair

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of proteins, MecA and MecB. Loss-of-function mutations in either *mecA* or *mecB* result in the overproduction of ComK and the derepression of competence genes (Dubnau and Roggiani 1990; Hahn et al. 1994; van Sinderen and Venema 1994). Because the sequence of *mecB* reveals that it is the *B. subtilis* version of the heat shock gene *clpC* (Msadek et al. 1994), we have adopted the latter nomenclature. Overexpression of MecA inhibits competence and the synthesis of ComK, even in a *clpC* mutant background, whereas the overexpression of ClpC has no effect (Kong and Dubnau 1994). In vitro experiments have demonstrated that MecA can bind to ComK. On the basis of these data it was proposed that ClpC may somehow facilitate the binding of MecA to ComK, resulting in the inactivation of the latter as a transcription factor (Kong and Dubnau 1994). Because *comK* transcription requires ComK, this would limit ComK synthesis and result in the failure of competence development.

ClpC is a heat-inducible protein of *B. subtilis*, which was independently identified as a general stress protein (Krüger et al. 1994; Msadek et al. 1994; Hecker et al. 1996). It is a member of a highly conserved and ubiquitous family of proteins, also known as the HSP100 family, which includes ClpA, ClpB, and ClpX (Parsell and Lindquist 1993; Schirmer et al. 1996). These proteins contain ATP-binding motifs, and certain members of the Clp family function in protein degradation or possess chaperone-like activities (Squires and Squires 1992; Parsell and Lindquist 1993; Wickner et al. 1994). *clpC* has been identified in several bacteria in addition to *B. subtilis* and in the chloroplasts of several higher plants (Squires and Squires 1992; Schirmer et al. 1996). Although competence development remains the only specific regulatory role that has been ascribed to a member of the widely distributed ClpC protein subfamily, other Clp proteins have been implicated in a variety of regulatory mechanisms (for review, see Schirmer et al. 1996).

The observations summarized above led us to a series of hypotheses that were tested in the experiments reported here. We propose that ClpC and MecA form a complex that binds to and inactivates ComK. In vivo experiments have suggested that ComS may exert its positive effect on ComK synthesis via the MecA/ClpC system (Hahn et al. 1996). We propose, therefore, that when ComS is synthesized it interacts with the ternary ClpC–MecA–ComK complex, causing the release of active ComK.

As a first step toward understanding the roles of ClpC and MecA in the signal transduction pathway of competence development, we have documented the ATP-dependent binding of ClpC to MecA in vitro. We then extended these studies to demonstrate the formation of a ternary complex of these proteins and ComK, and their influence on the DNA-binding activity of the latter protein. In the presence of ComS, ComK is released from this complex in a form that is active for DNA binding. These observations enable us to describe a new and unique molecular switch which consists of ClpC, MecA, and ComS.

Results

Binding of MecA to ClpC

To determine whether MecA and ClpC can form a complex, we used fusions of MecA and ClpC to glutathione S-transferase (GST). The MecA–GST fusion contained a thrombin cleavage site between the MecA and GST portions, whereas the ClpC–GST fusion contained a Factor Xa cleavage site at the corresponding location.

A glutathione–agarose column was loaded with the two fusion proteins as described in Materials and Methods. Factor Xa was then applied to specifically cleave the ClpC–GST fusion on the column. After extensive washing, the column was eluted with 5 mM glutathione to release the remaining MecA–GST, cleaved GST, and any ClpC that might have remained on the column by virtue of its association with MecA. The binding, washing, and elution steps were carried out in either the presence or the absence of ATP or the nonhydrolyzable ATP analog AMP–PNP.

The final wash and the glutathione elution samples were analyzed by Western blotting with anti-MecA and anti-ClpC antibodies (Fig. 1). MecA–GST was not present in the final wash but was eluted in the presence of glutathione (Fig. 1, lanes 1,2). ClpC coeluted with MecA–GST but only when ATP was present during binding and washing (Fig. 1, lanes 2,4). This experiment strongly suggested that MecA and ClpC are capable of associating in the presence of ATP. The failure to demonstrate this association in the presence of AMP–PNP (Fig. 1, lane 6) suggested that association requires ATP hydrolysis. The presence of cleaved ClpC even in the final wash sample in the presence of ATP (Fig. 1, lane 1) suggested that the ClpC–MecA complex was slowly dissociating on the column.

We have also demonstrated MecA–ClpC interaction using the yeast two-hybrid system (Fields and Song 1989), which is based on the GAL4 transcriptional acti-
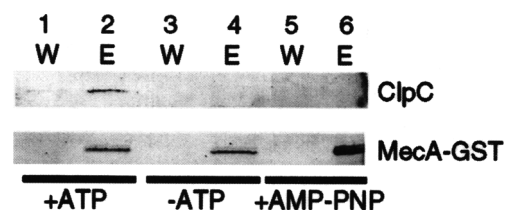
Figure 1. Binding of ClpC to MecA–GST. MecA–GST and ClpC–GST were incubated with Factor Xa on glutathione–agarose columns to release ClpC (as described in Materials and Methods). The columns were washed extensively and MecA–GST and GST were then eluted with wash buffer containing 5 mM glutathione. ATP or AMP–PNP (1 mM) was present in all solutions as indicated. Samples taken from the last wash fraction and from the glutathione elution fraction were analyzed by SDS-PAGE and Western blotting with anti-ClpC and anti-MecA antibodies. Lanes 1 and 2 show the result of the Western blot for the final wash (W) and elution (E) fractions in the presence of ATP, lanes 3 and 4 in the absence of ATP, and lanes 5 and 6 in the absence of ATP and in the presence of AMP–PNP.

vator (Phizicky and Fields 1995). GAL4 contains separable domains for DNA binding and the activation of transcription. Interacting proteins fused to the separated domains induce the transcription of a GAL4-inducible *lacZ* reporter gene. Using the plasmids pGBT9 and pGAD424, we cloned *mecA* and *clpC* as fusions to both the activator and DNA-binding domains of GAL4. Both combinations resulted in dark blue colonies when the recombinant plasmids were transformed into the appropriate yeast tester strain and tested for β-galactosidase activity on filters containing X-gal (data not shown). Control transformants lacking either the MecA or the ClpC domain remained white. This experiment provided confirmatory evidence documenting the ability of ClpC and MecA to stably interact.

