JOURNAL OF BACTERIOLOGY, Sept. 1994, p. 5753–5761
0021-9193/94/$04.00+0

Vol. 176, No. 18

The Regulation of Competence Transcription Factor Synthesis Constitutes a Critical Control Point in the Regulation of Competence in *Bacillus subtilis*

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Received 28 April 1994/Accepted 28 June 1994

***comK*, which encodes the competence transcription factor, is itself transcriptionally activated at the transition from exponential growth to stationary phase in *Bacillus subtilis*. MecA, a negative regulator of competence, also inhibits *comK* transcription when overexpressed, and a *mecA* null mutation results in *comK* overexpression. Although null mutations in *mecA*, as well as in another gene, *mecB*, are known to bypass the requirements for nearly all of the competence regulatory genes, the *comK* requirement is not suppressed by *mecA* inactivation. Various competence regulatory genes (*comA*, *srfA*, *degU*, *abrB*, *sin*, and *spo0A*) are shown to be required for the expression of *comK*. *srfA* transcription is shown to occur equally in cells destined for competence and those destined not to become competent. In contrast, *comK* transcription is restricted to the presumptive competent cells. These and other results are combined to describe a regulatory pathway for competence.**

The competence regulon of *Bacillus subtilis* is controlled by a complex signal transduction cascade (6). The late competence genes, which encode proteins required for the binding, processing, and transport of transforming DNA, are transcribed postexponentially when a specific DNA-binding protein (competence transcription factor [CTF]) is synthesized in response to the regulatory cascade (32). It is clear that CTF is necessary for this transcription to take place; it is not yet clear whether the presence of CTF is sufficient.

Both the expression of *comK*, monitored as a *comK-lacZ* fusion, and the DNA binding activity due to CTF (32) increase sharply at the time of transition to stationary phase (T_0), just when the late competence genes are induced (54). Recent evidence has established that the gene encoding CTF is *comK* (51), shown previously to be required in vivo for the expression of the late competence genes (52, 54).

An interesting aspect of competence regulation is its cell type specificity. At the transition to stationary phase, cultures in competence medium (CM) differentiate into two cell types, competent and noncompetent, which can be resolved by density gradient centrifugation (18, 22). Late competence genes are expressed only in the competent cell fraction (2). The factors that determine cell fate in this system are unknown, and it is not known whether ComK expression is cell type restricted.

The synthesis of the late gene products is negatively regulated by the products of *mecA* and *mecB* (10, 27, 28, 35, 44). It has been postulated that the Mec proteins serve to sequester or otherwise inactivate ComK (27, 35). Upon receipt of an appropriate signal from genes upstream in the signalling pathway, the Mec proteins are thought to release ComK from inhibition. In addition, van Sinderen and Venema (53) have shown that ComK is required for the transcription of *comK* itself. The release of ComK from Mec inhibition would therefore throw a transcriptional switch, leading to the increased expression of CTF and therefore of the late competence genes. In accordance with this model, MecA has been shown to bind directly to ComK in vitro (27). Additional support is provided by observations on the effects of *mecA* and *mecB* expression. Elimination of either MecA or MecB by mutation causes a dramatic overproduction of the late competence proteins (10, 27, 28). MecA overproduction represses the expression of late competence genes. In contrast, MecB overproduction does not repress competence. Finally, the overexpression of MecA down-regulates late competence gene expression even in a *mecB* loss-of-function mutant, whereas overexpression of MecB has no effect in a *mecA* null background. From these data, we have inferred that the signal instructing MecA to release ComK is probably mediated by MecB (27). Interestingly, the latter protein resembles members of the ClpC heat shock family, is probably a nucleotide-binding protein, and is involved in thermoprotection of *B. subtilis* (35).

A large number of additional genes (*comX*, *comA*, *comP*, *comQ*, *spo0K*, *srfA*, *spo0A*, *degU*, *abrB*, *spo0H*, and *sinR*) have been shown to be required for the expression of competence (2, 13, 17, 25, 29, 37, 38, 45, 54–57). *comQ*, *comX*, *spo0K*, *comP*, and *comA* are required to induce the transcription of *srfA* near the end of the exponential growth phase (15, 21, 38, 39, 54, 56). ComA is a response regulator protein that binds to the *srfA* promoter with increased affinity when phosphorylated and presumably acts as a positive transcription factor (21, 39, 43, 55). ComP is a membrane-localized histidine kinase that probably donates a phosphoryl group to ComA in vivo, presumably in response to an extracellular signal (57). ComX, ComQ, and Spo0K are believed to act upstream of ComP as parts of the signal-generating apparatus (29, 45, 56), although it is possible that they act on *srfA* independently of ComP and ComA-PO_4.

