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A Hydrophobic Mutant of *Rhizobium etli* Altered in Nodulation Competitiveness and Growth in the Rhizosphere

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We isolated and characterized CE3003, a Tn*5*-induced mutant with altered colony morphology derived from *Rhizobium etli* CE3. CE3003 produced domed colonies and was highly hydrophobic as indicated by its ability to partition into hexadecane, whereas its parent produced flat colonies and was hydrophilic. On bean plants, CE3003 induced nodules and reduced acetylene. CE3003 and CE3 grew at similar rates when they were grown separately or together in culture medium or inoculated singly onto bean seeds. However, when they were mixed at a 1:1 ratio and applied to seeds, CE3003 achieved significantly lower populations than CE3 in the rhizosphere. Five days after coinoculation of CE3 and CE3003, the population of the mutant was less than 10% of the population of CE3 in the bean rhizosphere. To determine the nodulation competitiveness of the mutant, it was coinoculated with CE3 at various ratios at planting, and the ratio of the nodules occupied by each strain was determined 21 days later. A 17,000-fold excess of CE3003 in mixed inocula was required to obtain equal nodule occupancy by the two strains. A genomic library of strain CE3 was mobilized into CE3003, and we identified a cosmid, pRA3003, that restored the parental colony morphology and hydrophilicity to the mutant. Restoration of the parental colony morphology was accompanied by recovery of the ability to grow competitively in the rhizosphere and to compete for nodulation of beans. The data show an association between cell surface hydrophobicity, nodulation competitiveness, and competitive growth in the rhizosphere in mutant CE3003.

Bacteria of the genus *Rhizobium* induce the formation of nitrogen-fixing nodules on the roots of legumes, entering a symbiotic association that contributes to crop productivity and soil fertility. This process requires a coordinated exchange of signals between the plant and the bacteria (15, 21, 26, 35, 43), in which both the bacterial and plant cell surfaces are thought to participate.

The major barrier to increasing the benefit derived from symbiotic nitrogen fixation for agriculture is nodulation competitiveness, which is defined as the relationship between the ratio of strains in the inoculum and the ratio of nodules occupied by those strains (5, 51). Highly competitive indigenous strains of *Rhizobium* spp. present in agricultural soils often nodulate plants to the exclusion of inoculant strains that are superior in nitrogen fixation (25, 31, 53). Although it is the key determinant in the success of inoculants for agriculture and also has intrinsic value as a problem in microbial ecology, nodulation competitiveness has been the focus of relatively few mechanistic studies and is poorly understood.

Recent work has begun to focus on the genetic basis for nodulation competitiveness, resulting in the identification of genetic changes and gene products that affect competitiveness (7, 11, 45, 50). The cell surface appears to play a significant role in determining nodule occupancy. Mutant analyses suggest that exopolysaccharides (EPS) (12, 28, 38, 42, 54) and lipopolysaccharides (LPS) (33) affect the nodulation competitiveness of certain *Rhizobium* species. These surface molecules also are essential for induction of normal nodules by some *Rhizobium* species on their respective hosts (4, 13, 14, 17, 18, 22, 23, 34).

To understand the role of the cell surface in nodulation competitiveness of *Rhizobium etli* (46), we isolated and studied mutants with altered colony morphology. In this report, we describe the cell surface and symbiotic properties of a hydrophobic mutant of *R. etli* CE3, which retained the ability to induce nitrogen-fixing nodules but was substantially reduced in nodulation competitiveness and competitive growth on roots of common bean plants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids are listed in Table 1. Bacterial stock cultures were stored in 10% dimethyl sulfoxide at −80°C. *R. etli* strains (46) were grown in yeast extract-mannitol (YEM) (52) or tryptone-yeast extract (TY) (10), and colony morphology was evaluated on solid BSM medium (9). *Escherichia coli* strains were grown in LB medium (36). Antibiotics were added to media at the following concentrations: spectinomycin (Sp) and streptomycin (Sm), 200 μg/ml; kanamycin (Km), 200 and 25 μg/ml for *R. etli* and *E. coli*, respectively; erythromycin (Em), 30 μg/ml; and tetracycline (Tc), 20 μg/ml. All solid media contained 1.5% agar (Sigma Chemical Co., St. Louis, Mo.). Growth in culture of *R. etli* strains was described by enumerating bacteria growing in YEM by dilution plating. For observation of encapsulation, suspensions made from bacteria grown on plates were spread on microscope slides, air dried, and gently heat fixed. Bacterial smears were then stained with crystal violet and observed at a magnification of ×1,000.

