Antirepression as a second mechanism of transcriptional activation by a minor groove binding protein

Wiep Klaas Smits,1 Tran Thu Hoa,2† Leendert W. Hamoen,1‡ Oscar P. Kuipers1 and David Dubnau2*
1Department of Genetics, University of Groningen, Kerklaan 30, 9751NN, Haren, the Netherlands.
2Public Health Research Institute, 225 Warren St, Newark, NJ 07103-3535, USA.

Summary

Competence for genetic transformation in the bacterium Bacillus subtilis is a bistable differentiation process governed by the minor groove DNA binding protein ComK. No detectable comK transcription occurs in the absence of an intact comK gene, indicating that ComK has auto-activating properties. ComK auto-stimulation, which is dependent on ComK binding to the comK promoter, is a critical step in competence development, ensuring quick and high-level expression of the late-competence genes. Auto-stimulation is also essential for the bistable expression pattern of competence. Here, we demonstrate that ComK acts as an activator at its own promoter by antagonizing the action of two repressors, Rok and CodY. Importantly, antirepression occurs without preventing binding of the repressing proteins, suggesting that ComK and the repressors might bind at distinct surfaces of the DNA helix. DegU, a DNA binding protein known to increase the affinity of ComK for its own promoter, potentiates the antirepression activity of ComK. We postulate that antirepression is primarily achieved through modulation of DNA topology. Although to our knowledge ComK is the only DNA binding protein shown to act in this novel fashion, other minor groove binding proteins may act similarly.

Introduction

In bacteria, the activation of transcription typically involves a DNA binding protein that recognizes a sequence upstream from the RNA polymerase (RNAP) binding site, resulting in the recruitment of RNAP to the promoter or stabilization of RNAP binding. Indeed, the latter mechanism obtains in the case of ComK, the master regulator of competence for genetic transformation (Susanna et al., 2004).

Competence, the ability to take up DNA from the environment and integrate it into the genome, is the end-point of a complex developmental process resulting in the expression of ComK (van Sinderen et al., 1995). This activator drives the expression of a multitude of genes, including those encoding the DNA uptake and integration machinery (Berka et al., 2002; Hamoen et al., 2002; Ogura et al., 2002). In contrast to the majority of DNA binding regulatory proteins, ComK activates transcription by binding in the minor groove of the DNA, upstream of its target genes (Hamoen et al., 1998).

In B. subtilis there is no detectable comK–lacZ expression in the absence of functional ComK, demonstrating that ComK has auto-activating properties (van Sinderen and Venema, 1994). ComK exerts this auto-activation by direct binding to its own promoter, PcomK (van Sinderen and Venema, 1994). This binding is stimulated by the presence of the priming protein DegU, which exerts a positive effect on competence development (Hamoen et al., 2000). Although the transcription of the downstream comG operon does not occur in vitro or in Escherichia coli unless ComK is provided, the transcription of comK itself occurs readily under both conditions in the absence of ComK (van Sinderen et al., 1995; below and Fig. S1). These apparent discrepancies suggest that ComK acts in vivo at PcomK by antagonizing the action of one or more repressor proteins. Three candidate repressors have been identified that directly repress transcription from the comK promoter; the transition state regulator AbrB (Hamoen et al., 2003), the nutritional regulator CodY (Serror and Sonenshein, 1996) and Rok (Hoa et al., 2002).

In this study, we demonstrate that ComK acts as an antirepressor at its own promoter in vivo as well as in vitro,
relieving Rok- and CodY-mediated repression. Strikingly, it does so without preventing binding of the repressors. The antirepression activity of ComK is potentiated by DegU, which also binds to the promoter of comK. Interestingly, ComK is able to activate transcription of recA by reversing the repression exerted by LexA (Hamoen et al., 2001). ComK thus activates transcription in two distinct manners; as a classical transcriptional activator at downstream competence genes such as comG, and as an antirepressor at PcomK and PprecA. To our knowledge, ComK is the only DNA binding protein proven to reverse transcriptional repression without eliminating the binding of the repressors, although this novel mechanism may reflect a property of other proteins that bind through the minor groove of DNA.

Results

Rationale for the antirepression hypothesis

ComK acts as a transcriptional activator at PcomK and at downstream competence promoters, such as the promoter of comG (PcomG). ComK is required for in vitro transcription from PcomG (Hamoen et al., 1998; Susanna et al., 2004) but when PcomK was incubated with nucleotides and RNAP in the absence of ComK, strong transcription was observed (see below). Additionally, a fusion of the lacZ coding sequence to PcomK was readily expressed in a heterologous host (E. coli), whereas a PcomG–lacZ fusion was not, unless comK was coexpressed (van Sinderen et al., 1995; Susanna et al., 2006). The observed expression from PcomK in E. coli could indicate that ComK acts as an antirepressor in B. subtilis, antagonizing one or more species-specific repressors (Supplementary Fig. S1). In fact, the E. coli genome (Blattner et al., 1997) contains no homologues of the previously identified repressors of comK (CodY, AbrB and Rok), as judged by a BLAST similarity search (http://www.ncbi.nlm.nih.gov/blast/). The hypothesis of antirepression makes a strong prediction; that removal of repressors would render transcription from PcomK in B. subtilis at least partially independent of ComK. To test this, we introduced mutations in the genes encoding these repressors, and evaluated the expression of an ectopic comK–lacZ reporter fusion.

