Transcriptional Organization of the Region Encoding the Synthesis of the Flagellar Filament in *Pseudomonas fluorescens*[†]

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***Pseudomonas fluorescens* F113 is motile by means of type b flagella. Analysis of the region encoding the synthesis of the flagellar filament has shown a transcriptional organization different from that of type a flagella. Additionally to the promoters driving *fliC*, *fliD*, and *fleQ* expression, we have found promoters upstream of the *flaG* gene and the *fliST* operon. These promoters were functional in vivo. Both promoters have been mapped and appear to be dependent on the vegetative sigma factor and independent of FleQ, the master regulator of flagellum synthesis.**

Pseudomonads are ubiquitous bacteria that can adapt to different lifestyles, with species that are either saprophytic or pathogenic for plants and animals. Motility is an important trait for pseudomonads, and it has been shown to be involved in rhizosphere colonization (7, 13, 14, 18), biofilm formation (9, 20, 27), and pathogenesis in plant (10, 22) and animal (4) models. Flagellar biosynthesis in pseudomonads is regulated in a hierarchical way, with the transcriptional activator *fleQ* on top of the regulatory cascade (2, 8). Besides, the alternative sigma factors encoded by *fliA* and *rpoN* are required for flagellum assembly (25, 26). The regulation of flagellar biosynthesis has been analyzed using DNA microarrays and *Pseudomonas aeruginosa* PAK (12), which contains type a flagella (24). Other pseudomonads, like *Pseudomonas fluorescens* F113 or *P. aeruginosa* PAO1, contain type b flagella and are characterized by different synteny in the region encoding the formation of the flagellar filament (7, 12, 24). In type b strains, this region contains *fliC*, encoding type b flagellin; *flaG*, encoding a protein of unknown function implicated in filament length (7); *fliD*, encoding the flagellar-cap protein (3); *fliS*, encoding a FliC chaperone required for flagellin export and assembly (7); *fliT*, encoding a protein of unknown function required for full motility and rhizosphere colonization in *P. fluorescens* F113 (7); *fleQ* (2, 8), encoding the master regulatory protein; and *fleSR*, encoding a two-component system required for flagellar biosynthesis (23).

Analysis of the transcriptional organization of a region implicated in the synthesis of the flagellar filament in *P. fluorescens* F113. In order to establish the transcriptional organization of the *fliC-fleQ* region of *Pseudomonas fluorescens* F113, the upstream regions of the *fliC*, *flaG*, *fliS*, *fliT*, and *fleQ* genes were amplified and cloned into the reporter vector pMP220 to form transcriptional fusions with a promoterless *lacZ* gene. These constructs were introduced into strain F113 by triparental

mating, and β -galactosidase activity was tested (19). Substantial activity was shown for the regions upstream of all genes (125 to 350 Miller units), except the region upstream of the *fliT* gene (<5 Miller units) (see Fig. S1 in the supplemental material). Previous studies of other pseudomonads have shown the existence of promoters upstream of the *fliC*, *fliD*, and *fleQ* genes. To our knowledge, no promoters have been found upstream of the *flaG* or *fliS* gene. These results indicated a monocistronic organization for the *fliC*, *flaG*, and *fliD* operons and the existence of a bicistronic *fliST* operon. To confirm this organization, primers for the coamplification of adjacent genes in this region were designed (Fig. 1A) from the genomic sequences of strain F113 and *P. aeruginosa* PAO1. PCR products of the expected sizes were obtained for all primer pairs from genomic DNA from strains F113 and PAO1 (data not shown). Total RNA from F113 and PAO1 was subjected to reverse transcription (RT)-PCR using these primers. In the case of F113 (Fig. 1B), only the *fliS-fliT* primer pair yielded an RT-PCR product, confirming the transcriptional organization defined by *lacZ* fusions. However, in the case of *P. aeruginosa* PAO1 (Fig. 1C), RT-PCR products were obtained with the *fliC-flaG* and *fliS-fliT* pairs, indicating that in *P. aeruginosa*, *fliC*, *flaG*, and *fliST* are transcribed as bicistronic operons.

