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comK Acts as an Autoregulatory Control Switch in the Signal Transduction Route to Competence in *Bacillus subtilis*

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The *comK* gene is a regulatory transcription unit which is essential for the development of genetic competence in *Bacillus subtilis*. The transcription of *comK* is under strict nutritional and growth phase-dependent control and has been shown to depend on the gene products of *comA* and *srfA*. In this report, we show that expression of *comK* is dependent on its own gene product as well as on the gene products of all other tested regulatory genes known to be involved in competence development (*abrB*, *comA*, *comP*, *degU*, *sin*, *spo0A*, *spo0H*, *spo0K*, and *srfA*). A *mecA* mutation is able to suppress the competence deficiency of mutations in any of these regulatory loci except for mutations in *spo0A* and, as we show here, in *comK*. Furthermore, we show that the presence of *comK* on a multiple copy plasmid leads to derepression of *comK* expression, causing an almost constitutive expression of competence in minimal medium as well as permitting competence development in complex medium. We infer from these results that the signals which trigger competence development, after having been received and processed by the various components of the competence signal transduction pathway, all converge at the level of *comK* expression. As soon as derepression of *comK* expression occurs, the positive autoregulation rapidly results in accumulation of the *comK* gene product, which subsequently induces competence.

A specific but poorly understood set of environmental signals triggers the global response in *Bacillus subtilis* which enables cells to take up exogenously added DNA, a property referred to as genetic competence (for a recent review, see reference 14). Since competence fully develops postexponentially only in minimal salts-based media containing glucose as the sole carbon source, these signals must convey information to the cells with respect to the growth stage and medium conditions of the culture. All inducing signals for competence development are believed to be sensed and assimilated through an elaborate signalling network which ultimately effects the transcription of the so-called late competence genes, encoding proteins which constitute the actual DNA-binding and uptake apparatus.

A large number of genetic loci have been implicated in the competence-specific signalling network, and it has become evident that this regulatory system is embedded into an even larger signalling network governing all known postexponential-phase adaptations such as sporulation, motility, antibiotic production, and degradative enzyme synthesis. Several of the regulatory genes required for competence are similar to sensor and effector members of the two-component signal transduction systems (40, 56). Upon reception of the appropriate signal(s), the sensor component becomes subject to autophosphorylation and subsequently transfers the phosphoryl group to its cognate effector, which usually acts as a transcriptional activator. The gene products of *comA* (67) and *comP* (69) resemble effector and sensor members, respectively. In concert with the gene products of *spo0K* (46, 51), *comQ* (68), and *comX* (34), ComA and ComP are postulated to constitute a sensing device detecting both nutritional signals (the availability of amino acids and the nature of the carbon source) as well as information concerning cell density (14, 20, 43). The similarities between the gene products of the *spo0K* locus and components of several oligopeptide permease systems gave rise to the idea that this system responds to cell density-dependent accumulation of an extracellular competence factor (19, 20, 51), which had been implicated previously (28) and was recently shown to be specified by *comX* (34). The primary role of ComA in competence development is to induce transcription of *srfA* (20, 43), a large operon involved in the nonribosomal synthesis of the lipopeptide surfactin (9, 42, 65). Only a small portion of the *srfA* locus is involved in competence development. This portion functions as an information assembly link within the regulatory network (20, 62). Null mutations in *spo0H*, encoding the minor sigma factor σ^H, also affect expression of *srfA* and, therefore, competence (1, 20, 52). DegS and DegU are sensor and effector members, respectively, regulating the synthesis of degradative enzymes (25, 31, 38). Only DegU is required for competence development, although certain mutations in *degS* also affect competence by altering the activity of the *degU* gene product (32, 38, 50). The gene product of *spo0A* is another effector member involved in several postexponential processes, including competence development (6, 52). The major if not the only role of *spo0A* in competence is to control the expression of AbrB (12, 13), a DNA-binding protein which acts both positively as negatively on competence (47, 48, 58). SinR is another DNA-binding protein regulating several late growth processes which is required for competence (2, 18).

Mutations in *mecA* and *mecB* (medium-independent expression of competence) permit the expression of the late competence genes and therefore competence itself in complex media (15, 50). These mutations also suppress the competence deficiency of mutations in all of the regulatory genes tested except *spo0A* (15, 50). Both *mec* loci have been cloned and sequenced, and it has been shown that the *mecA* and *mecB* phenotypes are due to loss of function mutations and that MecA and MecB act as negative regulators of competence (30, 41).

Expression of the late competence genes was shown to require *comK* expression, implying that this locus also has a

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regulatory function in competence development (64). In contrast to most other competence-required regulatory genes, which are not under strict nutritional or temporal control, expression of *comK* is induced postexponentially and exclusively so in competence-stimulating medium (64). Expression of *comK* was shown to be dependent on the gene product(s) of *srfA* (65).

