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RESEARCH ARTICLE

Dual Control of Flagellar Synthesis and Exopolysaccharide Production by FlbD-FliX Class II Regulatory Proteins in *Bradyrhizobium diazoefficiens*

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ABSTRACT *Bradyrhizobium diazoefficiens*, the N_2-fixing symbiont of soybean, has two independent flagellar systems: a single subpolar flagellum and several lateral flagella. Each flagellum is a very complex organelle composed of 30 to 40 different proteins located inside and outside the cell whereby flagellar gene expression must be tightly controlled. Such control is achieved by a hierarchy of regulators that ensure the timing of synthesis and the allocation of the different flagellar substructures. Previously, we analyzed the gene organization, expression, and function of the lateral flagellar system. Here, we studied the role of the response regulator FlbD and its *trans*-acting regulator FliX in the regulation of subpolar flagellar genes. We found that the LP-ring, distal rod, and hook of the subpolar flagellum were tightly controlled by FlbD and FliX. Furthermore, we obtained evidence for the existence of cross-regulation between these gene products and the expression of LafR, the master regulator of lateral flagella. In addition, we observed that extracellular polysaccharide production and biofilm formation also responded to these flagellar regulators. In this regard, FlbD might contribute to the switch between the planktonic and sessile states.

IMPORTANCE Most environmental bacteria switch between two free-living states: planktonic, in which individual cells swim propelled by flagella, and sessile, in which bacteria form biofilms. Apart from being essential for locomotion, the flagellum has accessory functions during biofilm formation. The synthesis of flagella is a highly regulated process, and coordination with accessory functions requires the interconnection of various regulatory networks. Here, we show the role of class II regulators involved in the synthesis of the *B. diazoefficiens* subpolar flagellum and their possible participation in cross-regulation with the lateral flagellar system and exopolysaccharide production. These findings highlight the coordination of the synthetic processes of external structures, such as subpolar and lateral flagella, with exopolysaccharides, which are the main component of the biofilm matrix.

KEYWORDS *Bradyrhizobium*, flagella, transcription, *flbD*, *fliX*

Most environmental bacterial species use flagella both as locomotion devices and as adhesins in the search for optimal living conditions. Thus, during their life cycles, bacteria may swim; adhere to surfaces, establishing biofilms; and detach from them to swim again, seeking new habitats (1–3). Although the types and multiplicity of flagella differ among bacterial species, all flagellar systems known to date share three common substructures: (i) the basal body, which includes the motor and the export apparatus embedded in the plasma membrane, (ii) the extracellular hook, which acts as a universal join with the filament, and (iii) the filament, which in most species projects to the surrounding medium with a length greater than that of the long axis of the

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cell and whose rotation propels the cell in liquid medium or over wet surfaces (4). In all species, these complex organelles are composed of tens of different proteins encoded by >30 genes (5).

Despite the high energy cost required for flagellar functioning and synthesis, some bacterial species possess two genetically, functionally, and structurally independent flagellar systems. In some species, such as *Vibrio* spp. and *Aeromonas* spp., each flagellar system possesses a sharply distinct function; thus, while a polar flagellar system is used for swimming in liquid medium, an inducible lateral flagellar system is used for swarming on wet surfaces (6, 7). Nevertheless, the two systems interact in *Vibrio* spp., and the polar flagellum, acting as a mechanosensor, induces the synthesis of the lateral flagellar system in response to the viscosity of the medium (2, 8). In other species, such as *Shewanella putrefaciens* and *Bradyrhizobium diazoefficiens*, both flagellar systems may be expressed simultaneously in liquid medium and used for swimming (9–13). In *B. diazoefficiens*, the N_2-fixing symbiont of soybean, two evolutionarily independent and functional flagellar systems coexist (14): a subpolar system, containing a single thick filament composed of four different 65-kDa flagellin subunits (FliC1234), and a lateral system with thin filaments formed by two different 33-kDa flagellin subunits (LafA12). While the former is constitutively expressed under the conditions studied so far, the latter is induced under certain environmental conditions, probably related to the oxic and energetic states of bacteria (15–18). As mentioned above for *Vibrio* spp., in *B. diazoefficiens*, the stability of the subpolar flagellum influences the expression of the lateral flagella (19). In addition to *B. diazoefficiens*, all *Bradyrhizobium* spp. sequenced possess the subpolar flagellum, which is encoded in at least six gene clusters that are scattered throughout the genome (see Fig. S1 in reference 20). In contrast, the lateral system is present only in the *Bradyrhizobium japonicum* phylogenomic group and is encoded in a single gene cluster, although its possible acquisition by horizontal gene transfer could not be confirmed (14).

The assembly of flagella is controlled with strict spatiotemporal coordination, which is achieved by a sequential and hierarchical regulation of gene transcription and translation by means of key regulators, assigned to class I (master), II, III, or IV according to their order of appearance in the hierarchy (21–23). In the group of alphaproteobacteria to which *B. diazoefficiens* belongs, two well-characterized and distinct flagellar regulatory models have been described, one in the marine bacterium *Caulobacter crescentus* and the other in the symbiotic bacterium *Ensifer meliloti* (24, 25). Whereas polar flagellum synthesis in *C. crescentus* is controlled by a cascade involving the regulatory proteins CtrA (master, or class I), FlbD-FliX (class II), and FlbT-FlaF (class III) (26), peritrichous flagellum synthesis in *E. meliloti* is regulated by VisNR-Rem (masters, or classes IA and IB) and FlbT-FlaF (class II) (27, 28). Nevertheless, CtrA is also involved in part of the regulatory cascade in *E. meliloti*, as demonstrated by CtrA control of *flaA*, *flaB*, and *flaC* flagellin transcription levels (29). In *B. diazoefficiens*, we found homologs to most of these regulatory genes in different clusters. We have reported previously that the synthesis of lateral flagella is regulated through LafR (class IB) and $FlbT_L$-$FlaF_L$ (class II), similarly to the model described for *E. meliloti* (30). However, the control of subpolar flagellum synthesis is still unknown. Comparison with *C. crescentus* showed that all CtrA, FlbD-FliX, and $FlbT_s$-$FlaF_s$ counterparts were found in *B. diazoefficiens* (9, 31–34), coincidentally located in three clusters related to the subpolar system (clusters 2, 5, and 6) (see Fig. S1 in reference 20). This finding led us to hypothesize that those genes might be responsible for the regulation of subpolar flagellum synthesis in *B. diazoefficiens*, whereby each *B. diazoefficiens* flagellar system might be regulated by a different mechanism.

