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Engineering the *nifH* Promoter Region and Abolishing Poly-β-Hydroxybutyrate Accumulation in *Rhizobium etli* Enhance Nitrogen Fixation in Symbiosis with *Phaseolus vulgaris*

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***Rhizobium etli*, as well as some other rhizobia, presents nitrogenase reductase (*nifH*) gene reiterations. Several *R. etli* strains studied in this laboratory showed a unique organization and contained two complete *nifHDK* operons (copies a and b) and a truncated *nifHD* operon (copy c). Expression analysis of *lacZ* fusion demonstrated that copies a and b in strain CFN42 are transcribed at lower levels than copy c, although this copy has no discernible role during nitrogen fixation. To increase nitrogenase production, we constructed a chimeric *nifHDK* operon regulated by the strong *nifHc* promoter sequence and expressed it in symbiosis with the common bean plant (*Phaseolus vulgaris*), either cloned on a stably inherited plasmid or incorporated into the symbiotic plasmid (pSym). Compared with the wild-type strain, strains with the nitrogenase overexpression construction assayed in greenhouse experiments had, increased nitrogenase activity (58% on average), increased plant weight (32% on average), increased nitrogen content in plants (15% at 32 days postinoculation), and most importantly, higher seed yield (36% on average), higher nitrogen content (25%), and higher nitrogen yield (72% on average) in seeds. Additionally, expression of the chimeric *nifHDK* operon in a poly-β-hydroxybutyrate-negative *R. etli* strain produced an additive effect in enhancing symbiosis. To our knowledge, this is the first report of increased seed yield and nutritional content in the common bean obtained by using only the genetic material already present in *Rhizobium*.**

The common bean (*Phaseolus vulgaris* L.) is the most important crop in Mexico after maize and represents the main protein source for large sectors of the population. Bean plants tolerate a wide range of environments and are cultivated from tropical to temperate regions covering up to 2-million hectares in Mexico and 22-million hectares in the rest of the world. Their seeds are consumed either fresh or dry (7). Most of the fields used for their cultivation are fertilized with agrochemicals.

Biological nitrogen fixation is an exclusively prokaryotic process in which atmospheric dinitrogen is converted in an easily assimilable metabolite, ammonia. *Rhizobium* bacteria, and related genera, induce nodules and fix nitrogen in the roots of legumes in a complex regulated process (12). Given the current world food demand, increasing biological nitrogen fixation offers economic, agricultural, and environmental benefits. Improvement of this process can be obtained by the use of genetically manipulated *Rhizobium* bacteria. Historically, researchers have had limited success in trying to improve the *Rhizobium*-legume relationship in agronomically important crops. Strategies used to enhance symbiotic nitrogen fixation include: (i) transgenic expression of hydrogenase uptake in *Rhizobium* strains (1), (ii) construction and expression of a hybrid nodulation regulatory *nodD* gene (31), (iii) increasing expression of NifA and C_4-dicarboxylic acid transport genes (3), and (iv) obtention of an acid-tolerant *R. leguminosarum* biovar trifolii strain (9). None of these strategies improved nitrogen fixation ability, compared with inoculation with the wild type, more than 20% for any parameter measured.

The common bean is nodulated by different species of *Rhizobium*; the majority of strains isolated from bean nodules in Mexican agricultural fields belong to *Rhizobium etli* (29). The *R. etli* type strain is CFN42. This strain contains three copies of the *nifH* gene (named a, b, and c) which code for the nitrogenase reductase component, two of them (a and b) are linked to the *nifDK* genes which code for dinitrogenase (23, 26). Reiteration c is linked to a truncated *nifD* homolog (*nifD**) gene (35). The three *nifH* copies are actively expressed during symbiosis although the *nifHDK* operons are expressed at lower levels than the third *nifHc* copy. The nitrogenase activity is encoded by only the two complete *nifHDK* operons in a gene dosage-dependent manner (27). All these genes are located on a 371-kb symbiotic plasmid (pSym) (14).

Both operons a and b are preceded by identical RpoN (σ54)-dependent promoters and canonical NifA (nitrogen fixation activator)-binding sites named upstream activator sequences located at 90 bp from the promoter (26). The third copy, *nifHc*, is preceded by an identical RpoN-dependent promoter and is activated by NifA bound to a nonconsensus-binding site 85 bp upstream (35). The asymetric arrangement of regulatory elements could contribute to the *nifH* differential expression observed during symbiosis (35).

Poly-β-hydroxybutyrate (PHB) is a poly-β-hydroxyalkanoate accumulated by a wide range of rhizobia as a carbon and

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reductive power storage polymer in free life (32, 34) and/or in symbiosis (18, 19, 38). *R. etli* produces PHB not only in free life but also during symbiosis (8, 10). Although the role of PHB in symbiosis is not well understood, mutation of the *R. etli phaC* gene, the product of which catalyzes the PHB polymerization step, produced a mutant with increased nitrogenase activity and a slight increase in bean seed yield compared to those of the wild-type strain CFN42 (8). Physiological characterization showed that the PHB$^-$ strain excreted a huge quantity of metabolites, mainly from the tricarboxylic acid (TCA) cycle as fumarate, malate, and 2-oxoglutarate, suggesting that the mutant is unable to oxidize the carbon source present in the growth medium. The PHB$^-$ strain showed a lower NAD^+/NADH ratio. The abundance of reduced cofactors is apparently related to the absence of a reductive power sink (PHB) (8). Encarnación et al. (10) proposed that in *R. etli*, PHB serves as a reductive power sequester, so that the TCA cycle continues functioning under microaerobic conditions. The PHB$^-$ strain shows an increased ability to fix nitrogen (at late stages of symbiosis), in contrast to the notion that PHB could help to prolong or sustain symbiotic nitrogen fixation as proposed by Bergersen et al. (2). In the case of *R. etli*, apparently part of the excess reducing power present in the PHB$^-$ strain is channeled to nitrogen fixation.

The main purpose of our work was to significantly improve the symbiotic efficiency in the *R. etli-P. vulgaris* relationship by an in vitro manipulation approach of the bacterial genetic material, specifically that which encodes nitrogenase enzyme production. In view of the previously mentioned knowledge about *nifH* transcriptional activation, we intended to improve nitrogen fixation efficiency by modifying the nitrogenase genes transcription rate. To increase this rate and at the same time to conserve NifA-dependent regulation, we constructed a chimeric complete nitrogenase *nifHDK* operon coupled to the strong *nifHc* promoter region and expressed it either on a stably inherited plasmid or in the symbiotic plasmid itself. We assessed the effects of such constructions on symbiosis with common bean plants in greenhouse experiments and compared them to those of inoculation with the parent strain. Additionally, the chimeric nitrogenase operon was expressed in a PHB$^-$ background to determine if the carbon and reducing power not stored in the polymer could be derived to fuel nitrogen fixation.