The precise roles of *degU*, *sinR*, *abrB*, and *spo0H* are not known, but all are required for the transcription of the late competence genes. Transcription of *srfA* from a regulatable promoter bypasses the need for ComQ, ComP, ComA, and Spo0K but not that for DegU, SinR, and AbrB (21, 39). It appears, therefore, that these proteins act after or in parallel with *srfA*. Sin (14) and AbrB (50) are known to be DNA-binding proteins, acting as transition state regulators during

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TABLE 1. Strains used

Strain	Genotype	Reference
BD1826	*hisB2 leu-8 metB5 comG-lacZ* (Km^r)[a]	This report
BD1974	*hisB2 leu-8 metB5 amyE::srfA-lacZ* (Cm^r)	This report
BD1991	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r)	This report
BD1992	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r) *abrB*::Km^r	This report
BD1993	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r) *sinR*Δ (Phl^r)	This report
BD1994	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r) P_{spac} *srfA* (Phl^r)[a]	This report
BD2026	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r) Δ(*degS degU*) (Km^r)	This report
BD2103	*hisB2 leu-8 metB5 mecA*Δ (Spc^r) *comG12-lacZ* (Cm^r)[a]	28
BD2104	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r) *mecA*Δ (Spc^r)	This report
BD2106	*metB5 leu-8 amyE::comK-lacZ* (Cm^r) $degU^h32$	This report
BD2125	*hisB2 leu-8 metB5 comK*Δ (Km^r) *mecA*Δ (Spc^r) *comG12-lacZ* (Cm^r)[a]	This report
BD2155	*hisB2 leu-8 metB5 amyE::comK-lacZ* (pKD73)	This report
BD2251	*hisB2 leu-8 metB5 amyE::srfA-lacZ* (Cm^r) *comK*Δ (Km^r) *mecA*Δ (Spc^r)	This report
BD2252	*hisB2 leu-8 metB5 amyE::srfA-lacZ* (Cm^r) *comK*Δ (Km^r)	This report
BD2253	*hisB2 leu-8 metB5 amyE::srfA-lacZ* (Cm^r) *mecA*Δ (Spc^r)	This report
BD2315	*hisB2 leu-8 amyE::comK-lacZ* (Cm^r) *spo0H*Δ*Hin*dIII	This report
BD2316	*hisB2 leu-8 amyE::comK-lacZ* (Cm^r) *spo0A*Δ*204*	This report
BD2317	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r) *comK*Δ (Km^r)	This report
BD2318	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r) *comK*Δ (Km^r) *mecA* (Spc^r)	This report
BD2319	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r) *comA*-Tn*917* (Em^r)	This report
BD2320	*hisB2 leu-8 metB5 amyE::comK-lacZ* (Cm^r) *comA*-Tn*917* (Em^r) *mecA*Δ (Spc^r)	This report
BD2323	*hisB2 leu-8 metB5 comK-lacZ*[a]	This report
BD2324	*hisB2 leu-8 metB5 srfA-lacZ*[a]	This report

[a] The construct was chromosomally inserted by Campbell-like (single-reciprocal) recombination. In all cases these were noninactivating recombination events, resulting in the construction of $comG^+$, $srfA^+$, or $comK^+$ strains.

the approach to stationary phase (47). DegU is a response regulator and probably a DNA-binding protein as well (42). *spo0H* encodes a minor sigma factor (σ^H) which is needed for sporulation as well as for competence (2, 11). Spo0A is a response regulator protein which plays a critical role in the regulation of many postexponential expression systems (23). One role of phosphorylated Spo0A is to down-regulate the transcription of *abrB* (41). We have suggested that the major, and perhaps only, role of Spo0A in competence development is to control the level of AbrB (5). This implies that AbrB plays a negative as well as a positive role in competence regulation, and this hypothesis has been confirmed (20).

With the exception of *spo0A*, the need for all of these genes is completely bypassed by loss-of-function mutations in either *mecA* or *mecB* (44). This finding has led to the hypothesis that the bypassed genes are required to signal the release of ComK inhibition by the Mec system and that they therefore act upstream of Mec and ComK in the signalling pathway. The failure of *mec* mutations to bypass the loss of *spo0A* suggests that the negative role of AbrB is exerted after Mec in the competence pathway.

These considerations lead to the prediction that *comA*, *comP*, *comQ*, *spo0K*, *srfA*, *degU*, *abrB*, *spo0H*, and *sinR* are necessary for the increased synthesis of ComK at T_0, which is thought to reflect the release of ComK by MecA. This has already been shown in the case of *srfA*, which is clearly required for *comK* induction (52, 54). A second prediction is that null mutations in *mecA* will fail to bypass the need for ComK in late gene induction. A third is that MecA elimination and overproduction should cause the overexpression and inhibition, respectively, of ComK synthesis, similar to the demonstrated effects on *comG*, a representative late competence gene (28). These predictions have been tested, and the results are presented in this report. We also show that overexpression of ComK feeds back to an earlier step in the pathway, inhibiting the transcription of *srfA*, and that *comK* is expressed preferentially in the competent cell fraction, whereas *srfA* is not.

MATERIALS AND METHODS

Bacterial strains. All strains used were derivatives of IS75 (*hisB2 leu-8 metB5*) and are listed in Table 1. The provenances of several of the mutations and constructs used are as follows. *abrB*::Km^r was a kind gift from T. Tanaka. It was prepared by the insertion of a neomycin resistance cassette into the *Bst*XI site of *abrB*. We have used kanamycin to select for this mutation. *sinR*Δ (Phl^r) was obtained from I. Mandic-Mulec and I. Smith (30). The P_{spac}-*srfA* construct was obtained from M. Nakano and P. Zuber and is described in reference 39. The Δ(*degS degU*) mutation was a gift from T. Msadek (34). The *mecA*Δ (Spc^r) mutation is described in reference 28 and was a gift from T. Msadek, F. Kunst, and G. Rapoport. The *comG12-lacZ* (Cm^r) Campbell construct was derived from strain BD1512 (1). $degU^h32$ was obtained from T. Msadek and is described in references 33 and 48. The *comK*Δ (Km^r) mutation was obtained from D. van Sinderen (52). The *comG-lacZ* (Km^r) construct is a transcriptional fusion of *lacZ* to *comG*, inserted at the *comG* locus by Campbell-like recombination. *spo0A*Δ*204* was obtained from P. Zuber. *spo0H*Δ*Hin*dIII was obtained from I. Smith and is described in reference 2. Plasmid pKD73 consists of pUB110 carrying an intact copy of *mecA* (28). BD2323 and BD2324 were constructed by the Campbell-like integration of pLGW312 and pLGW310, respectively, into IS75. These plasmids (kindly provided by D. van Sinderen) carry the regulatory regions of *comK* and *srfA*, respectively, fused to *lacZ*. The resulting recombinants are $srfA^+$ and $comK^+$ and express *lacZ* under transcriptional control of the *srfA* and *comK* regulatory elements.

