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Effects of *mecA* and *mecB* (*clpC*) Mutations on Expression of *sigD*, Which Encodes an Alternative Sigma Factor, and Autolysin Operons and on Flagellin Synthesis in *Bacillus subtilis*

MUHAMMAD HARUNUR RASHID, ATSUO TAMAKOSHI, AND JUNICHI SEKIGUCHI*

Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Ueda-shi, Nagano 386, Japan

Received 12 April 1996/Accepted 10 June 1996

The expression of the major vegetative phase-specific autolysin genes (*cwlB* [*lytC*] and *cwlG* [*lytD*]) was greatly reduced by *mecA* and *mecB* null mutations. In contrast to the negative effects on late competence genes (such as *comG*) and levansucrase gene (*sacB*) expression, this positive effect of *mec* genes on autolysin gene expression was not mediated through the ComK protein but apparently through the level of the SigD protein. The pleiotropic effects of the *mec* mutations, i.e., the reduction of *sigD* expression and the overexpression of the ComK protein, seem not to be interwoven since the SigD- and ComK-dependent functions are clearly separable in the *mec* mutants. We also show that the synthesis of the flagellin protein, which is encoded by the SigD-dependent *hag* gene, was similarly affected by the *mec* mutations. Complementation analysis with a SigD-overproducing plasmid, pHYSigD, in *mec* mutants revealed the reversion of almost all of the SigD-dependent phenotypes except motility. This finding suggested that Mec proteins act on motility genes at two levels, one of which is apparently SigD independent. Finally, we discuss the transcriptional regulation of the *sigD* gene by multiple regulators, i.e., MecA, MecB, SinR (FlaD), and DegS-DegU, and its implications for cells in a global context.

During vegetative growth, *Bacillus subtilis* produces a complement of autolytic enzymes (8), including two major (CwlB [LytC] and CwlG [LytD]) (14, 41) and two minor (CwlE and CwlF) autolysins (39). CwlB is a 50-kDa *N*-acetylmuramoyl-L-alanine amidase (14), and CwlG a 90-kDa endo-β-*N*-acetylglucosaminidase (41), but the bond specificities of 50-kDa CwlE and 35-kDa CwlF are unknown at present (39). The *cwlB* gene is part of a three-gene operon encoding a putative lipoprotein (LppX [LytA]), a modifier protein (CwbA [LytB]) that stimulates amidase activities, and CwlB, in that order (20, 21, 24). *lytR*, which is transcribed divergently from the *cwlB* operon, encodes a 35-kDa protein that acts as an attenuator of the expression of both the *cwlB* and *lytR* operons (24). The CwlG protein is encoded by a monocistronic operon, *cwlG* (27, 38). The expression of both the *cwlB* and *cwlG* operons mainly depends on expression of the *sigD* gene (22, 24, 27, 29). The *sigD* gene encodes the alternative sigma factor, σ^D, which directs the transcription of the flagellin structural gene, the motility genes *motA* and *motB*, and the *flgM* regulatory gene of *B. subtilis* (13, 28, 29). Additionally, the *cwlB* operon has another weak promoter recognized by the major housekeeping sigma factor, σ^A (22, 24). It was suggested by zymographic analysis that the gene encoding the minor autolysin, CwlE, is also transcribed by E-σ^D (39). Thus, the fact that three (CwlB, CwlG, and probably CwlE) of four vegetative autolysins utilize E-σ^D for their expression makes σ^D a key factor for autolysin synthesis in *B. subtilis* (36).

One of the several pleiotropic regulators of *B. subtilis*, SinR (FlaD), is required for competence, motility, and autolysin synthesis and is inhibitory for sporulation and exoprotease production (46). Regarding the negative roles of SinR (FlaD) in sporulation and exoprotease production, it is now known that SinR prevents the expression of *aprE* (structural gene for the major alkaline protease) and sporulation regulatory genes (*spo0A* and *spoIIA*) by directly binding to their upstream promoter regions (25, 26, 46). It was proposed by us that SinR controls, at least in part, autolysin synthesis through the expression level of the SigD protein, either directly or indirectly (22).

The DegS-DegU histidine protein kinase-response regulator pair is required for both the expression of genes encoding degradative enzymes and the development of genetic competence (18, 19, 33). However, no biochemical evidence for specific binding of the DegU protein to target sites has yet been obtained; thus, it remains possible that some as-yet-unidentified intermediates may be involved in the regulation of these processes (33). We previously reported that, like the SinR protein, the phosphorylated form of the DegU protein controls autolysin expression, probably through the expression level of the *sigD* gene, either directly or indirectly (47).

At the end of the exponential growth phase, *B. subtilis* has several alternative developmental processes to choose from, such as competence development, acquisition of motility, production of extracellular degradative enzymes, and finally sporulation, the ultimate response to starvation (4, 5, 19, 33, 35, 46). One of these phenomena, the development of genetic competence, is triggered by the binding of the competence transcription factor (CTF/ComK) to the upstream regions of several late competence genes (*comC*, *comD*, *comE*, *comF*, and *comG*) during the transition state, followed by the expression of these genes (3–6, 32, 48, 49). The ComK protein also has a positive regulatory effect on the levansucrase gene (*sacB*) expression and is required for its own synthesis (10, 34, 50). During exponential growth, negative regulatory effects on competence and levansucrase gene expression are exerted by the MecA and MecB proteins, which appear to form a complex

* Corresponding author. Mailing address: Department of Applied Biology, Faculty of Textile Science & Technology, Shinshu University, 3-15-1 Tokida, Ueda-shi, Nagano 386, Japan. Phone: 81-268-22-1215, ext. 278. Fax: 81-268-22-4079. Electronic mail address: jsekigu@giptc.shinshu-u.ac.jp.

