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Bacillus subtilis Pellicle Formation Proceeds through Genetically Defined Morphological Changes▽†

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Biofilms are structured multicellular communities of bacteria that form through a developmental process. In standing culture, undomesticated strains of *Bacillus subtilis* produce a floating biofilm, called a pellicle, with a distinct macroscopic architecture. Here we report on a comprehensive analysis of *B. subtilis* pellicle formation, with a focus on transcriptional regulators and morphological changes. To date, 288 known or putative transcriptional regulators encoded by the *B. subtilis* genome have been identified or assigned based on similarity to other known proteins. The genes encoding these regulators were systematically disrupted, and the effects of the mutations on pellicle formation were examined, resulting in the identification of 19 regulators involved in pellicle formation. In addition, morphological analysis revealed that pellicle formation begins with the formation of cell chains, which is followed by clustering and degradation of cell chains. Genetic and morphological evidence showed that each stage of morphological change can be defined genetically, based on mutants of transcriptional regulators, each of which blocks pellicle formation at a specific morphological stage. Formation and degradation of cell chains are controlled by down- and up-regulation of σ^D- and σ^H-dependent autolysins expressed at specific stages during pellicle formation. Transcriptional analysis revealed that the transcriptional activation of *sigH* depends on the formation of cell clusters, which in turn activates transcription of σ^H-dependent autolysin in cell clusters. Taken together, our results reveal relationships between transcriptional regulators and morphological development during pellicle formation by *B. subtilis*.

Biofilms are surface-associated, multicellular communities of bacteria that are thought to be the most common mode of bacterial growth in natural environments (29, 45). Biofilm formation is now recognized as a developmental process, which begins with attachment of planktonic cells to the surface of a substrate (45). Flagella and type IV pili have been shown to play important roles in the initial attachment of various bacteria to a surface (45). After surface attachment, bacteria adapt to surface growth and increase production of an extracellular matrix that consists of exopolysaccharides, proteins, and DNA (7). A mature biofilm is a three-dimensional structured community in which bacterial cells are covered and connected by the extracellular matrix. Biofilm structure varies with conditions; indeed, different forms of biofilms, such as plaques, slimes, pellicles, and colonies, have been observed under different environmental conditions. Most bacteria form biofilms in response to the activities of multiple genetic pathways, which enable bacteria to form biofilms under a wide variety of conditions (7, 45).

Bacillus subtilis is a soil bacterium that serves as a model organism for gram-positive bacteria. Previous studies have shown that six global regulators, AbrB, Spo0A, σ^H, CcpA, DegU, and SinR, affect biofilm formation (6, 11, 21, 33, 50, 51). Specifically, mutations in *spo0A*, *sigH* (σ^H), or *degU* abolish biofilm formation, and mutations in *abrB*, *ccpA*, or *sinR* enhance biofilm formation. AbrB is a global repressor of genes activated in stationary phase, whereas Spo0A represses the transcription of *abrB* after the onset of stationary phase. The function of Spo0A in biofilm formation is to repress *abrB* transcription; conversely, *abrB* inactivation can restore biofilm formation in a *spo0A* mutant background (22). AbrB plays a negative role in biofilm formation because it represses transcription of the *yqxM-sipW-tasA* operon. TasA is a major protein component of the extracellular matrix, and *yqxM* and *sipW* encode proteins required for secretion of TasA (4, 22). Although a clearly defined role for σ^H in biofilm formation has yet to be identified, σ^H is known to be required for full expression of the *yqxM-sipW-tasA* operon (52). The catabolite control protein CcpA has been shown to be a repressor of biofilm formation (50). DegU is a response regulator of the DegSU two-component system which activates the transcription of genes encoding secreted proteins, such as protease, α-amylase, and levansucrase. However, clear roles for CcpA and DegU in biofilm formation have not been established (50, 51).

SinR negatively regulates both the *yqxM-sipW-tasA* operon and the *eps* operon, the latter of which encodes proteins involved in exopolysaccharide biosynthesis (6, 11, 33). The small peptide SinI, the first gene product of the *sinIR* operon, antagonizes SinR activity by binding to the SinR protein (3). SinR-mediated repression is modulated by transcriptional activation of *sinI* and by the uncharacterized proteins YlbF and YmcA; however, the mechanism by which *sinI* is activated remains unclear (3, 33). SinR is also known to have a positive effect on the transcription of flagellar genes. Thus, the SinI/R system has been proposed as a master regulatory system that governs the transition from a planktonic state to a biofilm state

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TABLE 1. Partial list of *B. subtilis* strains used in this study[a]

Strain	Genotype	Source or reference[b]
168	*trpC2*	C. Anagnospoulos
ATCC 6051[c]	Wild type	American Type Culture Collection
W694	*epsH*::pMutin (Em^r)	YVERd (35)→ATCC 6051
W619	*swrA*::*cat*	This work
W954	*fliF*::*kan*	This work
W917	*motA*::*kan*	This work
W568	*hag*::*kan*	This work
W33	*cwlS*::pMutin (Em^r)	YOJLd (35)→ATCC 6051
W1016	*lytE*::*cat*	This work
W111	*lytC*::*cat*	This work
W673	*lytD*::*erm*	This work
W674	*lytF*::*kan*	This work
W678	*lytC*::*cat lytD*::*erm lytF*::*kan*	This work
W758	*amyE*::*hagp-gfp* (Cm^r)	This work
W1134	*amyE*::*cwlSp-gfp* (Cm^r)	This work

[a] Regulator mutants used in this study are listed in Table S1 in the supplemental material.

[b] Arrows indicate the direction of donor-to-recipient transformation for strain construction.

[c] ATCC 6051 is the same as NCIB 3610.

(11, 33). Moreover, many genes involved in biofilm formation have also been identified via genome-wide screening, using transposon insertions and the *Bacillus subtilis* functional analysis collection of mutants, but the precise roles of these genes in biofilm formation are still unknown (5, 10).

This study presents a comprehensive analysis of pellicle formation, with a focus on transcriptional regulators and morphological changes. Nearly all of the potential regulators that have been identified in the *B. subtilis* genome were disrupted, and the effects of mutations in these genes on pellicle formation were examined. As a result, we identified 19 regulators that are required for pellicle formation, and the results presented here reveal relationships between regulators and morphological development during pellicle formation by *B. subtilis*.

MATERIALS AND METHODS

Bacterial Strains. The *B. subtilis* strains used in this study are listed in Table 1 and in Table S1 in the supplemental material. The *B. subtilis* strain ATCC 6051 was obtained from the American Type Culture Collection. In all cases, *B. subtilis* strains were maintained on TBABM (33 g/liter tryptose blood agar base [Difco], 4 mg/liter $FeCl_3$, 0.2 mg/liter $MnSO_4$, 5.5 mg/liter $CaCl_2$ 1.7 mg/liter $ZnCl_2$, 0.43 mg/liter $CuCl_2 \cdot 2H_2O$, 0.6 mg/liter $CoCl_2 \cdot 6H_2O$ and 0.6 mg/liter $Na_2MoO_4 \cdot 2H_2O$) and 2× SG [16 g/liter of nutrient broth (Difco), 2 g/liter KCl, 0.5 g/liter $MgSO_4 \cdot 7H_2O$, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2 \cdot 4H_2O$, 1 μM $FeSO_4$, and 0.1% glucose] solidified by 1.5% agar. *Escherichia coli* HB101 was used for construction and maintenance of plasmids. Antibiotics were used at the following concentrations: ampicillin, 30 μg/ml; chloramphenicol, 5 μg/ml; erythromycin-lincomycin, 0.5 μg/ml and 25 μg/ml, respectively; and kanamycin, 10 μg/ml.

Construction of *B. subtilis* mutants. Mutant strains for each potential regulator gene were constructed using an overlap-extension PCR technique. The *cat* gene was amplified from the plasmid pCBB31 (34) by PCR with primers pUC-F (5′-GTTTTCCCAGTCACGACG-3′) and pUC-R (5′-GAATTGTGAGCGGATAAC-3′). Upstream and downstream regions of each regulator gene were amplified by PCR, using gene-specific primer sets F1/R1 and F2/R2 for each regulator (see Table S1 in the supplemental material). The 5′ ends of primers R1 and F2 are complementary to the pUC-R and pUC-F sequences, respectively. Three PCR fragments per gene were then mixed and used as templates for a second PCR with primers F1 and R2.

