***Rhizobium etli* Genetically Engineered for the Heterologous Expression of *Vitreoscilla* sp. Hemoglobin: Effects on Free-Living and Symbiosis**

Mario Ramírez,¹ Brenda Valderrama,² Raúl Arredondo-Peter,¹ Mario Soberón,² Jaime Mora,¹ and Georgina Hernández¹

¹Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Apartado Postal 565-A; ²Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apartado Postal 520-3, Cuernavaca, Morelos, México

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Oxygen concentration is an environmental signal that regulates nitrogen fixation in the *Rhizobium*-legume symbiosis. We investigated the effect of the heterologous expression of *Vitreoscilla* sp. hemoglobin (VHb), which is an oxygen-binding protein, in *Rhizobium etli*. The *vhb* gene and its native promoter were subcloned in the plasmid pMR4 and transformed into the *R. etli* strain CE3. Free-living cultures of engineered *R. etli* CE3 expressed the *vhb* gene, as shown by the CO-difference spectral and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analyses of cell extracts. The expression of *vhb* in free-living *R. etli* grown under most limiting oxygen concentrations resulted in an increase in respiratory activity, chemical energy content, and expression of the nitrogen-fixation gene *nifHc*. Bacteroids isolated from nodules of bean plants inoculated with the engineered *R. etli* CE3 expressed the *vhb* gene, as shown by RNA slot-blot analysis. Bean plants inoculated with the engineered strain exhibited higher nitrogenase activity and total nitrogen content (68% and 14 to 53%, respectively) than bean plants inoculated with the *R. etli* wild type. These results suggest that the synthesis of VHb in engineered *R. etli* stimulated the respiratory efficiency of free-living rhizobia, and also probably of symbiotic bacteroids, thus leading to higher levels of symbiotic nitrogen fixation.

Additional keywords: bacterial Hbs, rhizobial nitrogen fixation.

Rhizobia may live either aerobically in the soil or microaerobically in the internal environment of the root nodules of legume plants, where fixation of atmospheric nitrogen occurs. Oxygen concentration is a critical factor that regulates essential processes in the endosymbiotic rhizobia, such as nitrogen fixation and respiration (Batut and Boistard 1994). The apparent paradox of a low oxygen concentration that is required for nitrogen fixation and a high oxygen supply that is

required for bacteroid respiration is satisfied by the cooperation of both symbiotic partners (Batut and Boistard 1994; O'Brian and Maier 1989). In determinate nodules, such as those from soybean and bean plants, a physical cortical barrier exists that limits the diffusion of gases (Layzell and Hunt 1990). Oxygen concentration in the infected cells is about three orders of magnitude lower than the oxygen concentration in the rhizosphere (Layzell and Hunt 1990; Soupène et al. 1995). In nodules, facilitated diffusion of oxygen by leghemoglobin (Lb), a plant protein with high affinity for oxygen, is required to provide the necessary oxygen flux to the actively respiring bacteroids (Appleby 1984; O'Brian and Maier 1989). It has been shown that the addition of Lb to isolated bacteroids results in a 10-fold increase in nitrogenase activity and a twofold increase in the rate of oxygen uptake (Wittenberg et al. 1974).

Oxygen concentration is an important environmental signal that regulates the expression of *nif* genes, which are involved in rhizobial nitrogen fixation (Fischer 1994). In *Rhizobium meliloti*, transcription of *nif* genes is induced in oxygen-depleted conditions under the control of the two-component regulatory system FixL/J. FixL is a membrane oxygen-binding protein that autophosphorylates in the deoxygenated form, and that transmits a signal to FixJ via phosphorylation-dephosphorylation reactions (Gilles-Gonzalez et al. 1991). Further, in the phosphorylated (active) form FixJ will activate NifA protein, which is a transcriptional activator of the *nif* operon. In addition to the *R. meliloti* FixL/J system, a number of oxygen-sensing/regulating proteins have been described, such as Fnr and ArcA, that activate microaerobic metabolism in bacteria growing at low oxygen concentrations (Spiro and Guest 1991).