The improved symbiotic relationship obtained in this way is the highest reported for *R. etli* to date and involves the use of only genetic elements already present in the bacterial genome. Greenhouse experiments with the modified strains support their potential application to obtain better crop yields and more nutritive bean seeds.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. Plasmids and strains used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani complex medium (28). *R. etli* strains were grown, as described elsewhere, in peptone-yeast extract (PY) or minimal medium containing 1.2 mM K_2HPO_4, 0.8 mM $MgSO_4$, 10 mM succinic acid, 10 mM NH_4Cl, 1.5 mM $CaCl_2$, and 0.0005% $FeCl_3$, with the pH adjusted to 6.8 (5). The following antibiotics were added to the indicated final concentrations (in micrograms per milliliter): kanamycin, 30; nalidixic acid, 20; carbenicillin, 100; and tetracycline, 6 or 10. Plasmids were conjugated into either wild-type *R. etli* CFN42^T (or streptomycin-resistant derived strain CE3) or strain SAM100 (*phaC*) by triparental mating with pRK2013 as a helper plasmid (11).

DNA manipulations. DNA manipulations, such as isolation, transformation, restriction analysis, agarose gel electrophoresis, and hybridization, were performed by standard procedures (28). DNA fragments were purified from agarose gels with the use of the GeneClean kit (Bio101 Inc., Buena Vista, Calif.) or Wizard PCR Resin (Promega, Madison, Wis.). The Eckhardt method as modified by Hynes and McGregor (17) was used to determine plasmid profiles.

RNA isolation and dot blot hybridization. RNA from 18 days postinoculation (dpi) nodules or free-living cells was isolated by phenol extraction (28) and purified with a MicrobExpress kit (Ambion, Austin, Tex.) according to the manufacturer's instructions. For dot blot hybridization, the membrane was loaded with samples and fixed with UV light with a StrataLinker 1800 apparatus (Stratagene, La Jolla, Calif.). The *nifH* probe was a 300-bp fragment obtained by PCR with *nifH* forward and *nifH* reverse oligonucleotides (described below). A 16S rRNA gene probe was obtained by PCR with universal oligonucleotides fd1 and rd1 (37). The labeled probes were prepared with ^{32}P and a MegaPrime kit (Amersham, Little Chalfont, United Kingdom). Membranes were hybridized at high stringency at 65°C, washed three times with 0.1% sodium dodecyl sulfate in 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C, and then exposed to standard film (28) or on a Phosphor Imager screen for signal quantification in a Molecular Dynamics (Amersham, United Kingdom) scanner.

Construction of plasmid pHP55. To produce a chimeric *nifHDK* operon controlled by the *nifHc* promoter region (hereafter referred as pr c *nifHcDK*), the promoter region of the *nifHDKb* operon contained in plasmid pCQ12 (26) was replaced by the *nifHc* promoter region as follows (see Fig. 1). A 1.5-kb fragment containing the *nifHc* promoter region and part of the *nifHc* gene was isolated by digesting pCQ23 (26) with BglII. pCQ12 was digested with BglII and BamHI to eliminate the *nifHDKb* promoter and part of the *nifHb* gene, resulting in loss of a 1.8-kb segment. The largest fragment of that digestion was ligated with the 1.5-kb BglII fragment and a plasmid with the correct orientation was chosen and named pHP40. Since the nucleotide sequence of both *nifHb* and *nifHc* genes are identical, the *nifH* gene formed by the joined fragments remains functional.

The 4.5-kb EcoRI fragment carrying the pr c *nifHcDK* construction from pHP40 was cloned into plasmid pIC20H (22) to produce pHP50. The 4.5-kb SpeI fragment containing pr c *nifHcDK* from plasmid pHP50 was cloned into the XbaI restriction site of the *Rhizobium* stably inherited vector, pTR101, (36) to produce pHP55.

Strain HP55 was obtained by triparental mating with *E. coli* HB101/pHP55 as donor, *E. coli* HB101/pRK2013 (11) as helper, and *R. etli* CFN42 as recipient. Selection was made on PY plates plus nalidixic acid and tetracycline (10 μg ml^{-1}).

Construction of strain HP310 containing the pr c *nifHcDK* chimeric operon. To obtain double-recombinant *Rhizobium* strains containing the pr c *nifHcDK* construction, we ligated a suicide vector, pWS233 (30), digested with EcoRI, to the 4.5-kb EcoRI fragment carrying pr c *nifHcDK* from pHP40. The plasmid obtained was named pHP100. In the vector XbaI site, we cloned a 1.6-kb PstI-EcoRI fragment bordered by SpeI sites and containing a fragment of the *hemN* gene located downstream of *nifHcD** genes (35). The plasmid obtained, pHP789, was conjugated to *R. etli* CFN42 with pRK2013 as helper by selection on PY plates with nalidixic acid and tetracycline (6 μg ml^{-1}). Selected colonies were cultured overnight in liquid PY and again grown overnight in liquid PY with 10% sucrose, a condition under which cells containing vector sequences were lysed. Surviving cells were plated onto PY plates with nalidixic acid, and a colony was chosen and named HP310.

A *nifH-lacZ* fusion was obtained by cloning the *lacZ-kan* cassette from pKOK6 (20), digested with BamHI, into the BglII site of pHP789. A plasmid with the correct orientation was chosen and named pHP789 lac. Incorporation into HP310 was done by triparental conjugation by selection with tetracycline (6 μg ml^{-1}) and kanamycin (30 μg ml^{-1}), and colonies selected by growth in liquid PY with 10% sucrose. Surviving cells were selected on PY plates with nalidixic acid and kanamycin (30 μg ml^{-1}). A colony showing the incorporation of the cassette into the *nifHb* reiteration by a hybridization assay (data not shown) was chosen and named HP310 lac.

PCR assays and DNA sequencing. PCR assays were performed with a GeneAmp PCR kit (PerkinElmer Applied Biosystems, Foster City, Calif.) following the manufacturer's instructions. Primers used were *nifHc* EcoRV forward (5′-GGC CGG ATA TCG CCT GAG A), *nifHa* forward (5′-CCG TCT GTC GGC TTT GTC TG), intra-*nifH1* reverse (5′-GTA AAA TGC GAT TTG ACG C), intra-*nifH* forward (5′-GAG GAC GTG CTC AAG GCC GGC TAC), end-*nifH* reverse (5′-CAG CAC GCC GAG CTC AGG AAG ATG), *nifD* forward (5′-GGC GTG ATG ACG ATC CG), *nifD* reverse (5′-GCA TTC CGA CTG CAC GC), *nifK* forward (5′-CCA GGC TCT TCC CAT CG), *nifK* reverse

