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A Molecular Switch Controlling Competence and Motility: Competence Regulatory Factors ComS, MecA, and ComK Control σ^D-Dependent Gene Expression in *Bacillus subtilis*

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***Bacillus subtilis*, like many bacteria, will choose among several response pathways when encountering a stressful environment. Among the processes activated under growth-restricting conditions are sporulation, establishment of motility, and competence development. Recent reports implicate ComK and MecA-ClpC as part of a system that regulates both motility and competence development. MecA, while negatively controlling competence by inhibiting ComK, stimulates σ^D-dependent transcription of genes that function in motility and autolysin production. Both ComK-dependent and -independent pathways have been proposed for MecA's role in the regulation of motility. Mutations in *mecA* reduce the transcription of *hag*. encoding flagellin, and are partially suppressed by *comK* in both medium promoting motility and medium promoting competence. Reduced σ^D levels are observed in *mecA* mutants grown in competence medium, but no change in σ^D concentration is detected in a *comK* mutant. The *comF* operon, transcription of which requires ComK, is located immediately upstream of the operon that contains the *flgM* gene, encoding the σ^D-specific antisigma factor. An insertion mutation that disrupts the putative *comF-flgM* transcription unit confers a phenotype identical to that of the *comK* mutant with respect to *hag-lacZ* expression. Expression of a *flgM-lacZ* operon fusion is reduced in both *sigD* and *comK* mutant cells but is abolished in the *sigD comK* double mutant. Reverse transcription-PCR examination of the *comF-flgM* transcript indicates that readthrough from *comF* into the *flgM* operon is dependent on ComK. ComK negatively controls the transcription of *hag* by stimulating the transcription of *comF-flgM*, thereby increasing the production of the FlgM antisigma factor that inhibits σ^D activity. There likely exists another *comK*-independent mechanism of *hag* transcription that requires *mecA* and possibly affects the σ^D concentration in cells undergoing competence development.**

The gram-positive, spore-forming bacterium *Bacillus subtilis* will activate one of several developmental programs when it is confronted with a growth-restricting environment. As is the case with many bacterial species faced with nutritional stress, *B. subtilis* will produce several extracellular degradative enzymes and antibiotics. More elaborate responses include the establishment of motility and processes of cellular specialization such as sporulation and genetic competence. Molecular mechanisms exist which serve as switches that permit the cell to choose an appropriate developmental path in response to harsh environmental conditions (18). An example of such a mechanism is the SinR-SinI pair, which participates in the cell's decision to undergo either sporulation or competence and motility (3, 17, 29, 42). The phosphorylation state of the response regulator DegU is another determinant of whether cells produce degradative enzymes such as proteases or undergo competence establishment and become motile (26).

Although motility and genetic competence appear to be coregulated, recent studies have shown that there likely exists another molecular switch governing the cell's decision to choose one or the other of these pathways. Competence development is part of a complex signal transduction network influenced by the nutritional state of the environment and cell density (13, 18; Fig. 1). The key regulatory event in the establishment of genetic competence is activation of transcription factor ComK (46). ComK is required for transcription of the late competence operons (13, 35, 46) that encode, among other proteins, ComE (a DNA binding protein that functions in DNA uptake); ComGA, -B, and -C, which form a type IV pilus bundle that is thought to position ComE; and ComFA, an ATP-dependent helicase required for DNA import (6, 7, 10, 11). ComK is negatively controlled by MecA and ClpC by direct protein-protein interaction (24, 44). MecA-ClpC-dependent inhibition of ComK is overcome by ComS (9, 21, 44), a small protein encoded by the *srf* operon (8, 21, 36, 37, 45), which also encodes the enzyme surfactin synthetase, a peptide synthetase catalyzing the synthesis of the lipopeptide antibiotic surfactin (15, 32, 45, 47).

The transcription of genes that function in motility, including those that code for flagellum assembly, proteins functioning in chemotaxis, and autolysins, requires the alternative RNA polymerase sigma subunit σ^D (1, 20, 27, 31, 34, 41). FlgM functions as an antisigma factor that negatively controls σ^D (4, 14). MecA exerts opposite effects on competence development and motility (40, 43). MecA negatively controls the establishment of competence by interaction with ComK but is required for optimal expression of genes that are transcribed by the σ^D form of RNA polymerase. Thus, MecA may serve as part of a molecular switch governing the cell's decision to become motile or undergo genetic competence. Two independent reports provide conflicting views of how MecA-dependent positive

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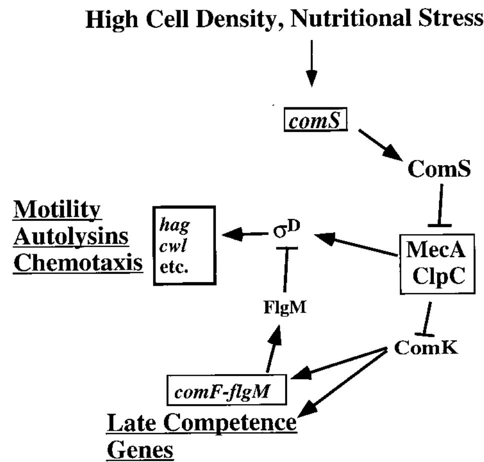


FIG. 1. ComK-dependent and -independent MecA control of *hag* expression. High cell density and nutritional stress stimulate expression of the *comS* gene. ComS interaction with MecA-ClpC results in release of ComK, which activates *comF-flgM* operon transcription, as well as the expression of other late competence operons. Antisigma factor FlgM negatively controls σ^D, resulting in reduced expression of *hag* and other genes of the σ^D-regulon. MecA affects the σ^D protein level, particularly in cells grown in medium that promotes competence development.

control is exerted. Rashid et al. presented data suggesting that MecA control is independent of ComK (43), while Ogura and Tanaka proposed that ComK negatively controls σ^D-dependent transcription and that the positive effect of MecA occurs solely through its interaction with ComK (40).

This report includes data showing that there exist both ComK-dependent and -independent mechanisms of MecA control of flagellar gene expression and that ComK negatively controls σ^D-dependent transcription by stimulating the transcription of the *flgM* gene encoding the σ^D-specific antisigma factor. MecA inhibits ComK but also affects the level of σ^D in a ComK-independent manner.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used in this study are listed in Table 1. All of the strains constructed during this study are derivatives of *B. subtilis* JH642 (from J. Hoch). DNA from HB1002 cells bearing a *hag-lacZ* translational fusion (5; from J. D. Helmann) was used to transform JH642 competent cells with selection for erythromycin resistance (Erm^r) to create strain LAB2607. To create *mecA* and *comK* mutant strains bearing a *hag-lacZ* translational fusion (strains LAB2722 and LAB2723, respectively), DNA from AG1312 (*mecA*::*spc*) (25) or 8G32 (*comK*::*kan*) (46) was used to transform LAB2607 competent cells with selection for spectinomycin (Spc^r) or neomycin (Neo^r) resistance. A *mecA comK* double mutant bearing a *hag-lacZ* translational fusion (LAB2724) was constructed by transforming LAB2607 competent cells with DNA from AG1312 and 8G32 with selection for Spc^r and screening for Neo^r.