It is not known if hemoglobins (Hbs), oxygen-binding proteins, exist in rhizobia. However, Hbs have been found in bacteria (Hardison 1998). Although little is known about the function of bacterial Hbs, some bacterial species synthesize Hbs under hypoxic conditions, and thus it is likely that under these conditions some species use Hbs for delivery of oxygen as the terminal electron acceptor (Hardison 1998). The first known, and a thoroughly studied, bacterial Hb was isolated from a *Vitreoscilla* sp. (Wakabayashi et al. 1986). Because the synthesis of *Vitreoscilla* sp. hemoglobin (VHb) increases un-

Corresponding author: Georgina Hernández; Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Apartado Postal 565-A, Cuernavaca, Morelos, México; Telephone or Fax: +52 (73) 11 67 10; E-mail: gina@cifn.unam.mx

der hypoxic conditions, it has been postulated that the function of Vhb is to bind and transport oxygen for the bacterial metabolism in microaerobic environments (Webster 1987). The gene that codes for Vhb has been cloned and sequenced (Dikshit and Webster 1988; Koshla and Bailey 1988), and the *vhb* gene together with the native promoter has been expressed in heterologous microbial hosts, such as *Escherichia coli* (Dikshit et al. 1990; Koshla and Bailey 1988), *Serratia marcescens* (Wei et al. 1998), *Streptomyces coelicolor* and *S. lividans* (Magnolo et al. 1991), *Acremonium chrysogenum* (DeModena et al. 1993), and *Saccharomyces cerevisiae* (Chen et al. 1994), as well as in the plant host *Nicotiana tabacum* (Holmberg et al. 1997). Effects resulting from the modification of aerobic metabolism by transgenic Vhb, such as increased cell densities, protein synthesis, and secondary metabolite production, have been reported for engineered *E. coli* and fungi (Koshla and Bailey 1988; Koshla et al. 1990; Khosravi et al. 1990; DeModena et al. 1993; Magnolo et al. 1991). Transgenic tobacco expressing *vhb* showed enhanced plant growth, reduced germination and flowering time periods, and higher chlorophyll and nicotine content (Holmberg et al. 1997).

Because the expression of *nif* genes and the efficiency of nitrogen fixation depend on the concentration of oxygen, we were interested in studying the effect of the heterologous expression of Vhb in *R. etli*, both in free-living conditions and in symbiosis with beans. Our results showed that engineered *R. etli* expressing Vhb had enhanced respiration rates and *nif* gene expression under low oxygen concentration. Also, engineered rhizobia exhibited higher nitrogenase activity during symbiosis, and nodulated plants exhibited higher total nitrogen content in foliage and seeds.

RESULTS

Expression of *vhb* in free-living *R. etli* CE3.

To express the *Vitreoscilla* sp. *vhb* gene in *R. etli* CE3, a 2.2-kb fragment from the plasmid pUC8:15 containing the *vhb* gene and its native promoter was subcloned into the stable vector pTR101 (Davis et al. 1992; Weinstein et al. 1992), thus generating the pMR4 plasmid. Plasmid pMR4 was conjugated to *R. etli* CE3 and was >99% stable when transconjugants were grown on PY or minimal medium without selection. Moreover, in contrast to the white color of the cell pellet of the *R. etli* CE3 wild type, the cell pellet of *R. etli* CE3/pMR4 had a reddish color after centrifugation of liquid cultures, which suggested that the *vhb* gene was expressed in the transformed cells.

The *Vitreoscilla* sp. *vhb* gene, regulated by its own promoter, has been expressed in heterologous bacterial hosts (Dikshit et al. 1990; Khosla et al. 1990; Magnolo et al. 1991). In order to determine whether the *vhb* gene was expressed in free-living *R. etli* CE3/pMR4, slot-blot analysis was performed with the bacterial RNA as template and the *vhb* gene as probe. The Vhb transcript was detected in *R. etli* CE3/pMR4 cells grown in microaerobic liquid cultures, and no hybridization signal was detected in the control wild-type strain *R. etli* CE3 (Fig. 1).