Genetic and molecular techniques. Transposon Tn*5* was introduced into *R. etli* CE3 by triparental spot matings (47). Transconjugants were selected simultaneously for kanamycin resistance and visually screened for altered colony morphology on solid BSM medium with streptomycin. Km^r transconjugants arose at a frequency of 1.65×10^{-5} per recipient. Isolation of genomic DNA from *R. etli* (19) and plasmid DNA from *R. etli* and *E. coli* (30) and transformation of *E. coli* (36) were performed as described previously. The genomic library derived from *R. etli* CE3 was constructed by using standard

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genetic characteristics	Colony morphology[a]	Reference
R. etli			
CE3	Spontaneous Sm^r mutant of CFN42	Flat, mucoid	40
CE8	Spontaneous Em^r mutant of CFN42	Flat, mucoid	40
CE3003	*hyd-1*::Tn*5* Sm^r Km^r	Domed, mucoid	This study
CE3003(pRA3003)	CE3003 containing cosmid pRA3003; Sm^r Km^r Tc^r	Flat, mucoid	This study
E. coli			
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*) *U169*	NA[b]	27
LE392	F$^-$ *hsdR574*(r_K^- m_K^+) *supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55*	NA	39
Plasmids			
pGS9	p15A::Tn*5*	NA	47
pRK2013	Tra$^+$ Km^r	NA	24
pJB3	R68.45-derivative; Tc^r	NA	16
pLA2917	Km^r Tc^r broad-host-range cosmid vector	NA	1

[a] Colony morphology on YEM plates incubated for 3 days at 28°C.
[b] NA, not applicable.

protocols (36). Inserts of an average size of 26.7 ± 1.0 kb (n = 29), obtained by partial digestion of CE3 genomic DNA with *Sau*3AI, were introduced into the unique *Bgl*II site of cosmid vector pLA2917 (1). Restriction digests and agarose gel electrophoresis were performed by standard methods. For Southern blot analyses, blotting, nonradioactive labelling of probes, and detection of DNA were performed as described previously (38). To determine whether the mutant contained a single Tn*5* insertion, we used the Tn*5*-carrying plasmid pGS9 (Table 1) to probe Southern blots of genomic DNA of the mutants and CE3 digested with *Eco*RI, which does not cut within the transposon. The linkage between the transposon insertion and the altered colony morphology was assessed by the marker mobilization method of Diebold and Noel (22) with strain CE8 (Em^r Km^s; Table 1) as recipient.

Assay for hydrophobicity. Cell surface hydrophobicity was qualitatively assayed by a test for partitioning into hexadecane (44). Each bacterial culture grown in broth was mixed 1:1 (vol/vol) with hexadecane, and the mixture was agitated with a vortex mixer at maximum speed for 1 min. The resulting emulsion was allowed to separate for 1 h at room temperature, and the turbidities of the aqueous and organic phases were observed visually.

LPS analysis. The LPS in whole-cell extracts were examined by polyacrylamide gel electrophoresis (PAGE) and silver staining as described by Cava et al. (17). For treatment with proteinase K, the boiled cell suspensions were cooled to 60°C, and 0.2 mg of proteinase K per ml in Tris-EDTA (pH 8.0) was added. The mixture was incubated at 60°C for 1 h and centrifuged for 1 min to remove cell debris. The supernatants were assayed for 2-keto-3-deoxyoctulosonic acid by the method of Karkhanis et al. (32). Aliquots containing equal amounts of 2-keto-deoxyoctulosonic acid equivalents were subjected to electrophoresis in 12% acrylamide gels at 10 mA.

