

# Rok (YkuW) regulates genetic competence in *Bacillus subtilis* by directly repressing *comK*

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## Summary

**The Rok (YkuW) protein acts as a negative regulator of *comK*, which encodes the competence transcription factor of *Bacillus subtilis*. In the absence of Rok, ComK is overproduced, and when excess Rok is present *comK* transcription is inhibited. Rok acts transcriptionally to repress *comK* expression but does not affect ComK stability, which is controlled by the MecA switch. Gel-shift assays show that Rok binds directly to a DNA fragment that contains the *comK* promoter. SinR and AbrB act negatively on *rok* transcription, and the inactivation of *rok* bypasses the positive requirements for *sinR* and *abrB* for the expression of *comK*. Therefore, the dependence of *comK* expression on SinR and AbrB may be a result of their repression of *rok* transcription. It has also been shown *in vivo* that Rok and ComK can individually repress *rok* transcription, and that Rok and ComK bind to the *rok* promoter *in vitro*. These interactions establish feedback loops, and the roles of these circuits are discussed.**

## Introduction

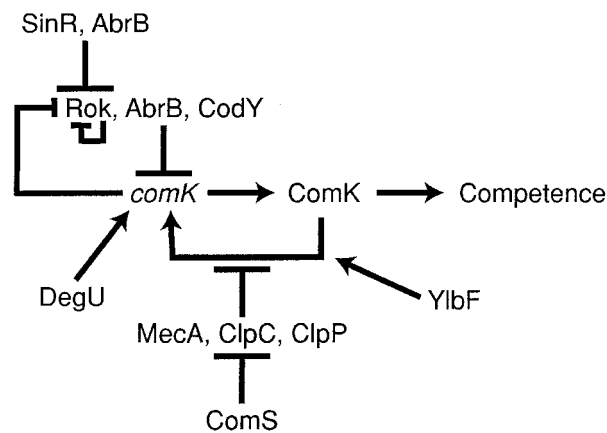
In *Bacillus subtilis*, genes encoding the proteins required for the binding and uptake of transforming DNA are transcribed only in the presence of the competence regulatory factor ComK (van Sinderen *et al.*, 1994;1995; Hamoen *et al.*, 1998). ComK synthesis, in turn, is regulated by a series of reactions that involve quorum sensing, as well as controls at the transcriptional and post-transcriptional levels (Fig. 1). Expression at the *comK* promoter is particularly complex. Two proteins, DegU and ComK itself, bind directly to the *comK* promoter (*PcomK*) and act positively. This action of ComK at its own pro-

moter establishes a positive feedback loop (van Sinderen and Venema, 1994), and imposes switch-like behaviour on the system. DegU, a response regulator, binds to *PcomK* and increases the affinity of ComK for its own promoter (Hamoen *et al.*, 2000). This co-operative binding is probably important early in competence development, when the concentration of ComK is still low. In addition, AbrB and CodY bind to *PcomK* and act directly as repressors (L. Hamoen, M. Marahiel and P. Serror, personal communication; Serror and Sonenshein, 1996). Other proteins act positively on *comK* transcription during the development of competence, including AbrB, which also acts negatively as noted above (Hahn *et al.*, 1996), and SinR. These two proteins activate the transcription of *comK*, although probably not by direct binding to *PcomK*. As AbrB acts as both an activator and a repressor of *comK*, its concentration in the cell must be within a specific range for competence to develop (Hahn *et al.*, 1995a).

In addition to the multiple controls at *PcomK*, ComK stability is regulated by modulation of the ClpCP protease, which degrades ComK before stationary phase and during the escape from the competent state. ComK cannot bind directly to ClpCP and is targeted for degradation by the two-domain adapter protein MecA (Turgay *et al.*, 1998). The N-terminal domain of MecA binds to ComK and the C-terminal domain to ClpC (Persuh *et al.*, 1999). The small protein ComS, synthesized as a result of a phosphorylation cascade initiated by the competence quorum-sensing system (Lazazzera *et al.*, 1999), binds to the N-terminal domain of MecA, causing the release of ComK, which is thereby protected from degradation. During the escape from competence, ComK is bound once again by MecA and is consequently degraded (Turgay *et al.*, 1998).

Another protein, YlbF, also acts positively on the synthesis of ComK (Tortosa *et al.*, 2000). YlbF acts post-transcriptionally to increase the synthesis of ComK, possibly by stabilizing ComS. In an attempt to elucidate the role of YlbF, we used mini*Tn10* mutagenesis to isolate a mutation that suppresses the *ylbF* requirement for competence. This screen yielded a knockout mutation in *ykuW*, a gene of previously unknown function. Although investigation of *ykuW* did not shed light on *ylbF*, we show here that YkuW acts as an additional direct repressor at *PcomK*, together with AbrB and CodY. We also show that inactivation of *ykuW* bypasses the requirements for SinR

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**Fig. 1.** Competence regulation. In this simplified summary diagram, arrowheads and perpendiculars represent positive and negative effects respectively. These effects are variously exerted on the transcriptional or post-transcriptional level as described in the text.

and AbrB for *comK* expression and that these two proteins act to repress the synthesis of YkuW. These observations suggest a hypothesis for the positive roles of AbrB and SinR in competence development. The interaction of YkuW with *PcomK* is demonstrated, and it is shown that YkuW is an autorepressor, probably acting directly on its own promoter.

## Results

### Isolation of a *rok* (*ykuW*) mutant

*YlbF* is required for the induction of genetic competence in *B. subtilis* (Tortosa *et al.*, 2000). In an attempt to explore the basis of this requirement, we used mini *Tn10* mutagenesis (Petit *et al.*, 1990) to isolate mutations that suppress the *ylbF* competence phenotype as described in *Experimental procedures*. One such suppressed strain carried an insertion after the second base of the seventh codon of the previously uncharacterized *ykuW* gene. Independent insertions from other suppressed clones were obtained in the *mecA* gene, consistent with our previous evidence that *mecA* inactivation bypasses the *ylbF* phenotype (Tortosa *et al.*, 2000).

As will be shown below, *ykuW* affects competence independently of *ylbF*. Although inactivation of *ykuW* bypasses the effect of *ylbF* mutation on competence, it does not relieve the sporulation phenotype of *ylbF* (not shown). The bypass of *ylbF* obtained in the *ykuW* null mutant is most probably as a result of derepression of *comK*, compensating for the loss of *ylbF*. Analysis of *ykuW*, therefore, does not inform us about the role of *ylbF*. Accordingly, the remainder of this report will be concerned only with the effect of *ykuW* on competence, and will not further address the role of *ylbF*. All of the experiments to be described were carried out in strains that are

