APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1987, p. 1959–1961
0099-2240/87/081959-03$02.00/0

Vol. 53, No. 8

Transposon Mutants of *Bradyrhizobium japonicum* Altered in Attachment to Host Roots†

STEPHEN J. VESPER,[1]* NASIR S. A. MALIK,[1] AND WOLFGANG D. BAUER[2]

Battelle-Kettering Laboratory, Yellow Springs, Ohio 45387,[1] and Department of Agronomy, Ohio State University, Columbus, Ohio 43210[2]

Received 19 March 1987/Accepted 24 May 1987

Transposon mutants of *Bradyrhizobium japonicum* 110 ARS were produced and screened for changes in attachment ability. Mutant CFK4 produced twice as many piliated cells, attached in 2.5-fold-higher numbers to soybean root segments, and colonized roots in about 2-fold-higher numbers than did the parental strain, 110 ARS. Mutants CFK35 and CFK38 were reduced in their attachment about 2-fold and 3.5-fold, respectively. This corresponded to reductions in piliated cells in their populations, reduced reaction with anti-pilus antiserum, and reduced hydrophobic attachment. Mutants CFK4 and CFK38 nodulated soybeans at about the same level as the parent strain, but CFK35 induced only pseudonodules. Two-dimensional gel analyses of the proteins from the mutants showed relatively few changes in proteins.

For many years, it has been known that some gram-negative bacteria bind to animal hosts by means of pili (fimbriae) (2, 3). Only recently has the role of pili in the attachment of bacteria to plant roots been established (5), including the firm attachment of *Bradyrhizobium japonicum* to soybean roots (12). Although the genetic control of pilus formation by certain animal pathogens has received extensive analysis (4, 7–9), little is known about the genetic control of pilus formation by bacteria attaching to plants.

B. japonicum USDA I-110 ARS (resistant to azide, rifampin, and streptomycin) was provided by L. D. Kuykendall, U.S. Department of Agriculture, Beltsville, Md. *Escherichia coli* SM10 was provided by A. Puhler, Bielefeld University, Bielefeld, Federal Republic of Germany. *E. coli* HB101 pRK2013 was provided by S. Long, Stanford University, Palo Alto, Calif. Transposon mutagenesis was done as described by So et al. (J. So, A. L. M. Hodgson, R. A. Haugland, M. Leavitt, Z. Falui, A. J. Nieuwkoop, and G. Stacey, Mol. Gen. Genet., in press). Mutants were screened for changes in attachment ability by three techniques: a qualitative microscopic assay (11), an enzyme-linked immunosorbent assay with anti-pilus antiserum (12), and hydrophobic attachment to polystyrene (12).

Manipulations of DNA such as total DNA purification, restriction endonuclease digests, Southern blotting, and hybridizations were done essentially as described by Maniatis et al. (6). Nick translation was done by using a nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as described in the instructions of the manufacturer.

Nodulation tests were done as previously described (1). Nodules produced by mutants CFK4 and CFK38 were examined after 1 week by making longitudinal sections through a random group of nodules and examining them with a dissecting microscope. Since no nodules were produced by CFK35, the swellings produced by CFK35 were examined after 3 weeks of growth.

Cultures of each mutant and the parent were harvested for two-dimensional gel electrophoresis at an A_{620} between 0.1 and 0.2 as described earlier (12). The bacterial cells were suspended, at 4°C, in a 5-ml extraction solution containing 4% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 5% sucrose, and 24 mg of insoluble polyvinylpyrrolidone. The bacterial cells were then broken by using a French pressure cell (American Instruments Co., Silver Spring, Md.), and the proteins were precipitated with acetone containing 10 mM 2-mercaptoethanol. The protein pellet was dissolved in isoelectric focusing loading solution containing 9.5 M urea, 5% 2-mercaptoethanol, and 2% ampholytes (LKB, Bromma, Sweden).

The bacterial proteins were separated by two-dimensional gel electrophoresis as described by Malik (N. S. A. Malik, Physiol. Plant., in press). The protein spots on the second-dimension slab gel were detected by silver staining (13). The assays used to measure attachment were as described earlier (11). Enzyme-linked immunosorbent assays were done as described earlier by using either anti-pilus antiserum (12) or whole-cell antiserum. Transmission electron microscopic observations and counts were made as described earlier (12).

Colonization was determined in a mixture of vermiculite and sandy loam soil (2:1) moistened with a quarter-strength Jensen plant nutrient solution (2:1 [wt/vol]), giving 95% field capacity. This material was thoroughly mixed, added to stainless steel pans (30 by 24 by 15 cm), and sterilized. Soybean seeds [*Glycine max* (L.) Merr. cv. Williams] were surface sterilized and germinated as described previously (1). Thirty 1-day-old seedlings were selected for uniformity and placed in 1.5-cm depressions in the amended soil, inoculated with 0.1 ml of an inoculum of approximately 10^9 cells per ml.