Analysis of the *flaG* and *fliST* promoters of *Pseudomonas fluorescens* F113. An analysis of the transcriptional organization of the flagellum synthesis region of *P. fluorescens* F113 revealed the presence of two previously unknown promoters controlling the transcription of the *flaG* gene and the *fliST* operon. In order to confirm the functionality in vivo of these two promoters, we used a complementation assay of *flaG* and *fliS* F113 mutants. Genomic DNA fragments containing each of the *flaG* and *fliS* genes with their putative promoter regions (as identified in the *lacZ* fusion assays) were cloned into the pFAJ1700 vector, which contains transcriptional terminators flanking both sides of the insertion site. Figure 2 shows the results of the complementation assay. Introduction of a cloned *fliS* gene under the control of its putative promoter restores wild-type motility to the nonmotile *fliS* mutant (7), demonstrating in vivo the functionality of the *fliS* upstream region as a promoter. Similarly, the introduction of a cloned *flaG* gene

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FIG. 1. RT-PCR determination of putative promoters in the flagellar-synthesis region. (A) Physical map of the region in type b pseudomonads. Numbered bars indicate the expected fragments amplified with each primer pair. Each fragment partially spans two adjacent genes. (B) Results from RT-PCR amplification from *P. fluorescens* F113 RNA. (C) Results from RT-PCR amplification from *P. aeruginosa* PAO1 RNA. DNA controls from both strains yielded the expected amplified fragments from all the primer pairs. Total RNA was extracted according to the instructions of the manufacturer (Roche Boehringer Mannheim) from *P. fluorescens* strains grown at 28°C in LB medium (5). RT-PCRs were carried out by using a one-step RT-PCR kit from Amersham Biosciences according to manufacturer's specified running conditions and by using a melting temperature of 59°C. Primers used are indicated in Table S1 in the supplemental material. The presence of DNA contamination was discarded by running a PCR under the conditions indicated above for the RNA samples. Strains, plasmids, and primers used are described in Table S1 in the supplemental material.

under the control of its own putative promoter reverts the hypermotility phenotype of a *flaG* mutant (7) to the wild-type level of motility, demonstrating that the upstream region of the *flaG* gene also contains a functional promoter.

In order to analyze the structure of the newly described promoters, rapid amplification of 5' cDNA ends (5'RACE) was performed. As shown in Fig. 3A, a single transcription start site was detected for the *flaG* transcript in the intergenic *fliC*-*flaG* region, 75 bp upstream of the translation start codon. Upstream of this transcriptional start site, a putative region containing -10 and -35 sites was observed. This region lies within the *fliC* coding sequence. Figure 3B shows the *fliST* promoter region. 5'RACE detected a single transcription start site in the *fliD*-*fliS* intergenic region, 55 bp upstream of the *fliS* translational start codon. A putative -10/-35 region was also observed for this promoter. The existence of these boxes, together with the lack of putative *fliA* and *rpoN* boxes, suggests that these two promoters use the housekeeping σ^{70} factor for their expression.

Regulatory analysis of the *flaG* and *fliST* operons in *Pseudomonas fluorescens*. To analyze the regulation of the two operons, we tested their expression in different genetic backgrounds by RT-PCR. Expression was analyzed in the wild-type strain and isogenic strains containing mutations in the master

FIG. 2. In vivo determination of *flaG* and *fliST* promoters in *P. fluorescens* F113. The *flaG* and *fliS* genes with their upstream regions were cloned into the pFAJ1700 vector, which contains transcriptional terminators flanking both sides of the insertion site. The constructs were used to complement the swimming motility phenotypes of *flaG* and *fliS* mutants, after introduction of the constructs by triparental mating. Swimming assays of organisms on LB medium were performed as previously described (7). (A) F113; (B) F113(pFAJ1700); (C) F113 *fliS* mutant; (D) F113 *fliS* mutant (pfliS); (E) F113 *flaG* mutant; (F) F113 *flaG* mutant (pflaG). Strains, plasmids, and primers used are described in Table S1 in the supplemental material.