The two-component system response regulator CtrA is also involved in cell cycle control (31, 35, 36), and for this reason, its deletion may be lethal. There are some exceptions, however, such as *Rhodobacter capsulatus*, *Rhodospirillum centenum*, and *Magnetospirillum magneticum*, where the control of flagellar synthesis via CtrA becomes independent of other essential processes (37–39). In *C. crescentus*, when CtrA

is phosphorylated by its cognate histidine kinase CckA, it initiates the transcription of some structural genes encoding the flagellar basal body as well as class II regulators FlbD-FliX and class III regulators FlaF-FlbT (26, 40). The response regulator FlbD interacts with the small protein FliX, and both together trigger the synthesis of another set of structural genes of the basal body and the hook (32, 34, 41–43). Then FlaF and FlbT posttranscriptionally regulate flagellin mRNA translation for subsequent filament formation (33, 44, 45).

The response regulator FlbD, a member of the NtrC-like family, possesses three characteristic domains: an N-terminal REC domain (sensor, susceptible to phosphorylation), a central AAA+ domain (ATPase, necessary for the switch to open the RNA polymerase holoenzyme complex), and a helix-turn-helix (HTH) C-terminal domain (DNA binding domain). This enhancer binding protein (EBP) acts through the RNA polymerase σ^{54} (RNAP-σ^{54}) subunit, activating class III gene transcription. In *C. crescentus*, FlbD can also act as an inhibitor of class II genes, which are activated by CtrA in the first step of flagellar construction (46). The function of FlbD is influenced and modified through the binding of its *trans*-acting activator FliX, which has been proposed to be the key component in the assembly checkpoint of early flagellar components (32, 43, 47). In this sense, FliX acts negatively on FlbD activity before the completion of class II protein assembly and positively on the transcription of class III and IV products. Nevertheless, there is no evidence for a mode of action of FliX on FlbD or for the mechanism by which the repression or activation switch occurs (26).

In this work, we describe the functional characterization of genes involved in the regulation of subpolar flagellum synthesis in *B. diazoefficiens* USDA 110. We focused on the role of the class II regulatory pair FlbD-FliX in subpolar flagellum synthesis by studying the effect of mutations in these genes. With this approach, we also observed that these mutations led to alterations in exopolysaccharide (EPS) formation, and we found new evidence for cross talk between the subpolar and lateral flagellar systems.

RESULTS

blr7003 to bll5837 encode FlbD-FliX class II regulators of the subpolar flagellum. In *B. diazoefficiens* USDA 110, the subpolar flagellum seems to be encoded in six clusters scattered in the genome (see Fig. S1 in reference 20). In clusters 2 (blr2191 to bll2207), 5 (bll5808 to bll5854), and 6 (blr6996 to blr7003), we found five open reading frames (bll2200, bll5837, bll5842, blr5847, and blr7003) with high sequence similarity to known flagellar synthesis regulators. To confirm their roles, we generated mutations in some of those putative regulators. Despite several attempts, we did not succeed in obtaining a *ctrA* mutant (bll2200), probably indicating that this gene is essential, as has been observed in other, related bacteria (35, 48). Since another approach will be necessary to confirm this result, we focused this work on the characterization of the FlbD-FliX regulatory pair.

We obtained *B. diazoefficiens* derivative strains with mutations in the putative class II regulator *flbD* (blr7003) and its proposed *trans*-acting factor *fliX* (bll5837) (see Fig. S2 in reference 20). These genes were located in two different clusters that included other structural components of the subpolar flagellum (see Fig. S1 in reference 20) and had a high degree of similarity to their counterparts in *C. crescentus* (32, 34). Mutations in both genes disrupted subpolar flagellum synthesis, as evidenced by the lack of all four FliC flagellins in liquid medium and reduced motility in swimming plates, a phenotype similar to that of the *fliC1234* mutant (Δ*fliC*) (Fig. 1) (19). In addition, possible overexpression of LafA flagellins was observed upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the *flbD* mutant (discussed further under "The *flbD* and *fliX* mutations do not affect bacterial growth but enhance the synthesis of LafA flagellins and the expression of the lateral flagellum master regulator *lafR*" below). Merodiploids were generated in both mutants by transferring the replicative plasmids pB3::*flbD* and pFAJ::*fliX*, which carry the wild-type genes under the control of the P*lac* or P*nptII* promoter. While the *fliX*::Km-pFAJ::*fliX* merodiploid was able to partially restore FliC production (Fig. 2A), there were significant differences in motility among

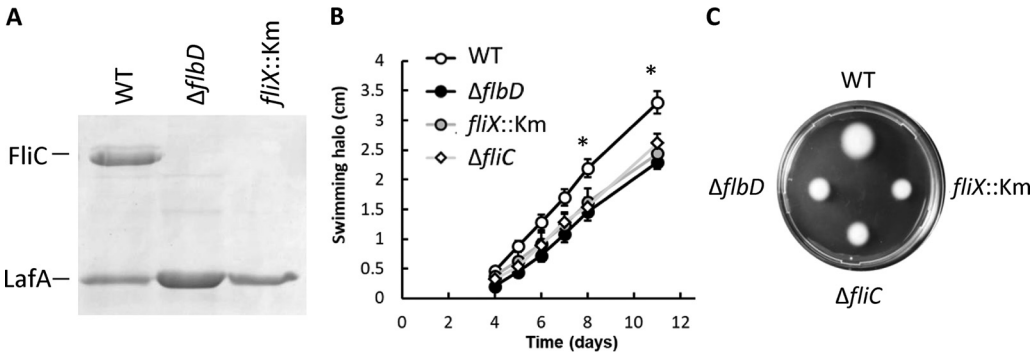

FIG 1 Extracellular proteins and swimming motility of *B. diazoefficiens* USDA 110. (A) SDS-PAGE of extracellular flagellins from the wild type (WT) and from the Δ*flbD* and *fliX*::Km mutants. The positions of subpolar (FliC1234) and lateral (LafA12) flagellins are indicated on the left. (B) Diameters of swimming halos of the WT, Δ*flbD*, *fliX*::Km, and Δ*fliC* strains over time in AG semisolid agar plates. Shown are averages ± standard deviations from three replicates (each strain in an individual plate) of one representative experiment. Asterisks indicate significant differences between the WT and mutants (P, <0.05 by analysis of variance). (C) Swimming plate on day 8 with the four strains together.