The synthesis of Vhb protein in engineered bacteria has been confirmed by obtaining the differential spectra (CO-dithionite reduced versus dithionite reduced) of cell extracts.

Characteristic signals of Vhb in CO-differential spectra have been reported with absorption maxima at 419, 532, and 570 nm and troughs at 436 and 554 nm (Webster and Liu 1974; Tyree and Webster 1978). In order to test for the presence of Vhb in *R. etli* CE3/pMR4, CO-differential spectra of cell extracts were obtained. Protein samples obtained from *R. etli* CE3 and CE3/pMR4 strains (see Materials and Methods) were reduced with dithionite, bubbled with CO, and the CO-differential (CO-reduced versus reduced) spectra were obtained. As a positive control, CO-differential spectra of partially purified Vhb were also obtained. The resulting CO-differential spectra showed differences between *R. etli* CE3/pMR4 (engineered) and CE3 (wild-type) strains (Fig. 2A). The CO spectrum of *R. etli* CE3/pMR4 showed maxima at 418, 534, and 567 nm and troughs at 436 and 551 nm, which are quite similar to peaks and troughs of the CO spectrum obtained for the partially purified Vhb (Fig. 2A). In contrast, the CO spectrum of the wild-type *R. etli* CE3 showed a small peak at 421 nm and a trough at 439 nm, which are different from the values observed for the *R. etli* CE3/pMR4 and Vhb CO spectra. These results indicated that Vhb had been synthesized in *R. etli* CE3/pMR4, which was further confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Figure 2B shows that a protein that co-migrates with the native Vhb in a polyacrylamide gel had been synthesized in *R. etli* CE3/pMR4 but not in *R. etli* CE3.

Respiratory activity and chemical energy content in free-living *R. etli* CE3/pMR4.

It has been proposed that the expression of Vhb in *E. coli* improves the efficiency, rather than the rate, of respiration under microaerobic conditions by increasing the amounts of dissolved oxygen and the efficiency of ATP production (Kallio et al. 1994). To determine the respiratory efficiency of engineered rhizobia, we evaluated the succinate oxidase activity of *R. etli* CE3 and CE3/pMR4 cells grown in microaerobic liquid cultures. Figure 3 shows that, when cultured microaerobically, *R. etli* CE3/pMR4 has two- to threefold higher respiratory rates than the *R. etli* CE3 wild-type strain. Moreover, we de-

Fig. 1. Slot-blot analysis of free-living *Rhizobium etli* RNA hybridized with the *vhb* gene probe. *R. etli* CE3 and CE3/pMR4 free-living cells were grown at 0.5% oxygen; bacteroids were isolated from 18-day-post-inoculation nodules. The RNA concentration loaded in each slot is indicated. **A**, 32 P-labeled *vhb* gene from pUC8:15 or **(B)** 16S rRNA-gene fragment from pKK3535 were used as the experimental and control probes, respectively.

terminated the ATP, ADP, NADH, and NADPH content in *R. etli* CE3 and CE3/pMR4 cells grown in microaerobic liquid cultures, and results showed that the nucleotide content was higher in *R. etli* CE3/pMR4 than in *R. etli* CE3 wild-type strain (Table 1). The observed increase in total chemical energy was 23, 82, and 69% at 0.25, 0.5, and 1% oxygen, respectively, relative to the wild-type strain (Table 1).

Activity of *nifHc* in free-living *R. etli* DEM233/pMR4.