ClpC possesses a MecA-dependent ATPase activity that is stimulated by ComK

The ability of ClpC to bind to MecA in the presence of ATP but not in the presence of the nonhydrolyzable ATP analog AMP–PNP, and the similarity of ClpC to ClpA, a chaperone with ATPase activity (Wickner et al. 1994; Seol et al. 1995), led us to seek a possible MecA-dependent ATPase activity of ClpC. For these experiments MecA and ClpC were purified as carboxy-terminal His$_6$-tag derivatives, prepared as described in Materials and Methods. The genes encoding both His$_6$-tag fusions were integrated into the *B. subtilis* chromosome and shown to function in vivo (data not shown).

ClpC alone exhibited a very low ATPase activity, but the presence of MecA induced an increase (Fig. 2A). As expected, MecA alone exhibited no ATPase activity (data not shown) and BSA, which was always present in the reaction mixture, could not induce the ATPase activity of ClpC. These experiments showed clearly that ClpC, whose amino acid sequence predicts the existence of two ATP-binding sites (Msadek et al. 1994), was responsible for the ATPase activity. When ComK was added, the ATPase activity was stimulated more than twofold, whereas in the absence of MecA, ComK had no detectable effect (Fig. 2A). Heat-denatured ComK had no influence (data not shown). The ATPase activity of ClpC depended on the concentrations of MecA and ComK. In additional experiments we varied the concentration of ATP and observed a dependence of ATPase activity on ATP concentration in the millimolar range; the activity was not measurable below 0.5 mM (data not shown). The experiments reported in this study employed 2 mM ATP. ADP strongly inhibited the MecA-dependent ATPase activity of ClpC, when added in a molar amount equal to that of ATP (data not shown). The experiments reported in Figure 2A demonstrate that MecA interacts with ClpC and that ComK also influences the ClpC ATPase but in a manner that depends on the presence of MecA.

ComK, MecA, and ClpC form a ternary complex

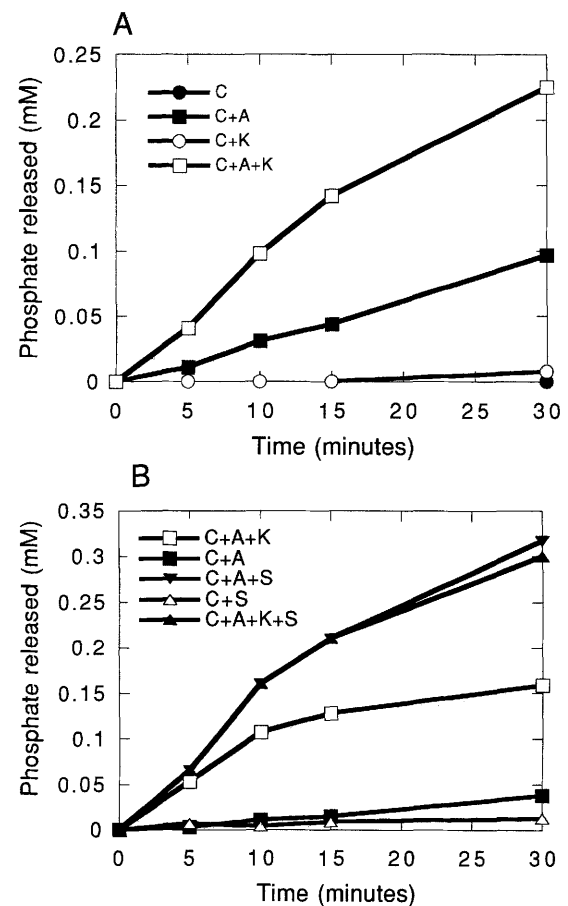The influence of ComK on the MecA-dependent ATPase of ClpC strongly suggested that ClpC, MecA, and ComK

Figure 2. ATPase assays. (*A*) ClpC (C) (0.3 μM) was incubated alone, with ComK (K) (0.3 μM), with MecA (A) (0.3 μM), and with MecA (0.3 μM) and ComK (0.3 μM), and the release of phosphate was followed as described in Materials and Methods. (*B*) ClpC (C) (0.3 μM) was incubated with ComS (S) (2.4 μM), with MecA (A) (0.3 μM), with MecA (0.3 μM) and ComK (K) (0.3 μM), with MecA (0.3 μM) and ComS (1.2 μM), and with MecA (0.3 μM), ComK (0.3 μM) and ComS (1.2 μM), and the release of Pi was followed as described in Materials and Methods.

interact, perhaps to form a complex. To test this possibility more directly, we incubated a mixture of ComK–MBP and ClpC with and without MecA and ATP. Amylose resin was then added to bind the ComK–MBP protein. The resin was washed extensively, and the ComK–MBP was eluted by the addition of 10 mM maltose to the wash buffer. The final wash and elution samples were then analyzed by Western blotting, using anti-ClpC, anti-MecA, and anti-ComK antibodies. Western blotting with the anti-ComK antibodies showed that the ComK–MBP was efficiently eluted by the addition of maltose (data not shown).