Repressor mutations result in a partial bypass of the ComK requirement in vivo

The requirement for ComK was not detectably bypassed by inactivation of codY or abrB, either individually or in combination (Fig. 1 and W.K. Smits et al., unpubl. obs.). Rok was recently identified as a major repressor of competence (Hoa et al., 2002). Therefore, we evaluated the effect of a rok mutation on PcomK expression in wild type (Fig. 1A) and comK backgrounds (Fig. 2B). In the presence of ComK, the deletion of rok leads to high levels of transcription, as was previously reported (Hoa et al., 2002). Importantly, there is significant comK–lacZ activity in the ∆comK ∆rok background, although it is about three- to fourfold lower than wild type and seven- to eightfold reduced compared with a comK+ background carrying a rok mutation. This result indicates that a rok mutation partially bypasses the in vivo requirement of ComK for its own expression. Interestingly, the bypass is stronger when both codY and rok are inactivated (Fig. 1B), despite the fact that a codY mutation alone does not lead to a detectable bypass.

Taken together, these results indicate that ComK reverses the action of Rok and strongly suggests a similar role for ComK towards CodY, thus supporting the hypothesis that ComK acts as antirepressor at its own promoter in vivo. The partial nature of the bypass suggests either that ComK plays a role at PcomK as a classical activator
as well as an antirepressor protein or that an additional repressor is involved. Such an additional repressor is unlikely to be AbrB, for reasons presented in the Discussion.

Rok and CodY repress transcription from PcomK in vitro

In order to further analyse the effects of individual transcription factors, we established in vitro transcription assays for the promoter of comK using purified B. subtilis RNAP holoenzyme (a kind gift from M. Salas). A template for these experiments was constructed by cloning a 186 bp fragment derived from sequences upstream of comK into plasmid pAN583 (Predich et al., 1992), as described in Experimental procedures. The resulting plasmid contains the sequence of the comK promoter that is required for binding of all known transcriptional regulators of the comK gene (Serror and Sonenshein, 1996; Hamoen et al., 1998; Hamoen et al., 2000; 2003; Hoa et al., 2002).

In contrast to the results obtained with pAN-G, a PcomG-containing derivative of pAN583 (Hamoen et al., 1998), there is a substantial level of transcription from PcomK under the conditions used (Figs 2 and 3, inset lanes marked with X). This level of transcription was not significantly augmented by the addition of ComK or DegU over a wide range of concentrations, either alone or in combination (Fig. 2A and B). A small but consistent increase (around 1.5-fold) was observed when only ComK was added to the reaction mixture, but this effect was not observed in the presence of DegU. In both cases, a decrease in transcription was evident at higher concentrations of ComK protein. A similar decrease with high ComK concentrations was observed when pAN-G was used as a template for the in vitro transcriptions (data not shown). The reason for this is not clear, but it may reflect non-specific inhibition by this DNA binding protein at high concentrations. These experiments indicate that strong auto-stimulation from PcomK is absent when ComK is added to the mixture, either alone or in the presence of DegU.

Next, we investigated the effects of Rok and CodY on the in vitro transcription from PcomK. The addition of Rok leads to strong repression, with no detectable transcrip-
The binding of CodY to the promoter of comK has been described previously (Serror and Sonenshein, 1996). However, the effects of the recently identified cofactors guanosine triphosphate (GTP) (Ratnayake-Lecamwasam et al., 2001) and branched chain amino acids (BCAAs) (Shivers and Sonenshein, 2004) have not been documented for PcomK. Although binding of CodY occurs in the absence of cofactors, despite the presence of an excess of non-specific competitor (poly-dIdC), we found that BCAAs stimulated binding of CodY to the ComK promoter (data not shown), in agreement with results obtained with other promoters (Shivers and Sonenshein, 2004). In contrast, GTP only stimulated binding detectably in the presence of BCAAs (data not shown). However, these effects on binding were relatively minor; affinity in the presence of both effector molecules increased only approximately twofold under our experimental conditions. In spite of this, we observed no transcriptional repression with CodY in the absence of effectors, under conditions that resulted in a fully retarded PcomK probe in the EMSA experiments (Fig. 3A). The addition of a mixture of BCAAs resulted in strong repression, with a cumulative repressive effect in the presence of GTP (Fig. 3A). We conclude that the effector molecules have a minor effect on the affinity of CodY for its target promoter, but are required for the repressor activity of CodY. The concentration of CodY required to achieve full repression is in good agreement with both EMSA experiments and with the concentration used for previously reported footprinting experiments (Serror and Sonenshein, 1996). As in the case of Rok, we observed that the addition of DegU to the in vitro transcription reactions exerted only a mild effect on CodY-mediated repression under the conditions tested (data not shown).