All *R. etli* strains characterized so far have three genes encoding nitrogenase reductase (*nifH*), denoted *nifHa*, *nifHb*, and *nifHc* (Quinto et al. 1985). The ex planta expression of *nifHa* and *nifHc* in *R. etli* is induced by microaerobiosis in minimal medium cultures (Valderrama et al. 1996). In microaerobic cultures and bean nodule bacteroids the *nifHc* gene is expressed at higher levels than the *nifHa* gene (Valderrama et al. 1996). To evaluate the effect of Vhb on the ex planta expression of *nifHc*, we used the *R. etli* DEM233 strain with the *nifHc-lacZ* gene fusion (Valderrama et al. 1996), which was engineered with the plasmid pMR4, and then we determined the β -galactosidase activity. *R. etli* DEM233 (control) and DEM233/pMR4 strains were grown under low oxygen concentrations. Figure 4 shows that *R. etli* DEM233 has high β -galactosidase activity in cultures grown between 0.25 and 1% oxygen, and that maximal β -galactosidase activity was detected at 1% oxygen concentration for *R. etli* DEM233 and DEM233/pMR4, but activities de-

clined 80% when the oxygen concentration was increased to 5%. Nevertheless, statistical analysis showed that the β -galactosidase activity was significantly higher (30%) in *R. etli* DEM233/pMR4 than in *R. etli* DEM233 when the strains were grown at 0.25, 0.5, and 0.75% oxygen (Fig. 4). The Vhb transcript was detected in every one of the oxygen concentrations used for this experiment (data not shown).

Symbiotic properties of *R. etli* CE3/pMR4.

Bean plants were inoculated with either *R. etli* CE3/pMR4, carrying the *vhb* gene, or with the *R. etli* CE3 control strain. The total bacterial population isolated from nodules elicited

Table 1. Chemical energy (Chem. en.) content of *Rhizobium etli* CE3 and CE3/pMR4 grown in microaerobic liquid cultures

Oxygen concentration	Strain	ATP	ADP	NADH	NADPH	Chem. en. ^a
		nmol per mg of protein				
0.25%	CE3	0.312	1.023	1.337	0.371	6.44
	CE3/pMR4	0.624	1.653	1.542	0.474	7.97
0.5%	CE3	0.132	0.577	2.001	0.491	8.37
	CE3/pMR4	0.430	1.624	3.573	0.830	15.28
1%	CE3	0.143	0.620	1.252	0.320	5.49
	CE3/pMR4	0.169	0.926	2.104	0.581	9.27

^a Chemical energy expressed as sum of ATP equivalents (1, 1/2, 3, and 4 for ATP, ADP, NADH, and NADPH, respectively) per mg of protein.

Fig. 2. Spectral and electrophoretic analyses of extracts obtained from *Rhizobium etli* CE3/pMR4 and *R. etli* CE3. **A**, CO-differential spectra (CO-reduced vs reduced) of partially purified *Vitreoscilla* sp. hemoglobin (Vhb; trace 1), *R. etli* CE3/pMR4 (trace 2), and *R. etli* CE3 (trace 3). **B**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of protein extracts obtained from *R. etli* CE3/pMR4 and CE3: lane 1, molecular mass markers (in kDa); lane 2, Vhb partially purified from *Escherichia coli*; lanes 3 and 4 (20 and 30 μ g of total protein, respectively), fractionated (45 to 65% saturation with ammonium sulfate) soluble protein obtained from *R. etli* CE3/pMR4; lanes 5 and 6, same as lanes 3 and 4, but obtained from *R. etli* CE3.

by *R. etli* CE3/pMR4 was Tc^r, thus indicating that the plasmid pMR4 was retained in bacteroids during symbiosis. Nodules elicited by *R. etli* CE3/pMR4 showed a more intense red color than those elicited by the wild-type strain CE3. Slot-blot hybridization at high stringency of the *vhb* gene probe with RNA isolated from *R. etli* CE3/pMR4 and CE3 showed that a hybridization signal was detected in *R. etli* CE3/pMR4 but not in

Fig. 3. Respiratory activity of *Rhizobium etli* CE3/pMR4 and CE3 in microaerobic liquid cultures. Oxidase activity present in the CE3 wild-type strain or the CE3/pMR4 strain was measured after 8 h of incubation in microaerobic liquid cultures under different oxygen concentrations.