The Western blots obtained with anti-ClpC and anti-MecA antibodies are shown in Figure 3. Use of the anti-MecA antibody showed that MecA coeluted with ComK–MBP in both the presence and absence of ATP (Fig. 3, lanes 1,2,7,8), confirming previous experiments that demonstrated MecA binding to ComK (Kong and
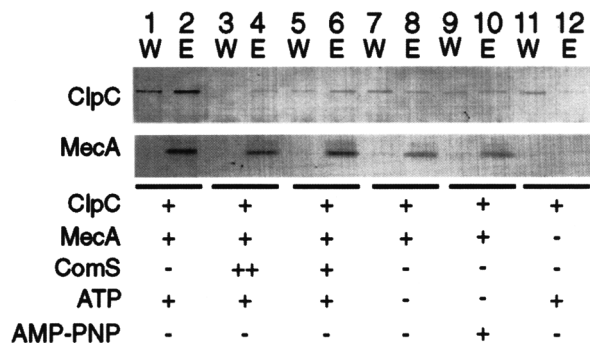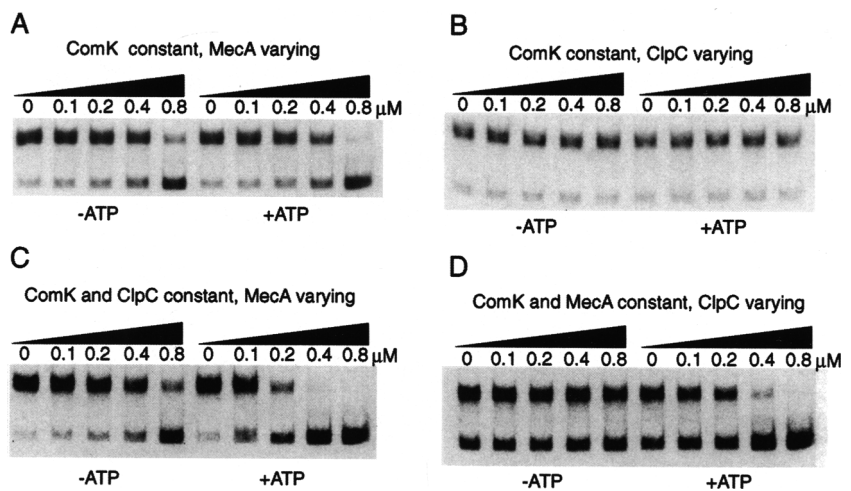
Figure 3. Binding of ComK–MBP to MecA and ClpC. ComK–MBP (0.34 μM) was incubated for 15 min at 37°C with ClpC (0.22 μM) and with or without MecA (0.20 μM), ComS (0.38 μM or 0.77 μM), ATP (2 mM), or AMP-PNP (2 mM) as described in Materials and Methods. The various incubation mixtures were then incubated for 30 min with amylose resin, washed extensively, and the ComK–MBP was then eluted with wash buffer containing 10 mM maltose. Samples from the final wash (W) and from the maltose elution (E) were analyzed by SDS-PAGE and Western blotting with anti-ClpC and Anti-MecA antibodies.

Dubnau 1994). On the other hand, very little ClpC coeluted with ComK–MBP when either ATP or MecA was absent (Fig. 3, lanes 7,8,11,12). AMP–PNP was unable to substitute for ATP in this reaction (Fig. 3, lanes 9,10). These experiments demonstrated that in the presence of hydrolyzable ATP (Fig. 3, lanes 1,2), a ternary complex of ClpC, MecA, and ComK was formed, and that the association of ClpC and ComK required the presence of MecA. This was consistent with the experiment shown in Figure 2A, demonstrating that ComK only stimulated the ClpC ATPase when MecA was present.

In the ternary MecA–ClpC–ComK complex, ComK is unable to bind the comK promoter

The autoregulatory activation of *comK* transcription by ComK is the critical event in competence development (Hahn et al. 1994; van Sinderen and Venema 1994; van Sinderen et al. 1995). We therefore measured the ComK-dependent gel retardation of a DNA fragment carrying the *comK* promoter to study the influence of MecA, ClpC, and ATP on the DNA-binding activity of ComK.

Figure 4A demonstrates that MecA alone could inhibit the ComK-dependent gel retardation of the probe fragment but only when present at the highest concentrations used. ATP had no effect on this inhibition, except when added at the highest concentration of MecA. We have no explanantion for this minor effect. Figure 4B shows that ClpC had no effect on the DNA-binding activity of ComK in either the presence or absence of ATP, when MecA was omitted. The in vitro data presented in Figure 4, A and B, correspond to and provide explanations for in vivo effects reported previously; when MecA is overproduced, competence and ComK synthesis are inhibited, whereas ClpC overproduction has no detectable effect (Kong and Dubnau 1994). The inhibiting effect of high MecA concentrations on the DNA-binding activity of ComK provides confirmatory evidence that MecA can bind to ComK.

Figure 4D presents an experiment in which a constant concentration of MecA, insufficient to inhibit the retardation, was supplemented by the addition of various amounts of ClpC in the presence and absence of ATP. The addition of ClpC enhanced the inhibition of retardation only in the presence of ATP. In effect, the addition of ClpC permitted the inhibition of DNA-binding activity of ComK to occur at a lower MecA concentration than would be required to obtain inhibition in the

Figure 4. Binding of ComK to the *comK* promoter fragment. (*A*) Influence of MecA and ATP. ComK (0.1 μM) was incubated with varying amounts of MecA, and a ^{32}P-end-labeled *comK* promoter fragment, with and without 2 mM ATP, and then analyzed by gel electrophoresis followed by autoradiography, as described in Materials and Methods. The concentrations of MecA are shown above the lanes. (*B*) Influence of ClpC and ATP. ComK (0.1 μM) was incubated with varying amounts of ClpC and a ^{32}P-end-labeled *comK* promoter fragment with and without 2 mM ATP, and then analyzed by gel electrophoresis followed by autoradiography, as described in Materials and Methods. The concentrations of ClpC are shown above the lanes. (*C*) Influence of MecA and ATP with constant ClpC. ComK (0.1 μM) and ClpC (0.8 μM) were incubated with vary-

ing amounts of MecA and with a ^{32}P-end-labeled *comK* promoter fragment, with and without 2 mM ATP, and analyzed by gel electrophoresis followed by autoradiography, as described in Materials and Methods. The concentrations of MecA are shown above the lanes. (*D*) Influence of ClpC and ATP with constant MecA. ComK (0.1 μM) and MecA (0.4 μM) were incubated with varying amounts of ClpC and with a ^{32}P-end-labeled *comK* promoter fragment with and without 2 mM ATP, and analyzed by gel electrophoresis followed by autoradiography, as described in Materials and Methods. The concentrations of ClpC are shown above the lanes.

absence of ClpC. Similarly, when ClpC was maintained at a constant concentration and MecA was varied, MecA inhibited the retardation at a lower concentration than was needed to obtain inhibition in the absence of ClpC (Fig. 4C). These results reflect and explain the in vivo situation; ClpC enhances the inhibition of ComK activity when MecA is normally expressed but is not needed for this inhibition when MecA is overproduced (Kong and Dubnau 1994). These data are also consistent with the ATPase data and the experiment reporting ternary complex formation, because ComK and ClpC only interact in the presence of MecA.