The *flgMΔ80* mutation is an in-frame deletion removing codons 6 through 85 of the *flgM* gene (34). To create a *flgMΔ80* SPβc2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan* strain, the transducing lysate of HB4041 [ZB307A SPβc2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*, obtained from J. D. Helmann (14)] was used to lysogenize CB149 (*flgMΔ80*) (34) with selection for chloramphenicol resistance (Cm^r), creating strain LAB2827. P*hag-cat-lacZ* is a transcriptional fusion containing the promoter region of *hag* with the upstream UP element deleted (14). The same lysate was used to transduce strains JH642, LAB2916, LAB2917, LAB2724, LAB2923, LAB2924, and LAB2925, thereby creating the wild type (LAB2819) and *mecA* (LAB2920), *comK* (LAB2921), *mecA comK* (LAB2922), *mecA flgMΔ80* (LAB2926), *comK flgMΔ80* (LAB2928), and *mecA comK flgMΔ80* (LAB2929) mutant strains bearing the phage-borne *hag-lacZ* transcriptional fusion, respectively. The presence of the *flgMΔ80* mutation was verified by PCR and agarose gel electrophoresis of the *flgM*-specific PCR fragment. The forward primer (UFlgM′) used for PCR amplification was a 22-mer with the sequence GCGA ATTCAGATCACTCATCTT, and the reverse primer (LFlgM′) was a 24-mer with the sequence GGGCTTTCTCCTTTTTTATTGCTT.

To create an insertion mutation at the site of P_{D-1} of the *comF-flgM* operon (34), a DNA fragment extending from 539 bp upstream to 329 bp downstream of the P_{D-1} transcription start site was synthesized by PCR amplification. The forward primer used to amplify the fragment was a 30-mer with the sequence AC GCGGATCCTCA ATCTGTTCATGCCGTAT, and the reverse primer was a 30-mer with the sequence TAAACTGCAGGGTATGCCAAATTAG GAAGA. The primers contained restriction sites for *Bam*HI and *Pst*I, respectively. These sites (underlined) were used to insert the cleaved PCR fragment into *Bam*HI-*Pst*I-cleaved plasmid pMMN13 (36), a pGEM4 derivative carrying a *cat* gene. The resulting plasmid was then introduced into wild-type strain JH642 by transformation with selection for Cm^r. The lysate of the SPβc2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan* strain was then used to lysogenize one of the transformants with selection for Neo^r.

Transformation and transduction. Competent *B. subtilis* cells were prepared as previously described (12). Specialized transduction with SPβ was done as described by Zuber and Losick (48).

Media. *B. subtilis* cells were routinely cultivated in 2XYT medium (36) to obtain cells for the preparation of DNA or to induce antibiotic resistance in

TABLE 1. Strains used in this study

Strain	Genotype	Reference or source
JH642	*pheA trpC2*	J. Hoch
HB1002	*hag*::pDM6330 *hag-lacZ erm*	5
HB4041	SPβc2Δ2 Tn*917*::Φ(*Phag-cat-lacZ*) *kan*	14
AG1312 (BD2092)	*trpC2 mecA*::*spc*	25
8G32	*trpC2 comK*::*kan*	46
CB149	*pheA trpC2 flgMΔ80*	33
LAB2607	*pheA trpC2 hag-lacZ erm*	This work
LAB2722	*pheA trpC2 hag-lacZ erm mecA*::*spc*	This work
LAB2723	*pheA trpC2 hag-lacZ erm comK*::*kan*	This work
LAB2724	*pheA trpC2 hag-lacZ erm mecA*::*spc comK*::*kan*	This work
LAB2916	*pheA trpC2 mecA*::*spc*	This work
LAB2917	*pheA trpC2 comK*::*kan*	This work
LAB2918	*pheA trpC2 mecA*::*spc comK*::*kan*	This work
LAB2819	*pheA trpC2* SPβc2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*	This work
LAB2827	*pheA trpC2 flgMΔ80* SPβ2c2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*	This work
LAB2920	*pheA trpC2 mecA*::*spc* SPβc2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*	This work
LAB2921	*pheA trpC2 comK*::*kan* SPβc2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*	This work
LAB2922	*pheA trpC2 mecA*::*spc comK*::*kan* SPβ2cΔ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*	This work
LAB2923	*pheA trpC2 mecA*::*spc flgMΔ80*	This work
LAB2924	*pheA trpC2 comK*::*kan flgMΔ80*	This work
LAB2925	*pheA trpC2 mecA*::*spc comK*::*kan flgMΔ80*	This work
LAB2926	*pheA trpC2 flgM*Δ80 *mecA*::*spc* SPβc2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*	This work
LAB2928	*pheA trpC2 comK*::*kan flgMΔ80* SPβc2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*	This work
LAB2929	*pheA trpC2 mecA*::*spc comK*::*kan flgMΔ80* SPβ2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*	This work
LAB2931	*pheA trpC2 comF-flgM*::pJL010 (*cat*)	This work
LAB2932	*pheA trpC2 comF-flgM*::pJL010 (*cat*) SPβc2Δ2 Tn*917*::Φ(P*hag-cat-lacZ*) *kan*	This work
LAB2944	*pheA trpC2 hag-lacZ erm Δsrf*::*cat*	This work
CB100	*trpC2 sigD*::*cat*	J. D. Helmann (14)
LAB2994	*E. coli* DH5α/pJL011 *bla*	This work
LAB2995	*trpC2 pheA1 flgM* (*orf139*)*-lacZ cat*	This work
LAB2996	*trpC2 pheA1 flgM* (*orf139*)*-lacZ comK*::*neo*	This work
LAB2997	*trpC2 pheA1 flgM* (*orf139*)*-lacZ cat sigD*::*erm*	This work
LAB2998	*trpC2 pheA1 flgM* (*orf139*)*-lacZ cat comK*::*neo sigD*::*erm*	This work

transformed cells. 2XYT and one-step competence medium (CM) (12, 38) were used to culture cells for assays of *lacZ* fusion-encoded β-galactosidase activity. *Escherichia coli* cells were propagated in 2XYT medium to obtain cells for plasmid isolation. The antibiotic concentrations used for selection of drug-resistant organisms were as follows: chloramphenicol, 5 μg/ml; erythromycin in combination with lincomycin, 1 and 25 μg/ml, respectively; neomycin, 5 μg/ml; spectinomycin, 75 μg/ml; ampicillin, 25 μg/ml. The antibiotic concentrations used to induce drug resistance were as follows: chloramphenicol, 0.5 μg/ml; erythromycin, 0.1 μg/ml.