all *fliX* mutant strains and the wild type (Fig. 2C). In addition, the Δ*flbD*-pB3::*flbD* merodiploid was unable to complement either FliC production or motility (Fig. 2A and B), possibly because the *flbD* transcript was not produced in the merodiploid. To address this hypothesis, we performed reverse transcription-PCR (RT-PCR) with RNA samples from the different strains and corroborated the presence of the *flbD* transcript in the merodiploid and its absence in the *flbD* mutant carrying the empty vector (see Fig. S3 in reference 20). Since *flbD* was transcribed correctly from the plasmid, the lack of complementation should be caused by something else. Another possible explanation was that FlbD overproduction/accumulation caused by constitutive expression under the control of the P*lac* promoter in the merodiploid might produce miscoordination in the regulatory cascade, thus impairing flagellar synthesis. To test this hypothesis, we constructed wild-type strains carrying the pB3::*flbD* plasmid (WT-pB3::*flbD*) or the empty vector (WT-pB3). As expected, overproduction of FlbD caused a defect in swimming performance (Fig. 3A). Also, the total amount of flagellin purified from each strain was analyzed by SDS-PAGE for a quantitative comparison. Uncontrolled expression of *flbD* caused a downregulation of subpolar filament synthesis in the wild-type strain, as was observed with the Δ*flbD*-pB3::*flbD* strain (Fig. 3B). Furthermore, the wild-type strain carrying pFAJ::*fliX* (WT-pFAJ::*fliX*) showed a similar effect of reduced motility (Fig. 3C), reinforcing the idea that FlbD and FliX acted together and that the uncontrolled and miscoordinated overproduction of these proteins interfered with the correct progression of the flagellar signaling cascade.

FlbD acts through both σ^{54} transcription factors present in *B. diazoefficiens* depending on the growth condition. The EBP FlbD acts in concert with the RNAP-σ^{54} subunit (41, 50). *Bradyrhizobium diazoefficiens* USDA 110 carries two genes encoding RNAP-σ^{54} homologs: *rpoN1* (blr1883), which is induced under low oxygen concentrations, and *rpoN2* (blr0723), which is present under both aerobic and anaerobic conditions (51, 52). To ascertain which of them was used by FlbD, we used SDS-PAGE to analyze the extracellular flagellins produced by Δ*rpoN1* and Δ*rpoN2* mutants, as well as those from the Δ*rpoN1* Δ*rpoN2* (Δ*rpoN1-2*) double mutant obtained from aerobic and anoxic liquid cultures. As expected, the Δ*rpoN1* mutant produced FliC and LafA flagellins under aerobic conditions, while the Δ*rpoN2* and Δ*rpoN1-2* mutants produced only LafA (Fig. 4A). In contrast, under anoxic conditions, both the Δ*rpoN1* and Δ*rpoN2* mutants produced the FliC flagellin, indicating that FlbD can use either RpoN copy to activate transcription (Fig. 4B).

FlbD-FliX-mediated transcriptional control of the LP-ring, distal rod, and hook genes. As shown in Fig. S1 of reference 20, several operons could be predicted in the different gene clusters encoding the subpolar flagellum. In order to assess the precise control points involving FlbD and FliX, the transcription levels of several flagellar genes

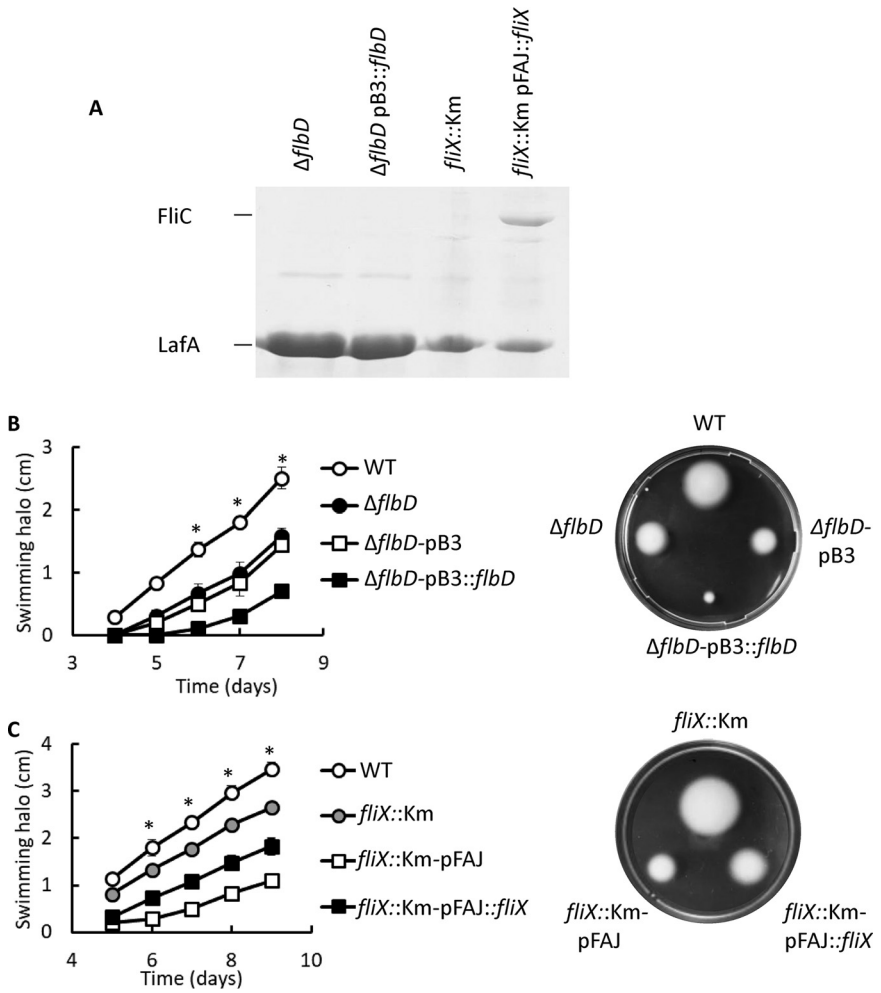

FIG 2 Flagellin production and swimming motility of *B. diazoefficiens* USDA 110. (A) SDS-PAGE of Δ*flbD* and *fliX*::Km mutants complemented in *trans* with the respective wild-type (WT) genes inserted into replicative plasmids. (B and C) Swimming halo diameters of WT, mutant, and complemented (Δ*flbD*-pB3::*flbD* and *fliX*::Km-pFAJ::*fliX*) strains recorded in AG semisolid agar at the indicated times. The mutants carrying empty plasmids (Δ*flbD*-pB3 and *fliX*::Km-pFAJ strains) were also included, since they have a small defect in swimming performance. Shown are averages ± standard deviations from three replicates of one representative experiment. Asterisks indicate significant differences by analysis of variance ($P < 0.05$) between the WT and the Δ*flbD*, Δ*flbD*-pB3, or Δ*flbD*-pB3::*flbD* strain in panel B and among all strains in panel C.

in the Δ*flbD* or *fliX*::Km mutants were compared with those in the wild-type strain by retrotranscribed quantitative PCR (RT-qPCR) (Table 1). From the analysis of the data, we found a group of genes that clearly were not under FlbD-FliX control. This group was composed mainly of genes that encoded part of the basal body components (*fliLM* and *pomAB*), part of the export apparatus (*flhA* and *fliOP*), and the proximal rod (*flgBC-fliE*). The changes observed in the transcription levels of the regulatory genes *ctrA*, *fliX*, *flaF*, and *flbT* were not present in the Δ*flbD* mutant (data not shown). As we expected, these data indicated that neither FlbD nor FliX acted in the early stage of flagellar synthesis.