Measurement of EPS accumulation. Bacteria were grown in 3 ml of YEM broth without selective antibiotics at 28°C for 3 days with aeration. Each culture was then diluted with sterile distilled water to an optical density at 600 nm of 0.1, and 100 μl was spread onto YEM plates without antibiotics. After 3 days of incubation at 28°C, bacterial lawns from five plates were collected in a known volume of sterile distilled water. EPS determinations were made on these suspensions as described previously (38). Cell numbers were estimated by dilution plating onto YEM plates containing the appropriate antibiotics. Extracellular polysaccharide (i.e., EPS) collected from each strain was assayed for hexose by the phenol-sulfuric acid assay of Ashwell (3). EPS and hexose assays were conducted on three separate cultures of each strain, the results were analyzed by one-way analysis of variance (ANOVA), and the means were compared by the honest significant difference test at an α equal to 0.05. For nuclear magnetic resonance analysis, EPS from each strain was dialyzed for 24 h with four changes of deionized water and lyophilized again, and the final product was resuspended at 5 mg/ml in D_2O. ^{1}H-nuclear magnetic resonance spectra were obtained with a Bruker AM-500I Aspect 3000 spectrometer.

Preparation of bacterial inoculum for plants. Inocula were prepared as described previously (5, 38). One milliliter of inoculum was applied to the seed immediately after planting. Inocula for competition experiments contained between 10^7 and 10^8 cells per ml. Inocula for nodulation and acetylene reduction assays contained about 10^3 cells per ml. For the nodulation kinetics studies, inoculum (10^4 to 10^5 cells per ml) was applied to the radicle after the seedlings were transferred to the growth pouches.

Conditions for plant growth. Seeds of bean (*Phaseolus vulgaris* L.) cultivar Puebla 152 were used in all experiments and supplied by the Department of Horticulture, University of Wisconsin-Madison, EMBRAPA-CNPAF, Goiânia, Goiás, Brazil, or Escuela Agricola Panamericana, El Zamorano, Honduras. For competition, nodulation, and acetylene reduction assays, surface-disinfected seeds were planted in sterilized sand and vermiculite (1.5:1 [vol/vol]) in glass tubes (2, 5). Plants were maintained in a growth chamber as described previously and watered with sterile nitrogen-free plant nutrient solution as needed (5).

Roots were tested for nitrogen fixation (29) and examined for nodules 28 days after planting and inoculation of seeds. The results were analyzed by one-way ANOVA, and the treatment means were compared by the honest significant difference test at an α of 0.05.

For assays of nodulation competitiveness, seeds were planted in glass tubes and inoculated with mixtures of parent and mutant bacteria of at least five different ratios. Twenty-one days after inoculation, the strains occupying the nodules were identified on the basis of antibiotic resistance as described by

Beattie and Handelsman (6). The nodulation competitiveness of each strain was determined by plotting its proportional representation in the nodules as a function of the inoculum ratios applied to the seeds at planting. The intersections of the curves in the competition plots indicate the inoculum ratio necessary for equal representation of the strains in the nodules. Two strains are considered equally competitive when they occupy equal proportions of nodules at an inoculum ratio of 1:1. Competitiveness indices (CIs) were calculated on the basis of the mathematical model derived by Beattie et al. (5) that relates the ratio of competing strains in a mixed inoculum to their ratio in the nodules:

$$\log\left[\frac{P_{\text{Parent}} + P_{\text{Both}}}{P_{\text{Mutant}} + P_{\text{Both}}}\right] = CI_{\text{Parent:Mutant}} + k \log\left[\frac{I_{\text{Parent}}}{I_{\text{Mutant}}}\right]$$

where P_{Parent} is the proportion of nodules occupied by only the parent strain, P_{Mutant} is the proportion of nodules occupied by only the mutant, P_{Both} is the proportion of nodules doubly occupied, $I_{\text{Parent}}/I_{\text{Mutant}}$ is the ratio of the parent to the mutant in the inoculum, $CI_{\text{Parent:Mutant}}$ is the CI, and k is a constant (the slope of the line). A CI of 0 is derived when the parent and the mutant occupy equal proportions of the nodules at an inoculum ratio of 1:1, and it is characteristic of a mutant that is equal in competitiveness to its parent.

For studies of the kinetics of nodulation, 10 growth pouches per strain (Northrup King, Inc., Minneapolis, Minn.) were planted with 2-day-old bean seedlings (5). Nodules were counted daily under a dissecting microscope for 15 days after inoculation. At each time point, the average numbers of nodules induced by each strain were compared by one-way ANOVA and by the honest significant difference test at an α of 0.05. Nodulation rates were compared by regression analysis.