regulatory gene *fleQ* and in the genes encoding the alternative sigma factors required for flagellar biosynthesis, *fliA* and *rpoN*. The expression of the *fliC* gene, encoding flagellin, was also monitored in the same backgrounds. As shown in Fig. 4, *fliC* expression is strictly dependent on *fleQ*, *fliA*, and *rpoN*. The requirement of these genes for *fliC* expression has already been shown for *P. aeruginosa* (12, 25, 26). Conversely, the expression of *flaG* and *fliST* was observed in all backgrounds, indicating that the expression of these genes does not require FleQ or the alternative sigma factor. This result and the presence of putative -10 and -35 boxes in the promoter regions strongly indicate that these promoters are dependent on the vegetative sigma factor. However, some effect of these regulatory proteins on their transcriptional regulation cannot be excluded. Since *fleQ* is also σ^{70} dependent in *P. aeruginosa* (11), it is likely that in *P. fluorescens*, *flaG* and *fliST* are at the same hierarchical level as *fleQ*. Previous studies of *P. aeruginosa* PAK, a type a strain, indicated that *fliC* and *flaG* (*fleL*) were cotranscribed (12). Here we have shown that this is also true for the b-type strain *P. aeruginosa* PAO1 but not for *P. fluorescens* F113. The lack of amplification by RT-PCR of a band spanning both genes and the in vivo complementation of a *flaG* mutant by a DNA fragment containing the *flaG* promoter clearly show that in *P. fluorescens* F113, *flaG* expression is totally independent of *fliC* expression. Similarly, in *P. aeruginosa* PAK14, *fliS* and *fliT* (*fleP*) seemed to be cotranscribed from a FleQ-dependent promoter upstream of *fliD* (12). Here, we present evidence that this is not the case in b-type strains. The results presented here show that flagellar biosynthesis is

FIG. 3. Physical map and sequences of the *fliG* and *fliST* promoters in *P. fluorescens* F113 as determined by 5'RACE. 5'RACE was performed using the RACE second-generation kit (Roche Applied Science). Primers used in each amplification are specified in Table S1 in the supplemental material. cDNA containing the transcription initiation site was subcloned into pDRIVE (Qiagen), and at least three clones were sequenced for the mapping of each promoter using universal primers. Putative -10 and -35 regions are boxed. Arrows indicate the determined transcription start site. The numbering corresponds to the sequence AF399739, deposited in GenBank. Strains, plasmids, and primers used are described in Table S1 in the supplemental material.

regulated differently in different *Pseudomonas* strains and even in strains containing the same type of flagella. Furthermore, genes implicated in important functions of flagellar synthesis, such as the regulation of flagellar length and flagellin secretion, are outside the regulatory four-tiered circuit and appear to depend on housekeeping regulation. The regulation of flagellar genes outside the typical hierarchy is uncommon but has been observed for other bacteria. In *Salmonella enterica* serovar Typhimurium, expression of the *flk* gene, encoding a regulator of flagellar-gene expression (1), was shown to be independent of the master regulatory factors FlhDC and did not require the σ^{28} factor (16). In *Caulobacter crescentus*, an operon containing the *flgBC* and *fliE* genes has been shown to

be regulated outside of the flagellar regulatory hierarchy (6). Finally, in several spirochetes, such as *Borrelia burgdorferi* (15), *Leptospira borgpetersenii* (17), and *Spirochaeta aurantia* (21), important genes implicated in flagellum synthesis and encoding flagellins and hook-associated proteins require a vegetative σ^{70} factor.

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FIG. 4. Expression analysis of the *fliG* and *fliST* promoters. Total RNA (10 ng) from F113 (lane 1), the F113 *fleQ* mutant (lane 2), the F113 *fliA* mutant (lane 3), and the F113 *rpoN* mutant (lane 4) were subjected to RT-PCR with primers to amplify the *fliG* and *fliST* fragments. 16S RNA RT-PCR was used as a control. RT-PCR conditions were as described for Fig. 1. The strains, plasmids, and primers used are described in Table S1 in the supplemental material.

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