Furthermore, in a second group of genes (shaded in Table 1), including putative operons *flgFGAH* (cluster 5), *flgEKL* (cluster 5), and *fliK-flgD* (cluster 6), encoding the LP-ring, the distal rod, and the hook, respectively, we found strong differences in transcription levels between each mutant and the wild-type strain. This result implies that FlbD and FliX are required for this control point, since the lack of just one of them significantly decreases the transcription levels of these genes. As can be seen in Table 1,

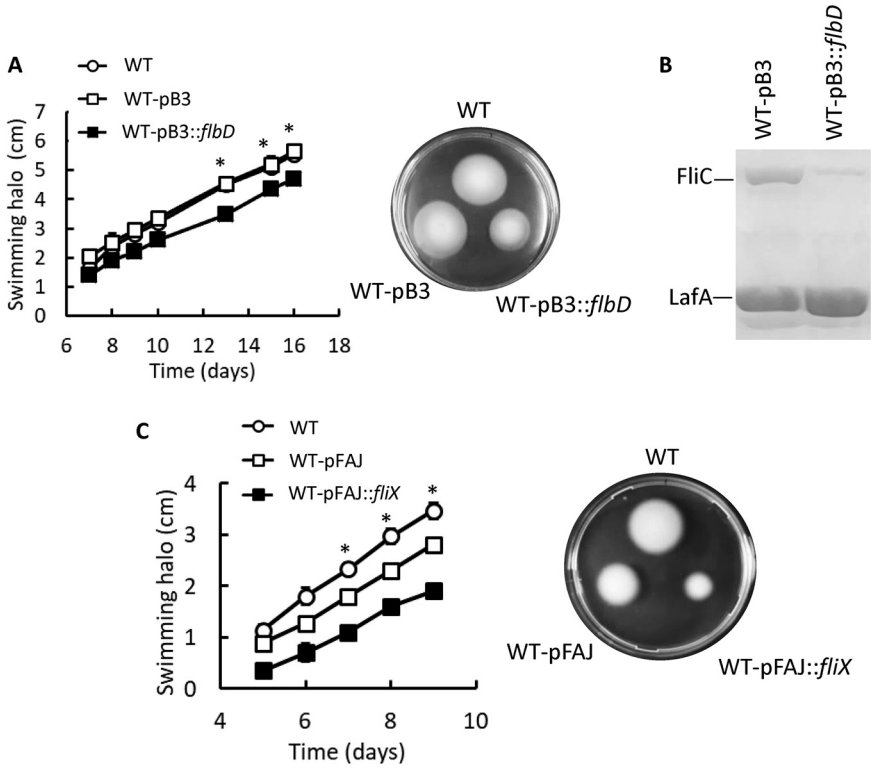

FIG 3 Overexpression of FlbD and FliX in the wild-type (WT) background. (A and C) Swimming assay in AG semisolid agar plates of the WT strain, the strain carrying the empty vector, and the strain overexpressing *flbD* (A) or *fliX* (C). Shown are averages ± standard deviations from three replicates of one representative experiment (error bars not shown are smaller than the symbols). Asterisks indicate significant differences by analysis of variance ($P < 0.05$) between the WT and WT-pB3 strains and the WT-pB3::*flbD* strain in panel A or among the three strains in panel C. (B) SDS-PAGE of the total amounts of extracellular flagellins obtained from 30 ml of PSY-Ara with WT-pB3 or WT-pB3::*flbD* at the same OD (OD_{500}, 3).

the transcriptional control of the genes that encode the hook is exerted on a single paralog. The *flgDE* operon in cluster 3, which encodes an extra copy of part of the hook, had similar expression in the wild type and the Δ*flbD* mutant.

Within the third group (underlined in Table 1), two operons encoded structurally related components that showed differences in transcription levels between the Δ*flbD* mutant and the wild type. Part of the cytoplasmic ring (the *fliFGH* operon in cluster 6) was positively regulated, while part of the secretion system (the *fliIJ* operon in cluster 2) was under negative regulation.

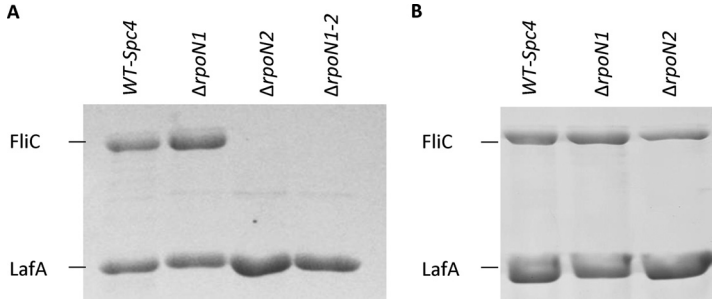

FIG 4 Roles of the two RNAP-σ^{54} factors in flagellar synthesis. (A) SDS-PAGE of extracellular flagellins of the wild-type strain (WT-*Spc4*) and the three derived mutants (the Δ*rpoN1* and Δ*rpoN2* mutants and the Δ*rpoN1-2* double mutant) in aerobic culture. (B) SDS-PAGE of extracellular flagellins obtained under anoxic growth conditions.

