

Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor

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Competence is a physiological state, distinct from sporulation and vegetative growth, that enables cells to bind and internalize transforming DNA. The transcriptional regulator ComK drives the development of competence in *Bacillus subtilis*. ComK is directly required for its own transcription as well as for the transcription of the genes that encode DNA transport proteins. When ComK is sequestered by binding to a complex of the proteins MecA and ClpC, the positive feedback loop leading to ComK synthesis is interrupted. The small protein ComS, produced as a result of signaling by a quorum-sensing two-component regulatory pathway, triggers the release of ComK from the complex, enabling *comK* transcription to occur. We show here, based on *in vivo* and *in vitro* experiments, that ComK accumulation is also regulated by proteolysis and that binding to MecA targets ComK for degradation by the ClpP protease in association with ClpC. The release of ComK from binding by MecA and ClpC, which occurs when ComS is synthesized, protects ComK from proteolysis. Following this release, the rates of MecA and ComS degradation by ClpCP are increased in our *in vitro* system. In this novel system, MecA serves to recruit ComK to the ClpCP protease and connects ComK degradation to the quorum-sensing signal-transduction pathway, thereby regulating a key developmental process. This is the first regulated degradation system in which a specific targeting molecule serves such a function.

Keywords: ClpC/ClpP/ComK/competence/MecA/
proteolysis

Introduction

Competence is defined as the ability to bind and internalize exogenous DNA. In *Bacillus subtilis*, competence is a physiological state, distinct from vegetative growth and sporulation, which occurs at the onset of late exponential growth under specific nutritional conditions. Competence is expressed in a distinct subpopulation of non-dividing cells which are arrested for DNA replication and the

synthesis of stable RNA (B.J.Haijema, M.Albano, J.Hahn and D.Dubnau, unpublished; Nester and Stocker, 1963; McCarthy and Nester, 1967; Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968; Dooley *et al.*, 1971). When resuspended in fresh growth medium, competent cells can escape this arrested state, but only after a pronounced lag (Nester and Stocker, 1963).

A set of proteins encoded by late competence genes are responsible for the binding, processing and internalization of transforming DNA, and are present only in the competent subpopulation (reviewed in Dubnau, 1997). The transcription factor ComK is needed for the transcription of these genes, and is active and expressed only in the cells fated to become competent (van Sinderen *et al.*, 1995; Hahn *et al.*, 1996). ComK also activates the transcription of genes needed for recombination and DNA repair (Haijema *et al.*, 1996). ComK synthesis and activity is a crucial checkpoint, committing a cell to competence development. ComK control is embedded in a complex signal transduction network, linking it to other developmental processes in *Bacillus* such as sporulation, and the synthesis of degradative enzymes and secondary metabolites (Dubnau, 1993; Grossman, 1995).

Since ComK is the factor that drives a cell to competence, and since competence is accompanied by the blockage of essential cell functions, it is no surprise that ComK synthesis is subject to a number of finely tuned and redundant regulatory circuits. The transcription of *comK* is activated by ComK itself and is controlled further by additional transcriptional regulators (Dubnau, 1993; Grossman, 1995). In addition, ComK is activated and overproduced in strains carrying null mutations in *mecA* or *clpC* (formerly known as *mecB*) (Dubnau and Roggiani, 1990; Roggiani *et al.*, 1990). MecA normally binds to ComK and inhibits its activity, while ClpC enhances this inhibition by binding to MecA. When sequestered in a ternary complex with MecA and ClpC, ComK is not able to activate its own transcription (Kong and Dubnau, 1994; Turgay *et al.*, 1997). Mutational inactivation of either *mecA* or *clpC* releases ComK and permits its overproduction.

ComS, a small protein of 46 amino acid residues, is necessary for competence development (D'Souza *et al.*, 1994; Hamoen *et al.*, 1995) and is expressed in response to the quorum-sensing pheromones ComX and CSF (Magnuson *et al.*, 1994; Solomon *et al.*, 1996; Lazazzera *et al.*, 1997). ComS induces the increased synthesis of ComK by dissociating its ternary complex with ClpC and MecA, thereby releasing active ComK which in turn activates its own transcription. Thus, ClpC, MecA and ComK, together with the signaling protein ComS, form a regulatory device controlling the activity of ComK. In cells where ComS releases ComK, a burst of ComK expression occurs, because of the positive autoregulation of *comK* expression by ComK (Hahn *et al.*, 1996; Turgay

et al., 1997). For unknown reasons, this release occurs only in the subpopulation of cells fated for competence.

MecB was shown to be a heat shock protein and was identified from its sequence as ClpC (Krüger *et al.*, 1994; Msadek *et al.*, 1994). ClpC is a member of the Hsp100 family of proteins which includes ClpA, ClpB, ClpX and ClpY in *Escherichia coli*, and Hsp104 in *Saccharomyces cerevisiae* (Schirmer *et al.*, 1996). In *E. coli*, ClpA and ClpX independently interact with the protease ClpP to form high molecular weight oligomers which can degrade specific substrate proteins (Gottesman *et al.*, 1997). Both ClpA and ClpX are ATPases which possess chaperone-like activity and are responsible for the substrate specificity of the ClpAP and ClpXP proteases (Wickner *et al.*, 1994; Wawrzynow *et al.*, 1995). ClpC, ClpE, ClpX and ClpY (CodW), but not ClpA or ClpB homologs, were identified as members of the Hsp100 family in the *B. subtilis* genome (Kunst *et al.*, 1997).