TABLE 1. *B. subtilis* strains used in this study

Strain	Relevant genotype(s)[a]	Source or reference
AC327	*purB his-1 smo-1*	1, 44
QB4658	*trpC2* Δ*mecA*::*spc* Δ(*degS-degU*)::*kan amyE*::(*sacB-lacZ erm*)	F. Kunst (34)
QB4662	*trpC2* Δ*mecA*::*kan amyE*::(*sacB-lacZ erm*)	F. Kunst (34)
QB4768	*trpC2* Δ*mecB*::*spc* Δ*comK*::*kan amyE*::(*sacB-lacZ cat*)	F. Kunst (34)
SU10	*purB his-1 smo-1* Δ*mecA*::*spc*	QB4658→AC327[b]
SU12	*purB his-1 smo-1* Δ*mecA*::*kan*	QB4662→AC327
SU14	*purB his-1 smo-1* Δ*mecB*::*spc*	QB4768→AC327
SU16	*purB his-1 smo-1* Δ*comK*::*kan*	QB4768→AC327
7TGL1	*purB his-1 smo-1* (*cwlG-lacZ cat*)	pDEB1G→AC327
SU100	*purB his-1 smo-1* Δ*mecA*::*kan* (*cwlG-lacZ cat*)	7TGL1→SU12
SU102	*purB his-1 smo-1* Δ*mecB*::*spc* (*cwlG-lacZ cat*)	SU14→7TGL1
SU104	*purB his-1 smo-1* Δ*comK*::*kan* (*cwlG-lacZ cat*)	SU16→7TGL1
SU106	*purB his-1 smo-1* Δ*mecB*::*spc* Δ*comK*::*kan* (*cwlG-lacZ cat*)	SU16→SU102
AC327SL	*purB his-1 smo-1* (*sigD-lacZ cat*)	22
SU200	*purB his-1 smo-1* Δ*mecA*::*kan* (*sigD-lacZ cat*)	AC327SL→SU12
SU202	*purB his-1 smo-1* Δ*mecB*::*spc* (*sigD-lacZ cat*)	AC327SL→SU14
SU204	*purB his-1 smo-1* Δ*comK*::*kan* (*sigD-lacZ cat*)	SU16→AC327SL
SU206	*purB his-1 smo-1* Δ*mecB*::*spc* Δ*comK*::*kan* (*sigD-lacZ cat*)	SU16→SU202
680GL20	*trpC2 lacA17 lacR1* (*cwlG-lacZ cat*)	38
327GL10	*purB his-1 smo-1* (*cwlG-lacZ cat*)[c]	680GL20→AC327
SU50	*purB his-1 smo-1* Δ*mecA*::*spc* (*cwlG-lacZ cat*)[c]	SU10→327GL10
SU52	*purB his-1 smo-1* Δ*mecB*::*spc* (*cwlG-lacZ cat*)[c]	SU14→327GL10
SU54	*purB his-1 smo-1* Δ*mecA*::*spc* Δ*comK*::*kan* (*cwlG-lacZ cat*)[c]	SU16→SU50
SU56	*purB his-1 smo-1* Δ*mecB*::*spc* Δ*comK*::*kan* (*cwlG-lacZ cat*)[c]	SU16→SU52
CB100	*trpC2 sigD*::*cat*	M. J. Chamberlin (13)
327SDC	*purB his-1 smo-1 sigD*::*cat*	CB100→AC327

[a] *cat*, *erm*, *kan*, and *spc* designate the chloramphenicol acetyltransferase gene, erythromycin resistance gene, kanamycin resistance gene, and spectinomycin resistance gene, respectively.
[b] Arrows indicate construction by transformation of the respective strains with either a plasmid or chromosomal DNA.
[c] Unfortunately, these *cwlG-lacZ* fusions do not occur in single copies, and therefore, these strains were not used for β-galactosidase measurements.

preventing positive autoregulation of *comK* gene expression (15). MecB (ClpC) belongs to the class of stress response-related Clp ATPases, and a null mutant of *mecB* (*clpC*) shows impaired tolerance for heat shock and salt stress, and filamentous-cell phenotype (17, 34).

Since regulation of competence gene expression, degradative enzyme synthesis, and autolysin gene expression are apparently interwoven in *B. subtilis*, we were interested in whether the MecA and MecB (ClpC) proteins affect autolysin gene expression in a fashion similar to that in which they affect competence and levansucrase gene expression. In this study, we show that the MecA and MecB (ClpC) proteins positively regulate autolysin gene expression and that this positive effect is apparently mediated through the SigD protein instead of the ComK protein. We also show that the MecA and MecB (ClpC) proteins are positive regulators for all SigD-dependent functions and that they affect motility on at least two levels.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. pDEB1G is an integrative plasmid of *B. subtilis* and contains a *cwlG-lacZ* transcriptional fusion gene (38). Plasmid pHY5HS is a derivative of shuttle vector pHY300PLK, which contains the *lppX-lacZ* translational fusion gene (20). pHYSigD, a multicopy plasmid used for overproducing σ^D, was constructed as follows. A 944-bp *Sph*I fragment of pSD (38) was blunt-ended with T4 polymerase and then inserted into the *Sma*I site of pHY300PLK (Takara). The 944-bp insert in pHYSigD contains the *sigD* structural gene and spans 70 bp upstream of the *sigD* start codon containing a C-terminal portion of OrfB and 112 bp downstream of the second *sigD* stop codon containing the N terminus of OfrC (13, 29). The orientation of the insert in pHYSigD, in which the *sigD* gene runs in the same direction as the plasmid-borne *tet* gene (tetracycline resistance gene), was determined by restriction analysis with *Eco*RI.

Media and antibiotics. Cultures were grown in either Luria-Bertani (LB) broth or on LB agar plates (43). The antibiotics added to the solid or liquid medium were chloramphenicol (5 μg/ml), kanamycin (50 μg/ml), spectinomycin (100 μg/ml), and tetracycline (20 μg/ml). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to LB plates at 100 μg/ml. Overproduction of levansucrase by strains carrying the *mecA* or *mecB* deletion mutation was detected on LB agar plates containing 4% (wt/vol) sucrose (incubation at 37°C for 12 to 16 h). The *comK* null mutation was checked for the loss of competence by the standard procedure (2). The *sigD* null mutation was confirmed by the filamentous growth and nonmotility of the resulting strain (28).

***B. subtilis* transformation.** Competent cells of *B. subtilis* were prepared as described by Anagnostopoulos and Spizizen (2), using the two-step protocol. Preparation, transformation, and regeneration of *B. subtilis* protoplasts were carried out as described previously (1).

Swarm plate assay. The swarm plate assay was performed on soft agar plates (0.3% agar) containing either tryptone or minimal medium as described previously (37, 38).

Observation of cell motility and morphology. Cell motility and morphology were investigated with a phase-contrast microscope during growth in liquid LB medium, as described previously (44, 45).

β-Galactosidase assay. Cells were precultured in liquid LB medium with antibiotics at 30°C for 12 h with shaking (140 shakes per minute). They were then inoculated into fresh LB medium to an optical density at 600 nm (OD_{600}) of 0.1 and cultured with shaking at 37°C. Samples were withdrawn at intervals to assay β-galactosidase activity. The frozen cells were suspended in Z buffer (30) containing lysozyme and Triton X-100 and then lysed as described previously (38). Measurements and calculations of β-galactosidase activity (expressed as units per unit of optical density at 600 nm) were carried out as described by Miller (30). T_o was defined as the point at which cell growth was no longer exponential. The results shown are representative of at least two independent experiments.