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristic(s)	Source or reference
R. etli		
CFN42	Wild-type strain, Sm^r Nal^r	5
DEM153	CFN42 with a *nifHa-lacZ* reporter fusion in pSym, Km^r	35
DEM233	CFN42 with a *nifHc-lacZ* reporter fusion in pSym, Km^r	35
SAM100	CFN42 derivative, *phaC* Km^r	8
HP55	CFN42 containing pHP55 plasmid, Tc^r	This work
HP310	CFN42 derivative containing chimeric operon pr c *nifHcDK* in pSym	This work
HP210	CFN42 derivative containing pHP210 plasmid (pTR101, *nifHc* with its own regulatory region), Tc^r	This work
HP220	CFN42 derivative containing pHP220 plasmid (pTR101, *nifHDKb* with its own regulatory region), Tc^r	This work
HP310 lac	HP310 derivative containing a *nifH-lacZ* fusion in the chimeric operon pr c *nifHcDK* in pSym, Km^r	This work
E. coli HB101	F^- *hsd* S20-*recA* 13	4
Plasmids		
pAM341	pTR101, with a fragment containing the *nifHc* promoter region cloned into the *Xba*I site, Tc^r	24
pRK2013	Helper plasmid, ColE1, *mob*$^+$ Tra^+ Km^r	11
pCQ12	pBR328, with a 4.5-kb EcoRI fragment containing the *R. etli nifHDKb* operon, Tc^r	26
pCQ23	pBR328, with a 4.2-kb EcoRI fragment containing the *R. etli nifHc* gene, Tc^r	26
pTR101	pTR100 (mini-RK2), with a 0.8-kb stability locus, Tc^r	36
pIC20H	Cloning vector, Amp^r	22
pKOK6	pSUP202, with a *lacZ-kan* cassette	20
pWS233	Mobilizable replicon ColE1, Gm^r Tc^r *sacRB*	30
pHP40	pCQ12, pr c *nifHcDK* construction in a 4.5-kb EcoRI fragment	This work
pHP50	pIC20H, with a 4.5-kb EcoRI fragment containing the pr c *nifHcDK* construct	This work
pHP55	pTR101, containing the pr c *nifHcDK* construct in a 4.5-kb fragment cloned on an XbaI site	This work
pHP100	pWS233, with a 4.5-kb EcoRI fragment containing the pr c *nifHcDK* construct cloned into the EcoRI site	This work
pHP789	pHP100, with a 1.6-kb PstI-EcoRI fragment from pCQ23, cloned downstream of the pr c *nifHcDK* construct	This work
pHP210	pTR101, with a 4.5-kb EcoRI fragment containing the *nifHc* gene with its own regulatory region	This work
pHP220	pTR101, with a 5-kb EcoRI fragment containing *nifHDKb* operon with its own regulatory region	This work

(5′-GGC CGG GTT CAC GAC C), and 238 reverse (5′-CGT TCC TGG TTG ATA TCG AGC CAA GGT GTC) located downstream to *nifK*. DNA sequencing of the pr c *nifHcDK* construct in pSym from strain HP310 was done on a 5-kb product obtained with primers *nifHc* EcoRV forward and 238 reverse with HP310 total DNA as template and then with all of the mentioned oligonucleotides as primers to obtain the sequence of the product with a PerkinElmer DNA sequencer.

Nodulation test, nitrogenase activity, and nitrogen content determination in bean plants and seeds. Seeds of *P. vulgaris* cv. Negro Jamapa were surface sterilized and germinated as previously reported (5). *R. etli* strains used for inoculation were grown overnight in PY complex medium, washed twice with a 0.85% NaCl solution, and diluted to an A_{540} of 0.05. Seedlings were planted in groups of five in autoclaved pots containing vermiculite as support material, and then each one was inoculated with 1 ml of bacterial suspension (approximately 10^6 cells per plant). As controls, experiments included noninoculated plants fertilized with 10 mM KNO_3–2 mM NH_4NO_3 or without added nitrogen. Plant growth and watering were carried out under aseptic conditions in a greenhouse.

Greenhouse conditions included temperature of 22 to 28°C and relative humidity of 50 to 60%. Groups of 10 plants for each experimental condition were harvested at 18, 25, and 32 dpi, and the nodule dry weight, nitrogenase activity, total plant dry weight, and nitrogen content were determined for each plant including the noninoculated (control) plants. Bacteria were isolated from nodules, and their identities verified by their antibiotic resistance patterns. Nitrogenase specific activity (expressed as μmoles of ethylene h^{-1} g of nodule dry $weight^{-1}$) was determined by incubating the detached root with 1/80 (vol/vol) acetylene. Ethylene production was estimated with a model 3300 gas chromatograph (Varian, Middelburg, The Netherlands). Plants or seeds were dried in an oven at 60°C for 3 days. Total nitrogen content of samples from dry plants or seeds was determined with a nitrogen analyzer (model ANTEK 9000; Antek Instruments, Inc., Houston, Tex.) and reported as milligrams of nitrogen per gram of dry plant or per gram of powdered seed. Nitrogen yield was calculated by multiplying the nitrogen content in seed times the yield and is expressed as milligrams of N in seed $plant^{-1}$. Statistical analysis was performed according to the method of Steel and Torrie (33).

β-Galactosidase activity determination in *R. etli* cultures and plant nodules. Cultures of *R. etli* strains were grown overnight in PY medium, collected, and washed with minimal medium as described above. Flasks containing minimal medium were inoculated at an initial A_{540} value of 0.05. Aliquots (20 ml) were injected into 150-ml bottles sealed with rubber stoppers, flushed with several volumes of 1% oxygen–99% argon mixture (analytical grade; Linde, Mexico City, Mexico), and incubated at 30°C with shaking at 200 rpm. Replicas of the cultures were simultaneously incubated in cotton-stoppered flasks to evaluate aerobic conditions. After 8 h, 1-ml samples were withdrawn, centrifuged at 10,000 × *g* at 4°C, and resuspended in 1 ml of cold Z buffer for β-galactosidase activity determination as described elsewhere (28). Replica 1-ml samples were pelleted and resuspended in 5% TCA, and their protein content was determined by the method of Lowry et al. (21). Specific activities were reported as nmoles of *o*-nitrophenol $minute^{-1}$ milligram of $protein^{-1}$.

Nodules from single plants were crushed in 1 ml of cold Z buffer (28) and centrifuged at 4°C for 5 min at 8,000 × *g* in a benchtop centrifuge, and a 0.05-ml aliquot of clear supernatant was transferred to a tube containing 0.95 ml of Z buffer and thoroughly mixed with 2 drops of chloroform. β-Galactosidase activity was measured in a Beckman DU7500 spectrophotometer at 420 nm as recommended by the manufacturer (28). Additional aliquots of the nodule extract (0.05 ml) were precipitated with 0.5 ml of 5% TCA, and the protein content was measured by the method of Lowry et al. (21). Specific activities were reported as nmoles of *o*-nitrophenol $minute^{-1}$ milligram of $protein^{-1}$.

Strain deposition. The chimeric pr c *nifHcDK* construct, strains containing it, and other relevant sequences have been submitted for patents. Strain HP310 was deposited under accession no. NRRL B-30606 in the Culture Collection of the USDA Agricultural Research Service, Peoria, Ill.