Rok reduces the affinity of RNAP for PcomK

Many repressors act by masking the −35 or −10 promoter elements that constitute the RNAP binding site. Indeed, DNase I footprinting experiments have revealed that AbrB and CodY bind to PcomK at sequences that partially overlap these elements (Serror and Sonenshein, 1996; Hamoen et al., 2003). However, the location of the binding site for Rok is unknown. Despite repeated attempts, we were unable to further define the binding site for Rok using DNaseI footprinting experiments or bioinformatic approaches (Albano et al., 2005). Using Rok and overlapping PcomK-probes in EMSA experiments, we narrowed the region encompassing the Rok binding site to 130 bp (Fig. 4). This segment includes the sequences that are protected by ComK and DegU in hydroxyl radical footprint experiments (Hamoen et al., 1998; 2000). Surprisingly, the Rok-binding site appears to lie upstream of the −35 and −10 sequences, making it unlikely that Rok occludes
the RNAP binding site in the comK promoter. Considering its function as a repressor, we were therefore interested to see whether Rok reduces the affinity of RNAP for PcomK, despite the location of its binding site.

EMSA experiments using purified RNAP and Rok demonstrated that Rok reduces the binding of RNAP (Fig. 5). The concentration of Rok required for half-maximal reversal of RNAP binding is comparable to the apparent KD of Rok-binding to PcomK in the absence of RNAP. Although this reversal would appear to be consistent with direct competition between RNAP and Rok for binding to PcomK, we also consistently observed a slightly super-shifted protein/DNA complex in the presence of both RNAP and Rok, evident when intermediate concentrations of Rok are used. This super-shifted band migrates slightly slower than the complexes of the DNA with RNAP or Rok alone (Fig. 5). It thus appears possible that, in contrast to AbrB and CodY, Rok does not compete directly with RNAP for binding to the DNA but relies on an alternative mechanism to reduce the affinity of RNAP for PcomK. Although this would be consistent with the unusual location of the Rok repressor binding site noted above and is not unprecedented (Perez-Martin et al., 1994; Rojo, 2001), it is a suggestion that deserves further confirmation.

**ComK reverses repression in vitro**

These experiments enabled us to directly test the antirepression hypothesis by determining whether ComK acts in vitro to reverse Rok and CodY repression. At 170 nM Rok, transcription from PcomK was reduced sixfold (Fig. 2B and C, dash-dotted line). The addition of ComK to the repressed promoter resulted in a moderate increase in transcription, although transcription was submaximal at concentrations greater than ~130 nM ComK protein (Fig. 2C). The observed decrease in transcription at high levels of ComK may be non-specific, as noted above, and was not investigated further. Previously, it was reported that DegU acts as a priming protein, enhancing the affinity of ComK for its own promoter (Hamoen et al., 2000).

Interestingly, this phenomenon is independent of protein-protein contacts, and presumably relies on modulation of
DNA topology (Hamoen et al., 2000). In light of these findings, we were interested to see if the antirepression by ComK would be enhanced in the presence of DegU. Results from in vitro transcription experiments indeed demonstrate that the transcription from PcomK is restored to approximately 80% of the unrepressed state, when DegU is present in the reactions (Fig. 2C). It thus seems that DegU potentiates the antirepressing activity of ComK.

During our in vitro transcription experiments, we observed that antirepression was strongest at concentrations of Rok that did not fully repress the ComK promoter. Previous observations of multiple DNA–protein complexes in EMSA experiments using the comK promoter (Albano et al., 2005) hint at the possibility that at high concentrations of Rok, the protein occupies multiple sites and may not be susceptible to antirepression. Thus, in vivo auto-stimulation might only occur within a narrow range of Rok levels, implying that the expression of Rok must be fine-tuned to allow competence development. Previously, it was reported that a rok-lacZ promoter fusion is expressed at an essentially constant rate throughout growth (Hoa et al., 2002). This conclusion is supported by measurements of fluorescence made with a Prok-gfp reporter in single cells showing that Prok-driven expression remains constant in minimal medium and demonstrates little variability between cells, whereas it increases in a rich medium (Fig. S2).

The in vivo data strongly suggest that ComK not only antagonizes repression by Rok, but also by CodY. To test this, we performed in vitro transcription reactions under conditions that support repression by CodY, i.e. in a binding buffer containing BCAAs (Fig. 3A). The addition of the amino acid mixture did not affect transcription in the absence of CodY (data not shown). The addition of 8 μM CodY in the absence, or of 4 μM CodY in the presence of additional GTP, reduced transcription around 10-fold compared with the unrepressed state (Fig. 3, dash-dotted line). From Fig. 3B it can be seen that the repression by CodY in the presence of BCAAs is readily reversed upon the addition of ComK protein. We consistently observed a four- to sixfold increase in transcription compared with the CodY-repressed state. As was the case for Rok, we observed stronger antirepression in the presence of DegU protein. Moreover, the concentrations of ComK required to achieve maximal derepression are comparable for both CodY and Rok (~130 nM). Taken together, these results demonstrate that ComK acts as an antirepressor at its own promoter towards both Rok and CodY.