Extraction of autolysins and flagellin proteins. *B. subtilis* cells cultured as described above were harvested by centrifugation (10,000 × *g*, 10 min, 4°C) as described in the figure legends for the extraction of autolysin and flagellin proteins. LiCl extracts (extracts A and B) for autolysins and a sodium dodecyl sulfate (SDS) extract (extract S) for flagellin proteins were prepared as described previously (38, 39), with some minor modifications. Briefly, after the cell pellet was washed with buffer T (25 mM Tris-HCl [pH 7.2]) containing 0.5 mM phenylmethylsulfonyl fluoride, the crude LiCl extract (extract A) was prepared by extracting the cell pellet with a 5 M LiCl-buffer T solution. Extract A was then diluted 10 times with the same buffer T solution containing 0.5 mM phenylmethylsulfonyl fluoride, and the SDS-purified *B. subtilis* cell wall was added to allow selective binding of autolysins to the cell wall. The cell-wall-binding proteins were then reextracted with a 5 M LiCl-buffer T solution to yield the cell wall extract (extract B). The proteins in extracts A and B were then further concentrated by precipitation with trichloroacetic acid (2% [vol/vol]).

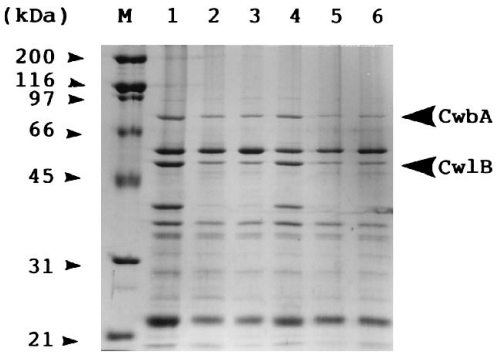

FIG. 1. Analysis of cell wall-binding proteins (extract B) from *mec* mutants by SDS-PAGE. Samples were prepared from cells grown in LB medium at 37°C and harvested at an OD_{600} of 1.0 as described in Materials and Methods. Cell wall-binding proteins corresponding to 70 OD_{600} units of cells were applied to each lane. SDS-PAGE (12% polyacrylamide gels) was performed as described by Laemmli (23). After SDS-PAGE, the proteins were stained with Coomassie brilliant blue. Lane M contains the protein standards (Bio-Rad), the masses of which are shown at the left. Lanes: 1, AC327 (wild type); 2, SU12 (*mecA*); 3, SU14 (*mecB*); 4, SU16 (*comK*); 5, SU54 (*mecA comK*); 6, SU56 (*mecB comK*). The positions of the CwlB and CwbA proteins are indicated by arrowheads on the right.

For preparation of the SDS extract sample (extract S), the cell pellet, after being harvested by centrifugation, was quickly and directly suspended in distilled water, and the SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer was added (23). After the cell suspension was boiled for 5 min, cell debris was removed by centrifugation (15,000 × *g*, 5 min, room temperature), and the supernatant was used as the SDS extract sample (extract S).

SDS-PAGE. Protein samples were analyzed by SDS-PAGE (23). All gels contained 12% (wt/vol) acrylamide, and proteins were visualized with Coomassie brilliant blue (R-250).

General methods. Chromosomal DNAs from *B. subtilis* and plasmid DNAs from *Escherichia coli* were prepared as described previously (43). Chromosomal rearrangements made by transformation with integrative plasmids were confirmed by Southern blot analysis (43). Probe labelling, hybridization, and detection were performed with a nonradioactive DNA labelling and detection kit (Boehringer Mannheim).

RESULTS

The MecA and MecB (ClpC) proteins are positive regulators for autolysin gene expression, but ComK is not involved in this process. Similarity of the nucleotide sequences upstream of the transcriptional start sites of the *cwlB* and *cwlG* autolysin operons (21, 38) to those of the *comC* and *comG* late competence operons (4, 32) initially suggested that the competence transcription factor ComK may activate autolysin gene expression. Therefore, we presumed that the MecA and MecB proteins may negatively regulate the transcription of the *cwlB* and *cwlG* operons through the level of the competence transcription factor ComK protein. To test this assumption, we constructed a set of strains bearing deletion mutations in the *mecA*, *mecB*, and *comK* genes, which were otherwise isogenic, and compared the amounts of CwlB and CwbA proteins in these strains with those in the wild-type strain. The cell wall-bound proteins were extracted with a 5 M LiCl-buffer T solution, resulting in a crude extract (extract A). The CwlB and CwbA proteins in extract A were then selectively purified with the SDS-purified *B. subtilis* cell wall extract (extract B). The proteins in extract B from an equal amount of cells were analyzed by SDS-PAGE, as shown in Fig. 1. Contrary to our expectations, we found that compared with that in the wild-type strain AC327 (lane 1), the synthesis of the CwlB and CwbA proteins was greatly reduced in strains SU12 (*mecA*) (lane 2) and SU14 (*mecB*) (lane 3) but remained relatively unaffected in strain SU16 (*comK*) (lane 4). From these data, we assumed that for autolysins, the ComK protein, if acting at all, may act as a repressor instead of an activator. To test this assumption, we constructed strains SU54 (*mecA comK*) and SU56 (*mecB comK*), and compared their CwlB and CwbA protein levels with those of the wild-type strain. CwlB and CwbA production remained low in these double mutants (lanes 5 and 6). The above results suggested that the MecA and MecB proteins do affect autolysin synthesis but that the ComK protein may not be involved in this process.

To elucidate whether the positive effects of the *mecA* and *mecB* proteins on CwlB synthesis are exerted at the transcriptional level or in a later process, we examined the expression of the *lppX* (first gene of the *cwlB* operon)-*lacZ* translational fusion gene in the multicopy plasmid pHY5HS (20) harbored by the wild-type strain (AC327) and *mecA*, *mecB*, and *comK* knockout strains (SU12, SU14, and SU16, respectively) (Table 1). The results showed that temporal expression of *lppX-lacZ* was greatly reduced in the *mecA* and *mecB* strains but remained virtually unaffected in the *comK* strain (data not shown). These results are in line with the levels of CwlB and CwbA proteins in these strains (Fig. 1) and suggest that MecA and MecB exert their effects on the *cwlB* gene at the transcriptional level.

To determine whether this is also the case with CwlG, we combined a chromosomal single-copy *cwlG-lacZ* transcriptional fusion with knockout mutations in the *mecA*, *mecB*, and *comK* genes, which resulted in strains SU100, SU102, and SU104, respectively (Table 1), and examined the *cwlG-lacZ* expression in the latter. The results showed that, like *cwlB* expression, *cwlG* expression was similarly reduced in strains SU100 (*mecA*) and SU102 (*mecB*) but remained virtually unaffected in strain SU104 (*comK*) (Fig. 2). To examine the involvement of ComK, we then constructed a double mutant bearing mutations in the *mecB* and *comK* genes (SU106) and examined its *cwlG-lacZ* expression. As shown in Fig. 2, *cwlG* expression remained at the same low level as that with the single mutation of either the *mecA* or *mecB* gene. From these results, we conclude that the MecA and MecB proteins positively regulate the autolysin genes (*cwlB* and *cwlG*) at the transcriptional level, either directly or indirectly, and that ComK is not involved in this regulatory pathway.