TABLE 1 Changes in the transcription levels of different subpolar flagellar genes in the Δ*flbD* and *fliX*::Km mutants relative to those in the wild-type strain[a]

Flagellar component(s)	Locus tag	Gene measured (operon gene[s])	Relative expression (fold change [mean ± SEM])	
			Δ*flbD* mutant	*fliX*::Km mutant
Export apparatus	blr2201	***fliI*** (*fliJ*)	2.29 ± 0.08	NC
	bll2207	***flhA***	NC	NC
	blr5816	*fliO* (***fliP***)	NC	NC
Motor/C-MS rings	bll5826	***fliL*** (*fliM*)	NC	NC
	blr5838	***flgI*** (*flgJ*-Hypoth.)	NC	NC
	blr6999	***fliF*** (*fliGH*)	−3.08 ± 0.07	NC
	blr3800	***pomA*** (*pomB*)	NC	NC
P-L rings/rod	blr5827	***flgF*** (*flgGAH*)	−55.95 ± 38.72	−13.95 ± 0.48
	bll5814	***flgB*** (*flgC-fliE*)	NC	NC
Hook	blr3696	***fliD*** (*fliS*)	NC	
	blr3699	***flgD*** (*flgE*)	NC	
	bll5854	***flgE*** (*flgKL*)	−36.11 ± 19.64	−31.13 ± 12.60
	blr6996	***fliK*** (*flgD*)	−41.25 ± 5.95	−55.30 ± 29.44

[a]Two independent RT-qPCR assays with three technical replicates were performed, and the results were analyzed using the $\Delta\Delta C_T$ method. Target genes that were measured by RT-qPCR are shown in boldface, and genes in parentheses were part of the same putative operon. Genes in group 2 are shaded, and genes in group 3 are underlined. NC, no change; Hypoth., hypothetical protein.

The *flbD* and *fliX* mutations do not affect bacterial growth but enhance the synthesis of LafA flagellins and the expression of the lateral flagellum master regulator *lafR*. Considering that cytokinesis defects have been reported in *flbD* and *fliX* mutants in *C. crescentus* (53), we analyzed the growth kinetics and morphology of both the Δ*flbD* and *fliX*::Km mutants. The results showed no differences in growth rate or cell elongation from the wild type, since no filamentous structures were detected (see Fig. S4A and B in reference 20).

As we mentioned above, LafA flagellins recovered from the culture medium of the *flbD* mutant seemed to be substantially more abundant than those of the wild type. In agreement with this observation, the quantification of electrophoretic band densities in the Δ*flbD* mutant relative to those in the wild type showed 2.1-fold-higher density of the lateral flagellin polypeptide band and 9.5-fold-higher expression of the *lafR* transcript (Fig. 5A). On the other hand, the lack of the master regulator of lateral flagella (Δ*lafR* mutant) did not induce changes in the amount of subpolar flagellins (FliC1 to FliC4 [FliC1-4]) (Fig. 5B).

***flbD* could be involved in the regulation of EPS production and biofilm formation.** The Δ*flbD* mutant showed higher mucoid secretion than the wild type when grown on yeast extract-mannitol (YEM) agar plates supplemented with Congo red (CR), suggesting that EPS synthesis might have been exacerbated (Fig. 6A). To confirm this phenotype, EPS production was quantified by precipitation from liquid culture. As shown in Fig. 6B, EPS production by the Δ*flbD* mutant was 2.5-fold more abundant than that by the wild type. EPS production by the *fliX*::Km mutant was not significantly different from that by the wild-type strain, even though there seemed to be a small increase. To go further, we used Calcofluor staining and a CR binding assay to characterize the EPSs produced by the different strains. In agreement with the results presented above, the Δ*flbD* mutant showed a 1.25-fold increase in relative absorbance with the CR binding assay, greater than that of the wild type (see Fig. S5A and B in reference 20) (54). In contrast, Calcofluor staining showed a decrease in fluorescence for the Δ*flbD* mutant (Fig. 6C), suggesting a change in EPS composition (55). These effects were not observed in the *fliX*::Km mutant (Fig. 6C) (see Fig. S5A and B in reference 20). To evaluate whether biofilm formation was affected in these class II mutants, we measured the adhesion of bacteria to glass tubes. As can be seen in Fig.

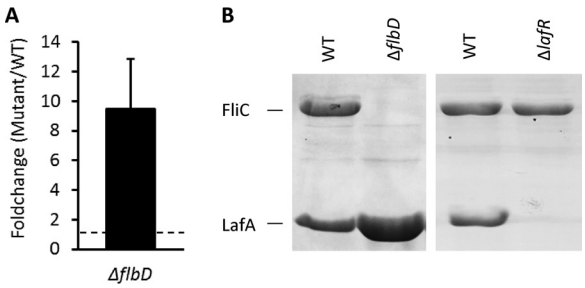

FIG 5 Role of FlbD in lateral flagellar synthesis. (A) Relative quantification of the mRNA of the lateral flagellar master regulator LafR by use of the $\Delta\Delta C_T$ method. The value represents the mean of the fold changes ± SEM for two independent assays (each with three technical replicates). (B) SDS-PAGE of extracellular flagellins in the wild-type strain and the two regulatory-gene mutants (the Δ*lafR* mutant and the Δ*flbD* mutant).

6D, the Δ*flbD* and *fliX*::Km mutants showed delayed biofilm formation under the conditions tested.

The phenotype of symbiosis with soybean plants was not affected in the Δ*flbD* or *fliX*::Km mutant. Since EPSs have an influence on early symbiosis with soybean plants, a nodulation assay was conducted to test whether the association of the Δ*flbD* or *fliX*::Km mutants with soybean plants was altered. No differences were observed in the number of nodules per plant or the dry weight of shoots among plants inoculated with either mutant and those inoculated with the wild type (data not shown).