RESULTS

Construction and transcriptional expression analysis of the chimeric pr c *nifHcDK* operon. *R. etli* contains three copies of *nifH* that encode nitrogenase reductase (26, 27, 35). One of these, *nifHc*, is expressed at higher levels than the other two and is induced during nodule development in a NifA-dependent manner (35). Its regulatory region contains an unusual NifA-binding site upstream of the RpoN-dependent promoter,

which differs from the canonical NifA-binding site located upstream from the *nifHa* and *nifHb* copies (35). To construct a chimeric pr c *nifHcDK* operon, the *nifHDKb* genes lacking their promoter were cloned downstream of the *nifHc* promoter and subcloned into the stable vector plasmid pTR101 or the suicide plasmid pWS233 for conjugation, as described in Materials and Methods (Fig. 1). The construct was cloned into plasmid pTR101, which is stably inherited in *R. etli*, and the plasmid obtained was named pHP55 and introduced to CFN42 by a triparental mating. To integrate the construct into the pSym of CFN42, a mating was made with *E. coli* cells containing suicide plasmid pHP789 with the construct. Recombinant cells were grown on PY plates with tetracycline and then grown in PY liquid medium plus sucrose for positive selection of double recombinants, presumably containing no vector sequences. A colony was chosen and named HP310.

To determine the expression of the chimeric pr c *nifHcDK* construct in *R. etli* CFN42, a *nifH-lacZ* fusion was created by inserting a *lacZ-kan* cassette into the BglII site of pHP789 and then introduced by triparental mating into strain HP310. β-Galactosidase activity of the strain containing this fusion, named HP310 lac, was determined in free-living cultures under a low-oxygen atmosphere (1% oxygen, 99% argon) and in symbiosis. This fusion presented a 4.4-fold induction respect to aerobic conditions. Low oxygen is a well-known physiological condition for the NifA-mediated induction of *nifH* (35). For comparison, strains DEM153 (*nifHa-lacZ* in pSym) and DEM233 (*nifHc-lacZ* in pSym) were used (35), and as described above, the *nifHc-lacZ* fusion was more highly expressed (21-fold induction) than the *nifHa-lacZ* fusion (6.5-fold induction) under microaerobic conditions relative to aerobic conditions (Table 2).

β-Galactosidase activity from nodules formed by *R. etli* strains carrying the reporter gene fused to *nifH* under the transcriptional control of pr c (HP310 lac and DEM233 strains) had the highest values during the early days of symbiosis (11 and 18 dpi). For the latter day, two independent experiments were conducted which gave similar results, and one is shown in Table 2. Activity of the fusion in strain HP310 lac was similar to that of DEM233 at 18 dpi (Table 2).

Construction and genetic characterization of strains containing the pr c *nifHcDK* chimeric operon. The chimeric pr c *nifHcDK* operon contained on pHP55 plasmid was transferred to *R. etli* CFN42 and SAM100 (*phaC*) (8) as described in Materials and Methods. Plasmid DNA was extracted from the transconjugants, and the BamHI digestion profile was found to be identical to that of pHP55 (data not shown). An *R. etli* strain with the chimeric operon incorporated into pSym was made by a triparental mating with *E. coli* HB101/pHP789 as donor, *E. coli* HB101/pRK2013 as helper, and *R. etli* CFN42 as recipient, as described in Materials and Methods.

To confirm genetic exchange, we carried out a PCR assay with an upper oligonucleotide designated *nifHc Eco*RV forward, which specifically hybridizes with the *nifHc* promoter region, and a lower oligonucleotide, *nifD* reverse, corresponding to the 3′ end of *nifD.* This segment is absent in the wild-type *nifHcD** reiteration. Only the pHP789 plasmid (Fig. 2A) and strains derived from the mating mentioned contained the expected 1.8-kb fragment; one of these, designated HP310, was selected (Fig. 2A). In contrast, strain CFN42 did not produce a PCR product with this oligonucleotide combination (Fig. 2A). The 1.8-kb fragment was sequenced on both ends, and adequate priming was confirmed (data not shown). The recombinant *nifHDK* operon was obtained by PCR with strain HP310 DNA as template with *nifHc* EcoRV forward and 238 reverse (located downstream of *nifK*) oligonucleotides, and its nucleotide sequence was obtained. The sequence revealed that the *nifHDK* operon was coupled to an intact *nifHc* promoter region (data not shown).

To determine the genetic modifications in *nifHDKb* operon caused by the double-recombination process with plasmid pHP789, we hybridized total DNA digested with BamHI from

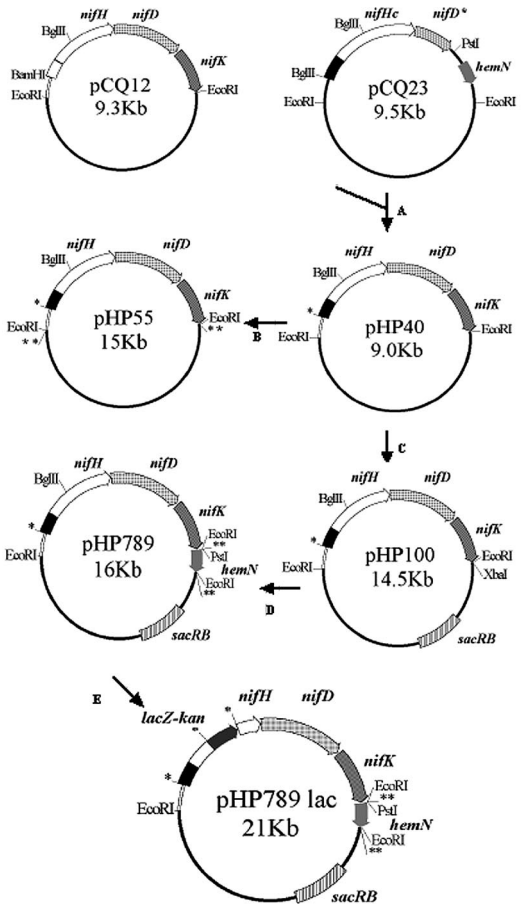

FIG. 1. Scheme of plasmid construction. (A) pCQ12 was digested with BamHI and BglII; the largest fragment was ligated to a 1.8-kb BglII fragment from pCQ23. The promoter region of *nifHDKb* (pr b) is represented by an open box, and the promoter region of *nifHc* (pr c) is represented by a closed box. (B) pHP40 was digested with EcoRI, and the fragment containing pr c *nifHcDK* was cloned into the EcoRI site of pIC20H, which was then digested with SpeI, and the fragment of interest was cloned into the XbaI site of pTR101. (C) pHP40 was digested with EcoRI, and the 4.5-kb fragment containing pr c *nifHcDK* was cloned into the EcoRI site of pWS233, generating pHP100 plasmid. (D) pHP100 was digested with XbaI and ligated into a 1.6-kb SpeI fragment containing part of the *hemN* gene from pCQ23 digested with PstI and EcoRI, obtaining pHP789 plasmid. (E) pHP789 was digested with BglII and ligated to a 5-kb BamHI fragment containing *lacZ-kan* genes. *, site formed by BamHI-BglII joining; **, site formed by XbaI/SpeI joining; plasmids are not drawn to scale.