Culture conditions and β-galactosidase assay. Cells precultured in Difco sporulation (DSM) agar plates or 2XYT broth at 37°C overnight were used to inoculate CM or 2XYT broth. Cultures were grown in 300-ml baffled sidearm flasks (MRA, Clearwater, Fla.) at 37°C in a shaking water bath. Samples were collected and assayed for β-galactosidase activity by the methods described previously (37, 49).

Protein extraction and Western immunoblot analysis. Samples were harvested at T_0 (at the end of exponential growth) and T_2 (2 h after the end of exponential growth) by centrifugation at 4°C. The cells were washed once in phosphate-buffered saline, centrifuged again, and stored at −70°C. The thawed pellets were resuspended in 20 mM Tris-HCl (pH 7.5)–5 mM EDTA–1 mM dithiothreitol–1.5 mM phenylmethylsulfonyl fluoride. Whole cell extracts were prepared with a French press, diluted in sodium dodecyl sulfate (SDS) sample buffer, and boiled for 5 min. Samples with the same protein concentrations, as determined with a Bio-Rad protein assay kit, were applied to an SDS–12% polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to nitrocellulose and probed with anti-σ^D antibodies (obtained from J. D. Helmann), followed by a secondary rabbit antibody conjugated with alkaline phosphatase as recommended by the manufacturer (GIBCO Bethesda Research Laboratories). The intensity of each band was determined with the NIH-Image computer program.

Construction of the *flgM′-lacZ* fusion. The same PCR product as described in the construction of the *comF-flgM* insertion mutation was cleaved with *Pst*I followed by T4 DNA polymerase to render the ends flush. The blunt-ended PCR fragment was digested with *Bam*HI. The digested PCR fragment was then inserted in front of a promoterless *lacZ* gene in plasmid pTKlac (23), which was cut with *Hin*dIII, treated with T4 DNA polymerase to fill in the *Hin*dIII ends, and then cleaved with *Bam*HI. The resulting plasmid (pJL011) was then introduced into JH642 competent cells by transformation. To examine the effects of mutations in *comK*, *sigD*, or both *comK* and *sigD* on the expression of *flgM′-lacZ*, *comK* mutant, *sigD* mutant, or *comK sigD* double mutant cells bearing *flgM′-lacZ* were constructed by transformation with DNA from the three mutant strains, using the wild-type strain carrying *flgM′-lacZ* as the recipient. In the case of the *sigD* mutation, the *cat* insertion marker had to be replaced with the *erm* marker of plasmid pCm::Er of strain ECE72 (*Bacillus subtilis* Genetic Stock Center, Columbus, Ohio), which was used to transform CB100 cells with selection for Erm^r and screening for Cm^s.

Reverse transcription-PCR (RT-PCR). Wild-type and *comK* mutant cells grown in CM were harvested at $T_{0.5}$ to isolate RNA. Isolation of RNA was performed as previously described (39). To ensure that no contaminating DNA was present in the RNA preparation, about 4 to 5 μg of the RNA sample (in 15 μl) was incubated at 37°C for 1 h with 30 U of RNase-free DNase and 0.5 μl (20 U) of RNase inhibitor (Promega) in a 50-μl volume. DNase-treated RNA samples still containing contaminating DNA were treated again as described above until no DNA contamination was detected by PCR. The treated RNA samples were recovered by using RNaid in accordance with the protocol recommended by the manufacturer (Bio 101, Inc.). PCR was performed to check for contaminating DNA (for the locations of the primers used, see Fig. 8). The nucleotide sequences of the downstream and upstream primers are as follows, respectively: 5′-GCACCTTTCACAAGGGTATGCAAATTAG-3′ (primer 1 [see Fig. 8]) and 5′-ACGCGGATCCTCAATCTGTTCATGCCGTAT-3′ (primer 2 [see Fig. 8]).

RT was conducted as described previously (2). The purified RNA was used as a template to synthesize cDNA strands by using avian myeloblastosis virus reverse transcriptase (Promega) and the antisense downstream primer shown above that was designed to anneal to *orf139* mRNA. The resulting cDNA was then used as a template to create an amplified RT-PCR fragment by using Vent polymerase (New England BioLabs). The downstream and upstream primers were the same as those used as mentioned above to check for contaminating DNA, while another upstream primer with the nucleotide sequence 5′-ATGGGA GAACTGGCTAATTGTCCGAAATGCA-3′ (primer 3 [see Fig. 8]), which starts at the translational initiation codon of *orf139*, was used to detect both the readthrough *comF-flgM* operon transcript and the transcript initiating at the P_{D-1} promoter. The resulting RT-PCR products were identified by agarose gel electrophoresis (1%, wt/vol), while the PCR product from a template of chromosomal DNA from wild-type *B. subtilis* JH642 was applied as a positive control.

RESULTS

***mecA*, *comK*, and *comS* affect the expression of *hag-lacZ*.** To reexamine the roles of MecA and ComK in the regulation of the σ^D regulon, the *mecA* and *comK* mutations were introduced by transformation into cells bearing a translational *hag-lacZ* fusion plasmid integrated at the *hag* locus (14). A fusion-bearing strain containing both of the *mecA* and *comK* mutations was also constructed. Expression of *hag* in the three mutant strains was examined in cultures grown in 2XYT and in CM. Rich medium conditions, such as those existing in 2XYT, promote expression of genes of the σ^D regulon but do not promote competence due to the Mec-dependent inhibition of ComK. This inhibition is relieved in CM by the *comS* gene product. High levels of *hag-lacZ* activity were observed in wild-type cells grown in 2XYT, with expression increasing as the culture reached the end of exponential growth (Fig. 2A). A *mecA* mutation resulted in substantially lower *hag-lacZ* activity throughout the growth curve. The *comK* mutation did not change the level of expression from that observed in wild-type cells, but introduction of a *mecA* mutation into the *comK* background resulted in a modest but reproducible decrease in expression. MecA positively influences *hag-lacZ* expression