ComK does not prevent binding of the repressing proteins at PcomK

The binding site of ComK (partially) overlaps those of the repressor proteins acting on PcomK as judged by the results from DNA footprinting experiments (Fig. 4) (Serror and Sonenshein, 1996; Hamoen et al., 1998; 2000; 2003), raising the possibility that the prevention of repressor binding to the DNA is responsible for the antirepression observed in this study. In fact, it was recently reported that CcpA activates transcription of the ilvB operon in B. subtilis by preventing the binding of the transcriptional repressor CodY (Shivers and Sonenshein, 2005). Therefore, EMSA experiments were carried out to test this hypothesis.

The addition of Rok, DegU and ComK individually, resulted in significant retardation of a fragment of PcomK encompassing all three binding sites (Fig. 6A), confirming earlier findings (van Sinderen et al., 1995; Hamoen et al., 2000; Hoa et al., 2002). DegU can bind simultaneously with Rok or ComK, as is evident from the super-shifted bands (Fig. 6A), and stimulates the binding of ComK (Hamoen et al., 2000), but probably not Rok. Surprisingly, the presence of both Rok and ComK results in a super-shifted band, indicating that there is no obvious competition between the two proteins for binding to PcomK. In fact, a difference in mobility of the probe (super-shift) was
observed between the lanes with two proteins and the lane in which all three transcription factors (Rok, ComK and DegU) were present (Fig. 6A; W.K. Smits et al., unpubl. obs.). Moreover, the addition of DegU to a mixture containing Rok and ComK increased the total amount of probe shifted. These observations demonstrate that Rok, ComK and DegU can simultaneously interact with P

Analogous to the situation for Rok, we investigated whether competitive binding occurs between CodY and ComK. Strikingly, the addition of CodY to a mixture that contains ComK resulted in a super-shift as well, both in the presence and absence of DegU protein (Fig. 6B). This indicates that ComK, DegU and CodY are also able to bind simultaneously to the same fragment of the comK promoter. Although in vitro repression by CodY only occurred in the presence of BCAAs, the super-shift results obtained in the presence of BCAAs and/or GTP were comparable to those in the absence of effector molecules (data not shown). The presence of ComK appears to release some CodY from the comK promoter (Fig. 6B, compare lanes Y and YK), and we cannot exclude the possibility that these proteins interact directly. This negative effect of ComK on CodY binding was not observed in the presence of DegU and does not affect the conclusion that CodY, ComK and DegU can interact simultaneously with the same fragment of DNA.

Rok binding is inhibited by major groove binding drugs

ComK is able to reverse Rok- and CodY-imposed repression at its own promoter, without reversing the binding of these repressor proteins. Simultaneous binding of proteins to the DNA is possible if they bind to different surfaces of the DNA helix. ComK is known to bind through the minor groove of the DNA (Hamoen et al., 1998) and based on the published crystal structure, CodY is thought to be a major groove binding protein (Levdikov et al., 2006). Because the DNA binding domain of Rok and its binding characteristics are largely unknown, we addressed whether Rok binds to the major or minor groove of the DNA. To this end, we performed gel shift experiments in the presence of drugs that interfere with binding in either the major (methyl green) or minor (chromomycin A3) groove of the DNA. We found that the addition of methyl green strongly reduced the affinity of Rok for the comK promoter, whereas no effect was observed for chromomycin A3 at relevant concentration for these drugs (Fig. 7). These results are precisely the opposite of those obtained for ComK binding to the same promoter (Hamoen et al., 1998). Similarly, the addition of actinomycin D, another minor groove binding drug, did not interfere with Rok-binding (data not shown). These data strongly suggest that Rok is a major groove binding protein, like CodY, and thus might bind to PcomK without interfering with ComK-binding through the minor groove of the DNA.

Discussion

The expression of comK in B. subtilis demonstrates an absolute requirement for ComK. In contrast to comG, however, the gene is readily expressed in vitro or in a heterologous host (van Sinderen and Venema, 1994; van Sinderen et al., 1994). These data led to the prediction that there are two distinct mechanisms for transcriptional activation by ComK. The first involves direct activation of genes that show low (or no) basal levels of transcription in...
the absence of ComK, such as comG. Indeed it has been shown that ComK directly activates transcription from PcomG, by stabilizing the binding of RNAP (Susanna et al., 2004). For the second class of genes, which includes recA (Hamoen et al., 2001), ComK reverses the repression of promoters that demonstrate a significant basal level of transcription in the absence of transcription factors. The present study substantiates this hypothesis and establishes comK as a representative example of this second class of activated genes.

