Stripping Bacillus: ComK auto-stimulation is responsible for the bistable response in competence development

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Summary

In Bacillus subtilis competence for genetic transformation develops only in a subpopulation of cells in an isogenic culture. The molecular mechanisms underlying this phenotypic heterogeneity are unknown. In this study, we stepwise simplify the signal transduction cascade leading to competence, yielding a strain devoid of all regulatory inputs for this process that has been identified so far. We demonstrate that auto-stimulation of ComK, the master regulator for competence development, is essential and in itself can be sufficient to generate a bistable expression pattern. We argue that transcriptional regulation determines the threshold of ComK to initiate the auto-stimulatory response, and that the basal level of ComK (in a wild-type strain governed by MecA-mediated proteolytic control) determines the fraction of cells that reach this threshold, and thus develop competence.

Introduction

The response of bacteria to internal and external stimuli is co-ordinated by complex arrays of sensing and regulatory proteins. The majority of these signal transduction cascades are robust, but some are notorious for their variable output, and resulting heterogeneous phenotypes. Examples of this non-genetic population heterogeneity include phase-variation in a number of pathogenic bacteria (Kim and Weiser, 1998; Henderson et al., 1999), production of colicin K and phage-lambda induced lysis in Escherichia coli (Arkin et al., 1998; Mulec et al., 2003), gene expression during infection and suboptimal lac-induction in Salmonella (Tolker-Nielsen et al., 1998; Hau-tefort et al., 2003), and cellular differentiation in Bacillus subtilis (Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968; Chung et al., 1994).

Despite it being a rather common phenomenon, little is known about the molecular mechanisms underlying the heterogeneous outcome of the regulatory processes involved. It may be random, and based on stochastic fluctuations of regulatory proteins, as has been proposed for phage-lambda lysis in E. coli (Arkin et al. (1998)). Alternatively, it is conceivable that a specific mechanism has evolved which leads to the separation into subpopulations, considering the assumption that phenotypic variability contributes to the fitness of the species (Booth, 2002; Sumner and Avery, 2002). It has been proposed, for instance, that the presence of positive auto-regulation can be sufficient to evoke a separation into two subpopulations (a so-called bistable response) (Ferrell Jr, 2002). Recently, theoretical modelling has substantiated this supposition and biological support came from the use of simple, well defined gene regulatory systems showing positive feedback architecture, such as the ones derived from bacteriophage lambda of E. coli (Hasty et al., 2000), and a tetracycline-responsive transactivator system in yeast (Becskei et al., 2001).

In B. subtilis, a complex signal transduction cascade directs the induction of genetic competence, but only a small fraction of the cells will eventually reach this state (Hamen et al., 2003). In the present study we investigate the mechanisms that could be responsible for the differentiation into competent and non-competent cells. Competent cells express a complex DNA-binding, -uptake, and -integration machinery, and undergo severe metabolic changes, whereby replication and synthesis of macromolecules are arrested (Dubnau, 1991). These cells are physically different from non-competent cells and can be separated by means of density gradient centrifugation (Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968). It is therefore not surprising that the development of competence is a strongly controlled process. Under laboratory conditions the fraction of cells that become
Bistability in competence of B. subtilis

competent is limited to 10–20% of a culture (Haijema et al., 2001). Only in this small subpopulation the key regulator, ComK, is expressed. ComK activates over a hundred genes, among which the genes encoding the DNA-uptake and recombination systems (Berka et al., 2002; Hamoen et al., 2002; Ogura et al., 2002). In Fig. 1, a simplified scheme of the competence signal transduction cascade is depicted (Hamoen et al., 2003). Premature transcription of comK is prevented by three different repressors: AbrB, CodY and Rok, which all bind to the comK promoter region. In addition, ComK is captured by the adaptor protein MecA, which targets ComK to the ClpCP protease complex, resulting in its proteolytic degradation (Turgay et al., 1998). One of the reasons for this apparent redundancy of control mechanisms is that ComK binds to its own promoter region and strongly stimulates its own expression (van Sinderen and Venema, 1994). This auto-stimulatory response is a critical step in the establishment of competence and requires the response regulator DegU, which stabilizes the binding of ComK (Hamoen et al., 2000).

