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Bistability in *the Bacillus subtilis* K-state (competence) system requires a positive feedback loop

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Summary

High expression of the transcriptional activator ComK occurs in 10–20% of the cells in stationary phase cultures of *Bacillus subtilis* strain 168. ComK drives the expression of more than 100 genes constituting the semidormant K-state, distinct from sporulation and vegetative growth. Among the genes so activated are those that permit competence for genetic transformation. We have addressed the origin of bistability in expression of ComK. We show that bistability requires positive autoregulation at the promoter of *comK*, but not a potential toggle switch, in which ComK represses the promoter of *rok* and Rok represses the promoter of *comK*. We further address the source of the noise that results in the stochastic selection of cells that will express *comK*. A revised model for the regulation of *comK* expression is proposed that partially explains bistability.

Introduction

Phenotypic population heterogeneity and, in some cases, cell fate may be viewed as noise-driven processes (reviewed in Rao *et al*., 2002). The soil bacterium *Bacillus subtilis* responds to environmental stress with an arsenal of survival strategies, some of which are expressed heterogeneously and are likely to be probabilistically invoked. *B. subtilis* can become motile, secrete degradative enzymes or antibiotics, produce spores or become competent for genetic transformation. The heterogeneity during competence development will be considered in this report.

In *B. subtilis*, competence is part of a physiological state, the K-state (Berka *et al*., 2002), distinct from vegetative growth and sporulation, that develops during late exponential growth under specific nutritional conditions. Remarkably, competence is expressed in only 10–20% of the cells in laboratory strains (Nester and Stocker, 1963; Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968). Transcriptional profiling has revealed that well over 100 genes in this distinct subpopulation, many of which have no obvious role in transformation, are induced concomitantly with the development of transformability (Berka *et al*., 2002; Hamoen *et al*., 2002; Ogura *et al*., 2002). It has been proposed that the competent cells have embarked on a survival strategy, of which transformability is only one feature. This competence-associated survival mode has been called the K-state (Berka *et al*., 2002), because it depends on the synthesis of the master regulator, ComK (van Sinderen *et al*., 1995). As there are two clearly distinguishable cell types, competent and non-competent, we find it useful to describe this system as exhibiting bistability. This is not a mathematically rigorous usage, in part because the expression of competence occurs during the transition to stationary phase when the system is not at steady state. However, the term ‘bistability’ is a useful approximation, leading to the consideration of mechanisms that have been invoked elsewhere to explain true bistability.

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Theoretical studies have described two such regulatory arrangements that can exhibit bistability (reviewed in Ferrell, 2002). In the first, cell-signalling systems containing positive feedback loops together with a cooperative (nonlinear) response to an activator can exhibit noise-driven bistability. Only those random cells with higher than average concentrations of the activator initiate the loop. In the second arrangement, a system containing two mutually repressing negative feedback loops can lead to population heterogeneity. In this case, random cells with a high concentration of the first repressor, will have a decreased amount of the second one, leading to the expression of a set of target genes. Artificial bistable systems of these two types have been engineered in *Escherichia coli* and *Saccharomyces cerevisiae* and their properties have been explored (Gardner *et al.*, 2000; Becskei *et al.*, 2001). In fact, a naturally occurring bistable system involving a positive feedback loop was studied nearly 50 years ago in *E. coli* (Novick and Weiner, 1957).

In *B. subtilis,* genes encoding proteins required for the binding and uptake of DNA are transcribed in the presence of the positively acting transcription factor ComK (van Sinderen *et al.*, 1995), which is synthesized only in the competent (K-state) fraction of cells (van Sinderen and Venema, 1994; Haijema *et al.*, 2001). ComK synthesis, in turn, is regulated by a complex series of reactions that involve quorumsensing, as well as controls at the transcriptional and post-transcriptional levels (summarized in Fig. 1A, reviewed in Hamoen *et al.*, 2003a). ComK is required for the transcription of its own gene and binds to the promoter of *comK* (P_{comK}) as a dimer of dimers (Hamoen *et al.*, 1998). The system therefore contains positive feedback and involves a non-linear response at the promoter of *comK*.

Additional proteins bind to the promoter of *comK* and act as activators or repressors. DegU increases the affinity of ComK for P_{comK} (Hamoen *et al.*, 2000), whereas AbrB, CodY and Rok bind to P_{comK} and act as repressors (Serror and Sonenshein, 1996; Hoa *et al.*, 2002; Hamoen *et al.*, 2003b). AbrB must be maintained within a narrow window of concentrations (Hahn *et al.*, 1995a), as it acts to repress both the promoters of *comK* and *rok* (Hoa *et al.*, 2002). In addition to this transcriptional regulation, the stability of ComK is precisely controlled (Turgay *et al.*, 1997; Turgay *et al.*, 1998). Prior to the onset of stationary phase, ComK is bound by the adapter protein MecA, which targets it for degradation by the ClpC-ClpP protease. As cell density increases during growth and the cells approach stationary phase, a process initiated by quorum-sensing (Solomon *et al.*, 1995) leads to the synthesis of the small protein ComS, which binds to MecA, preventing the targeting of ComK for degradation.

As noted in this brief summary, a positive feedback loop exhibiting a non-linear response to the cellular concentration of ComK is central to competence regulation. The essential features of a bistable system of the first type are therefore present. However, the features required for a toggle switch mechanism are also apparently present. It has been shown that Rok represses P_{comK} and that ComK represses P_{rok} (Hoa *et al.*, 2002). This arrangement hints at the possible existence of a bistable system of the second type (Fig. 1B). In this model, cells with higher concentrations of ComK would repress *rok* expression and therefore produce even more ComK. When a threshold is passed, this situation would become ‘irreversible’ and the cells would enter the competent state. Conversely, cells with less ComK would express more *rok* and remain in the non-competent state.

Although these qualitative features are suggestive, their function in the cell depends on the relevant binding and rate constants, on the concentrations of active proteins and on the cell-to-cell variations (noise) in these concentrations. In other words, the mere presence of reciprocally acting repressors or of a positive feedback loop with nonlinearity does not prove

that these features are responsible for bistability, and an experimental approach to decide this question is required.