Growth conditions and competence. Cultures were grown in either Luria broth (LB) (46) or CM (2). Antibiotics added to solid or liquid media were chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), spectinomycin (100 μg/ml), and phleomycin (1 μg/ml).

For determination of β-galactosidase as a function of growth, cultures were grown in CM through the one-step regimen as described previously (2). For the Renografin

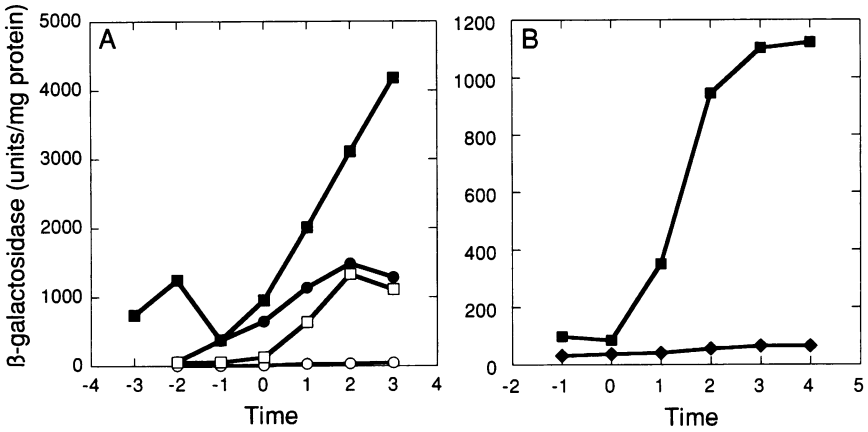

FIG. 1. Effects of MecA deficiency (A) and overexpression (B) on the expression of *comK-lacZ*. (A) ○, *mecA*$^+$ *comK-lacZ* strain (BD1991) grown in LB; □, *mecA*$^+$ *comK-lacZ* strain (BD1991) grown in CM; ●, *mecA*Δ *comK-lacZ* strain (BD2104) grown in LB; ■, *mecA*$^+$ *comK-lacZ* strain (BD1991) grown in CM; ◆, *mecA*$^+$ *comK-lacZ* strain (BD2155), carrying pKD73 (multicopy *mecA*), grown in CM.

centrifugation experiments, the two-step regimen (8) was used. With the one- and two-step protocols, transformation frequencies for single markers were approximately 0.1 and 1%, respectively.

Renografin gradient separation. Cultures grown through the two-step competence regimen (7) were resolved on Renografin gradients as described by Haseltine-Cahn and Fox (22) and modified by Joenje et al. (26).

β-Galactosidase assays. β-Galactosidase determinations were carried out as described previously, and the results are expressed as units of enzyme activity per milligram of total protein (16).

Plasmid and strain constructions. Markers were introduced by transformation except that when it was necessary to use a competence-deficient recipient, phage PBS1 transduction was used. To construct translational fusions to *lacZ* at the *amyE* locus, plasmid pAC5 was used. pAC5 contains homology to the *amyE* locus and carries a promoterless copy of *lacZ*. It was kindly provided by I. Martin-Verstraete and is described more fully in reference 28.

A *srfA-lacZ* fusion integrated at the *amyE* locus was constructed as follows. A 562-bp PCR fragment including the regulatory region and the first six codons of *srfA* was cloned between the *Eco*RI and *Bam*HI sites of plasmid pAC5, creating a translational fusion to *lacZ*. The PCR primers 5′CG GAATTCGGTTTTTGCGCGGTACACAT and 5′CGGGAT CCAAAGGGTAAAAAGTTATTTCC are flanked by *Eco*RI and *Bam*HI sites, respectively (underlined in the primer sequences). The resulting plasmid was used to transform IS75 with selection for Cm^r. Transformants were screened for integration into the *amyE* locus by growth on starch plates and flooding with KI.

To construct a *comK-lacZ* fusion, a 591-bp PCR fragment encompassing the regulatory region and the first six codons of *comK* was cloned into pAC5 to create an in-frame fusion with *lacZ*. The PCR primers 5′TCCCCCGGGGGAACAATTGT GAACGGATAA and 5′CGGGATCCCAGTCTGTTTTCTG ACTCATATT have *Sma*I and *Bam*HI sites, respectively (underlined in the primer sequences), and these were used for cloning into pAC5. DNA from one such clone was used for transformation into IS75, and Cm^r transformants were screened as described above for amylase production.