Although the regulatory pathways involved in competence development are well characterized, the molecular mechanisms responsible for the heterogeneous induction of ComK remain elusive. It is tempting to consider the competence signal transduction cascade simply as a network with positive feedback architecture, and thus explain the bistability. For several reasons, however, this conclusion is premature. First of all, positive feedback is no guarantee for bistable behaviour (Becskei et al., 2001; Ozbudak et al., 2004) and bistability is not limited to systems employing positive auto-regulation (Walz and Caplan, 1995). Second, it does not take into account the abundant negative regulatory mechanisms that are present in the competence regulatory cascade. Third, many of the regulators are only present, activated or deactivated, during a certain period in the growth of a culture (Hamoen et al., 2003). The limited presence of one, or maybe a few, of these regulators may ultimately be the cause of the non-uniform synthesis of ComK in a culture. Moreover, it is known that the absence of a functional MecA protein results in synthesis of ComK in nearly all cells of a culture (Haijema et al., 2001), while the positive feedback loop is still present in such a strain. This last observation suggests that the heterogeneity of competence is specifically established at the level of MecA-mediated proteolytic control of ComK. However, the absence of MecA leads to such strong overproduction of ComK that the transcription factors DegU, SinR and AbrB are no longer required for comK expression (Hahn et al., 1996). Therefore, it is not justified to discard the possibility that the complex regulation of the comK promoter determines the heterogeneous output of the competence signal transduction cascade.

The abundance and intertwinement of the different regulatory pathways complicates the use of a simple genetic mutagenesis approach to determine the role of the individual regulatory mechanisms in the establishment heterogeneity. Therefore, we decided to stepwise simplify the signal transduction pathway leading to competence development. First, we uncoupled the comK expression from the transcriptional control exerted by the transcription factors that bind to the promoter of comK, leaving the auto-stimulatory loop, and the MecA/ComS-mediated post-translational control, intact. To this end, the comK promoter region (PcomK) was replaced by a promoter region which only requires ComK for its activation, i.e. the comG promoter (PcomG) (Fig. 1). Second, we combined the PcomG-driven comK locus with a mecA mutation, thus creating a strain devoid of all regulatory inputs of competence development identified so far. In addition, a strain was constructed in which ComK auto-stimulation is removed. In subsequent experiments it was shown that the comK auto-stimulatory loop is essential, and in itself can be sufficient to generate a bistable expression of comK. Finally, a schematic model for the effect of individual regulatory factors on bistability in competence is presented.

Results

A single cell reporter for competence

To visualize competence development in individual cells, we constructed a fusion between the promoter region of comG and the 5' end of the gene coding for the green fluorescent protein, GFP (comG-gfp). This construct was introduced by Campbell-type integration into B. subtilis,
leaving the original comG operon intact. Fluorescence microscopy was used to measure the expression of GFP in individual cells. To validate the use of this system for the analysis of competence development at the single cell level, H-plots (see Experimental procedures) were generated for a wild-type and a mecA strain containing the comG-gfp reporter (Fig. 2A and B, respectively). The fraction of competent cells and expression patterns are in good agreement with previously reported expression studies (Hahn et al., 1995; 1996; Haijema et al., 2001). To verify that the production of GFP reflects the production of ComK in these experiments, Western blot analyses were performed using ComK- and GFP-specific antibodies (Fig. 3). Both in the wild-type strain and the mecA mutant, the GFP signal appeared slightly later than the ComK signal, which is in good agreement with previous studies showing that expression of comG starts about an hour after comK induction (van Sinderen and Venema, 1994). The strong signal observed in the Western blot for both GFP and ComK in the mecA mutant correlates well with the strong GFP signals observed in the H-plot in Fig. 2B. Taken together, we can conclude that the comG-gfp fusion provides a reliable method for monitoring ComK production in single cells.