ComS liberates ComK from the ternary MecA–ClpC–ComK complex

On the basis of in vivo data, we have suggested that ComS may provide a signal that acts through MecA and ClpC to activate ComK synthesis (Hahn et al. 1996). To test this idea in the in vitro system developed in this study, we have used chemically synthesized ComS.

When synthetic ComS was added to the ATPase assay it increased the MecA-dependent ATPase activity of ClpC (Fig. 2B). The stimulatory effect was dependent on the presence of MecA, and its magnitude depended on the ComS concentration (data not shown). In this respect the ComS effect on the ClpC ATPase activity resembled that of ComK (Fig. 2A).

We then tested the effect of ComS on formation of the ternary complex among ComK, ClpC, and MecA. As noted before, Figure 3, lanes 1 and 2, shows that ClpC coeluted with ComK–MBP when ComS was absent. When ComS was included in the reaction mixture, very little coelution of ClpC with ComK–MBP was detectable (Fig. 3, lanes 3–6), suggesting that ComS had caused the dissociation of the ClpC–MecA–ComK complex or prevented its formation. ComS had little effect on the association of MecA with ComK (Fig. 3, lanes 4,6), although the amount of MecA coeluting with the ComK–MBP

was slightly decreased compared with the absence of ComS (Fig. 3, lane 2).

Finally, an experiment was performed to determine the effect of ComS on the inhibition of DNA-binding activity of ComK caused by ClpC and MecA in the presence of ATP. The addition of ComS reversed this inhibition in a concentration-dependent manner (Fig. 5A, lanes 3–7). We then addressed the reversibility of ComK DNA-binding and of the inhibition of this binding by MecA and ClpC. In this experiment (Fig. 5B), subsequent samples were loaded on the same gel, and the successive samples therefore have migrated less than ones loaded previously. When radiolabeled DNA probe was incubated with ComK, ClpC, and ATP for 20 min, gel retardation was noted when an aliquot of the reaction mixture was tested (Fig. 5B, lane 2). To the remaining reaction mixture, MecA was added and incubation was continued for an additional 20 min. Analysis of an aliquot taken at this point revealed that a nearly complete inhibition of DNA-binding activity of ComK had occurred (Fig. 5B, lane 4). ComS was then added to half of the remaining reaction mixture. After 20 min further incubation the sample that had not received ComS still showed the inhibition of DNA-binding activity of ComK (Fig. 5B, lane 7), but retardation was partially restored in the portion that had been incubated with ComS (Fig. 5B, lane 6). This experiment demonstrated the reversibility of the interactions reported in this study; the addition of MecA, ClpC, and ATP was able to dissociate the specific complex formed between ComK and its binding site upstream from the comK promoter, and ComS could dissociate the ternary complex formed between ComK, MecA, and ClpC, freeing ComK for interaction with its specific DNA target.

Discussion

A reasonable interpretation of previous observations was that ClpC acts to assist the binding of MecA to ComK, thereby preventing ComK from binding to its own pro-
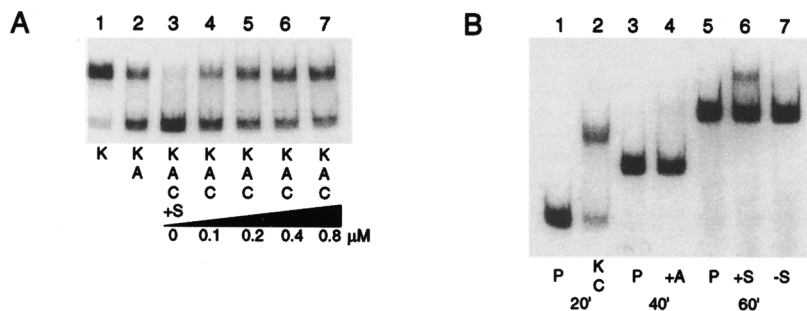

Figure 5. Influence of ComS on DNA-binding activity of ComK. (A) Influence of ComS with constant MecA, ClpC, and ATP. ComK (K) (0.1 μM) with or without ClpC (C) (0.8 μM) or MecA (A) (0.5 μM) and in the presence of 2 mM ATP, was incubated with varying amounts of ComS (S) and with a ^{32}P-end-labeled comK promoter fragment and analyzed by gel electrophoresis followed by autoradiography, as described in Materials and Methods. The concentrations of ComS are indicated below the lanes. (B) ComS reverses the inhibition of ComK DNA binding. ComK (0.1 μM) and ClpC (1.2 μM) were incubated with a ^{32}P-end-labeled comK promoter fragment for 20 min at 37°C as described in Materials and Methods. A portion was loaded on a polyacrylamide gel (lane 2). MecA (0.4 μM) was added to the remainder of the reaction mixture and incubation was continued for a further 20 min, while electrophoresis of the first sample was under way. A portion of this incubation mixture was then loaded on the same gel (lane 4). ComS (0.4 μM) was added to half of the remaining reaction mixture and incubation was continued for an additional 20 min. Both samples (with and without added ComS) were then loaded in lanes 6 and 7 of the gel. The labeled ComK promoter probe fragment (P) was also loaded at each time (lanes 1, 3, and 5). The gel was analyzed by autoradiography.

moter (Kong and Dubnau 1994; Msadek et al. 1994). In this model, ComS, synthesized in response to a quorum-sensing signal transduction pathway, might cause the release of ComK from association with MecA, permitting it to bind upstream from the *comK* promoter. Although these hypotheses were consistent with the available data, they were in no way compelling, because the in vivo data did not demonstrate that the observed effects were attributable to direct interactions among the known protein components of this system. It had been shown that MecA and ComK could associate in vitro (Kong and Dubnau 1994), but the effects of ClpC and ComS might have been exerted through unknown intermediate molecules.