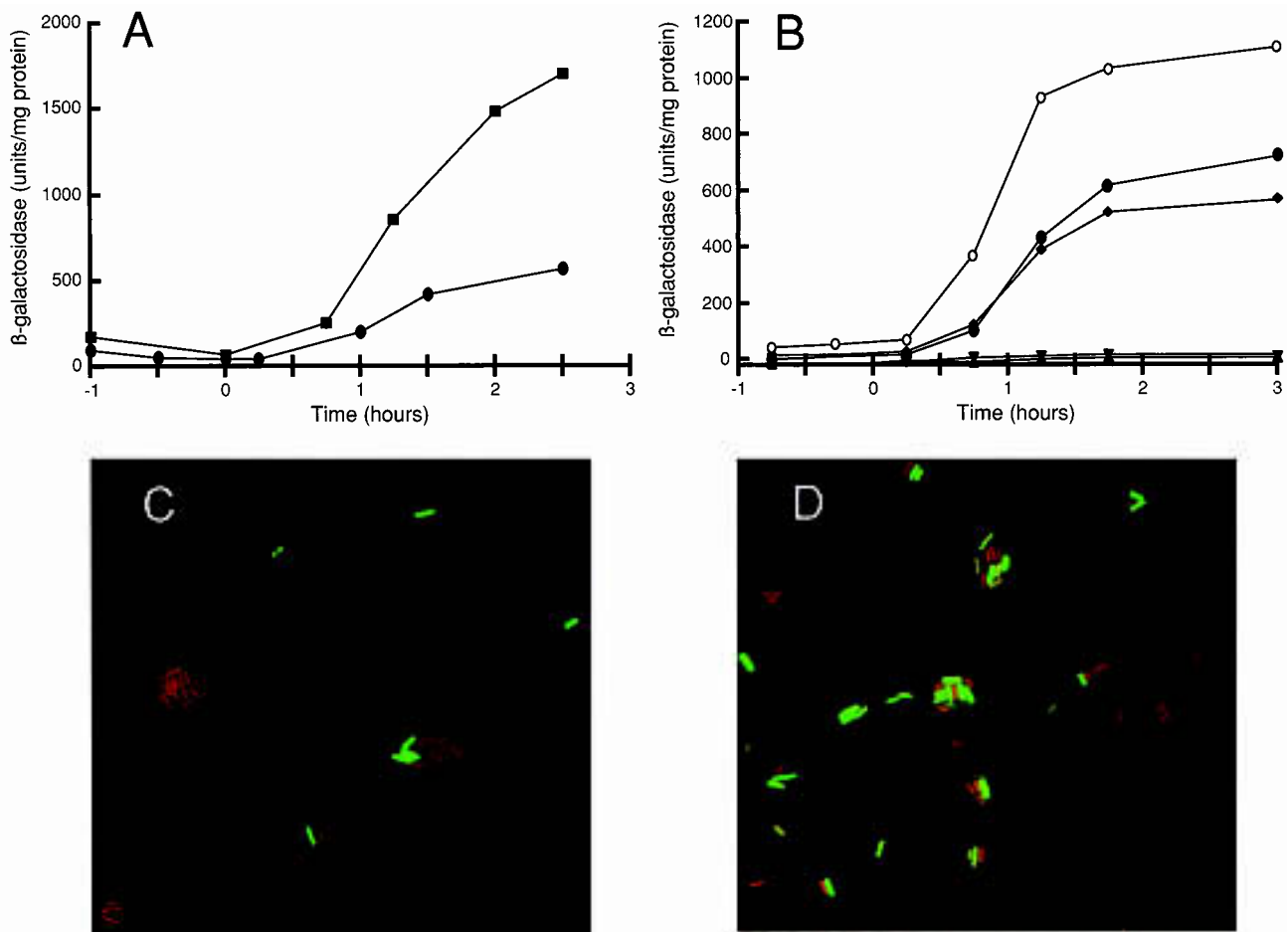
wild type with respect to *ylbF*. As the central finding of this study is that *ykuW* encodes a direct repressor of *comK*, we will refer to *ykuW* with the more descriptive name *rok* (repressor of *comK*).

The *rok* open reading frame (ORF) encodes a predicted protein with 191 amino acid residues. Hydropathy analysis predicts Rok to be a soluble protein. Surprisingly, no protein with significant similarity to Rok was found by BLAST searches in any organism, including the close relatives *Bacillus anthracis*, *Bacillus stearothermophilus* and *Bacillus halodurans*. Upstream of *rok* on the *B. subtilis* chromosome lies *ykuV*, a gene of unknown function. Orthologues of *ykuV* are present in *B. halodurans*, *B. stearothermophilus* and *B. anthracis*. Downstream of *rok*, and transcribed in the reverse direction, is *yknT*. No orthologues of this gene are found in any of the low-GC Gram positives. Finally, downstream from *yknT* is *mobA*, which encodes a protein involved in molybdopterine-guanine dinucleotide biosynthesis. *mobA* orthologues are found in *B. halodurans*, *B. stearothermophilus* and *B. anthracis*. It appears that both *rok* and its neighbour *yknT* are unique to *B. subtilis* among the sequenced bacteria.

### *Rok* acts as a repressor of *comK* expression

Figure 2A shows that the *rok* null mutation increased the transcription of a *comK-lacZ* reporter two- to threefold. To determine the effect of *rok* overexpression, we attempted to construct a pUB110 derivative carrying *rok*. This attempt was repeatedly unsuccessful, suggesting that excess expression of *rok* may be lethal. Instead, we placed *rok* under the control of *Pspac*, which can be regulated by the addition of isopropyl thio- $\beta$ -D-galactoside (IPTG). This inducible construct was integrated at the *rok* locus so as to inactivate the wild-type copy of *rok* (BD3094). Figure 2B shows that in the absence of IPTG the expression of the *comK-lacZ* reporter was increased, although not quite to the level obtained in the *rok* null mutant (compare with Fig. 2A and B), probably because the *Pspac* promoter is leaky. As the IPTG concentration was increased, the reporter expression decreased, even in the presence of 10  $\mu$ M IPTG. We conclude that Rok acts negatively on the expression of *comK*. As expected, almost identical results were obtained using a *comG-lacZ* reporter (not shown), as the expression of the *comG* operon is ComK dependent.

This negative effect of Rok on *comK* expression was examined further by fluorescence microscopy. Figure 2C shows the fluorescence of strains carrying a fusion of *comK* to the green fluorescent protein (GFP). In a wild-type background (BD2716), only about 10% of the cells were fluorescent, because competence and *comK* expression occur in a minority of cells (Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968; Haijema



**Fig. 2.** Effects of Rok dosage on the expression of *comK-lacZ* during growth in competence medium.

A. Expression of *comK-lacZ* in BD1991 (*comK-lacZ*,  $\bullet$ ) and in BD3085 (*comK-lacZ rok*,  $\blacksquare$ ).  
 B. Expression of *comK-lacZ* in BD2594 (*comK-lacZ*,  $\bullet$ ) and in BD3099 (*comK-lacZ pSpac-rok*) in the absence ( $\circ$ ), or the presence of, 0.01 mM ( $\blacklozenge$ ), 0.05 mM ( $\blacktriangledown$ ) or 0.1 mM IPTG ( $\blacktriangle$ ). In panels A and B, time is given in hours before or after the transition to stationary phase  $T_0$ .  
 C and D. Fluorescence microscopy of BD2716 which carries a *comK-gfp* fusion construct in a wild-type background (C) and of BD2958 (D) which carries the same fusion in a *rok* background. The GFP signal is pseudo-coloured green. In both panels, the cells are stained with propidium iodide (pseudo-coloured red) to visualize the total cell population.

*et al.*, 2001). However, in a *rok* mutant (BD2956), the *comK-gfp* fluorescence was expressed in most of the cells in the culture (>60%) (Fig. 2D). Thus, Rok serves to limit the fraction of cells that express competence.