Three days after inoculation, the seedlings were gently removed from the growth medium, placed in 700 ml of sterile distilled water in bottles (Wheaton Industries, Millville, N.Y.), and gently shaken to remove soil particles. The seedlings were then transferred to another Wheaton bottle containing 700 ml of water and rinsed by vigorously shaking the bottles for 10 s. The seedlings were then placed in a sterile pan and grouped for uniformity in size. Three groups of 6 to 10 uniform (±0.5 cm) roots (between 8 and 11 cm) were cut into 2-cm sections starting from the root tip. The sections from each group were combined and placed in 2 cm of water plus Tween 20 (1 drop per 100 ml). The roots were sonicated to release attached bacteria, and the released

* Corresponding author.
† Contribution C-923 of the Battelle-Kettering Laboratory.

TABLE 1. Attachment of parent and mutants to soybean roots as determined by plate count and microscopic assays

Strain	Plate count assay (cells attached/segment)[a]	Quantitative microscopic assay (cells attached)[b]
110 ARS	$5.3 \times 10^2 \pm 0.2$	1.0
CFK4	$1.3 \times 10^3 \pm 0.1$	2.2
CFK35	$3.0 \times 10^2 \pm 0.1$	0.13
CFK38	$1.5 \times 10^2 \pm 0.05$	0

[a] Results are the averages ± standard deviation of three to six experiments with an average of three groups of 10 segments per treatment for each experiment. Results were normalized to an initial inoculum of 10^6 cells per ml.

[b] Because the mutants were tested on different days (always with a 110 ARS control), each mutant is compared on a relative basis with the 110 ARS control, which is given the value 1.

bacteria were enumerated as described previously (11). The number of bacteria attached per gram (wet weight) of root was calculated.

About 10,000 Tn*5* mutants of 110 ARS were generated for this study, and about 1,200 were randomly selected for testing. Three mutants, CFK4, CFK35, and CFK38, were found to differ from the parental strain in attachment ability. These mutants were confirmed to be 110 serogroup with an enzyme-linked immunosorbent assay by using 110 ARS antiserum (data not given). They were shown to contain a single Tn*5* insertion by Southern blot hybridizations with a Tn*5* probe (10).

Mutant CFK4 attached in twofold-higher numbers than the parental strain (Table 1), and this correlated with an increase in the subpopulation with pili (Table 2). CFK38 cells attached in about 3.5-fold-lower numbers than did 110 ARS (Table 1). The reduced attachment ability was correlated with reduced levels of piliated cells, a reduction in reaction to the anti-pilus antiserum, and reduced hydrophobicity (Table 2). No polar-attached cells were found attached to root hairs even after 6 h (Table 2). The population of CFK35 cells with pili was lower than the parental population (Table 1), and this correlated with reduced attachment and hydrophobicity (Table 2).

The inoculum dosage versus nodulation response of soybeans in growth pouches for CFK4 and CFK38 were similar to those obtained for 110 ARS (Fig. 1). These dosage-dependent nodulation curves are typical of strains of *B. japonicum* in growth pouches (T. V. Bhuvaneswari, personal communication). The CFK35 mutant failed to nodulate soybean plants. Only small swellings were occasionally produced, which even after 3 weeks failed to enlarge and develop. When these swellings were examined, they were white inside, whereas nodules produced by CFK4 and CFK38 had the typical pink color of *B. japonicum* nodules.

TABLE 2. Reaction of cultures with anti-pilus antiserum, culture hydrophobicity, and number of cells with pili for parent and mutant populations

Strain	ELISA[a]	Hydrophobicity[b]	% of cells with pili[c]
110 ARS	0.26 ± 0.03	20%[d]	5[d]
CFK4	0.24 ± 0.03	6.2 ± 0.8	9–13
CFK35	0.09 ± 0.02	10.3 ± 2.8	0–1
CFK38	0.07 ± 0.02	4.2 ± 1.1	0–1

[a] Results are average optical density ± standard deviation based on duplicate tests with three replicates each. ELISA, Enzyme-linked immunosorbent assay.

[b] Hydrophobicity was determined as previously described (12). The results are the averages ± standard deviation of two or three experiments with three replicate plates for each test.

[c] The range is given for random triplicate counts of 100 cells. All cultures examined were early log-phase (optical density between 0.1 and 0.2 at 620 nm).

[d] These estimates come from earlier work (12).