Here we show that ComK autoregulation is necessary for bistability. We also show that the mutually repressing Rok–ComK interactions are probably not major factors in establishing bistability. Finally, we discuss the possible sources of the noise that presumably determines which cells express the K-state. A similar study has independently concluded that the ComK autoregulatory loop is needed for bistability (Smits *et al.*, 2005).

Results

Bistability in competence development

To study the heterogeneity of cultures expressing the K-state, we utilized an in frame fusion of the *comK* promoter, ribosomal binding site and first seven amino acid residues to the green fluorescent protein (GFP). Transcription from this promoter is completely dependent on the binding of ComK itself (van Sinderen and Venema, 1994). Hence, the fluorescence signals exhibited by cells containing this fusion provide a readout for the level of active ComK and of the K-state, as this transcription factor is both necessary and sufficient for induction of K-state genes (Hahn *et al.*, 1996). We have shown elsewhere that the cells expressing *comK-gfp* are the ones expressing the competence protein *comEA* (Haijema *et al.*, 2001) and are also the cells binding and internalizing DNA (J. Hahn, B. Maier and D. Dubnau, unpubl.). For the experiment shown in Fig. 2, which illustrates bistability, the *comK-gfp* fusion was placed in the *comK* locus by Campbell-like recombination, placing both the fusion and an intact copy of *comK* under control of the native promoter (Fig. 2A). In this strain (BD2711, Haijema *et al.*, 2001), all the other relevant regulatory genes remain intact.

At T_0, the time of transition to stationary phase, nearly all of the cells in the culture exhibited near background levels of fluorescence (Fig. 2B), characteristic of strains lacking a GFP reporter. At 30 and 60 min following the end of exponential growth, a number of cells with intermediate levels of fluorescence were evident (Fig. 2B), although the high-expressing class is also clearly represented. Many of these are likely to be cells that are approaching the maximal expression level. Later, at T_2, the cellular distribution of fluorescent intensities was clearly bimodal, with 11% of the cells in a high-expressing subpopulation. In several independent experiments using this strain, the total high-expressing class consisted of 10–20% of the total population. Within the lower intensity class of cells at T_2, a fraction (about 7.5% of the total) exhibited fluorescence higher than the background (Fig. 2B). This fraction may consist of cells that will go on to enter the high-expressing class, which would then comprise about 18.5% of the total population. It is also possible that these cells represent a distinct subgroup and that the system is more complex than a two-state model would assume.

This experiment demonstrates that the expression of *comK,* and hence of the K-state, is bistable and that ComK-GFP fluorescence provides a suitable readout for the study of bistability.

Positive autoregulation of comK is essential for bistability

We next asked if the positive autoregulatory loop is essential for bistability. For this, we placed a copy of *comK* under control of the Phyperspank (P_{hs}) promoter, at the ectopic *amyE* locus. This promoter (kindly provided by Dr David Rudner, Harvard Medical School, Boston, MA), is inducible by isopropyl-β-D-thiogalactoside (IPTG) and exhibits stronger induction and less leakiness than previous versions of IPTG-inducible promoters for use in *B. subtilis*. At the *comK* locus, we placed two different constructs. In the first (Fig. 3A), an

intact copy of *comK* driven by its native promoter was present, together with the same *comK-gfp* fusion used for BD2711 (Fig. 2A). This strain retains a positive feedback loop, because expression of the *comK* copy at the native locus activates its own transcription. The second strain (Fig. 3B) was isogenic with this one, but the copy of *comK* at the native site was inactivated by insertion of a kanamycin-resistance cassette. This strain does not exhibit positive auto-regulation as it lacks an intact copy of *comK* driven by the promoter of *comK.* As a copy of *comK* under P_{hs} control is present in both strains, it is possible to prime the system by the addition of the inducer, IPTG. Most important, the use of this inducible promoter provided active ComK in the absence of autoregulation. In the strain that retains the positive feedback loop, growth to T_2 in the absence of IPTG permits the expression of GFP fluorescence in 16–18% of the cells, demonstrating that the normal regulatory machinery remains intact and functional (not shown).

Figure 4 presents the results of a typical experiment in which the two strains were induced during logarithmic growth with varying concentrations of IPTG and harvested for microscopy 30 min later, prior to T_0. At this time, in the wild-type strain (BD2711), no fluorescent cells were detectable (Fig. 2B), demonstrating that the fluorescent signals presented in Fig. 4 reflect the presence of the P_{hs}–*comK* construct. The upper panels (Fig. 4A–D) show superimposed fluorescent and bright field images of cells from the control strain that lacks the positive loop, as well as frequency distributions of fluorescent intensities in the population (Fig. 4E). At intermediate IPTG concentrations (Figs 4B and C), nearly all of the cells were fluorescent, with intensities that were intermediate between those of the uninduced cells and those induced with higher IPTG concentrations. In other words, as the concentration of IPTG was increased, the cells responded in a graded manner, exhibiting a unimodal population response.

When the autoregulatory loop was intact however, a strikingly different pattern was evident. Most notably, the cells responded in a distinctly bimodal fashion, qualitatively similar to the wild-type response shown in Fig. 2B. It is also evident that this strain exhibited a hypersensitive response to IPTG, compared to the control lacking the autoregulatory loop. In the absence of IPTG, occasional cells with near maximal fluorescence signals were observed and these were not seen in the control strain without the loop. These fluorescent cells may be seen both in the representative micrograph (Fig. 4F) as well as in the frequency distribution (Fig. 4J). In fact, 1.2% of the cells exhibited a near maximal level of fluorescence in the absence of IPTG. This expression may be as a result of the slight leakiness of the P_{hs} promoter. We conclude from these observations that K-state bistability requires feedback regulation of *comK*. A similar conclusion, regarding the need for auto-stimulation at the promoter of *comK*, has been reached independently (Smits *et al.*, 2005). We cannot conclude, however, that the positive autoregulatory loop rather than a toggle switch is at work, because the control construct depicted in Fig. 3B also eliminates the toggle switch configuration shown in Fig. 1B. This is because Rok presumably cannot repress at P_{hs}.