The resultant PCR fragment was used for transformation of *B. subtilis* strain 168, and the mutations were subsequently transferred to the undomesticated *B. subtilis* strain ATCC 6051 by transformation with chromosomal DNAs prepared from the 168 mutants. Transformation of strain ATCC 6051 was carried out using a standard procedure (15), except that incubation after the addition of DNA was prolonged to 2 h. Strain 168 contains mutations in at least *degQ* and *swrA*, which are involved in biofilm formation (32, 51). In the ATCC 6051 background, the *degQ* and *swrA* mutations produce an easily distinguished smooth colony phenotype on 2× SG. Thus, to eliminate the possibility of congression of these mutations with the regulator deletion mutations, colony morphologies of at least 20 transformants of each ATCC 6051 regulator deletion strain were carefully examined on TBABM and 2× SG plates.

Since *comA* is close to *degQ* on the genome, it was difficult to introduce only the *comA* mutation into ATCC 6051. To circumvent this problem, a *comA*::*cat degQ*::*erm* double mutant was constructed in strain 168, and then only the *comA*::*cat* mutation was introduced into ATCC 6051 by screening for erythromycin-sensitive (Em^s) and chloramphenicol-resistant (Cm^r) transformants. Similarly, the *ykvB* mutation was transferred to ATCC 6051 by using chromosomal DNA from a *ykvB*::*cat swrA*::*erm* double mutant. When several types of transformants with different colony morphologies appeared, backcross analysis was carefully carried out. Moreover, several independent transformants were isolated and used in the analyses presented below. Frozen stocks of the ATCC 6051 strains were prepared without prolonged cultivation.

Flagellar and autolysin mutants were constructed using an overlap-extension PCR technique. Primers used for construction are shown in Table S2 in the supplemental material. The Em^r and kanamycin resistance (Km^r) cassettes were amplified by PCR, using the primers pUC-F and pUC-R, with the plasmids pAE41 for Em^r (34) and pDG780 for Km^r (19) as templates.

Construction of *hag-gfp* and *cwlS-gfp* transcriptional fusions. Promoter-*gfp* fusions were constructed using the plasmid pDCG-1. Construction of pDCG-1 was done as follows. The Cm^r cassette was amplified from pCBB31 by PCR, using the primers cat-amyE-F (5′-AAGATGATATCAGATCTCTAGAGTCGACCTGCAGGCATGCAAGCTTACCATGATTACGAATTCGAGCTCGGTACC-3′) and cat-amyE-R (5′-AAGATGATATCACTAACGGGGCAGGTTAGTG-3′). The 5′ region of the primer cat-amyE-F contains multiple cloning sites (underlined). The plasmid pDL2 is an integration vector harboring sequences upstream and downstream of *amyE* (17). After digestion with EcoRV, the PCR-amplified *cat* fragment was ligated into pDL2 treated with Bpu1102I, SmaI, and PolI. The resultant plasmid, pDC, contains multiple cloning sites and the *cat* cassette in the same orientation as the *amyE* sequences. Next, the *gfp-uv4* gene (28) was amplified from pGFPuv4 (28) by a PCR using the primers GFPuv4-tc-F (5′-AATCTTCTAGATTGAGGAGGTCTTGTAAACATGAGTAAAGGAGAAGAACTTTTCACTGG-3′) and GFPuv4-R (5′-AAAGAAGATCTGGGTAACTATTGCCGGGATC-3′). The 5′ region of the primer GFPuv4-tc-F contains a Shine-Dalgarno sequence from *groEL* (underlined). After digestion with SphI and BglII, the resultant PCR product was inserted into pDC to generate pDCG-1. The promoter regions of *hag* and *cwlS* were amplified by PCR, using chromosomal DNA prepared from ATCC 6051 as a template, with the following primer sets: for *hag*, hag-P-F (5′-GAAGAATTCTGCGGTTGAAGGGGATCAAG-3′) and hag-P-R3 (5′-AAGAAGCTTGTTCAGTGTGTTAAGCGCTG-3′); and for *cwlS*, cwlS-P-F (5′-GAAGAATTCGGCGACCAATTTGTTGTCGC-3′) and cwlS-P-R (5′-AAGCAGCATGCGAAACAGCCAAGCCGGCTAC-3′). The resultant PCR fragments were digested with EcoRI and HindIII or EcoRI and SphI and then inserted into pDCG-1, generating pDCGhag and pDCGcwlS, respectively. DNA sequencing was performed at each step in order to confirm that the expected sequence was amplified.

Pellicle formation. The wild-type and mutant strains were grown overnight at 30°C on TBABM plates supplemented with antibiotics when appropriate. Next, a fresh small colony was used to inoculate 10 ml of 2× SGG (2× SGG is 2× SG supplemented with 1% [wt/vol] glycerol) into one well of a six-well plate (BD Falcon). Each plate was then incubated at 30°C, and pellicle formation was recorded at 24 and 48 h. Pellicle formation in a standing culture of minimal MSgg medium was examined according to the method described by Branda et al. (6). Wild-type and mutant strains were grown to mid-log phase in LB medium, and 12 μl of culture was used to inoculate 12 ml of MSgg medium in one well of a six-well plate. Each plate was then incubated at 23°C for 72 h.

Swarming motility assay. To detect swarming motility, 14-cm-diameter LB swarm plates with 0.7% agar (30) were dried at room temperature overnight. A fresh small colony of each *B. subtilis* strain was inoculated onto the center of the LB swarm plate by use of a toothpick, and swarming motility was recorded after 12 h of incubation at 30°C in a humidified chamber.

Microscopic observation. Flagella were stained using a modified version of the method described by Kodaka et al. (36). Cells were sampled from the edge of a swarm colony with a toothpick and transferred to 10 μl of water on a glass slide. Next, the sample was dried in a laminar flow hood. Subsequently, 10 μl of Ryu

solution (36) was spotted onto the dry sample. After the sample was incubated for 30 s, it was covered with a 24- by 24-mm coverslip, and excess solution was removed using a paper towel. Cells were observed via phase-contrast microscopy, using a DMRE-HC microscope (Leica) combined with a digital charge-coupled device camera (1300Y; Roper Science).

For analysis of cellular morphology during pellicle formation, a fresh small colony grown on a TBABM plate was used to inoculate 5 ml of 2× SGG medium in one well of a 12-well plate (BD Falcon). Plates were incubated at 30°C, and 20 μl to 2 ml of culture was collected. When necessary, cells were pelleted and resuspended in 20 μl of medium. For membrane staining, a 1/10 volume of 20-μg/ml FM4-64 was mixed with the cell suspension. Cellular morphology was observed via phase-contrast microscopy, using a Leica microscope as described above. Fluorescence signals of FM4-64 and green fluorescent protein (GFP) were visualized using the appropriate filters from the L5 filter set (Leica). Image acquisition and processing were carried out using Metamorph software (Universal Imaging Corporation).

Northern blot analysis. Fresh colonies of the wild-type and mutant strains were used to inoculate 10 ml of 2× SGG in the wells of a six-well plate. After incubation at 30°C for 14 h, cells of each strain were collected from three wells of culture (total, 30 ml) by centrifugation and immediately frozen in liquid nitrogen. Total RNA was isolated according to the method described by Igo and Losick (26). Each RNA sample (0.7 μg) was separated using a formaldehyde–1.2% agarose gel and then transferred to a positively charged nylon membrane (Roche) with a vacuum blotter (model 785; Bio-Rad). To quantify the RNA in each lane, the nylon membrane was stained with staining solution (0.04% methylene blue, 0.5 M sodium acetate [pH 5.2]), and then the membrane was washed with water until rRNA bands became visible. For preparation of digoxigenin (DIG)-labeled RNA probes, DNA fragments were amplified by PCR, using the primers described below. Each reverse primer contained the T7 promoter sequence at its 5′ end (underlined). PCR fragments were purified by polyethylene glycol precipitation and used as templates for synthesis of DIG-labeled RNA probes. RNA probes were synthesized using DIG RNA labeling mix (Roche) and T7 RNA polymerase (Roche). Hybridization and detection were performed according to the manufacturer's instructions (Roche). The primers used were as follows: lytD-N-F, 5′-CAGCCGCGTATACCGACTAC-3′; lytD-N-T7R, 5′-TAATACGACTCACTATAGGGCGAAGACTGAATGGTTGTGACAG-3′; lytF-N-F, 5′-GCATCTGCGATTGTCGGCAC-3′; lytF-N-T7R, 5′-TAATACGACTCACTATAGGGCGAATATGTTCCCGTAGAAGATG-3′; cwlS-N-F, 5′-AAAGCGGTGACTCTCTTTGG-3′; cwlS-N-T7R, 5′-TAATACGACTCACTATAGGGCGATGGAACCGCTGTTTCCGTTC-3′; sigH-N-F, 5′-AGTTGGAGGACGAGCAGGTC-3′; sigH-N-T7R, 5′-TAATACGACTCACTATAGGGCGAATCCAGCAGCGTTCGGTCTG-3′; abrB-N-F, 5′-GTATCTCTTGGGAGGAGAATG-3′; abrB-N-T7R, 5′-TAATACGACTCACTATAGGGCGATTTAAGGTTTTGAAGCTGGTTTTGG-3′; aprE-N-F, 5′-AATGAGTGCCATGAGTTCCG-3′; and aprE-N-T7R, 5′-TAATACGACTCACTATAGGGCGACTTCCTGTTGAATCAAGCAC-3′.