TABLE 2. Transcriptional activity of *nifH-lacZ* fusions in free-living cultures and nodules

Strain	Sp act of β-galactosidase (nmol of ONP min^{-1} mg of $protein^{-1}$) in[a]:			
	Free living cultures		Nodules	
	1% O_2	20% O_2	11 dpi	18 dpi
CFN42	7 ± 2	9 ± 2	NA	65 ± 15
HP310 lac (*nifHc-lacZ*)	363 ± 75	82 ± 10	492 ± 137	929 ± 136
DEM153 (*nifHa-lacZ*)	136 ± 25	20 ± 3	276 ± 57	372 ± 190
DEM233 (*nifHc-lacZ*)	426 ± 40	21 ± 5	1,005 ± 143	1,158 ± 358

[a] Data from free-living cultures are the averages of three different experiments. Nodules from five plants per strain per day were analyzed. Values are means ± standard deviations. NA, not analyzed; ONP, *o*-nitrophenol.

HP310 and CFN42 against *nifH* and *nifK* probes. With BamHI digestion, strain CFN42 presents three *nifH* signals of approximately 9.0, 6.3 and 4.5 kb (26). The first two correspond to *nifHDK* nitrogenase operons a and b. However, in strain HP310, the *nifH* hybridization showed that the wild-type *nifHDKb* band (6.3 kb) was absent and instead the strain contained a 4.8-kb band, very close to the 4.5-kb band corresponding to wild-type *nifHc* (Fig. 2B). A *nifK* hybridization demonstrated that the 4.8-kb band was a complete *nifHDK* operon (Fig. 2B).

Incorporation of the chimeric construct in the *nifHDKb* reiteration was not unexpected because plasmid pHP789 was constructed based on the *nifHDKb* reiteration and a 300-bp fragment belonging to region b remained upstream of the pr c *nifHcDK* construct (Fig. 1). It is possible that this 300-bp segment participated in the recombination process. All isolates obtained by mating with pHP789 and analyzed by hybridization showed the chimeric construct always incorporated into the *nifHDKb* reiteration (data not shown). Furthermore, we hybridized total DNA digested with BamHI from HP310 and CFN42 against a cosmid collection which covers the entire CFN42 pSym sequence (13). In strain HP310 we observed a band of 4.8-kb instead of the 6.3-kb band in the *nifHDKb* region, while the rest of the symbiotic plasmid appeared intact (data not shown). By sequencing downstream of the end of *nifKb*, a single change of one base, which created a BamHI site, was found and was not present in the wild-type sequence (data not shown). The latter explained the reduction in band length.

R. etli CFN42 contains six high-molecular-weight plasmids (with DNA sizes of 150 to 600 kb), named p42a to p42f. The symbiotic plasmid is p42d (371 kb) (14). Plasmids p42b and p42a have similar sizes (150 kb) and appear as a doublet (Fig. 2C). Eckhardt plasmid profile analysis revealed that the symbiotic plasmid of HP310 was similar in size to that of CFN42, but that p42a was absent (Fig. 2C). However, the rest of the plasmids appeared intact. Curing of p42a could be due to additional recombination events originated by reiterated identical sequences shared by both replicons p42a and p42d. It has been previously shown that curing of p42a from the wild-type strain does not alter its symbiotic properties (6). A 7-kb fragment of the symbiotic plasmid, upstream of pr c *nifHcDK* in strain HP310, was sequenced and found to be identical to that reported for CFN42 pSym (14), except for a 20-bp deletion located close to a transposase (data not shown). This 7-kb sequence is also present in p42a (G. Dávila and V. González, unpublished data).

Symbiotic performance of an *R. etli* strain overexpressing nitrogenase. The nitrogenase expression-enhanced pr c *nifHcDK* operon harbored on plasmid pHP55 was introduced into strain CFN42, and its symbiotic effectiveness was evaluated on bean plants (Fig. 3). A control CFN42 strain harboring plasmid pAM341 (strain AM341 [24]), containing only the *nifHc* promoter region (pr c) cloned into pTR101, was included in all experiments, and no differences were observed in comparison

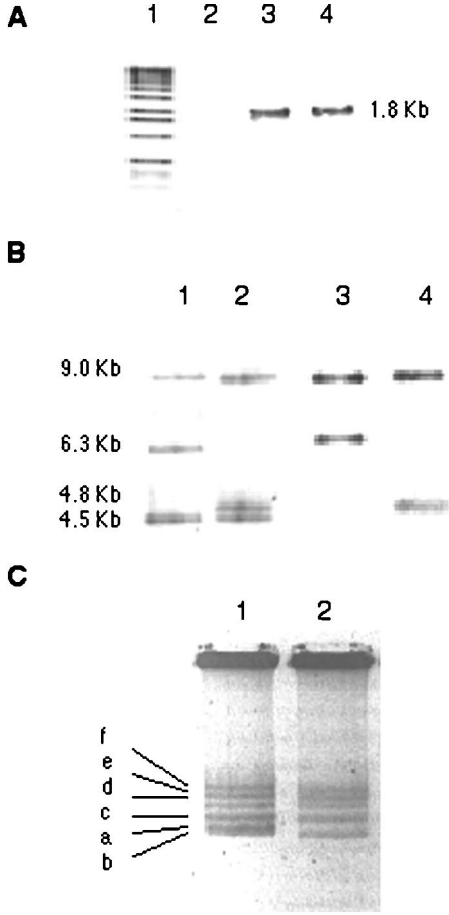

FIG. 2. Genetic characterization of strain HP310. (A) PCR with oligonucleotides *nifHc* EcoRV forward and *nifD* reverse. Lanes: 1, DNA size marker; 2, CFN42; 3, HP310; and 4, plasmid pHP789. (B) Southern hybridization using as probes an intra-*nifH* PCR product (lanes 1 and 2) and an intra-*nifK* PCR product (lanes 3 and 4). Lanes: 1 and 3, CFN42; 2 and 4, HP310. (C) Eckhardt plasmid profile of strains CFN42 (lane 1) and HP310 (lane 2). a to f, plasmids p42a to p42f, respectively.

with CFN42 (data not shown). Three independent assays with each modified strain were performed in the greenhouse. Data presented correspond to a representative experiment. There were no differences between strains with regard to number of nodules and internal (determined by optical microscopy) or external morphology (data not shown). Plants inoculated with HP55 had increases of about 23, 38, and 120% in nitrogenase activity at 18, 25, and 32 dpi, respectively, compared with plants inoculated with the parent strain CFN42, although it was only significantly different at 32 dpi ($P < 0.05$) (Fig. 3A).

Correlating with the higher nitrogenase activity observed in bean plants inoculated with strain HP55, there was an increase of 25% in plant weight (mean ± standard deviation, 0.56 ± 0.11 and 0.70 ± 0.13 g plant^{-1} for CFN42 and HP55, respectively) at 32 dpi. For nitrogen content in plants, HP55 had an increase of 15% (24.5 ± 5.9 mg of N plant^{-1}) with respect to CFN42 (21.3 ± 4.7 mg of N plant^{-1}) at 32 dpi. A major difference was observed when seed yields were compared. Plants inoculated with strain HP55 produced a significant increase of 39% (at $P < 0.05$) in seed yield (1.49 ± 0.16 g of seed plant^{-1}) compared with plants inoculated with the parent strain CFN42 (1.07 ± 0.21 g of seed plant^{-1}) (Fig. 3B). Furthermore, it is noteworthy that not only was seed yield increased by the expression of the recombinant pr c *nifHcDK* operon but also the nitrogen content of seeds was increased by 16% (from 43 ± 2 to 50 ± 3 mg of N g of seed^{-1} in plants inoculated with strains CFN42 and HP55, respectively) (Fig. 3C). As a result of the increase in yield and nitrogen content in seeds, an increase of 62% in nitrogen yield was obtained with strain HP55 (74.5 mg of N in seed plant^{-1}) compared to that with CFN42 (46.0 mg of N in seed plant^{-1}). The growth responses of *P. vulgaris* plants (45 dpi) inoculated with strain HP55 compared with plants inoculated with wild-type strain CFN42 are shown in Fig. 3D. More than 99% of the bacteria isolated from strain HP55-induced nodules retained the pHP55 plasmid after 25 or 32 dpi.

