A Molecular Switch Controlling Competence and Motility: Competence Regulatory Factors ComS, MecA, and ComK Control $\sigma^D$-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 $\sigma^D$-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 $\sigma^D$ levels are observed in mecA mutants grown in competence medium, but no change in $\sigma^D$ 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 $\sigma^D$-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 $\sigma^D$ activity. There likely exists another comK-independent mechanism of hag transcription that requires mecA and possibly affects the $\sigma^D$ 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 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 $\sigma^D$ (1, 20, 27, 31, 34, 41). FlgM functions as an antisigma factor that negatively controls $\sigma^D$ (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 $\sigma^D$ 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
High Cell Density, Nutritional Stress

Motility

Autolysis

Chemotaxis

Late Competence

Genes

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 $\sigma^D$-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 $\sigma^D$-dependent transcription by stimulating the transcription of the $flgM$ gene encoding the $\sigma^D$-specific antisigma factor. MecA inhibits ComK but also affects the level of $\sigma^D$ 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 $B. subtilis$ HB1012 (from J. Hoch). DNA from HB1002 cells bearing a $hag$-$lacZ$ translational fusion (5; from J. D. Helmann) was used to transform HB1042 competent cells with selection for erythromycin resistance ($Em^R$) 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 $Neo^R$. The $flgM$::orf139::lacZ translational fusion (LAB2725) was constructed by transforming LAB2607 competent cells with DNA from HB1012 and 8G32 with selection for $Spc^R$ and screening for Neo$^R$.

The $flgM$::orf139 mutation is an in-frame deletion removing codons 6 through 85 of the $flgM$ gene (34). To create a $flgM$::orf139::lacZ strain, the transducing lysate of HB4041 (ZB307A SPc::flgM22 Tn917::$\Phi$(Phag-cat-lacZ)) kan strain, the transducing lysate of HB4041 (ZB307A SPc::flgM22 Tn917::$\Phi$(Phag-cat-lacZ)) kan, obtained from J. D. Helmann (14) was used to lysogenize CB149 ($flgM$::orf139) with selection for chloramphenicol resistance (Cm$^R$), creating strain LAB2827. Phag-cat-lacZ is a translational fusion containing the promoter region of $hag$ with the upstream UP element deleted (14). The same lysate was used to transduce strains HB642, LAB2916, LAB2917, LAB2724, LAB2923, LAB2924, and LAB2925, thereby creating the wild type (LAB2819) and mecA (LAB2920), comK (LAB2921), mecA comK (LAB2922), mecA $flgM$ (LAB2926), comK $flgM$ (LAB2928), and mecA comK $flgM$ (LAB2929) mutant strains bearing the phage-borne $hag$-$lacZ$ translational fusion, respectively. The presence of the $flgM$::orf139 mutation was verified by PCR and agarose gel electrophoresis of the $flgM$-specific PCR fragment. The forward primer (U1F) was used for PCR amplification was a 22-mer with the sequence ACCCAATGCACTTACCTGCT, and the reverse primer (U2F) was a 24-mer with the sequence CCCTTTCCCTTACTTTAT.

To create an insertion mutation at the site of $P_{flgM}$, of the comF-$flgM$ operon (34), a DNA fragment extending from 539 bp upstream to 329 bp downstream of the $P_{flgM}$ transcription start site was synthesized by PCR amplification. The forward primer used to amplify the fragment was a 30-mer with the sequence ATCGCCGATCCCTCAATCTGTTGATGGCGCAGTCGATT and the reverse primer was a 30-mer with the sequence TACACTGAGCAGGTATGCGCAATTACG GAGAG. The primers contained restriction sites for BamHI and PstI, respectively. These sites (underlined) were used to insert the cleaved PCR fragment into BamHI-PstI-cleaved plasmid pMM13 (36), a pGEM4 derivative carrying a cat gene. The resulting plasmid was then introduced into wild-type strain HB642 by transformation with selection for Cm$^R$. The lysate of the $spc$ (spc-comK) (25) or $flgM$ (25) (LAB2928) 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 SP8 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 the appropriate $B. subtilis$ strains.