Influence of regulatory factor mutations on K-state development. Is the toggle switch required?

To gain further insight into the roles of various regulatory genes in the regulation of *comK*, and to test whether the toggle switch plays a role in bistability, we have introduced knockout mutations in regulatory genes and determined their effects on *comK* expression. For measurements in bulk cultures, we used a *comK–lacZ* reporter, inserted ectopically at *amyE*. To determine the effect on bistability, we utilized the *comK–gfp* fusion described above, in the strain BD2711 background (Fig. 2A). Figure 5A presents the results of β-galactosidase measurements in samples withdrawn during growth. Inactivation of the repressor genes (*codY* or *rok*) had little or no effect prior to T_0, but increased the final level of β-galactosidase in the culture. Even a double *codY rok* mutant did not result in the premature

expression of *comK–lacZ*, although it further increased the plateau level of β-galactosidase expression (T.T. Hoa and D. Dubnau, unpubl.). Overexpression of *degU* on a multicopy plasmid had little or no effect on the plateau level, again without any detectable effect before T_0. These results were entirely confirmed using the *comK–gfp* fusion; representative microscopic fields are shown in Fig. 5B–E and G–J. At T_2, in a typical experiment, the fractions of cells expressing ComK-GFP in the wild-type, *rok*, *codY* and *degU* overexpressing strains were 10 ± 2%, 60± 11%, 41 ± 18% and 10 ± 4% respectively.

A markedly different result was obtained when *comS* was overexpressed on a multicopy plasmid (Fig. 5A and Hahn *et al.*, 1995b). In this case the timing of expression was dramatically altered, with substantial β-galactosidase activity and frequent fluorescent cells detected before T_0 (Fig. 5F). At T_{-1}, T_0 and T_2, the percentage fluorescent cells were 10%, 28% and 72% respectively. Similar results have been obtained with inactivation of *mecA* and *clpC*, two other genes on the pathway that regulates ComK stability (not shown and Hahn *et al.*, 1995b; Hoa *et al.*, 2002).

We conclude that CodY, Rok and DegU do not affect the timing of competence development, but that the levels of CodY and Rok in the wild-type strain act negatively to limit the final fraction of the population that expresses ComK. The fact that *rok* and *codY* mutants still exhibit bimodal distributions in *comK-gfp* expression, shows that these genes are individually not required for bistability and in the case of *rok*, provides strong evidence that the putative toggle switch is not needed.

The putative toggle switch is not required for bistability in strain BD4010

To further test whether the toggle switch contributes to bistability, we introduced a knockout of *rok* into the strain described in Fig. 3A that retains the positive autoregulatory loop (BD4010). Figure 6 demonstrates that in this strain, the bimodal response is retained in the absence of Rok. Furthermore, the response to IPTG is hypersensitive compared to the results seen with BD4010, because of the absence of Rok-repression at the promoter of *comK*. This experiment shows that Rok is not required for bistability, in this synthetic construct. This result supports the observations reported in Fig. 5 and further suggests that the ComK autoregulatory loop rather than the postulated toggle switch mechanism is essential for bistability.

We have also asked whether Rok and CodY might somehow function redundantly to effect a toggle switch-like mechanism, although this seems unlikely, as there is no evidence that ComK can act at the promoter of *codY*. To test this possibility, we have examined the fluorescence of a *rok codY comK-gfp* strain (BD4042) grown to T_2. In four experiments with this strain, an average of 78 ± 11% of the cells were fluorescent, demonstrating that even in this double knockout strain, *comK-gfp* is expressed heterogeneously.

Discussion

It has been known for many years (Nester and Stocker, 1963; Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968) that competence in the domesticated laboratory strains of *B. subtilis*, is expressed in 10–20% of the cells in a given culture (Fig. 2B). In natural isolates of *B. subtilis*, the fraction of cells expressing competence is markedly lower than this, presumably because these strains have not been artificially selected for high transformability. In one such isolate, only about 1% of the cells express a *comK–gfp* fusion, but in these rare cells, expression is at a high level (J. Hahn, H. Maamar and D. Dubnau, unpubl.) This dramatic example of population heterogeneity may have evolved so that few cells in a clone will commit to a particular fitness-enhancing strategy. As the prolonged semidormancy that accompanies the K-state (Haijema *et al.*, 2001) poses a potential

challenge to survival, this strategy serves to minimize risks to the genotype. If, on the other hand, the few cells expressing the K-state happen to enjoy an advantage, the chances that the genotype will survive will be enhanced. Presumably the heterogeneity mechanism has evolved to maximize the benefit-to-risk ratio. There may be many examples of population heterogeneity selected by evolution in single celled organisms (see for instance Balaban *et al.*, 2004), and an understanding of the mechanisms that regulate this heterogeneity would be of general interest.

It has been shown, both experimentally and from modelling studies, that biological systems can achieve bistability using at least two distinct regulatory circuits (Gardner *et al.*, 2000; Becskei *et al.*, 2001; Ferrell, 2002). The first employs a positive feedback loop and the second uses reciprocally active repressors. Although the *comK* regulatory system contains all of these elements, we have shown in two different genetic backgrounds that the Rok repressor and the putative toggle switch are not needed for bistability and that instead, the first of these mechanisms plays a central role in generating the bimodal expression pattern of competence and the K-state.

Figure 5 suggests that in wild-type cells, the MecA mediated degradation pathway will eliminate any ComK that is synthesized prior to T_0. The degradative machinery has the capacity to suppress the effect of fluctuations in the level of CodY and Rok, because even the total absence of each of these proteins (Fig. 5) or of both of them simultaneously (T.T. Hoa and D. Dubnau, unpubl.) has little or no effect on expression before T_0. The same appears to be true of cells that express an enhanced level of DegU. As cells approach T_0, enough ComS is synthesized in at least 10–20% of the cells to protect ComK from degradation. In contrast to the situation before T_0, elimination of *rok* or *codY* by mutation, causes an increase in the final fraction of ComK-expressing cells, showing that these factors normally limit activation of the positive auto-regulatory loop.