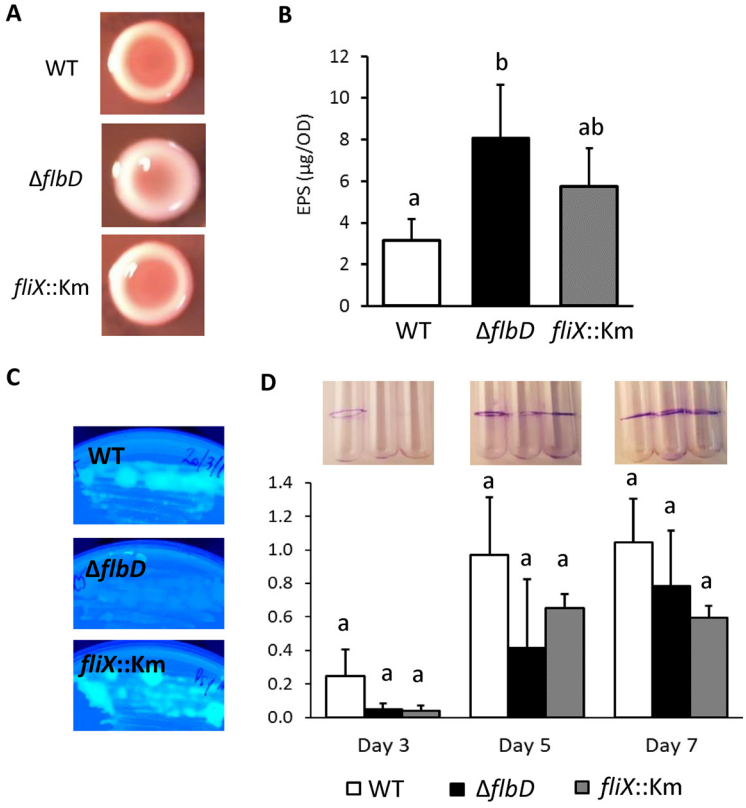

FIG 6 EPS synthesis and biofilm formation in the Δ*flbD* and *fliX*::Km mutants. (A) Colony morphologies of strains grown on YEM agar plates supplemented with Congo red. (B) EPS quantification using glucose as the standard. Bars represent means ± standard deviations from three independent assays. Different letters indicate statistically significant differences ($P < 0.05$) according to analysis of variance. (C) Calcofluor binding assay in PSY-Ara plates. (D) (Bottom) Biofilm formation (expressed as the mean absorbance at 595 nm ± standard deviation), determined by measuring bacterial adhesion in glass tubes on days 3, 5, and 7. (Top) Each tube in the photographs corresponds to the bar directly below it (showing the result for a particular strain) and is representative of three replicates.

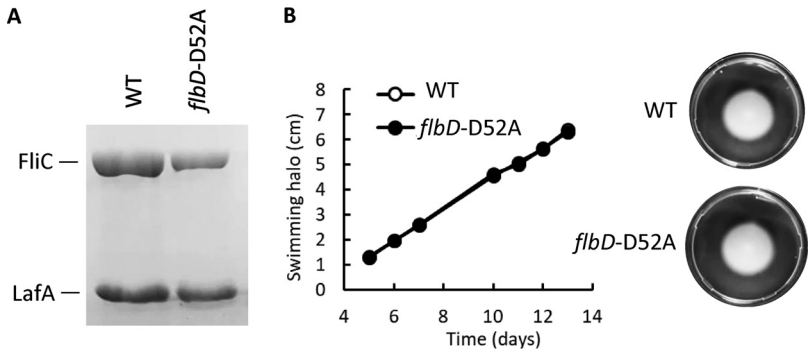

FIG 7 Flagellin expression and swimming performance in the *flbD*-D52A mutant. (A) SDS-PAGE of extracellular flagellins of the wild-type (WT) strain and the *flbD*-D52A mutant. (B) Diameters of swimming halos of the WT and the D52A mutant recorded over time in AG semisolid agar plates. (Left) Data points on the graph are means ± SEM from three replicates of one representative experiment (error bars not shown are smaller than the symbol; open and filled symbols overlap). (Right) Representative swimming plates on day 10 of incubation.

FlbD did not need phosphorylation in the conserved D52 residue. Although FlbD has an atypical REC domain, it conserves the aspartic acid residue (D52) that is susceptible to phosphorylation. In *C. crescentus*, this aspartic acid residue is phosphorylated *in vitro*, and its replacement by alanine (D52A) produces a mutant that is unable to complement the motility defect in *flbD* mutants (56). However, the role of D52 *in vivo* is still unclear (43). To determine whether this posttranslational modification in FlbD was necessary for motility in *B. diazoefficiens*, we generated a D52A mutant and tested its ability to swim and produce flagellins. We found that this mutant was able to synthesize both subpolar and lateral flagellins and that its swimming performance was not affected (Fig. 7). Together, these results indicated that D52 was not required for motility. Nevertheless, in *B. diazoefficiens*, other aspartic residues conserved in FlbD homologs are present at positions 47 and 56 near the conserved D52 (see Fig. S6 in reference 20), and they could be implicated in phosphorylation.

DISCUSSION

In this work, we obtained evidence in support of the hypothesis that the synthesis of the subpolar flagellum is regulated similarly in *B. diazoefficiens* and *C. crescentus*. In agreement with observations in the latter species and *E. meliloti* (35, 48), we could not suppress CtrA function, suggesting that it might be essential in *B. diazoefficiens*. Nevertheless, more studies will be needed to corroborate this hypothesis.

After CtrA activation, the class II regulators FlbD and FliX continue the flagellar regulatory cascade (25, 26). Our results indicated that *B. diazoefficiens* FlbD activated the transcription of σ^{54}-dependent flagellar genes using RpoN2 in aerobiosis and either RpoN1 or RpoN2 in low-oxygen environments. In addition, motility control by FlbD would not depend on the posttranslational modification in the conserved aspartic acid residue D52.

In *C. crescentus*, FlbD activity depends on the interaction with FliX, a small protein that is proposed to modulate FlbD promoter activity by acting as a checkpoint between early assembly products and middle-stage flagellar gene transcription (26, 32, 34, 43, 57). Here, we observed that two flagellar gene clusters seemed to be controlled by FlbD in a FliX-independent way. Genes encoding the MS ring (*fliFGH*) were regulated in a positive manner, while *fliIJ* genes were negatively regulated. This effect might be associated with a fine-tuning control mechanism mediated by FlbD. In this sense, FliX would probably redirect FlbD to middle-stage gene promoters when the early basal body products are assembled. In order to clarify the role of FliX in the basal body checkpoint, it will be necessary (i) to identify the mechanism by which FliX is able to sense the state of construction of the flagellum and (ii) to determine which other

proteins might be associated with this process (26). It should be noted that FlbD and FliX have control over only one of the operons that encode the hook (*flgE* in cluster 5 and *flgD* in cluster 6). The paralogous copies of *flgDE* (in cluster 3) showed no changes in their transcription levels. We might consider this cluster a nonfunctional gene duplication, although these copies could be necessary in other situations not tested here. As an example, the *motCD* genes in *Pseudomonas* are involved in swarming motility and swimming near liquid-solid interfaces, but they are not used for swimming in the bulk liquid (58, 59).