Symbiotic contribution of *nifHc* or *nifHDKb* overexpression in *R. etli*. To determine the contribution to symbiotic performance of overexpression of *nifHc* or *nifHDK*, and to compare with that produced by pr c *nifHcDK*, we cloned into plasmid pTR101 the respective reiterations of strain CFN42. The plasmids obtained, pHP210 (pTR101, *nifHc*) and pHP220 (pTR101, *nifHDKb*), were incorporated by triparental mating into CFN42 strain and assayed in the greenhouse. The numbers of nodules and the morphology formed by all these strains appeared normal and were similar to those for the wild-type strain CFN42 (data not shown). A dot blot hybridization was made with mRNA extracted from 18-dpi nodules, showing that the *nifH* transcript was more abundant in nodules obtained for HP210 and HP55 inoculation (122 and 106%, respectively) than those formed by CFN42. HP220 presented 25% more *nifH* transcript than CFN42 (Fig. 4). The relative intensity signal was calibrated with use of the 16S rRNA gene.

Nitrogenase activity in bean plants produced by inoculation with strains HP210 and HP220 was slightly increased by 20 and 13%, respectively, while strain HP55 had a significant increase of 68% (at P of <0.05), compared with that for strain CFN42 at 18 dpi (Table 3). In plant weight determination (at 32 dpi), HP210 and HP220 produced increases of 39 and 22%, respectively, compared with CFN42. HP55 produced a significant increase of 50% against CFN42. In regard to seed yield, HP210 and HP220 had increases of 21 and 9%, respectively, compared to CFN42 (Table 3). However, HP55 inoculation produced 2.50 ± 0.23 g plant^{-1}; this is a significant increase of 75% compared to that of CFN42. As expected, nitrogen-fertilized plants produced the highest value (2.60 ± 0.48 g plant^{-1} [Table 3]). With regard to nitrogen content in seed, HP220 had higher values than HP210. In this parameter, strain HP55 had an increase of 29% compared to CFN42 strain and 21% more than the nitrogen-fertilized plants. With regard to nitrogen yield, strains HP220 and HP210 had increases of 11 and 33% (64.6 and 54.0 mg of N in seed plant^{-1}, respectively) compared with CFN42 (48.5 mg of N in seed plant^{-1}), while HP55 increased 125% (109.0 mg of N in seed plant^{-1}) compared to CFN42 and 16% above nitrogen-fertilized plants (93.9 mg of N in seed plant^{-1}). As observed, symbiotic overexpression of pr c *nifHcDK* (HP55) in *R. etli* produced the highest increases in all parameters measured in comparison with results for overexpression of *nifHDKb* (HP220) or *nifHc* (HP210).

Symbiotic performance of an *R. etli* PHB$^-$ strain expressing the pr c *nifHcDK* construction. A PHB$^-$ *R. etli* strain showed 5- to 21%-higher nitrogenase activity compared with that for wild-type strain CFN42 in late stages of symbiosis with *P. vulgaris* (8). Additionally, increases in seed yield (8%) and nitrogen content in seed (15%) were observed (8). To determine if an additive effect could be obtained by combining the expression of the chimeric pr c *nifHcDK* construct and a PHB$^-$ background, plasmid pHP55 containing pr c *nifHcDK* was introduced by conjugation into strain SAM100 (8). Strain SAM100 had increases of 29, 13, and 87% in nitrogenase activity at 18, 25, and 32 dpi (Fig. 3A), 4% in plant weight (0.58 ± 0.11 g plant^{-1}), 34% in nitrogen content per plant (28.6 ± 6.6 mg of N plant^{-1}), 60% in seed yield (1.71 ± 0.34 g plant^{-1}), 12% in nitrogen content in seed (Fig. 3C), and 46% in nitrogen yield (78.7 mg of N in seed plant^{-1}) with respect to its parent strain CFN42. Nitrogenase activities in bean plants inoculated with SAM100/pHP55 were higher than and significantly different (at P of <0.05) from those in plants inoculated with its parent strain SAM100 at 25 and 32 dpi by 82 and 42%, respectively (Fig. 3A). Furthermore, increases of 19% in plant weight (0.69 ± 0.09 g plant^{-1}), 12% in nitrogen content per plant (31.7 ± 5.5 mg of N plant^{-1}), 18% in seed yield (Fig. 3B), 19% in nitrogen content in seed (Fig. 3C), and 32% in nitrogen yield (104.3 mg of N in seed plant^{-1}) were observed for SAM100/pHP55 inoculation compared to SAM100 inoculation.

Symbiotic effect of an *R. etli* strain with the pr c *nifHcDK* construct incorporated into pSym. We assessed the symbiotic effect on bean plants inoculated with strain HP310, which contains the pr c *nifHcDK* construct in pSym. In the greenhouse, plants inoculated with HP310 had increases of 25, 97, and 44% in nitrogenase activity at 18, 25, and 32 dpi, respectively, compared with plants inoculated with parent strain CFN42 (Fig. 5A), with significant differences obtained at 25 and 32 dpi ($P < 0.05$). HP310 had a significant increase of 38% in plant weight (0.76 ± 0.08 g plant^{-1}) compared with CFN42 (0.55 ± 0.06) (Fig. 5B) at 32 dpi. For comparison, results for noninoculated (0.47 ± 0.08) and fertilized (1.11 ± 0.21 g plant^{-1}) plants are shown in Fig. 5B. Seed yield of plants inoculated with strain HP310 produced a significant increase