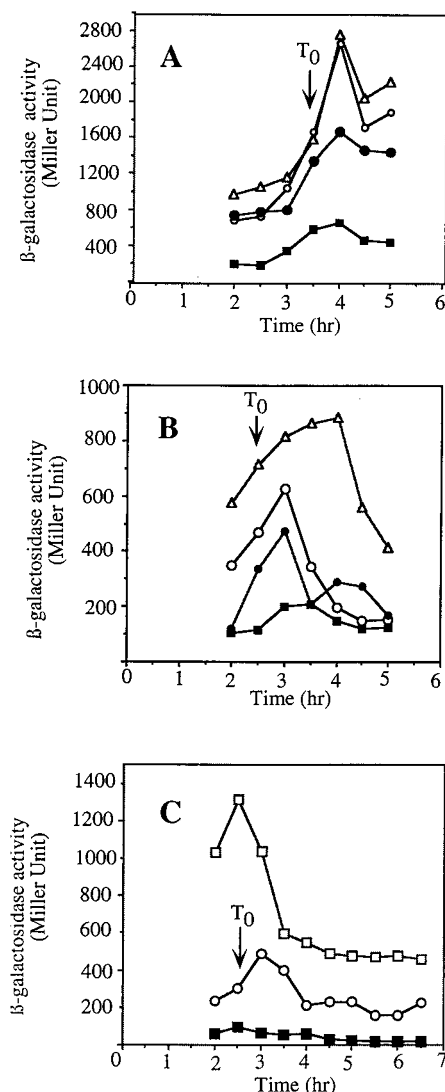


FIG. 2. Expression of *hag-lacZ* in the wild type and *mecA*, *comK*, and *mecA comK*, and *srf* (*comS*) mutants. Cells of each strain were grown in 2XYT (A) or CM (B and C), and samples were collected at the indicated times. *hag*-directed β-galactosidase activity was determined as described in Materials and Methods and in accordance with published protocols. Symbols: △, LAB2723 (*comK hag-lacZ*); ○, LAB2607 (*hag-lacZ*); ●, LAB2724 (*comK mecA hag-lacZ*); ■, LAB2722 (*mecA hag-lacZ*); □, LAB2944 (*hag-lacZ srf*).

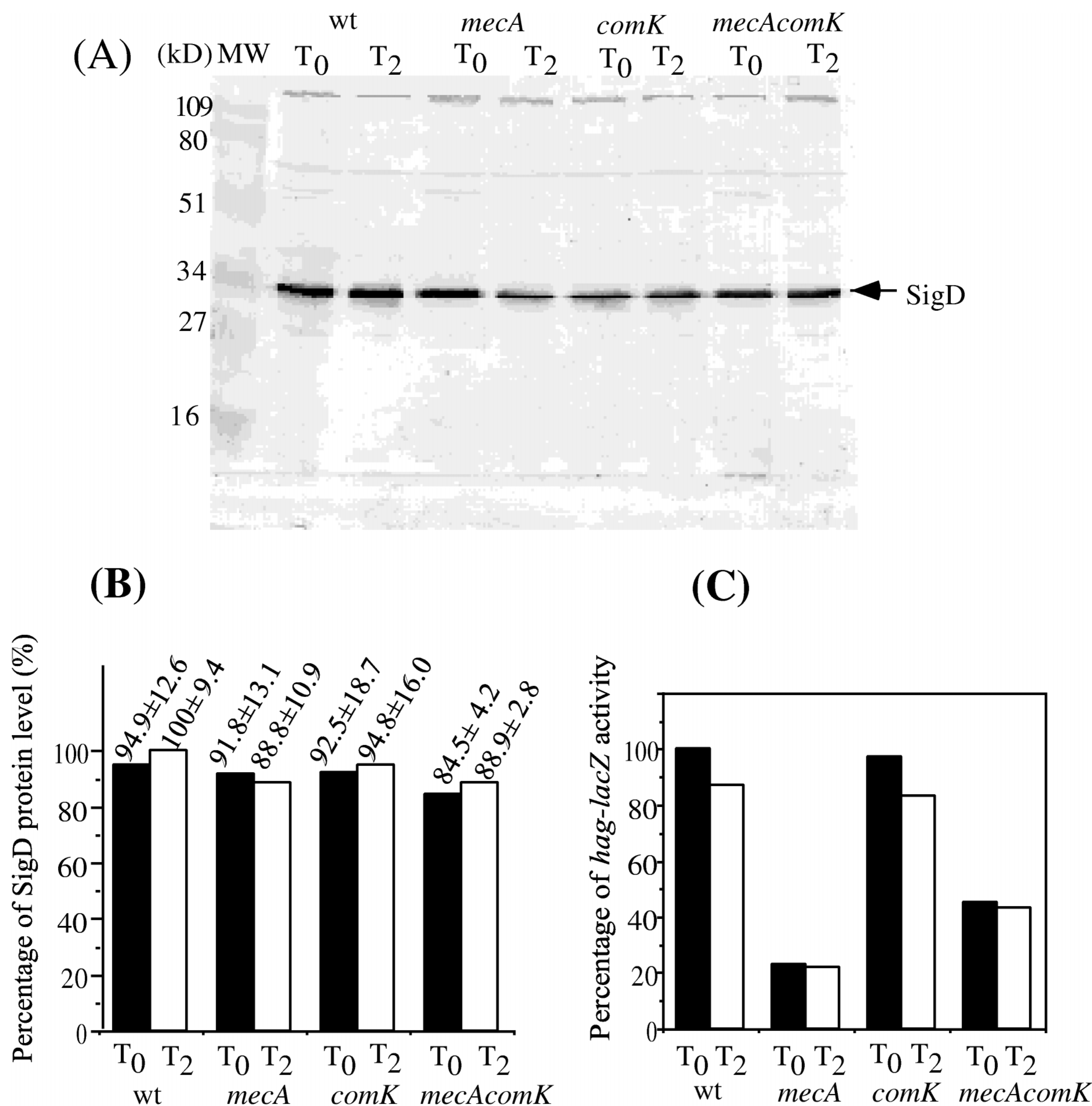


FIG. 3. Level of σ^D protein in the wild type (wt) and *mecA*, *comK*, and *mecA comK* mutants grown in rich medium. Cells precultured in 2XYT liquid medium were grown in 2XYT medium at 37°C. Samples were harvested at T_0 and T_2 (at the end of exponential growth and 2 h after the end of exponential growth, respectively). Cell extracts with equal protein concentrations were applied to SDS–12% polyacrylamide gels and subjected to electrophoresis. The resolved protein was electrotransferred to nitrocellulose and analyzed by the Western blotting procedures described in Materials and Methods. (A). Lane MW contained molecular size markers. The other lanes contained samples from cultures of LAB2607 (wild type), LAB2722 (*mecA*), LAB2723 (*comK*), and LAB2724 (*mecA comK*) cells collected at T_0 and T_2 of the growth curve. (B) Western blot band intensity determined by scanning of the image of the stained blot and quantification by the NIH-Image computer program. The values presented are percentages of the level of protein in the wild-type strain at T_2. The standard deviation was calculated from three independent experiments. (C) Levels of *hag*-directed β-galactosidase in cultures used to obtain extracts for Western blot analysis.

primarily by inhibiting ComK activity but may play some additional role in *hag* expression.