As Rok appears to act negatively on the expression of *comK*, we tested the effect of the *rok::Tn10* mutation on transformability. Inactivation of *rok* increased the frequency of transformation fivefold compared with the isogenic wild-type strain (BD630). We also determined the effects of *rok* inactivation and overexpression on sporulation. The *rok* knockout strain (BD2955) decreased the sporulation frequency about fivefold. When the strain described above, with *rok* under *Pspac* control (BD3094), was tested in the absence of IPTG, a lesser depression (about twofold) was observed, consistent with the results with the knockout strain, and again indicating that the *Pspac-rok* construct is leaky. Overproduction of Rok in

the presence of 0.1 mM IPTG resulted in a 30-fold decrease in the sporulation frequency. These relatively minor effects, which suggest that both under- and over-expression of Rok were deleterious to sporulation, were not investigated further.

The results obtained in this study were all obtained with the transposon-inactivated *rok* mutation. A knockout deletion of *rok* was also constructed with the insertion of a kanamycin resistance marker. This mutation conferred a phenotype that was identical to that of the transposon-inactivated *rok* mutation with respect to competence and *comK* expression (not shown).

#### *The effect of Rok is exerted at the comK promoter*

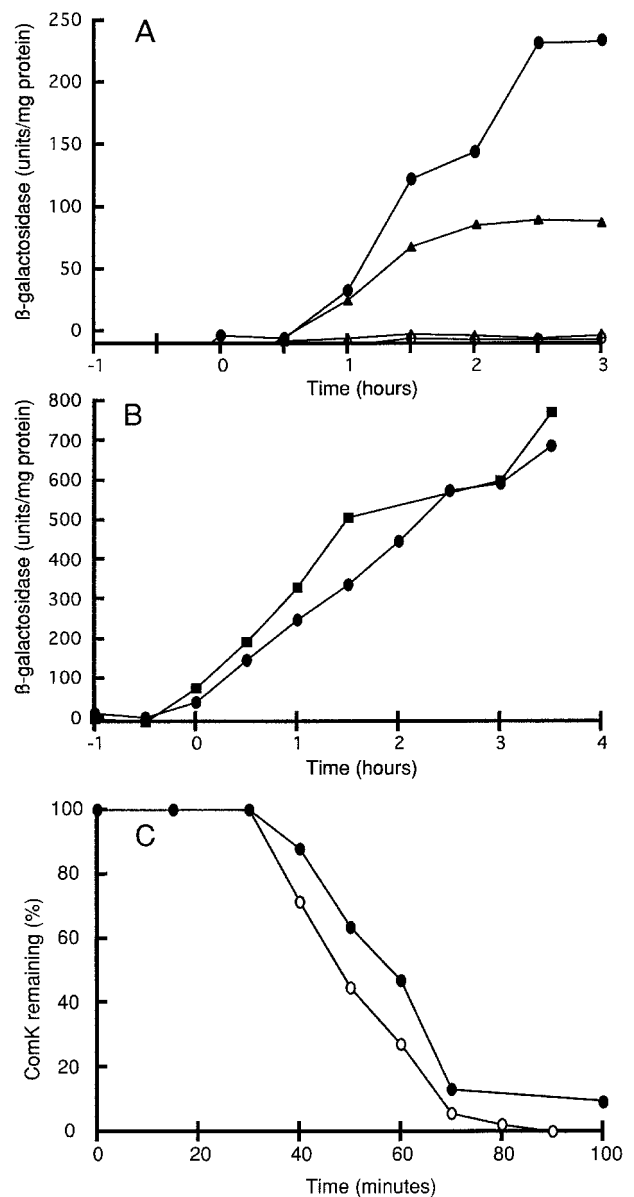
As ComK is required for the expression of its own gene (van Sinderen and Venema, 1994), the effect of Rok on

*comK-lacZ* transcription may be as a result of an effect at the *comK* promoter, or an effect on the activity or stability of ComK. To distinguish these possibilities, we used a construct in which *comK* is under control of the xylose-inducible *Pxyl* promoter and we also inactivated the *comK* locus (Hahn *et al.*, 1996). The only source of *comK* in the resulting strain was from the *Pxyl-comK* construct. Also present was a *comG-lacZ* reporter for ComK activity; the transcription of *comG* is completely dependent on the presence of ComK (van Sinderen *et al.*, 1995). If the effect of *rok* inactivation is exerted post-transcriptionally on *comK*, we would expect to see an increase in *comG-lacZ* expression when *rok* is inactivated in this strain. Figure 3A shows that there was no increase. In fact, the *rok* mutation resulted in a twofold decrease in *comG-lacZ* expression. This effect might be exerted on the activity of ComK or directly on the *comG* promoter by some unknown mechanism. Although this decrease is unexplained, we conclude that the negative effect of Rok on *comK* transcription is probably exerted at *PcomK* (Fig. 1).

This conclusion was buttressed by additional experiments. *comK* is regulated post-transcriptionally by a proteolytic mechanism (Turgay *et al.*, 1998). The end-product of the competence quorum-sensing pathway (Lazazzera *et al.*, 1999) is a small protein, ComS, that prevents the targeting of ComK to the ClpCP protease. *comS* is transcribed as part of the *srfA* operon (D'Souza *et al.*, 1994; Hamoen *et al.*, 1995). If Rok was acting as a repressor of *srfA*, its elimination would result in excess synthesis of ComS, which would increase the level of ComK by inhibiting ComK degradation. However, if Rok acts on the transcription of ComK, the simplest model would predict that its absence would have little or no effect on *srfA-lacZ* transcription. Figure 3B confirms this prediction. Finally, if Rok acts via the promoter of *comK*, the stability of ComK should not be affected by the absence of Rok. To test this prediction, we used Western blotting to measure the cellular content of ComK, at various times during the incubation of competent cells in the presence of tetracycline and rifampicin to prevent further ComK synthesis. As expected from previous results, in the wild-type background the content of ComK decreased steadily (Fig. 3C; Turgay *et al.*, 1998). In the *rok* strain, nearly identical decay kinetics were observed, confirming the prediction.

#### Rok binds directly to PcomK

These experiments demonstrate that Rok acts negatively on transcription from *PcomK*. However, the effect of Rok might be as a result of direct interaction with *PcomK* or might be indirect, acting upstream of another transcription factor. To determine whether Rok binds to the *comK* promoter, we first constructed a His-tagged version of *rok* and used it to purify Rok-His<sub>6</sub> by affinity chromatography.



**Fig. 3.** Rok acts on the transcription of ComK.

A. Expression of *comG-lacZ* in BD2739 (*comG-lacZ comK pxyl-comK*) in the absence (○) and presence (●) of xylose, and of BD2957 (*comG-lacZ comK Pxyl-comK rok*) in the absence (△) and presence (▲) of xylose.

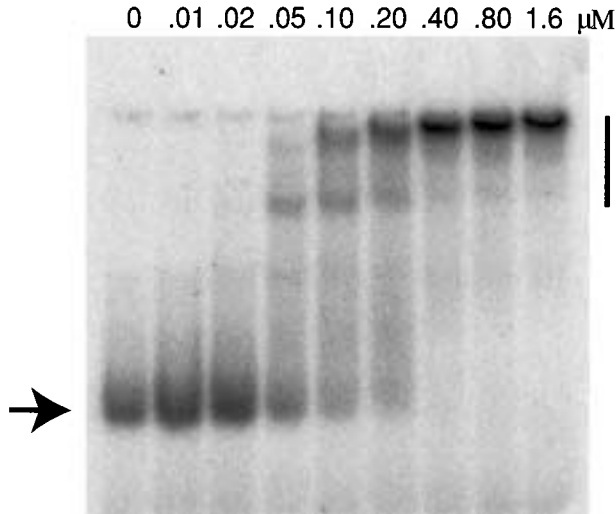
B. Expression of *srfA-lacZ* in BD1890 (*srfA-lacZ*, ●) and in BD3102 (*srfA-lacZ rok*, ■).