In this study, we show that *comK* expression is dependent not only on the gene products of all the regulatory loci tested (*abrB*, *comA*, *comP*, *degU*, *sin*, *srfA*, *spo0A*, *spo0H*, and *spo0K*) but also on its own gene product. Furthermore, we show that *mec* mutations do not bypass a *comK* deletion mutation. Cells harboring *comK* on a multicopy plasmid developed medium-independent competence that rendered them almost constitutively competent in minimal media. The position of *comK* in the competence-specific signal transduction route is discussed.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. The construction of strain 8G32 places the *comK-lacZ* transcriptional fusion just upstream of the *comK* gene, as described previously (64). Strain 8G40 was constructed as follows. Upon transformation of strain 8G5 with plasmid pBTW312 (Fig. 1A), which was linearized with *Sca*I, chloramphenicol-resistant Amy$^-$ transformants which carried the *comK-lacZ* transcriptional fusion within the interrupted *amyE* gene, as verified by Southern hybridization, were obtained (results not shown). One of these transformants was designated 8G40. The various mutations in strain 8G32 or 8G40 were introduced by (in some cases successive) chromosomal transformations and selection for the mutation-associated antibiotic resistance. Strain 8G328 was constructed as follows. First, 8G5 was transformed with plasmid pDR1, resulting in chloramphenicol-resistant transformants in which the *spo0K* locus was interrupted. Chromosomal DNA was subsequently isolated from one of these transformants and used to introduce the *spo0K* mutation into strain 8G32. Double and triple mutants containing *mec* mutations were constructed in two steps: the *mecA42*, *mecB23*, and *mecB31* mutations were, in combination with *comG12*::Tn*917lacZ*, introduced into the 8G5 genetic background by congression using simultaneous selection for erythromycin resistance, conferred by the *comG12*::Tn*917lacZ* mutation, and the blue colony morphology of the *mec* phenotype on TY agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The *comK* disruption mutation was subsequently moved into these double mutants by phage PBS1 transduction and selection for kanamycin. The presence of the various *degU* and *degS* mutations was confirmed by using the appropriate plate assay to screen for levansucrase and protease production (see below). The genetic background of all strains used was that of 8G5 (*trpC2 his met tyr-1 ade nic purA ura*). *Escherichia coli* MC1000 was used as a cloning host for the construction of pBTW312.

Construction of plasmids. Plasmids used are listed in Table 2. Plasmid pBTW312 (Fig. 1A) was constructed by cloning the *comK-lacZ* transcriptional fusion described previously (55) into plasmid pBT213 (1a), a ptrpBG1 derivative capable of integration by replacement recombination into the *B. subtilis* *amyE* locus (54). Plasmid pGSP12 (Fig. 1B) was obtained as follows. First, the *comK*-containing *Sma*I-*Hin*dIII fragment of pGSP300 (64) was ligated into *Sma*I-*Hin*dIII-restricted pHP12 (22). This ligation mixture was subsequently used to transform protoplasts of *B. subtilis* PSL1 with selection on erythromycin. Restriction analysis of plasmid preparations of several erythromycin-resistant transformants confirmed the presence of the expected and desired plasmid pGSP12.

TABLE 1. Bacterial strains used

Strain	Relevant genotype or characteristics[a]	Reference or source
B. subtilis		
0G1	Prototrophic	5
8G5	Com$^+$	5
8G32	*comK-lacZ* (Km^r) Com$^+$	64
8G321	*comK-lacZ* (Km^r) *comA*::*cm*	D. Dubnau, 64
8G322	*comK-lacZ* (Km^r) *comP*::*cm*	64, 69
8G323	*comK-lacZ* (Km^r) *sin*::*cm*	17, 64
8G324	*comK-lacZ* (Km^r) *abrB*::Tn*917* (Em^r)	48, 64
8G325	*comK-lacZ* (Km^r) *spo0A*::*cm*	20, 64
8G326	*comK-lacZ* (Km^r) *abrB*::Tn*917* (Em^r) *spo0A*::*cm*	20, 48, 64
8G327	*comK-lacZ* (Km^r) *spo0H*::*cm*	20
8G328	*comK-lacZ* (Km^r) *spo0K*[pDR1] (Cm^r)	51
8G59	*comK-lacZ* (Km^r) *srfA*::*cm*	65
8G40	*amyE*::*comK-lacZ* (Cm^r) Com$^+$	This work
8G401	*amyE*::*comK-lacZ* (Cm^r) *comK*::*km*	This work, 64
8G402	*amyE*::*comK-lacZ* (Cm^r) *degS*Δ (Km^r)	This work, 39
8G403	*amyE*::*comK-lacZ* (Cm^r) *degS degU*::*km*	This work, 39
8G404	*amyE*::*comK-lacZ* (Cm^r) *degU*::*emr*	This work, F. Kunst and T. Msadek
8G405	*amyE*::*comK-lacZ* (Cm^r) *degU146* (Km^r)	This work, 10
8G406	*amyE*::*comK-lacZ* (Cm^r) *degU32*(Hy) (Km^r)	This work, 39
8G407	*amyE*::*comK-lacZ* (Cm^r) *mecA*::*spc*	This work, 30, 32
8G408	*amyE*::*comK-lacZ* (Cm^r) *mecA*::*spc comK*::*km*	This work, 30, 32, 64
8G1248	*comG12*::Tn*917lacZ*$^+$ (Em^r)	15
8G1722	*mecA42 comG12*::Tn*917lacZ*$^+$ (Em^r)	15
8G1722K	*mecA42 comG12*::Tn*917lacZ*$^+$ (Em^r) *comK*::*km*	15, 64
8G1720	*mecB23 comG12*::Tn*917lacZ*$^+$ (Em^r)	15
8G1720K	*mecB23 comG12*::Tn*917lacZ*$^+$ (Em^r) *comK*::*km*	15, 64
8G1721	*mecB31 comG12*::Tn*917lacZ*$^+$ (Em^r)	15
8G1721K	*mecB31 comG12*::Tn*917lacZ*$^+$ (Em^r) *comK*::*km*	15, 64
PSL1	*recE4*, restriction deficient	45
E. coli MC1000	F$^-$ Δ*lacX74 rpsL thi*	70