The mutational inactivation of *mecA* or *clpC* results in ComK overexpression in all of the cells in a culture grown to stationary phase and the appearance of cells which exhibit defects in nucleoid segregation and reduced viability (Hahn *et al.*, 1995). It was proposed that ComK was responsible for the arrest in DNA replication which normally accompanies competence development and that MecA is essential for the escape from competence. Our earlier work led us to postulate that cells escape from the competent state simply by the binding of ComK in a complex with MecA and ClpC. However, as reported in this paper, ComK is present in the competent cells in vast excess over the levels of MecA and ClpC. This observation, and the importance of ComK for competence development, led us to examine the *in vivo* stability of ComK. We show that ComK is rapidly degraded during the escape from competence, and that this degradation requires MecA, ClpC and ClpP. Using purified proteins we demonstrate *in vitro* that the ATP-dependent degradation of ComK by ClpCP requires MecA and occurs more rapidly in the absence of ComS. Furthermore, when ComS is present, MecA is degraded by ClpCP, as is ComS itself. These observations suggest that the regulation of competence development as well as the escape from competence, rely on regulated proteolysis. In this novel system, MecA serves to recruit ComK to the ClpCP protease, and connects ComK degradation to the quorum-sensing signal-transduction pathway, thereby regulating a key developmental process.

Results

In vivo amounts of ComK, MecA and ClpC

Since MecA and ClpC are involved in the control of ComK synthesis and activity, we measured the cellular content of these three proteins by quantitative Western blotting, reasoning that such data would provide insight into the *in vivo* operation of this system. It became clear that ComK is present in considerable excess over MecA and ClpC, thus excluding models for the escape from competence simply by the rebinding of ComK to MecA/ClpC.

These measurements are shown in Table I. The number of MecA molecules/cell was constant, whereas ClpC doubled in amount from t_0 to t_2 . ComK was not detectable

at t_0 , and then increased dramatically, consistent with its positive autoregulation. MecA (Kong and Dubnau, 1994), and presumably ClpC, were expressed in all of the cells in a competent culture. ComK, on the other hand, was only expressed in ~5–10% of the cells and a 10-fold correction was therefore applied in Table I. ComK was undetectable at t_0 , and there were 12 000 molecules per competent cell at t_1 and 91 000 at t_2 . Since ComK binds to DNA as a tetramer (Hamoen *et al.*, 1998), the number of active ComK entities per cell at t_2 could be estimated as 22 750. The high content of ComK at t_2 is unusual for a regulatory protein, and is roughly comparable to the content of HU molecules (30 000 or 15 000 dimers) in the smaller cells of *E. coli* (Pettijohn, 1996). MecA is present in solution as a dimer (M. Persuh and D. Dubnau, unpublished), leading to an estimate of 700 entities/cell at t_2 . We postulate that ClpC is present in a complex similar to those containing ClpA or ClpX in *E. coli* and that there are therefore either 6 or 12 ClpC monomers per complex, since ClpAP and ClpXP structures have been observed containing either single or double rings of the ATPase subunits (Kessel *et al.*, 1995; Grimaud *et al.*, 1998). This leads to an estimate of 650–1300 active units of ClpC per cell, comparable to the estimate for MecA. This excess of ComK over MecA and ClpC led us to consider a model for the regulation of competence which involves the degradation of ComK.

ComK is degraded during the escape from competence

The data presented below demonstrate that ComK is rapidly degraded during the escape from competence and that MecA and ClpC are needed for this degradation. Cells were grown to t_2 in competence medium and then diluted 1:20 into fresh competence medium containing rifampicin and tetracycline. This regime permitted the reversal of competence-inducing signals and prevented the synthesis of new ComK. Samples were taken at intervals after dilution, and extracts were analyzed by Western blotting for the presence of ComK. ComK was degraded at a constant rate and was eliminated after ~2 h (Figure 1). In *mecA* and *clpC* backgrounds on the other hand, ComK was stable. In a strain carrying a multicopy *comS* plasmid the decay rate of ComK was decreased ~2-fold. Since ComK was stabilized in strains lacking MecA and ClpC, we propose that these proteins are part of the degradative machinery. In contrast, since ComK degradation was slowed in a strain which overexpresses ComS, this protein must be involved in a mechanism which decreases the rate of ComK degradation. The experiment shown in Figure 1 was performed in the presence of antibiotics to block transcription and translation. Similar results were obtained in the absence of these antibiotics, suggesting that little additional ComK synthesis was occurring.

Neither ClpC nor MecA are known to have proteolytic activities, nor were they able to degrade ComK *in vitro* (Turgay *et al.*, 1997). One obvious candidate for a proteolytic component involved in the degradation of ComK is ClpP, since this protein is known to form proteasome-like complexes with ClpA or ClpX in *E. coli*. It seemed likely from these results that binding to MecA and ClpC targets

Table I. Cellular content of competence regulatory proteins^a

	t_0	t_1	t_2	Oligomericity	Molecules/cell (corrected for oligomericity)
ClpC	3800	4100	8000	6 or 12 ^b	670–1300
MecA	1300	1200	1400	2 ^c	700
ComK	<300	1200	9100	4 ^d	2275
ComK (corrected) ^e	<3000	12 000	91 000	4	22 750

^aThe numbers represent molecules/cell determined by quantitative Western blotting at the indicated times during growth in competence medium. t_0 , t_1 and t_2 refer to the time of transition to stationary phase, and 1 and 2 h thereafter.

^bWe assume that each ClpCP molecule contains either one or two hexamer rings of ClpC.

^cDetermined by *in vitro* cross-linking (M.Persuh and D.Dubnau, unpublished).

^dHamoen *et al.* (1998).

^eWe have enumerated the competent cells in a population using a *comK-gfp* fusion as well as by immunofluorescence with anti-ComK or anti-ComEA antibodies (B.J.Haijema, J.Hahn and M.Albano, unpublished). These measurements yield estimates for the percentage of competence-expressing cells in a typical population of ~5–10%. The numbers for ComK in this table were therefore corrected by a factor of 10. We assume that ClpC, which is not under competence control, is expressed in all the cells, and we know that this is true for MecA (Kong and Dubnau, 1994).