C. Degradation of ComK in BD2955 (*rok*, ●) and BD630 (wild type, ○).

In panels A and B, time is given in hours before or after the transition to stationary phase  $T_0$ . In panel C, time is given in minutes after dilution of  $T_2$  cultures into fresh medium containing rifampicin and tetracycline.

The purified Rok-His<sub>6</sub> protein was then used in gel-shift experiments with a DNA fragment that encompassed the sequence from -290 to +18 (relative to the start site of *comK* transcription). Figure 4 demonstrates that Rok-His<sub>6</sub>

bound to the probe fragment with a half-maximum concentration of protein of about  $0.05\ \mu\text{M}$ . This experiment was carried out in the presence of excess poly(dI-dC). Especially with higher concentrations of Rok ( $>0.05\ \mu\text{M}$ ), a second more shifted band appeared, suggesting that more than one molecule of Rok may bind to a given probe fragment. These results, together with those demonstrating that Rok acts negatively on *comK* transcription, suggest strongly that its effect is exerted by binding directly to *PcomK*.



**Fig. 4.** Binding of Rok to *PcomK*. A radiolabelled fragment from the promoter region of *comK* was incubated with Rok-His<sub>6</sub> at the indicated concentrations, before resolution by polyacrylamide gel electrophoresis (PAGE). A vertical bar indicates the positions of the shifted bands. A solid arrow to the left of the figure indicates the position of the radiolabelled probe.

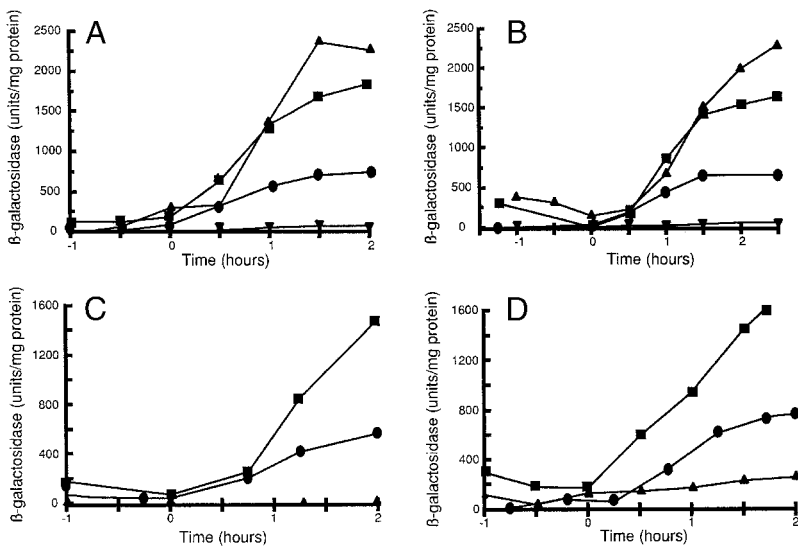
*rok* bypasses loss-of-function mutations in *sinR* and *abrB*

As Rok acts negatively on the transcription of *comK*, we determined whether the *rok* null mutation was able to bypass the negative effects of *abrB*, *sinR* or *comS* inactivation, or of MecA overproduction on the expression of *comK*. Figure 5 displays the results of these experiments, in which the expression of a *comK-lacZ* reporter was measured. Panels A and B demonstrate that the inactivation of *rok* permits the expression of *comK-lacZ* in the presence of individual knockouts of *sinR* or *abrB* to at least the same level as obtained in the isogenic *rok* strain that lacked these inactivating mutations. In the absence of the *rok* mutation, little or no expression of *comK-lacZ* was obtained in *sinR* or *abrB* knockouts, as previously reported (Hahn *et al.*, 1996). We conclude that inactivation of *rok* completely bypasses the need for these two genes for competence expression (Fig. 1).

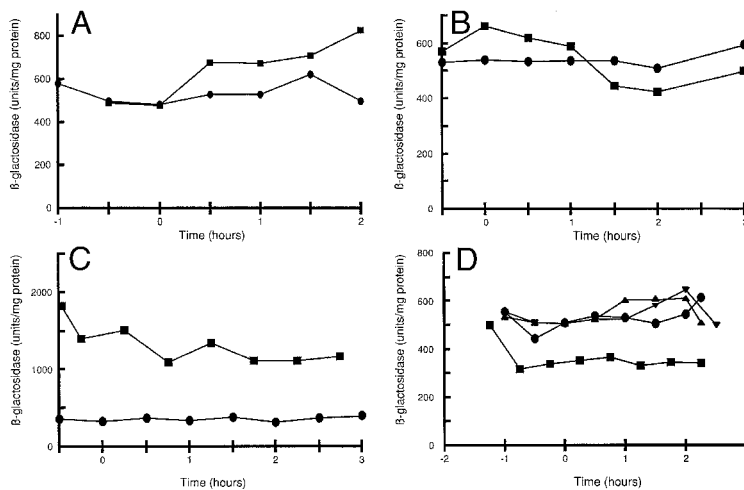
In contrast, *rok* inactivation bypassed neither the inhibitory effects of MecA overproduction (Fig. 5C) nor of *comS* inactivation (Fig. 5D). As described above, ComS and MecA function post-transcriptionally to regulate *comK* (Fig. 1). The failure of the *rok* mutation to bypass the absence of ComS, or the overproduction of MecA, is consistent with the action of Rok on *PcomK*, and confirms that the MecA switch regulating ComK stability is functional in the *rok* mutant.

#### Control of *rok-lacZ* expression

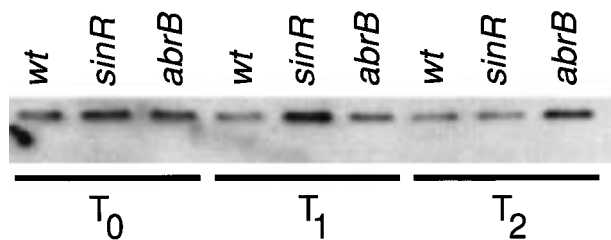
To study the regulation of *rok*, we constructed a fusion of *lacZ* to the *rok* promoter. This construct contained the sequences from  $-960$  to  $+20$  (with  $+1$  defined as the beginning of the ORF) and we assumed that this 980 bp



**Fig. 5.** Bypass of competence regulatory mutations by inactivation of *rok*. In each panel, the expression of *comK-lacZ* from BD1991 (*comK-lacZ*) is represented by a solid dot (●) and that from BD3085 (*comK-lacZ rok*) by a solid square (■). In addition, *comK-lacZ* expression in the following strains is presented: A. BD3090 (*comK-lacZ rok sinR*, ▲), BD1993 (*comK-lacZ sinR*, ▼). B. BD3086 (*comK-lacZ rok abrB*, ▲), BD1992 (*comK-lacZ abrB*, ▼). C. BD3088 (*comK-lacZ rok multicopy mecA*, ▲). D. BD3087 (*comK-lacZ rok comS*, ▲). In panels C and D the curves for the *comK-lacZ multicopy mecA* and *comK-lacZ comS* strains are omitted for clarity, as these expressed little or no  $\beta$ -galactosidase. Time is given in hours before or after the transition to stationary phase  $T_0$ .