[a] All strains except 0G1 are isogenic with 8G5 (*trpC2 his met tyr-1 ade nic purA ura*). The colons represent disruptions by insertion of the antibiotic markers indicated (*cm*, *km*, *emr*, and *spc*), conferring resistance to chloramphenicol, kanamycin, erythromycin, and spectinomycin, respectively. Strain 8G402 harbors a deleted *degS* gene, a mutation which does not influence the expression of *degU*. Strain 8G328 contains the Campbell-integrated plasmid pDR1 within the *spo0K* locus. When a mutation is linked with an antibiotic cassette, the required antibiotic resistance is represented between brackets (Em^r, Km^r, and Cm^r represent resistance to erythromycin, kanamycin, and chloramphenicol, respectively).

Media. *B. subtilis* minimal medium (competence medium [CM]) consisted of Spizizen's minimal salts (55) supplemented with glucose (1%), potassium glutamate (0.2%), ferric ammonium citrate (2.2 mg/liter), and casein hydrolysate (0.02%; Difco Laboratories). Amino acids, nucleotides (20 μg of each per ml), or vitamins (0.4 μg/ml) were added if required. Minimal agar consisted of minimal salts supplemented with 1% glucose, 0.2% potassium glutamate, the required growth factors, 2.2 mg of ferric ammonium citrate per liter, and 1.5% agar. TY medium and TY agar were prepared as described by Biswal et al. (3). The liquid complex medium of Schaeffer et al. (53) was used as the sporulation medium for *B. subtilis*. Amylase production was tested by growing colonies overnight on minimal agar plates containing 1% starch and checking for

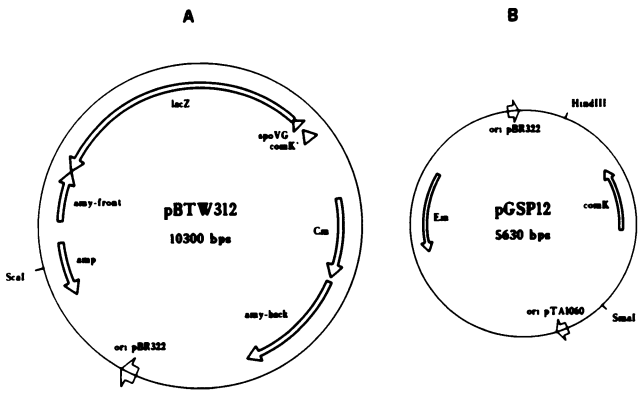

FIG. 1. Plasmids pBTW312 (A) and pGSP12 (B). Construction of the plasmids is described in Materials and Methods. Plasmid pBTW312 (±10,300 bp) carries a pBR322 replicon (ori pBR322), an ampicillin resistance gene (amp), and a chloramphenicol resistance gene (Cm). The *spoVG-lacZ* translational fusion is preceded by the promoter containing the 5′ end of the *comK* gene and flanked by the 5′ and 3′ ends of the *B. subtilis amyE* gene. Plasmid pGSP12 (5,630 bp), containing the replication origins of pBR322 and pTA1060, confers resistance to erythromycin (Em) and carries the complete *comK* transcription unit.

halo formation (54). Overproduction of proteases by the strain carrying the *degU32*(Hy) mutation was detected on skim milk plates (38). Absence of levansucrase production by strains carrying the *degU146* point mutation or the *degS* and *degU* deletion mutations was detected on ST plates (31), using Seakem HGT Agarose (18 g/liter; FMC) instead of agar. Levansucrase-negative mutants have an easily distinguishable phenotype since they lack the mucoid, shiny, slimy appearance of levan-producing colonies. The antibiotic used for selection of transformants was erythromycin (1 μg/ml), kanamycin (50 μg/ml), chloramphenicol (5 μg/ml), ampicillin (100 μg/ml), or spectinomycin (50 μg/ml). X-Gal was added to TY or minimal plates at 80 μg/ml.

Chemicals and enzymes. The chemicals used were of analytical grade and were obtained from Merck or BDH. Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (Boehringer).

TABLE 2. Plasmids used

Plasmid	Description[a]	Source or reference
pBT213	Amp^r Cm^r *amyE-lacZ* insertion vector	J. Alonso and S. Chai
pLGW312	Km^r, pLGW300 derivative, integration vector, contains *comK-lacZ* transcriptional fusion	64
pBTW312	Amp^r Cm^r, *amyE-lacZ* insertion vector, contains *comK-lacZ* transcriptional fusion	This work
pHP12	Em^r, *E. coli-B. subtilis* shuttle vector	22
pGSP12	Derivative of pHP12, contains *comK*	This work
pGSP300	Cm^r Km^r, *E. coli* low-copy-number vector, contains *comK*	64
pDR1	Cm^r, integration plasmid, contains internal fragment of the *spo0K* operon	51

[a] Abbreviations: Amp^r, Cm^r, Km^r, and Em^r, resistance to ampicillin, chloramphenicol, kanamycin, and erythromycin, respectively.

Isolation of DNA. Chromosomal (66) and plasmid (27) DNAs were purified as described previously.