Bacterial growth in the rhizosphere. Two-day-old broth cultures grown in YEM were diluted with sterile distilled water to a concentration of 10^5 cells per ml. Surface-disinfected bean seeds were placed on top of the sterilized sand and vermiculite mixture in glass tubes. One milliliter of inoculum consisting of a single strain or a mixture of two strains at a 1:1 ratio was applied to the seed, and then the seed was pushed into the mixture of sand and vermiculite. Inoculated tubes were maintained in a growth chamber as described above. The germinating seed and its roots were removed from four tubes at each sampling time, and each seedling was placed in 9 ml of sterile water and sonicated in a model 2200 ultrasonic bath (Branson Ultrasonics Corp., Danbury, Conn.) for 30 s. After the seedlings had emerged, the aerial parts were aseptically removed before the root systems were transferred into sterile water for sonication. Suspensions were then serially diluted and plated onto YEM plates containing the appropriate antibiotics to determine bacterial populations on the root and seed.

RESULTS

Isolation and characterization of mutant CE3003. We screened Tn*5* mutants of *R. etli* CE3 for altered colony morphology. We found only one mutant, designated CE3003, with a domed colony morphology (Table 1; Fig. 1) among 2,000 Km^r transconjugants screened. We designated the mutation in CE3003 *hyd-1*::Tn*5*. In the same group of Tn*5* mutants, nonmucoid, EPS-deficient mutants were detected at a frequency of 6.5×10^{-3} per Km^r transconjugant. The cells of CE3003 were highly hydrophobic since they partitioned to hexadecane in a hexadecane separation test (Fig. 2). CE3 and CE3003 did not differ in exponential growth rate when they were grown separately or mixed in broth culture, although CE3 achieved a higher population size in the late stationary phase (Fig. 3).

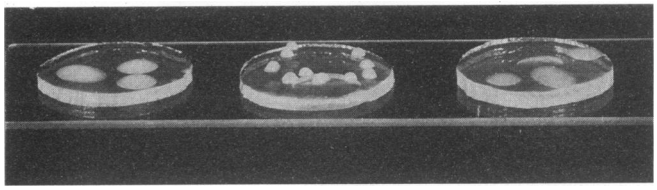

FIG. 1. Colony morphology of *R. etli* CE3 (left), CE3003 (center), and CE3003(pRA3003) (right) on YEM plates.

Southern blot analysis of genomic DNA indicated that CE3003 contained a single transposon insertion, and the transposon was integrated into a 1.8-kb *Eco*RI restriction fragment. When the transposon was transferred to *R. etli* CE8 with the mobilizing plasmid pJB3, each of 1,107 Km^r colonies had the domed colony morphology, suggesting that the transposon insertion was closely linked to the determinant of altered colony morphology. A genomic library derived from strain CE3 and constructed in the vector pLA2917, which confers Tc^r, was introduced into CE3003. Two of 500 Tc^r transconjugant colonies had the flat colony morphology characteristic of CE3. One of the two colonies with restored colony morphology was streaked for purity and stored for further analysis, and its cosmid was designated pRA3003. Loss of the cosmid by the restored mutant when it was grown in the absence of tetracycline selection was accompanied by the reappearance of the mutant phenotype, indicating that the cosmid pRA3003 was responsible for restoration of the parental phenotype.

pRA3003 did not alter the colony morphology of CE3 nor did it restore the parental colony morphology to a collection of 19 nonmucoid mutants of CE3 that were deficient in EPS (data not shown). Southern blot analysis indicated that pRA3003 contained an insert of 22 kb. In *Eco*RI digests of total genomic DNA from CE3 and CE3003 probed with pRA3003, a fragment present in CE3 was missing in CE3003 and was replaced with a fragment in CE3003 that was 5.6 kb larger than the missing fragment. This suggests that the transposon-containing fragment in CE3003 contained sequences in common with the insert in pRA3003.