Fig. 4. Induction of the *nifHc* gene from *Rhizobium etli* strains in microaerobic liquid cultures. The β-galactosidase activity present in an *R. etli* DEM233 wild-type strain containing a *nifHc-lacZ* gene fusion and in the same strain but bearing the pMR4 plasmid (*R. etli* DEM233/pMR4) was measured after 8 h of growing in microaerobic liquid cultures under different oxygen concentrations. Values are mean ± standard deviations of Miller units from four different cultures assayed in duplicate.

CE3, thus indicating that the *vhb* gene was expressed in *R. etli* CE3/pMR4 bacteroids (Fig. 1).

Nodulation, nitrogenase activity, and nitrogen content were determined in bean plants inoculated with *R. etli* CE3 or CE3/pMR4 strains. Nodules elicited by the CE3/pMR4 strain emerged at the same time as those elicited by the wild-type strain, and the nodule dry weight was similar among plants inoculated with the engineered and wild-type strains. Figure 5A shows the results of time-course determinations of nitrogenase-specific activity. At early stages of the symbiosis, 15 and 20 days post inoculation (dpi), when nitrogenase-specific activity peaks, nodules elicited by *R. etli* CE3/pMR4 exhibited nitrogenase activity 68% higher than the values exhibited in nodules formed by the wild-type strain. At 25 dpi and later stages, the nitrogenase activity declined in both strains, although the activity in nodules formed by CE3/pMR4 was slightly higher.

Fig. 5. A, Nitrogenase specific activity and (B) foliage total nitrogen content of *Phaseolus vulgaris* plants inoculated with *Rhizobium etli* CE3 or CE3/pMR4 strains. Values are mean ± standard deviation of eight plants for each condition. Results shown are for one representative experiment out of five performed.

Because a higher nitrogenase activity during the period of nitrogen fixation could result in a higher accumulation of nitrogen in the plant, we determined the total nitrogen content in the foliage of plants inoculated with CE3/pMR4 or CE3 strains (Fig. 5B). At every time point, the total nitrogen content in the foliage of plants inoculated with the engineered strain was higher, ranging from 14 to 53%, than the values of plants inoculated with the wild-type strain (Fig. 5B). At later time points, the foliage dry weight of plants inoculated with CE3/pMR4 was slightly higher (10 to 20%) than that of plants inoculated with the wild-type strain. We also observed that the plants inoculated with CE3/pMR4 flowered and formed pods about 8 to 12 days earlier than the control plants that had been inoculated with *R. etli* CE3. Moreover, seeds were harvested from plants inoculated with the engineered or wild-type strains, and the total nitrogen content was determined. The total nitrogen content of seeds from plants inoculated with *R. etli* CE3/pMR4 was 16% higher (0.088 mg of N per mg of dry weight) than that of seeds from *R. etli* CE3 inoculated plants (0.076 mg of N per mg of dry weight).

DISCUSSION

Symbiotic nitrogen fixation in *Rhizobium* spp. is regulated by oxygen concentration. In this work, we attempted to modify the regulation of nitrogen fixation in *R. etli* by expressing the *vhb* gene, which codes for a bacterial hemoglobin from *Vitreoscilla* spp., and to determine its effect on free-living and symbiotic cells.

VHb was synthesized in engineered *Rhizobium etli* (CE3/pMR4) as shown by spectral and SDS-PAGE analyses. The spectral properties of VHb synthesized by *R. etli* were almost identical to those of VHb synthesized by a *Vitreoscilla* sp. (Fig. 2A); also, VHb was detected in cell extracts of *R. etli* CE3/pMR4 by SDS-PAGE (Fig. 2B). These data are evidence that the *vhb* gene was expressed in *R. etli* CE3/pMR4, and that the VHb protein was synthesized in the engineered bacteria.