RESULTS AND DISCUSSION

Identification of transcriptional regulators required for pellicle formation. Pellicle formation is a late-growth-phase phenomenon and may be controlled by induction or repression of specific regulators. At least 288 regulators and potential regulators in the *B. subtilis* genome are listed in the BSORF database (http://bacillus.genome.jp/; see Table S1 in the supplemental material). The *B. subtilis* laboratory strain 168 is amenable to genetic manipulation; however, strain 168 produces only an extremely thin, featureless pellicle in standing culture (6). Therefore, it seemed appropriate to construct deletion strains for each of these regulators by using the undomesticated strain ATCC 6051, which produces a thick, structured pellicle in standing culture. Strain ATCC 6051 is the same as NCIB 3610, which has been used by many labs for the study of biofilm formation (4, 5, 6, 12, 33). ATCC 6051 has low competence, and thus it is difficult to transform it directly with plasmid DNA or PCR products. Thus, we first constructed regulator mutants by using strain 168, and then the deletions were transferred to strain ATCC 6051 by transformation with chromosomal DNAs prepared from strain 168 mutants (see Materials and Methods). As a result, 285 regulator genes were disrupted in strain 168, and all mutations were successfully introduced into ATCC 6051. The notable exceptions were the three essential regulator genes *dnaA*, *sigA*, and *yycF*, for which it was not possible to construct deletion strains. Although at least two sequence differences between laboratory and undomesticated strains have been reported (32, 51), for the transformation of ATCC 6051 the transformation frequencies were about the same for these 285 alleles, indicating that the genomic structures of the ATCC 6051 and 168 genomes lack large differences.

Pellicle formation by undomesticated strains of *B. subtilis* has been analyzed in MSgg minimal medium (4, 5, 6, 12, 33). However, we examined pellicle formation in the rich medium 2× SGG because, in minimal medium, many mutants might show defects in pellicle formation due to an indirect effect, such as nutrient requirements. In 2× SGG standing culture, wild-type ATCC 6051 formed a thick, structured pellicle within 24 h (see Fig. S1 in the supplemental material). Time course analysis of pellicle formation in 2× SGG showed that a structured pellicle was formed as follows (see Fig. S2 in the supplemental material): (i) a flat, thin pellicle was formed (10 h); (ii) the pellicle became thick and then many folds appeared on the pellicle (12 h and 14 h); and (iii) the folds grew up and became a microscopic architecture (15 h to 24 h). By formation of a microscopic architecture on a pellicle, pellicle thickness may be controlled so that the pellicle is not too thick, which may be important for maintaining proper aerobic conditions in the pellicle. Thus, the shape and density of the microscopic architecture formed on a pellicle are indicators of the degree of pellicle development. In addition, the pellicle formed uniformly over the medium surface, not from the edge of a well (see Fig. S2 in the supplemental material). This observation suggests that pellicle formation by *B. subtilis* is independent of adhesion of cells to a substrate.

To examine the ability of each mutant to form pellicles, each mutant was inoculated into a standing culture of 2× SGG and incubated at 30°C for 48 h. Twenty-three of the regulator mutants formed unusual pellicles or did not form pellicles at all. Of these, 19 mutants exhibited growth rates comparable to that of the wild-type strain in agitated culture. Thus, we concluded that 19 of the 285 potential regulators were required for pellicle formation (Table 2; see Fig. S1 in the supplemental material). Six of these were previously reported to be involved in biofilm formation (6, 11, 22, 33, 50, 51). Specifically, mutations in *degU*, *spo0A*, or *sigH* abolish biofilm formation, and mutations in *abrB*, *ccpA*, or *sinR* enhance biofilm formation. However, the phenotypes observed for mutations in *abrB*, *ccpA*, and *sinR* were slightly different from those stated in previous reports. Mutations in *abrB* and *ccpA* had a moderate effect on pellicle formation in 2× SGG medium. In previous studies, AbrB and CcpA have been shown to be repressors of biofilm formation, and *abrB* and *ccpA* mutants form normal biofilms (21, 50). The *ccpA* and *sinR* mutants showed a delayed pellicle formation phenotype that has not been observed in previous work (33, 50).

To directly compare our results with previously published results, the pellicle formation of mutants that were defective in pellicle formation in 2× SGG was examined in MSgg minimal

TABLE 2. Transcriptional regulators required for pellicle formation and/or swarming motility

Regulator	Pellicle formation[a]	Swarming motility[b]	Function (reference)
abh	+	++	σ^X-related regulator (25)
abrB	+	−	Repressor of biofilm formation (21, 22)
ahrC	Delay	+	Repressor of *roc* operon (43)
alsR	+	++	Activator of *alsSD* (47)
ccpA	+, delay	+	Catabolite repression regulator (13, 50)
codY	Delay	+	Global regulator of nitrogen and amino acid metabolism (39)
comA	+	++	Two-component regulator required for competence development (14)
degU	−	−	Two-component regulator required for biofilm formation (40, 51)
hpr	++	+	Repressor of degradative enzymes (46)
purR	++	+	Repressor of the *pur* operon (55)
resD	−	++	Two-component regulator required for aerobic and anaerobic respiration (41)
sigD	Delay	−	Sigma factor required for flagellar and autolysin (1, 10)
sigH	−	++	Sigma factor required for biofilm formation (6)
sigX	+	+	Sigma factor required for modification of cell envelope (9)
sinR	Delay	−	Repressor of the *eps* operon and the *sipW* operon (11, 33)
slr	−	++	Unknown[c]
spo0A	−	++	Two-component regulator required for biofilm formation (6, 21)
spo0J	++	+	Required for chromosome segregation and sporulation (27)
ydcN	+	+	Putative phage repressor
glvR	++	+	Activator of the *glvAR-malP* operon (56)
yvrH	+	++	Two-component regulator required for cell surface proteins and the *sigX* operon (48)
cysL	+	++	Activator of the *cysJI* operon (20)
rok	+	++	Regulator of cell surface proteins and extracellular proteins (2)

[a] Pellicle formation in 2× SGG was scored as equivalent to that of ATCC 6051 (++), unusual pellicle formation (+), no pellicle formation (−), or delayed pellicle formation (delay).
[b] Swarming motility was scored as equivalent to that of ATCC 6051 (++), poor (+), or nonexistent (−).
[c] *slr* is annotated as an activator of sporulation and competence in the SubtiList database (http://genolist.pasteur.fr/SubtiList/), but no literature exists.

medium. Many of these mutants also showed a defect in pellicle formation in MSgg medium, but several phenotypic differences were observed. Mutations in *abh*, *ahrC*, *rok*, and *yvrH* had a moderate effect on pellicle formation in 2× SGG but not in MSgg medium. In MSgg medium, the *abrB* mutant formed a pellicle with an excessive architecture compared with that of the pellicle formed by the wild-type strain, suggesting that AbrB acts as a repressor of pellicle formation in MSgg. The *abrB* phenotype is thus dependent on the medium conditions. In MSgg medium, the wild-type strain formed a pellicle within 72 h after inoculation, and none of the mutants showed a delay in pellicle formation in this medium. For example, the *sinR* mutant showed a delay in pellicle formation only in rich medium (2× SGG). In contrast to a previous report (50), the *ccpA* mutant reduced pellicle formation in both the 2× SGG rich medium and MSgg minimal medium. This difference could be due to differences in the assay conditions or the parental strain, but we did not pursue it further in this study.