Biochemical characterization of mutant CE3003. The LPS

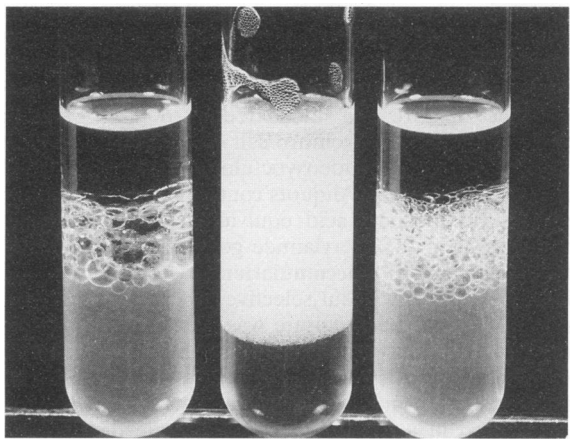

FIG. 2. Partitioning of cells of strains CE3 (left), CE3003 (center), and CE3003(pRA3003) (right) to hexadecane.

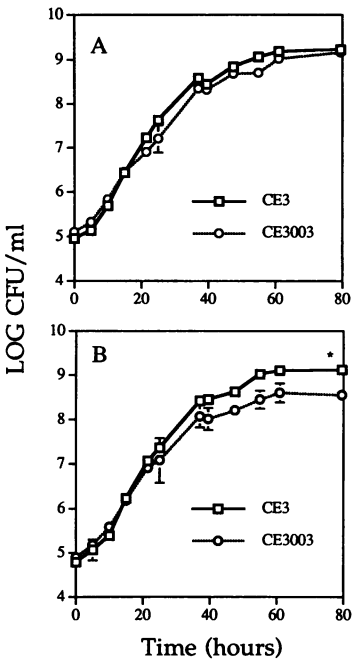

FIG. 3. Growth of CE3 and CE3003 in separate cultures (A) or in mixed culture (B) in YEM broth. Bacteria were enumerated by dilution plating on YEM plates with either streptomycin or streptomycin and kanamycin to differentiate between the two strains. An asterisk indicates significant difference ($P \leq 0.01$).

profile of CE3003 was different from those of CE3 and CE3003(pRA3003), which were indistinguishable from each other. CE3003 lacked a band corresponding to a high-molecular-weight form of LPS that was present in CE3 and CE3003(pRA3003). A second band that was prominent in CE3 and CE3003(pRA3003) was barely detectable in CE3003. Treatment of the cell extracts with proteinase K prior to electrophoresis resulted in the appearance of an LPS band in CE3003 that comigrated with the high-molecular-weight form of LPS in extracts of CE3 and CE3003(pRA3003). Protease treatment did not affect the LPS profiles of CE3 and CE3003(pRA3003) (data not shown). Sodium dodecyl sulfate (SDS)-PAGE analysis of the proteins of membrane fractions indicated no differences among strains in the protein composition of the outer membrane (data not shown).

We studied several other traits commonly associated with cell surface hydrophobicity. Microscopic observation of bacterial smears stained with crystal violet revealed that both CE3 and CE3003 were encapsulated (data not shown). CE3003 accumulated 55% of the EPS accumulated by CE3 (1.8 mg of EPS per 10^9 cells) when the bacteria were grown on YEM plates, but the difference between strains was not significant (by ANOVA, $\alpha = 0.05$). The proportions of the EPS that were accounted for by glucose equivalents (milligrams of hexose per milligram of EPS) were comparable for the two strains and not significantly different, and ^{1}H-nuclear magnetic resonance spectroscopy analysis indicated that all of the peaks that were detected in the EPS from CE3 also were detected in the EPS from CE3003 (data not shown).

We observed clumping when CE3003 was grown in broth for 3 days, and the clumps were dispersed by agitating the culture in a vortex mixer at high speed for 1 min. Microscopic observation of bacterial suspensions after agitation confirmed that the cell clumps had been completely dispersed. The clumps formed by CE3003 were not dispersed by incubation with 10 U of cellulase per ml at 37°C for 30 min (37), showing that the clumps of CE3003 were not due to cellulose fibrils. Microscopic observation showed that both the mutant and the parent were motile.

