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Effect of Naturally Occurring *nif* Reiterations on Symbiotic Effectiveness in *Rhizobium phaseoli*

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Most naturally occurring strains *of Rhizobium phaseoli* possess reiteration *of* the nif genes. Three regions contain nitrogenase structural genes in strain CFN42. Two *of* these regions (a and b) have copies *of* nifH, nifD, and nifK, whereas the third region (c) contains only nifH. Strains containing mutations in either nif region a or nif region b had significantly diminished symbiotic effectiveness compared with the wild-type strain on the basis *of* nodule mass, total nitrogenase activity per plant, nitrogenase specific activity, total nitrogen in the shoot, and percentage *of* nitrogen. A strain containing mutations in both nif region a and nif region b was totally ineffective. These data indicate that both nif region a and nif region b are needed for full symbiotic effectiveness in *R. phaseoli.*

Bacteria of the genus *Rhizobium* induce nitrogen-fixing nodules on the roots of their host plants. For fast-growing *Rhizobium* species, it has been shown that the genes required for nodulation *(nod)* and nitrogen fixation *(nif)* are located on large plasmids (12). The nitrogenase enzyme complex is composed of two enzymes, nitrogenase (Mo-Fe protein) and nitrogenase reductase (Fe protein). The Mo-Fe protein is composed of two α subunits and two β subunits encoded by the *nifD* and *nifK* genes, respectively. The Fe protein is composed of two identical subunits encoded by the *nifH* gene.

For *Rhizobium phaseoli,* the symbiont of *Phaseolus vulgaris,* we reported the reiteration of nitrogen fixation gene sequences (13). Three regions homologous to the *nifH* gene were identified in *R. phaseoli* CFN42. These regions are located on a single plasmid (13). Heteroduplex and nucleotide sequencing studies of each region established the presence of complete *nifH* genes in each of the three regions (14). Hybridization studies using as probes *Klebsiella pneumoniae nifD* and *nifK* genes indicated that both genes are present in *nif* regions a and b, while only *nifH is* present in *nif* region c (14). Conceivably, *nif* regions a and b are *nifHDK* operons by analogy to the arrangement found in other *Rhizobium spp. (1).* A mutational study indicated that at least two of the reiterated regions *(nif* regions a and b) are functionally expressed (14).

Reiteration of nitrogenase structural genes appears to be a widespread feature among *R. phaseoli* isolates. Martinez et al. (5) reported that, among 50 isolates, 95% contained reiteration of nitrogenase structural genes. Reiteration of nitrogenase structural genes has been found also in *R. fredii (11), R. trifolii (21),* and *Rhizobium sp.* strain ORS571 (8, 9).

Given the widespread occurrence of repeated *nif* genes in *R. phaseoli,* a hypothesis explaining the kind of selective pressure that maintains these reiterations must be sought. Maybe reiterated *nif* gene sequences are necessary for optimal expression of nitrogen fixation activity. Assuming that only *nif* regions a and b possess the potential to produce a functional nitrogenase enzyme complex, the hypothesis leads to the following predictions. (i) Strains having single mutations in any of the regions carrying complete *nifHDK* reiterations *(nif* region a or *nif* region b) must have a diminished symbiotic effectiveness compared with the wildtype strain. (ii) Strains carrying mutations in both *nif* region a and *nif* region b must be totally ineffective. Our results provide evidence which supports this hypothesis.

R. phaseoli CE-3 is a spontaneous streptomycin-resistant derivative of wild-type strain CFN42 (14). Strain CFN2202 is a derivative of CE-3 which carries an insertion of a kanamycin resistance interposon into the *nifH* gene located in region b (14). Strain EM407 is a derivative of CE-3 which carries a kanamycin and spectinomycin resistance interposon into the *nifH* gene located in region a (E. Morett and G. Espin, manuscript in preparation). Strain CFN2210 is a derivative of CE-3 carrying cointegrates of intermediary vectors pLS151 and pLS153. These vectors are derived from plasmid pSUP205 (15). pLS151 carries a spectinomycin resistance interposon in the *nifH* coding frame of *R. phaseoli* CE-3 *nif* region a (19). pLS153 carries a 2-kilobase *HindIII*BamHI fragment from *nif* region a (14) with a kanamycin resistance interposon from plasmid pGV97 (4) in the *nifK* gene. As these plasmids carry only fragments internal to the reiterations, their cointegration in *nif* regions a and b leads to a block of the expression of each *nifK* gene. This strain was shown to be stable, maintaining its phenotypic and genotypic characteristics through nodulation (data not shown).