Competence, transformation, and transduction. *E. coli* was made competent and transformed by the method of Mandel and Higa (35). Competent cells of *B. subtilis* were prepared as described by Bron and Venema (5), using the two-step protocol. In some cases, a one-step protocol was used to facilitate the correlation between enzyme production and competence of a given strain by omitting the second dilution step (1). When competence development in sporulation medium was tested, samples were washed and resuspended in CM before transforming DNA was added. Preparation, transformation, and regeneration of *B. subtilis* protoplasts were performed as described previously (7). PBS1 transductions were performed as described by Hoch et al. (26).

Determination of β-galactosidase activity. Cultures were grown as described previously (65). Measurements and calculations of β-galactosidase units (expressed as units per unit of optical density at 600 nm) were carried out as described by Miller (36).

Southern hybridization. After electrophoresis in agarose gels, the DNA was transferred to a GeneScreen Plus membrane (Dupont, NEN Research Products) by using a modified Southern protocol (8). Random-primed DNA was labelled with digoxigenin-dUTP, using a Boehringer nonradioactive DNA labelling and detection kit, and subsequently denatured for 10 min at 100°C. The hybridization and staining steps were carried out as recommended by the manufacturer.

Antibody preparation and Western blotting (immunoblotting). ComK protein was overproduced by using the expression vector pET7 (60), in which *comK* was cloned behind the T7 promoter. A cell lysate of the ComK-overproducing strain was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the ComK-containing region was excised from the gel. SDS was subsequently removed by dialysis from the gel slices. Antiserum reacting against ComK was obtained from a rabbit which was periodically injected with Freund adjuvant containing crushed gel slices. After transfer of proteins to a nitrocellulose membrane (Schleicher & Schuell), protein-antibody complexes were made visible by using the Western-Light chemiluminescent protein detection system (Tropix).

Protein determination. Protein concentrations were determined by the method of Bradford (4), with bovine serum albumin as the standard.

RESULTS

Expression of *comK* is growth stage and growth medium specific. To study the expression of *comK*, we made use of strains 8G32 and 8G40 (Table 1), in which the expression of a promoterless *lacZ* gene is driven by the *comK* promoter. The strains harbor, at different positions on the chromosome, a *comK-lacZ* fusion which is associated with an antibiotic resistance marker, conferring resistance to either kanamycin (strain 8G32) or chloramphenicol (strain 8G40), to facilitate the construction of the various strains described below. Strain 8G32 carries the *comK-lacZ* fusion just upstream of the *comK* gene (64). Strain 8G40 harbors the *comK-lacZ* transcriptional fusion within the interrupted *amyE* gene (see Materials and Methods). In both strains, expression of β-galactosidase activity is sharply increased just after the transition from the exponential to the stationary growth phase (defined as T_0) but exclusively so in CM, as reported previously (64). This growth

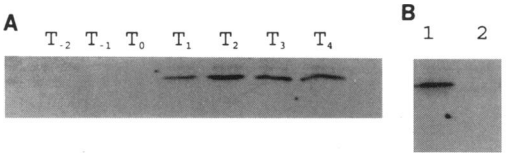

FIG. 2. Western blotting of ComK in extracts of *B. subtilis* 8G5 as a function of growth in CM (A) (time is in hours before and after T_0) or in different media (B) (lanes 1 and 2 are blotted extracts of cells grown to T_2 in CM and sporulation medium, respectively). Equal amounts of protein were applied to all lanes.

phase- and growth medium-dependent β-galactosidase activity pattern faithfully reflects the actual *comK* accumulation, as was demonstrated in Western blotting experiments using anti-ComK antibodies (Fig. 2).

Effects of various mutations on competence in the 8G5 genetic background. Mutations in many regulatory genes prevent full expression of competence. To measure the effect on competence development in the genetic background used in our laboratory, we moved various mutations by transformation or transformational congression to the 8G5 genetic background and determined the transformability of the resulting strains (Table 3). The observed transformation-deficient phenotypes are in good agreement with those reported previously (reviewed in reference 14). As can be seen from Table 3, all of these mutations except the *comK* disruption still permitted a residual level of competence which varies with the mutation tested. Neither the *degU146* mutation, which inactivates the phosphorylation site of *degU* (10), nor deletion of *degS* had a noticeable effect on competence development, confirming the hypothesis that only unphosphorylated *degU* is required for competence (38). Mutations in either *abrB* or *spo0H* decreased the transformation frequency only moderately. Furthermore, we observed that introduction of the *abrB* mutation into a strain harboring the *spo0A* disruption partially suppressed the competence-deficient phenotype associated with the *spo0A* mutation, as was noted previously (1, 61).

Effects of regulatory mutations on the expression of *comK-lacZ*. By means of the *comK-lacZ* fusion, the epistatic effects of the regulatory mutations on *comK* expression were examined during growth in CM (Fig. 3). All of the mutations tested affected expression of the *comK-lacZ* fusion in accordance with their effects on competence development (Table 3 and Fig. 3): null mutations of *comA*, *comP*, *sin*, *spo0A*, *spo0K*, *srfA*, and *degU* and the *degU32*(Hy) mutation severely lowered expression of the *comK-lacZ* fusion; the *abrB*, *spo0H*, and *abrB spo0A* mutations caused a moderate decrease, while the *degS* deletion and the *degU146* mutation hardly affected expression of the fusion. A *comK* disruption severely reduced expression of the *comK-lacZ* fusion, indicating that the ComK protein has a positive effect on its own transcription (Fig. 3F). These results imply that the gene products of all tested regulatory loci required for competence exert their effects before or at the point of *comK* expression, acting either on the level of *comK* transcription or posttranscriptionally by preventing *comK* autoregulation.