Antirepression at the comK promoter

Three repressors of comK have been identified: AbrB (Hamoen et al., 2003), CodY (Serror and Sonenshein, 1996) and Rok (Hoa et al., 2002). In vitro transcription experiments demonstrate that ComK can act as an antirepressor towards the latter two (Figs 2 and 3). This is supported by the observation that the in vivo requirement of ComK for its own expression is partially bypassed by inactivation of rok, and that this bypass is stronger when codY is inactivated as well (Fig. 1B). However, the levels of transcription from PcomK in a codY rok comK triple mutant do not reach those of a wild-type strain (Fig. 1B). The residual requirement of ComK may be due to the small but significant increase in transcription through the action of ComK itself (Fig. 2A). Alternatively, the action of another repressor, such as AbrB, might be responsible. Repeated attempts to construct an abrB codY rok comK–lacZ strain failed, both in a comK+ and a ΔcomK background, suggesting that knocking out all three pleiotropic repressors is lethal. AbrB acts both positively and negatively in the competence regulatory cascade (Hahn et al., 1995) (Hahn et al., 1996), but its positive requirement is bypassed by a rok mutation (Hoa et al., 2002). When the effect of a rok abrB codY triple mutation on comK–lacZ expression was assessed, only a marginal increase in transcription compared with a rok comK double mutant was observed (data not shown). Furthermore, in vitro transcription results suggest that ComK is unable to act as an antirepressor with respect to AbrB (Hamoen et al., 2003; W.K. Smits et al., unpubl. results). Although we cannot exclude the possibility that yet another repressor of comK which is antagonized by ComK remains to be identified, this is unlikely to be AbrB.

The priming protein DegU exerts its positive influence on competence development by stimulating ComK binding to its own promoter (Hamoen et al., 2000). Mutation of degU reduces the overall expression of comK (van Sinderen and Venema, 1994; Hahn et al., 1996) and leads to a decrease in the fraction of competent cells (W.K. Smits, unpubl. obs.). In this study, we report that DegU potentiates ComK as an antirepressor (Figs 2 and 3). It is noteworthy that the EMSA results demonstrate that CodY and Rok do not prevent binding of DegU (Fig. 6). Had this been the case, antirepression would have been severely hampered. As might be expected from its effect on the affinity of ComK-binding to PcomK, the presence of DegU permits slightly higher levels of antirepression at lower concentrations of ComK than are needed in the absence of DegU (Fig. 2).

Previous work has shown that even a slight increase in the native levels of Rok is sufficient to completely abolish competence development (Hoa et al., 2002). In addition, AbrB and SinR, which exert minor negative effects on rok expression and Rok protein levels are both positively required for competence development unless rok is mutated (Hoa et al., 2002). It appears that small increases in the in vivo concentration of Rok have major effects on the expression of comK. In the course of this study, we observed that in vitro we could only observe antirepression when transcription from PcomK was not fully repressed, and, our in vivo measurements of Prok-gfp levels support the notion that under conditions that sustain competence, rok expression is kept constant. Together, this evidence suggests that Rok is a critical determinant for competence development by determining the potential for auto-activation by ComK. The competence regulatory system is maintained on a knife-edge in which minor perturbations of protein concentrations can drive the system towards dramatically increased expression, or prevent the initiation of competence altogether. The importance of Rok in controlling the level of comK expression is reinforced by observations that complete reversal of repression in vitro cannot be achieved by the addition of ComK (Fig. 2) and that a rok knockout mutant expresses higher levels of fluorescence per competent cell from comK-gfp than the isogenic rok+ strain (data not shown). The rok gene itself is subject to complex regulation (Hoa et al., 2002) and more detailed investigations are necessary to address how Rok levels are regulated.

A model for the action of ComK early in competence development

Based on the data presented in this study, the following sequence of events can be proposed for early events in the development of competence for genetic transformation. During the exponential growth phase, transcription from the comK promoter is prevented through the repression exerted by AbrB, CodY and Rok (Fig. 8A). The little ComK that is produced is trapped by the adaptor protein MecA, and targeted for degradation by the ClpCP protease (Turgay et al., 1997; 1998). At the end of the exponential growth phase, quorum sensing stimulates the release of ComK from the proteolytic complex, after which it is able to exert its function as a transcription factor in the cell (Fig. 8B). Consistent with this sequence
Fig. 8. Model for the regulatory events early in competence development. Thickness of the lines indicates the strength of the regulatory interaction. Positional information is not conserved in this representation. A, AbrB; K, ComK; P, proteolytic complex; R, Rok; Rp, RNA polymerase holoenzyme; U, DegU; Y, CodY.

A. The comK promoter is repressed by Rok, AbrB and CodY. DegU is present. Residual ComK is trapped by the proteolytic complex.

B. Quorum sensing events cause limited release of ComK. In the presence of DegU, ComK acts as an antirepressor towards Rok and CodY, without preventing their binding.