ComK auto-stimulation is functional in a PcomG-comK background

The first step in the removal of regulatory inputs for competence development was the replacement of PcomK by PcomG. The comG operon codes for several proteins which are involved in the formation of the DNA-binding and -uptake machinery, and in vitro and in vivo transcription experiments strongly suggest that ComK is the only protein required for the activation of the comG promoter (Hamoen et al., 1998). A region between the transcription start (+1) of the original promoter of comK and the upstream located yhxC gene was replaced by the promoter region of the comG operon, in such a way that the transcriptional start (+1) of the comG promoter was fused in frame with the transcriptional start of the original comK promoter. This new fusion mutant was designated PcomG-comK. Theoretically, the promoter swap removes

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**Fig. 2.** H-plot analyses of GFP intensities in single cells of strains containing the comG-gfp reporter.
A. Wild-type.
B. mecA.
C. Pwt-comK Pxyl-comK.
D. PcomG-comK Pxyl-comK.
E. PcomG-comK mecA.

Strains were grown in the presence of 2% xylose. Time is given in hours relative to the transition point between the exponential and stationary growth phase (T0). H-plots were generated as described in Experimental procedures.
the control of the transcription factors that bind to PcomK, but leaves the auto-stimulatory induction of comK and the proteolytic control by MecA/ClpCP intact (Fig. 1). In order to check whether the auto-stimulatory loop in the PcomG-comK strain is still intact, we monitored the activity of a comG-lacZ reporter fusion in this background. This reporter has proved to be a reliable indicator for ComK levels in the cell (van Sinderen and Venema, 1994). To our surprise, no comG-lacZ expression could be detected. Previous work has shown that there is almost no detectable transcription from the comG promoter when ComK is absent (Hamoen et al., 1998). Apparently, cells have to generate a substantial basal level of ComK, in order to initiate the auto-stimulatory response. To achieve this, we introduced an additional copy of comK, under the control of a xylose-inducible promoter, in the ectopic amyE locus (Pxyl-comK). The same construct was used by Hahn and coworkers to study the regulation of comK expression (Hahn et al., 1996). They showed that, to obtain wild-type kinetics of comK expression, the presence of a native copy of comK (indicated here as Pwt-comK) is required. As shown in Fig. 4, the xylose induction of Pxyl-comK in a PcomG-comK background resulted in a strong induction of comG-lacZ expression, with β-galactosidase levels twice as high as in a wild-type strain, and much higher than in a Pxyl-comK strain without a native copy of comK. In a strain harbouring both a native comK locus and the xylose-inducible comK (Pwt-comK Pxyl-comK), the expression of comG-lacZ began 1 h earlier and reached an even higher level (Fig. 4). These results indicate that the auto-stimulatory loop, as predicted, is still functional. It is noteworthy that the mutant strains harbouring the Pxyl-comK locus still display growth-phase dependent expression of ComK, despite the presence of xylose in the medium from the beginning of growth. Similar results were obtained by Hahn and coworkers, who demonstrated that this is resulting from MecA-dependent proteolytic control, and that deletion of mecA leads to kinetics similar to that of the Pxyl-promoter (Hahn et al., 1996).

PcomG-comK uncouples comK-transcription from transcription factors

To verify that transcription of comK became independent of the transcription factors that act on PcomK (Fig. 1), we looked at the effect of mutations in these genes on comG-lacZ expression in a PcomG-comK Pxyl-comK background. As expected, a mutation in degU did not alter the expression of comG-lacZ, when comK expression was driven from the comG promoter. The fact that a Pwt-comK Pxyl-comK construct still shows an influence of this transcription factor (Hahn et al., 1996) indicates that the observed uncoupling is not resulting from the presence of xylose-inducible copy of comK. In contrast, when comS was mutated in a PcomG-comK Pxyl-comK background, comG-lacZ expression was abolished. ComS is required for the inactivation of the MecA/ClpC/ClpP proteolytic complex, and this finding is thus consistent with the fact that the proteolytic control of ComK is still functional in a PcomG-comK Pxyl-comK strain. In some cases (CodY, Rok) the lacZ study did not unambiguously demonstrate uncoupling of comK transcription, which could be resulting from an effect of these regulators on the post-translational control of ComK (e.g. the MecA/ClpCP complex). There-

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**Fig. 3.** Detection of ComK and GFP protein. Equal amounts of protein were loaded in each lane. Samples were collected during the fluorescence microscopy experiments. Time is given in hours relative to the transition point between the exponential and stationary growth phase (T0). ON indicates samples taken after prolonged stationary phase growth.