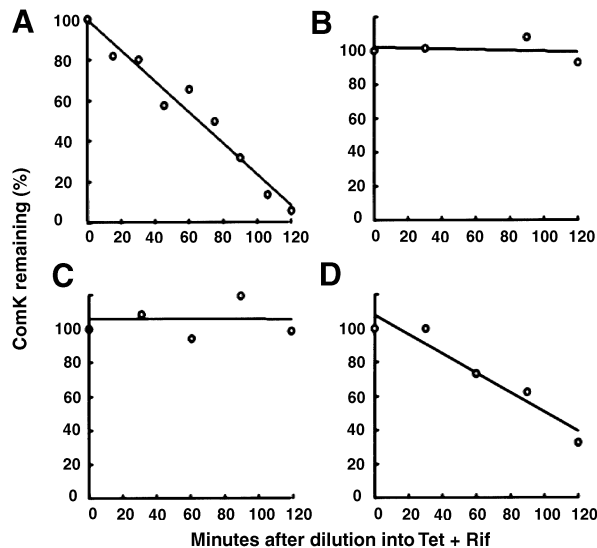


Fig. 1. Stability of ComK *in vivo*. A competent culture was diluted 20-fold into fresh competence medium in the presence of rifampicin and tetracycline. Samples were withdrawn at intervals and analyzed by Western blotting with anti-ComK antibodies. The resulting films were scanned and the signals were normalized to the initial values. (A) Wild-type strain; (B) *mecA* strain; (C) *clpC* strain; (D) ComS-overexpressing strain.

ComK for degradation by ClpP. We therefore decided to test the effect of a *clpP* mutation on competence.

MecA accumulates in *clpP* and *clpC* mutants

To determine the effect of a *clpP* mutation on the stability of ComK, we insertionally inactivated the *clpP* gene of *B.subtilis* and tested the resulting strain for its ability to become competent and express *comK-lacZ*. Unexpectedly, the *clpP* mutant strain was not competent, exhibited no *comK-lacZ* activity and had no detectable ComK, as determined by Western blotting. Transcription of *comK-lacZ* could be partially restored in the *clpP* mutant strain by the introduction of a *mecA* mutation (data not shown). Similar results have recently been reported independently (Msadek *et al.*, 1998). These observations can be explained if ClpP is responsible for the degradation of MecA, since ComK acts as an activator of its own transcription, and an excess of MecA prevents this positive autoregulatory activity by binding ComK (Kong and Dubnau, 1994). To

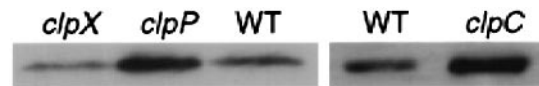


Fig. 2. Effect of *clp* mutations on the accumulation of MecA. Samples were taken from wild-type (WT), *clpX*, *clpC* and *clpP* competent cultures at t_2 , and were analyzed by Western blotting.

determine whether MecA accumulates in a *clpP* strain, extracts of cells grown to t_2 in competence medium were analyzed by Western blotting. Excess MecA accumulated in the *clpP* strain, as well as in the *clpC* strain (Figure 2). In contrast, a null mutation in *clpX* had no effect. Mutations in other ATPase/protease loci [*codW* (*clpY*), *codX* (*clpQ*)] and *lonA* also had no detectable effects on the level of MecA (not shown). These results suggest that ClpC and ClpP act to degrade MecA, possibly acting together in a ClpC–ClpP complex. Although inactivation of either *clpP* or *clpC* results in the accumulation of excess MecA, only the *clpP* mutation results in a failure to synthesize ComK. This is presumably because in the absence of ClpC the affinity of MecA for ComK is lowered (Turgay *et al.*, 1997), and the accumulated MecA therefore has little or no effect on the level of active ComK in the cell.

ClpP, ClpC and MecA are required *in vivo* for the degradation of ComK

Using a genetic background that avoids the inhibitory effect of MecA on ComK synthesis, we demonstrated that ClpP, as well as MecA and ClpC, were necessary for the *in vivo* degradation of ComK. To accomplish this, we used a strain carrying a *Tn10* insertion in the ComK-Box of the *comK* promoter (Luttinger *et al.*, 1996; Hamoen *et al.*, 1998), thereby completely disrupting transcription from the *comK* locus. An additional copy of the *comK* gene under the control of the inducible *Pxyl* promoter was placed in the chromosomal *amyE* locus. In this strain (BD2524), the expression of ComK is dependent on the presence of xylose but not on the *PcomK* promoter (Hahn *et al.*, 1996). BD2524 was grown in competence medium with xylose induction, and at hourly intervals samples were taken and processed for Western blot analysis. In this background, ComK was detected at t_0 and at t_1 but was barely detectable after t_2 (Figure 3). This is different from the wild-type situation in which the amount of ComK peaks at t_2 under the same conditions (not shown). ComK

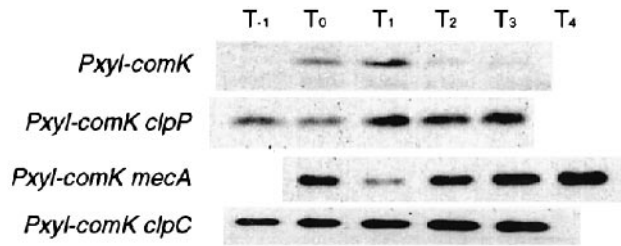


Fig. 3. Synthesis and stability of ComK in the *PxyI-comK comK::Tn10* background. *mecA*, *clpP* and *clpC* mutations were introduced into strain BD2524. The strains were grown to competence in the presence of xylose to induce *comK* transcription and samples taken at the indicated times were analyzed by Western blotting.