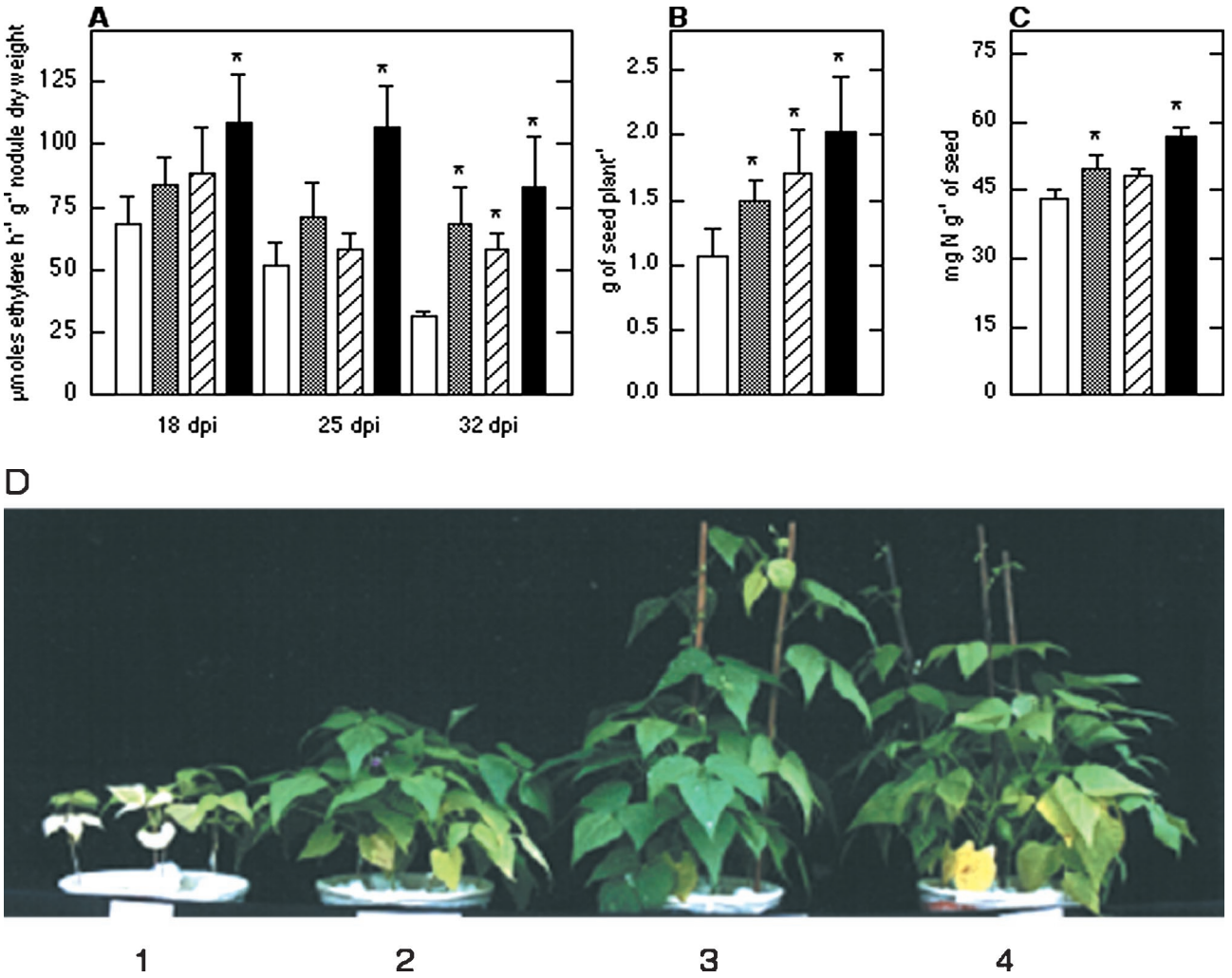

FIG. 3. Symbiotic performance of *R. etli* strains with modified nitrogenase expression construct in greenhouse experiments. (A) Specific nitrogenase activity □ Strains. □, CFN42; ▨, HP55 (pTR101, pr c *nifHcDK*); ▨, SAM100 (*phaC*); ■, SAM100/pHP55 (*phaC*, pTR101, pr c *nifHcDK*). Values are means ± standard deviations of a representative experiment with 10 *P. vulgaris* plants for each condition and time ($n = 30$). (B) Seed yield from 10 plants. (C) Nitrogen content in seeds from five plants. Asterisks indicate that the means of the samples are different at (P of <0.05) with respect to CFN42. (D) Growth response of *P. vulgaris* plants (45 dpi) inoculated with *R. etli* strains in the greenhouse. Images: 1, Noninoculated nonfertilized; 2, inoculated with CFN42; 3, inoculated with HP55; 4, noninoculated fertilized with 10 mM KNO_3–2 mM NH_4NO_3.

($P < 0.05$) of 33% (1.34 ± 0.3 g of seed plant^{-1}) compared with that in plants inoculated with the parent strain CFN42 (1.01 ± 0.2 g of seed plant^{-1}). In this case, nitrogen content in seed produced by HP310 was significantly increased by 34% compared with that produced by wild-type strain CFN42 (59 ± 3 and 44 ± 2 mg of N g of seed^{-1}, respectively). Nitrogen yield obtained with HP310 was 81% higher than that obtained with CFN42 (79 and 44 mg of N in seed plant^{-1}, respectively). Plants inoculated with HP310 had an appearance similar to those inoculated with HP55 (data not shown).

DISCUSSION

Functional analysis of the elements located upstream of the reiterated *nifH* genes in *R. etli* CFN42 revealed an asymmetric arrangement of the regulatory regions of the two *nifHDK* operons (copies a and b) and the third reiterated *nifH* copy (35). Copies a and b are activated by NifA bound to a canonical binding site, while *nifHc* is activated by NifA bound to a divergent site. This asymmetric arrangement involves a dissimilar facing of the NifA-binding sites located in these promoter regions, which may imply a particular initiation complex architecture resulting in different transcription levels (35). By sequence alignment, a similar arrangement can be found in the reiterated *nifH* regulatory regions of *R. leguminosarum* biovars phaseoli and trifolii and *Rhizobium sp.* strain NGR234, where the NifA binding site in one copy differs by about a half helical turn in distance to its promoter with respect to another copy(ies) (G. Guerrero and J. Mora, unpublished results).

We have found that all strains of *R. etli* analyzed to date carry three *nifH* reiterations, two of them in *nifHDK* operons and the third reiteration linked to a truncated *nifD** gene (35; B. Valderrama, unpublished results). The third *nifH* gene has been analyzed in two strains closely related to *R. etli* isolated from bean nodules, and the corresponding upstream region

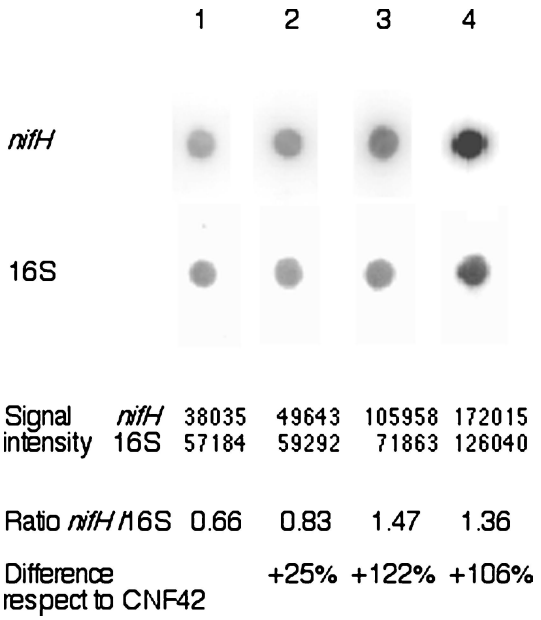

FIG. 4. Dot blot hybridization of mRNA extracted from *P. vulgaris* nodules inoculated with *R. etli* strains at 18 dpi. Lanes: 1, CFN42; 2, HP220; 3, HP210; 4, HP55. Hybridization was done with an intra *nifH* PCR product or 16S DNA as a probe. Intensity signal (in counts) was obtained by exposure in a PhosphorImager screen.

sequence highly resembles that from CFN42 (data not shown). It is important that polar insertions in *nifHc* have no obvious symbiotic phenotype (35).