The CO-differential spectra of extracts from *R. etli* CE3/pMR4 and the partially purified VHb showed absorption maxima in the Soret region at 418 and 419 nm, respectively. Although the 418 and 419 nm maxima were absent in the spectra of extracts from the *R. etli* wild type (CE3) (Fig. 2A), it was interesting to note that the CO-differential spectrum of the wild-type strain showed a maximum at 421 nm, which is also a signal in CO spectra of Hbs (Wood 1984).

Transgenic VHb has been synthesized in diverse heterologous hosts, including bacteria (Dikshit et al. 1990; Kallio et al. 1994; Magnolo et al. 1991; Wei et al. 1998) and plants (Holmberg et al. 1997), and it has been proposed that the expression of VHb in industrially or agriculturally important microorganisms may yield commercial benefits (Dikshit et al. 1990). However, the effects of VHb in agricultural processes, such as symbiotic nitrogen fixation, have not been studied so far. In this work, we studied the effects of VHb in free-living *R. etli* under microaerobic conditions, since it has been shown that in heterologous hosts the expression of *vhb* is enhanced under oxygen limitation (Kallio et al. 1994; Khosla et al. 1990; Wei et al. 1998). Bailey (1995) has postulated that in engineered cells VHb functions by transporting oxygen, and hypothesized that under these condi-

tions the concentration of intracellular oxygen increases, which affects the cell metabolism by increasing the rate of ATP turnover and the redox potential (i.e., the interior of the cell becomes more oxidizing). Our data showed that, under most limiting oxygen concentrations, the engineered *R. etli* strain expressing VHb had increased respiratory activity (Fig. 3), chemical energy content (Table 1), and expression of the *nifHc* gene (Fig. 4), compared with the wild-type strain. In addition, *vhb* was expressed in *R. etli* CE3/pMR4 bacteroids (Fig. 1). It is likely that the total oxygen concentration in engineered bacteroids is higher than the concentration in control bacteroids, and that this promotes a higher synthesis of ATP. Therefore, we propose that the expression of VHb, which is an oxygen-transporting protein, stimulates the efficiency of respiration in microaerobic, free-living *R. etli* by increasing the efficiency of ATP production, and that this also might occur in symbiotic bacteroids, thus leading to the higher levels of nitrogenase activity and total nitrogen content that were observed in bean plants inoculated with *R. etli* CE3/pMR4 (Fig. 5).

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultures.

The bacterial strains and plasmids used in this study are listed in Table 2. Batch liquid cultures of *R. etli* strains were grown at 30°C in PY (peptone-yeast) complex medium, shaken at 200 rpm in a LAB-Line orbit shaker No. 3590 (Lab-Line Instruments, Melrose Park, IL). Microaerobic liquid cultures of *R. etli* strains were obtained as follows. Cells from exponentially growing cultures of *R. etli* in PY medium were collected by centrifugation, washed, and resuspended in minimal medium containing 10 mM Na-succinate as the carbon source and 10 mM ammonium chloride as the nitrogen source (Mendoza et al. 1995); absorbance was adjusted to $A_{540} = 0.05$. Aliquots of 20 ml of the above cultures were injected into sealed, 150-ml bottles previously flushed with several volumes of the appropriate oxygen-argon mixture (Valderrama et al. 1996). Final oxygen concentrations in microaerobic cultures were 5, 1, 0.75, 0.5, and 0.25%.

Escherichia coli strains were grown at 37°C on either solid or liquid LB (Luria-Bertani) complex medium. Antibiotics were added in the following final concentrations (per ml): 10 µg of tetracycline (Tc), 20 µg of nalidixic acid (Nal), 200 µg of streptomycin (Sm), 30 µg of kanamycin (Km), and 100 µg of ampicillin (Ap). Plasmid pMR4 (below) was conjugated to *R. etli* CE3 and DEM233 by triparental mating with pRK2013 as a helper plasmid and selecting for Nal Tc colonies to generate the strains *R. etli* CE3/pMR4 and DEM 233/pMR4, respectively. Plasmid stability in *R. etli* CE3/pMR4 was estimated by resistance to Tc. Colonies of *R. etli* CE3/pMR4 strain from liquid cultures, or isolated from bean nodules, were grown on solid PY Nal medium, replicated on solid PY Tc medium, and the percentage of Tc^r colonies was determined.