**Fig. 6.** Effect of regulatory mutations on the expression of *rok-lacZ*. In each panel the expression of  $\beta$ -galactosidase from BD3091 (*rok-lacZ*) is represented by solid dots (●). In addition, *rok-lacZ* expression from the following strains is shown: A. BD3104 (*rok-lacZ abrB*, ■). B. BD3103 (*rok-lacZ sinR*, ■). C. BD3127 (*rok-lacZ rok*, ■). D. BD3110 (*rok-lacZ mecA*, ■), BD3115 (*rok-lacZ mecA comK*, ▼), BD3106 (*rok-lacZ comK*, ▲). Time is given in hours before or after the transition to stationary phase  $T_0$ .



**Fig. 7.** Effects of *sinR* and *abrB* mutations on the Rok signal in Western blots. Extracts from mutant and wild-type strains were analysed by Western blotting using anti-Rok antiserum. Samples were taken at the indicated times. Equal amounts of total protein were loaded on each lane.

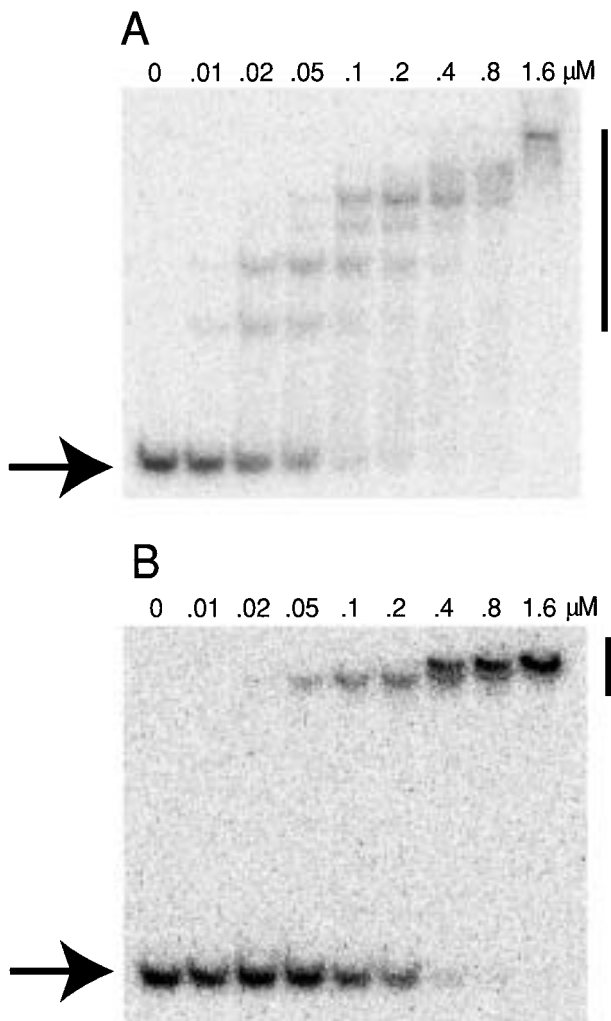
fragment contained all of the *rok* regulatory sequences. The *rok-lacZ* construct was integrated at the ectopic *amyE* locus. The various panels in Fig. 6 show that in competence medium and in the wild-type background, the transcription of *rok-lacZ* did not vary appreciably during growth. In complex medium (Luria broth) the specific activity of  $\beta$ -galactosidase decreased steadily during growth and following the onset of stationary phase, from about 800–200 units  $\text{mg}^{-1}$  protein (not shown).

Additional experiments, carried out in competence medium, were performed to determine the effects of several mutations on the expression of *rok-lacZ*. Figure 6A reveals that, when *abrB* was inactivated, *rok-lacZ* expression was moderately elevated after  $T_0$ . When *sinR* was inactivated, *rok-lacZ* expression was slightly elevated before about  $T_1$  (Fig. 6B). Although these effects are not large, they were noted repeatedly. These data suggested that the increases in *rok-lacZ* expression observed in the *abrB* and *sinR* strains might explain the bypass of the inactivating mutations by *rok* inactivation. If the products of these genes down regulate *rok* expres-

sion, in their absence Rok will be overexpressed and *comK* expression will be inhibited. These conclusions were supported by the results of Western blotting experiments using Rok antiserum. Figure 7 shows that Rok is overproduced in the *abrB* background and that the effect is most evident at  $T_2$ . In the *sinR* background, Rok overproduction is most evident at  $T_0$  and  $T_1$ . These results are consistent with the *rok-lacZ* expression data in Fig. 5A and B and, together with the bypass experiments (Fig. 5), suggest that AbrB and SinR may exert their positive effects on competence by negatively controlling the cellular level of Rok protein (Fig. 1).

*rok-lacZ* expression was elevated in a *rok* background, suggesting that Rok may act negatively at its own promoter (Fig. 6C). In addition, when *mecA* was inactivated, a decrease in *rok-lacZ* expression was noted, and this was reversed when *mecA* and *comK* were inactivated together (Fig. 6D). These data demonstrate that Rok is an autorepressor and that excess ComK represses *rok* transcription. Although overproduction of ComK decreased *rok-lacZ* expression about twofold, eliminating *comK* had little or no effect on *rok-lacZ* expression (Fig. 6D). This observation rules out a possible explanation for the slightly increased expression of *rok-lacZ* noted in the *abrB* and *sinR* mutants. As ComK is apparently a repressor of *rok*, it might be supposed that the decreased level of ComK in *abrB* and *sinR* mutants would result in increased *rok* expression. However, as just noted, the complete inactivation of *comK* does not increase *rok-lacZ* expression, excluding this explanation.

To determine whether the negative effects of Rok and ComK on *rok* expression might be a result of direct binding to the *rok* promoter, gel-shift assays were performed using a radiolabelled fragment with sequences from the putative *rok* promoter region and purified Rok-



**Fig. 8.** Binding of Rok and of ComK to *Prok*. A radiolabelled fragment from the promoter region of *rok* was incubated with Rok-His<sub>6</sub> (A) or with ComK-MBP (B) at the indicated concentrations, before resolution by PAGE. Vertical bars indicate the positions of the shifted bands. In both panels, a solid arrow to the left of the figure indicates the position of the radiolabelled probe.

His<sub>6</sub> or ComK fused to the maltose-binding protein (ComK-MBP). Figure 8 shows that both Rok-His<sub>6</sub> and ComK-MBP can indeed bind to *Prok*. The apparent  $K_D$  for these interactions are about 0.05 and 0.1  $\mu$ M for Rok and ComK respectively. Rok-His<sub>6</sub> binding to *Prok* results in multiple shifted bands, as in the case of Rok binding to *PcomK*, again suggesting that more than one Rok molecule can bind to a given target site.

## Discussion

This report establishes that Rok is a direct repressor at *PcomK* and its role in the regulation of competence is

schematically represented in Fig. 1. In its absence, ComK synthesis is enhanced, and when it is overexpressed the synthesis of ComK is depressed. When *rok* is inactivated, more than half of the cells become competent and the transformation frequency is correspondingly increased. We have observed that mutations that enhance ComK synthesis, including *mecA* and *codY* null mutations (unpublished data and Hahn *et al.*, 1995b) and ComS-overexpressing constructs (Hahn *et al.*, 1996), also increase the percentage of cells that reach competence. ComK is a positively autoregulatory transcription factor and the regulation of *comK* is sensitively poised to respond dramatically to the concentration of ComK itself. Rok is the third protein, in addition to AbrB and CodY, shown to bind directly to *PcomK* and act as a repressor (Figs 1, 2 and 3). The failure of *rok* inactivation to bypass the loss of *comS*, or the overexpression of *mecA* (Fig. 5), is consistent with the conclusion that Rok acts by binding directly to *PcomK*, as the ComS-MecA pathway exerts its effect post-transcriptionally on the stability of ComK. Also consistent are the observations that Rok does not affect ComK stability (Fig. 3C) and has little or no effect on the transcription of *srfA-lacZ* (Fig. 3B).