Flagella are required for normal progression of pellicle formation. Swarming motility is another type of multicellular behavior and was shown to have control mechanisms that overlapped with those governing biofilm formation in *B. subtilis* (12). This background led us to examine the swarming ability of the regulator mutants. Thirteen of the mutant strains showed defects in swarming motility (Table 2; see Fig. S1 in the supplemental material). To further analyze these defects, cells of nine mutants with strong defects in swarming motility were picked from the edges of colonies on swarm plates, and their flagella were stained and visualized. The *ccpA*, *codY*, *degU*, *sigD*, and *sinR* mutants had few or no flagella, and the *abrB* mutant contained a smaller number of flagella than that observed for the wild type (Fig. 1A). In spite of having a defect in swarming motility, the *ahrC*, *hpr*, and *purR* mutants had flagellum numbers that were comparable to that of wild-type cells. The *hpr* and *purR* mutants produced normal pellicles, indicating that swarming ability is not essential for pellicle formation.

Interestingly, four of the flagellum-defective mutants, the *ccpA*, *codY*, *sigD*, and *sinR* mutants, showed a similar delay in pellicle formation. These mutants had not formed pellicles after 24 h of incubation but had formed pellicles by 48 h. The *sigD* and *sinR* genes have been reported to be required for transcription of flagellar genes. The *sigD* gene encodes a flagellum-associated sigma factor, σ^D, which directs transcription of the class three flagellar genes encoding the flagellin, motor, and chemotaxis proteins (1). Although a direct role for SinR in flagellar regulation has not been revealed, *sinR* mutation does reduce the expression of σ^D-regulated genes (16). Thus, although swarming motility is not strictly required for pellicle formation, flagella appear to play some role in pellicle formation. In order to further address the role of flagella in pellicle formation, four flagellar genes belonging to different hierarchies in flagellar regulation were disrupted. The product of the *swrA* gene is a regulator required for transcription of the *fla-che* operon (8, 31, 32). The product of the *fliF* gene is the M-ring protein, a component of the flagellar basal body (1). *motA* and *hag* belong to the class three genes and encode a motor protein and flagellin, respectively (1). As shown in Fig. 1B, mutations in any of the four flagellar genes caused a delay in pellicle formation, supporting the idea that flagellar formation is required for the proper progression of pellicle forma-

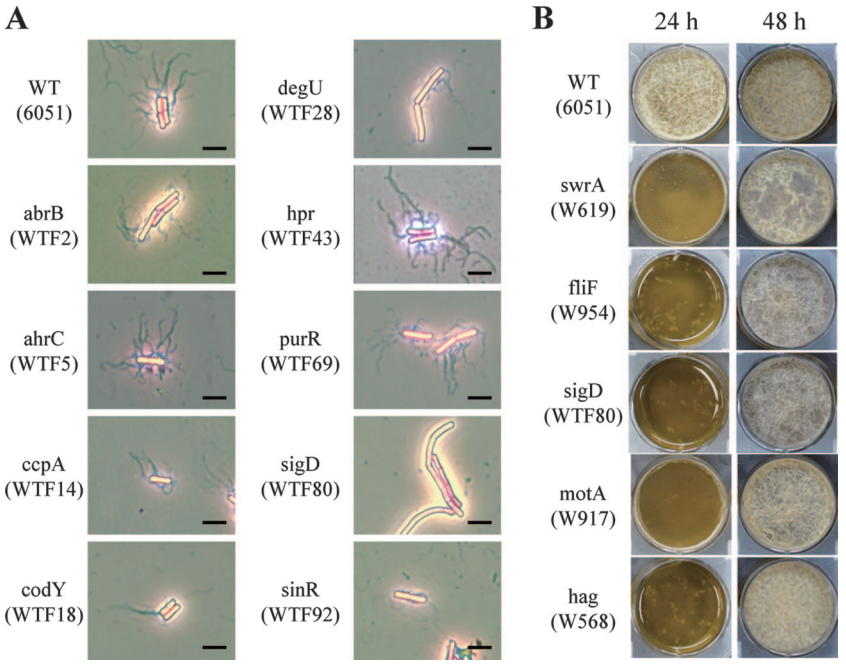

FIG. 1. Flagella are required for efficient formation of a pellicle by *B. subtilis*. (A) Flagellum formation in mutants defective in swarming motility. Cells were sampled from the edges of colonies on swarm plates and stained to visualize flagella. Cells were observed by phase-contrast microscopy combined with a charge-coupled device camera. Bars, 5 μm. (B) Pellicle formation in flagellar mutants. A fresh colony from each mutant grown on TBABM overnight was used to inoculate 2× SGG medium in a six-well plate, and the cultures were incubated at 30°C without agitation. A pellicle formed by each mutant was photographed 24 h and 48 h after inoculation. The diameter of each well is 3 cm.

tion. Moreover, these data are consistent with the idea that the delay in pellicle formation in the *ccpA*, *codY*, *sigD*, and *sinR* mutants can be attributed to the defect in flagellar formation. In nature, bacteria may fluctuate between a motile state and a sessile, biofilm-forming state. Although the delay of pellicle formation in flagellar mutants was observed only in rich medium (2× SGG), this phenotype is important because it points to the existence of a regulatory interaction between flagellar formation and biofilm formation.

Morphological changes during pellicle formation. The morphology of cells in a pellicle is markedly different from the morphology of planktonic cells. Planktonic cells exist as one or two independent cells, whereas cells in a pellicle form aggregates in which cells are regularly aligned and tightly bound together. The regular alignment of cells in an aggregate suggests that aggregate formation may be guided by a specific developmental program rather than resulting from random aggregation of cells. In addition, a previous study has also indicated that the formation of such aggregates is an important process in pellicle formation (6).

To investigate this hypothesis, cells were collected from the bottoms of culture wells during pellicle formation, and cellular morphology was observed. Since the cells in standing cultures do not grow synchronously, cell morphology was slightly different depending on the location of cells in the well, and representative results are presented below. Two to four hours after inoculation, most of the cells were typically observed as one or two cells that moved rapidly (Fig. 2A). At 6 h, most cells lost motility and were 4 to 10 times longer than planktonic cells (Fig. 2B). Visualization of membranes by FM4-64 revealed that these long cells had a clear division plane, suggesting that long "cells" were actually cell chains (Fig. 2C). After 7 to 8 h, the number of cell chains increased (Fig. 2D), and the chains began to form aggregates in which several chains were tightly connected to form clusters (Fig. 2E). These clusters of cell chains frequently formed a woven string-like structure (Fig. 2F to H). At 9 to 10 h, each cell in the clusters became visible, indicating that cell separation had occurred (Fig. 2I). The structure of cell aggregates at this time was quite similar to that observed for pellicles that form on the air-medium interface at 20 h (Fig. 2K). However, at 9 to 10 h, cells could still be observed as a haze at the bottom of the well (Fig. 2J). Thus, the ordered structure of cells observed in the pellicle appears to form before the cells float to the surface of the medium.

Morphology of cells with mutations in regulator genes. The next set of experiments was designed to explore the relationship between morphological changes and regulator mutations that prevent pellicle formation. To do this, the morphology of each regulator mutant that was defective in pellicle formation was analyzed 10 h after inoculation. However, the putative phage regulator YdcN was excluded from the analysis. We found that 18 of the regulator mutants could be classified into four groups, depending on the stage at which pellicle formation was blocked (Fig. 3; see Fig. 6 for a summary).