RESULTS

Expression of *comK*. To study the expression of *comK* during growth, a translational fusion of *comK* to *lacZ* was constructed. This construct was then integrated by replacement into the *amyE* locus of the host chromosome, yielding a strain that expressed β-galactosidase under the control of *comK* regulatory sequences.

The *comK-lacZ* strain (BD1991) was grown in CM, and samples were taken at hourly intervals to measure β-galactosidase synthesis (Fig. 1A). During exponential growth, a low level of expression was detected, which increased dramatically at T_0. *comK* expression is therefore growth stage regulated. These expression patterns parallel the development of competence and are consistent with the results of van Sinderen et al. (52, 54). Since the latter were obtained with a *comK-lacZ* construct that had been integrated at the *comK* locus by Campbell-like recombination, thereby preserving the entire *comK* upstream regulatory sequence, we can conclude that the fragment placed upstream of *comK* in our *lacZ* fusion strain must contain the essential regulatory sequences.

MecA deficiency does not bypass a null mutation in *comK*. It has been shown that null mutations in *mecA* bypass the requirements for most of the known competence regulatory genes (*comA*, *comP*, *comQ*, *degU*, *abrB*, *sinR*, *srfA*, *spo0K*, and *spo0H*) (44). To determine whether a *mecA* deletion also bypasses the competence requirement for *comK*, we have introduced a *comK* deletion into a *mecA*Δ strain also carrying a *comG-lacZ* fusion, to create BD2125. When streaked on CM or on LB containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), this strain was white, revealing that the MecA deficiency does not bypass the loss of *comK*. An isogenic *comK*$^+$ strain (BD2103) was blue when streaked simultaneously as a control.

The product of *mecA* inhibits *comK* expression. The mutational inactivation of *mecA* increases the expression of late competence genes (10, 28). In addition, overexpression of MecA results in a severe decrease in late competence gene expression (28). Since *comK* is a regulatory gene required for late competence gene expression (51, 54), it was of interest to determine the effect of MecA deficiency on *comK* expression. A *mecA* deletion was moved into a *comG-lacZ* strain (BD2104), and β-galactosidase levels were assayed during

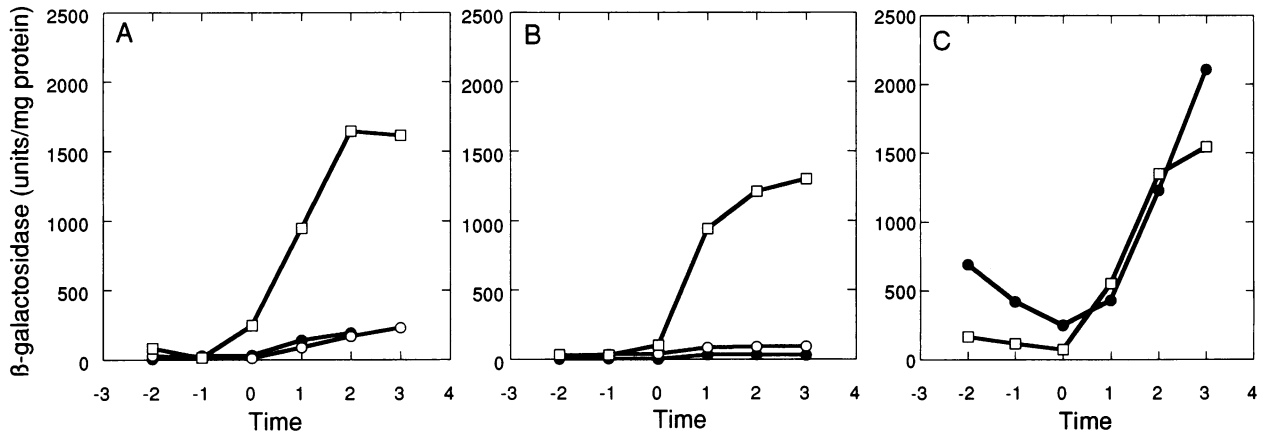

FIG. 2. Epistatic effects of competence regulatory mutations on *comK-lacZ* expression. (A) □, *comK-lacZ* strain (BD1991); ●, *comK-lacZ abrB*::Km^r strain (BD1992); ○, *comK-lacZ sinR*Δ strain (BD1993). (B) □, *comK-lacZ* strain (BD1991); ●, *comK-lacZ spo0AΔ204* strain (BD2316) and *comK-lacZ degU^{hy}32* strain (BD2106) (the values were nearly identical); ○, *comK-lacZ* Δ(*degS degU*) strain (BD2026). (C) □, *comK-lacZ* strain (BD1991); ●, *comK-lacZ spo0H*Δ*Hin*dIII strain (BD2315). All cultures were grown in CM.

growth in CM. Figure 1A shows that MecA deficiency causes a three- to fourfold increase in *comK* expression. *comG-lacZ* expression is very low in complex media but is derepressed in a *mecA* loss-of-function mutant background (10). Similarly, *comK-lacZ* expression was not observed in complex medium (LB) in the *mecA*$^+$ background, but elevated expression occurred in the *mecA* mutant. The derepression of *comK* in a *mecA*Δ background has also been shown by Western blotting (immunoblotting) using an anti-ComK serum (27). We have determined the effect of MecA overproduction on *comK-lacZ* expression by introducing a multicopy plasmid carrying *mecA*. In this strain (BD2155), expression of *comK-lacZ* was almost completely abolished (Fig. 1B).