The *rok* null mutation does not bypass the inactivation of *comS*, although it was isolated as a *ylbF* bypass mutation. Although *ylbF* and *comS* operate on the same regulatory pathway, the effect of *ylbF* inactivation is not as severe as that of a *comS* mutation, and we have proposed that YlbF may stabilize ComS or play some other auxiliary role in the MecA switch (Tortosa *et al.*, 2000). Thus, the *rok* mutation may succeed in bypassing *ylbF* simply by increasing the expression of ComK several fold, whereas this effect is not sufficient to compensate for the loss of ComS, an essential effector of the MecA pathway. An alternative possibility to consider is that YlbF, as SinR and AbrB, acts as a repressor of *rok*. In a *ylbF* mutant, *rok* would be overexpressed, inhibiting ComK synthesis, and this effect would be suppressed by inactivation of *rok*. This hypothesis cannot be true, as the effect of *ylbF* on competence is post-transcriptional and is not exerted at the *comK* promoter (Tortosa *et al.*, 2000).

It has been appreciated for several years that the transition state regulators SinR and AbrB are required for the expression of *comK* (Hahn *et al.*, 1996), although the reasons for these requirements were unknown. The present data demonstrate that inactivation of *sinR* or *abrB* result in the modestly increased expression of *rok*, as shown by the use of a *rok-lacZ* reporter (Fig. 6) and by Western blotting (Fig. 7). These observations suggest that the accumulation of Rok, when *abrB* or *sinR* are inactivated, leads to the repression of *comK*. In support of this, we have observed that *rok* inactivation leads to the bypass of the *sinR* and *abrB* requirements (Fig. 5). It has

also been suggested that SinR acts to potentiate the expression of *comS* (Liu *et al.*, 1996). It is possible, then, that SinR plays a dual role in competence development, although the bypass shown in Fig. 5 is essentially complete. If our hypothesis for the positive roles of AbrB and SinR is correct, the magnitude of the effects shown in Figs 5 and 6 would suggest that *comK-lacZ* transcription is sensitive to small perturbations in the cellular concentration of Rok.

It is not known whether AbrB and SinR act directly to repress *rok*. However, it appears that at least two proteins do act negatively at *Prok*; namely ComK and Rok itself (Fig. 5C and D), and the circuitry involved in the control of Rok synthesis is evidently complex. The repression of *Prok* by Rok, establishes a feedback loop that serves to control the level of *rok* expression. This effect may be important to the cell, as our failure to construct a strain carrying a multicopy *rok* plasmid suggests that excess *rok* expression is lethal.

The ability of ComK to repress *rok* transcription suggests that ComK accumulation, during the development of competence, limits the synthesis of Rok. The consequence of such a regulatory pathway is to establish a two-element positive feedback loop at *comK*. In a sense, this is redundant, as ComK also activates directly at its own promoter. However, if the system functions to ensure a rapid increase in ComK synthesis, it may be important to limit or even to decrease the amount of Rok. In the *rok-lacZ* experiments shown in Fig. 6, inhibition of *rok-lacZ* expression was evident when ComK was overproduced by *mecA* inactivation, whereas we could not detect an effect of *comK* inactivation on *rok* expression measured with a *lacZ* fusion. The apparent  $K_D$  of ComK binding to *Prok* is about 0.1  $\mu\text{M}$  (Fig. 8B). There are about 22 000 ComK tetramers per competent cell at  $T_2$  (Turgay *et al.*, 1998) and the volume of a competent cell can be calculated as about  $0.29 \times 10^{-9} \mu\text{l}$  (Haijema *et al.*, 2001). From this, we infer that the concentration of ComK tetramers in the competent cell is in excess of 100  $\mu\text{M}$ . Why then, is the *rok-lacZ* construct essentially unresponsive to the inactivation of *comK*, as at 100  $\mu\text{M}$  ComK, *Prok* should be saturated with ComK tetramers? One possibility is that this promoter is subject to positive controls that override repression because of ComK. A more satisfying explanation is provided by the fact that in these non-growing cells, measurements of *rok-lacZ* expression (Fig. 6) will reflect  $\beta$ -galactosidase expression occurring before, as well as after,  $T_0$ , obscuring the effect of repression owing to ComK, which is only produced after  $T_0$ . In contrast, *mecA* inactivation leads to substantial ComK synthesis during growth and, in such a mutant, we readily detect repression owing to ComK. In fact the Western blot in Fig. 7 does reveal a decrease in the level of Rok protein from  $T_0$  to  $T_2$ , and this may be because of

repression by ComK.

In any event, it appears that in addition to its role as a positive activator, ComK is capable of acting negatively on transcription, as ComK represses *rok* transcription and binds to *Prok* (Figs 5 and 7). This is the first example in which ComK has been shown to act as a repressor. A search of the sequence upstream from the *rok* coding sequence has revealed a potential ComK binding site. ComK has been shown to bind at dual AAAANNNNTTTT motifs separated by 8, 18 or 31 bases. The characterized ComK binding sites have up to three mismatches from this consensus but no deviations from these spacings (Hamoen *et al.*, 1998). Upstream from *rok*, and separated from its start codon by 109 bases, is the sequence TGAAAAATAAAACATTTTCA GAAAATAAGGAATTTTT, in which the boldface type represents putative ComK boxes, separated from one another by eight bases.

It is striking that regulation at the *comK* promoter is highly redundant. Three proteins (AbrB, CodY and Rok) act directly to repress the expression of *comK*. This serves to underscore the importance to the cell of restricting ComK synthesis, as this protein, present at extraordinarily high concentrations in the competent cell, causes a block in cell division (Hahn *et al.*, 1995b; Haijema *et al.*, 2001). It is likely that each of these repressors responds to an independent signalling pathway, making ComK synthesis, in turn, responsive to multiple signals. *abrB* transcription is repressed by SpoOA~PO<sub>4</sub>, and is, therefore, regulated by the multiple signals that control the phosphorylation of this master regulator (Grossman, 1995; Jiang *et al.*, 2000). CodY acts as a GTP sensor, monitoring the metabolic status of the cell (Ratnayake-Lecamwasam *et al.*, 2001). It is not clear how the effect of Rok on competence development is regulated. Although the total amount of Rok protein in the cell does not vary markedly during growth, it does decrease somewhat after  $T_0$  (Fig. 7). The data obtained with the *Pspac-rok* construct (Fig. 2B) suggest that even a modest change in the concentration of Rok may have a marked effect on *comK* expression. It is also possible that the activity of Rok may be regulated, in addition to its amount, perhaps decreasing at  $T_0$ .

## Experimental procedures

### *Strains and microbiological procedures*

All the *B. subtilis* strains used were isogenic derivatives of BD630, and are listed in Table 1. Transformation, selective and growth media, and the growth of strains to competence are described or referenced in Albano *et al.* (1987). For the measurement of transformation, DNA from strain BD170 was used with selection for leucine prototrophy.