The first group of regulator genes consisted of *sigH*, *slr*, and *spo0A*; strains with mutations in these genes could not form cell chains. Spo0A and σ^H (a product of *sigH*) are master regulators for stationary-phase phenomena and have previously been reported to be required for biofilm formation (6, 21). A function of Spo0A in biofilm formation is to repress *abrB* transcription (21). Slr is annotated as being an activator of sporulation and competence in the SubtiList database (http:

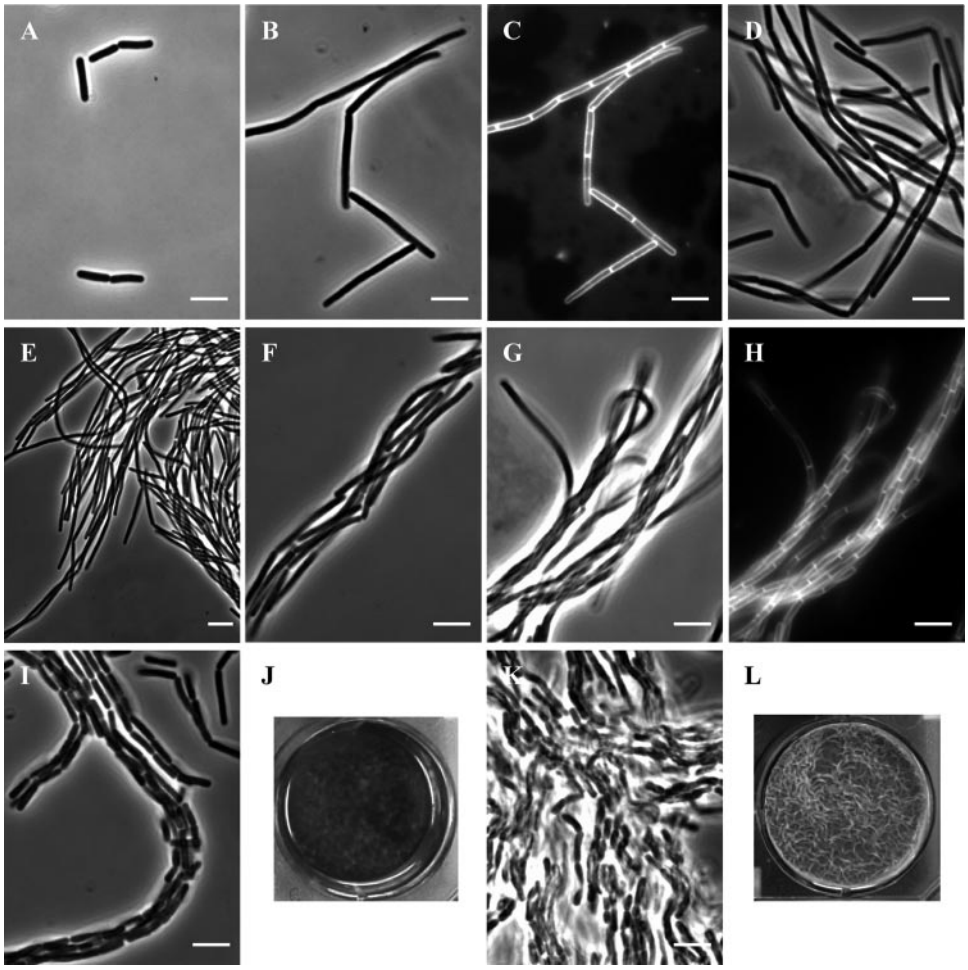

FIG. 2. Morphological development of *B. subtilis* during pellicle formation. Strain ATCC 6051 was grown under the conditions described for Fig. 1. Cells were withdrawn from the bottom of a well 2 h (A), 6 h (B to D), 8 h (E to H), or 10 h (I) after inoculation or withdrawn from pellicles formed at the air-medium interface 20 h after inoculation (K). (C and H) Cells were stained with FM4-64, a dye that visualizes membranes. Pellicles at 10 h (J) and 20 h (L) are also shown. Bars, 5 μm.

//genolist.pasteur.fr/SubtiList/), but no supporting literature exists. The function of *slr* in biofilm formation is unknown.

The second group of regulator genes consisted of *abh*, *alsR*, *sigX*, *yvrH*, and *cysL*. Cells with mutations in these genes form cell chains, but the chains do not form cell chain clusters. The function of these regulators in pellicle formation is unknown. AlsR has been shown to activate transcription of the *alsSD* operon, which is required for acetoin synthesis (47). In rich medium, *B. subtilis* produces a large amount of pyruvate by carbon catabolism. Pyruvate is converted to acetate, acetoin, and lactate, which are excreted into the medium. The production of acetoin is probably important for maintaining the intercellular pH, and thus the *alsR* mutant strain slightly reduces the growth rate at the late exponential phase in agitated cultures in 2× SGG (data not shown). Therefore, the effect of the *alsR* mutation on pellicle formation is possibly caused by a deficiency in carbon metabolism or pH homeostasis.

It is expected that the *abh*, *sigX*, and *yvrH* gene products share a set of one or more common targets. Abh is transcribed by the σ^X form of RNA polymerase (25) and positively regulates σ^X-regulated genes (unpublished data). Moreover, it was shown that the *yvrH* mutation reduces transcription of the *sigX* operon (48). Thus, mutations in both *abh* and *yvrH* reduce the transcription of σ^X-regulated genes. The extracytoplasmic function type of sigma factor σ^X directs the transcription of genes involved in modification of the cell envelope (9). These observations raise the possibility that the cell envelope structure is important for the formation of cell chain clusters.

In biofilms, cells are covered with an extracellular matrix that consists of exopolysaccharides, proteins, and DNA. It has previously been shown that the *B. subtilis eps* operon encodes proteins involved in the biosynthesis of exopolysaccharides and, furthermore, that mutation of the *eps* operon abolishes biofilm formation (6). Therefore, we were interested in examining at which stage the mutation of an *eps* gene blocks pellicle formation. Microscopic observation showed that *epsH* mutant cells form cell chains but not clusters of cell chains. Thus, exopolysaccharides appear to be required for the formation or maintenance of cell chain clusters during pellicle formation. Because both the *sigX* and *epsH* mutations prevent pellicle formation at the same stage, the cell envelope is likely to be required for fixation of the extracellular matrix around cells.

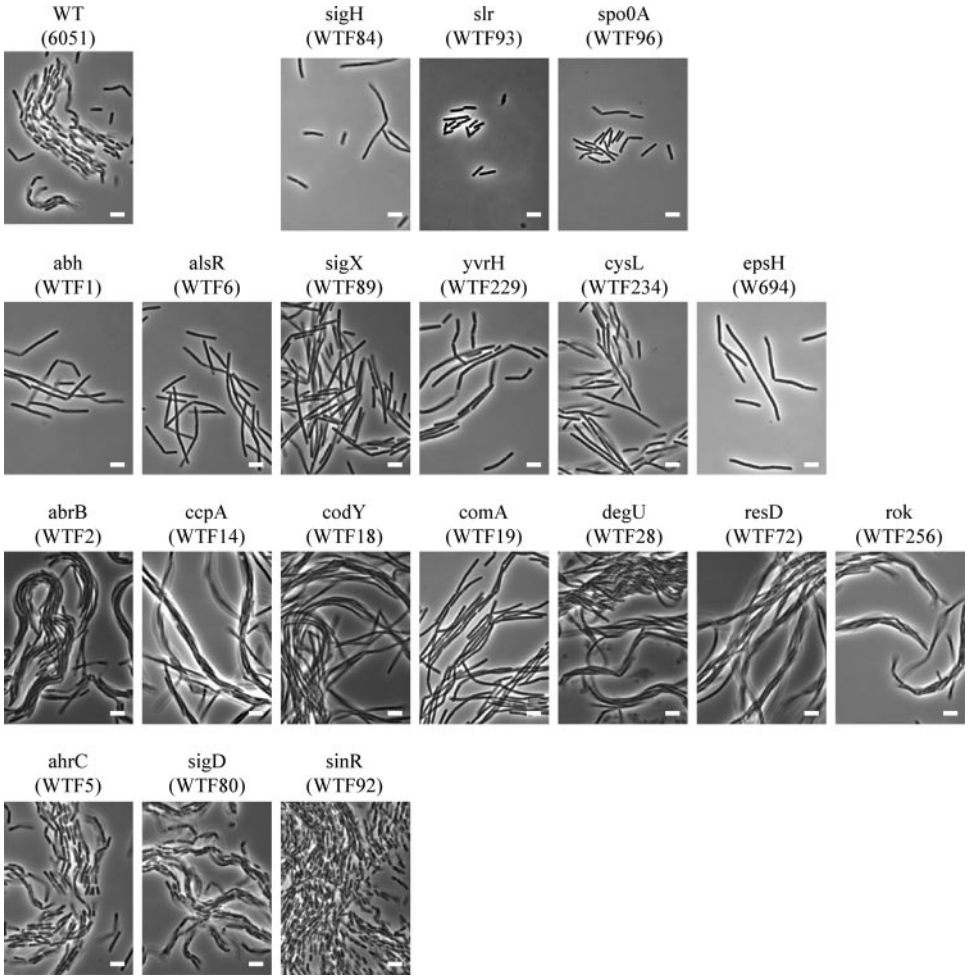

FIG. 3. Morphology of cells defective in pellicle formation. Mutant strains were grown in standing cultures as described for Fig. 1. Cells were withdrawn from the bottom of a well 10 h after inoculation. Bars, 5 μm.