In cells grown in CM, ComK levels are higher due to reversal of Mec-dependent inhibition by ComS (19, 28, 44). Thus, *comK* more profoundly influences *hag-lacZ* expression. As shown in Fig. 2B, the expression of *hag-lacZ* in a *comK* mutant is significantly higher than in wild-type cells. As in 2XYT medium, *mecA* cells exhibit low levels of *hag-lacZ* activity. This repression of *hag* is only partially reversed by the *comK* mutation. The repression of *hag* expression caused by a *mecA* null mutation is, in part, *comK* dependent, but MecA plays some other role in the positive control of *hag*.

As further evidence for MecA-ComK-dependent control of *hag* expression and the involvement of the so-called early *com* regulators, the expression of *hag-lacZ* in an *srf* deletion mutant lacking the *comS* gene was examined (Fig. 2C). Significantly higher levels of *hag-lacZ* expression were observed in *comS* mutant cells grown in CM than in wild-type cells, in accordance with the observed *comK*-dependent control of *hag*. If ComS served to release ComK from MecA-ClpC-dependent inhibition, then it, too, would be expected to exert a negative influence on *hag* expression.

***mecA* and *comK* mutations have little effect on σ^D levels in rich medium, but *mecA* mutant cells grown in CM contain a**

reduced concentration of σ^D. How do MecA and ComK exert their effects on the regulation of *hag*? As had been shown previously, expression of the class III flagellar regulon genes, such as *hag*, requires the σ^D form of RNA polymerase (31, 33). It was conceivable, therefore, that competence regulatory factors regulate the expression of the *sigD* gene. Cells of the wild-type and *comK*, *mecA*, and *comK mecA* mutant strains bearing a translational *hag-lacZ* fusion (5) were grown in 2XYT medium and, in a separate experiment, CM. Samples were collected at T_0 and T_2 (0 and 2 h after the end of the exponential growth phase, respectively) for the measurement of σ^D protein levels by immunoblot analysis, while *hag* expression was quantified by measuring *hag*-directed β-galactosidase activity. There was little significant change in the level of protein observed in the four 2XYT cultures, as shown by a computer-aided scan of the developed immunoblot (Fig. 3A and B). This is in contrast to the level of *hag-lacZ* expression observed in the cell culture samples. In the *mecA* and *comK* mutant cells, there was a significant difference in the level of *hag-lacZ* expression (Fig. 3C) but virtually identical concentrations of σ^D protein. This suggested that ComK might affect the activity of σ^D rather than the σ^D concentration.

In CM, the *mecA* mutation modestly influenced the level of σ^D protein. While wild-type and *comK* mutant cells contain similar concentrations of σ^D protein, *mecA* and *mecA comK* mutant cells show reduced levels of σ^D (Fig. 4). The ComK-independent positive control of *hag* expression exerted by MecA could be directed, at least in part, at the concentration of σ^D protein.

A *comF-flgM* insertion mutation results in heightened expression of *hag-lacZ*. Because a mutation in *comK* appeared to affect the activity of σ^D, it was possible that the σ^D-specific antisigma factor FlgM was involved. It was noticed that the *flgM* operon, consisting of four genes in the order *orf139 flgM orf160 fliK* (31, 34), resides immediately downstream of *comF*, a late competence operon, transcription of which requires ComK (30, 46). We reasoned that *comF* and *flgM* could lie in the same transcription unit and that ComK functions in the negative control of *hag* by stimulating the transcription of *flgM*, thereby increasing the level of the σ^D-specific antisigma factor. To test this hypothesis, a plasmid insertion mutation was constructed to separate upstream ComK-dependent transcription from σ^D-dependent *flgM* operon transcription. If *comK* functioned in the negative control of *hag* by activating *comF-flgM* transcription, then the disruption of ComK-dependent transcription of *flgM* by the plasmid insertion would confer the same phenotype, with respect to *hag* expression, as a *comK* mutation. An 868-bp fragment encompassing the 3′ end of *comFC*, the P_{D-1} promoter, and the 5′ end of *orf139* (Fig. 4) was generated by PCR. The P_{D-1} promoter is utilized by the σ^D form of RNA polymerase and is located between the putative transcriptional terminator of the *comF* operon and the start codon of *orf139*. The fragment was inserted into integration vector pMMN13. The resulting plasmid, pJL010, was used to transform cells of strain JH642. The transformant obtained had undergone a Campbell-type recombination event, yielding a strain bearing an integrated plasmid at the *comF-flgM* junction (Fig. 5). This strain was lysogenized with SPβP*hag-lacZ* to yield strain LAB2932. The P*hag-lacZ* fusion is a transcriptional fusion between a promoterless *lacZ* gene and a derivative of the *hag* promoter that lacks the upstream UP element; hence, the levels of *hag*-directed β-galactosidase activity are lower in strains bearing this fusion than in those carrying the translational fusion. The patterns of *hag-lacZ* expression in the *comK* and *comF-flgM* insertion mutants are nearly identical, with higher levels of expression in the stationary phase in CM than that observed in wild-type cells (Fig. 6).

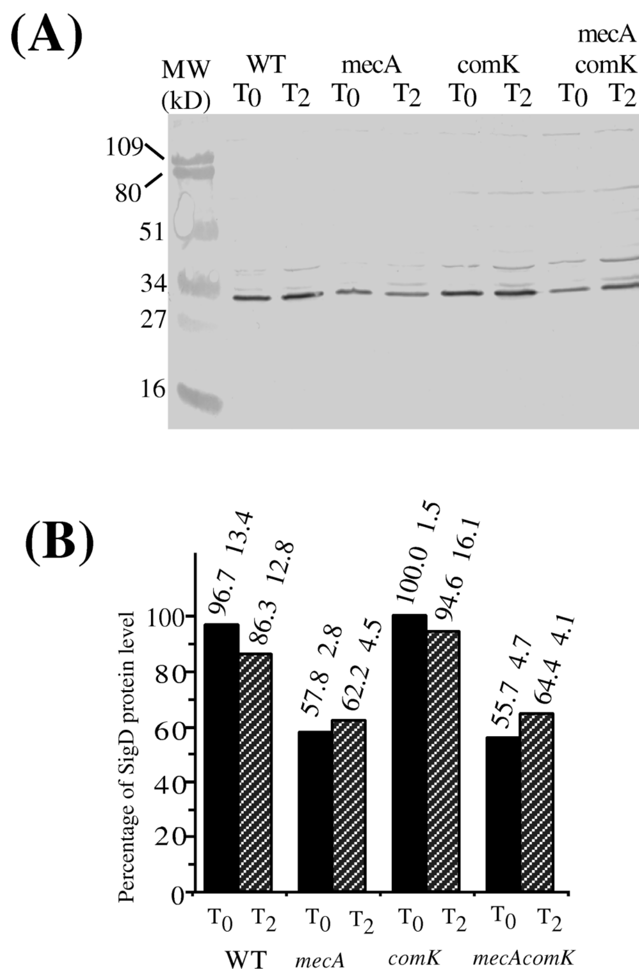


FIG. 4. Levels of σ^D protein in the wild type (WT) and *mecA*, *comK*, and *mecA comK* mutants grown in CM. Cells precultured in DSM agar plates were grown in CM at 37°C. Samples were harvested at the same time points as in Fig. 3. Analysis of the protein extracts was conducted as described in the legend to Fig. 3. (A) Western blot of extracts of LAB2607 (wild type [WT]), LAB2722 (*mecA*), LAB2723 (*comK*), and LAB2724 (*mecA comK*) cell samples collected at T_0 and T_2 of the growth curve. (B) Western blot band intensity determined and presented as described in the legend to Fig. 3.