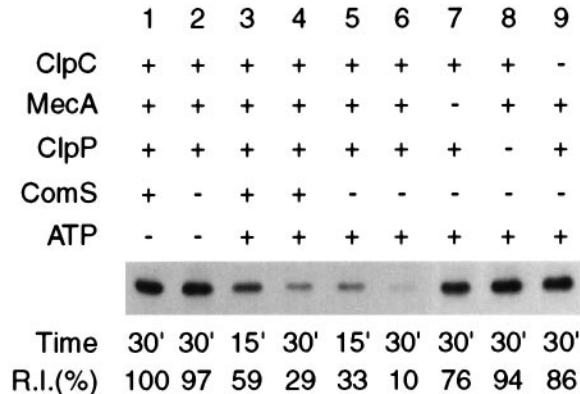


Fig. 4. Stability of ComK *in vitro*. ComK was incubated with or without ClpC (0.56 μ M), MecA (0.13 μ M), ClpP (0.62 μ M), ComS (1.3 μ M) and with an ATP-generating system as indicated and described in Materials and methods. After 15 or 30 min of incubation the samples were analyzed by Western blotting with anti-ComK antibodies. R.I. indicates the relative intensities of the bands, determined by scanning and densitometric analysis.

disappears earlier in the *PxyI* strain background because the *PxyI* promoter is weak and the net synthesis of ComK is low in this background (Hahn *et al.*, 1996). Mutations in *clpP*, *mecA* and *clpC* were introduced into the same xylose-inducible background and these strains were similarly grown and analyzed (Figure 3). The amount of ComK in these mutant strains was markedly increased and ComK was stabilized, consistent with the experiment presented in Figure 1. Mutations in *codW* (*clpY*), *codX* (*clpQ*) and *lonA* did not stabilize ComK in similar experiments (data not shown). We conclude that ClpP, as well as MecA and ClpC, is required for ComK degradation.

ClpCP degrades ComK, MecA and ComS *in vitro*

Using purified proteins, we next demonstrated *in vitro* that the ATP-dependent degradation of ComK requires MecA, ClpP and ClpC and is decreased in rate in the presence of ComS. To obtain ClpP protein we fused the *B.subtilis* *clpP* to a sequence encoding a C-terminal His₆ tag, and expressed this construct in *E.coli* for purification. When this fusion construct was integrated into the *B.subtilis* chromosome, it was able to substitute for a wild-type *clpP* gene for competence development (not shown).

We first tested the *in vitro* stability of ComK and the dependence of this stability on ClpP, MecA, ClpC, ComS and ATP. Degradation of ComK occurs *in vitro*, and is dependent on the presence of ATP (Figure 4, compare lane 2 with lane 6), ClpC (Figure 4, lanes 5 and 9), ClpP

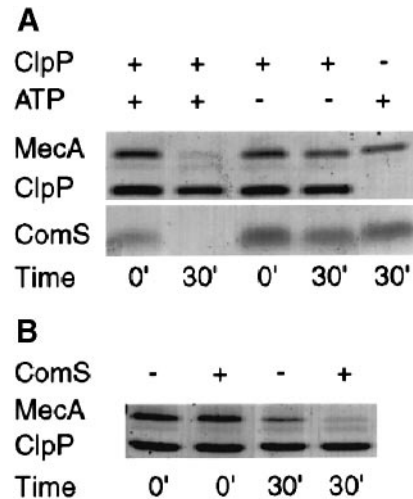


Fig. 5. Stability of MecA and ComS *in vitro*. (A) MecA (0.13 μ M), ClpC (0.6 μ M) and ComS (5.2 μ M) were incubated with or without ClpP (0.6 μ M) or ATP as indicated. (B) The same concentrations of MecA, ClpP, ClpC and ATP were incubated with and without ComS (1.3 μ M) as described in Materials and methods. Samples taken at the times indicated were analyzed by SDS-PAGE followed by staining with Coomassie Blue. The faint bands visible between MecA and ClpP are from the pyruvate kinase added with the ATP-generating system. When ATP was omitted [in (A)] the pyruvate kinase and phosphoenolpyruvate were still included. The data in (A) are from a single gel, but the irrelevant central portion was omitted.

(Figure 4, lanes 5 and 8) and MecA (Figure 4, lanes 5 and 7). The presence of ComS reduces the rate of degradation of ComK significantly (Figure 4, compare lanes 3 and 4 with 5 and 6). These results reflect those obtained *in vivo* with appropriate mutants.

Figure 5A and B presents an experiment testing the *in vitro* stabilities of MecA and ComS, and the dependence of their stabilities on ClpP, ComS and ATP. MecA is degraded if ComS, ClpP and ATP are present, but is stable if ClpP or ATP are absent. Figure 5B shows that the degradation of MecA is stimulated in the presence of ComS, although MecA is still degraded when ComS is omitted. The *in vivo* stabilities of ComK and MecA are therefore affected in opposite ways by the presence of ComS. In an independent experiment (not shown) we have found that when ClpC is omitted, MecA is also not degraded, as expected from the dependence of its degradation on ATP. In additional experiments in which pyruvate kinase and phosphoenolpyruvate were omitted, we have observed that ADP cannot replace ATP for the degradation of either ComK or MecA (not shown).

It is also apparent that ComS is degraded along with MecA (Figure 5A). This degradation requires MecA and ClpC (not shown), as well as ClpP and ATP. In several experiments we have noted that the rate of ComS degradation is greater than that of MecA (not shown).

Interaction of ClpP with ClpC

We reported previously that the addition of MecA to ClpC stimulates the ATPase activity of ClpC and that the addition of ComS causes a further stimulation, but only when MecA is present (Turgay *et al.*, 1997). ClpC by itself exhibits very low ATPase activity. We have examined the effect of ClpP on the ClpC ATPase in various combina-

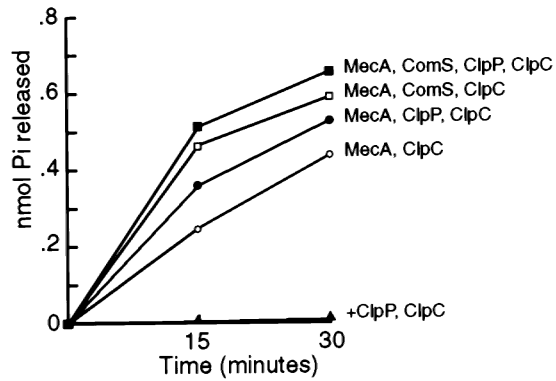


Fig. 6. ClpC ATPase activity. The ATPase activity of ClpC was measured in the presence of the proteins indicated in the figure present at the following concentrations: ClpC (0.56 μ M); MecA (0.26 μ M); ClpP (0.62 μ M); and ComS (1.3 μ M).

tions with ComS and MecA (Figure 6). When ClpP alone was added to ClpC, little ATPase activity was observed. However, when ClpP was added to ClpC in the presence of MecA or of MecA plus ComS, a further increase was observed over that observed when these components were added in the absence of ClpP. This is comparable to the results obtained with ClpAP in *E.coli*, as discussed below.