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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 colonies 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 sporation (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 (New Brunswick Scientific, Inc., Edison, N.J.) or 2-liter Erlenmeyer flasks (Corning, Corning, N.Y.) 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 $t_o$ (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$^5$ 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 PstI followed by T4 DNA polymerase to render the ends flush. The blunt-ended PCR fragment was digested with BamHI. The digested PCR fragment was then inserted in front of a promoterless lacZ gene in plasmid pT1Klac (23), which was cut with HindIII, treated with T4 DNA polymerase to fill in the HindIII ends, and then cleaved with BamHI. 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 C600 cells with selection for Erm$^R$ and screening for Cm$^R$.

Reverse transcription-PCR (RT-PCR). Wild-type and comK mutant cells grown in CM were harvested at $t_o$ 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 suspending 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′-GCCACTTTCAGGTTAGGAAATGAAATAG-3′ (primer 1 [see Fig. 8]) and 5′-ACCGCGATCTCAATCGT TacGCGTTCCGATCG-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 above to determine whether contaminating DNA, while another upstream primer with the nucleotide sequence 5′-AGTGGA GAAAGGGCTGCTAAATGTCCGAAATGCA-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$_{P+1}$ promoter. The resulting RT-PCR products were identified by agarose gel electrophoresis (1%), 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$^5$ 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$^5$ 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.
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 α3 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 likl (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 lysosgenized with SPβPhag-lacZ to yield strain LAB2932. The Phag-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 mutant strains are nearly identical, with higher levels of expression in the stationary phase in CM than that observed in wild-type cells (Fig. 6).

Table 2 summarizes the effects of flgMΔ80 in wild-type and comK and mecA mutant genetic background cells grown in CM. flgMΔ80 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 flgMΔ80 comK mecA triple mutant showed a level of hag-lacZ expression similar to that of a flgMΔ80 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-
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.

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 a RT-PCR product of 885 bp derived from the comF-flgM read-through transcript. The other reaction utilized a primer hybrid-
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 $\sigma^D$ regulon. It participates in both comK-dependent and comK-independent mechanisms of regulation. The expression of hag is reduced in mecA mutants.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Mean $\beta$-galactosidase activity (Miller units) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB2819</td>
<td>Wild type</td>
<td>70.5 ± 3.5</td>
</tr>
<tr>
<td>LAB2827</td>
<td>flgMΔ80</td>
<td>127.5 ± 23.2</td>
</tr>
<tr>
<td>LAB2926</td>
<td>flgMΔ80 mecA</td>
<td>59 ± 0.4</td>
</tr>
<tr>
<td>LAB2928</td>
<td>flgMΔ80 comK</td>
<td>129 ± 5.7</td>
</tr>
<tr>
<td>LAB2929</td>
<td>flgMΔ80 mecA comK</td>
<td>69.5 ± 6.4</td>
</tr>
<tr>
<td>LAB2920</td>
<td>mecA</td>
<td>34.5 ± 6.4</td>
</tr>
</tbody>
</table>

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

**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 $\beta$-galactosidase activity. Symbols: ○, LAB2995 [flgM (orf139)-lacZ]; ■, LAB2997 [sigD flgM (orf139)-lacZ]; ▲, LAB2996 [comK flgM (orf139)-lacZ]; ●, LAB2998 [sigD comK flgM (orf139)-lacZ]; ▼, LAB2999 [comK sigD flgM (orf139)-lacZ]; □, LAB2998 [comK sigD flgM (orf139)-lacZ].

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,
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 σD 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 σD protein level was observed between wild-type and comK mutant cells. This suggested that σD activity was altered in the comK mutant. A likely target for MecA-ComK-dependent control was FlgM, the σD-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 read-through 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-oriF139 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 σD 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 the σD 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 σD protein.

Rashid et al. reported that the MecA-CIcC-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 σD-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 autolysis, all processes requiring the σD 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 σD 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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