MecA and MecB proteins exert their effects on autolysin genes through the level of the SigD protein. Since the MecA and MecB proteins exert their effects on the late competence and levansucrase genes indirectly (15, 34), and since they are not typical transcription regulators (10, 17, 50), we assumed that they exert their effects on autolysin genes also indirectly. ComK is not involved in this positive regulation by Mec proteins (as described above). Both the *cwlB* and *cwlG* operons are mainly transcribed by E-σ^D, although the *cwlB* operon also has a weak σ^A-type promoter (22, 29). For *cwlB*, we previously demonstrated that the *flaD1* (*sinR*) point mutation and various *degU* mutations (either a null mutation leading to a deficient phenotype or point mutations leading to either deficient or hyperproduction [Hy] phenotypes with respect to degradative enzyme production) interfere with the expression of the *cwlB* operon by depressing the expression of the *sigD* gene (22, 47). We obtained similar results for *cwlG-lacZ* fusion expression in the above-mentioned mutants (40). To study this possibility with the *mec* mutants, we measured the single-copy *sigD-lacZ* translational fusion gene expression in strains SU200 (*mecA*), SU202 (*mecB*), and SU204 (*comK*) (Fig. 3). It became clear that a mutation in either the *mecA* or *mecB* gene greatly depressed the temporal expression of the *sigD* gene, but the *comK* mutation did not affect its expression significantly. To explore whether ComK is involved in the Mec-mediated reg-

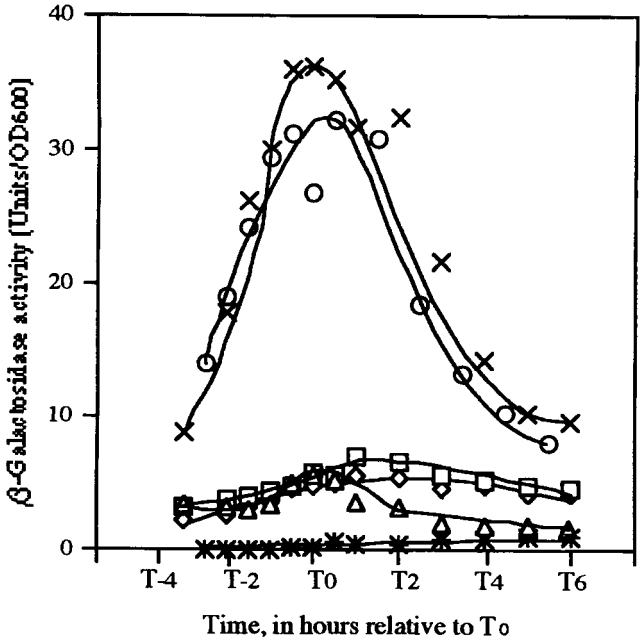

FIG. 2. Effects of *mec* mutations on β-galactosidase expression driven by the single-copy *cwlG-lacZ* transcriptional fusion constructed in the *B. subtilis* chromosome. The strains were grown in LB at 37°C, and at various intervals aliquots were removed for the assay of β-galactosidase activity. T_o is defined as the time at which cells leave the exponential phase of growth. Symbols: ○, 7TGL1 (wild type); △, SU100 (*mecA*); □, SU102 (*mecB*); ×, SU104 (*comK*); ◇, SU106 (*mecB comK*); ∗, AC327 (control without fusion).

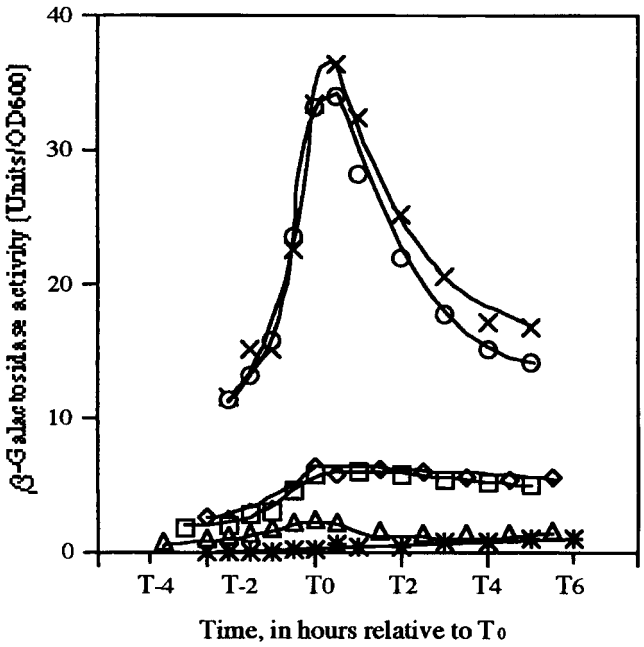

FIG. 3. Effects of *mec* mutations on single-copy *sigD-lacZ* translational fusion gene expression. The growth conditions and the measurement of β-galactosidase activity are described in the legend to Fig. 2. Symbols: ○, AC327SL (wild type); △, SU200 (*mecA*); □, SU202 (*mecB*); ×, SU204 (*comK*); ◇, SU206 (*mecB comK*); ∗, AC327 (control without fusion).

ulation of *sigD* expression, we constructed strain SU206 (*mecB comK*) in the *sigD-lacZ* background (Table 1). *sigD* expression was not elevated in the double mutant compared with that in the *mecA* or *mecB* single mutant (Fig. 3), suggesting that ComK does not interfere with the expression of the *sigD* gene. These observations are consistent with the results of studies on the expression of the *cwlB* and *cwlG* genes (Fig. 1 and 2). The MecA and MecB proteins affect the transcription of the *sigD* gene, either directly or indirectly. As a result, the downstream *cwlB* and *cwlG* genes are repressed. We prefer the latter idea of an indirect action by Mec proteins on the *sigD* gene because, as stated above, they are not typical transcription regulators, and MecB is regarded as a regulatory subunit of an ATP-dependent protease-like complex (17, 34).