Table 1. Strains.

Name	Genotype <sup>a</sup>	Source
BD170	<i>trp thr</i>	–
BD630	<i>his leu met</i>	–
BD1826	<i>comGA12::Tn917-lacZ</i> (Cbd <sup>b</sup> ) ( <i>kan</i> )	Albano and Dubnau (1989)
BD1890	<i>srfA-lacZ::Tn917-lacZ</i> ( <i>ery</i> )	Jaacks <i>et al.</i> (1989)
BD1991	<i>comK-lacZ::amyE</i> ( <i>cat</i> )	Hahn <i>et al.</i> (1994)
BD1992	<i>comK-lacZ::amyE</i> ( <i>cat</i> ) <i>abrB</i> ( <i>kan</i> )	Hahn <i>et al.</i> (1994)
BD1993	<i>comK-lacZ::amyE</i> ( <i>kan</i> ) <i>sinR</i> ( <i>phl</i> )	Hahn <i>et al.</i> (1994)
BD2594	<i>comK-lacZ::amyE</i> ( <i>spc</i> )	Turgay <i>et al.</i> (1998)
BD2716	<i>comK-gfp</i> (Cbd <sup>b</sup> ) ( <i>cat</i> )	Haijema <i>et al.</i> (2001)
BD2739	( <i>xylR P<sub>xyl</sub>-comK::amyE</i> ( <i>ero</i> ) <i>comG-lacZ</i> (Cbd <sup>b</sup> ) ( <i>cat</i> ) <i>comK</i> ( <i>kan</i> ))	Hahn <i>et al.</i> (1996)
BD2779	<i>comG-lacZ</i> (Cbd <sup>b</sup> ) ( <i>kan</i> ) <i>ylbF::Tn10</i> ( <i>spc</i> )	Tortosa <i>et al.</i> (2000)
BD2955	<i>rok::Tn10</i> ( <i>spc</i> )	This work
BD2957	( <i>xylR P<sub>xyl</sub>-comK::amyE</i> ( <i>ery</i> ) <i>comG-lacZ</i> (Cbd <sup>b</sup> ) ( <i>cat</i> ) <i>comK</i> ( <i>kan</i> ) <i>rok::Tn10</i> ( <i>spc</i> ))	This work
BD2958	<i>comK-gfp</i> (Cbd <sup>b</sup> ) ( <i>cat</i> ) <i>rok::Tn10</i> ( <i>spc</i> )	This work
BD3085	<i>comK-lacZ::amyE</i> ( <i>cat</i> ) <i>rok::Tn10</i> ( <i>spc</i> )	This work
BD3086	<i>comK-lacZ::amyE</i> ( <i>cat</i> ) <i>rok::Tn10</i> ( <i>spc</i> ) <i>abrB</i> ( <i>kan</i> )	This work
BD3087	<i>comK-lacZ::amyE</i> ( <i>cat</i> ) <i>rok::Tn10</i> ( <i>spc</i> ) <i>comS</i> ( <i>tet</i> )	This work
BD3088	<i>comK-lacZ::amyE</i> ( <i>cat</i> ) <i>rok::Tn10</i> ( <i>spc</i> )(pKD93, multicopy <i>mecA</i> )	This work
BD3090	<i>comK-lacZ::amyE</i> ( <i>cat</i> ) <i>rok::Tn10</i> ( <i>spc</i> ) <i>sinR</i> ( <i>phl</i> )	This work
BD3091	<i>rok-lacZ::amyE</i> ( <i>cat</i> )	This work
BD3094	<i>Pspac-rok</i> (Cbd <sup>b</sup> ) ( <i>cat</i> )	This work
BD3099	<i>comK-lacZ::amyE</i> ( <i>spc</i> ) <i>Pspac-rok</i> (Cbd <sup>b</sup> ) ( <i>cat</i> )	This work
BD3101	<i>comK-lacZ::amyE</i> ( <i>cat</i> ) <i>rok::Tn10</i> ( <i>spc</i> ) <i>comK</i> ( <i>kan</i> )	This work
BD3102	<i>srfA-lacZ::Tn917lacZ</i> ( <i>ery</i> ) <i>rok::Tn10</i> ( <i>spc</i> )	This work
BD3103	<i>rok-lacZ::amyE</i> ( <i>cat</i> ) <i>sinR</i> ( <i>phl</i> )	This work
BD3104	<i>rok-lacZ::amyE</i> ( <i>cat</i> ) <i>abrB</i> ( <i>kan</i> )	This work
BD3106	<i>rok-lacZ::amyE</i> ( <i>cat</i> ) <i>comK</i> ( <i>kan</i> )	This work
BD3110	<i>rok-lacZ::amyE</i> ( <i>cat</i> ) <i>mecA</i> ( <i>ery</i> )	This work
BD3115	<i>rok-lacZ::amyE</i> ( <i>cat</i> ) <i>mecA</i> ( <i>ery</i> ) <i>comK</i> ( <i>kan</i> )	This work
BD3127	<i>rok-lacZ::amyE</i> ( <i>cat</i> ) <i>rok::Tn10</i> ( <i>spc</i> )	This work

a. All of the strains listed below BD630 are in the *his leu met* background and are, therefore, isogenic.

b. Cbd, inserted by Campbell (single reciprocal) recombination.

### General procedures

Molecular biological methods were essentially as described in Sambrook *et al.* (1989). Western blotting was carried out as described previously (Kong and Dubnau, 1994).

### Isolation of suppressors of ylbF

BD2779 (*ylbF*(*spc*) *comG-lacZ*(*kan*)) was transformed using the *Tn10* delivery plasmid pHV1248, with selection for chloramphenicol (Cam) resistance. Although the *ylbF* mutation reduces the transformation frequency, sufficient transformability is present to permit this selection. The *ylbF* mutation in BD2779 decreases induction of the *comG-lacZ* reporter, and this strain, therefore, forms white colonies on minimal medium (MM) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal). The pHV1248 plasmid carries a copy of *Tn10* marked with *cat* and is temperature-sensitive for replication. At the non-permissive temperature, the plasmid is cured, and selection for Cam resistance ensures that a copy of *Tn10* has inserted into the chromosome. Cultures mutagenized in this way were plated on minimal plates, containing Cam and Xgal, and blue colonies were isolated. Chromosomal DNA was isolated from these suppressed clones, and the Cam<sup>R</sup> marker was then out-crossed into BD1826 (*comG-lacZ::kan*). The resulting strain was transformed with pIC216 (Steinmetz and Richter, 1994) with

selection for spectinomycin (Spc) resistance. This plasmid replaces the *cat* determinant of the original mini *Tn10* with Spc resistance, and inserts a pUC replication origin in the mini *Tn10* construct. The insertion sites of the *Tn10* element were identified as follows. DNA from several of these transformants was treated with *EcoRI* or *BamHI* which each cut once within the inserted element. The cleaved DNA was self-ligated and used to transform *Escherichia coli*. Plasmid DNA was then isolated from the *E. coli* transformants. Sequencing of several such plasmids from one suppressed strain determined that the insertion had occurred in the previously uncharacterized *ykuW* gene.