What selects cells for the K-state? The choice of K-state-expressing cells must be stochastic, because the cultures used for these experiments are well shaken (exposed to uniform environments) as well as clonal (genetically uniform). We propose that those cells exceeding a threshold concentration of ComK because of noise in the synthesis or stability of this protein are the ones that express the K-state. Once a cell has achieved a certain level of *comK* expression, the positive autoregulatory loop will be activated and the switch will be thrown. In other words, the basal amount of ComK itself ultimately selects a cell for high-level expression. In accordance with this notion, we have observed that any mutation enhancing ComK synthesis, including the overexpression of *comS*, or the inactivation of *mecA*, *clpC*, *rok* or *codY*, increases the percentage of cells that express the K-state (this study and Hahn *et al.*, 1995b; Hoa *et al.*, 2002). In the cultures of BD2711 represented by Fig. 2B, rare cells could be detected that express high levels of GFP fluorescence, even during exponential growth (not shown), consistent with the occasional noise-driven activation of the ComK loop.

Noise can originate in the random nature of the initiation of transcription or translation (intrinsic noise) or may be as a result of variations in the activities of cellular components that influence gene expression (extrinsic noise) (Elowitz *et al.*, 2002; Swain *et al.*, 2002). In the case of *comK*, five transcription factors are known to bind to the promoter (CodY, Rok, DegU, AbrB and ComK) and it is possible that fluctuations in one or more of these determine which cells express *comK*. In addition, the post-translational mechanism that regulates the stability of the ComK protein may be a source of extrinsic noise in the expression of ComK, as the levels of MecA, ClpC, ClpP and ComS may vary from cell to cell. Such variations would influence the basal concentration of ComK at T_0, which would then alter the rate of transcription at P_{comK}. At one extreme, it is conceivable that the

variation in *comK* expression that selects cells for the K-state is dominated by noise in a single one of these many factors. At the other extreme, the variation may be determined by the joint probability that all of these factors vary to raise the expression of *comK* above a threshold value in 10–20% of the cells in a culture.

It is instructive to consider three models for the source of the noise that determines the K-state. These models are heuristic, and clearly all three may be partially valid. In the first model, the intrinsic noise in expression of *comK* selects cells for the K-state. A cell that randomly fires P_{comK} more often may achieve a critical level of ComK, thereby activating the loop. The role of ComS synthesis may be to simply permit the survival of ComK after T_0.

In the second model, like the first, all the cells achieve a high enough level of ComS after T_0 to protect ComK from degradation, but variations in the proteins that act at P_{comK} select cells for expression. The use of GFP fusions to CodY and Rok shows that these proteins are expressed in all the cells (not shown), but it is possible that only those cells at the low end of the CodY and/or Rok distribution activate the ComK loop. Another protein, AbrB, represses at P_{comK} (Hamoen *et al.*, 2003b), but it is unlikely that variation in AbrB helps select cells for *comK* expression. As cells approach T_0, the concentration of AbrB decreases, because of repression by the phosphorylated form of the master regulator Spo0A (Strauch *et al.*, 1990). However, AbrB also represses at P_{rok}, thereby exerting a positive effect as a repressor of a repressor (Hoa *et al.*, 2002). As a result, the effect on *comK* expression of variations in the concentration of AbrB should be buffered by opposing effects. This buffering has been observed experimentally (Hahn *et al.*, 1995a). Consequently, we are left with CodY and Rok as candidates for the second hypothesis.

The third model proposes that noise in the level of ComS, or another component of the degradation mechanism, selects cells for the K-state. It is known that transcription at the *comS* promoter takes place in all the cells (Hahn *et al.*, 1994) and that a ComS-YFP fusion protein is actually synthesized in all the cells in a culture (J. Hahn, H. Maamar and D. Dubnau, unpubl.). However, there is undoubtedly noise in the expression of *comS* and this may provide the variability that selects cells for stabilization of ComK. Although this model seems attractive, because of the important effects of ComS over-production (Fig. 5) or of *mecA* and *clpC* inactivation before T_0, it is actually no more compelling than the first two models.

We may summarize our current understanding of the regulation of the K-state as follows. As cells grow and divide exponentially, the expression of *comK* is limited by redundant mechanisms. CodY, Rok and AbrB repress expression at P_{comK}, and any ComK synthesized despite this repression will be targeted to the ClpC/ClpP proteosome and rapidly degraded. The concentration of ComK in the cell will be low under these conditions, and the positive feedback loop that acts at P_{comK} will not be activated. As a culture approaches T_0, the concentration of pheromone in the medium rises, and consequently ComS is produced. ComS can act to prevent the degradation of ComK, by releasing it from binding to MecA. We propose that at this point, the system is delicately balanced, because at least some ComS is produced in all the cells. Any variation that further increases the concentration of ComK (high ComS, low MecA, ClpC, CodY or Rok or a stochastic increase in the transcription or translation of *comK*) may potentially activate the positive feedback loop. The expression of *comK* will then rapidly increase, favoured by the non-linear response of P_{comK} to ComK.

This model formulated above does not explain why all the cells do not eventually enter the K-state. At least two factors potentially act temporally to limit the expression of *comK*. As noted above, *comK* is only expressed within a narrow range of AbrB concentration (Hahn *et*

al., 1995a). As cells enter stationary phase, the amount of AbrB drops continually (Strauch *et al.*, 1990), thus eventually mitigating *comK* expression. During this period, the activity of SinR also decreases (Shafikhani *et al.*, 2002), potentially activating rok transcription and further repressing *comK* expression (Hoa *et al.*, 2002). Thus, the K-state may develop only within a temporal window of opportunity, restricted stochastically to the cells that 'get there first'.

A major unanswered question is whether the noise in a single extrinsic factor or the intrinsic noise in *comK* expression dominates the selective process, or if the selection results from multiple variations.