Table 2 summarizes the effects of *flgM*Δ*80* in wild-type and *comK* and *mecA* mutant genetic background cells grown in CM. *flgM*Δ*80* in combination with a *comK* mutation does not result in *hag-lacZ* expression higher than that observed in the *flgM* mutant. As with the *comK* mutation, the *flgM* deletion did not show complete suppression of *mecA*. The *flgM*Δ*80 comK mecA* triple mutant showed a level of *hag-lacZ* expression similar to that of a *flgM*Δ*80 mecA* mutant. That the suppression of *mecA* by *flgM* and *comK* mutations is not significantly higher than that of each mutation alone suggests that *comK* and *flgM* operate within a common genetic pathway, consistent with the hypothesis that *comK* regulates *flgM* expression. The incomplete suppression of *mecA* caused by a *flgM* mutation indicates that the *comK*-independent function of *mecA* in regulating *hag* expression does not involve FlgM.

Expression of the *flgM* operon is dependent on both *sigD* and *comK*. The phenotype of the *comF-flgM* insertion mutation suggested that the transcription of the *flgM* operon was controlled by *comK*. To test this prediction, a *flgM* operon-*lacZ* transcriptional fusion was constructed by inserting the same *comFC-orf139* PCR fragment used to make the insertion mu-

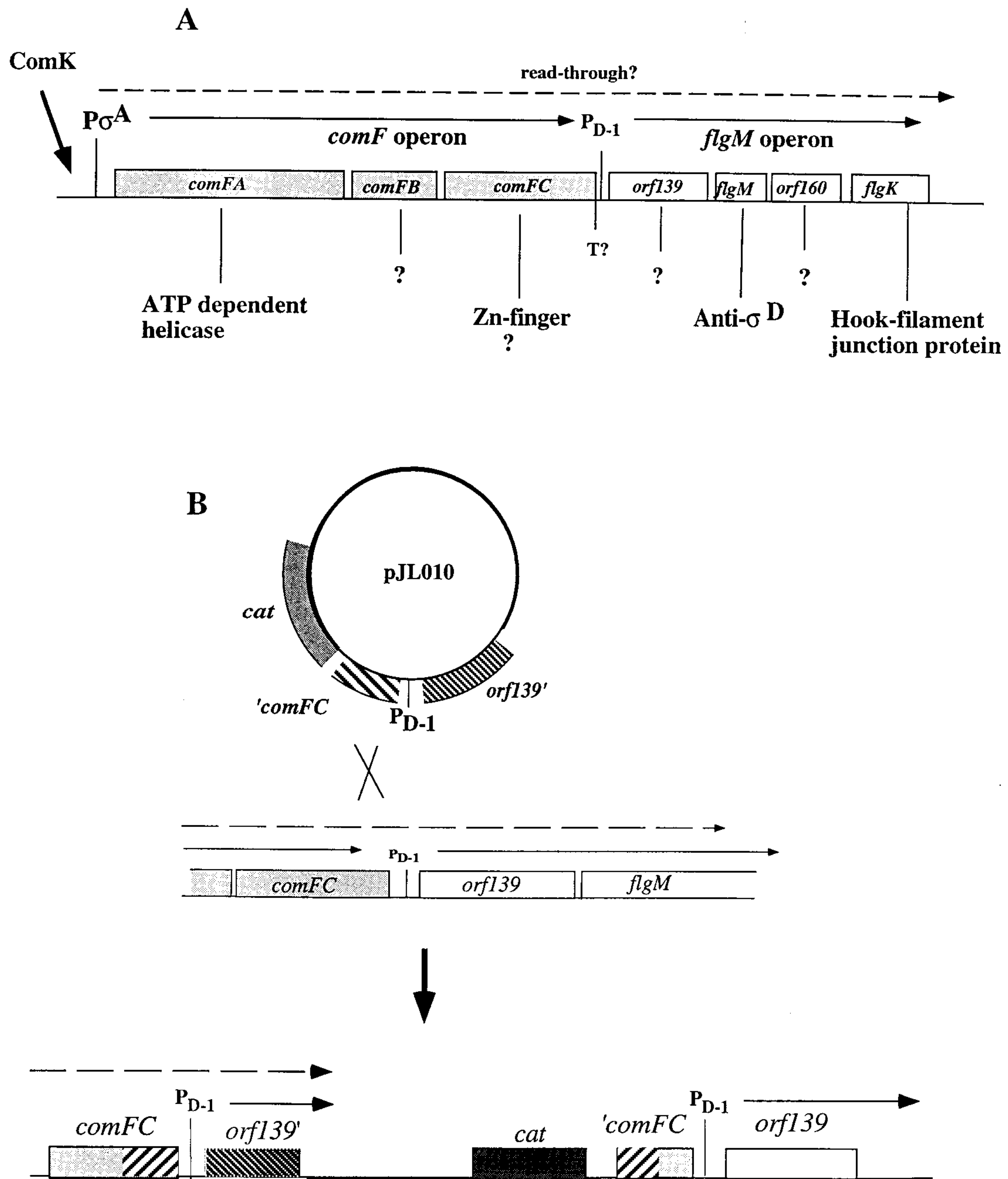


FIG. 5. Structure of the *comF-flgM* operon and construction of a *comF-flgM*::pJL010 insertion mutant. (A) *flgM* is located within an operon containing the promoter P_{D-1}, immediately downstream of the *comF* operon. *comF* is a *B. subtilis* late competence operon. Transcription of *comF* is driven by a single σ^A-type promoter, utilization of which is dependent on ComK. The *flgM* operon contains *orf139*, *flgM*, *orf160*, and *flgK*. (B) The mutant was constructed by insertion of a plasmid (pJL010) carrying *comFC* and *orf139* fragments by a single-recombination mechanism into the region containing the junction between the *comF* and *flgM* operons.

tation upstream of a promoterless *lacZ* gene. The construction was introduced into JH642 competent cells, in which the plasmid would insert by a single recombination event into the putative *comF-flgM* operon. Mutant derivatives of the fusion-bearing strains were constructed by transformation with *sigD* and *comK* mutant DNA, thereby creating *sigD*, *comK*, and *sigD comK* mutants all carrying the *flgM* operon-*lacZ* fusion.