In this study compelling evidence has been presented showing that ClpC and MecA form a complex in the presence of ATP. We have also shown that ClpC and MecA can form a ternary complex with ComK in the presence of ATP and that this complex inhibits the binding of ComK to the *comK* promoter. We conclude therefore that ClpC, bound to MecA in the presence of ATP, acts to increase the affinity of MecA for ComK, enabling MecA to prevent ComK from binding to its own promoter. Finally, ComS has been shown to cause the reactivation of ComK by dissociating the ternary complex, permitting ComK to bind to its specific DNA target. This system therefore acts as a switch composed of MecA and ClpC, acting on ComK. The switch is thrown by ComS, and the positive autoregulation of ComK transcription acts to amplify the output.

Our in vitro results faithfully reflect the in vivo behavior of the ComK regulation system. For instance, it has been reported that loss-of-function mutations in either *mecA* or *clpC* have similar phenotypes, resulting in the overproduction of ComK (Hahn et al. 1994; van Sin-

deren and Venema 1994). We show here that the omission of either MecA or ClpC permits ComK binding to its DNA target to occur (Fig. 4). When MecA is overproduced in vivo, even in a *clpC* mutant, ComK synthesis is inhibited completely (Hahn et al. 1994). Our in vitro results show that high concentrations of MecA prevent ComK–DNA binding in the absence of ClpC (Fig. 4). Finally, *comS* mutations prevent the induction of ComK synthesis in vivo (D'Souza et al. 1994; Hamoen et al. 1995), and ComS has now been shown in vitro to cause the release of ComK from association with ClpC and MecA (Figs. 4 and 5).

Figure 6 summarizes the current model for ComK regulation as well as the principal findings of the present study. Although all of the proteins involved are shown arbitrarily as monomers, we have no information at present concerning their oligomeric states. We have chosen to show MecA as binding to ClpC and ComK using separate sites. This is based on the observation that MecA can bind independently to either potential partner (reactions 1 and 4 in Fig. 6). However we cannot exclude the possibility that ClpC enhances the interaction of MecA and ComK by contacting both proteins simultaneously.

We have tested the possibility that the inhibition of ComK activity by ClpC and MecA involves a phosphorylation step, by incubating the three proteins using our standard conditions (see Materials and Methods) in the presence of [γ-^{32}P]ATP. No phosphorylation of ClpC, ComK, or MecA was detected by autoradiography of polyacrylamide gels (data not shown). Because proteins resembling ClpC have been shown to work in concert with ClpP to effect the degradation of target molecules, and because a weak similarity between ClpP and MecA has been reported (Kong and Dubnau 1994), we sought evidence that ClpC and MecA caused the degradation of

Figure 6. The molecular switch for ComK regulation. The figure depicts our present understanding of the inhibition of ComK (K) activity by ClpC (C) and MecA (A) in the presence of ATP, and the influence of ComS (S) on the regulatory system. MecA binds to ComK (reaction 4) or to ClpC in the presence of ATP (reaction 1). When all three proteins are present together with ATP, the ternary complex is favored (reactions 2 and 3), resulting in an inhibition of ComK DNA binding. ComS can resolve this complex by interacting with MecA (reaction 5), and ComK is then released, activating its own transcription (reaction 6) and that of the late competence genes.
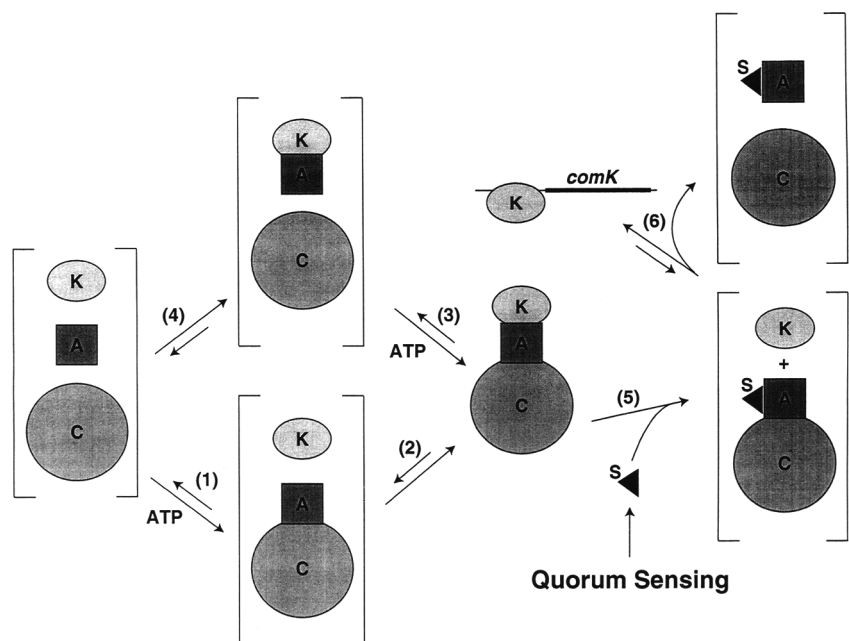
ComK. Using our standard conditions (see Materials and Methods) we incubated the three proteins in the presence of ATP and examined the incubation mixture by SDS-PAGE followed by silver staining (data not shown). No loss of any of the three proteins was detected even after 4 hr of incubation at 37°C. In addition, the experiment shown in Figure 5B demonstrates that the inhibition of ComK DNA-binding activity caused by ClpC and MecA was reversible. We conclude that the inhibition of ComK DNA binding does not require the degradation of ComK by MecA and ClpC.