**Fig. 4.** Effects of comK promoter replacement on comG-lacZ expression. comG-lacZ expression in wild-type (●), Pwt-comK Pxyl-comK in presence (△) and absence of 2% xylose (▲), PcomG-comK Pxyl-comK in presence (■) and absence of 2% xylose (□), and comK::sp Pxyl-comK (△). Time is given in hours relative to the transition point between the exponential and stationary growth phase (T0). β-Galactosidase activity is given in nmole min⁻¹ OD₆₀₀⁻¹.
fore, electrophoretic mobility shift assays (EMSAs) were performed, which ruled out the possibility that the transcription factors bind to PcomG. A detailed description of these experiments is available as Supplementary Material (Appendix S1 and Fig. S1). Finally, we also tested the effect of a mutation in the comZ gene on comG-lacZ expression in the PcomG-comK Pxyl-comK strain. comZ is reported to affect comG-expression independent of comK (Ogura and Tanaka, 2000), but we observed no difference in β-galactosidase activity in our strain under the conditions tested (data not shown). This indicates that the comZ gene does not influence the outcome of our experiments. Taken together, these results suggest that replacement of the comK promoter by the comG promoter results in a bypass of the regulatory inputs that normally govern the activity of the comK promoter.

Heterogeneous expression of comK and Pxyl-comK

Theoretically, the PcomG-comK strain could give an indication of the importance of the transcriptional regulation of comK for heterogeneity in competence, because the proteolytic control of ComK and the auto-stimulatory loop are intact, whereas the transcriptional control by a number of transcription factors is removed. However, in order to achieve competence, it was necessary to introduce the Pxyl-comK locus. Although previous work (Hahn et al., 1996) and the results from this study (Fig. 4) indicate that the temporal regulation of comK expression is similar to wild-type, the expression levels are significantly higher in strains that contain the Pxyl-comK in the presence of the ComK auto-stimulatory loop. The effect of xylose-induction on the heterogeneous expression of comK is not documented so far. Therefore, we introduced the comG-GFP reporter construct in a Pwt-comK Pxyl-comK and a PcomG-comK Pxyl-comK strain. As can be seen in the ensuing H-plots (Fig. 2C and D), in both strains the percentage of cells expression comG-gfp is significantly higher than in wild-type cells. At T5, 80% of the cells in a PcomG-comK Pxyl-comK strain express the reporter, and a Pwt-comK Pxyl-comK strain even resembles a mecA mutant at later time points, with nearly all cells expressing ComK. The increased expression of GFP and ComK in the Pxyl-comK mutants is also observed in a Western blot analysis (Fig. 3). It seems that the xylose-induced comK expression almost completely abolishes the heterogeneity that is observed in wild-type. Thus, it is impossible to evaluate the contribution of transcriptional control on PcomK to the heterogeneity of competence.

Stripping B. subtilis of all known external regulators of ComK

Above, it was shown that in a PcomG-comK strain the ComK auto-stimulatory loop is still intact, comK transcription is uncoupled from the transcription factors that bind to PcomK, and the proteolytic control of ComK is functional (see also Fig. 1). By combining the PcomG-comK locus with a mecA mutation, we remove the last known regulatory input for competence development, leaving only the ComK auto-stimulatory loop intact. The mutant produced normal levels of ComK in a growth-phase dependent manner, as demonstrated by the Western blot analysis shown in Fig. 3. The fact that a PcomG-comK strain containing a mecA deletion is able to initiate competence supports the idea that PcomG-driven expression of comK is too low to escape from MecA-mediated proteolysis, as was suggested by the previous experiments. It should be emphasized that in the PcomG-comK mecA strain, no xylose-inducible comK is present, which could influence the heterogeneity. When competence was monitored at the single cell level, we found that fluorescence of the comG-gfp reporter was still limited to a subpopulation of cells (Fig. 2E). Even after prolonged incubation, this fraction did not exceed 50% of the population, despite the fact that all known regulatory inputs of ComK activity were removed in the PcomG-comK mecA strain.