These results demonstrate that *comK* is under nutritional as well as growth stage-related control and suggest that the *mecA* gene product acts negatively on *comK* expression. The negative effect of MecA on late competence gene expression (10, 27, 28) can therefore be most simply explained as an effect on *comK* expression, preventing the autoregulatory buildup of ComK. The MecA deficiency relieves the nutritional requirements for competence and for *comK* expression. However, the overexpression obtained in LB was not as great as that in CM (Fig. 1A), suggesting that some nutritional regulation may take place even in the *mecA* null background. *comK* expression in the null *mecA* background still exhibits an increase during the approach to stationary phase, suggesting that growth stage-related regulation persists.

Epistatic dependencies in the regulation of *comK* expression. The transcription of late competence genes is dependent on the products of many regulatory genes that function as components of a signal transduction pathway. To test the dependence of *comK* expression on known competence regulatory genes, we have combined mutations in each of the genes to be tested with the *comK-lacZ* translational fusion. This fusion was inserted at the *amyE* locus, and the strains to be tested were therefore *comK*$^+$. This is important since van Sinderen and Venema (53) have shown that *comK* is required for its own transcription. Figure 2 presents the results of β-galactosidase measurements performed on these strains during growth in CM. It is clear that mutations in all of the regulatory genes tested, with the exception of *spo0H*, are required for the expression of *comK-lacZ*, as predicted by the hypothesis that they are required for the release of ComK from Mec inhibition.

Although *abrB* and *sinR* loss-of-function mutations (in strains BD1992 and BD1993, respectively) are required for *comK-lacZ* expression (Fig. 2A), the residual β-galactosidase synthesis in the *abrB*::Km^r and *sinR*Δ strains is easily measurable. This is consistent with the observation that AbrB deficiency leads to only a 50-fold decrease in transformability (2). The transformability of a *sinR* null mutant cannot be accurately determined because *sinR* strains clump in liquid culture. In the accompanying report (53), the effect of AbrB deficiency on *comK-lacZ* expression is found to be no more than twofold. This quantitative discrepancy may be due to a strain difference.

The dramatic dependence of *comK* expression on *spo0A* (BD2316; Fig. 2B) and the failure of *mecA* and *mecB* loss-of-function mutations to bypass Spo0A deficiency (44) are consistent with the hypothesis that AbrB acts negatively after Mec in the regulatory pathway, with Spo0A acting as a repressor of AbrB synthesis (49).

DegS deficiency has little or no effect on competence development, whereas the loss of *degU* has a strong effect on late competence gene expression (reviewed in reference 42). The decrease in *comK* expression in the Δ(*degS degU*) background (BD2026; Fig. 2B) is therefore due to the absence of DegU. *degU^{hy}32* is a so-called *degU*(Hy) allele that leads to a competence deficiency (33, 48). We have shown that this effect is manifested at the level of *srfA* transcription (21), and it is therefore not surprising that it affects the induction of *comK* (BD2106; Fig. 2B).

Finally, Fig. 2C shows that a *spo0H* loss-of-function mutation (in strain BD2315) has no effect on *comK-lacZ* expression. In fact, in several additional experiments (data not shown), Spo0H deficiency seemed to result in a slight increase in β-galactosidase synthesis. This result is somewhat paradoxical. Since *mecA* loss of function bypasses the *spo0H* dependency of late competence gene expression (44), we had expected that σ^H would act before MecA and therefore before ComK.

Although these results will be discussed further below, we can conclude that AbrB probably acts negatively on *comK* expression after the point of Mec action, as implied by the severe *spo0A* dependency of *comK* expression and the failure of *mec* mutations to bypass this dependency. We can further

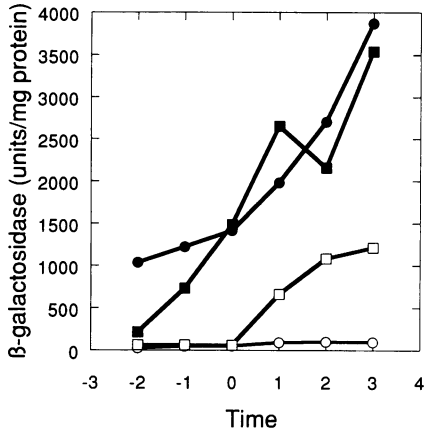

FIG. 3. MecA deficiency bypasses the ComA requirement for *comK-lacZ* expression. □, *comK-lacZ* strain (BD1991); ○, *comK-lacZ comA* strain (BD2319); ■, *mecAΔ comK-lacZ* strain (BD2104); ●, *mecAΔ comA comK-lacZ* strain (BD2320). All cultures were grown in CM.

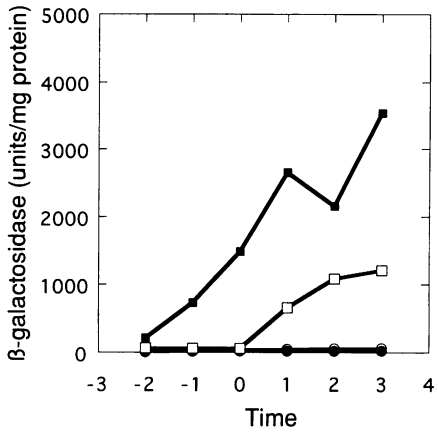

FIG. 4. MecA deficiency does not bypass the requirement of ComK for its own expression. □, *comK*$^+$ *comK-lacZ* strain (BD1991); ■, *mecAΔ comK*$^+$ *comK-lacZ* strain (BD2104); ○, *comKΔ comK-lacZ* strain (BD2317); ●, *mecAΔ comKΔ comK-lacZ* strain (BD2318). All cultures were grown in CM.