Experimental procedures

General methods and materials

The *B. subtilis* strains used are all derivatives of strain BD630 and are described in Table 1. *Bacillus* strains were grown either in liquid competence medium (Albano *et al.*, 1987) supplemented with glucose (0.5%), L-histidine, L-leucine and L-methionine (50 μg ml^{-1}) or on tryptose blood agar base (TBAB, Difco) supplemented with chloramphenicol, erythromycin or kanamycin (5 μg ml^{-1}) or spectinomycin (100 μg ml^{-1}). *B. subtilis* competent cells were prepared as described previously (Albano *et al.*, 1987). *E. coli* XL1 blue (Stratagene) was used for cloning *comK* into the P_{hs} vector, and transformants were selected on Luria–Bertani (LB) plates containing ampicillin (100 μg ml^{-1}). DNA manipulation and other molecular biological procedures were performed using standard protocols.

Construction of P_{hs}-comK

The comK gene including the open reading frame and ribosome binding site was synthesized by polymerase chain reaction (PCR) using the primers, comKFor 5′-CCCAAGCTTAACAGATGATAGATTATTAGTA-3′ and comKRev 5′-ACATGCATGCATTGACATCTCAGGTATATGG-3′. The product was cut with *Hin*dIII and *Sph*I (sites are underlined above) ligated to pdr111 hyper-SPANK, previously digested with the same enzymes and transformed into *E. coli* XL1 blue, with selection for ampicillin resistance. This plasmid, a generous gift from D. Rudner (Harvard Medical School), is derived from pDR66, and therefore carries front and back sequences of *amyE*. It also contains the hyper-SPANK promoter (P_{hs}), inducible by IPTG, and is not capable of replication in *B. subtilis*. The recombinant plasmid was transferred to BD630 with selection for spectinomycin resistance and the transformants were screened for the absence of amylase activity.

Microscopy

The strains with P_{hs}-*comK* at *amyE* were grown in competence medium to mid-log phase. IPTG was added to the indicated concentrations, and samples were taken thereafter for microscopy. For non-inducible strains, samples were taken throughout growth as indicated. Cells were permitted to attach to polylysine-coated slides. Cross walls were visualized by staining RNA and DNA with propidium iodide (PI). Samples were mounted in Slow Fade (Molecular Probes). Microscopy was performed with a Zeiss Axiovert 135 M microscope equipped with an Orca Digital Camera (Hamamatsu), and a Zeiss 1.3 NA Plan Neo-Fluor 100 X oil immersion objective. Openlab software (Improvision) and Adobe Photoshop were used for image acquisition and processing. Three images were captured for each field of cells: phase contrast and fluorescence images for GFP and PI. Omega Optical filter sets XF23 and XF34 were used for GFP and PI respectively.

To calculate the fluorescence intensity of single cells, the average pixel value of non-GFP-containing cells (BD630) was subtracted from the measured pixel value of each *gfp*-containing cell. When negative values were obtained, these were set to zero. The Improvision lasso selection tool was used to delimit cell boundaries in the propidium iodide fields and the average pixel intensities were recorded in the corresponding GFP fields.

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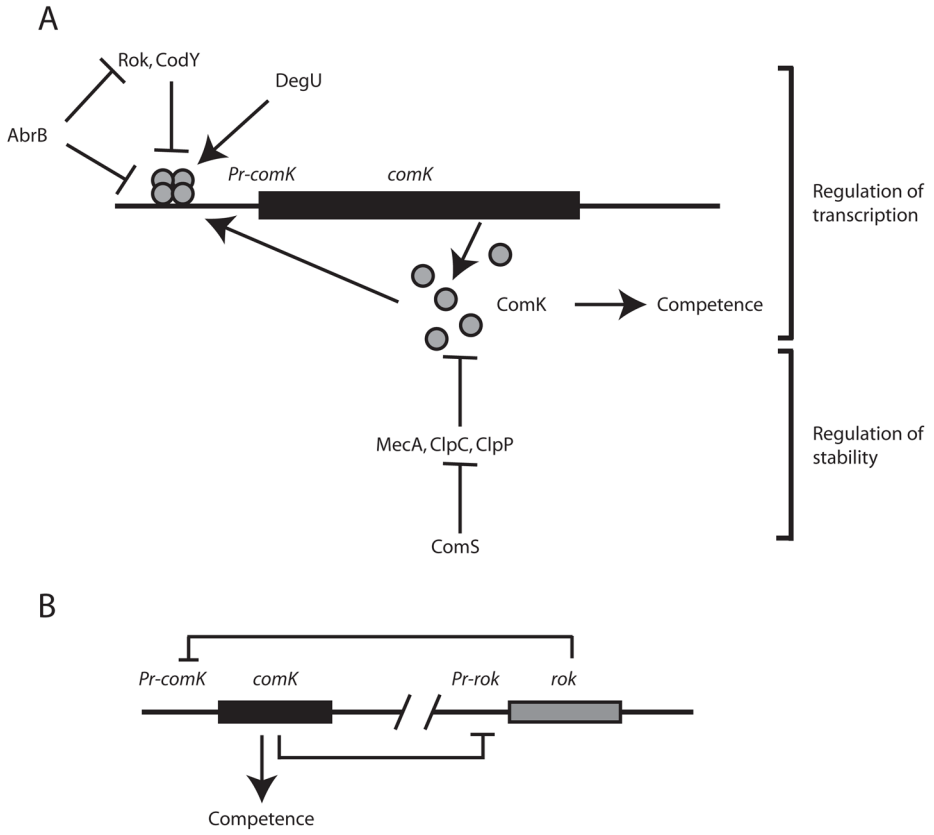

Fig. 1.

Regulation of competence.

A. Summary of competence regulation. Arrows and perpendiculars represent positive and negative regulation respectively. ComK binds to its promoter as a dimer of dimers.

B. Illustration of the putative toggle switch, in which ComK represses the transcription of *rok* and Rok represses the transcription of *comK*.