Removal of ComK auto-stimulation

The results described above strongly suggest a critical role for ComK auto-stimulation in establishing a bimodal distribution in comG-expression during competence development. To analyse the function of ComK auto-stimulation in more detail, a strain was constructed in which the only source of ComK is derived from the xylose-inducible promoter (comK::sp Pxyl-comK). comG-gfp expression in the comK::sp Pxyl-comK background was analysed by flow cytometry. The comK::sp Pxyl-comK strain still shows growth-phase dependent expression (data not shown), which was found to be dependent on MecA in a similar study using a lacZ-reporter fusion (Hahn et al., 1996). However, from Fig. 5A, it can be seen that removal of the auto-stimulatory loop leads to a monomodal distribution in fluorescence. In contrast, the reference strain comG-gfp, with normal regulation of ComK activity, displays a bimodal distribution with a non-fluorescent and a fluorescent population, corroborating the H-plot data (Fig. 2A). Similar results were obtained using a range of inductions (data not shown). Fluorescence in the comK::sp Pxyl-comK background does not reach the levels observed in strains where the auto-stimulatory loop is present. Most likely, this is caused by the limited strength of Pxyl compared to PcomK, because inductions with up to 0.5% xylose yield a graded response (Fig. 5B). Together, these results demonstrate that the ComK auto-stimulatory loop is essential, and can in itself
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be sufficient to obtain phenotypic heterogeneity in a competent B. subtilis culture.

Discussion

**Competence development as a bistable response**

During competence for genetic transformation, a wild-type B. subtilis culture clearly displays a bistable pattern of comK expression (Figs 2A and 5A). In this study, we set out to investigate which factors of the competence signal transduction cascade could be responsible for the phenotypic variability. In order to do so, we stepwise simplified the signal transduction cascade for competence development, by replacing PcomK with PcomG, and subsequently deleting the meca gene. Interestingly, in the resulting strain, which is stripped of all its normal regulatory inputs, comK is still expressed in only a subpopulation of cells (Fig. 2E). Apparently, the expression of comK, in conjunction with the auto-stimulatory loop, is sufficient to generate phenotypic heterogeneity. Moreover, simple induction of comK in the absence of the auto-stimulation resulted in a monomodal distribution of fluorescence (Fig. 5), demonstrating that the positive feedback-loop is essential for bistability. In addition, it demonstrates that MecA-mediated proteolysis (which is intact in this strain) is not the major determinant for bistability, which is consistent with the data obtained from the PcomG-comK meca strain. ComK auto-stimulation thus represents the key-determinant for the heterogeneity in competence. We postulate that the stochastic fluctuations (noise) in comK transcription and translation together with ComK auto-stimulation are sufficient to generate the bistable distribution of comK expression. This finding is in good agreement with recent modelling and studies on artificial gene regulatory networks, in which it was proposed that the presence of positive feedback architecture can lead to a bistable response (Hasty et al., 2000; Becskei et al., 2001). In the absence of transcriptional and post-translational control, it may seem strange that growth-phase dependent expression of comK is still observed in a PcomG-comK meca strain (Figs 2E and 3). Although we cannot exclude the possibility that a comG-specific growth-phase dependent regulator exist, current data do not provide any evidence for the existence of such a regulator. However, the growth-phase dependency can be explained by the continuous dilution of ComK-levels as a consequence of division of the cells during logarithmic growth. Only when cell-growth retards and ultimately ceases there will be time to accumulate a sufficient amount of ComK to initiate the auto-stimulatory reaction.

**Transcriptional control modulates ComK threshold levels**

Considering competence development as a bistable response allows for an alternative interpretation of the regulatory mechanisms acting on comK transcription. It has been shown that the bistable behaviour of an auto-activating gene regulatory network requires a substantial threshold of the activating protein (Hofer et al., 2002). As such, the transcriptional regulators acting on PcomK (DegU, AbrB, CodY and Rok) serve to modulate ComK threshold levels. The presence of DegU in the system, for example, lowers the concentration of ComK required to elicit the auto-stimulatory response, by enhancing binding of ComK to its own promoter (Hamoen et al., 2000). Removal of DegU thus leads to an increase of the threshold of ComK and subsequent low levels of competence.
(Ogura and Tanaka, 1996). Oppositely, removal of Rok from the system lowers the threshold of ComK required for auto-stimulation, and leads to an increase of the fraction of competent cells (Hoa et al., 2002).