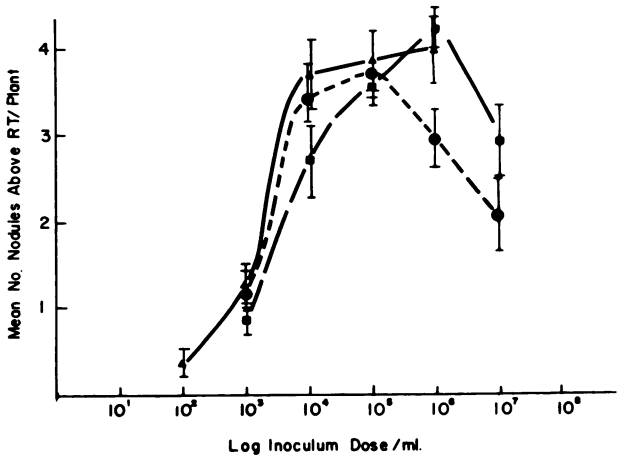

FIG. 1. Nodulation dose response of parent and mutants in growth pouches. Experimental protocol was as previously described (1). All values are the average ± standard error of at least 40 plants from two or three experiments. Symbols: ●, 110 ARS; ■, CFK4; ▲, CFK38.

Cells of mutant CFK4 were found colonizing most of the root surface in twice the numbers of the parent. Only the 2-cm section closest to the point of inoculation was equally colonized by mutant and parent. For every one cell of 110 ARS, piece 1 (first 2 cm from the root tip) possessed 2.6 ± 0.12 CFK4 cells, piece 2 had 1.7 ± 0.24 cells, piece 3 had 2.1 ± 0.22 cells, and piece 4 (closest to the site of inoculation) had 0.86 ± 0.02 CFK4 cells. These results represent the averages and standard deviations of two separate experiments with three replicate groups in each.

Proteins isolated from 110 ARS were compared with those from each mutant. Generally, only two to five protein losses or gains occurred as a result of the introduction of the transposon. These changes were usually in low-molecular-weight proteins.

In this report, we have shown that the percentage of piliated cells in a culture of *B. japonicum* can be increased or decreased by single Tn*5* insertions. These changes in piliated cell numbers correlate with the ability of these mutants to attach firmly to soybean roots. Mutant CFK4 attaches in greater numbers than its parent, 110 ARS, but nodulation is unaffected. However, colonization of soybean roots can be improved by increasing the population of *B. japonicum* cells which firmly attach. Mutant CFK38, which does not attach in a polar manner, still nodulates soybeans. It may be that polar attachment is not necessary for nodulation. Mutant CFK35, which does attach in a polar manner, does not nodulate soybeans. Thus, there is no simple relationship between firm attachment and nodulation. Characterization of these mutants and the protein changes observed should provide further insights into the genetic control of pili on *B. japonicum*.

This work was supported in part by grant DE-AC02-81ER10895 A003 from the U.S. Department of Energy.

LITERATURE CITED

1. **Bhuvaneswari, T. V., K. K. Mills, D. K. Crist, W. R. Evans, and W. D. Bauer.** 1983. Effects of culture age on symbiotic infectivity of *Rhizobium japonicum*. J. Bacteriol. **153**:443–451.
2. **Gaastra, W., and F. K. deGraaf.** 1982. Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. Microbiol. Rev. **46**:129–161.
3. **Jones, G. W., and R. E. Isaacson.** 1982. Proteinaceous bacterial adhesins and their receptors. Crit. Rev. Microbiol. **10**:229–260.
4. **Kehoe, M., R. Sellwood, P. Shipley, and G. Dougan.** 1981. Genetic analysis of K88-mediated adhesion of enterotoxigenic *Escherichia coli*. Nature (London) **291**:122–126.
5. **Korhonen, T. K., E. Tarkka, H. Ranta, and K. Haahtela.** 1983. Type 3 fimbriae of *Klebsiella* sp.: molecular characterization and role in bacterial adhesion to plant roots. J. Bacteriol. **155**:860–865.
6. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Maurer, L., and P. E. Orndorff.** 1987. Identification and characterization of genes determining receptor binding and pilus length of *Escherichia coli* type 1 pili. J. Bacteriol. **169**:640–645.
8. **Orndorff, P. E., and S. Falkow.** 1984. Organization and expression of genes responsible for type 1 piliation in *Escherichia coli*. J. Bacteriol. **159**:736–744.
9. **Orndorff, P. E., and S. Falkow.** 1984. Identification and characterization of a gene product that regulates type 1 piliation in *Escherichia coli*. J. Bacteriol. **160**:61–66.
10. **Regensburger, B., L. Meyer, M. Filser, J. Weber, D. Studer, J. W. Lamb, H.-M. Fisher, M. Hahn, and H. Hennecke.** 1986. *Bradyrhizobium japonicum* mutants defective in root-nodule bacteroid development and nitrogen fixation. Arch. Microbiol. **144**:355–366.
11. **Vesper, S. J., and W. D. Bauer.** 1985. Characterization of *Rhizobium* attachment to soybean roots. Symbiosis **1**:139–162.
12. **Vesper, S. J., and W. D. Bauer.** 1986. Role of pili (fimbriae) in attachment of *Bradyrhizobium japonicum* to soybean roots. Appl. Environ. Microbiol. **52**:134–141.
13. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**:197–203.