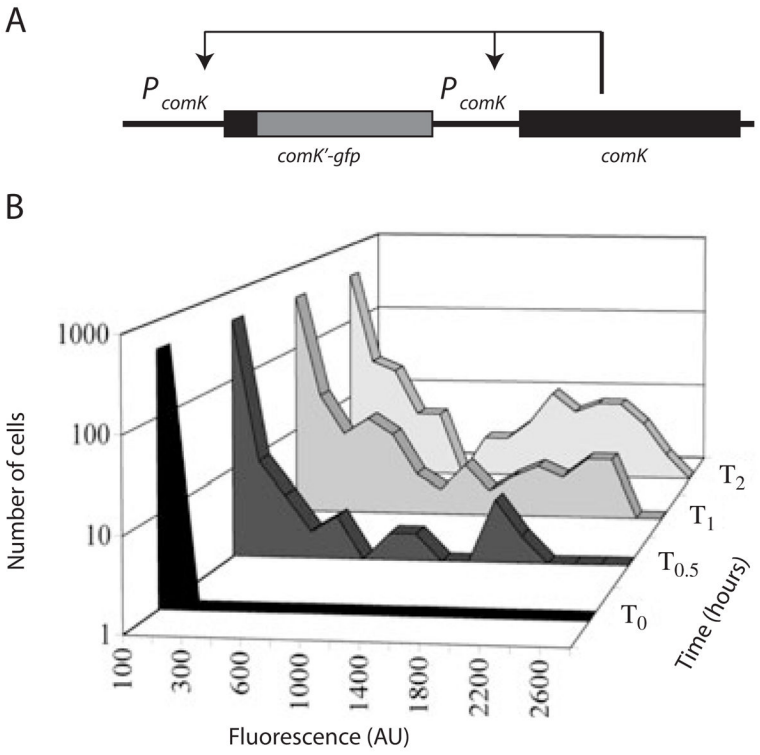

Fig. 2.

Bistability during the development of competence.

A. The construction (BD2711) used to demonstrate bistability. The arrows represent positive regulation.

B. The frequency distributions of fluorescence intensities in cells carrying *comK-gfp* (strain BD2711) are plotted at various times during the development of competence. T_0 refers to the time of departure from exponential growth. At each time point, the fluorescent intensities of 500 cells were evaluated.

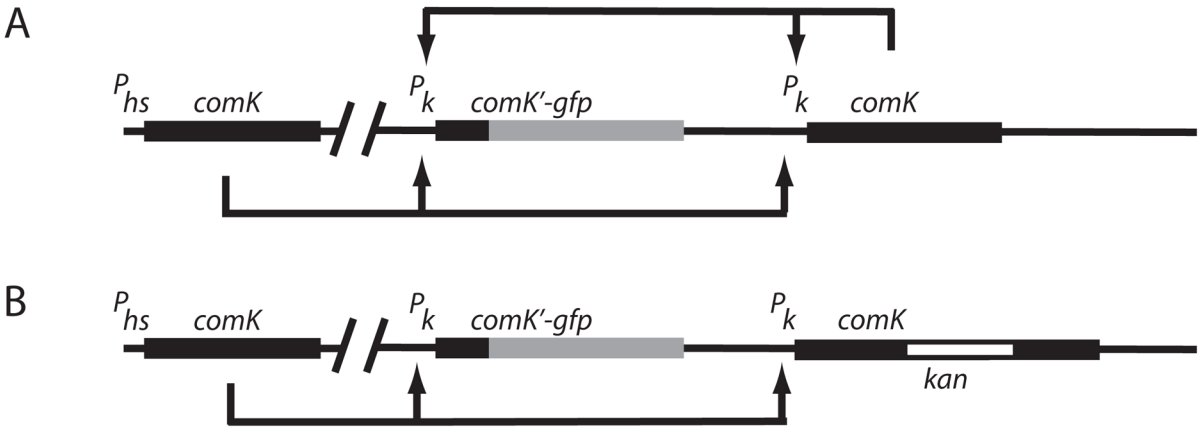

Fig. 3.

Constructs used to test the requirement for the autoregulatory loop for bistability. The arrowheads indicate positive regulation.