Although a ternary complex clearly forms when all three proteins are incubated with ATP, the order of binding is not clear. In particular, reaction 3 (Fig. 6) has not been demonstrated directly, and it is possible that MecA must first associate with ClpC before the ternary complex is formed. In vivo, in any event, it is likely that the ternary complex is formed following reactions 1 and 2, because MecA and ClpC are constitutively present at relatively high concentrations compared with ComK, and the ATP concentration used in all of our experiments (2 mM), is in the physiological range reported for *Bacillus* (1–3 mM) (Jolliffe et al. 1981; Guffanti et al. 1987). In a preliminary experiment using a crude wild-type extract, MecA was found to elute from a gel filtration column at the position expected for a ClpC–MecA complex, but at the position of MecA monomer when an extract from a *clpC* mutant was used (L. Kong and D. Dubnau, unpubl.).

ComS must cause the release of ComK from the ternary complex (reaction 5 in Fig. 6), because the gel retardation experiments (Figs. 4 and 5) show that the position of the gel-shifted band after the addition of ComS is the same as that obtained with ComK alone. ComS has been shown to bind to MecA in a cross-linking study (L. Liu and P. Zuber, pers. comm.). This direct evidence that ComS interacts with MecA is strongly supported by our observation that stimulation of the ATPase activity of ClpC by ComS addition is dependent on the presence of MecA (Fig. 2B). We can envisage three modes of action of ComS. ComS might act by directly competing with ComK for a binding site on MecA, it might alter the conformation of MecA causing the release of ComK, or it might cause the release of ClpC, thereby indirectly causing a decrease in the affinity of MecA for ComK. Figure 3 (lanes 4,6) demonstrates that ComS did not completely prevent the association of MecA with ComK–MBP. This suggests that ComS and ComK may not compete for the same site on MecA but, rather, that the effect of ComS may be to decrease the affinity of ComK for MecA. Also, in Figure 5, A and B, the addition of ComS does not completely reverse the inhibition of DNA-binding activity of ComK but, rather, seems to restore approximately the level of inhibition obtained with MecA alone. It is possible, therefore, that the interaction of MecA with ComS causes the dissociation of the ClpC–MecA complex, which in turn leads to the release of ComK.

The roles of ATP and ATP hydrolysis in these reactions are not understood. Because ClpC has two potential ATP-binding sites (Msadek et al. 1994), ATP is likely to play more than one role. It is possible that ATP hydrolysis is required for the association of ClpC and MecA, because AMP–PNP cannot support this binding (Fig. 1). In the DnaK–DnaJ–GrpE system, ATP-binding is required for the association of DnaK with a target peptide and ATP hydrolysis and the release of ADP is required for dissociation (Hartl 1996). In our system the only evidence that ATP hydrolysis is required for protein–protein association is that AMP–PNP cannot support either ClpC–MecA interaction or formation of the ternary complex. This experiment needs to be supported by additional data, because AMP–PNP is an ATP analog that may bind poorly to one of the ClpC ATP sites. However, if ATP turnover proves to be essential for complex formation, this may reflect a cycling of MecA between an active ClpC-bound state and an inactive unbound form accompanied by ATP hydrolysis. Our data (Fig. 3) demonstrates the formation of a ternary complex but does not exclude the possibility that this complex is continually undergoing association and dissociation.

In any event, the ATPase activities detected are a valuable tool, because the stimulation of ATPase upon addition of ComK or ComS provides strong support for the interaction of these molecules with the ClpC–MecA complex. In a preliminary experiment (data not shown) we have determined that ADP is a potent inhibitor of the ATPase activity of ClpC. We have also found that the ATPase activity of ClpC varies with the ATP concentration in the low millimolar range. These observations raise the possibility that alterations in the energy charge (the ratio of ATP to ADP) might play a regulatory role in this system, as it has been proposed to do in the control of σ-factor activity in *B. subtilis* (Alper et al. 1994).

The experiment shown in Figure 5 represents an attempt to test the reversibility of several of the interactions. This experiment revealed that MecA and ClpC could remove ComK from its DNA target after it was bound and that ComS could reverse the inhibition of ComK binding to DNA after the ternary complex was formed. The latter reversal mimics the dynamic situation likely to obtain in a culture as it approaches stationary phase. The low basal level of ComK in the cells will be present in a complex with MecA and ClpC. ComS will be synthesized in response to a signal transduction pathway that senses population density (Solomon et al. 1995), and as a result ComK will be liberated and available to bind upstream from its own promoter, thereby initiating *comK* transcription and that of the competence genes responsible for DNA binding and uptake by competent cells.

As a general stress protein, the *B. subtilis* ClpC is presumably involved in a variety of interactions, such as those required for heat tolerance and the regulation of SigD activity (Krüger et al. 1994; Msadek et al. 1994; Hecker et al. 1996; Rashid et al. 1996). In the switch that controls ComK activity, the general stress protein ClpC, which is related to a family of chaperones, is involved in a specific regulatory event. The competence switch harnesses ClpC by using MecA to confer specific molecular recognition, mediating binding to ComK and to ComS.

The enzymatic capabilities of chaperones, which enable them to bind and alter proteins, also enable them to participate in specific regulatory mechanisms (Hendrick and Hartl 1993; Schirmer et al. 1996).

Materials and methods

General methods

The Western blotting was carried out by standard methods as described previously (Kong and Dubnau 1994). Anti-ClpC antibodies were prepared by immunizing guinea pigs with purified ClpC–GST protein as described for the anti-ComK antibodies (Kong and Dubnau 1994). β-Galactosidase was assayed as described previously (Albano et al. 1987). DNA manipulations, cloning, and standard molecular biological methods were carried out as described in Sambrook et al. (1989). The ClpC–GST fusion (see below) was purified on a glutathione column and used by Liyun Kong to immunize guinea pigs as described for the anti-MecA and anti-ComK antibodies (Kong and Dubnau 1994). Protein concentrations were determined using the Bradford (Bradford 1976) assay (Bio-Rad).