Effects of *mec* mutations on other SigD-dependent functions. To examine whether the *mec* mutations also affect other SigD-dependent functions, we tested the motility of these strains on semisolid agar media containing tryptone. As shown in Fig. 4A, the *mecA* and *mecB* mutants are nonmotile whereas the *comK* mutant is not defective in motility. The double mutants (*mecA comK* and *mecB comK*) also remained immotile (Fig. 4B). Essentially similar results were obtained on minimal swarm plates containing mannitol as an attractant (data not shown). To determine whether this apparent nonmotility on swarm plates was due to a lack of flagellin, the building block of flagella, encoded by the σ^D-dependent *hag* gene (31), we examined the flagellin levels of these strains. Proteins were extracted with the SDS-PAGE sample loading buffer (23), resulting in an SDS extract (extract S), and then analyzed by SDS-PAGE. As shown in Fig. 5, synthesis of the flagellin protein was greatly reduced in the *mecA* and *mecB* mutants (lanes 2 and 3) but was not reversed in double mutants, *mecA comK* and *mecB comK* (lanes 5 and 6). In the *comK* mutant, flagellin synthesis was not affected, compared with that in the wild-type strain, AC327 (lanes 1 and 4). Thus, examination of the flagellin protein levels completely supported the results obtained on the semisolid agar plates. Since SigD-deficient cells grow as long filaments of unseparated but septated cells during exponential growth (28), we then investigated the morphology of the *mec* mutant strains during exponential growth in LB medium at 37°C. Almost all of the cells of either the *mecA* or *mecB* strain grew as long filaments of cells, whereas the *comK* mutant grew as normal rods as in the wild-type strain (data not shown). This phenotype of the *mec* mutants also was not reversed in the double mutants (data not shown). On the basis of these results, we conclude that the *mecA* and *mecB* mutations do not specifically affect autolysin expression but generally affect the SigD-dependent functions.

Complementation analysis of SigD-dependent functions in *mec* mutants with a σ^D-overproducing plasmid. The data presented in the previous sections strongly suggest that *mec* mutations prevent the acquisition of motility and autolysin synthesis through the level of *sigD* gene expression. Since Mec proteins are pleiotropic regulators, we asked whether *sigD* is the only gene through which the Mec system inhibits the above processes. If the Mec system acts on motility and autolysin only through *sigD*, then overexpression of the SigD protein from a *sigD* gene lacking its normal promoter should render *mec* mutant cells that are motile, rod shaped, and autolysin proficient. To examine this possibility, we constructed a plasmid, pHYSigD, as described under Materials and Methods. In this plasmid, the expression of the *sigD* gene occurs through read-through transcription from the plasmid-borne *tet* (tetracycline) promoter. When introduced into strain 327SDC (*sigD*), this plasmid complemented all SigD-dependent functions completely, i.e., 327SDC harboring pHYSigD became motile, grew as normal rods (data not shown), and produced normal levels of autolysin and flagellin proteins.

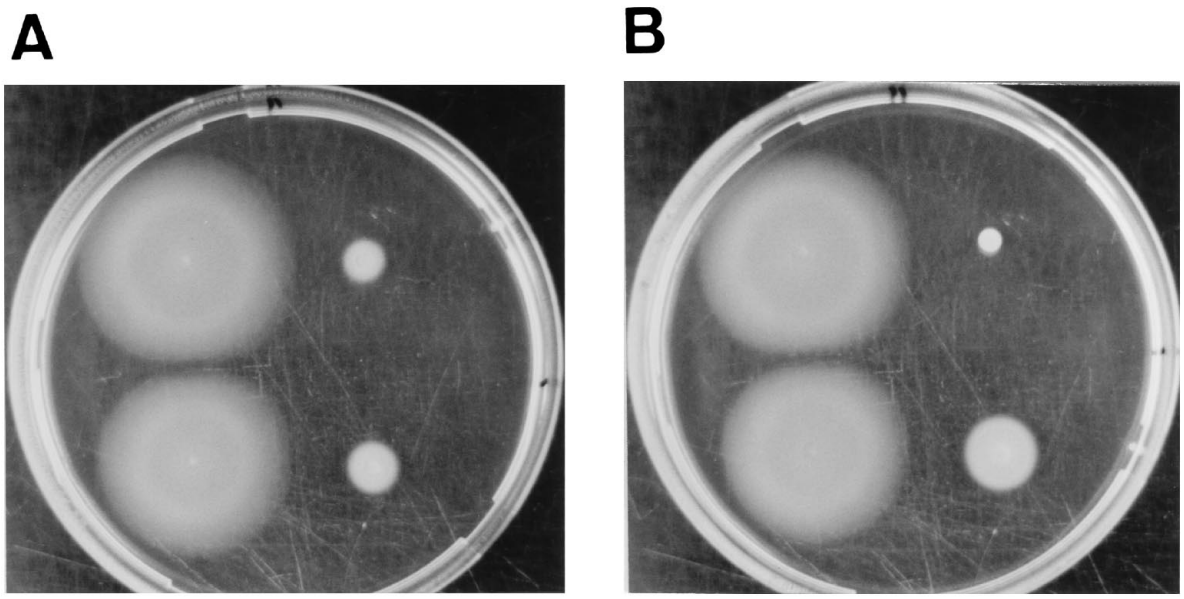

FIG. 4. Motility of *mec* mutants on tryptone swarm plates. The strains (1 μl of each suspension) were spotted onto swarm plates containing 0.3% agar and then incubated at 37°C for 10 h before being photographed. The strains are (clockwise from the upper left corner) AC327 (wild type), SU12 (*mecA*), SU14 (*mecB*), and SU16 (*comK*) (A) and AC327 (wild type), SU54 (*mecA comK*), SU56 (*mecB comK*), and SU16 (*comK*) (B).

The *mecA* or *mecB* strains were transformed with pHYSigD or, as a control, with pHY300PLK. The resulting strains were cultured with tetracycline (20 μg/ml), and then the levels of the CwlB and CwbA proteins were investigated. Figure 6 shows that the CwlB and CwbA protein levels were completely restored to normal by pHYSigD (compare lanes 5 with 6 and lanes 7 with 8). To determine if *cwlG* transcription levels are restored in *mec*-deficient cells overproducing σ^D, we measured *cwlG-lacZ* expression in *mecA* and *mecB* mutants harboring pHYSigD. As a control, we also measured *cwlG-lacZ* expression in the wild-type strain (7TGL1) harboring pHYSigD. As shown in Fig. 7, complementation of *cwlG* expression in the pHYSigD-bearing *mecA* and *mecB* strains was about 1.5- and 2.0-fold, respectively, compared with that in the wild-type strain without the plasmid. These values are within the range of 2.3-fold stimulation of *cwlG* expression by pHYSigD in the wild-type background. Microscopic observation of *mec* cells carrying pHYSigD showed that long chains of filamentous cells became mixtures of short filaments and elongated rods (data not shown), suggesting correction of the cell separation defect to some extent by a putative SigD-dependent autolytic enzyme (CwlE may be one such enzyme) (39). Interestingly, *mec* mutants harboring pHYSigD were not motile, as judged by the examination of strains on semisolid agar plates containing either tryptone (Fig. 8B) or mannitol in the case of minimal plates (data not shown). Determination of the flagellin protein level by SDS-PAGE revealed that pHYSigD did not cause restoration of flagellin synthesis significantly in these strains (Fig. 9). This result suggests the possibility that the MecA and MecB proteins simultaneously act positively on the *sigD* gene and on some other motility genes, including the *hag* gene (31). The effects on some other motility genes may be direct or indirect. However, we favor the idea of an indirect affect, probably through at least one unknown intermediate other than SigD and ComK.