TABLE 3. Transformability of regulatory mutants

Mutation[a]	Transformation frequency[b]	Mutation	Transformation frequency
Com$^+$	1.0	*spo0A::cm*	1.6×10^{-4}
comA::cm	2.1×10^{-3}	*spo0A::cm abrB*::Tn*917*	4.0×10^{-2}
comP::cm	3.6×10^{-3}	*sin::cm*	4.0×10^{-3}
spo0K[pDR1]	4.2×10^{-3}	*degS degU::km*	3.0×10^{-5}
abrB::Tn*917*	2.0×10^{-2}	*degS*Δ (Km^r)	8.0×10^{-1}
srfA::cm	2.4×10^{-3}	*degU::emr*	4.0×10^{-5}
comK::km	$<10^{-6}$	*degU146*	7.0×10^{-1}
spo0H::cm	6.0×10^{-2}	*degU32*(Hy)	2.0×10^{-3}

[a] The various mutations were introduced into the 8G5 genetic background (see Table 1 for a description and the source of each mutation).

[b] Transformability was determined by using the two-step competence regimen and selection for Trp$^+$ transformants. The transformation frequency for Trp$^+$ was calculated as the average from several independent experiments and was normalized to the average transformation percentage determined for the Com$^+$ strain (0.3%).

A *comK* null mutant is not bypassed by *mec* mutations. Expression of *comK* as well as competence development normally only occur when a culture is grown in CM (64). Mutations in *mecA* or *mecB*, however, permit expression of competence in all media tested (15). Furthermore, *mec* mutations suppress the effects of all tested regulatory *com* mutations except *spo0A*, not only bypassing the dependencies of late competence gene expression on these regulatory gene products but also restoring the associated competence-deficient phenotype (50). To determine whether *mec* mutations were able to bypass a *comK* disruption, the *mecA42*, *mecB23*, and *mecB31* mutations were first introduced into the 8G5 genetic background in combination with the *comG12*::Tn*917lacZ* mutation to allow selection for the *mec* phenotype as well as monitoring of the transcription of *comG*, specifying a component of the DNA uptake apparatus. Subsequently, triple mutants that contained a *comK* disruption, in addition to a specific *mec* mutation and *comG12*::Tn*917lacZ*, were constructed. Figure 4 clearly shows that none of the *mec* mutations was able to suppress the effect of the *comK* mutation on the expression of this late competence gene, indicating that ComK acts later in the competence signalling cascade than the *mecA* and *mecB* gene products.

A *mecA* knockout mutation causes overexpression of ComK. MecA is thought to act as a negative regulator of competence, since MecA overproduction inhibits expression of *comG*, a late competence gene (30). In addition, it was observed that a *mecA* deletion mutant caused overexpression of *comG* as well as other late competence genes (30). The *mecA* null mutation dramatically increased the expression of *comK* in CM, as demonstrated by measuring β-galactosidase activity of a *comK-lacZ* fusion (Fig. 5) and by Western blot analysis (Fig. 6A), and relieved medium-dependent repression of *comK* in complex medium (Fig. 6B and results not shown). However, expression of the *comK-lacZ* fusion in the *mecA* deletion strain still increased at T_0, indicating that the *mecA* mutation did not relieve the growth phase dependency of *comK* expression. Figure 5 also demonstrates that expression of the *comK-lacZ* fusion in CM is severely inhibited in a strain harboring a *mecA comK* double mutation, confirming our previous result that a *mecA* mutation is not able to suppress the *comK* disruption.

Effects of *comK* present in multiple copies. Regulation of *comK* expression appears to be dependent on all gene products which take part in the competence-specific signalling pathway. If their sole function in competence development is to activate or derepress *comK*, overexpression of ComK is expected not only to bypass the need for these gene products but also to render competence development independent of nutritional and growth phase-specific signals. In addition, since ComK acts positively on its own promoter, ComK overexpression would increase expression of the *comK-lacZ* transcriptional fusion. To test this hypothesis, plasmid pGSP12, a pHP12 derivative with a copy number of about five per chromosome equivalent (22), containing the complete *comK* transcription unit, was