The third group of regulator genes consisted of *abrB*, *ccpA*, *codY*, *comA*, *degU*, *resD*, and *rok*. Mutations in these genes blocked cell separation after the formation of cell clusters. Among these genes, *degU* has been shown to be required for biofilm formation. DegU activates transcription of the *pgs* operon, which is involved in γ-polyglutamic acid production. γ-Polyglutamic acid has been shown to be a component of extracellular matrixes produced by undomesticated *B. subtilis* strains (40, 51). The ComA-dependent gene *degQ* has also been shown to be required for transcription of the *pgs* operon (51). Since the relationship between DegU and DegQ has been shown genetically (23), ComA possibly affects DegU activity through the transcriptional activation of *degQ*. However, mutation in the *pgs* operon does not affect pellicle formation, at least in ATCC 6051 (4; data not shown), and thus the target(s) of DegU in biofilm formation is still unclear.

CcpA is a global regulator of carbon catabolite regulation (13). It has been shown that mutation in *ccpA* affects the expression of genes involved in the central pathway of carbon catabolism in rich media (53). Specifically, the *ccpA* mutant strain is unable to activate glycolysis or carbon overflow metabolism or to repress Krebs cycle enzymes (53). ResD is an activator of genes for aerobic and anaerobic respiration (41). Mutation in *abrB* affects pellicle formation only in a rich medium (see Fig. S1 in the supplemental material), and it has been shown that AbrB is a repressor of the *cta* and *qcr* operons, which encode cytochrome oxidases (22). Thus, a possible explanation is that mutations in these regulators may cause unbalanced expression of catabolic genes, which in turn prevents pellicle formation.

The final group of regulator mutants was comprised of *ahrC*, *sigD*, and *sinR*. Mutations in these genes did not affect the formation of cell clusters, suggesting that the genes are involved in a later stage, such as floating of cell clusters to the surface of the medium. Although the morphology of the *sigD* and *sinR* mutants was comparable to that of the wild type at 10 h, these mutants made a lump of cell aggregates, which was also observed in the motility-defective *abrB*, *ccpA*, *codY*, and *degU* mutants (data not shown; see below).

Cell separation is controlled by phase-specific autolysins. A characteristic feature of pellicle formation is the formation and degradation of cell chains via the control of cell separation. Cell separation is dependent on the activity of a cell wall hydrolase, autolysin. At least six autolysins, LytC, LytD, LytE,

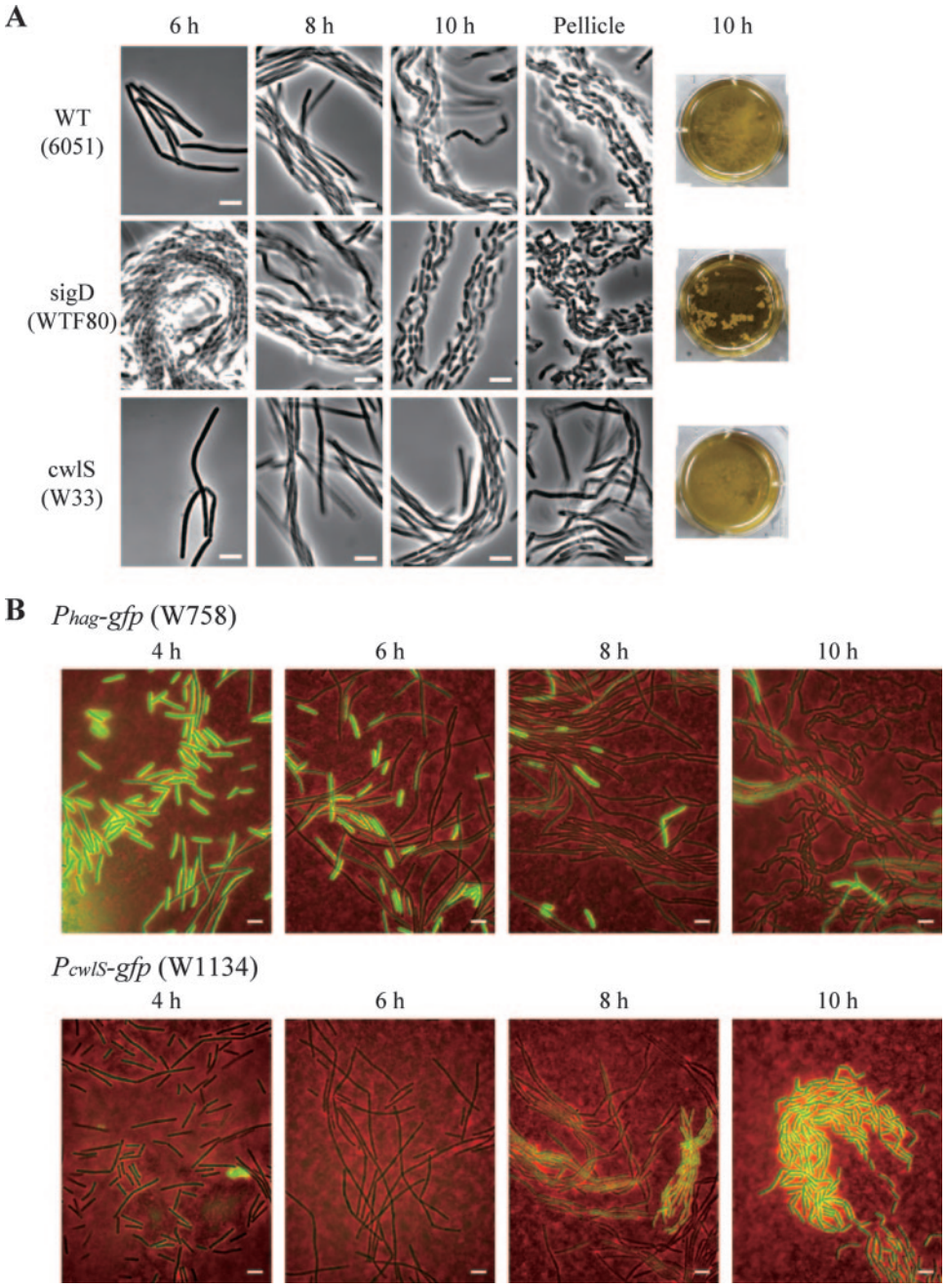

FIG. 4. Phase-specific autolysins are required for formation and degradation of cell chains during pellicle formation. (A) Time course analysis of cellular morphology of the *sigD* and *cwlS* mutants. The mutant strains were grown in standing cultures as described for Fig. 1. Cells were withdrawn from the bottom of a well (6 to 10 h after inoculation) or from pellicles (24 h after inoculation for the wild-type and *cwlS* mutant strains and 48 h after inoculation for the *sigD* mutant strain). In addition, cultures at 10 h after inoculation are shown in the rightmost panels. The *sigD* mutant forms large lumps of cell aggregates in standing culture. The diameter of each well is 3 cm. Bars, 5 μm. (B) Expression of *hagp-gfp* and *cwlSp-gfp* during pellicle formation. Strains with promoter-*gfp* fusions at the *amyE* locus were grown in standing cultures in 2× SGG medium. Samples were withdrawn from the bottom of the wells at the indicated times. Pictures are merged images with phase-contrast (false-colored red) and GFP (false-colored green) fluorescence shown. Bars, 5 μm.

LytF, LytG, and CwlS, have been reported to affect cell separation in *B. subtilis* (18, 24, 44, 56). In particular, the autolysins LytE, LytF, and CwlS are thought to be specific for cell separation, because these enzymes localize at cell separation sites (18, 57).