conclude that DegU, Sin, and AbrB act positively on *comK* expression, consistent with the complete bypass of null mutations in these genes by loss-of-function *mecA* or *mecB* mutations (44). Since the major roles for *comA*, *comP*, *comQ*, and *spo0K* in competence are to induce the transcription of *srfA* (21, 39) and since *srfA* is needed for the expression of *comK* (54), we can conclude that the former genes should also exert a positive effect on *comK* expression. As shown above, a *mecA* deletion does not bypass the *comK* requirement for competence gene expression. We can therefore confidently predict that the bypass of the early regulatory genes by *mecA* mutation will be manifested in the expression of *comK*. This prediction is confirmed for *comA* by the experiment shown in Fig. 3. Although ComA is required for the expression of *comK-lacZ* in a *mecA*$^+$ background (BD2319), it is not needed when *mecA* is inactivated (BD2320).

MecA deficiency does not bypass the requirement of *comK* for its own expression. As noted above, van Sinderen and Venema (53) have shown that *comK* is required for its own expression. Although loss of MecA does not bypass the ComK requirement for *comG* expression, it does bypass the *srfA*, *degU*, *sinR*, and *abrB* requirements for *comK* expression. ComK may act in its own expression indirectly at a point prior to that of MecA action or after MecA, possibly binding directly to its own promoter. In the former case, a null *mecA* mutation would be expected to permit the expression of *comK-lacZ* in the absence of an intact copy of *comK*. Figure 4 shows that this is not the case. In a null *comK* background, no *comK-lacZ* expression was observed either in a *mecA* deletion mutant (BD2318) or in the isogenic *mecA*$^+$ strain (BD2317). We conclude that ComK is needed for its own synthesis after the point of MecA activity, possibly acting directly as a positive autoregulatory transcription factor.

ComK acts negatively on *srfA* expression. Figure 5 shows that in a *mecA* null mutant strain (BD2253), a *srfA-lacZ* reporter construct is repressed two- to threefold. Since one consequence of *mecA* inactivation is the overproduction of ComK (Fig. 1A), we tested the effect of inactivation of *comK* as well as of *mecA* (BD2251). In this background, *srfA-lacZ* expression was elevated to a level even higher than that of the wild type. Therefore, ComK may act negatively on *srfA* transcription, although we have no evidence that this is a direct effect. *comK* deficiency does not significantly elevate *srfA-lacZ* transcription in a *mecA*$^+$ background (BD2252; Fig. 5).

Expression of *srfA-lacZ* and *comK-lacZ* in Renografin-separated cells. Competent cultures of *B. subtilis* are heterogeneous and exhibit cell-type-specific gene expression. Only about 10% of the cells reach competence, and these can be resolved from the noncompetent fraction by virtue of their reduced buoyant density (18, 22). Although the physiological basis for this density difference is unknown, equilibrium sedimentation in gradients of Renografin provides a useful tool for isolation of the competent and noncompetent subfractions. In this way, it has been shown that late competence genes are expressed preferentially in the light (competent) Renografin-separated cells (2). Since the first open reading frame of the *comG* operon is uniquely required for competent cells to achieve reduced buoyant density, we would expect that all of

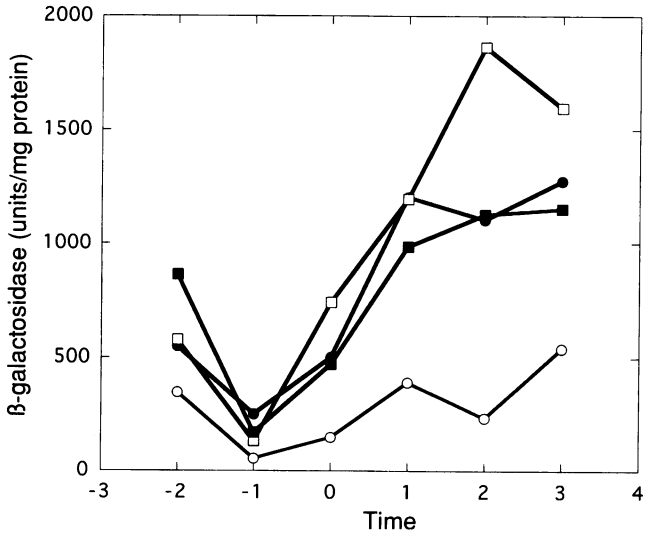

FIG. 5. Effect of ComK overproduction on *srfA* transcription. ■, *srfA-lacZ* strain (BD1974); ○, *mecAΔ srfA-lacZ* strain (BD2253); ●, *comKΔ srfA-lacZ* strain (BD2252); □, *mecAΔ comKΔ srfA-lacZ* strain (BD2251). All cultures were grown in CM.

TABLE 2. Expression of *comK-lacZ* and *srfA-lacZ* in Renografin-separated cells[a]

Strain and fraction	Viable counts/ml	No. of transformants/ml	% Transformation (Leu$^+$)[b]	β-Galactosidase (U/mg of protein)
BD2324 (*srfA-lacZ*)				
Light	3.2×10^7	4.8×10^6	15	1,183
Heavy	1.7×10^9	1×10^7	0.58	971
BD2323 (*comK-lacZ*)				
Light	4.8×10^7	9×10^6	18.8	2,154
Heavy	1.7×10^9	1×10^7	0.6	189

[a] After recovery from Renografin gradients, the light and heavy cells were resuspended in 1 ml each. All values in the second and third columns are normalized to this volume.