To explore further the interaction of ClpP and ClpC, we used immunoaffinity chromatography with ClpP columns to demonstrate the ATP-dependent interaction of ClpP with ClpC. This experiment demonstrated an interaction (not shown). Under the conditions of the experiment the binding of ClpP to ClpC was apparently not strong, but was ATP-dependent. It is likely that when attached to the column material ClpP cannot form an appropriate ring structure (Wang *et al.*, 1997), and that its binding to ClpC is therefore suboptimal. *In vitro* we have observed ATP-dependent proteolysis of MecA, ComS and ComK, dependent on the presence of ClpC and ClpP (Figures 4 and 5). We conclude that ClpC and ClpP must interact to form a proteolytic complex analogous to that formed by ClpA–ClpX and ClpP in *E.coli*.

Discussion

A decisive step leading to competence development in *B.subtilis* is the control of the activity, and consequently the synthesis, of the positively autoregulated competence transcription factor ComK. In this study we show that as a result of the explosive synthesis of ComK which begins at t_0 , the number of ComK tetramers increases to ~20 000 per cell, which can be compared to the content of the histone-like HU protein in *E.coli*. ComK has a high binding affinity for certain sites on DNA, and as is true of other DNA-binding proteins, also exhibits non-specific DNA binding (Hamoen *et al.*, 1998). We have observed by immunofluorescence with anti-ComK antibodies that ComK is associated with the nucleoid of competent cells (B.J.Haijema and D.Dubnau, unpublished). ComK is known to bend DNA in the vicinity of its binding sites (Hamoen *et al.*, 1998). The large number of ComK molecules bound to the nucleoid DNA (potentially ~1 tetramer per 200 base pairs) may not only cause the activation of transcription of the late competence, recombination and DNA repair genes, but may also have structural

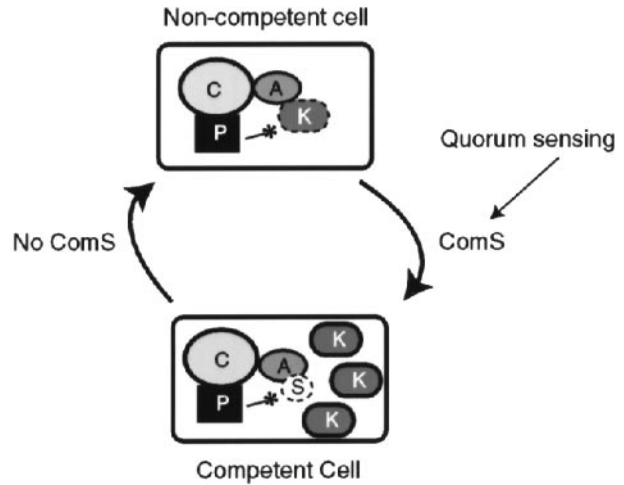


Fig. 7. Model for regulation of competence by proteolysis. In the non-competent cell any ComK (K) synthesized is bound to MecA (A) and targeted for degradation by ClpCP. ComS (S) is synthesized as a consequence of the quorum-sensing pathway and binds to MecA, altering its conformation and resulting in the release of ComK. As a result ComK is stabilized and ComS degradation is accelerated. Instability is indicated by dashed lines and proteolysis by a line terminated by an asterisk.

consequences for the chromosomes, and ComK binding might directly cause the replication arrest and transcriptional silencing characteristic of competent cells (Nester and Stocker, 1963; McCarthy and Nester, 1967).

Proteolysis during the escape from competence

Clearly, the synthesis of ComK has a profound effect on the physiology of competent cells and these cells can only escape competence when MecA is present (Hahn *et al.*, 1995). The present study demonstrates that ComK is degraded as the cells escape from competence and that MecA is needed to target ComK for this degradation. We have demonstrated *in vitro* that the degradation of ComK is accomplished by ClpC and ClpP, and that this degradation is ATP-dependent. ComS, which is known to cause the release of ComK from binding to ClpC and MecA (Turgay *et al.*, 1997), protects ComK from degradation both *in vivo* and *in vitro*. In addition, MecA and ComS are themselves degraded by ClpCP *in vitro*, in the presence of ATP. ComK degradation is needed for the escape from competence to rid the cell of this toxic molecule. However, it is probable that an interruption of ComK synthesis plays a part in this escape. It was noted above that ComK degradation was also observed in the absence of added antibiotics, suggesting that ComK synthesis was essentially turned off. This inference is supported by experiments using an in-frame *comK-lacZ* fusion, which demonstrated a marked decrease in the rate of *comK* expression at $\sim t_2$ (Hahn *et al.*, 1994). Once the rate of ComK degradation exceeds its rate of synthesis, the ComK level in the cell will continue to decline, because ComK is needed for its own transcription.

Proteolysis during the development of competence

These observations add an important new dimension to the regulation of competence (Figure 7). During exponential growth, any ComK synthesized is bound by MecA and

ClpC, and is thereby prevented from acting as a transcription factor (Turgay *et al.*, 1997). The present results suggest that the bound ComK is also targeted for degradation by ClpCP. In the *Pxyl-comK clpP* strain, a clear ComK signal appears before t_0 , whereas in the *clpP*⁺ strain a signal is not apparent until t_0 (Figure 3). In addition, we have observed that in the *Pxyl-comK* background, the mutational inactivation of *clpP* raises the expression of a *lacZ* fusion to the late competence gene *comG* during exponential growth (not shown). Since *comG* transcription is ComK-dependent, this experiment shows that the *clpP* strain contains an excess of functional ComK. These observations demonstrate that in this background, in the *clpP* mutant, an increased amount of ComK is present before t_0 . This implies that the degradative pathway is normally needed to fully restrain the synthesis of ComK prior to t_0 .

