Journal of Bacteriology, Aug. 1996, p. 5017–5019
0021-9193/96/$04.00+0 Vol. 178, No. 16
Copyright © 1996, American Society for Microbiology

Expression of the Structural Gene, *laf1*, Encoding the Flagellin of the Lateral Flagella in *Azospirillum brasilense* Sp7

SARA MOENS,[1] MICHAEL SCHLOTER,[2] AND JOS VANDERLEYDEN[1]*

F. A. Janssens Laboratory of Genetics, Katholieke Universiteit Leuven, B-3001 Heverlee, Belgium,[1] and GSF-Forschungszentrum für Umwelt und Gesundheit, GmbH, Institut für Bodenökologie, Gesellschaft für Strahlenforschung-Forschungszentrum für Umwelt und Gesundheit, 85758 Oberschleißheim, Germany[2]

Received 4 April 1996/Accepted 6 June 1996

The induction of the lateral flagella of *Azospirillum brasilense* Sp7 was studied by using a translational fusion between the *laf1* promoter and *gusA*. The fusion was induced when cells were grown on solid media but not when they were grown in broth. The fusion was also induced by incubation of liquid-grown cells with an anti-polar flagellum polyclonal antiserum. Hindrance of polar-flagellum rotation is suggested to be the signal for this induction.

Azospirillum brasilense is a nitrogen-fixing soil bacterium that colonizes plant roots (16). It displays mixed flagellation: one polar flagellum is present under all growth conditions, while synthesis of lateral flagella is induced when cells are grown on solid media (18). The polar flagellum is used for locomotion in liquid media (swimming); the lateral flagella are involved in swarming, which can be described as active surface spreading (3, 5). For a long time, swarming behavior was considered to be a trait of only a few bacterial species, but recently this phenomenon has been observed for many more species (4). In many cases, only one type of flagellum is observed on both swimmer and swarmer cells. The induction of lateral flagella and swarming has been studied best in *Vibrio parahaemolyticus*, a bacterium that also shows a mixed flagellation pattern (9).

Previously, the gene encoding the lateral flagellin, *laf1*, was isolated and a knock-out mutant was constructed (14). In this paper, we report on the construction of a translational *laf1*::*gusA* fusion and its use to study the induction of *laf1* gene expression. Unless stated otherwise, the materials and methods used in this communication are as described previously (14).

Construction and expression of pFAJ0210. A translational fusion between the *laf1* promoter region and the reporter gene *gusA* was constructed (Fig. 1A). A 0.8-kb *Nae*I fragment, containing the *laf1* promoter and the 5′ end of *laf1*, was cloned into the *Sma*I site of a pUC18 derivative containing a promoterless *gusA* gene (available in our laboratory; derived from pBI101.3 [7]). Restriction analysis was used to check the orientation of the insert. DNA sequence analysis of the fusion, starting from a *gusA* primer, confirmed that the fusion was in frame (Fig. 1B). This construct was then cloned as an *Eco*RI-*Hin*dIII fragment in the broad-host-range vector pLAFR3, yielding pFAJ0210. This plasmid was transferred to *Escherichia coli* S17-1, from which it was mobilized to *A. brasilense* Sp7.

Sp7(pFAJ0210) was tested for β-glucuronidase activity after growth in broth or on an agar surface. The β-glucuronidase activity of Sp7(pFAJ0210) was compared quantitatively (using the substrate *p*-nitrophenyl-β-D-glucuronide [6]) with that of Sp7(pFAJ31.13). pFAJ31.13 contains a fusion of a constitutive *Azospirillum* promoter and *gusA* (19). It is clear from Table 1 that the pFAJ0210 fusion is induced only on the agar surface. Expression was also monitored qualitatively using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc). Sp7(pFAJ0210) colonies turned blue on plates containing X-Gluc that were solidified with agar at concentrations ranging from 1.5 to 0.3% (wt/vol). With 0.4% agar, the cells spreading into the medium also became blue, while with 0.3% agar, only surface-grown cells became blue. The cells in the 0.3% agar medium remained white. This indicates that at agar concentrations lower than 0.4%, the spreading in the medium is mainly due to swimming.

Madi and colleagues (8) observed lateral flagella on *A. brasilense* Cd grown in static liquid conditions. We were not able to detect β-glucuronidase activity in *A. brasilense* Sp7 harboring pFAJ0210 when this organism was grown under the same static liquid conditions. However, Sp7(pFAJ31.13) also did not give measurable β-glucuronidase activity under these conditions, suggesting that the density of the cell culture grown under such conditions is too low for measurement of β-glucuronidase activity.