The flagellum-associated sigma factor σ^D controls expression of the *lytC*, *lytD*, and *lytF* genes, and mutation in *sigD* causes the production of cell chains in agitated culture, a phenomenon that is not observed for the *sigD*$^+$ strain (44). In standing culture, the *sigD* mutant produced a lump of cells large enough to be seen by eye (Fig. 4A). Microscopic observation revealed that the lump was a large aggregate of cell

chains. Moreover, the *sigD* mutant strain produced clusters of cell chains at an early time, when wild-type strains were still in a planktonic form. However, as time progressed, *sigD* mutant cells did separate, and the overall morphology became comparable in size and shape to that for clusters of cell chains in wild-type cultures grown for the same length of time. Although mutation in any one of the σ^D-dependent autolysin genes, *lytC*, *lytD*, and *lytF*, did not have any effect on cell separation, the triple mutant strain showed a defect in cell separation that was comparable to what was observed for the *sigD* mutant strain (see Fig. S3 in the supplemental material). These observations suggest that σ^D-dependent autolysins play a role in cell separation of planktonic cells, whereas other autolysins might be involved in cell separation after cell cluster formation. Moreover, the autolysin triple mutant did produce a normal pellicle, indicating that the defect in cell separation during planktonic growth does not disrupt the later processes that lead to pellicle formation.

In an attempt to identify autolysins that act in cell aggregates, the effects of mutations in *lytE* and *cwlS* were examined. The mutation in *cwlS*, but not that in *lytE*, had an effect on the later stage of pellicle formation. The *cwlS* mutant strain produced cell chains and clusters of cell chains, but individual cells in the clusters did not separate and remained in chain form even in the pellicle. These observations suggest that the CwlS protein is the cell separation enzyme that acts after the formation of clusters of cell chains.

Because *cwlS* is transcribed by the σ^H form of RNA polymerase (18), it seems probable that cell length during pellicle formation is controlled by the ordered action of σ^D- and σ^H-dependent autolysins, an effect that could be achieved via stage-specific activation of the two sigma factors during pellicle formation. To determine if the two sigma factors are activated in a stage-specific manner, the σ^D-dependent *lytF* promoter and the σ^H-dependent *cwlS* promoter were fused to *gfp*, and promoter-*gfp* fusions were introduced at the *amyE* locus of the *B. subtilis* genome so that the transcriptional activity of the sigma factors could be monitored over time. However, we were unable to detect GFP signals in cells harboring the *lytFp-gfp* fusion, and therefore we used the *hagp-gfp* fusion to monitor σ^D activity. Four hours after inoculation, most cells were planktonic cells in which the *hagp-gfp* fusion was expressed strongly (Fig. 4B). With the progression of time, the number of planktonic cells decreased, and cells formed cell chains and cell clusters in which expression of the *hagp-gfp* fusion decreased (Fig. 4B, 6h to 10 h). In contrast, *cwlSp-gfp* activity was low in planktonic cells and cell chains (Fig. 4B, 4h and 6 h) and increased in cell clusters, especially in aggregates in which cell separation occurred (Fig. 4B, 8h and 10 h). As shown above, σ^H activity is required for progression from planktonic cells to cell chains, indicating that σ^H activity is low in planktonic cells and increases in cell clusters. These results suggest that cell separation during pellicle formation is controlled by autolysins whose expression is determined by phase-specific activities of sigma factors.

Regulatory cascade for pellicle formation. We showed that regulators involved in pellicle formation can be classified into four groups based on the morphology of the mutant cells during pellicle formation. To address whether this classification of regulators was significant for pellicle formation, we analyzed the transcription of genes under the control of these regulators by Northern blotting. Total RNAs were purified from cells of wild-type and mutant strains grown to the point just before the formation of pellicles in standing cultures in 2× SGG. At this time, almost all cells of the wild-type strain formed cell clusters. Mutants of *abrB*, *sigD*, and *sinR* were removed from this analysis because we could not isolate sufficient RNA from these mutants.

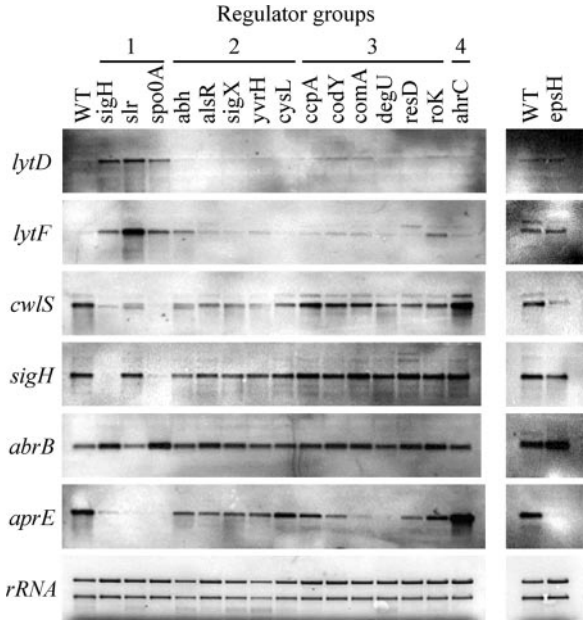

FIG. 5. Northern blot analysis of *lytD*, *lytF*, *cwlS*, *sigH*, *abrB*, and *aprE*. Total RNAs were prepared from cells of wild-type and mutant stains grown to just before the formation of pellicles in standing cultures in 2× SGG. Each RNA sample (0.7 μg) was separated in a formaldehyde–1.2% agarose gel, and RNAs were transferred to a nylon membrane. Transcripts were detected with DIG-labeled gene-specific RNA probes. As a control, the membrane stained with methylene blue to visualize rRNA is also shown (bottom).

As expected for mutants of the group 1 regulators, transcription of *lytD* and *lytF* was observed at a high level compared with that of the wild-type strain (Fig. 5). Introduction of the *sigD* mutation into strains carrying the *sigH*, *slr*, or *spo0A* mutation restored cell chains but not pellicles (data not shown). These results suggest that the group 1 regulators are important for repression of σ^D activity and the level of σ^D-dependent autolysins. The constitutive expression of σ^D-dependent autolysins may prevent the formation of cell chains in mutants of the group 1 regulators.

Mutations of the group 1 regulators severely affected the transcription of *cwlS* (Fig. 5). To our surprise, mutations of the group 2 regulators also reduced the transcription of *cwlS* (Fig. 5). This is consistent with the observation that mutant cells of the group 2 regulators appeared as cell chains 10 h after inoculation, at which time wild-type cells separated in a CwlS-dependent manner. Since *cwlS* is transcribed by the σ^H form of RNA polymerase, transcription of *sigH* was examined. We found that transcription of *sigH* decreased in mutants of the group 1 and group 2 regulators; in particular, transcription of *sigH* was completely abolished in the *spo0A* mutant (Fig. 5). The negative impact of mutations of the group 2 regulators on

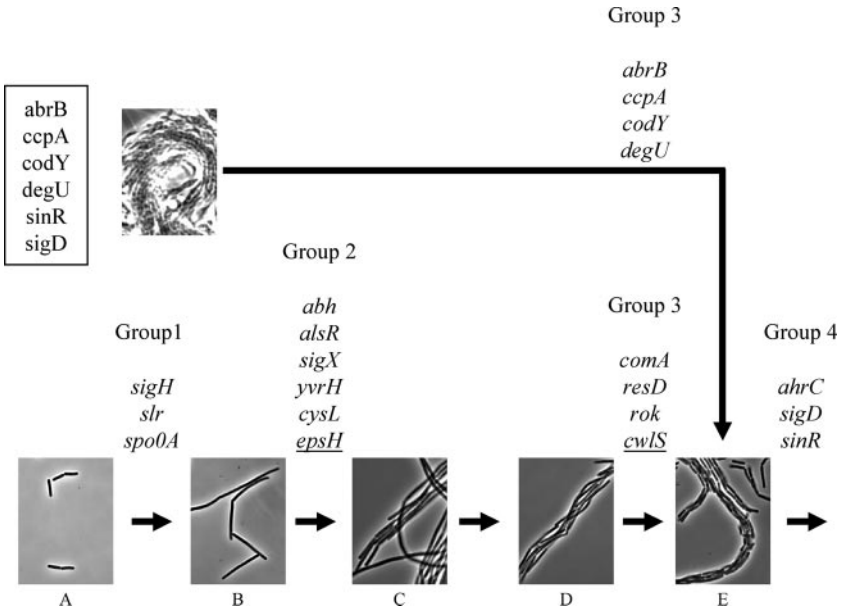

FIG. 6. Schematic model of cell clustering during pellicle formation. A temporal progression is shown from left to right. Cell cluster formation begins with freely floating planktonic cells (A). Planktonic cells lose motility and form cell chains in a process that appears to be induced by a decrease in the levels of σ^D-dependent autolysins (B). The number of cell chains increases, and chains come together, forming clusters (C). The clusters of cell chains often make a woven string-like structure (D). Finally, the σ^H-dependent autolysin CwlS induces cell separation, and the cells in a cluster become clear (E). Genes shown above arrows are required for the indicated stages; *epsH* and *cwlS* are underlined. Mutant strains defective in flagellum formation are indicated with a box; these mutant cells form clusters via a different route.