TABLE 2. Symbiotic characteristics of *R. etli* CE3 and its mutants

Strain	Symbiotic properties 28 days after planting[a]	
	No. of nodules/ plant	ARA[b]
CE3	24.2 ± 2.8	0.46 ± 0.09
CE3003	14.8 ± 2.5	0.27 ± 0.08
CE3003(pRA3003)	18.8 ± 2.7	0.40 ± 0.07

[a] Values are means (± standard error) of 16 plants per strain and are representative of two separate experiments conducted in glass tubes.
[b] ARA, acetylene reduction activity in micromoles of C_2H_4 per plant per hour.

Symbiotic properties of mutant CE3003. Between 0 and 16 days after planting, there were no significant differences ($P > 0.05$) in the number of nodules on plants inoculated with CE3, CE3003, and CE3003(pRA3003), except at 9 days after planting ($P < 0.05$) when plants inoculated with CE3003 had significantly fewer nodules. We applied regression analysis to compare the nodulation rates for the three strains, and we observed that the relationship between the number of nodules and time after planting was linear and could be described by a single regression model for all three strains ($R^2 = 0.87$) as follows: $y = -28.0 + 5.07x$, where y is the number of nodules and x is the time (in days) after planting in pouches. CE3003 induced fewer nodules on plants grown in glass tubes ($P = 0.052$) than did CE3 and CE3003(pRA3003), which were indistinguishable in nodulation, although the difference in nodule numbers of CE3 and CE3003 was not accompanied by a significant difference ($P = 0.23$) in acetylene reduction (Table 2).

We studied the ability of CE3, CE3003, and CE3003 (pRA3003) to grow in the rhizosphere when inoculated individually or in pairwise mixtures. The three strains grew equally well on roots when applied to the seeds singly (Fig. 4A). When CE3 and CE3003 were applied to seeds in a mixture at a 1:1 ratio, the final ratio of the two strains was 14:1 (Fig. 4B). The difference between the populations was significant ($P < 0.05$). Restoration of the flat colony morphology to CE3003 (pRA3003) was accompanied by recovery of the ability to grow competitively with CE3 in the rhizosphere (Fig. 4C). The difference in populations could not be explained by clumping of CE3003 since the root samples were sonicated prior to plating, and direct microscopic observation showed that sonication completely dispersed clumps of bacteria.

We measured the nodulation competitiveness of CE3003 and CE3. CE3003 was substantially less competitive ($CI_{CE3:CE3003} = 0.143$; $P < 0.01$) in nodulation than CE3 (Fig. 5A). A 17,000-fold excess of CE3003 in the inoculum was required to obtain equal nodule occupancy by CE3003 and CE3. Restoration of the parental colony morphology to CE3003 was accompanied by restoration of its ability to compete for nodulation ($CI_{CE3:CE3003pRA3003} = 0.002$; $P = 0.78$), since an inoculum ratio of 1:1 of CE3 and CE3003(pRA3003) resulted in equal representation of the two strains in the nodules (Fig. 5B).