Optimal expression of the *lacZ* fusion was dependent on both the *comK* and *sigD* genes (Fig. 7), with the double mutant *comK sigD* cells exhibiting nearly undetectable levels of *lacZ* expression, supporting the hypothesis that *flgM* operon transcription is positively controlled by ComK.

RT-PCR was employed to determine if ComK-dependent read-through transcription from *comF* into the *flgM* operon could be detected. RNA was purified from both wild-type (JH642) and *comK* mutant (LAB2917) cells at 30 min after the end of exponential growth. Two different RT-PCRs were assembled. Both utilized an oligonucleotide corresponding to a sequence within the *orf139* open reading frame to prime reverse transcriptase-catalyzed cDNA synthesis from *flgM* operon RNA. In one reaction, amplification was carried out by using a primer hybridizing to *comFC* sequences within the cDNA to obtain an RT-PCR product of 885 bp derived from the *comF-flgM* read-through transcript. The other reaction utilized a primer hybrid-

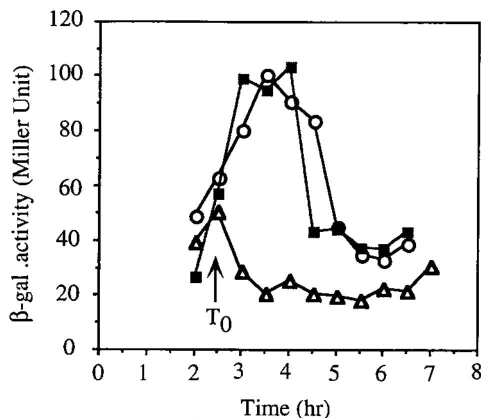


FIG. 6. Expression of *hag-lacZ* in wild-type and *comK* and *comF-flgM*::pJL010 mutant cells grown in CM. Cells were precultured on DSM agar plates and then grown in CM liquid at 37°C. *hag*-directed β-galactosidase (β-gal) specific activity of samples collected at the indicated time points was determined as described in Materials and Methods and in the legend to Fig. 3. Symbols: △, LAB2819 (SPβ*hag-lacZ*); ○, LAB2921 (SPβ*hag-lacZ comK*); ■, LAB3932 (SPβ*hag-lacZ comF-flgM*::pJL010).

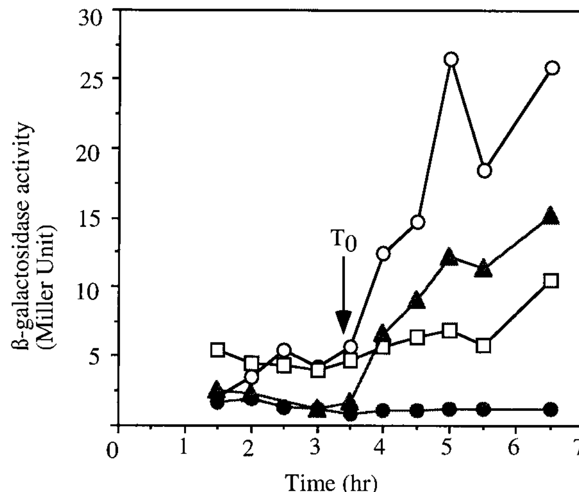


FIG. 7. *flgM* operon (*orf139*)-lacZ fusion expression in *comK* and *sigD* mutant cells. Cells of the wild-type and the *comK*, *sigD*, and *comK sigD* mutant strains bearing plasmid pJL011 [*flgM* (*orf139*)-*lacZ*] integrated at the *flgM* locus were grown in CM. Samples were collected at 30-min intervals for assay of β-galactosidase activity. Symbols: ○, LAB2995 [*flgM* (*orf139*)-*lacZ*]; ▲, LAB2997 [*sigD flgM* (*orf139*)-*lacZ*]; □, LAB2996 [*comK flgM* (*orf139*)-*lacZ*]; ●, LAB2998 [*sigD comK flgM* (*orf139*)-*lacZ*].

izing to the region of the cDNA corresponding to the amino-terminal coding end of *orf139*, the first gene of the *flgM* operon. Amplification of the cDNA yields an RT-PCR product of 270 bp derived from both the readthrough transcript and the RNA synthesized from the P_{D-1} promoter. Control reaction mixtures containing RNA, primer, and DNA polymerase were included to determine if contaminating DNA remained in the RNA after DNase treatment.

As shown in Fig. 8, an RT-PCR product corresponding to a readthrough *comF-flgM* operon transcript could be detected in wild-type cells (lane 9) but not in the *comK* mutant cells (lane 5), whereas the 270-bp product is observed in reaction mixtures containing either *comK* mutant or wild-type cellular RNA (lanes 6 and 10). The results described above confirm that *comF-flgM* transcription is *comK* dependent. More of the RT-PCR product was detected in the reaction mixture producing the 270-bp fragment than in that producing the 885-bp product derived from the *comK*-dependent transcript. This might be due to the possibility that the RNA was harvested before the time in the growth curve when the ComK concentration and, hence, ComK-dependent transcription, was at a maximum.

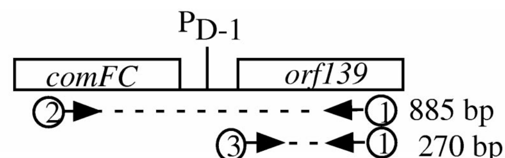
DISCUSSION

The *mecA* gene product is required for the optimum expression of genes that constitute the σ^D regulon. It participates in both *comK*-dependent and *comK*-independent mechanisms of regulation. The expression of *hag* is reduced in *mecA* mutants grown in both 2XYT and CM. A mutation in *comK* has no effect on the level of *hag* expression in 2XYT, a rich medium that does not promote competence, but when a *comK* mutation is introduced into a *mecA* mutant, nearly complete suppression of the *mecA* mutation is observed. This indicates that in medium that does not promote competence, the major function of MecA in stimulating *hag* expression is to inhibit ComK. In CM,

TABLE 2. Effect of *flgM* mutation on *hag* expression

Strain	Genotype	Mean β-galactosidase activity (Miller units) ± SD[a]
LAB2819	Wild type	70.5 ± 3.5
LAB2827	*flgM*Δ*80*	127.5 ± 23.2
LAB2926	*flgM*Δ*80 mecA*	59 ± 0.4
LAB2928	*flgM*Δ*80 comK*	129 ± 5.7
LAB2929	*flgM*Δ*80 mecA comK*	69.5 ± 6.4
LAB2920	*mecA*	34.5 ± 6.4

[a] Samples were collected at $T_{0.5}$ from cultures of wild-type and mutant cells grown in CM.