There were no differences in growth behavior or cell morphology between the Δ*flbD* or *fliX*::Km mutants and the wild type, suggesting that cell cycle regulation was not affected. Instead, a possible connection with other regulatory pathways was found. One such connection might be related to the transition between motile and sessile lifestyles. Besides the importance of flagella for motility in the planktonic state, the control of their expression is very important during biofilm formation. In *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*, slowed-down flagellar rotation can serve as an initial signal to start the transition to surface living (2, 60–62). In *Agrobacterium tumefaciens*, functional flagella would be necessary to initiate the first steps in biofilm formation (63). Furthermore, in *Aeromonas* spp., flagella function primarily as adhesins (64). During biofilm development, flagellar synthesis is transcriptionally halted, and synthesis is reinitiated during the senescence stage to enable escape from the matrix (1). Hence, in these two states, motile and biofilm-associated regulons are expressed in opposite manners (2). In some bacteria, this counterregulation is exerted by the same regulator, activating certain genes and inhibiting others. In *P. aeruginosa*, the flagellum master regulator FleQ, which is a σ^{54}-dependent EBP like FlbD, also controls EPS synthesis (65, 66). The second messenger c-di-GMP, which is involved in the switch between motile and sessile lifestyles, binds directly to the AAA+ domain of FleQ as part of this network signaling (66–69). In *B. diazoefficiens*, inactivation of the regulatory protein FlbD impaired subpolar flagellum synthesis and at the same time increased EPS production, similarly to FleQ in *P. aeruginosa*. In the Δ*flbD* mutant, not only did the amount of EPS produced increase, but its composition seemed to change. According to P. Wood (55), some (1→3)-β-D-glucans showed a strong interaction with CR and a weak interaction with Calcofluor. We might speculate that the lack of the FlbD regulator may repress the synthesis of a new cryptic polysaccharide. Besides that, biofilm formation was delayed in both class II mutants (the Δ*flbD and fliX*::Km mutants), suggesting a role for subpolar flagella as adhesins in the early steps of biofilm formation in *B. diazoefficiens* (12) or maybe in the surface-sensing process (2, 62).

In addition to impairing subpolar flagellar synthesis, the mutation of *flbD* increased the expression of the lateral flagellar system at the level of transcription of its master regulator *lafR* and stimulated the production of lateral flagellins. Although each flagellar system has its own regulatory circuit, we found some evidence of a connection between the regulatory pathways of the two flagellar systems through FlbD. In contrast to the effects of *flbD* mutation, the mutation in the lateral flagellar master regulator *lafR* did not affect subpolar flagellin synthesis, suggesting unidirectional cross-regulation, from the subpolar to the lateral flagellar system.

In some way, the subpolar flagellum, which appears to be constitutively expressed, might partially repress the lateral system under conditions that allow the simultaneous expression of both appendages. This might be related to the balance of energy expenditures under such conditions and indicates a close relation of the two flagellar systems in *B. diazoefficiens*. Furthermore, a role for the subpolar system as a mechanosensor cannot be ruled out, since the lack of its function could be related to a missing signal and therefore to derepression of the lateral flagellar system.

The existence of coordination in the regulation of the two systems might be a suitable explanation for the maintenance of the lateral flagellar system along the evolution of the *B. japonicum* phylogroup.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this work are summarized in reference 20, Table S1. *Bradyrhizobium diazoefficiens* was grown routinely in solid yeast extract-mannitol (YEM) (70) at 28°C. For swimming assays and flagellin extraction, the strains were grown in arabinose-gluconate-supplemented HM salts (AG). The composition of AG was the same as that of HMY-Ara, published previously (19), except for using arabinose (1 g · liter^{-1}) and gluconate (1 g · liter^{-1}) as the carbon sources. For RNA extraction, exopolysaccharide (EPS) quantification, and biofilm assays, bacteria were grown in peptone-salt-yeast extract supplemented with 1% (wt/vol) arabinose (PSY-Ara) (19, 71). Anoxically conditioned cultures were prepared according to the method of Jiménez-Leiva et al. (72) by using AG as the culture medium but replacing NH_4Cl with 10 mM KNO_3 (73). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (74) at 37°C.

Antibiotics were added at the following concentrations: 150 and 25 μg ml^{-1} kanamycin (Km), 100 and 10 μg ml^{-1} gentamicin (Gm), and 100 and 10 μg ml^{-1} tetracycline (Tc) for *B. diazoefficiens* and *E. coli*, respectively; 200 μg ml^{-1} ampicillin (Ap) for *E. coli*; and 20 μg ml^{-1} chloramphenicol (Cm) for *B. diazoefficiens*.

Cloning procedures. All the cloning procedures, including DNA isolation, digestion, ligation, and strain transformation, were carried out as described previously (74).

The oligonucleotide primers used for cloning procedures and mutant check-up were designed using Primer-BLAST and are listed in reference 20, Table S2. DNA was amplified by PCR with *Taq* or *Pfu* DNA polymerase (Productos Bio-Lógicos, Quilmes, Argentina). Electroporation was performed with a Gene Pulser (Bio-Rad, Hercules, CA) at 1.5 V, 25 μF, and 200 Ω in a 0.1-cm-gap-width electroporation cuvette. *Escherichia coli* S17-1 was used for biparental matings with *B. diazoefficiens* USDA 110 as described previously (74). DNA and/or plasmid samples were sequenced by Macrogen Corp. (Seoul, South Korea).

To construct the Δ*flbD* mutant, a 1,395-bp fragment from *B. diazoefficiens* USDA 110 (positions 7711305 to 7712680 according to the GenBank database [27375111]) was amplified using primers 7003FwE and 7003RvH. The fragment was purified, digested with the EcoRI and HindIII restriction enzymes, and cloned into pK18*mobsacB* to obtain the pK*sacB*::*flbD* construct. This plasmid was digested with the AvaI restriction enzyme (whose restriction sites are inside the *flbD* coding sequence, surrounding the AAA+ domain [see Fig. S2 in reference 20) and was religated to obtain the final plasmid pK*sacB*::Δ*flbD*, carrying the *flbD* fragment with the desired deletion (bp 7711926 to 7712280). This construct was electroporated into *E. coli* S17-1 and was moved by mating into *B. diazoefficiens* USDA 110. Single recombinants were obtained by selecting Km-resistant colonies and were checked by PCR. The double-crossover event was forced by the addition of 10% (wt/vol) sucrose. The resistant clones were selected and checked using E7003Fw and E7003Rv, which are external primers of the recombination regions. The clones that amplified the 1,297-bp fragment were chosen, and the PCR products were sequenced to corroborate the deletion.

To construct the *fliX*::Km mutant, a 1,001-bp blunt-ended fragment from the *B. diazoefficiens* USDA 110 genome (bp 6404484 to 6405484) was amplified using primers 5837Fw and 5837Rv. The fragment was first digested with EcoRV and then cloned into pBlueScript SK(+). Only the construct with the PCR product cloned in the same orientation as the P*lac* promoter was named pBS::*fliX* and was selected for further work. This plasmid was digested with EcoRI and HindIII, and the fragment was subcloned into pG18*mob2* for pG::*fliX* construction. Finally, the Km-resistant gene (previously digested from the pUC4K plasmid by using BamHI) was inserted into the same restriction site present in the *fliX* insert of pG::*fliX*, yielding the pG::*fliX*::Km vector. This plasmid was moved by mating into *B. diazoefficiens* USDA 110. Transconjugants were selected by Km resistance and Gm sensitivity on YEM agar plates. The mutation was confirmed by PCR with an external primer from the recombination zone and a primer that hybridized in the Km cassette (E5837Fw/KmFw and E5837Rv/KmRv). These products were confirmed by sequencing.