[b] The transformabilities of the unfractionated BD2324 and BD2323 cultures were 1.52 and 1.50%, respectively.

the regulatory genes needed for the transcription of *comG* would also be required for Renografin separation. This has been directly confirmed in the case of *comA* (19). To further characterize the cell-type-specific expression of competence genes, we examined whether either of the regulatory genes which are induced during the development of competence (*srfA* and *comK*) exhibits enhanced expression in the light buoyant density fraction. Since we would expect null mutations in these genes to prevent density gradient resolution, we used fusions to *lacZ* integrated at the respective homologous sites by Campbell-like recombination. These strains are phenotypically Com$^+$.

Table 2 shows the results of an experiment in which the *srfA-lacZ* and *comK-lacZ* fusion strains were grown to competence, the cells were resolved on Renografin gradients, and the light and heavy fractions were assayed for transformation and for β-galactosidase specific activity. The transformation frequencies of both strains, as well as *comK-lacZ* transcription, were markedly enhanced in the light cell fraction. *srfA-lacZ* specific activities, on the other hand, were nearly equal in the light and heavy fractions. The low recovery of protein (not shown) and of viable cells in the light fractions may indicate some trapping of light material in the more voluminous heavy fractions. If so, we have underestimated the enrichment of transformability and of *comK-lacZ* transcription in the light fractions. Since *srfA* transcription is turned on at T_{-1}, and since the appearance of the light buoyant density cell fraction begins at T_0, we conclude that the transcription of *srfA* occurs equally in presumptive competent and noncompetent cells, whereas the increased *comK* transcription that occurs at T_0 is restricted to the developing competent cells.

DISCUSSION

This study assists in ordering several regulatory gene products in the competence control pathway. *degU*, *sinR*, *abrB*, and *spo0A* are required for the expression of *comK* (Fig. 2), which encodes CTF (51). It had been reported previously that *srfA* (*comL*) is needed for *comK* expression (54), and in the accompanying report, van Sinderen and Venema (53) document the finding that ComK is required for its own expression. Since *comA*, *comP*, *comQ*, and *spo0K* are needed for the transcription of *srfA*, we would expect that these genes are also needed to express *comK*; indeed, this has been confirmed for *comA* (Fig. 3).

MecA is a negative regulator of competence (28). Strains that harbor loss-of-function *mecA* mutations bypass the requirements for all of the regulatory genes with the exceptions of *spo0A* and, as shown in this report, *comK*. We have suggested elsewhere (5) that the major role for *spo0A* in competence is to prevent the accumulation of excess AbrB, a negative regulator of competence (2). Since the AbrB requirement is bypassed by *mecA*Δ but repression by AbrB (reflected in the *spo0A* requirement) is not (44), we can conclude that AbrB acts positively prior to MecA and negatively after or at the same time as MecA.

The relationships just described are summarized in the scheme shown in Fig. 6. At least three main branches appear to regulate *comK*, two of them converging at Mec. The first branch includes *srfA* and the genes that regulate the transcription of this operon (reviewed in reference 9). The second includes AbrB, SinR, and DegU. These three proteins are grouped for convenience because they exhibit several common features. They do not operate via branch I or III, and their modes of action converge at Mec. However, it is not known

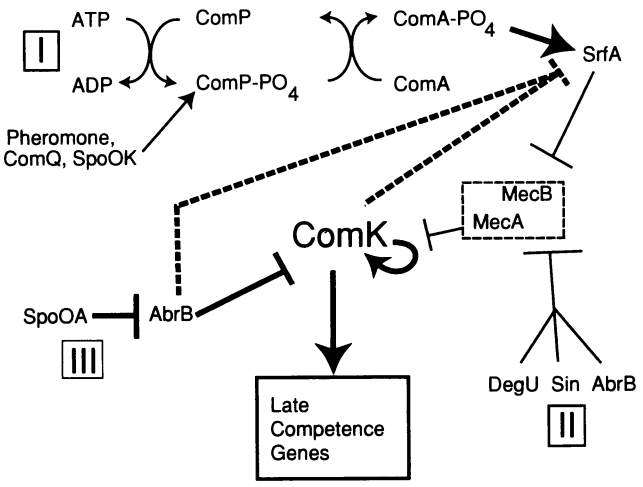

FIG. 6. Competence regulatory pathway. This scheme is based on the work in this report and on previously published data cited in the text. ComX (competence pheromone), ComQ, and Spo0K are on the pathway leading to transcription of *srfA*, and they are believed to act via the ComP-ComA cascade. SrfA, Sin, AbrB, and DegU act positively on *comK*, by participating in the generation of signals instructing MecA to release ComK or otherwise cease the inhibition of ComK activity. These signals are probably mediated by MecB (27). MecA and MecB are enclosed in a box because they appear to work together in inhibiting ComK activity. There is no evidence that the product of SrfA, Sin, AbrB, or DegU acts directly on the Mec proteins. In addition, it is not known whether SrfA, Sin, AbrB, and DegU converge to generate a single signal which acts on the Mec system or whether separate signals converge at Mec itself. Arrows indicate positive effects, and perpendiculars represent negative effects. The dashed lines indicate relatively minor effects. Heavy lines indicate effects that are known to be exerted transcriptionally. The three main branches of the regulatory pathway are identified by boxed roman numerals.

how they operate or whether they cooperate to deliver a single signal or act independently of one another. The negative effect of AbrB on *comK* expression mediates the third main branch. It is likely that this involves a direct effect on *comK* transcription, since in vitro binding of AbrB to the *comK* promoter region has been detected (21a). A minor component of AbrB repression is probably also exerted earlier, since *srfA* transcription is lowered about two- to fourfold in a *spo0A* mutant (21, 25). Since all of the known signals converge on *comK*, we consider that the regulation of this gene is the key control point in the competence pathway.