Yeast two-hybrid system

The two-hybrid assay (Bartel et al. 1993) was performed using the Matchmaker Two-Hybrid System (Clontech Laboratories), according to the manufacturer's recommendations. The *mecA* and *clpC* coding sequences were obtained by PCR with the primers MA1 (5'-TTGGATCCAAATGGAAATTGAAAGAAT-TAACG-3') and MA2 (5'-AACTGCAGTTGCTATGATG-CAAAGTGTTT-3'), for *mecA*, and MB1 (5'-TTGGATCC-ATATGATGTTTGGAAGATTTAC-3'), and MB2 (5'-AACTGCAGCCGTCTTCTATATTAATTCGTT-3') for *clpC*, using chromosomal DNA from *B. subtilis* strain IS75 as template. Both coding sequences were inserted in the yeast expression vectors pGBT9 (*TRP1* marker) and pGAD424 (*LEU2* marker), using the underlined *Bam*HI and *Pst*I sites. Cloning into pGBT9 resulted in a translational fusion between the GAL4 DNA-binding domain sequence and the *mecA* or *clpC* coding sequence. Cloning into pGAD424 resulted in a translational fusion between the GAL4 activation domain sequence and the *mecA* or *clpC* coding sequence. Yeast strain SFY526 was transformed with a combination of pGBT9 and pGAD424 and the different derivatives of both plasmids. The plasmid pair pVA3/pTD1, provided by the manufacturer, was included as a positive control. Transformants were selected on minimal medium lacking *TRP* and *LEU*. The positive colonies were then assayed on filters for β-galactosidase activity, using X-gal.

Strains and plasmids

The MecA–GST and ComK–MBP constructs were described previously (Kong and Dubnau 1994). A ClpC–GST fusion was constructed by Liyun Kong using the primers M1 (5'-GGGAATTCGTCTTCTATATTAATTCG-3') and M2 (5'-CGGGATCCATGATGTTTGGAAGATTTACAG-3') in the plasmid pGEX-2T (Pharmacia) with the help of the underlined *Eco*RI and *Bam*HI restriction sites. The MecA–GST fusion was constructed with the plasmid pGEX-3X (Pharmacia) These constructs contain cutting site for thrombin and Factor Xa, respectively.

Carboxy-terminal His₆-tag fusions to ClpC and MecA were constructed by PCR cloning. Oligonucleotide primers used to amplify *mecA* were QMA1 (5'-GGCCCATGGAAATT-GAAAGAATTAAG-3') and QMA2 (5'-GGTGGATCCTGATG-

CAAAGTGTTT-3'). Primers used to amplify *clpC* were QSMB1 (5'-TCGGCATGCTGTTTGGAAGATTTACAG-3') and QMB2 (5'-CTGGATCCATTCGTTTTAGCAGTCG-3'). These primers contained restriction sites for *Nco*I, *Bam*HI, *Sph*I, and *Bam*HI, respectively. These sites (underlined) were used to clone ClpC and MecA as carboxy-terminal His₆-tag fusions in the expression plasmids pQE60 (for MecA) and pQE70 (for ClpC) (Qiagen). The resulting plasmid with the *mecA* gene pQMA was used to express MecA–His₆ in *E. coli*. To test the function of the MecA–His₆ fusion in *B. subtilis*, the last 657 bp of the *mecA* gene with the His₆-tag fusion was cloned in a pUC18 plasmid carrying kanamycin resistance cassette for selection in *B. subtilis*. This construct, pAK1, was used to transform *B. subtilis* BD1960 [*comG:lacZ* (*amyE*) (Cm^R)], a derivative of IS75 (*his leu met*) to achieve a Campbell-like integration at the *mecA* locus, which inactivated the resident *mecA* gene and placed the *mecA–his₆* fusion under control of the *mecA* promoter. Kanamycin-resistant colonies were found to have a Mec⁺ phenotype on X-gal plates and by comparing the β-galactosidase activity of this strain BD2583 to that of BD1960.

The ClpC–His₆ protein was not stable in *Escherichia coli*. To replace *clpC* by *clpC–his₆* on the *Bacillus* chromosome so that ClpC–His₆ could be isolated from that organism, we modified the ClpC–His₆ fusion plasmid pBQ, by replacing the *Xho*I–*Cla*I fragment bearing the promoter of pQE70 and the first 830 bp of *clpC*, by a kanamycin resistance cassette. The resulting plasmid pBK1 was used to transform *B. subtilis* BD1960 [*comG:lacZ* (*amyE*) (Cm^R)] to achieve a Campbell-like integration at the *clpC* locus, creating BD2490. The resident *clpC* gene was inactivated in this strain. Kanamycin-resistant colonies were shown to have a Mec⁺ phenotype on X-gal plates and by comparing the β-galactosidase activity of BD2490 to that of BD1960. ClpC–His₆ was produced by *B. subtilis* BD2490 as revealed by binding to Ni–NTA resin and Western blot analysis. The production of ClpC–His₆ by *B. subtilis* BD2490 was found to be heat inducible (Krüger et al. 1994).

Interaction of MecA–GST with ClpC

Crude extracts of *E. coli* strains carrying the MecA–GST and ClpC–GST fusion plasmids were prepared as described in Kong and Dubnau (1994). They were mixed and loaded on GST–agarose (Sigma) columns. The columns were washed and treated as if to purify the GST fusion protein (Kong and Dubnau 1994) but without the final elution step. The columns were washed with buffer X (100 mM NaCl, 50 mM Tris-HCl at pH 8.0, 1 mM CaCl₂) with or without 1 mM ATP or AMP–PNP and with 5 mM MgCl₂. One column volume of buffer X containing 0.01 µg/µl of factor Xa (New England Biolabs) was applied to the column and left overnight at room temperature. The columns were washed with 10 column volumes of buffer X with or without ATP or AMP–PNP and containing 5 mM MgCl₂. The MecA–GST and GST proteins were then eluted with buffer X containing 5 mM glutathione. Samples of the wash and elution steps were analyzed by SDS-PAGE and Western blot analysis with anti-MecA and anti-ClpC antibodies.