C. In stationary growth phase and upon nutrient limitation AbrB and CodY repression are relieved (see text for details).

D. Phosphorylation reduces the affinity of DegU for PcomK, causing it to dissociate.

E. ComK antirepression with respect to Rok still occurs in the absence of other factors.
of events is the observation that disruption of the proteolytic complex leads to an altered timing of competence development, whereas deletion of a repressor does not markedly alter the timing (Maamar and Dubnau, 2005). Low levels of ComK, through the priming action of DegU, are able to initiate the reversal of repression by Rok and CodY (Figs 2, 3 and 8B). Additionally, ComK might stimulate its own transcription directly. Levels of activated Spo0A rise in late exponential growth phase, thereby efficiently downregulating the transcription of AbrB (Fujita et al., 2002; Ogura et al., 2005) and this is the sole reason for the Spo0A requirement for the development of competence (Hahn et al., 1995). Therefore, reversal of repression by AbrB in vivo occurs independently of ComK. Nutritional repression by CodY is relieved when depletion of nutrients occurs, affecting the energy status of cells (Serrtor and Sonenshein, 1996; Ratnayake-Lecamwasam et al., 2001). This may explain why mutation of codY has a minor effect in the bypass of the ComK requirement in vivo, because it is already partially inactivated by this physiological mechanism. This notion is also consistent with the strong effect of Rok, because no downregulation of rok expression in minimal medium has been documented (Hoa et al., 2002 and Fig. S2). Together, antirepression and derepression at the comK promoter (Fig. 8B and C) lead to a strong increase in ComK levels, allowing transcription of the ComK regulon (Berka et al., 2002; Hamoen et al., 2002; Ogura et al., 2002).

The mechanisms of antirepression

Antirepression in bacteria can occur by at least three distinct mechanisms. In one, the antirepressing protein binds to the repressor preventing its binding to DNA. This mechanism has been documented in a number of cases, for instance in the regulation of SinR activity in B. subtilis (Bai et al., 1993) and MogR regulation in Listeria by GmaR (Shen et al., 2006). A second mechanism of antirepression in bacteria involves occlusion of the repressor binding site by other DNA binding proteins. For example, in B. subtilis, the binding of CcpA to the ilvB promoter prevents binding of the nutritional repressor CodY (Shivers and Sonenshein, 2005). In E. coli, several instances have been identified where antirepression involves interactions between multiple proteins, resulting in the release of the repressors (Browning et al., 2004; 2005; 2006). Here, we demonstrate a third mechanism in which ComK does not interfere with binding of the repressor proteins Rok and CodY. How does ComK act as an antirepressor?

The fact that ComK (Hamoen et al., 1998) and the repressors (Levdikov et al., 2006 and Fig. 7) bind through different grooves of the DNA may permit simultaneous binding by placing the proteins on distinct surfaces of the DNA helix even when they bind to the same area of the promoter. Minor groove binding proteins are frequently architectural proteins that sometimes bind nonspecifically to DNA (Bewley et al., 1998). It has been noted (van Sinderen et al., 1995) that ComK demonstrates limited similarity to the male sex determining factor SRY and T cell factor 1 (TCF-1), activators belonging to the high mobility group of minor groove binding proteins (Bewley et al., 1998). Binding of SRY to DNA induces strong bending, due to insertion of a hydrophobic wedge of the protein into the helix, forcing it from the B-form to the A-form (Bewley et al., 1998). Virtually all minor groove binding proteins, including ComK (Hamoen et al., 1998), bend or distort the DNA. This change in topology has been implicated in the assembly of higher-order DNA–protein complexes such as the transcriptional machinery (Bewley et al., 1998; Carey, 1998).

We suggest that this known ability of ComK to bend DNA is at the heart of its action as both an antirepressor and an RNAP recruitment protein. In the case of the comK promoter, we have suggested above that Rok may reduce the affinity of RNAP for PcomK through a mechanism other then occlusion of the core promoter elements (Fig. 5). If Rok alters the DNA topology such that the RNAP–DNA interaction is weakened, ComK-induced bending of the DNA might counteract these effects without preventing the binding of the repressor. If instead Rok represses by competing for RNAP binding, then ComK may reverse this effect by inducing a bend in DNA that reduces steric clashes between Rok and RNAP. A similar mechanism may obtain in the case of CodY.

In a detailed investigation of the mechanism of transcriptional activation of PcomG, it was reported that ComK does not seem to interact directly with the alpha subunit of RNAP (Susanna et al., 2004). Instead, it was postulated that ComK-induced bending wraps the upstream DNA around RNAP, thus stabilizing the interaction (Bewley et al., 1998; Susanna et al., 2004). We thus propose that ComK acts as an activator at both types of promoters by modulating DNA topology.

Other minor groove binders may act similarly to ComK as antirepressors. For instance, the phage protein p4G that presumable recognizes specific AT-tracts in the minor groove of the DNA has also been postulated to act as an antirepressor, antagonizing an unknown repressor protein (Horcajadas et al., 1999). Another interesting case involves regulation of the aceBAK operon by the integration host factor (IHF) (Goodrich et al., 1990; Resnik et al., 1996). IHF, a minor groove binding architectural protein that binds to an AT rich consensus sequence (Yang and Nash, 1989; Sun et al., 1996), interacts with two sites upstream of PaceBAK, and relieves repression by IclR. Because these two proteins bind to distinct sites, it is possible that IHF, like ComK, can relieve repression without prevention of repressor binding.
Perspective on ComK-mediated antirepression

ComK also antagonizes the repression by LexA on PrecA without directly displacing the repressor (Hamoen et al., 2001) and indeed other cases of antirepression by ComK may exist. Recently, it was reported that the ComK-activated gene rapH (Berka et al., 2002; Hamoen et al., 2002; Ogura et al., 2002) is under control of the repressor RghR (Hayashi et al., 2006). As with PrecA and PcomK, expression from PrapH occurs readily in the absence of ComK when RghR is absent (Hayashi et al., 2006). In addition, we found that RghR and ComK can bind simultaneously to a promoter fragment of rapH (W.K. Smits, C. Bongiorni, J.W. Veening, L.W. Hamoen, O.P. Kuipers and M. Perego, submitted for publication). It seems likely that further characterization of ComK-activated genes will reveal additional examples of antirepressed genes.