Seeds of *P. vulgaris* cv. Bush Bountiful were surface sterilized in 2% sodium hypochlorite for 2 min, rinsed, and germinated in sterile vermiculite. Seedlings were planted when the radicles were 2 to 3 cm long. Plants were grown in 1-liter plastic pots (Lab-Tek Products) filled with wet vermiculite as described by Singleton and Tavares (18). Three germinated seedlings were planted in each pot. The bacterial inocula were grown in yeast extract-mannitol broth (20). Immediately after planting, each seedling was inoculated with 10^6 to 10^7 viable cells of the appropriate strain which was suspended in 0.5 ml of sterile water. Viable counts were determined by the drop plate method (20). Four pots were left uninoculated as controls. Plants were grown in a greenhouse in a randomized complete-block design with four replications. The plants were thinned to 2 per pot 10 days after planting. Each pot received a daily application of 100

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TABLE 1. Growth, nodulation, and nitrogen fixation traits for *P. vulgaris* inoculated with *R. phaseoli* strains carrying mutations in *nif* reiterations[a]

Strain	Active *nif* reiteration[b]	Nodule dry wt (mg · pot^{-1})	Total nitrogenase activity[c]	Nitrogenase sp act[d]	Shoot dry wt (g · pot^{-1})	Total N in shoot (mg · pot^{-1})	% N
CE-3	a, b, c	290a	16.34a	113.0a	2.1a	66.8a	3.0a
EM407	b, c	184b	3.83b	43.9b	2.0a	36.6b	1.8b
CFN2202	a, c	160b	4.19b	64.5b	1.9a	38.3b	1.9b
CFN2210	c	161b	0.03c	0.6c	1.8a	19.8c	1.1c
Uninoculated control		0c	0.00c	0.0c	1.8a	20.9c	1.1c
+N control					6.4	219.3	3.4

[a] Means within the same column flanked by the same letter are not significantly different ($P = 0.05$) as determined by the Duncan multiple range test.
[b] Letters refer to the different *nif* reiterations; nomenclature is as described previously (14).
[c] μM C_2H_4 · plant^{-1} · h^{-1}.
[d] μM C_2H_4 · g of nodule^{-1} · h^{-1}.

ml of N-free nutrient solution (16). Micronutrients were provided from a commercial micronutrient concentrate (Monterey Chemical Co.). Four pots each received a daily application of 100 ml of nutrient solution containing 100 mg of N liter^{-1} as a measure of yield potential in the system.

At harvest, on day 28 after planting, acetylene-reduction assays were performed by incubating the excised root systems in 5% (vol/vol) acetylene for 45 min. Ethylene production was measured with a Varian Aerograph 940 gas chromatograph. After incubation, the nodules were removed from the roots and oven-dried at 70°C for 72 h for dry weight determinations. Shoots were cut at the surface of the growth medium and oven-dried in the same fashion. The dried shoots were weighed and milled, and samples (0.25 g) were digested with concentrated sulfuric acid after pretreatment with 5 ml of H_20_2. N content of the digests was determined by the colorimetric method of Mitchell (7). The data (positive N controls not included) were analyzed statistically as described by Gomez and Gomez (2). Correlation coefficients were calculated from entry means.

Growth, nodulation, and nitrogen fixation data for plants inoculated with strain CE-3 or its mutant derivatives are shown in Table 1. Plants nodulated by strains carrying mutations in *nif* region a (EM407) or in *nif* region b (CFN2202) or in both *nif* region a and *nif* region b (CFN2210) showed a lowered level of nodulation as compared with the wild-type strain (CE-3). The dry weight of nodules induced by strains EM407, CFN2202, and CFN2210 was 41% lower than that of nodules induced by the wild-type strain. These values were statistically significant at the 5% level. Differences in nodule dry weight were expected because these strains had lowered nitrogen fixation activity (see below). A direct correlation has been found between nitrogen fixation activity and nodule mass (10, 17).