At, or just before t_0 , the quorum-sensing pathway induces the synthesis of ComS (D'Souza *et al.*, 1994; Hamoen *et al.*, 1995). The presence of ComS prevents the binding of ComK to complexes containing MecA and ClpC. ComS appears to act by binding directly to MecA (Liu *et al.*, 1996; Turgay *et al.*, 1997) presumably altering the conformation of MecA and reducing its affinity for ComK. When ComS binds to MecA, the rate of ComK degradation by ClpCP decreases, because ComK is released (Figure 7). More ComK is now available to activate the transcription of its own gene, resulting in the synthesis of additional active ComK, until it is present in excess over MecA and ClpC. At this point the synthesis of ComK is free to increase dramatically. In this mechanism the degradation rate of ComK is adjusted in accordance with the input concentration of ComS, but ComK synthesis is regulated in a switch-like fashion, due largely to the positive autoregulation of ComK transcription.

Although ClpCP is needed to prevent the accumulation of excess MecA (Figure 2), it is not certain that the enhanced degradation of MecA in the presence of ComS is a regulatory process. On the other hand, the failure of the *clpP* strain to synthesize ComK suggests that the proteolytic role of ClpCP is necessary to restrain the accumulation of MecA. An alternative explanation, that in a *clpP* mutant, *mecA* transcription is somehow increased, was refuted by the use of a *mecA-lacZ* transcriptional fusion (Msadek *et al.*, 1998).

The *in vitro* degradation of ComS by ClpC and ClpP in the presence of MecA and ATP is highly suggestive. We believe that this represents a timing mechanism, placing a limit on the synthesis of ComK. The degradation of ComS would ensure that as the level of active ComK increases, ComS decreases in parallel. As the ComS level decreases, ComK will be increasingly rebound by ClpCP-MecA and targeted for degradation (Figure 7). We have obtained independent evidence that ComS is quite unstable *in vivo* (not shown), consistent with this hypothesis and with our *in vitro* data.

Protein-protein interactions and substrate recognition during proteolysis

Since the addition of ClpP to ClpC does not stimulate its very low intrinsic ATPase activity (Figure 6B), the interaction of the ATPase subunit with ClpP is clearly different from its interaction with potential substrates.

This can be compared to the result obtained in the *E.coli* system; when ClpP was added to ClpA, a decrease in ATPase activity was noted (Hwang *et al.*, 1988). In the presence of ClpP, the behavior of the ClpC ATPase upon the addition of MecA alone or in combination with ComS was similar to that described previously in the absence of ClpP (Turgay *et al.*, 1997), except that a further stimulation was observed. When ClpP is absent, the stimulation of ClpC ATPase upon addition of potential substrates probably reflects the chaperone-like activity of ClpC. In the absence of ClpP this activity is in a sense futile, since it does not lead to degradation. The additional stimulation noted when ClpP was present may therefore reflect the coupling of ATP hydrolysis to degradation. The stimulation of the ATPase activity of ClpCP when MecA alone or in combination with ComS was added is comparable to the published results obtained with ClpP and ClpA when the substrate casein was present (Hwang *et al.*, 1988).

The *B.subtilis* ClpP not only associates with ClpC, but also with ClpX (Y.-I.Kim and T.Baker, personal communication) and possibly with ClpE (Msadek *et al.*, 1998). This may explain the wide range of phenotypes of a *clpP* strain, affecting heat shock, motility, degradative enzyme synthesis, sporulation and competence (Gerth *et al.*, 1998; Msadek *et al.*, 1998).

MecA recognizes ClpC, ComK and ComS, and is needed to target the latter two proteins for degradation by ClpCP. MecA therefore diverts the proteolytic activity of ClpCP from its role in general stress management functions, such as degrading aggregated or misfolded proteins, to a regulatory function in competence development. MecA connects signaling via the quorum-sensing system with the regulation of ComK degradation. In this way the quorum-sensing two-component signal-transduction cascade modulates the stability of a key regulatory protein. It has been shown *in vivo* in *E.coli* that the degradation of σ^s by ClpXP is influenced by the response regulator SprE (RssB). SprE modulates the degradative activity of ClpXP toward σ^s in response to environmental signals (Zhou and Gottesman, 1998).

The use of a specific targeting molecule such as MecA seems to be unique at least among the prokaryotic proteolysis systems. In *E.coli*, a wide variety of substrate proteins for the ATP-dependent proteases ClpAP, ClpXP, Lon and FtsH have been identified (Gottesman *et al.*, 1997). It has been demonstrated that some of these have C-terminal sequences which can be recognized directly by ClpA or ClpX (Levchenko *et al.*, 1997) and the 'N-end rule' has also been implicated in targeting degradation by ClpAP (Tobias *et al.*, 1991). Substrate recognition by the eukaryotic proteasome occurs by a variety of mechanisms. The eukaryotic 26S proteasome is built from multiple protein subunits, including a 19S regulatory complex, which contain components necessary for protein substrate binding. Some of these components may play a role in targeting analogous to that of MecA and ClpC. The main substrate proteins for the proteasome are targeted for degradation by ubiquitination although other recognition mechanisms exist (Coux *et al.*, 1996).