Induction of pFAJ0210 with an anti-polar flagellum polyclonal antiserum. Previously, the production of a polyclonal antiserum against the polar flagellum of *A. brasilense* Sp7 was described (1). This antiserum, when added to a broth culture, induces formation of rosettes of a few cells and groups of many cells bound together at one pole. This can be observed microscopically (reference 1 and unpublished observations). Overnight incubation of broth-grown cells of Sp7(pFAJ0210) with various dilutions of this antiserum resulted in induction of β-glucuronidase activity up to a dilution of 1:100. Incubation with the preimmune serum did not induce this activity (Fig. 2). Incubation with a monoclonal antibody recognizing an outer membrane protein of *A. brasilense* Sp7 (17) also did not induce β-glucuronidase activity (data not shown). The polyclonal antiserum, which is capable of tethering cells by their polar flagella, is specifically inducing transcription of the *laf1* gene. The same phenomenon was observed earlier for *V. parahaemolyticus* (9). Increasing the viscosity of the medium and tethering the cells with a polar-flagellum antiserum very likely have the same effect on the polar flagellum: rotation becomes more difficult. Therefore, it seems that in *A. brasilense*, the signal of impaired rotation sensed by the polar flagellum is transduced

* Corresponding author. Mailing address: F. A. Janssens Laboratory of Genetics, Katholieke Universiteit Leuven, Willem de Croylaan 42, B-3001 Heverlee, Belgium. Phone: (32) 16 322403. Fax: (32) 16 322990. Electronic mail address: Janssens@agr.kuleuven.ac.be.

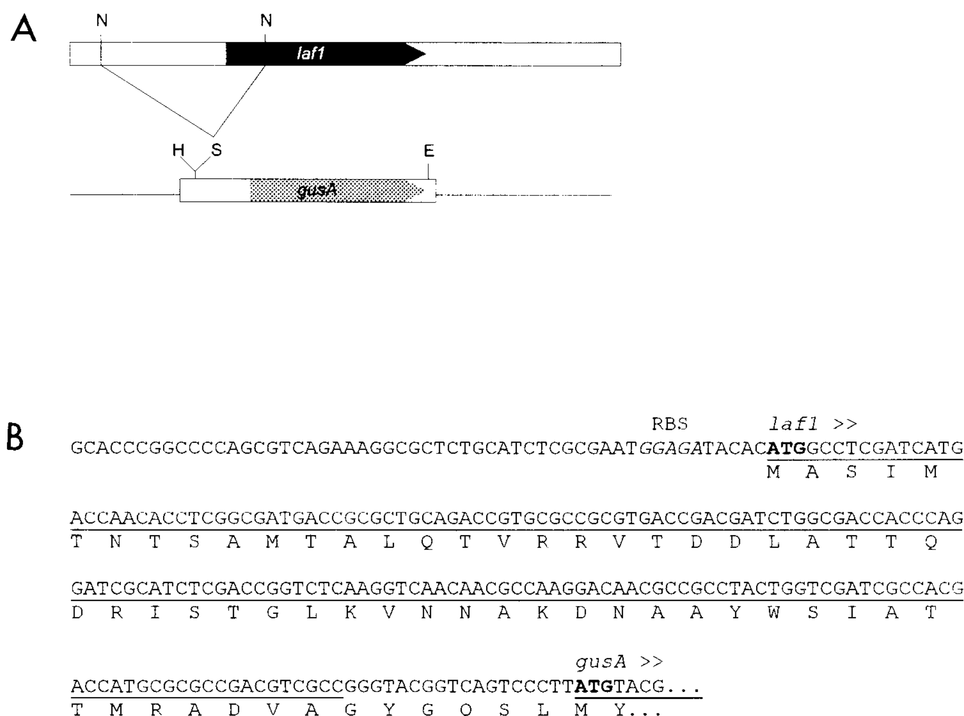

FIG. 1. (A) Construction of pFAJ0210. N, *Nae*I; H, *Hin*dIII; S, *Sma*I; E, *Eco*RI. (B) The fusion portion of pFAJ0210: DNA sequence and amino acid sequence of the fusion protein. The start codons of *laf1* and *gusA* are indicated in boldface, and the amino acids resulting from translation of *laf1* and *gusA* are underlined. RBS, ribosome binding site.

in the cell and leads to transcription of the structural gene for the lateral flagellin.