Because ComK can act as either an antirepressor or as a more classical activator protein, it might appear that some common promoter sequence element determines the mode of action of ComK in each case. Inspection of the various ComK-dependent promoters does not suggest any obvious explanatory feature, and perhaps the relevant difference is that some promoters like PcomG have relatively weak affinities for RNAP and need ComK to recruit this enzyme while PcomK and PrecA do not. Unlike PcomG, both of the latter promoters instead bind repressors and have therefore evolved to permit antirepression by ComK. It may be then that the two classes of promoters are distinguished by their relative affinities for RNAP, and by the presence or absence of repressor binding sites. Promoters subject to antirepression by ComK would be further characterized by the precise disposition of the repressor binding sites with respect to the ComK boxes so that the ComK-induced bending of DNA will prevent the repressors from interfering with the action of RNAP.

In conclusion, we propose that ComK exerts its function as an antirepressor without preventing binding of the repressors, based on its ability to induce bending of DNA, thereby stabilizing the binding of RNAP. We suggest that a remarkable choreography of DNA bending at PcomK, mediated by the binding of multiple repressors, activators and RNAP, regulates comK expression in response to upstream signal transduction pathways. Most minor groove binding proteins induce bending of DNA (Bewley et al., 1998) and it is tempting to speculate that antirepression or direct activation by other minor groove binding proteins relies on similar mechanisms.

Experimental procedures

Bacterial strains and media

All Bacillus subtilis strains are derivatives of the reference strain B. subtilis 168 (Kunst et al., 1997), are isogenic derivatives of strain BD630 (his leu met) or 168 (trpC2) and are listed in Table S1. Transformation, as well as selective and growth media are described or referenced in Albano et al. (1987) and Hamoen et al. (2002). Plasmids were maintained in E. coli strains as indicated in Table S1. Strain ProkG was constructed as follows. The promoter region of the rok gene was amplified by polymerase chain reaction (PCR) using primers Prok-F-new-KpnI and Prok-R-new-EcoRI and chromosomal DNA of strain 168 as a template. The 1543 bp amplified fragment was digested with KpnI and EcoRI and cloned into similarly digested pSG1151 (Lewis and Marston, 1999), yielding plasmid pSG1151-Prok(NN). This plasmid was used to transform B. subtilis, and transformants were selected after overnight growth at 37°C on TY with chloramphenicol. Campbell-type integration of the plasmid was verified by PCR (data not shown). Strain KGFP (comK-gfp) was obtained by transformation of plasmid pcomK-gfp (Haijema et al., 2001) into B. subtilis strain 168. Multiple loci were combined by transformation with chromosomal DNA.

Expression and purification of proteins

Rok-his6 (Albano et al., 2005), ComK (Susanna et al., 2006) and DegU-his6 (Haijema et al., 2000) were purified as described previously. CodY-his6 was isolated from E. coli strain KS272 harbouring the pKT1 plasmid (Kim et al., 2003) as follows. An overnight culture was diluted 1:100 into fresh TY with appropriate antibiotics. Growth was continued until OD 0.70 with continuous shaking (250 r.p.m., 37°C). At that moment, expression of CodY-his6 was induced by the addition of 0.1% arabinose, and continued for 1 h. Subsequently, cells were pelleted by centrifugation (10 min, 8000 r.p.m., 4°C), and stored at −80°C. The pellet was resuspended in 5 ml of buffer A (20 mM Tris-HCl pH 8.0, 0.2 M NaCl, 10 mM MgCl₂, 7% glycerol, 1 mM β-mercaptoethanol), supplemented with Complete Mini Protease Inhibitor (Roche), and cells were disrupted by sonication. Cellular debris was removed by centrifugation (10 min, 14 000 r.p.m., 4°C), and the supernatant fraction was incubated with 2 ml of equilibrated Superflow NiNTA resin (Qiagen) in a total volume of 15 ml of buffer A for 2 h with continuous mixing. The column material was packed in a Poly Pre Chromatography Column (Bio-Rad) and washed by gravity flow with 10 column volumes buffer A and 10 column volumes buffer B (identical to buffer A, but with 20 mM of imidazole). The protein was eluted from the column with buffer C (identical to buffer A, but with 500 mM imidazole), and 0.5 ml fractions were collected. Fractions were checked for protein content and purity by SDS-PAGE. Protein was quantified using the RC/DC protein determination kit (Bio-Rad), using a commercial bovine serum albumin solution (New England Biolabs) as a standard. The presence of a C-terminal 6xHis-tag on the DegU protein does not interfere with its function in competence development (Haijema et al., 2000) and CodY-his6 is functional in vivo (Ratnayake-Lecamwasam et al., 2001), but the effects of the tag on Rok are not documented. Although the presence of the 6xHis-tag might interfere with protein–protein interactions, it does not affect the binding of the Rok to the DNA or its ability to repress transcription. © 2007 The Authors