Total nitrogenase activity values allow a separation of the inoculant strains into three different groups (Table 1). Group 1 contains wild-type strain CE-3, which had the highest value for total nitrogenase activity. Group 2 contains EM407 and CFN2202, which had 25% of the total nitrogenase activity of group 1. Group 3, which contains CFN2210, was totally ineffective for N_2 fixation. Wild-type strain CE-3 ranked highest for nitrogenase specific activity. Strains carrying mutations in *nif* region a (EM407) or *nif* region b (CFN2202) ranked intermediate to CE-3 and CFN2210, with 49% of the nitrogenase specific activity of the latter strains. Strain CFN2210, carrying mutations in both *nif* region a and *nif* region b, was ineffective. A significant linear correlation was observed between nitrogenase specific activity and number of active *nif* reiterations ($r = 0.983$, significant at the 5% level). These results indicate that nitrogen fixation responds to the gene dosage level of the *nif7HDK* reiterations. Results of dry weight and nitrogen analyses are shown in Table 1. No significant differences between treatments were observed for shoot dry weight. However, significant differences between strains were found for both percentage of nitrogen and total nitrogen in the shoot. Wild-type strain CE-3 had the highest value for total nitrogen in the shoot. Strains EM407 and CFN2202 were intermediate, with values falling between those for CE-3 and CFN2210. Strain CFN2210 was ineffective for symbiotic nitrogen fixation and had the lowest value for total nitrogen in the shoot, which was not significantly different from that of the uninoculated control. Values for the positive nitrogen control indicated that the main limitation in our growth system was nitrogen availability from N_2 fixation. Values for percentage of nitrogen were also significantly different between strains. This difference was expected, because values for total nitrogen in the shoot showed that significance and dry weight values were uniform among treatments. This trend still holds (Table 1). Strain CE-3 showed the highest percentage of nitrogen, strains EM407 and CFN2202 ranked intermediate, and strain CFN2210 was indistinguishable from the uninoculated control in percentage of nitrogen.

These data clearly indicate that both *nif* region a and *nif* region b, carrying *nifHDK* reiterations, are needed for optimal nitrogen fixation in *P. vulgaris,* indicating a gene dosage effect for these reiterations.

It was not possible to analyze the effect of mutations in *nif* region c on nitrogen fixation, since the mutants available appear to be extremely unstable (unpublished data). However, *nif* region c alone is unable to confer any nitrogen fixation ability, since mutant CFN2210 is totally ineffective (Table 1). In a previous report (14), we were unable to detect differences in nitrogen fixation ability analogous to those reported here. However, those tests were carried out under conditions restrictive to plant growth (nitrogen-free nutrient agar) and for shorter durations (17 versus 28 days), which made it difficult to detect the differences reported here.

Reiteration of nitrogenase structural genes has been found in other members of the genus *Rhizobium,* such as *R. fredii* (11) and *Rhizobium* sp. strain ORS571 (8, 9). For *R. fredii,* a correlation between the loss of plasmid-borne *nif* gene copies and a reduction in symbiotic effectiveness has been found (6). However, in that study the loss of *nif* reiterations was achieved by plasmid curing, thus making it difficult to ascribe the observed differences in symbiotic effectiveness solely to the loss of *nif* reiterations. For *Rhizobium* sp. strain ORS571, a functional study of the two *nif* reiterations

indicates that both are needed to achieve optimal nitrogen fixation (9). Reiteration *of nodD* genes has been found in R. *meliloti,* and a mutational study indicates that at least two *nodD* genes are necessary for efficient nodulation *of* alfalfa (3). We conclude that both *nif* region a and *nif* region b, carrying *nifHDK* reiterations, are needed for *full* symbiotic effectiveness in *R. phaseoli,* indicating a gene dosage effect for these reiterations.

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