the transcription of *sigH* was weak, but this effect was reproducible, except for with the *cysL* mutant (data not shown). The effect of the *cysL* mutation on *sigH* transcription varied in every experiment and was uncertain (data not shown). The *sigH* gene is transcribed from a σ^A-dependent promoter that is negatively regulated by AbrB. Since Spo0A represses transcription of *abrB*, the *spo0A* mutation elevates the AbrB level drastically; this, in turn, reduces transcription of *sigH*. In fact, transcription of *abrB* increased in the *spo0A* mutant (Fig. 5). However, transcription of *abrB* did not increase in mutants of *slr* and the group 2 regulators (Fig. 5). These results suggest that transcription of *sigH* is controlled by an unidentified factor(s) in addition to AbrB.

Although the group 2 regulators σ^X and YvrH have been shown to regulate genes involved in modification of the cell envelope (9, 48), this does not account for the observation that mutations of these regulators reduce the transcription of *sigH*. Mutants of the group 1 and 2 regulators do not form cell clusters, and CwlS-dependent cell separation occurs in cell clusters. In addition, mutants of the group 3 and 4 regulators, which do form cell clusters, did not affect the transcription of *sigH* (Fig. 5). These observations implied that activation of *sigH* transcription might be dependent on the formation of cell clusters. To address this hypothesis, we examined transcription of *cwlS* and *sigH* in the *eps* mutant, which is defective in the synthesis of exopolysaccharides required for cell cluster formation. As shown in the rightmost panels of Fig. 5, transcription of *cwlS* and *sigH* decreased in the *eps* mutant. Thus, there is a mechanism for activation of *sigH* transcription coupled with the formation of cell clusters.

Transcription of *cwlS*, but not *sigH*, was reduced in mutants of *degU*, *resD*, and *rok* (Fig. 5). Thus, these regulators may be involved in transcription of *cwlS*, directly or indirectly. Alternatively, mutations of these regulators may affect σ^H activity, since σ^H is subject to complex posttranslational control, including temperature- and pH-dependent degradation (37, 42). Although the function of these regulators with respect to activation of σ^H has not yet been reported, it seems likely that some of these regulators may affect such activation.

We further analyzed the transcription of *aprE*, which is activated by DegU. Transcription of *aprE* disappeared in mutants of the group 1 regulators and decreased in mutants of the group 2 regulators, except for the *cysL* mutant (Fig. 5). Since transcription of *aprE* disappeared in the *sigH* mutant, the reduction of *aprE* transcription in the group 1 and 2 mutants may be caused in part by a reduction of *sigH* transcription. This consideration is supported by the observation that transcription of *aprE* decreased in the *eps* mutant (Fig. 5, right panels). Transcription of *aprE* also decreased in three mutants of group 3 regulators, namely, the *codY*, *comA*, and *resD* mutants. As described above, ComA possibly affects DegU activity through transcriptional activation of *degQ*. The roles of CodY and ResD in activation of *aprE* transcription are unknown.

Mutation of the group 4 regulator *ahrC*, which does not affect the formation of cell clusters, did not affect the transcription of *lytD*, *lytF*, *sigH*, and *abrB*. However, transcription of *cwlS* and *aprE* increased in the *ahrC* mutant. These observations indicate that unbalanced expression of σ^H- and DegU-dependent genes possibly prevents pellicle formation in the *ahrC* mutant.

These transcriptional analyses reveal that the classification of regulators based on the morphology of mutant cells represents the functional differences of these regulators in pellicle formation. Each group of regulators seems to act in order with

morphological development. Although the relationship between these regulators is largely unclear, these results suggest that these regulators compose a regulatory cascade that is coupled with morphological development in pellicle formation.

Concluding remarks. In this study, we have shown that pellicle formation by *B. subtilis* is accompanied by dramatic morphological changes that occur stepwise and can be defined genetically. Pellicle formation is controlled by a regulatory cascade that is coupled with morphological changes. Our tentative model of pellicle formation is illustrated in Fig. 6.

In planktonic cells, σ^D is highly active, inducing cell separation and motility (Fig. 6A). Pellicle formation may be initiated by a reduction in σ^D activity and in the levels of σ^D-dependent autolysins, thereby inducing the formation of cell chains (Fig. 6B). In this step, the group 1 regulators, Spo0A, Slr, and σ^H, play a critical role. Mutant strains deficient in these regulators continue expressing the σ^D-dependent autolysins, and thus these mutants do not form cell chains. On the other hand, the flagellum-defective mutants do not express σ^D-dependent autolysins, and thus these mutants form large aggregates (Fig. 6, top).

At this time, the synthesis of the extracellular matrix may be induced or up-regulated, since exopolysaccharides are required for the formation of cell clusters. The number of cell chains increases with growth, and the cell chains group to form cell clusters (Fig. 6C), which are often observed as a woven string-like structure (Fig. 6D). Mutations of the group 2 regulators prevent the formation of cell clusters. Although an obvious role for these regulators in cell cluster formation is unclear, σ^X, YvrH, and Abh probably affect the cell envelope structure, suggesting that this structure is important for fixation of the extracellular matrix.

In the cell chain clusters, CwlS production is induced by the σ^H form of RNA polymerase, which leads to cell separation (Fig. 6E). We showed that the *cwlS* promoter is highly active in cell clusters and that mutations that prevent the formation of cell clusters reduce the transcription of *sigH*. These observations strongly suggest that activation of *sigH* transcription is coupled to cell cluster formation. The timing of cell elongation, matrix synthesis, and expression of σ^H-dependent CwlS may be important for the formation of cell clusters. The transcriptional activation of *sigH* is dependent on the formation of cell clusters, which may be a mechanism for coordination of these stages as pellicle formation progresses.

Mutations of the group 3 regulators also prevent cell separation in cell clusters, although *sigH* transcription is induced normally in these mutants. Mutations of *degU*, *resD*, and *rok* reduce the transcription of *cwlS*. As described above, these regulators are probably required for transcription of *cwlS* directly or for the posttranscriptional activation of σ^H. Mutations of *ccpA*, *codY*, and *comA* prevent cell separation, but not the transcription of *cwlS*. These mutations probably affect CwlS activity or its localization.

Finally, the cell clusters float to the surface of the medium and grow, forming the pellicle. This step is prevented by mutations in the group 4 regulators, i.e., *ahrC*, *sigD*, and *sinR* (Fig. 6). As shown in Fig. 5, mutation in *ahrC* does not prevent the activation or repression of transcription of the genes tested, but the *ahrC* mutation causes overexpression of *cwlS* and *aprE*. This observation indicates that unbalanced expression of σ^H- and DegU-dependent genes seems to prevent pellicle formation in the *ahrC* mutant. The effects of the *sigD* and *sinR* mutations on pellicle formation are caused by a defect in flagellum formation. Mutations in flagellar genes cause a delay in pellicle formation, despite the fact that an early stage in pellicle formation appears to be a reduction of σ^D activity. Mutations in flagellar genes affect pellicle formation only in a rich medium such as 2× SGG. These observations suggest that the flagella or flagellum-associated proteins may have a regulatory role rather than a direct role, such as adhesion and cell-cell interaction, in pellicle formation. Thus, mutations in flagellar genes may also cause unbalanced expression of some regulons.

Each stage can be defined genetically by the set of regulator mutations, but no mutation was identified that blocks formation of the woven string-like structure. However, this structure still could be defined genetically, with the mutation(s) simply not yet having been found. Given the limited number of mutants tested in this study, another genetic screen needs to be designed.

Taken together, the results presented here reveal the relationships between regulators and the morphological changes associated with pellicle biofilm formation. Future studies will include the identification of the target genes of each regulator and the role(s) of those genes in pellicle formation in order to gain a further understanding of how these multicellular communities are established.

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