Purification of ClpC–His₆

2× YT medium (1.5 liter) with chloramphenicol (5 µg/ml) and kanamycin (5 µg/ml) was inoculated with an overnight culture of BD2490, which carries the *clpC-his₆* construct. The cells were grown for 4 hr at 37°C and then transferred to a water bath at 50°C for another 30 min of shaking to induce ClpC–His₆ synthesis. The following procedures were performed at 4°C. The cells were collected by centrifugation, resuspended in 15 ml

buffer A (200 mM NaCl, 20 mM Tris-HCl at pH 8.0, 10 mM MgCl$_2$, 1 mM β-mercaptoethanol) and broken with a French press. The extracts were centrifuged for 20 min at 20,000 rpm. The supernatants were diluted to 50 ml with buffer A and applied to a column of Ni–NTA Superflow resin (Qiagen) equilibrated with buffer A on an FPLC System (Pharmacia). The column was washed with buffer A and then with buffer A containing 40 mM imidazole and the protein was then eluted with a gradient of 40–250 mM imidazole in buffer A. The fractions with the highest concentrations of ClpC–His$_6$ (judged by SDS-PAGE) were pooled and dialyzed against buffer C (200 mM KCl, 20 mM Tris-HCl at pH 8.0, 10 mM MgCl$_2$, 1 mM β-mercaptoethanol). The protein was aliquoted and stored at –70°C.

Purification of MecA–His$_6$

To isolate MecA–His$_6$, 200 ml of 2× YT medium supplemented with 100 µg/ml of ampicillin and 25 µg/ml of kanamycin was inoculated with an overnight culture of *E.coli* strain M15 carrying pQA1 and pREP (Qiagen). After 4 hr of growth, 1 mM IPTG was added, and growth was continued for an additional 2 hr at 37°C. Cells were harvested and the MecA–His$_6$ protein was prepared essentially as described above for the ClpC–His$_6$ protein. Both proteins were purified to homogeneity as judged by SDS-PAGE and staining with Coomassie.

Purification of ComK

ComK was purified as a MBP–ComK fusion protein on an amylose (New England Biolabs) column and separated from MBP by means of cleavage with protease Factor Xa as previously described (van Sinderen et al. 1995). After the cleavage was completed, Factor Xa was inactivated by the addition of 1 mM PMSF. To separate ComK from MBP, the protein mixture was loaded onto a DEAE column (Pharmacia) equilibrated with 20 mM Tris-HCl at pH 8.0, 1 mM EDTA, and 0.5 mM DTT. ComK was eluted with a Na$_2$SO$_4$ gradient, dialyzed against 20 mM Tris-HCl at pH 8.0, 1 mM EDTA, 0.5 mM DTT (or 1 mM β-mercaptoethanol), 100 mM Na$_2$SO$_4$, aliquoted, and stored at –70°C. Purification and cleavage were followed by SDS-PAGE.

Synthesis of ComS

The 46-residue ComS peptide was chemically synthesized by Research Genetics and purified by HPLC. The identity and purity of the product was proven by amino acid analysis and mass spectrometry.

ATPase assay

The ATPase assay is based on measuring the concentration of the released orthophosphate colorimetrically (Lanzetta et al. 1979; Lill et al. 1990). The assay was carried out with the addition of 0.3 µM ClpC, MecA, and ComK as indicated. ComS was added at 1.2 µM. In the negative control (ClpC with only ComS) 2.4 µM ComS was included. The final buffer composition in the incubation mixtures was 100 mM KCl, 25 mM Tris-HCl at pH 8.0, 5 mM MgCl$_2$, 2 mM ATP, 0.5 mM DTT, 0.1 mM EDTA, 0.5 µg/µl of BSA, and 0.015 µg/µl of bacteriophage λ DNA. The λ DNA was included to stabilize ComK. The assay was performed in final volumes of either 30 or 50 µl, and incubation was at 37°C for the indicated times. The reactions were stopped by the addition of color solution (10.5 grams per liter of ammonium molybdate, 0.0034% malachite green, 0.1% Triton X-100) followed by 34% citric acid, and the OD at 650 nm was determined. The data were corrected for zero time values.

Complex of ComK–MBP, MecA, and ClpC

ComK–MBP (0.45 µM) was incubated for 15 min at 37°C with ClpC (0.3 µM) and with or without MecA (0.3 µM), ComS (0.5 µM and 1 µM), ATP (2 mM), or AMP–PNP (2 mM) in a volume of 150 µl and under the same buffer conditions as described above for the ATPase assay. After the addition of 50 µl of a 50% (vol/vol) slurry of amylose resin (New England Biolabs) preincubated in the washing buffer (100 mM KCl, 25 mM Tris-HCl at pH 8.0, 5 mM MgCl$_2$, 0.1 mM EDTA), the various incubation mixtures were then incubated for 30 min. The resin was washed extensively (3 × 200 µl). A final wash with 60 µl of buffer was then taken as the wash sample. The ComK–MBP was then eluted with 60 µl wash buffer containing 10 mM maltose. Equal volumes from the final wash and from the maltose elution samples were analyzed by SDS–PAGE and Western blotting with anti-ClpC, anti-ComK, and anti-MecA antibodies.

Gel-retardation analysis

Gel retardation with ComK was carried out essentially as described (van Sinderen et al. 1995). The promoter region of *comK* was chosen as DNA probe. This sequence was isolated by PCR using primers K1 (5′-CCGGAATTCAGAATCCCCCCAAT-GCC-3′) and K2 (5′-CGCGGATCCCTAATAATCTAT-CATCTG-3′) and chromosomal DNA from *B. subtilis* strain IS75 as template. The resulting 254-bp fragment extends from –254 to +1 relative to the transcriptional start of *comK* (van Sinderen et al. 1995). The probe was end-labeled with T4 polynucleotide kinase using [γ-^{32}P]ATP. The purified proteins and probe were premixed on ice in 20 mM Tris-HCl at pH 8.0, 100 mM KCl, 5 mM MgCl$_2$, 1 mM EDTA, 0.5 mM DTT, 0.05 µg/µl of BSA, 0.05 µg/µl of poly[d(I-C)] (Boehringer). After 20 min of incubation at 37°C, one-fourth volume of a 50% glycerol solution was added and the samples were loaded on a 4% polyacrylamide gel. Gels were run in TAE buffer (40 mM Tris-acetate at pH 8.0, 2 mM EDTA) for 20 min at 150 V, dried, and autoradiographed. The conditions used for this assay were chosen to be nearly identical to those used in the ATPase assays.

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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.

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