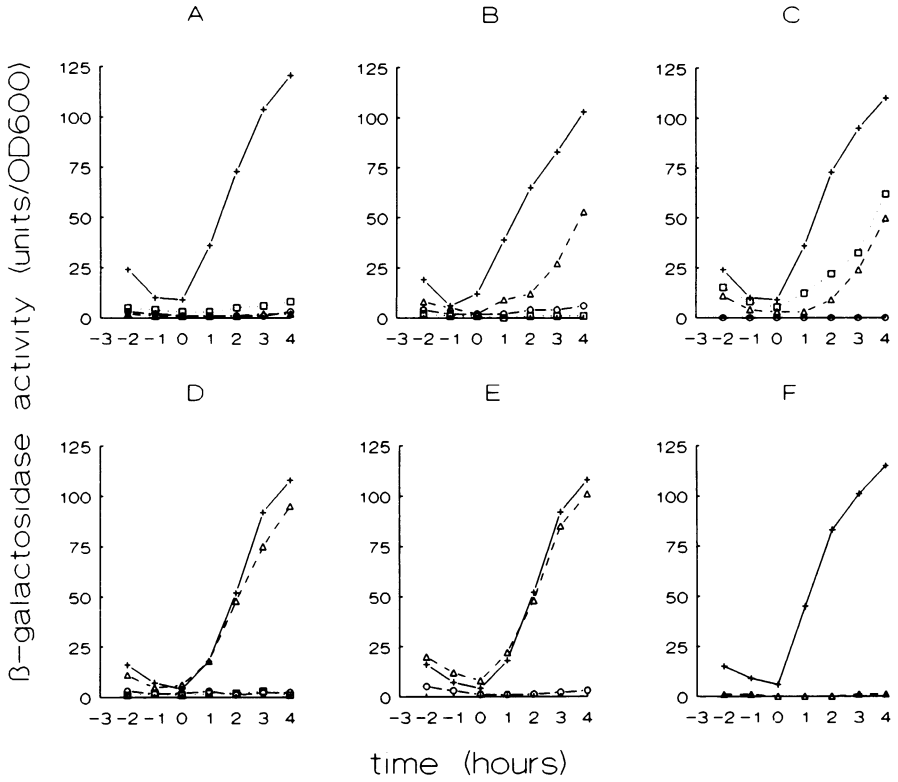

FIG. 3. Epistatic interactions of competence regulatory genes on *comK* expression. Expression of β-galactosidase was driven by the *comK-lacZ* transcriptional fusion in the wild-type transformable strains 8G32 (A to D) and 8G40 (E and F) as a function of growth in CM (+). The effects of deletion, disruption, or point mutations in regulatory genes on the expression of *comK* are shown. (A) *comP* (8G322) (△), *comA* (8G321) (○), and *spo0K* (8G328) (□); (B) *spo0H* (8G327) (△), *sin* (8G404) (○), and *srfA* (8G59) (□); (C) *abrB* (8G324) (△), *spo0A* (8G325) (○), and *abrB spo0A* (8G326) (□); (D) *degS* (8G402) (△), *degU* (8G404) (○), and *degS degU* (8G403) (□); (E) *degU146* (8G405) (△) and *degU32*(Hy) (8G406) (○); (F) *comK* (8G401) (△). The time scale refers to hours before and after the transition from the exponential to the stationary growth phase (defined as T_0). OD600, unit of optical density at 600 nm.

introduced into 8G40 (*comK-lacZ*). β-Galactosidase expression of 8G40 in the presence of either pGSP12 or pHP12 in CM and sporulation medium is shown in Fig. 7. In CM, the pGSP12-harboring strain gave rise to a significantly higher level of *comK-lacZ* expression compared with the control strain (Fig. 7A). In contrast to the control strain, the presence of pGSP12 in strain 8G40 also permitted expression of *comK-lacZ* in sporulation medium (Fig. 7B) as well as in all other rich media tested (veal invusion broth, tryptone-yeast medium, and Luria broth; results not shown). Similar results were obtained

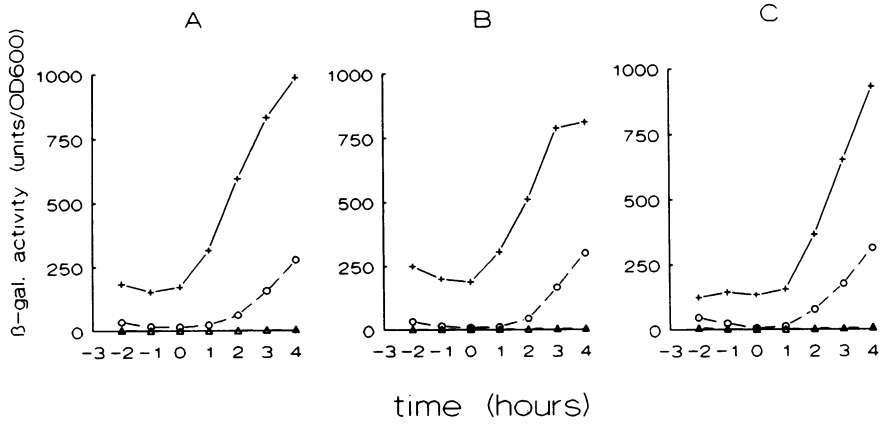

FIG. 4. β-Galactosidase (β-gal.) expression driven by the *comG12*::Tn*917lacZ* fusion in strains containing *mecA42* (+; A), *mecB23* (+; B), and *mecB31* (+; C) mutations and the effect of *comK* disruption in each of these *mec* strains (△) as a function of growth in CM. The expression of enzyme in the *mec*$^+$ strain containing the *comG12*::Tn*917lacZ* fusion is also shown (○). The time scale is as in Fig. 2. OD600, unit of optical density at 600 nm.

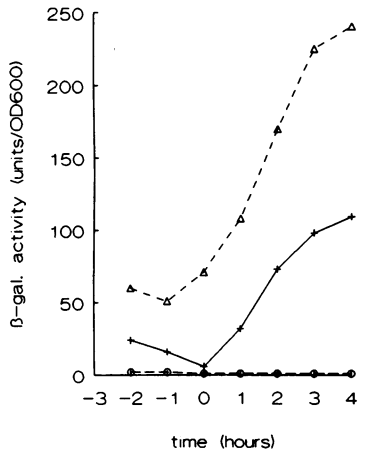

FIG. 5. Effects of *mecA* (△) and *mecA comK* (○) on β-galactosidase expression driven by the *comK-lacZ* transcriptional fusion as a function of growth in CM. Expression of enzyme in an otherwise wild-type strain is also shown (+). The time scale is as in Fig. 2. OD600, unit of optical density at 600 nm.