A. Construct containing the loop (strain BD4010).

B. Construct lacking the loop (strain BD4011).

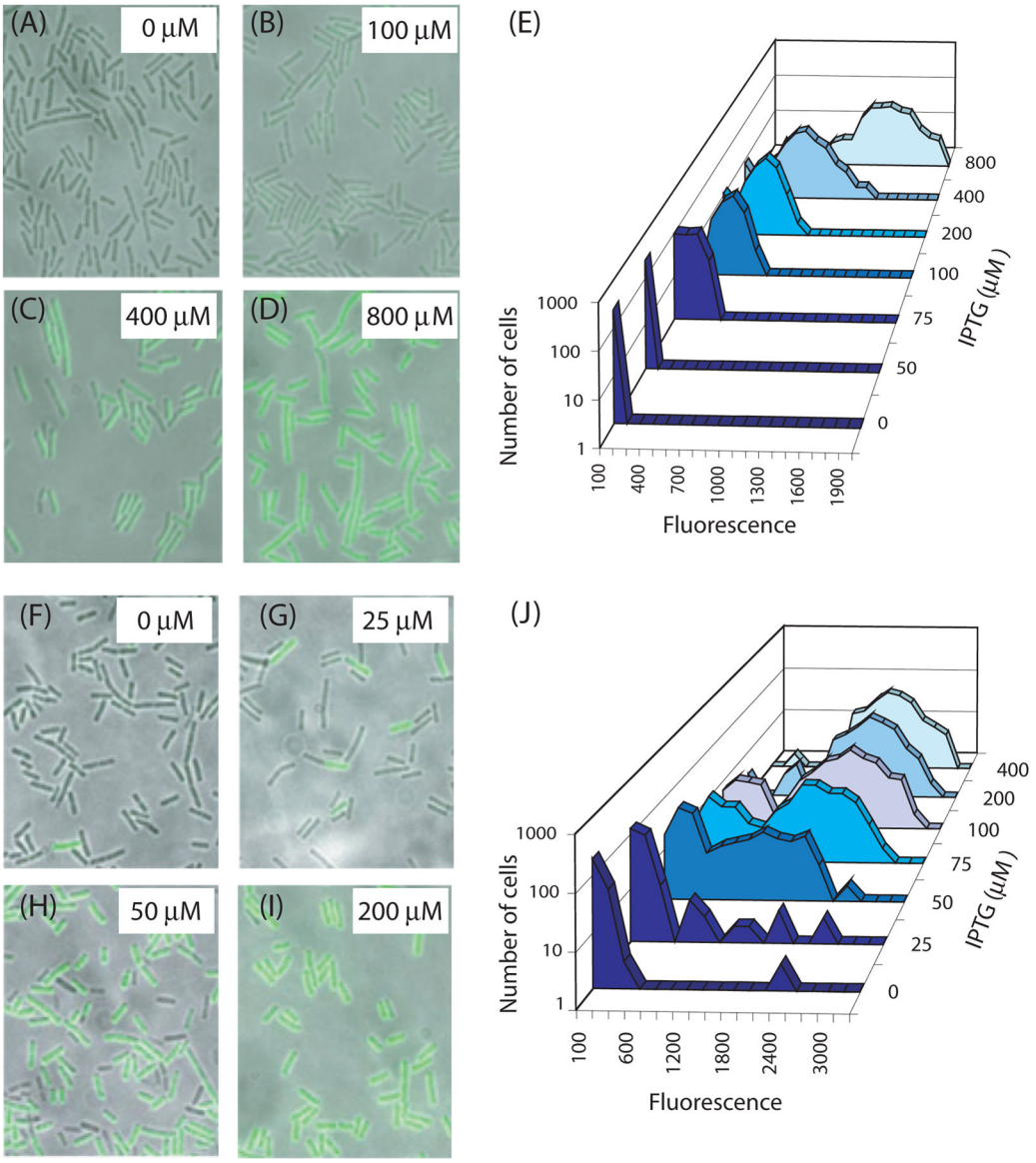

Fig. 4.

Distributions of ComK-GFP in cells without (A–E, strain BD4011) and with (F–J, strain BD4010) the ComK autoregulatory loop. The representative micrographs consist of fluorescent images overlaid with bright field images. All of the micrographs were recorded just before T_0. In panel F, the arrow points to a rare cell expressing *comK-gfp*. The concentrations of IPTG used for each panel are indicated. For each IPTG concentration, the fluorescent intensities of 250 cells were determined.

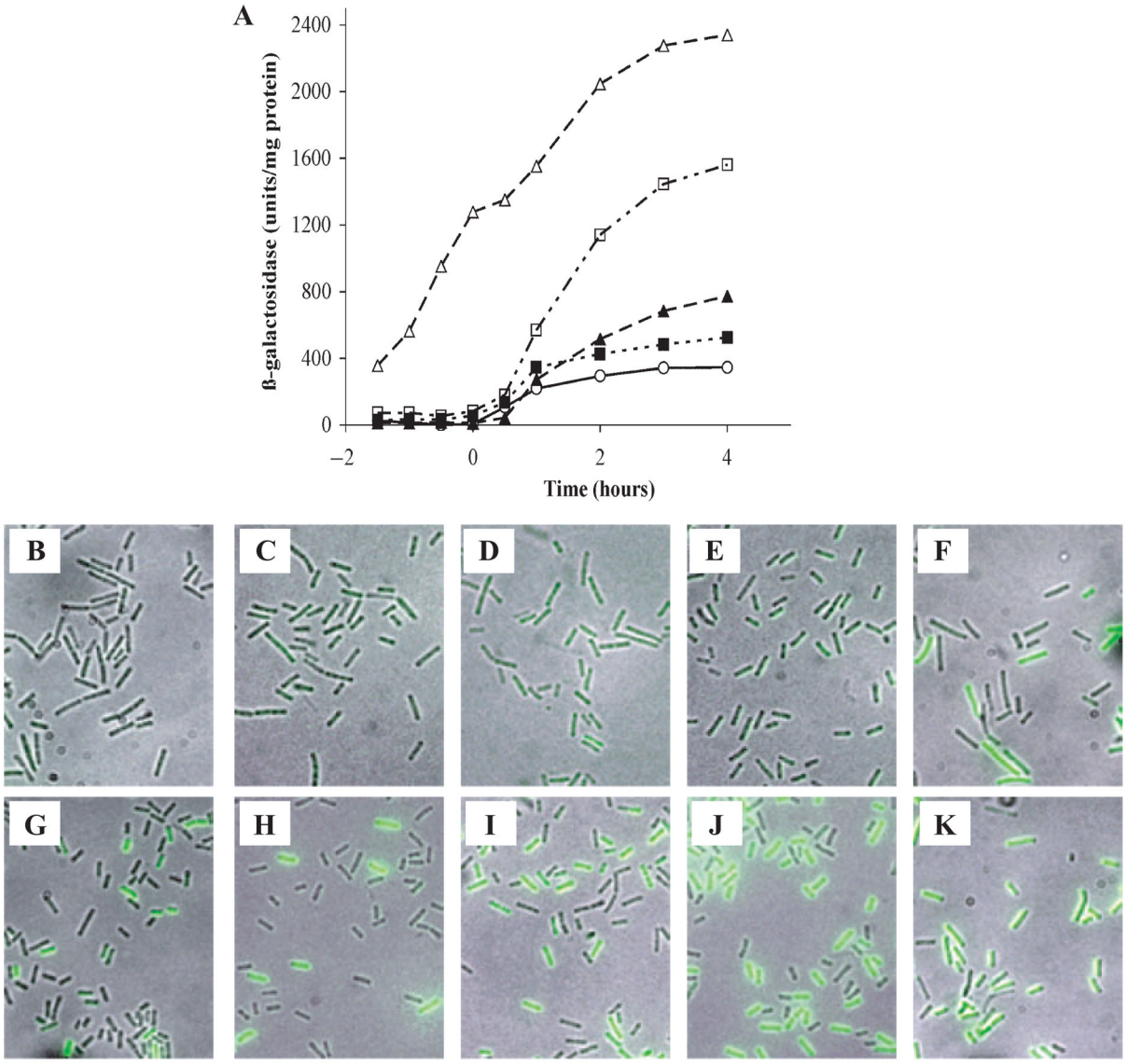

Fig. 5.