**Basal levels of ComK influence bistability**

The introduction of a xylose-inducible copy of comK, or the deletion of mecA, quickly leads to a more or less uniform induction of comK and abolishment of the bistable pattern in a wild-type background, as seen in Figs 2B, 2C and 5A. Yet, in these systems, the ComK auto-stimulatory loop is still intact, and essential for full induction of comK expression (Hahn et al., 1996). The observed absence of heterogeneity may therefore seem contradictory to the hypothesis that the auto-regulation is responsible for the establishment of heterogeneity. However, it was shown that by modulating basal levels of the auto-activating protein, using an inducible system for instance, different balances can be obtained between the high- and low-expressing states of a bistable system. When highly induced, all cells will initiate the auto-stimulatory loop and eventually be in the high-expressing state (Becskei et al., 2001). Thus, when the basal level of ComK in the cells is raised through induction of the Pxylo-comK locus, more cells will reach the threshold level required for initiating the auto-stimulatory loop. Similarly, in a mecA mutant, the premature expression of comK and the continuous slow accumulation of ComK protein because of lack of the proteolytic control, lead to a situation where almost all cells in the culture reach the threshold level of ComK for the initiation of the auto-stimulatory response. Yet, in a PcomG-comK mecA mutant heterogeneity was not abolished, despite the absence of proteolytic control, and the subsequent increase in ComK levels. The reason for this could be that the comK promoter works rather differently from the majority of general ComK-activated promoters, such as the comG promoter. In vitro transcription experiments demonstrate, for instance, that PcomK is leaky and shows significant transcription in the absence of an activator (unpubl. observation), whereas PcomG requires ComK (Hamoen et al., 1998; Susanna et al., 2004). When mecA is mutated, the relatively high basal level of transcription from PcomK is apparently enough to accumulate ComK to levels that initiate the auto-stimulatory loop in all cells of the population, whereas this is not the case for PcomG.

**Fluctuations in regulatory proteins not required for bistability**

Theoretically, stochastic fluctuations in the regulatory pathways governing competence development could be responsible for the bistable expression pattern of comK. However, prokaryotes employ abundant negative feedback mechanisms. This kind of regulation reduces the variability in gene expression (Becskei and Serrano, 2000; Thattai and Van Oudenaarden, 2001) and thus provides robustness to gene regulatory networks. In addition, it was recently reported that noise in the protein production rate generally is detrimental to the fitness of an organism and therefore subject to natural selection (Fraser et al., 2004).

Importantly, we demonstrate in this study that differences in threshold levels or basal levels of protein between cells are not necessary to generate a bistable expression pattern during competence development. We find that the ComK auto-stimulatory loop in itself is essential, and can be sufficient, to obtain to generate phenotypic heterogeneity. The mechanism represents a noise-based switch based on stochasticity in transcription and translation (Becskei et al., 2001; Elowitz et al., 2002; Ozbudak et al., 2002), in which transcriptional control of comK and the basal level of ComK influence the outcome of the bistable response, by modulating the threshold level required for ComK auto-activation, and determining the fraction of cells that reach this threshold, respectively. This is schematically depicted in Fig. 6. Meddling with either one of these variables will have a large impact on the percentage of competent cells, as demonstrated in the previous paragraphs, but will most likely not lead to conclusive answers with respect to the mechanism responsible for the separation into subpopulations. In order to do so, it would be necessary to monitor the levels of a multitude of regulatory proteins, as well as their activation state, on a single cell level; something which is not possible with the current state of technology.

**Concluding remark**

To our knowledge, this study provides one of the first
single cell analyses of a natural signal transduction cascade in bacteria that underscores the importance of a positive feedback loop for the establishment of a bistable pattern in gene expression. Many post-exponential and stress-related processes employ positive feedback loops in their regulation. It is assumed that these ensure a rapid response to changing environments. However, based on the observations above, it is tempting to speculate that organisms by employing positive feedback regulation specifically generate phenotypic diversity under adverse conditions, thereby increasing the fitness of the species. It would be interesting to see whether bistability is indeed a common phenomenon in processes that rely on positive auto-regulation, such as the production of antimicrobial peptides or stress responses.

**Experimental procedures**

**General materials and methods**

In this study, *B. subtilis* strain 8G5 (Bron and Venema, 1972) was used as a reference strain (wild-type) because of its increased transformability. The strains and plasmids used in this study are listed in Table S1, which is available as Supplementary material. The table also contains the sequence of the primers, described hereafter.