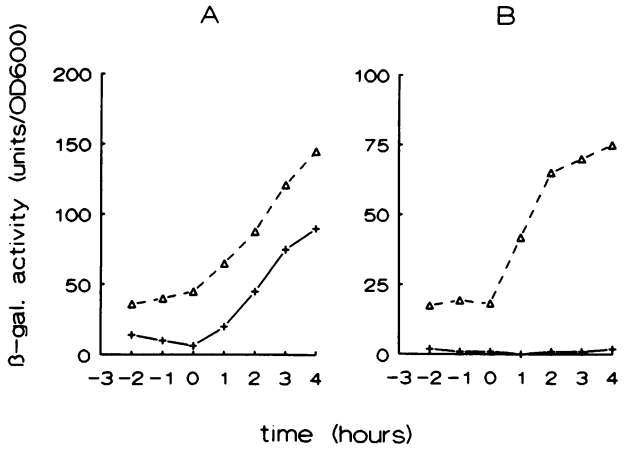

FIG. 7. Effects of multiple *comK* copies on *comK-lacZ* expression. β-Galactosidase expression, driven by the *comK-lacZ* fusion, was measured as a function of growth in the presence of the *comK*-containing plasmid pGSP12 (△) or the control plasmid pHP12 (+) in CM (A) or sporulation medium (B). The time scale is as in Fig. 2. OD600, unit of optical density at 600 nm.

when strain 8G1248 (*comG-lacZ*) instead of strain 8G40 (*comK-lacZ*) was used (results not shown). Western blot analysis confirmed that ComK in the pGSP12-carrying strain is overexpressed to a level comparable to that of a strain carrying a *mecA* mutation (Fig. 6). The effect of the presence of *comK* in multiple copies on the development of competence in CM and sporulation medium is shown in Fig. 8. In CM, the control strain carrying pHP12 exhibited normal postexponential expression of competence. In contrast, the presence of pGSP12 in strain 8G40 gave rise to an almost constitutive high level of competence during exponential growth which slowly declined when cells entered the stationary growth phase (Fig. 8A). In sporulation medium, the pGSP12-harboring strain developed competence postexponentially to a level which was at least 2 orders of magnitude higher than the very low level of competence developed in this medium by the control strain (Fig. 8B). This result indicates that overexpression of *comK* overcomes growth medium- and, in CM, growth stage-specific control of competence.

DISCUSSION

This study demonstrates that *comK* plays a pivotal role in the regulation of competence development in *B. subtilis*. The observed epistatic interactions between ComK and other (regulatory) *com* genes, combined with unpublished data and results from previous publications (in particular references 20 and 50), give insight into the hierarchical structure of the signal transduction network controlling competence development. A schematic picture of this information flow is presented in Fig. 9.

Null mutations in *degU* cause a competence-deficient phenotype and prevent the expression of *comK* (Table 3 and Fig. 3), confirming the role of this gene in regulation of competence (25, 31, 40). Although the gene product of *degU* is an effector member (40, 56), its cognate sensor, the gene product of *degS*, does not seem to be involved in competence development (Table 3). Our data (Fig. 3 and Table 3) are in agreement with the assumption (20, 32, 38) that the unphosphorylated DegU is required for competence development, whereas the phosphorylated equivalent, activating degradative enzyme production, has a negative effect on competence.

The gene products of *comA* (67), *comP* (69), *comQ* (68), *comX* (34), and *spo0K* (46, 51) are believed to constitute a sensing device required for the postexponential transcription

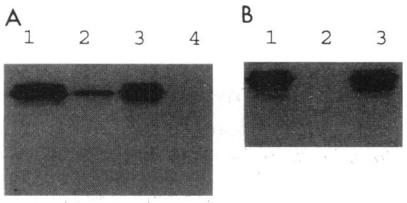

FIG. 6. Western blotting of ComK in extracts obtained from various strains grown to T_2 in CM (A) or sporulation medium (B). Extracts were prepared from strains 8G407 (*mecA*Δ; lane 1), 8G40 harboring plasmid pHP12 (control strain; lane 2); 8G40 harboring plasmid pGSP12 (*comK* on plasmid pHP12; lane 3); and 8G401 (*comK*Δ; lane 4). Equal amounts of protein were applied to all lanes.

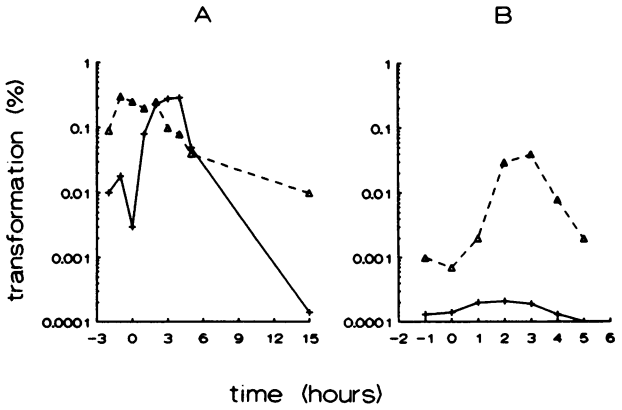

FIG. 8. Development of competence of strains 8G40(pGSP12) (△) and 8G40(pHP12) (+) in CM (A) or sporulation medium (B). The time scale is as in Fig. 2.