Proteolysis and bacterial development

The competence system is one of three bacterial systems in which degradation of a key regulatory molecule is

Table II. Strains

Strain	Genotype	Source or reference
<i>Escherichia coli</i> :		
SG22189	MC4100 $\Delta clpA$ <i>clpP::cat malP::lacI^Q</i>	S.Gottesman
<i>Bacillus subtilis</i> :		
IS75	<i>hisB2 leu-8 metB5</i>	I.Smith
BD2123	<i>hisB2 leu-8 metB5 mecA::spc amyE:: (comG-lacZ cat)</i>	Kong and Dubnau (1994)
BD2243	<i>hisB2 leu-8 metB5 clpC::spc (comG-lacZ cat)^a</i>	Kong and Dubnau (1994)
BD2524	<i>hisB2 leu-8 metB5 (comK::Tn10 cat) (comG-lacZ kan)^a amyE:: (PxylA-comK xylR cat::spc)</i>	Hahn <i>et al.</i> (1996)
BD2528	<i>hisB2 leu-8 metB5 (multicopy comS plasmid)</i>	Hahn <i>et al.</i> (1996)
BD2571	<i>trpC2 unk::spc $\Delta codX$</i>	Slack <i>et al.</i> (1995)
BD2572	<i>trpC2 unk::spc</i>	Slack <i>et al.</i> (1995)
BD2573	<i>trpC2 unk::spc $\Delta codW$</i>	A.L.Sonenshein
BD2574	<i>hisB2 leu-8 metB5 clpX::spc</i>	A.Grossman
BD2590	<i>hisB2 leu-8 metB5 clpP::cat</i>	this work
BD2594	<i>hisB2 leu-8 metB5 amyE:: (comK-lacZ cat::spc)</i>	this work
BD2595	<i>hisB2 leu-8 metB5 clpP::cat amyE:: (comK-lacZ cat::spc)</i>	this work
BD2597	<i>hisB2 leu-8 metB5 clpX::spc amyE:: (comK-lacZ cat)</i>	this work
BD2600	<i>hisB2 leu-8 metB5 mecA::erm clpP::cat amyE:: (comK-lacZ cat::spc)</i>	this work
BD2665	<i>hisB2 leu-8 metB5 lonA::cat (comK::Tn10 cat::spc) (comG-lacZ kan)^a amyE:: (PxylA-comK xylR cat::erm)</i>	Riethdorf <i>et al.</i> (1994); this work
BD2666	<i>hisB2 leu-8 metB5 clpX::spc (comK::Tn10 cat) (comG-lacZ kan)^a amyE:: (PxylA-comK xylR cat::erm)</i>	this work
BD2667	<i>hisB2 leu-8 metB5 clpP::cat (comK::Tn10 cat::spc) (comG-lacZ kan)^a amyE:: (PxylA-comK xylR cat::erm)</i>	this work
BD2668	<i>trpC2 unk::spc $\Delta codX$ (comK::Tn10 cat) amyE:: (PxylA-comK xylR cat::erm)</i>	this work
BD2669	<i>trpC2 unk::spc $\Delta codW$ (comK::Tn10 cat) amyE:: (PxylA-comK xylR cat::erm)</i>	this work
BD2676	<i>trpC2 unk::spc (comK::Tn10 cat) amyE:: (PxylA-comK xylR cat::erm)</i>	this work
BD2674	<i>hisB2 leu-8 metB5 mecA::kan (comK::Tn10 cat) amyE:: (PxylA-comK xylR cat::erm)</i>	this work
BD2711	<i>hisB2 leu-8 metB5 (comK-gfp cat)^a</i>	B.-J.Hajjema and D.Dubnau
BD2725	<i>hisB2 leu-8 metB5 (comK-gfp cat)^a (clpP his₆ clpP::kan)</i>	this work
BD2726	<i>hisB2 leu-8 metB5 clpC::spc (comK::Tn10 cat) (comG-lacZ kan)^a amyE:: (PxylA-comK xylR cat::erm)</i>	this work

^aThese constructs were chromosomally inserted by Campbell-like (single-reciprocal) recombination. In all cases these were non-inactivating recombination events resulting in the construction of *comG*⁺ or *comK*⁺ strains.

known to control a developmental program. In each case development yields two distinct cell types. During sporulation in *B.subtilis*, the mother-cell-specific sigma factor σ^E is degraded in the forespore compartment, and a regulatory phosphatase, required for the activation of the forespore sigma factor σ^F is preferentially degraded in the mother-cell compartment (Pogliano *et al.*, 1997). The proteases involved in these processes have not been identified and the nature of the regulatory mechanism has not been elucidated, although compartment-specific proteolysis requires the chromosome transport protein SpoIII^E. The activity of the transcriptionally active response regulator CtrA, which inhibits DNA replication in *Caulobacter crescentus*, is regulated by phosphorylation and the level of CtrA in the cell is regulated by proteolysis (Domian *et al.*, 1997; Quon *et al.*, 1998). In this case ClpXP is responsible for the proteolysis, which occurs in the portion of the pre-divisional cell destined to give rise to the stalked daughter. Although the nature of the mechanism that controls proteolysis is not yet known, additional factors that regulate proteolysis were postulated (Domian *et al.*, 1997). Such molecules might have roles analogous to those of MecA or ComS.

Competence also involves the generation of two cell types, characterized by the differential synthesis and accumulation of a regulatory molecule. Since overproduction of ComK (in a *mecA* strain) causes all the cells in a population to follow the competence developmental pathway, it is logical to infer that the synthesis of ComK

is a competence-determining event. As in the cases of sporulation and *Caulobacter* development, the crucial molecule is expressed in a cell-type-specific manner. Overexpression of ComS results in a shift of the developmental program in the direction of competence in most cells in a population (M.Albano and D.Dubnau, unpublished; Hahn *et al.*, 1996; Liu *et al.*, 1996). Differential transcription of *comS* is not the basis of this developmental decision, since its promoter is transcribed in all the cells of a pre-competent population (Hahn *et al.*, 1994). It is possible that differential degradation of ComS may be a cell-type-determining event in competence development.

Materials and methods

General methods

DNA manipulations and standard molecular biological methods were as described in Sambrook *et al.* (1989). Growth of *B.subtilis* in competence medium, transformation and β -galactosidase activity measurements were as previously described (Albano *et al.*, 1987). The strains used are described in Table II. Electrophoresis was carried out with standard SDS-polyacrylamide (Laemmli, 1970) or Tricine gels (Schagger and von Jagow, 1987). Protein was determined with Bio-Rad reagents (Bradford, 1976). Western blotting was as described previously (Kong and Dubnau, 1994). For signal quantitation, films were scanned and analyzed densitometrically.