Chemicals used were of analytical grade and, unless indicated otherwise, obtained from Merck, or Bakers Chemical Co. Enzymes were purchased from Roche, and used according to the supplier's instructions.

*Bacillus subtilis* strain 8G5 was grown in minimal medium (Hamoen et al., 2002) or TY (10 g L⁻¹ trypton, 5 g L⁻¹ yeast extract, 5 g L⁻¹ NaCl, pH 7.2) broth. For flow cytometric analysis of the *comK*:sp *pxyl-comK* strain, minimal medium was used in which glucose was replaced with the same concentration fructose, to relieve catabolite repression of the P*pxyl*-promoter (Hahn et al., 1996) and allow for lower xylose-concentrations for induction. Where necessary, media were supplemented with the appropriate antibiotics (Roche, Sigma) at a final concentration of: ampicillin 50 µg ml⁻¹ (*E. coli*), chloramphenicol 5 µg ml⁻¹ (*B. subtilis*), kanamycin 50 µg ml⁻¹ (*E. coli*) or 5–10 µg ml⁻¹ (*B. subtilis*), erythromycin 150 µg ml⁻¹ (*E. coli*) or 0.5 µg ml⁻¹ (*B. subtilis*), spectinomycin 100 µg ml⁻¹ (both *E. coli* and *B. subtilis*) and tetracycline 15 µg ml⁻¹ (*E. coli*) or 6 µg ml⁻¹ (*B. subtilis*).

Chromosomal DNA from *B. subtilis* was isolated as described (Bron and Venema, 1972). Mini-preparations of plasmid DNA from *E. coli* were obtained by the alkaline lysis method (Sambrook et al., 1989). All cloning procedures were carried out according to Kim et al. (1996). Polymerase chain reaction (PCR) products were purified using the Roche High Pure PCR purification Kit (Roche). Southern blot analyses were performed using the non-radioactive ECL labelling and detection system, according to the instructions of the manufacturer (Amersham).

**Construction of the PcomG-comK strain**

A part of the yhxC gene was amplified using primers yhxC1 and yhxC2, carrying a SphI and a BamHI restriction site, respectively. After digestion, this fragment was ligated into SphI-BamHI-cut pUC19 plasmid, resulting in pUC-Y. A spectinomycin resistance cassette (Guerrout-Fleury et al., 1995) was obtained by PCR using primers sp3 and sp2. After restriction the marker was introduced in BamHI-EcoRI digested pUC-Y plasmid, resulting in pUC-YS. Next, a 476 bp fragment, starting at the (+1) transcription start point of the *comK* gene, was amplified by PCR using primers K11 and K2, carrying half an EcoRV and a EcoRI restriction site, respectively. This fragment was digested and ligated into EcoRV-EcoRI digested pUC-YS plasmid, resulting in plasmid pUC-YSK. The *comG*-promoter region was amplified using the primers G7 and G12. The obtained fragment was subsequently ligated into EcoRV-EcoRI digested pUC-YKS, and the construct was checked for correct orientation. The obtained plasmid, pUC-YKSG, was used to replace the *comK* promoter region by the *comG* promoter region on the *B. subtilis* chromosome through a double cross-over event, and transformants were checked for correct integration by Southern blotting.

**Construction of a comG-gfp fusion strain**

A translational fusion between the *comG*-promoter region and *gfp* was constructed as follows. A 594 bp fragment of the promoter region of *comG* was amplified by PCR using primers comGprom1 and comGprom2, carrying a HindIII and an EcoRI restriction site, respectively. The fragment was digested and ligated into HindIII-EcoRI digested pSG1151 (Lewis and Marston, 1999). The resulting plasmid, pSG-ComGA, was digested with HindIII and XbaI to cut out the comG-gfp fragment. The fragment was isolated from gel and ligated into HindIII-XbaI digested pUC18 plasmid, after which a kanamycin resistance cassette was introduced in the Scal site, resulting in plasmid pGA-GFP. The plasmids with the *comG-gfp* fusion were used to transform *B. subtilis*. Transformants were checked for fluorescence on minimal medium by fluorescence microscopy.