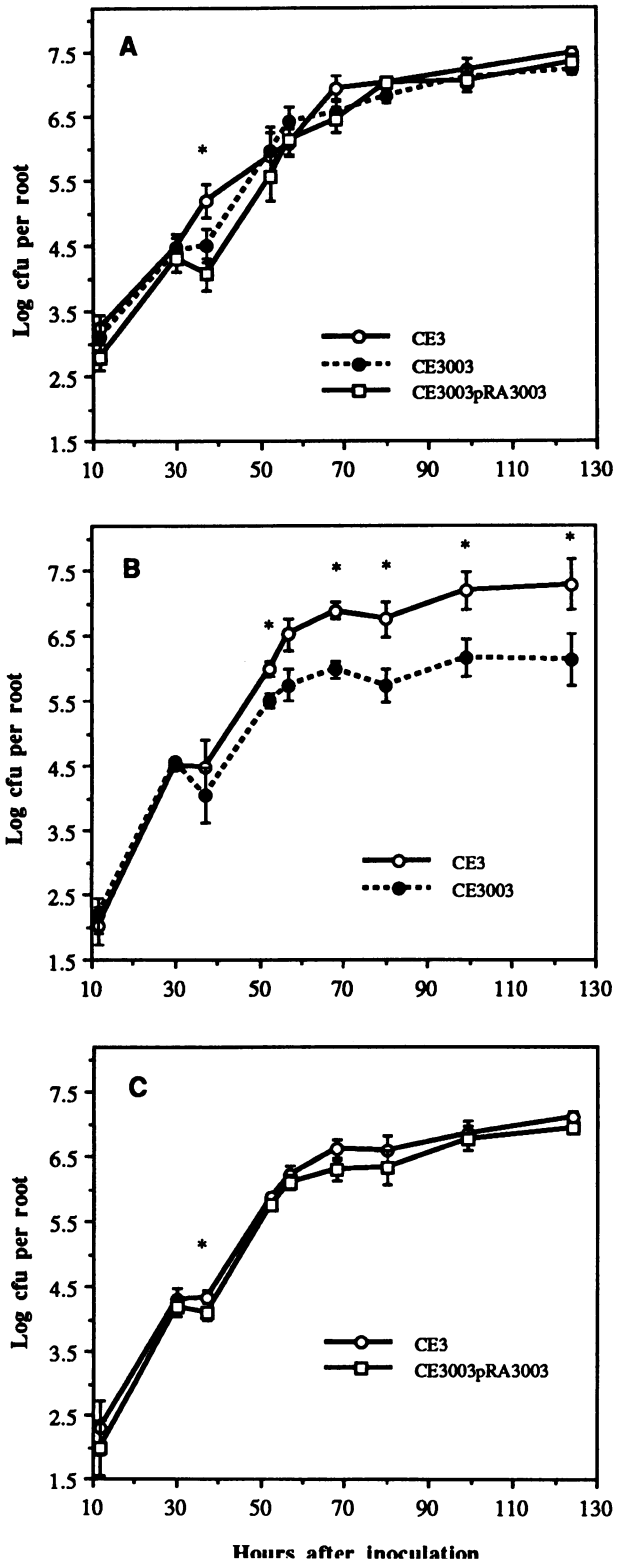

FIG. 4. Growth on roots by *R. etli* CE3, CE3003, and CE3003(pRA3003) when inoculated singly (A) or in pairwise combinations of CE3/CE3003 (B) and CE3/CE3003(pRA3003) (C) at a 1:1 inoculum ratio. Each value represents the average of populations on four plants per strain per time point. The data are representative of two similar experiments. Error bars are shown for all time points. An asterisk indicates significant difference between strains at that time point (by ANOVA) at an α of 0.05.

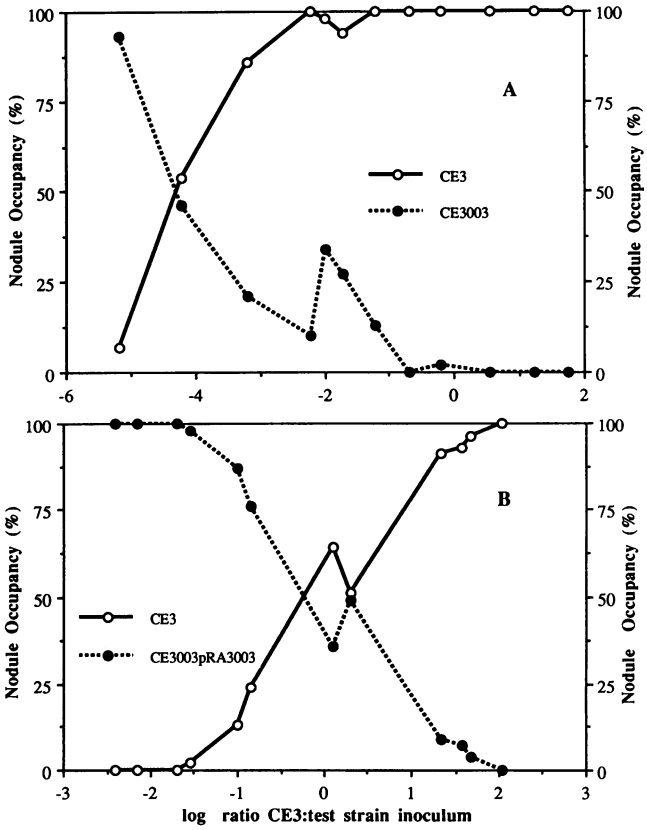

FIG. 5. Competition for nodulation by CE3, CE3003, and CE3003pRA3003 applied to plants in pairwise combinations of CE3/CE3003 (A) and CE3/CE3003(pRA3003) (B) over a range of inoculum ratios. The proportion of nodules occupied by each strain at each inoculum ratio represents the occupancy of 48 nodules from a total of six plants. Nodules with double occupancy were added to both strains in each pair. The data from two similar experiments were combined and are representative of a third experiment.