Strain construction and protein purification

An internal *clpP* fragment was amplified by PCR from chromosomal IS75 DNA using the primers P1 (5'-CGG AAT TCA TTG AAC AAA CGA AAC CGC-3') and P2 (5'-CGG AAT TCT TCA CTG TTT GGA

AGC GC-3'). This fragment was cloned into the *EcoRI* site of pUCCM18 (Inamine and Dubnau, 1995), resulting in pED221. The *clpP* strain BD2590 was constructed by transforming pED221 into IS75, leading to a disruption of *clpP*.

clpP was amplified by PCR from chromosomal IS75 DNA using the primers QC1pPSph (5'-GGA GGC AGC ATG CAA TTA ATA CCT ACA GTC-3') and QC1pPBam (5'-GTG GGA TCC CTT TTT GTC TTC TGT GTG AG-3'). The product was cloned into pQE70 (Qiagen) using the underlined *BamHI* and *SphI* sites. The structure of the resulting plasmid (pClpP11) was verified by sequencing. pClpP11 was introduced together with pRep (Qiagen) into SG22189 (Table II). The resulting strain was used to express and purify ClpP-His₆ as described previously for MecA purification (Turgay *et al.*, 1997). To test the function of the ClpP-His₆ fusion in *B. subtilis*, the last 394 bp of the *clpP* gene, including the sequence encoding the His₆-tag fusion, were cloned in a pUC18 plasmid carrying a kanamycin-resistance cassette. This construct, pK1, was used to transform *B. subtilis* BD2711 (Table II), to achieve a Campbell-like integration at the *clpP* locus, inactivating the resident *clpP* gene and placing the *clpP*-his₆ fusion under the control of the *clpP* promoter. The resulting strain BD2725 was competent and expressed ComK-green fluorescent protein (GFP) at the wild-type level (not shown). ClpC, MecA, ComK and ComS were obtained as described previously (Turgay *et al.*, 1997).

Calibrated Western blotting

All antibodies (Kong and Dubnau, 1994; Turgay *et al.*, 1997) were immunoaffinity purified on protein-coupled Affigel-15 columns (Harlow and Lane, 1988). *Bacillus subtilis* IS75 cells were grown in competence medium (Albano *et al.*, 1987) to *t*₀, *t*₁ and *t*₂. To determine the number of cells in these samples, an aliquot was fixed with 0.37% formaldehyde and counted in a Petroff-Hauser chamber. The cells were lysed in a French pressure cell and the extracts were boiled for 5 min in sample buffer (2% SDS, 125 mM Tris pH 6.8, 5% β-mercaptoethanol, 15% glycerol, 0.005% bromophenol blue) and analyzed by Western blotting. On each gel, known amounts of purified ClpC, ComK or MecA were included to construct a standard curve. The concentrations of the protein stock solutions were determined by amino acid analysis (Protein Chemistry Facility, Columbia University).

Determination of in vivo ComK and MecA stabilities

The *B. subtilis* strains were grown in competence medium to *t*₂ and diluted 1:20 into fresh competence medium with the addition of tetracycline (50 μg/ml) and rifampicin (5 μg/ml). The cultures were incubated at 37°C and 50 ml samples were taken. The cells were centrifuged, resuspended in 1 ml STM (50 mM NaCl, 25% sucrose, 50 mM Tris-HCl pH 8, 5 mM MgCl₂), washed and resuspended in STM + lysozyme (300 μg/ml). The cells were protoplasted by incubation at 37°C for 10 min. Sample buffer was added and the samples were boiled for 5 min. The remaining extract was used to determine protein concentration. The protein extracts (20 μg/lane) were analyzed by Western blotting. ComK was also investigated in the BD2524 background. In this case samples were harvested hourly during growth in competence medium containing xylose (2% w/v) and treated as described for the previous experiment. To test the stability of MecA in the wild-type and mutant strains, cells were harvested at *t*₂. Extracts were prepared and analyzed as described above.

In vitro degradation and ATPase assay

To determine the *in vitro* stability of ComK we used buffer A (100 mM KCl, 25 mM MOPS pH 7.0, 5 mM MgCl₂, 0.5 mM DTT) containing 4 mM ATP and 2 mM PEP with 1.17 μM pyruvate kinase (Boehringer Mannheim). The assay was performed in final volumes of 50 μl and incubation was at 37°C. Seventeen microliters of 4× sample buffer was added to stop the reaction, the mixture was boiled for 5 min, and 3 μl was applied to a 12% SDS-polyacrylamide gel and analyzed by Western blotting.

The *in vitro* stabilities of MecA and ComS were determined in buffer A containing 4 mM ATP, 2 mM PEP and 1.17 μM pyruvate kinase. The reaction volumes were 50 μl and samples were taken as described above for the ComK analysis. In the experiment shown in Figure 5, 10 μl aliquots were run on a Tricine (Figure 5A) or a 12% SDS-polyacrylamide gel (Figure 5B) and stained with Coomassie Blue.

The ATPase assays were carried out in buffer A as described previously (Turgay *et al.*, 1997).

ClpP-ClpC interaction

ClpP (2 mg/ml) was dialyzed against 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄ and crosslinked to activated Affigel-

10 (Bio-Rad) as recommended by the manufacturer. Two identical 200 microliter columns were poured with this material and washed extensively with buffer A with and without 2 mM ATP. ClpC (0.93 μM) in 200 μl of buffer A with and without ATP was loaded on each column and incubated for 80 min at room temperature. Two-hundred microliters of buffer A was applied as the first wash, with or without ATP. The columns were then washed with a total of 5 ml of buffer A with or without ATP. A final additional wash of 200 μl was collected. Three 200 μl aliquots of the elution buffer (500 mM NaCl, 5 mM EDTA) were then applied and the eluates were collected. The columns were washed with an additional 1 ml of elution buffer, and then with a final aliquot of 200 μl which was collected. Ten microliters of each sample were analyzed by Western blotting.

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