Effect of competence regulatory mutations on the expression of *comK*-lacZ (A) and of *comK-gfp* (B–K). In A, the following strains are shown: wild-type (○; strain BD1991), multicopy *degU* (■; strain BD4017), *codY* (▲; strain BD2607), *rok* (□; strain BD4016) and multicopy *comS* (△; strain BD4018). B–F exhibit cells taken at T_0 and G–K exhibit cells taken at T_2. The strains used were wild-type for regulatory genes (B, G; strain BD2711), carried a multicopy plasmid with *degU* (C, H; strain BD4013), were *codY* (D, I; strain BD4014), *rok* (E, J; strain BD4012) or carried a multicopy plasmid with *comS* (F, K; strain BD4015).

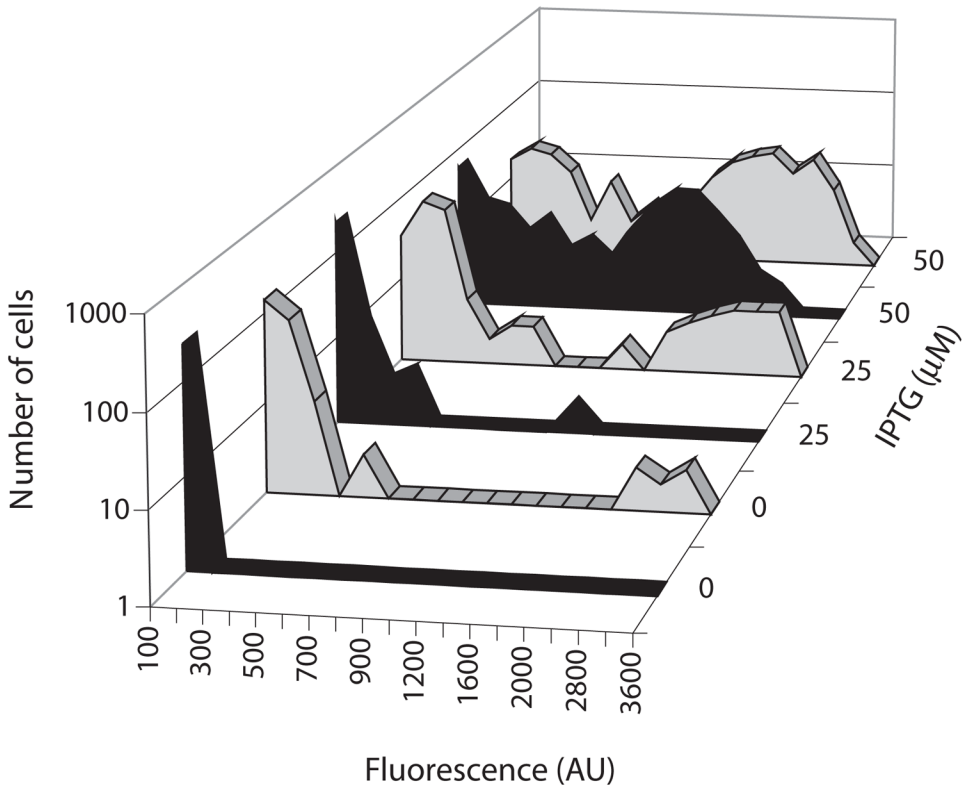

Fig. 6.

Effect of *rok* knockout mutation on bistability in the strain BD4010 background (Fig. 3A). The distributions for the *rok* (BD4019, shaded) and *rok*$^{+}$ (BD4010, black) strains are shown. The fluorescent intensities of 250 cells were evaluated for each IPTG concentration.

Table 1

B. subtilis strains used in this study.

Strain	Genotype	Source
BD630	*his leu met*	–
BD1833	*his leu met multicopy degU (kan[a])*	Hahn *et al.* (1996)
BD1991	*his leu met amyE:: comK-lacZ (cat[a])*	Hahn *et al.* (1994)
BD2121	*his leu met comK::kan[a]*	D. Van Sinderren
BD2589	*his leu met codY::erm[a]*	L. Sonenshein and P. Serror
BD2711	*his leu met comK-gfp (CBL[b], cat[a])*	Haijema *et al.* (2001)
BD3196	*his leu met rok::kan[a]*	I. Chen
BD3836	*his leu met amyE:: P_{hs}–comK (spc[a])*	This study
BD4010	*his leu met amyE:: P_{hs}–comK (spc[a]) comK-gfp (CBL[b], cat[a])*	This study
BD4011	*his leu met amyE:: P_{hs}–comK (spc[a]) comK-gfp (CBL[b], cat[a]) comK::kan[a]*	This study
BD4012	*his leu met comK-gfp (CBL[b], cat[a]) rok::kan[a]*	This study
BD4013	*his leu met comK-gfp (CBL[b], cat[a]) multicopy degU (kan[a])*	This study
BD4014	*his leu met comK-gfp (CBL[b], cat[a]) codY::erm[a]*	This study
BD4015	*his leu met comK-gfp (CBL[b], cat[a]) multicopy comS (kan[a])*	Hahn *et al.* (1996)
BD4016	*his leu met amyE:: comK-lacZ (cat[a]) rok::kan[a]*	This study
BD4017	*his leu met amyE:: comK-lacZ (cat[a]) multicopy degU (kan[a])*	This study
BD2607	*his leu met amyE:: comK-lacZ (cat[a]) codY::erm[a]*	J. Hahn
BD4018	*his leu met amyE:: comK-lacZ (cat[a]) multicopy comS (kan[a])*	This study
BD4019	*his leu met amyE:: P_{hs}–comK (spc[a]) comK-gfp (CBL[b], cat[a]) rok::kan[a]*	This study
BD4042	*his leu met comK-gfp (CBL[b], cat) rok::kan[a], codY::erm[a]*	This study

[a] *kan, cat, erm* and *spc* stand for resistance to kanamycin, chloramphenicol, erythromycin and spectinomycin respectively.

[b] Inserted by Campbell like integration.