### Genetic constructions

Primers used for the various constructions, and their sequences, are listed in Table 2. The *rok-lacZ* fusion was constructed as follows. A fragment, assumed to contain the promoter and regulatory sequences of *rok* (960 nt preceding the *rok* initiation codon, and extending through the first 20 nt of the *rok* ORF) was amplified from BD630 chromosomal DNA by polymerase chain reaction (PCR), using the primers *ykuWps* and *ykuWpa*. The PCR product was cleaved with *EcoRI* and *BamHI*, and inserted into *EcoRI*- and *BamHI*-cleaved pAC5 (constructed by I. Martin-Verstraete) to produce pED425. pAC5 carries a promoterless copy of *lacZ* and is designed to insert at *amyE* by replacement recombination.

**Table 2.** Primer sequences.

Primer	Sequence <sup>a</sup>
ykuWps	CGGAATTCGCTAGCATATTCGTTAGGGC
ykuWpa	CGGGATCCGCTTCTCTTTCATTAACAT
ykuWSma	TCCCCCGGGGAAGATGTTAATTTTGATAG
ykuWpDHa	ACATGCATGCAACGTTGTCATATTAGGG
ykuWyfps	GGAAATTCCTGGACAGAAATGGAAGCCCTT
ykuWyfpa	CTCGAGTTCGTTTGCTGATTCTGCAGATTC
ykuWcs	CATGCCATGGTTAATGAAAGAGAAGC
ykuWca	CGGGATCCCTTCGTTTGCTGATTCTG
K1	CCGGAATTCAGAATCCCCCAATGCC
K2	CGGGATCCCAGTCTGTTTTCTGACTCATATT
rokps	CGGAATTCGATGTTTTCTCAATTTTAG

a. The underlining indicates restriction sites referred to in the text.

The *rok-lacZ* strain BD3091 was constructed by transforming BD630 with pED425 DNA, leading to the insertion of the *rok-lacZ* construct in the *amyE* locus.

To construct a strain with *rok* driven by a modified *Pspac* promoter, a fragment containing 29 nt preceding the *rok* initiation codon, including the natural ribosome binding site, and extending through the first 157 of the 191 codons of *rok*, was amplified from BD630 chromosomal DNA by PCR, using the primers ykuWSma and ykuWpDHa. The PCR product was cleaved with *SmaI* and *SphI*, and cloned into *SmaI* and *SphI* cleaved pJQ43, to produce pED426. The *rok-lacZ* strain BD3094 was constructed by transforming pED426 into BD630, leading to its insertion at the *rok* locus by single reciprocal recombination with the simultaneous inactivation of the wild-type *rok* gene. The modified *Pspac* promoter in pJQ43, a gift from Alan Grossman, is isopropyl thio- $\beta$ -D-galactoside (IPTG)-inducible and expresses more strongly than the original *Pspac* promoter.

The *comK-gfp* fusion strain (BD2716) was constructed as described previously (Haijema *et al.*, 2001) except that a modified *gfp* (*gfpmut1*) was used, carried in plasmid pSG1151 (Lewis and Marston, 1999).

To construct an affinity-tagged *rok-his<sub>6</sub>* fusion, the coding sequence of *rok* was amplified from BD630 chromosomal DNA by PCR using the primers ykuWcs and ykuWca. The resulting fragment was cleaved with *NcoI* and *BamHI* and cloned into pQE60 (Qiagen). This resulted in a C-terminal, His-tagged *rok* construct (pED428) under regulation of an IPTG-inducible promoter. The structure of pED428 was verified by sequencing and used to synthesize Rok-His<sub>6</sub> fusion protein in *E. coli* M15 (Qiagen).

#### Determination of in vivo ComK stability

Determination of the *in vivo* stability of ComK was carried out as described by Turgay *et al.* (1998). The strains to be tested were grown in competence medium to 2 h past the end of exponential growth, and diluted 1:20 into fresh competence medium with the addition of tetracycline (50  $\mu$ g ml<sup>-1</sup>) and rifampicin (5  $\mu$ g ml<sup>-1</sup>). The cultures were incubated at 37°C and 50 ml samples were taken for each time-point. The cells were centrifuged, washed with STM (50 mM NaCl, 25% sucrose, 50 mM Tris HCl pH 8, 5 mM MgCl<sub>2</sub>) and then resuspended in 1 ml of STM containing lysozyme (300  $\mu$ g ml<sup>-1</sup>).

The cells were protoplasted by incubation at 37°C for 10 min. Sample buffer was added and the samples were boiled for 5 min. Equal volumes of protein extracts were loaded on gels and analysed by Western blotting. The bands were scanned with a flatbed scanner using Adobe PHOTOSHOP to acquire the data, and quantified using Scan ANALYSIS 2.56 software (Biosoft).

#### Protein purification

To prepare Rok-His<sub>6</sub>, 1000 ml of LB medium, supplemented with ampicillin (100  $\mu$ g ml<sup>-1</sup>) and kanamycin (25  $\mu$ g ml<sup>-1</sup>), was inoculated with *E. coli* M15 carrying pED428. The culture was grown to an OD<sub>600</sub> of 0.5, induced with 1 mM IPTG, and growth was continued for an additional 3 h at 37°C. Cells were collected by centrifugation and washed in lysis buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8; 300 mM NaCl; 10 mM imidazole). Pellets were frozen and stored at -20°C. The cells were then resuspended in lysis buffer, broken with a French press and centrifuged at 18 000 g for 30 s, at 4°C, to remove unbroken cells and debris. The supernatant was mixed with 2 ml of nickel nitrilotriacetate (NTA) resin (Qiagen) which had been equilibrated with lysis buffer, and stirred on ice for 2 h. The resin was washed several times with 50 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 8), 300 mM NaCl, 20 mM imidazole. The fusion protein was then eluted with elution buffer C (50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8; 300 mM NaCl; 200 mM imidazole). During isolation and purification, buffers were supplemented with 0.2 mM phenylmethylsulphonyl fluoride (PMSF) and with protease inhibitor cocktail components (Boehringer). Purification was monitored by SDS-PAGE, and fractions were checked for the absence of contaminating DNA by agarose-gel electrophoreses and ethidium bromide staining. Purified Rok was verified by N-terminal sequencing. ComK-MBP was prepared as described previously (van Sinderen *et al.*, 1995).

#### Gel retardation

Gel retardation analyses were carried out as described by Hamoen *et al.* (1998). The *comK* promoter region was isolated by PCR using primers K1 and K2 and *B. subtilis* BD630 chromosomal DNA as a template. The resulting 308 bp fragment contains 290 nt, preceding the *comK* initiation codon as well as the downstream 18 nt. For gel retardation with the *rok* promoter region, primers rokps and ykuwpa were used to isolate a fragment that extended from -306 to +21 with respect to the *rok* start codon. The probe fragments were end-labelled with T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]-ATP. Purified protein and approximately 0.05 ng  $\mu$ l<sup>-1</sup> probe were premixed on ice in binding buffer (20 mM Tris HCl, pH 8; 100 mM KCl; 5 mM MgCl<sub>2</sub>; 0.5 mM DTT; 10% (v/v) glycerol with 0.05% non-idet P-40 and 0.05 mg ml<sup>-1</sup> (BSA), containing 0.05 mg ml<sup>-1</sup> poly(dI-dC) in a total volume of 10  $\mu$ l. After 20 min at 37°C, samples were loaded on a non-denaturing 6% polyacrylamide gel. Gels were run in TAE buffer (40 mM Tris acetate, pH 8; 2 mM EDTA) at 7 V cm<sup>-1</sup>, using a Bio-Rad minigel system, and the signals were recorded with a phosphorimager (Molecular Dynamics).

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