Rhizobium bacteria undergo a complex differentiation process once they infect legume roots. Bacteroids present a particular structural and physiological adaptation to the nodule environment. One of these physiological changes is nitrogenase induction, mediated by the regulatory protein NifA. It has been shown that NifA is produced constitutively even under ex planta conditions, but since it is intrinsically oxygen-sensitive, it is active only under microaerobic or symbiotic conditions (25).

In order to acquire higher expression levels of nitrogenase while preserving its NifA-dependent regulation, we modified such expression by placing one of the reiterated *nifHDK* operons under the control of the stronger *nifHc* promoter region. It is important that all sequences used in this work are derived from *R. etli*'s own symbiotic plasmid and that no exogenous DNA other than that of the vector was added.

As reported above, the chimeric construct pr c *nifHcDK* was functional under the tested conditions of a low-oxygen atmosphere and in symbiosis (Table 2). It is important that the *nifH* sequence was not altered by the substitution of the promoter region (Fig. 1).

The expression of the chimeric pr c *nifHcDK* operon, either on a *Rhizobium* stably replicating plasmid or incorporated into pSym, produced a better symbiotic performance with *P. vulgaris* plants. The parameters used to assess the symbiosis were nitrogenase activity, dry plant weight, seed yield, and nitrogen content in plants and seeds as described above (Fig. 3 and 5). Furthermore, plant appearance confirmed the enhancement of the symbiotic ability of modified strains HP55 (Fig. 3D) and HP310 (data not shown).

The role of PHB in rhizobial symbiosis is still controversial. The symbiotic relationship between *S. meliloti* and alfalfa (*Medicago sativa*) is very successful, given that the plant derives 80% of its nitrogen requirement from symbiotic nitrogen fixation (15). Since *S. meliloti* does not accumulate PHB in symbiosis (16), reductive power not used for PHB synthesis could be used for nitrogen fixation. However, *R. etli* produces PHB in free life and also in symbiosis (8, 10). An *R. etli* PHB^- mutant produced increased nitrogenase activity in symbiosis and a moderate augmentation in seed yield in comparison with wild-type strain CFN42 (8). Apparently, in this case, part of the reducing power present in the strain was channeled to nitrogenase. By this token, in order to further increase the symbiotic performance of an *R. etli* strain expressing the pr c *nifHcDK* construct, we intended to derive the reducing power excess, produced by the *phaC* mutation, to energize nitrogenase catalysis. As observed above, by combining the latter two characteristics, we obtained a strongly enhanced symbiotic relationship, which gave the highest values of nitrogen fixation reported to date in *R. etli* (Fig. 3). Apparently, this nitrogen fixation effectiveness is the sum obtained by nitrogenase overexpression plus the *phaC* mutation.

According to the results presented, carbon supply to the bacteroid is always in excess under normal nitrogenase activity. The rest of the processes involved in the synthesis of the

TABLE 3. Symbiotic performance of *R. etli* strains with modified nitrogenase expression constructs in greenhouse experiments[a]

Strain or treatment	Nitrogenase activity[b]	Plant dry wt (g $plant^{-1}$)[c]	Seed yield (g $plant^{-1}$)[d]	N content in seed (mg of N g^{-1})[e]
CFN42 (wild type)	64.5 ± 9.2A	0.54 ± 0.13A	1.43 ± 0.13A	33.9 ± 2.3A
HP220(pTR101, *nifHDKb*)	72.7 ± 12.0A	0.66 ± 0.21AB	1.56 ± 0.32AB	41.4 ± 7.5B
HP210(pTR101, *nifHc*)	77.3 ± 18.5A	0.75 ± 0.29AB	1.73 ± 0.24A	31.2 ± 3.1A
HP55(pTR101, pr c *nifHcDK*)	108.2 ± 8.9B	0.81 ± 0.16B	2.50 ± 0.23B	43.6 ± 9.1B
With added N		1.22 ± 0.22C	2.60 ± 0.48B	36.1 ± 2.3AB

[a] Values are means ± standard deviations. Different letters represent significant differences ($P < 0.05$).
[b] Nitrogenase activity is expressed as micromoles of ethylene $hour^{-1}$ gram of $nodule^{-1}$.
[c] Dry weight was measured at 18 dpi. Ten plants were dried and weighed at 32 dpi.
[d] Seed yield from 10 plants (80 days old).
[e] Seeds from 5 plants were evaluated.

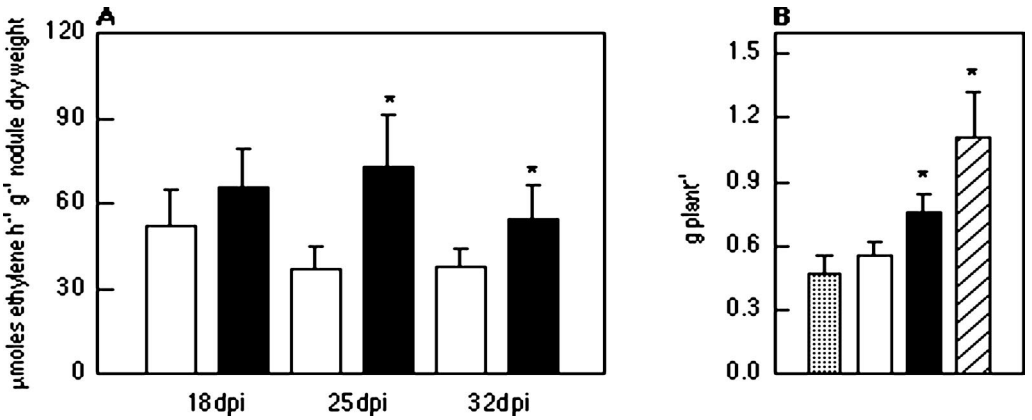

FIG. 5. Symbiotic performance of an *R. etli* strain with a modified nitrogenase expression construct incorporated into pSym. (A) Specific nitrogenase activity of *P. vulgaris* plants inoculated with CFN42 or HP310; (B) plant weight at 32 dpi. Values are means ± standard deviations for 10 plants for each condition and time ($n = 30$). Asterisks indicate that the means of the samples are different at $P < 0.05$. □, inoculation with CFN42; ■, inoculation with HP310; ▨, noninoculated nonfertilized; ▨, noninoculated fertilized with 10 mM KNO_3–2 mM NH_4NO_3.

nitrogenase structural proteins and their assembly are not limited. In addition, it is possible to derive carbon and reductive power to obtain energy for nitrogenase catalysis by abolishing the synthesis of the polymer PHB.

Field testing of the modified strains presented in this work may determine their potential use as a biofertilizer, which could reduce the cost incurred with the application of chemical fertilizers.

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