Induction of pFAJ0210 with WGA and other lectins. The polar flagellum was shown to be involved in an early step in wheat root colonization in vitro, the reversible adsorption phase (1, 10). The flagellin of the polar flagellum, Fla1, was shown to be a glycoprotein (15). It was therefore hypothesized that the adsorption could be mediated by the lectin wheat germ agglutinin (WGA), which is known to be expressed on the surface of wheat roots (13) and to bind to the cell surface of *A. brasilense* (2). When Sp7(pFAJ0210) was incubated with various concentrations of WGA, no induction of β-glucuronidase activity was observed (data not shown). Addition of WGA to broth-grown cells does not induce agglutination of cells, in contrast to what was observed microscopically after addition of the polyclonal antiserum. This could suggest either that WGA does not bind the sugar residues on Fla1 and that the adsorption step is not mediated by Fla1-WGA binding or that binding of WGA does not impair rotation of the polar flagella.

A number of other lectins with various sugar specificities were also assayed for their ability to induce β-glucuronidase activity in Sp7(pFAJ0210). None of these lectins caused induction of this activity (data not shown).

Expression of pFAJ0210 in *A. brasilense* mutants. Knocking out genes that encode the polar flagellum in *V. parahaemolyticus* results in induction of lateral flagella. For *A. brasilense*, no polar-flagellum genes have yet been identified, but all the available Tn*5*-induced mutants lacking the polar flagellum also lack the lateral flagella. Although we do not know the identities of the genes mutated in the flagellation mutants, introduction of pFAJ0210 into some of these mutants failed to show expression of the *laf1*::*gusA* fusion.

An *ntrA* mutant of Sp7 lacks both polar and lateral flagella (11). pFAJ0210 was also mobilized into this mutant, and β-glucuronidase activity of the plasmid-carrying mutant was quantitatively compared with that of Sp7(pFAJ0210). It is clear from Table 1 that expression of the fusion is many times lower in this nonflagellated mutant. It is not yet known how *ntrA* exerts its influence on the expression of flagellar genes, but transcription of *laf1* is blocked in an *ntrA* mutant.

TABLE 1. Induction of β-glucuronidase activity in various strains grown in broth and on agar

Strain	β-Glucuronidase activity[a] after growth	
	In broth	On agar surface (1.5% agar)
Sp7(pFAJ0210)	29.67 (1.48)	204.60 (3.56)
Sp7(pFAJ31.13)	289.29 (4.27)	200.89 (3.49)
Sp7	17.74 (1.65)	0
Sp7*ntrA*(pFAJ0210)	ND[b]	4.04 (0.23)

[a] β-Glucuronidase activity was measured using the substrate *p*-nitrophenol-β-D-glucuronide as described previously (6). Units were calculated as defined by Miller for β-galactosidase activity (12). Values obtained represent the means of at least three measurements; standard deviations are given in parentheses.

[b] ND, not determined.

This work was supported by grants from the Fonds voor Geneeskundig Wetenschappelijk Onderzoek (F.G.W.O. no. 3.0095.93) and the Flemish government (G.O.A. 1993). S.M. is a recipient of a fellowship from the Nationaal Fonds voor Wetenschappelijk Onderzoek (N.F.W.O.).

The lectins were kindly provided by W. Peumans.