The failure of *spo0HΔHin*dIII to decrease *comK* expression (Fig. 2C) is unexplained. This deletion, which lowers transformability and the expression of late competence genes about 20-fold (2), is bypassed by the loss-of-function *mecA42* mutation (44). We would therefore have expected Spo0H to act prior to MecA in the pathway and to be required for the full expression of *comK*.

The complexity of the competence regulatory pathway provides an opportunity for the integration of multiple input signals. The precise identities of the molecular signals involved are largely unknown, but some insights have been achieved. The *comA-srfA* pathway (branch I) appears to respond to cell density (15, 29). There is also circumstantial evidence that ComP and ComA respond to nutritional signals (57). Several genes not required for competence also depend for their transcription on ComP and ComA and can be induced by glucose starvation (36) or by starvation for nitrogen or phosphate (34).

The proteins grouped in the second branch may also respond to signalling. DegU is phosphorylated by DegS for the transcription of degradative enzymes, but DegS is not needed for competence (33). Excessive levels of DegU-PO_4, such as those achieved in the *degU*^{h}*32* mutant, are clearly inhibitory for competence (33, 48). This inhibition is seen on the level of *srfA* transcription, and it may be that DegU-PO_4 can directly bind to the *srfA* regulatory sequences as a repressor, although it is not known whether this is indicative of a normal control mechanism. The activity of another protein in the second branch, SinR, is negatively regulated by binding to SinI, and the concentration of the latter responds positively to Spo0A phosphorylation (3, 12). Thus, the SinR requirement for competence and the ability of this protein to inhibit sporulation provide an example of a competence-sporulation switch, which is thrown in favor of sporulation when the concentration of Spo0A-PO_4 exceeds a threshold level.

The *spo0A-abrB* pathway (branch III) potentially responds to multiple signals, nutritional and DNA replication related (24). Whether these signals function in triggering competence is not known. However, the dual role of AbrB presents an interesting case. When the conditions are appropriate for sporulation, Spo0A is highly phosphorylated in response to multiple signals, the concentration of AbrB in the cell drops precipitously, and stage II sporulation genes are induced (23). Both competence and sporulation ordinarily occur postexponentially, and we would expect to have overlapping but nonidentical sets of both signals and signalling proteins governing these two responses. Thus, the presence of glucose acts positively on competence and negatively on sporulation. Excess AbrB negatively regulates both processes, but competence, unlike sporulation, requires that a low level of AbrB be present in the cell. Thus competence and sporulation may be induced by a graded response, with a quantitative difference determining qualitatively distinct outcomes; intermediate concentrations of Spo0A-PO_4 may induce competence, while higher concentrations favor sporulation.

Perhaps the most poorly understood aspect of competence regulation concerns the cell-type-specific expression of the late competence genes. While the transcription of *srfA* occurs in all of the cells, including those destined to be noncompetent, *comK* transcription is restricted to the competent cell fraction, suggesting that the synthesis of this transcription factor distinguishes competent from noncompetent cells. Since a product of the *srfA* operon is required for *comK* transcription, the cell-type-determining event must occur in the regulatory pathway between the transcription of *srfA* at T_{-1} and that of *comK* at T_0.

Competence induces profound physiological changes. Competent cells have ceased DNA replication and stable RNA synthesis (4, 31). They are altered in buoyant density (18, 22) and exhibit a long lag before resuming growth (40). In fact, we have observed that the overproduction of ComK that results from MecA inactivation leads to the loss of colony-forming ability as a culture reaches stationary phase, possibly because the cells are irreversibly locked into the competent state (20). A mechanism, analogous to those that provide for exit from other globally induced states (e.g., SOS and heat shock), must also exist to permit the escape from competence and to permit the resumption of DNA replication and growth. It is likely that this mechanism involves the inactivation or removal of ComK, perhaps via the reactivation of MecA. Since in wild-type cells there may be a distribution of MecA inactivation levels, as well as of AbrB concentrations, it is possible that a subpopulation of cells will potentially synthesize dangerously excessive amounts of ComK. The inhibition of *srfA* transcription by ComK overexpression (Fig. 5) may provide a feedback loop that serves to protect this subpopulation of the competent cells from dead-end differentiation by interrupting signal generation.

ACKNOWLEDGMENTS

We acknowledge valuable discussions with the other members of our laboratory group as well as with I. Smith, J. Dubnau, L. Hamoen, and D. van Sinderen. We also thank A. Grossman, F. Kunst, I. Mandic-Mulec, T. Msadek, M. Nakano, G. Rapoport, D. van Sinderen, I. Smith, T. Tanaka, and P. Zuber for generous gifts of strains and D. van Sinderen for providing information prior to publication.

This work was supported by NIH grant AI10311.

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