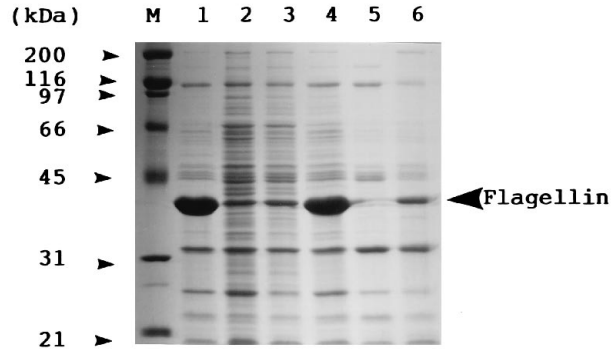

FIG. 5. SDS-PAGE analysis of flagellin protein in *mec* mutants. SDS extract (extract S) samples were prepared from cells grown in LB at 37°C and harvested at an OD_{600} of 2.0 as described in Materials and Methods. The electrophoretic conditions are described in the legend to Fig. 1. Each lane contained materials corresponding to 2 OD_{600} units of cells. Lanes: M, protein standards (Bio-Rad); 1, AC327 (wild type); 2, SU12 (*mecA*); 3, SU14 (*mecB*); 4, SU16 (*comK*); 5, SU54 (*mecA comK*); 6, SU56 (*mecB comK*). The flagellin protein is indicated by an arrowhead on the right.

DISCUSSION

The Mec system controls autolysin and motility gene expression in a ComK-independent and SigD-dependent manner through the level of the SigD protein. *mecA* and *mecB* were originally identified as genes in which mutations allowed late competence gene expression in complex media (7). They were also found to bypass the requirements for most of the competence regulatory genes (42). Subsequently, the *mecA* and *mecB* genes were cloned and sequenced (16, 34). Experiments with strains with chromosomal disruptions of these genes revealed that they act as negative regulators of competence (16, 34). It was also shown that *mec* mutations lead to the overexpression of the degradative enzyme, levansucrase (*sacB*), bypassing the DegS-DegU requirement (34). In this study, we extended these observations to include the pleiotropic effects of *mec* mutations on the regulation of SigD-dependent functions in *B. subtilis.*

For the regulation of competence gene expression and deg-

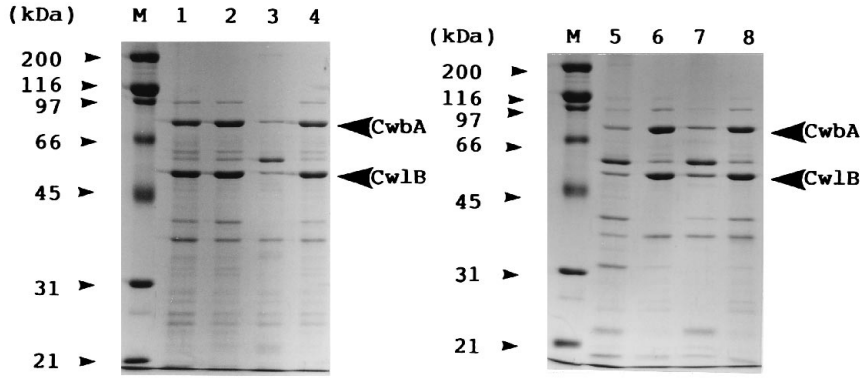

FIG. 6. Restoration of CwlB and CwbA synthesis by the σ^D-overproducing plasmid, pHYSigD, in *mec* mutants. Cells harboring either pHY300PLK (as a control) or pHYSigD were grown in LB containing tetracycline (20 μg/ml) at 37°C and then harvested at an OD_{600} of 1.0 for sample preparation. Proteins (extract B) extracted from 35 OD_{600} units of cells were applied to each lane. The electrophoretic conditions are described in the legend to Fig. 1. Lanes: M, protein standards (Bio-Rad); 1 and 2, AC327 (wild type); 3 and 4, 327SDC (*sigD*); 5 and 6, SU50 (*mecA*); 7 and 8, SU52 (*mecB*). Odd- and even-numbered lanes contain samples from cells harboring pHY300PLK and pHYSigD, respectively.

radative enzyme synthesis by the Mec system, it has been shown that the MecA and MecB proteins act as negative regulators of ComK, which is a competence transcriptional activator required for the expression of late competence genes and degradative enzyme synthesis (15, 34). Inactivation of either *mecA* or *mecB* results in overproduction of ComK (10, 50). It was hypothesized that transcription of the *comK* gene is inhibited by MecA through sequestration or otherwise inactivation of ComK at the protein level, thus, interfering with the positive autoregulating function of ComK (15). MecB is postulated to function prior to MecA by receiving an unknown signal from upstream regulatory genes and then relaying this signal to MecA to release ComK from its inhibition (15). Regarding the positive roles of Mec proteins in autolysin synthesis and the acquisition of motility, ComK was found to be unrelated. It was found that the Mec system acts positively on autolysin expression and, in part, on motility through the level of the SigD protein. It is not known whether the transcriptional repression of the *sigD* gene by the Mec system is exerted through the same mechanism as that for *comK*.

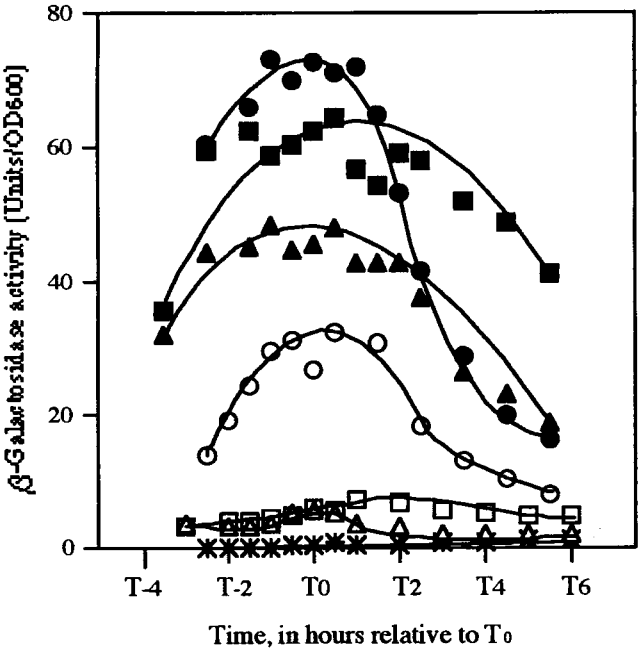

FIG. 7. Effects of SigD overproduction on *cwlG-lacZ* expression in *mec* mutants. Strains harboring pHYSigD were cultured in LB medium at 37°C with tetracycline (20 μg/ml). Symbols: ●, 7TGL1 (pHYSigD) (wild type); ▲, SU100 (pHYSigD) (*mecA*); ■, SU102 (pHYSigD) (*mecB*); ○, 7TGL1 without the plasmid (wild type); △, SU100 without the plasmid (*mecA*); □, SU102 without the plasmid (*mecB*); ∗, AC327 (control without any fusion and plasmid). For easier comparison, the data for strains without the plasmid from Fig. 2 are included.