Construction of plasmid pMR4.

Plasmid pUC8:15 (Dikshit and Webster 1988), containing the *Vitreoscilla* sp. *vhb* gene and the native promoter in a 2.2-kb *Hind*III fragment, was digested and then ligated into the *Hind*III-linearized pTR101 (Weinstein et al. 1992) to generate the plasmid pMR4.

Isolation of RNA and slot-blot analysis.

RNA from *R. etli* CE3 or CE3/pMR4 free-living cells or bacteroids was isolated with TRIzol Reagent (GIBCO BRL Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Bacteroids were isolated from 18-day-old nodules by centrifuging nodule extracts on a sucrose gradient as previously reported (Soberón et al. 1989). For RNA slot-blot hybridization, isolated RNA was blotted to a Hybond-N⁺ membrane (Amersham International, Buckinghamshire, UK) with the Minifold II Slot Blot system (Schleicher and Schuell, Keene, NH). Membranes were subsequently hybridized with the randomly primed, ³²P-labeled probe *vhb*, and the 16S-rRNA gene obtained from the pKK353 vector (Brosius et al. 1981). Membranes were hybridized at high stringency at 65°C, washed three-times with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) / 0.1% SDS at 60°C, and then the blot was autoradiographed by standard procedures (Sambrook et al. 1989).

Spectral detection of VHb in recombinant *R. etli* CE3/pMR4.

Engineered and non-engineered *R. etli* strains (CE3/pMR4 and CE3, respectively) were grown in 14 liters of PY broth at 30°C for 20 h, and 25 to 35 g of cell paste was obtained after centrifugation, which was resuspended in 100 ml of 100 mM Na-phosphate buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by sonication at maximum power (five cycles of 1 min each in ice), the resulting solution was cleared by centrifugation at 22,100 × g for 40 min at 4°C, and the supernatant was fractionated with liquid ammonium sulfate between 45 and 65% saturation. The resulting pellet was resuspended in 20 mM Tris-HCl (pH 8) and dialyzed for 18 h against the same buffer to remove the excess of salts. The dialyzed solution was used for spectral and electrophoretic analysis with the VHb isolated from *E. coli* (below) as a positive control. The amount of protein used for the spectral analysis was 46.5 and 44.2 g for extracts from CE3/pMR4 and CE3 strains, respectively.

Aliquots of the dialyzed solution and the partially purified VHb isolated from *E. coli* were subjected to spectral analysis. Samples were reduced by adding a few crystals of dithionite (Fluka, Buchs, Switzerland), and the differential CO-reduced versus reduced spectra were obtained in a spectrophotometer (model DU650; Beckman, Palo Alto, CA). Also, electrophoretic analysis of aliquots from the engineered and wild-type rhizobia, and the partially purified VHb isolated from *E. coli*, was performed by SDS-PAGE (Laemmli 1970). Total protein

was determined by a dye-binding assay (Bio-Rad, Hercules, CA) with bovine serum albumin as standard.

As a positive control, the VHb synthesized in recombinant *E. coli* was partially purified. Briefly, recombinant *E. coli* cells were grown in LB medium, disrupted, and cleared by centrifugation, and the supernatant was fractionated with ammonium sulfate as above. The red pellet was resuspended in 20 mM Tris-HCl (pH 8), dialyzed for 18 h against the same buffer, and then chromatographed through a DE52-cellulose (Whatman, Hillsboro, OR) resin. Upon protein absorption to the resin, the VHb was eluted with a 0 to 100 mM NaCl gradient in the above buffer. Fractions containing the VHb were pooled and concentrated by ultrafiltration with Amicon (Beverly, MA) concentrators. Preparations containing the VHb were checked by SDS-PAGE and by spectrophotometry in the visible region (350 to 700 nm).