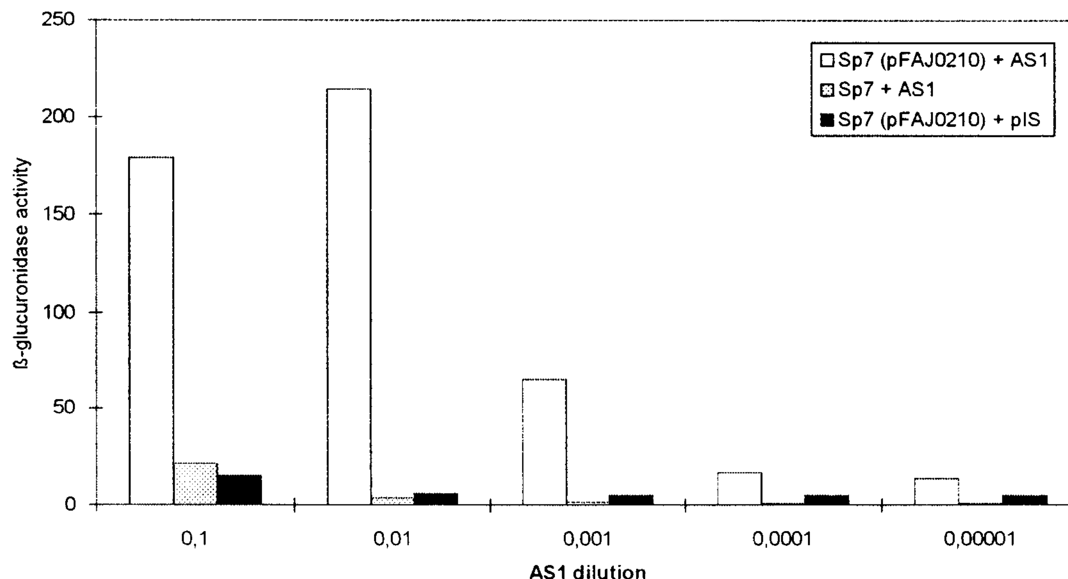

FIG. 2. Induction of pFAJ0210 with the polyclonal antiserum against the polar flagellum (AS1) and the preimmune serum (pIS). β-Glucuronidase activity was measured using the substrate *p*-nitrophenol-β-D-glucuronide as described previously (6). Units of activity were calculated as defined by Miller for β-galactosidase activity (12).

REFERENCES

1. **Croes, C. L., S. Moens, E. Van Bastelaere, J. Vanderleyden, and K. W. Michiels.** 1993. The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. J. Gen. Microbiol. **139:**960–967.
2. **Del Gallo, M., M. Negi, and C. A. Neyra.** 1989. Calcofluor- and lectin-binding exocellular polysaccharides of *Azospirillum brasilense* and *Azospirillum lipoferum*. J. Bacteriol. **171:**3504–3510.
3. **Hall, P. G., and N. R. Krieg.** 1983. Swarming of *Azospirillum brasilense* on solid media. Can. J. Microbiol. **29:**1592–1594.
4. **Harshey, R. M.** 1994. Bees aren't the only ones: swarming in Gram-negative bacteria. Mol. Microbiol. **13:**389–394.
5. **Henrichsen, J.** 1972. Bacterial surface translocation: a survey and a classification. Bacteriol. Rev. **36:**478–503.
6. **Jefferson, R. A.** 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. **5:**387–405.
7. **Jefferson, R. A.** 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6:**3901–3907.
8. **Madi, L., M. Kessel, E. Sadovnik, and Y. Henis.** 1988. Electron microscopic studies of aggregation and pellicle formation in *Azospirillum* spp. Plant Soil **109:**115–121.
9. **McCarter, L., M. Hilmen, and M. Silverman.** 1988. Flagellar dynamometer controls swarmer cell differentiation of *V. parahaemolyticus*. Cell **54:**345–351.
10. **Michiels, K. W., C. L. Croes, and J. Vanderleyden.** 1991. Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. J. Gen. Microbiol. **137:**2241–2246.
11. **Milcamps, A., A. Van Dommelen, J. Stigter, J. Vanderleyden, and F. J. de Bruijn.** 1996. The *Azospirillum brasilense rpoN* gene is involved in nitrogen fixation, nitrate assimilation, ammonium uptake and flagellar biosynthesis. Can. J. Microbiol. **42:**467–478.
12. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 354–358. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Mishkind, M., N. V. Raikhel, B. A. Palevitz, and K. Keegstra.** 1982. Immunocytochemical localization of wheat germ agglutinin in wheat. J. Cell Biol. **92:**753–764.
14. **Moens, S., K. Michiels, V. Keijers, F. Van Leuven, and J. Vanderleyden.** 1995. Cloning, sequencing, and phenotypic analysis of *laf1*, encoding the flagellin of the lateral flagella of *Azospirillum brasilense* Sp7. J. Bacteriol. **177:**5419–5426.
15. **Moens, S., K. Michiels, and J. Vanderleyden.** 1995. Glycosylation of the flagellin of the polar flagellum of *Azospirillum brasilense* Sp7, a Gram-negative nitrogen-fixing bacterium. Microbiology **141:**2651–2657.
16. **Okon, Y.** 1994. *Azospirillum*/plant root associations. CRC Press, Boca Raton, Fla.
17. **Schloter, M., S. Moens, C. Croes, G. Reidel, M. Esquenet, R. De Mot, A. Hartmann, and K. Michiels.** 1994. Characterization of cell surface components of *Azospirillum brasilense* Sp7 as antigenic determinants for strain-specific monoclonal antibodies. Microbiology **140:**823–828.
18. **Tarrand, J. J., N. R. Krieg, and J. Döbereiner.** 1978. A taxonomic study of the *Spirillum lipoferum* group with description of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. **24:**967–980.
19. **Vande Broek, A.** 1994. Histochemical and genetic analysis of the *Azospirillum brasilense*-wheat root association. Ph.D. thesis. Katholieke Universiteit Leuven, Leuven, Belgium.