In another study, the MecB (ClpC) protein was identified as a general stress protein (Gsp12) which belongs to the Clp ATPase family of proteins (17). MecB (ClpC) is required for cell survival at high temperatures (34). The thermosensitive phenotype of the *mecB* strain is not due to ComK overexpression, and the MecA protein is not required for growth at high temperatures (34). It has been reported that *mecB* mutant cells are elongated and form long cell chains during exponential growth (17). From this, it has been inferred that MecB (ClpC) plays a role in cell division (17). Recently, Hahn et al. (9) reported that *mecA* cells are impaired in nucleoid partitioning and cell division and that this mutation leads to loss of the colony-forming ability of cells. They found that these phenotypes of *mecA* inactivation were due to ComK overexpression in competence medium (9). We found in LB medium that both the *mecA* and *mecB* mutations lead to filamentous growth during the exponential phase. The filamentous phenotype of *mec* cells apparently results from two types of defects: (i) the nucleoid separation defect (9) and (ii) the cell separation defect (this study). Although the nucleoid separation defect was corrected in *mecA comK* cells (9), the cell separation defect was still uncorrected in *mecA comK* and *mecB comK* cells (this study), suggesting that these two defects can be separated genetically to some extent.

In a *mecA* or *mecB* mutant, phenotypes resulting from the overexpression of *comK* (i.e., overexpression of *sacB* and expression of late competence genes in complex media, the nucleoid separation defect) and phenotypes due to the reduced expression of *sigD* (i.e., nonmotility, autolysin deficiency, and cell separation defect) are genetically separable. Additionally, only *mecB* (*clpC*) is involved in the multiple stress tolerance of *B. subtilis* (17).

During the early stages of growth, the Mec proteins seem to activate SigD-dependent functions, including the acquisition of motility and autolysin production, and simultaneously repress the ComK-dependent functions including the expression of

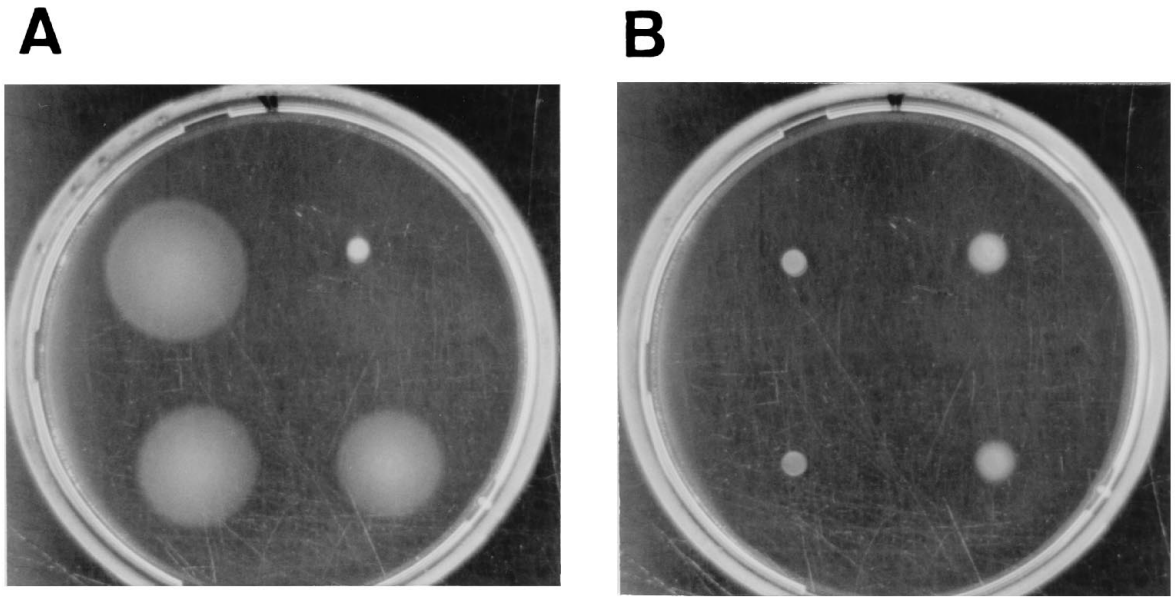

FIG. 8. Motility of *mec* mutants harboring pHYSigD. The growth medium and culture conditions are described in the legend to Fig. 4 except for the addition of tetracycline (20 μg/ml) to the medium. The strains are, (clockwise from the upper left corner) AC327 (pHY300PLK) (wild type), 327SDC (pHY300PLK) (*sigD*), 327SDC (pHYSigD) (*sigD*), and AC327 (pHYSigD) (wild type) (A) and SU50 (pHY300PLK) (*mecA*), SU52 (pHY300PLK) (*mecB*), SU52 (pHYSigD) (*mecB*), and SU50 (pHYSigD) (*mecA*) (B).

competence and degradative enzyme synthesis. On transition from exponential growth to the stationary phase (T_o), expression of competence and degradative enzyme synthesis are derepressed in response to an unknown signal, which presumably also shuts down the SigD-dependent functions. Thus, the MecA/MecB regulatory pair is involved in the decision made by cells to undergo one of at least three alternative developmental pathways, i.e., competence development, acquisition of motility, and production of extracellular degradative enzymes for further survival.

Transcriptional regulation of *sigD* and downstream autolysin genes by various regulators. The observed epistatic interactions between SigD and at least four pleiotropic regulators (MecA, MecB [ClpC], DegS-DegU, and SinR [FlaD]), combined with unpublished data and results from previous publications (22, 47), provide an insight into the transcriptional regulation of *sigD* and its effect on downstream genes in *B. subtilis* (Fig. 10). Regarding the positive role of SinR (FlaD) in *sigD* transcription, we recently found that a *sinR* null mutation depressed the *sigD-lacZ* expression about two- to fourfold, supporting our previous result with the *flaD1* (*sinR*) point mutation (22). Moreover, a SigD-independent route involving control of the Pa promoter of the *cwlB* operon by the FlaD1 protein may exist, as the Pa transcript was also missing in the *flaD1* mutant (22).