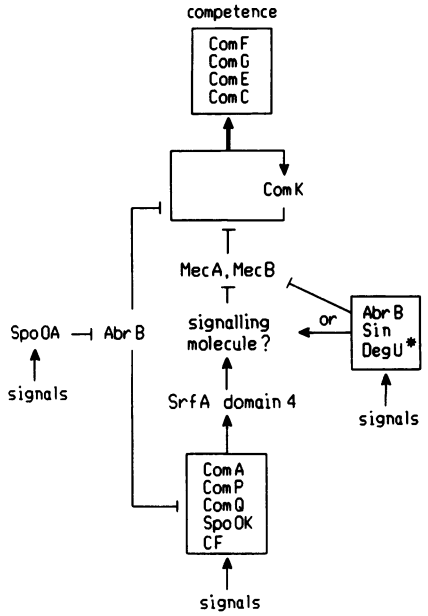

FIG. 9. Proposed scheme of information flow in the competence signal transduction pathway. The lines terminated by arrowheads indicate positive effects, and those terminated by perpendiculars indicate negative effects. CF, competence factor specified by *comX*; DegU*, DegU in its unphosphorylated state. For a detailed explanation, see the text.

activation of *srfA* (20, 34, 43), assembling information concerning carbon source, amino acid availability, and cell density (14, 20, 34, 43). Their effect on *comK* expression is therefore likely to be mediated through the action of *srfA*. Binding of the response regulator ComA to the *srfA* promoter region, with enhanced affinity when ComA is present in a phosphorylated form, has been demonstrated (49). Probably this binding facilitates *srfA* transcription through localized bending of its target DNA (44). The *srfA* operon not only plays a role in competence development but also is responsible for the nonribosomal synthesis of the lipoheptapeptide surfactin (20, 42, 56, 58). Recently, it has been shown that a small gene, designated *comS*, located within the portion of *srfA* that specifies the valine activation domain (62), is implicated in competence development (11, 23).

Mutations in *mecA* (30) and *mecB* (41) suppress the requirement for regulatory genes in expression of late competence genes and the development of competence itself, with the exceptions of *spo0A*, which is only partly bypassed (14, 15, 50), and *comK* (Fig. 4 and 5). Since *mec* mutations cause ComK overexpression (Fig. 5 and 6), the *mec* system apparently functions as a repressor of *comK* expression and ComK must act downstream of MecAB in the signal transduction route. Therefore, the primary role of the early competence genes *comA*, *comP*, *comQ*, *comX*, *degU*, *abrB*, *spo0H*, *spo0K*, *sin*, and *srfA* is to counteract the negative action of MecAB on *comK* expression (Fig. 9). Kong and Dubnau (29) have recently demonstrated that ComK is inactivated by a protein-protein interaction between ComK and MecA. These authors hypothesize that in response to a signal(s) delivered by the previously acting regulatory components, MecB relieves MecA-mediated inactivation of ComK by modulating the activity of MecA.

A *spo0A* disruption causes approximately a 1,000-fold decrease in transformation efficiency and severely depresses *comK* expression (Fig. 3 and Table 3). *spo0A* mutants have been shown to overproduce AbrB (59), a repressor of several genes which are normally induced after the transition to the stationary growth phase (reviewed in reference 57). An *abrB* null mutation restores transformability as well as *comK* expression of a *spo0A* mutant to the same level as a strain carrying an *abrB* mutation alone (Fig. 3 and Table 3). These results imply, as has been postulated previously (12, 13), that the role of Spo0A in competence development is to down regulate the expression of *abrB* at T_0, preventing overexpression but allowing a level of AbrB sufficient for optimal competence. AbrB is believed to exert its negative action at multiple points in the competence signalling route (20, 50). One point of AbrB repression is at the level of *srfA* expression (20), although *srfA* expression is not completely abolished in a *spo0A* mutant. The *spo0A* disruption almost completely abolishes *comK* expression (Fig. 3), suggesting that another point of AbrB repression occurs at the level of *comK* expression. The finding that AbrB binds to the *comK* promoter indicates that this repression is provoked by a direct impediment to *comK* transcription (24).

The partial dependence of competence development and *comK* expression on *spo0H*, which encodes the RNA polymerase sigma factor σ^H (16), indicates that the transcription of at least one, as yet unknown, regulatory competence gene is (partly) dependent on this alternative form of RNA polymerase. Bypass studies have demonstrated that *spo0H* acts both before and after *srfA* transcription (20) but before the *mec* system (50).

comK has been shown to be required for the expression of all tested late competence genes (33, 64), which specify components of the DNA uptake apparatus or proteins involved in the assembly of this device (reviewed in reference 14). Expression of *comK*, as demonstrated by Western blotting and β-galactosidase fusion studies, is growth phase, growth medium, and as shown in the accompanying report (21), cell type specific. These findings imply that apparently all regulatory signals required for competence development have been activated before or at the point of *comK* expression. The observation that *comK* expression is dependent on its own gene product indicates the existence of an autoregulatory loop in the competence signal transduction pathway. This could serve two purposes. First, it could provide the competence regulatory machinery, in addition to transcriptional regulation, with an extra means to posttranslationally control *comK* functioning, as has been proposed for the *mec* system (29). Second, *comK* autoregulation could serve as a means to rapidly increase the ComK concentration necessary for the quick synthesis and assembly of the DNA uptake apparatus. We have recently obtained evidence that the gene product of *comK* is the previously inferred competence transcription factor, which is responsible for the transcription activation of the late competence genes (37, 63). It therefore seems that the regulatory function of *comK* is to couple the competence regulatory network to the assembly of the DNA-binding and uptake apparatus.

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