DISCUSSION

To determine whether the cell surface plays a role in the nodulation competitiveness of *R. etli*, we studied a Tn*5*-induced mutant of strain CE3 with altered colony morphology. We chose mutant CE3003 because of its unusual domed colony morphology on solid medium. CE3003 was highly hydrophobic in a hexadecane separation test. Although CE3003 nodulated and reduced acetylene on bean plants when applied to bean plants singly, it was substantially affected in competitive growth in the rhizosphere and in nodulation competitiveness. The wild-type colony morphology, hydrophilicity, competitive growth in the rhizosphere, and nodulation competitiveness were all restored to CE3003 by a single cosmid clone containing an insert of DNA from CE3.

LPS mutants of certain bacterial species have been shown to be highly hydrophobic (20). The hydrophobicity of CE3003 might be due to either a change in LPS or an LPS-protein complex, since the LPS of the mutant had a profile that was different from that of the parent strain in the absence of protease treatment, but the LPS profiles were indistinguishable after protease treatment. Further work will determine whether LPS-protein interactions in CE3003 are responsible for its hydrophobic cell surface.

EPS capsules decrease the hydrophobicity of cells of other

bacterial species (8, 41). Although CE3003 accumulated less EPS than did CE3, the difference was not statistically significant, and both CE3 and CE3003 were encapsulated, suggesting that the hydrophobicity of the cells of CE3003 was not due to the absence of a capsule. Bacteria with hydrophobic cell surfaces tend to adhere to one another, resulting in the formation of clumps (49), and the overproduction of cellulose fibrils increases clumping and hydrophobicity of other *Rhizobium* strains (48). The clumps formed by CE3003 grown in broth were held together weakly since they were dispersed by agitation. The clumps were not susceptible to treatment with cellulase, showing that cellulose fibrils were not responsible for clumping and increased hydrophobicity.

The data may indicate that the hydrophobic cell surface of CE3003 reduces the ability of the mutant to compete for resources and that the inability of CE3003 to grow competitively in the rhizosphere reduces nodulation competitiveness. Alternatively, it is possible that although the competitive growth, nodulation competitiveness, and hydrophobic phenotypes share a common genetic and biochemical basis, they may not be causally related. Experiments are in progress to determine whether competitive growth in the rhizosphere contributes directly to nodulation competitiveness. The relatively small difference in competitive growth in culture and on roots is unlikely to account fully for the vast difference in nodulation competitiveness between CE3 and CE3003.

Changes in bacterial cell-surface properties have been shown previously to alter the competitiveness of strains of *Rhizobium meliloti* (28, 33), *Rhizobium fredii* (54), *Rhizobium tropici* (38), and *Bradyrhizobium japonicum* (12, 42). Mutant CE3003 differs quantitatively from mutants described in previous studies. The magnitude of the impact of the *hyd-1*::Tn*5* mutation on competitiveness of CE3003 is greater than the effects of other cell surface alterations on competitiveness (28, 33, 38, 42). A 17,000-fold excess of CE3003 over the parent strain was required for CE3003 to occupy 50% of the nodules, whereas other cell surface mutants occupy most of the nodules when they are present at 100-fold excess or less in the inoculum. Quantitative phenotypes such as competitiveness are notoriously difficult to study by genetic approaches because of the inherent variability in the measurement of the phenotype, which makes mutant identification and characterization challenging. The difference in competitiveness between CE3 and CE3003 is sufficiently large that variability has a negligible impact on interpretation of the data; therefore, the *hyd-1* locus is attractive for genetic analysis. An understanding of its role in the structure of the cell surface and symbiotic properties of CE3003 should lead to insight into the relationship between growth on the host root and nodulation competitiveness of *Rhizobium* spp. Improving our understanding of nodulation competitiveness may lead to better strategies to harness symbiotic nitrogen fixation for crop productivity in the agroecosystem.

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