The global concentration of the phosphorylated DegU protein (DegU-p) seems to be critical for the regulation of *sigD* transcription (47). When this concentration is equal to or less than the wild-type concentration (in the case of the *degU146* or *degU*::*tet* mutant), it appears that DegU-p acts as a positive regulator for *sigD* expression. When the DegU-p level is higher than the wild-type level (in the case of the *degU32*(Hy) mutant), it seems to act negatively on *sigD* transcription. The positive and negative roles of DegU-p in *sigD* transcription,

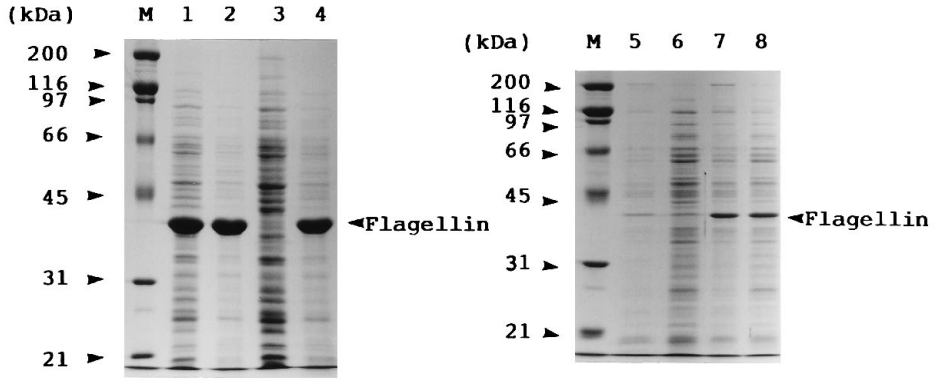

FIG. 9. Analysis of flagellin protein levels in *mec* mutants harboring pHYSigD. The growth and cell harvesting conditions are described in the legend to Fig. 6. SDS extract samples were prepared as described in Materials and Methods, and proteins from 1 OD_{600} unit of cells were applied to each lane. The lanes are same as those in the legend to Fig. 6.

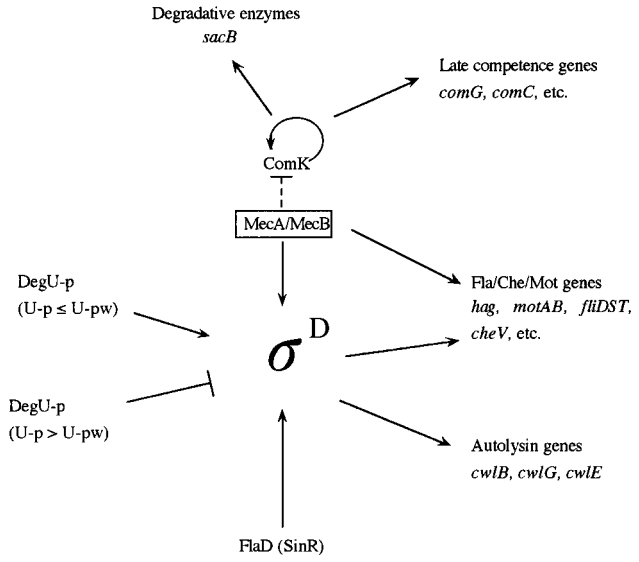

FIG. 10. Regulation of *sigD*, encoding an alternative sigma factor, and genes downstream of SigD by various pleiotropic regulators. This model is based on the work reported here and on our previously published data (22, 47). MecA and MecB (ClpC) are enclosed in a box because they appear to work together in inhibiting ComK activity, but it is not known whether this is also the case for *sigD*. Arrows indicate positive effects, and perpendicular bars represent negative effects. The dotted perpendicular bar represents a negative effect at the protein-protein level. The MecA, MecB (ClpC), SinR (FlaD), and DegU-p proteins act positively on transcription of the *sigD* gene, but there is no evidence that they act directly. In addition, it is not known whether they converge at an intermediate gene(s) to affect *sigD* transcription or whether they act separately on *sigD*. The MecA and MecB (ClpC) regulatory pair seems to affect flagellar/chemotaxis/motility genes at two levels, one of which is apparently SigD independent. DegU-p, DegU protein in its phosphorylated state; U-p, same as DegU-p; U-pw, DegU-p at the wild-type level.

depending on its concentration, are reminiscent of the roles of AbrB in competence. At a lower concentration, AbrB acts on competence positively, while at a higher level it acts negatively, and its negative effects are exerted at multiple points in competence regulation (6, 11). With the *cwlB* operon, it seems that the effect of DegU-p is mediated solely through the SigD protein, as the Pa transcript was unaffected in various *degU* mutants (46).

Finally, the Mec proteins act on *sigD* transcription positively without the involvement of ComK. Although it has not been determined by primer extension analysis whether the Pa transcript of the *cwlB* operon is affected by *mec* mutations, complementation analysis with pHYSigD strongly suggested that the Pa transcript, like the *degU* mutants, is probably unaffected in *mec* mutants. Whether the effects on *sigD* transcription of FlaD (SinR), DegU-p, MecA, and MecB (ClpC) are exerted directly, i.e., through the binding of the respective proteins upstream of the *sigD* structural gene, or indirectly, i.e., by activating/inhibiting the expression of an intermediate gene(s), remains to be elucidated.

Mec proteins act at multiple points in the pathway leading to the acquisition of motility. It is interesting that flagellin synthesis and motility were not reversed in *mec* mutants harboring pHYSigD, although autolysin synthesis and cellular filamentation were reversed. These results imply that SigD-dependent genes are differentially regulated by the same pair of regulators. The Mec proteins appear to play at least two roles in motility. They act positively on *sigD* to affect motility (Fig. 3). On the other hand, they simultaneously act on some other motility genes, including *hag* (Fig. 9), either directly or indirectly, and this action is independent of SigD. It is not uncommon for *B. subtilis* regulators to work at two levels in the same pathway. SinR is a direct repressor of the key sporulation regulatory gene, *spo0A* (25). It also directly represses stage II sporulation genes (e.g., *spoIIA*) that require Spo0A (26). AbrB is a direct repressor of *spo0H*, which encodes a minor sigma factor, σ^H, and of the *spoVG* gene, which is under the control of the σ^H regulon (12).

Why is the acquisition of motility controlled through redundant mechanisms? To obtain nutrition through the chemotactic movement of flagella, cells require the coordinated expression of many genes, which represents a substantial burden in terms of energy expenditure (35). Therefore, multiple and redundant control mechanisms may prevent cells from unjustified motility and be advantageous for the cells in the proper selection of overlapping, alternative developmental pathways.

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