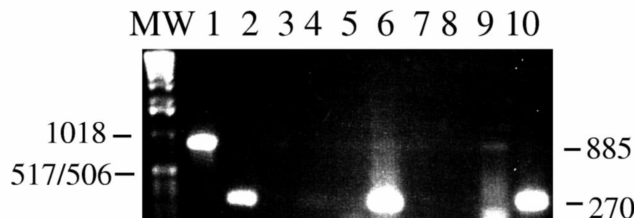


FIG. 8. RT-PCR products from *comF-flgM* and *flgM* operon transcripts. At the top is a diagram of the *comFC-orf139* region of the *comF-flgM* operon. P_{D1} indicates the location of the *flgM* promoter utilized by the σ^D form of RNA polymerase. The arrows indicate the oligonucleotide primers (numbered 1 through 3) used to prime RT and to amplify cDNA and PCR products. The bottom panel is a photograph of an ethidium bromide-stained 1% agarose gel on which the PCR and RT-PCR products were resolved. In lanes 1 and 2, the template for PCR was JH642 chromosomal DNA. In lanes 3 to 6, the template for PCR and RT-PCR was RNA from strain LAB2917 (*comK*::*neo*). In lanes 7 to 10, the template for PCR and RT-PCR was from JH642 (wild-type) cells. MW, molecular weight markers. Lanes: 1, PCR product of primers 1 and 2 (no reverse transcriptase); 2, PCR product of primers 1 and 3; 3, PCR using primers 1 and 2; 4, PCR using primers 1 and 3; 5, RT-PCR using primers 1 and 2; 6, RT-PCR using primers 1 and 3; 7, PCR using primers 1 and 2; 8, PCR using primers 1 and 3; 9, RT-PCR using primers 1 and 2; 10, PCR using primers 1 and 3.

comK mutant cells exhibit heightened *hag* expression which is above that observed in wild-type cells. In this medium, as opposed to 2XYT, active ComK is produced and wild-type cells show a level of *hag* expression lower than that observed in 2XYT-grown cells, in which ComK is absent. A mutation in *mecA* causes a dramatic reduction of *hag* expression in CM, and it is incompletely suppressed by a *comK* mutation. In CM, MecA functions to inhibit ComK but also stimulates *hag* expression through a ComK-independent mechanism. The *comS* mutation has nearly the same effect a *comK* mutation has in CM; both cause higher-level expression of *hag*, supporting the conclusion that MecA stimulates *hag* expression, in part, by negatively controlling ComK.

The examination of σ^D protein levels revealed no change in *sigD* expression in the *comK mecA* mutant and wild-type cells grown in 2XYT. In CM, little, if any, difference in the σ^D protein level was observed between wild-type and *comK* mutant cells. This suggested that σ^D activity was altered in the *comK* mutant. A likely target for MecA-ComK-dependent control was FlgM, the σ^D-specific antisigma factor. It was noticed that the *flgM* operon was located downstream from the *comF* operon, the transcription of which had been shown to require ComK (46). Optimal *flgM* transcription might require readthrough from the *comF* operon, although a sequence resembling a factor-independent terminator was identified at the end of *comFC* (34). This hypothesis was tested by creating an insertion mutation that separated *comF* from the *flgM* operon by introducing a plasmid bearing the *comFC-orf139* intergenic region by homologous recombination. The insertion mutation produced the same phenotype with respect to *hag-lacZ* expression as the *comK* mutation. These results strongly suggest that *flgM* transcription is dependent, in part, on ComK and that this is the basis of ComK-dependent negative control of *hag* and other genes of the σ^D regulon that had been shown to require MecA for their expression. This hypothesis was further supported by data showing that *flgM* operon expression was positively controlled by *comK* and by RT-PCR data indicating the presence of a *comF-flgM* operon transcript. This mechanism of control operates in cells grown in CM and 2XYT, but Western blot analysis of σ^D protein levels in cells grown in CM showed that MecA also affects *sigD* expression, by affecting either the transcription or translation of *sigD* or the stability of the σ^D protein.

Rashid et al. reported that the MecA-ClpC-dependent control of *hag* was independent of *comK* (43) when cells were grown in a rich medium that does not promote competence. However, our results not only implicate *comK* in the negative control of *hag* but also provide a reasonable explanation for the role of *comK*, i.e., to activate transcription of the *flgM* operon. Ogura and Tanaka showed that MecA stimulated σ^D-transcribed gene *degR* by inhibiting ComK (40), but their experiments did not examine the effect of *mecA* and *comK* mutations in cells grown in medium that promoted competence, a condition in which MecA exerts positive control of *hag* independently of *comK.*

The ComS-MecA-ComK system can be viewed as a molecular switch that is thrown in the direction favoring competence at high cell density, when the ComS peptide is present in abundance (Fig. 1). At a low cell density, when the ComS concentration is low, the switch is thrown in the other direction, that favoring motility, chemotaxis, and production of autolysins, all processes requiring the σ^D form of RNA polymerase. The activation of late competence operons when motility genes are down regulated is reminiscent of the opposing controls associated with toxin-coregulated pilus (TCP) production and motility in the intestinal pathogen *Vibrio cholerae* (16). The expression of motility genes is repressed when *tcp* is expressed, a situation reflecting the motility-dependent penetration of the intestinal mucous layer followed by the TCP-dependent attachment of vibrios to the cells lining the intestine. Both the TCP protein and the *B. subtilis* late Com products encoded by the *comG* operon are of the type IV pilus family (10, 22). It is possible that the transcriptional control mechanisms activating *tcp* expression directly or indirectly down regulate motility gene expression, perhaps through a mechanism involving *flgM* and a σ^D homolog. Why might there be mechanisms of control that favor type IV pilus production while suppressing the expression of motility functions? One possibility is that the two structures, the type IV pilus and the flagellum, are not compatible within the bacterial cell wall. Another explanation is based on the conditions under which the two structures are utilized. The function of the pilus, whether used in genetic exchange or in cell-cell contact, is associated with conditions of high local cell density that are conducive to cell-cell interaction, whether for genetic exchange or for coordination of the activity of a concentrated population of bacteria. Motility might be expected to be characteristic of bacteria encountering stress in a low cell density environment since individual bacteria are less likely to impact their immediate environment for the purpose of responding appropriately to stressful conditions and to utilize their mechanism of genetic exchange. Hence, their ability to relocate to a less stressful environment or one inhabited by a large population of their own species would necessitate flagellum formation.

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