Respiratory activities.

Oxidase activity was assayed in samples of *R. etli* CE3 or CE3/pMR4 grown microaerobically for 8 h at 30°C. The reaction vessels contained 3 ml of 50 mM potassium phosphate (pH 7.5) and cells (0.4 mg of protein), and oxygen consumption was measured with an oxygen meter (model 52; Yellow Springs Instruments, Yellow Springs, OH). For succinate oxidase activity, 40 mM Na-succinate was preincubated for 5 min with cells before oxygen consumption was measured.

Determination of nucleotides.

Nucleotide separation was performed by ion-exchange chromatography (Radial-Pak Sax Cartridge 8 mm × 10 cm; Waters, Milford, MA) from processed samples of *R. etli* microaerobic liquid cultures after 6 h of incubation, as described previously (Cevallos et al. 1996).

Estimation of β-galactosidase activity.

β-Galactosidase activity was determined in 1.5-ml samples of *R. etli* DEM233 or DEM233/pMR4 grown in microaerobic liquid cultures. Samples were incubated for 8 h, and an increase in A₄₂₀ in the presence of *o*-nitrophenyl β-D-galactopyranoside (ONPG) was determined (Valderrama et al. 1996). Activity was expressed as Miller units.

Nitrogenase activity and nitrogen content in bean plants.

Phaseolus vulgaris cv. Negro Jamapa seeds were surface sterilized and germinated as reported (Mendoza et al. 1995). *R. etli* CE3 or CE3/pMR4 strains used as inoculum were grown overnight in PY liquid medium, washed twice with

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>Rhizobium etli</i>		
CE3	Smr derivative of wild-type CFN42; Nal ^r	Noel et al. 1984
DEM233	<i>nifHc-lacZ</i> derivative of CE3	Valderrama et al. 1996
<i>Escherichia coli</i>		
HB101	Host strain for plasmids	Boyer and Roulland-Dussoix 1969
Plasmids		
pRK2013	Helper plasmid; Km ^r	Figurski and Helinski 1979
pUC8:15	pUC8 with a 2.2-kb <i>Hind</i> III fragment containing the <i>Vitreoscilla</i> sp. <i>vhb</i> gene plus the native promoter; Ap ^r	Dikshit and Webster 1988
pTR101	pTR100 mini-RK2 vector with the 0.8-kb <i>st</i> ability locus; Tc ^r	Weinstein et al. 1992
pMR4	pTR101 with the <i>vhb</i> gene (2.2-kb <i>Hind</i> III fragment) cloned in the <i>Hind</i> III site; Tc ^r	This work

10 mM MgSO₄ and diluted to an $A_{540} = 0.05$. Germinated seedlings were planted in autoclaved plastic pots (5 seedlings per pot) containing vermiculite as support, and then inoculated with 1 ml of the bacterial suspension per plant (approximately 10⁷ cells per plant). Groups of eight plants inoculated with each *R. etli* strain were harvested at 15, 20, 25, 30, 40, and 50 dpi, and the nodule dry weight, nitrogenase activity, and foliage nitrogen content were determined from each plant. Experiments with the same number of samples per treatment were done five times and similar results were obtained. Nitrogenase activity was determined in detached, nodulated roots by the acetylene reduction method, and were expressed as mol of ethylene per h per g of nodule (dry weight). Total nitrogen content of dried plants was determined with a nitrogen analyzer (ANTEK 7000; Antek Instruments, Houston, TX) and was reported as mg of nitrogen per plant (Cevallos et al. 1996). Times of plant flowering and pod filling were recorded for each experiment. A group of 25 plants inoculated with each strain was grown until seeds were harvested (approximately 65 dpi). Total nitrogen content was determined in powdered seeds (Cevallos et al. 1996) and was expressed as mg of nitrogen per mg of seed (dry weight).

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