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**Electrophoretic mobility shift assays**

Primers used to generate fragments for use in this EMSA experiments are listed in Table S2. EMSAs were performed as described (Albano et al., 2005), except for samples containing RNAP polymerase. For these samples, the protocol was adapted as follows. Reaction mixtures containing 5000 cpm labelled double stranded DNA were incubated at 37°C for 45 min before loading. Free probe and DNA/protein complexes were separated on a 6% non-denaturing polyacrylamide gel prepared with 1× TAE (40 mM Tris acetate [pH 8.0], 2 mM EDTA), in a 0.5–2.0× TAE gradient, for 2 h at 70 V. Subsequently, gels were dried and signals were recorded as described previously (Albano et al., 2005). To quantify signals, unprocessed captured images were analysed using Quantity One software (Bio-Rad). Data were imported into Microsoft Excel for further analysis and graphs for publication were prepared in Corel Graphics Suite 11.

**In vitro transcription assays**

In vitro transcription experiments were carried out as described before (Harnoen et al., 1998), using 150 ng B. subtilis RNAP holoenzyme (a kind gift of M. Salas) and non-linearized pAN-K plasmid as a template. The pAN-K plasmid was constructed as follows. Plasmid pAN583 (Predich et al., 1992) was cut with PvuII and EcoRI. The fragment carrying the comK promoter was isolated by PCR using primers pCK2 and Kprom1 (Table S2) and chromosomal DNA of BD630 (Browning et al., 2004) as a template. The product was ligated into the SmaI site of pAN583-inverse, yielding pAN583-inverse. The fragment carrying the comK promoter was cloned into the Smal site of pAN583-inverse, resulting in plasmid pAN-K. The resulting plasmid allows the generation of a terminated transcript derived from the comK promoter. Radiolabelled RNA from the in vitro transcription experiments was loaded onto a 6% denaturing polyacrylamide gel prepared with 1× TBE (40 mM Tris acetate, 0.5 M NaCl, 2 mM EDTA), in a TBE gradient, for 2 h at 70 V. Signals were recorded from unprocessed gels using phosphorscreens and a Cyclone PhosphorImager (Packard). Signals were recorded as cpm labelled double stranded DNA were incubated at 37°C, and chromosomal DNA of BD630 (Browning et al., 2004) as a template. The fragment carrying the comK promoter was cloned into the Smal site of pAN583-inverse, resulting in plasmid pAN-K. The resulting plasmid allows the generation of a terminated transcript derived from the comK promoter. Radiolabelled RNA from the in vitro transcription experiments was loaded onto a 6% denaturing polyacrylamide gel prepared with 1× TBE buffer (100 mM Tris borate [pH 8.3], 10 mM EDTA) for 1 h at 150 V. Signals were recorded from unprocessed gels using phosphorscreens and a Cyclone PhosphorImager (Packard). To quantify signals, unprocessed captured images were analysed using Quantity One software (Bio-Rad). Data were imported into Microsoft Excel for further analysis and graphs for publication were prepared in Corel Graphics Suite 11.

**β-Galactosidase assays**

β-Galactosidase reporter assays were carried out as described previously (Albano et al., 2005).

**Fluorescence microscopy**

Fluorescence of individual cells was determined by fluorescence microscopy and image analysis. Samples were taken at hourly intervals and cells were prepared for microscopy as described previously (Albano et al., 2005). Images were captured using the same setting for all time points. To quantify fluorescent signals from individual cells, captured images were imported into Quantity One software (Bio-Rad). A grid for all cells was generated based on the phase-contrast image, and subsequently overlaid on unprocessed images from the GFP-channel. Data were imported into Microsoft Excel for further analysis and graphs for publication were prepared in Corel Graphics Suite 11.

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**References**


**Supplementary material**

The following supplementary material is available for this article online:

**Fig. S1.** Heterologous expression and anti-repression. (A) Schematic depiction of the behavior of P<sub>comK</sub> when ComK acts as an anti-repressor or an activator. RNA polymerase is depicted as a square (Rp), ComK as a diamond (K). Expression in a heterologous host, *E. coli*, is given in the absence of functional ComK protein. (B) Expression from P<sub>comK</sub> in the absence of ComK in *B. subtilis* (KGFP<sub>DK</sub>) and *E. coli* (ED232) (Hajjema *et al.*, 2001). Cells harboring a comK-gfp reporter construct were grown, harvested and visualized according to Materials and methods.

**Fig. S2.** Fluorescence from a Prok-gfp reporter fusion. Fluorescence from single cells was quantified as described in Materials and Methods. Error bars show the standard deviation. The number of cells (n) on which the calculated mean and standard deviations are based is shown below each bar. Time is indicated in hours relative to the transition between exponential and stationary growth phase (T0).

**Table S1.** Strains and plasmids used in this study.

**Table S2.** Oligonucleotides used in this study.

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