**Construction of sinR, comS and abrB mutants**

Primers used to construct these mutants are; AbrB1 (EcoRI), AbrB2 (HindII), AbrB3 (HindII), AbrB4 (HindIII). *psin-B1* (BamHI), *psin-N1* (5’Nru), *psinN2* (Nru), *psin-P1* (PstI), *srfaA8* (EcoRI) and *srfaA9* (BamHI). The PCR fragments for making the *abrB* and *sinR* mutants were cloned into pUC18 plasmid and an erythromycin or tetracycline resistance marker (Guerrout-Fleury et al., 1995) was introduced in the Scal restriction site by blunt-end ligation. The mutants were obtained by double cross-over recombination into the *B. subtilis* chromosome. The *srfaA4* PCR fragment was cloned into pUC19T plasmid, carrying a tetracycline resistance marker (K. Leenhouts, unpubl.) and introduced by Campbell-type integration into the chromosome of *B. subtilis*. All strains were checked for correct integration by Southern blotting.

**β-Galactosidase assays and protein detection**

To assay cellular β-galactosidase levels, overnight cultures were diluted into fresh medium to an optical density of 0.01
at 600 nm (OD_{600}) and samples were taken at hourly intervals. The β-galactosidase assays and the calculation of β-galactosidase activity were performed as described (Bolhuis et al., 1999). SDS-PAGE and Western blotting of cell lysates harvested during fluorescent microscopic measurements was performed as described (Bolhuis et al., 1999). Chemiluminescent detection of bound ComK- (Kong and Dubnau, 1994) or GFP-specific antibodies (Molecular Probes) was performed with horseradish peroxidase-conjugated antirabbit IgG and the ECL Western blotting analysis system (Amersham).

Fluorescence microscopy and data analyses
Starter stock cultures at −80°C of all GFP-containing strains used in this study were made as follows. After overnight culturing in minimal medium, cells were diluted into fresh minimal medium to an OD_{600} of 0.01, and the cultures were grown for approximately 2 h at 37°C. Aliquots containing a volume of these cultures, yielding an OD_{600} of 0.01 when added to 50 ml of medium, were then frozen at −80°C in 8% glycerol. For each experiment, one tube per strain was taken from the −80°C batches and inoculated into 50 ml prewarmed minimal medium containing 2% xylose and grown at 37°C under vigorous shaking. Samples for fluorescence microscopy were taken at hourly intervals from the transition point into stationary growth phase (T0) until T5. Also, a sample was taken after prolonged growth in the stationary phase (overnight). In case of the mecA mutant, samples were taken from T2 until T4. Cells were stained with DAPI at a final concentration of 10 μg ml⁻¹ to visualize DNA and enable counting of the total number of cells. Aliquots of 2 μl were put on a slide covered with 1% agarose. The fluorescence of DAPI and GFP was visualized with a Zeiss Axiopt microscope, using appropriate filters. For each strain and time point, two pictures containing between 100 and 1000 cells each were taken using an AxioVision camera and AxioVs20 software (Zeiss). The total number of cells and the intensity of GFP fluorescence in individual cells were analysed using Image2D Master 2D Elite software v3.1 (Amersham Pharmaacia Biotech). The fluorescent intensity of individual cells was plotted against the number of cells counted. The total number of cells was normalized to a value of 100, so that the x-axis of the Microsoft Excel-generated graphs intuitively depicts the percentage of cells showing fluorescence. In the text of the article, these graphs are referred to as heterogeneity plots or H-plots.

Flow cytometric analyses
Cells were diluted 12.5–50x in 0.2 μM filtered starvation medium (Hamoen et al., 2002) and directly analysed on Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, NL) operating an argon laser at 488 nm. For each sample 20 000 cells were analysed. GFP signals were collected through an FITC filter with the photomultiplier voltage set between 700 and 800 V. Data were captured using EXPO32 software (Beckman Coulter) and further analysed using WinMDI 2.8 (http://facs.scripps.edu/software.html). Figures were prepared for publication using WinMDI 2.8 and Corel Graphics Suite 11. To distinguish background fluorescence from GFP specific fluorescence, parental strain B. subtilis 8G5 was analysed with each flow cytometric experiment.

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Supplementary material
The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4488/mmi4488sm.htm


Table S1. Strains, plasmids and primers used in this study.

Appendix S1. PcomG-comK uncouples comK-transcription from transcription factors.

References