To complement the mutations in *trans*, the complete *flbD* and *fliX* genes were amplified with *Pfu* DNA polymerase (Thermo Fisher Scientific, Waltham, MA). The *flbD* gene was cloned into the pBBR1MCS3 plasmid under the control of the P*lac* promoter in the PstI-XbaI restriction sites, producing the pB3::*flbD* plasmid. The *fliX* gene was subcloned from pBS::*fliX* with its own promoter into pFAJ1708 in the XbaI-KpnI restriction sites to yield the pFAJ::*fliX* plasmid. The constructs were moved into the corresponding mutant strains by biparental mating.

The single-nucleotide directed mutation in the triplet that encodes the conserved residue susceptible to phosphorylation (Asp52) (see Fig. S2 in reference 20) was performed as described previously (29), with minor modifications. PCR primers were designed over the mutation site with a single base mutation to change the GAC codon, which codes for Asp, to GCC, which codes for Ala. These primers were named 7003D52AFw and 7003D52ARv. Then a fusion PCR protocol was followed (75). For PCRs 1 and 2, primers 7003FwE/7003D52ARv and 7003D52AFw/7003RvH were used. PCR 3 was carried out using the PCR 1 and 2 products as templates, along with primers 7003FwE and 7003RvH. The 1,395-bp PCR 3 product with the single-base substitution in the Asp52 residue was cloned into the pK18*mobsacB* plasmid, producing pK*sacB*::*flbD*-D52A. Because the position of the point mutation overlapped with a SalI restriction site in the wild-type sequence, the clones were screened by digestion with this endonuclease. Clones resistant to digestion were then corroborated by DNA sequencing. This plasmid was transferred by mating to the wild-type strain, and simple crossovers were selected by Km resistance. Resolution of the plasmid was forced by plating the Km-resistant colonies in YEM agar supplemented with 10% (wt/vol) sucrose. The resulting clones were corroborated by PCR amplification, SalI digestion of the fragment, and sequencing.

Swimming assays. Semisolid AG plates (25 ml of 0.3% [wt/vol] agar in 90-mm plates) were inoculated with sterile toothpicks from solid cultures in three separate plates and were incubated at 28°C. The motility halo diameter was recorded for 2 weeks. At the same time, the strains were inoculated in the same agar plate and were photographed.

Flagellin purification and analysis. For flagellin preparation, *B. diazoefficiens* was grown in AG or PSY-Ara liquid medium (30 ml) until the stationary phase (optical density at 500 nm [OD_{500}], 3) was reached. Supernatants were precipitated as described elsewhere (11), and each sample was resuspended in the same volume. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (76). For relative quantification, the total amount of purified flagellins extracted from the culture medium at the same OD was loaded into the SDS-PAGE gel, and the intensity of the band was estimated using ImageJ (19).

RNA extraction, cDNA synthesis, and reverse transcription-quantitative PCR (RT-qPCR) assays. For expression analysis, the PSY-Ara bacterial culture was harvested at an OD_{500} of 0.7 and was cooled immediately. RNA and cDNA samples were prepared as described previously (19, 30).

The primers designed for RT-qPCR are listed in reference 20, Table S2. The *sigA* gene was used as the endogenous control. The iQ SYBR green Supermix (Bio-Rad, Hercules, CA) was used as an indicator of DNA amplification. Fluorescence was detected with qTOWER, version 2.2 (Analytik Jena, Jena, Germany), and was analyzed using the $\Delta\Delta C_T$ method (77). The fold change was calculated as Rq_{mut}/Rq_{wt}, where Rq_{mut} represents $2^{-\Delta\Delta CT}$ for the mutant strain and Rq_{wt} represents $2^{-\Delta\Delta CT}$ for the wild-type strain, with three technical replicates. The results were reported as averages ± standard errors of the means (SEM) from at least two independent assays, each with three technical replicates.

EPS quantification, Calcofluor staining, and CR binding assay. PSY-Ara cultures were harvested at the stationary phase (OD_{500}, 3). EPS was purified and quantified as described previously (78). For quantification, the anthrone method was used (0.2% [wt/vol] anthrone in 96% [wt/vol] sulfuric acid), with glucose (1 mg ml^{-1}) as the standard.

Calcofluor brightness was tested in PSY-Ara agar plates supplemented with 0.02% (wt/vol) Calcofluor white. The plates were incubated for 7 days at 28°C and were irradiated with UV light for photo shooting. The CR binding assay was performed according to the method of Spiers et al. (54) with minor modifications. Bacteria were grown in PSY-Ara to the exponential phase (OD_{500}, 1), and three 5-μl drops were cultured in PSY-Ara agar plates for 3 days at 28°C. Then the drops were resuspended in 1 ml of CR solution (0.005% [wt/vol]). After 2 h of incubation at 37°C, the cells were separated by centrifugation, and the absorbance of the supernatant was measured at 490 nm. The relative absorbance (Abs_{rel}) of each mutant was calculated as $Abs_{mutant}/Abs_{wild\ type}$.

Biofilm assays (adhesion to glass tubes). Biofilm formation was estimated by measuring the bacterial rings formed on the walls of glass tubes. Briefly, PSY-Ara-grown strains (OD_{500}, 0.08) were inoculated in test tubes and were incubated at 100 rpm for 3, 5, and 7 days at 28°C. After incubation, the tubes were washed, and the bacteria that adhered to the glass were stained with 0.1% (wt/vol) crystal violet for 30 min. The tubes were washed; the materials from bacterial rings were suspended in 33% (vol/vol) acetic acid; and the absorbance at 595 nm was measured.

Microscopy analysis. Cells were heat-fixed and stained with 0.1% (wt/vol) crystal violet for 15 min. Slides were washed and dried for optical microscopy. Images were taken at ×1,000 using the Nikon Eclipse E400 microscope equipped with a Nikon Coolpix 4500 digital camera.

Plant assay. DonMario 4800 soybean seeds were surface-sterilized by immersion in 96% (vol/vol) ethanol for 5 s and then in 20% (vol/vol) commercial bleach for 10 min, followed by six washes in sterile distilled water. Seeds were germinated on 1.5% (wt/vol) aqueous agar in the dark at 28°C. The nodulation assay was carried out in pots filled with perlite as support, which were initially watered with a mineral solution (Fåhraeus). After 21 days of culture in a chamber at 26°C with a 16-h photoperiod, the total number of nodules and the dry weight of the aerial part per plant were determined.

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