APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1999, p. 2015–2019 Vol. 65, No. 5
0099-2240/99/$04.00+0

Enhanced Nitrogen Fixation in a *Rhizobium etli ntrC* Mutant That Overproduces the *Bradyrhizobium japonicum* Symbiotic Terminal Oxidase cbb_3

MARIO SOBERÓN,[1]* OSWALDO LÓPEZ,[1] CLAUDIA MORERA,[1] MARIA DE LOURDES GIRARD,[2] MARIA LUISA TABCHE,[1] AND JUAN MIRANDA[1]

Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62271,[1] and Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Morelos,[2] México

Received 3 December 1998/Accepted 11 March 1999

The *ntrC* gene codes for a transcriptional activator protein that modulates gene expression in response to nitrogen. The cytochrome production pattern of a *Rhizobium etli ntrC* mutant (CFN2012) was studied. CO difference spectral analysis of membranes showed that CFN2012 produced a terminal oxidase similar to the symbiotic terminal oxidase of bacteroids in free-living cells under aerobic conditions, with a characteristic trough at 553 nm. CFN2012 produced two *c*-type cytochromes with molecular masses of 27 and 32 kDa, in contrast with the wild-type strain, which produced only a 32-kDa *c*-type cytochrome. The expression levels of the *R. etli fixNOQP* operon, which codes for terminal oxidase cbb_3, were not affected by the *ntrC* mutation. However, the production levels of the two *c*-type cytochromes (27 and 32 kDa) were enhanced at least eightfold when the *Bradyrhizobium japonicum fixNOQP* operon was expressed in CFN2012 from the *nptII* promoter (pMSfix^c), suggesting that these proteins are subunits FixO (27 kDa) and FixP (32 kDa) of cbb_3 and that CFN2012/pMSfix^c overproduced this terminal oxidase. CFN2012/pMSfix^c showed a significant increase in its symbiotic performance as judged by the determination of nitrogenase activities of plants inoculated with this strain, suggesting that the overproduction of cbb_3 terminal oxidase correlates with an enhancement in symbiotic nitrogen fixation.

In free-living diazotrophic bacteria combined nitrogen regulates the expression of the nitrogenase structural genes, inhibiting nitrogen fixation (10). The general nitrogen regulatory system (*ntr* system) activates the transcription of nitrogenase structural genes when combined nitrogen is not available (3). In contrast, in symbiotic nitrogen-fixing bacteria (genera *Bradyrhizobium* and *Rhizobium*) combined nitrogen has no effect on the expression of nitrogenase structural genes (8). Bacteria of these genera may establish a specific symbiotic relationship with their legume host plant. These bacteria elicit the formation of new organs, i.e., root nodules, in which bacteroids reduce atmospheric nitrogen to ammonia and supply the host plant with combined nitrogen. For the induction of root-nodules, members of *Rhizobium* express *nod* genes which code for enzymes involved in the formation of lipooligosaccharide factors (nod factors). However, in the presence of combined nitrogen, the formation of the root nodules is inhibited. It has been demonstrated that nitrogen negatively regulates bacterial *nod* gene expression (6, 7, 14, 27). In *Rhizobium etli* nitrogen repression of *nod* gene expression is mediated by the *ntr* system (14).

Symbiosis requires a respiratory chain that has a high affinity for O_2 and is efficiently coupled to ATP production since nitrogen fixation is an energy-consuming process, requiring up to 20 ATP molecules to reduce just one molecule of N_2. The genes of *Bradyrhizobium japonicum* which code for the bacteroid terminal oxidase have been identified as the *fixNOQP* operon (20). The sequence analysis of these genes, and biochemical characterization of the purified enzyme, showed that they code for a three-subunit terminal oxidase (cbb_3) (11, 20, 21). FixN is a *b*-type heme- and copper-containing subunit, FixO is a single-heme-containing *c*-type cytochrome, and FixP is a diheme-containing *c*-type cytochrome (11, 20, 21). In *R. etli*, mutants that produce the cbb_3 terminal oxidase under free-living conditions showed enhanced symbiotic nitrogen fixation (15).

In an attempt to study if nitrogen regulation affects respiration in *R. etli*, we analyzed the cytochrome production pattern of an *R. etli* mutant with change affecting the *ntr* system. A mutation in *ntrC*, which codes for a transcriptional activator protein that modulates gene expression in response to nitrogen (16), was analyzed. This analysis showed that the *ntrC* mutant produces a terminal oxidase of the cbb_3 type in free-living cultures. Analysis of the expression of the *R. etli fixNOQP* genes showed that the expression of these genes was not affected by the *ntrC* mutation. However, expression of the *B. japonicum fixNOQP* operon from a constitutive promoter in the *R. etli ntrC* mutant greatly enhanced the production level of the cbb_3 terminal oxidase and also the symbiotic performance of this strain. These data indicate that symbiotic nitrogen fixation can be improved by the overproduction of the symbiotic terminal oxidase cbb_3.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used are listed in Table 1. *R. etli* cells were cultured in minimal medium (MM), peptone yeast extract (PY) medium (17), or yeast extract succinate (YS) (15). To achieve microaerobic cultures (O_2 pressure, 2 kPa) 2 ml of active culture was used to inoculate 40 ml of medium. These cultures had previously been evacuated and flushed with a 1,200-ml · min^{-1} sterile N_2 stream for 10 min. Calculated volumes (9.8 ml) of sterilized, high-purity commercial air were injected by making use of disposable syringes, following extraction of the same volume of N_2. *Escherichia coli* was grown in Luria broth medium. Antibiotics

* Corresponding author. Mailing address: Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, U.N.A.M., Apdo Postal 510-3, Cuernavaca, Morelos 62271, México. Phone: 52 5 6227618. Fax: 52 73 172388. E-mail: mario@ibt.unam.mx.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference
Rhizobium etli		
CE3	Str^r derivative of CFN42	16
CFN2012	*ntrC*::Tn*5* mutant of CE3	15
Plasmids		
cGD101	Cosmid clone from *R etli* symbiotic plasmid	8
pMSfix^c	pTR101 containing the *B. japonicum fixNOQP* operon fused to the *nptII* promoter	8
pMP220	Transcriptional *lacZ* fusion vector; Tc^r	24
pOLfix10	pMP220::1.1-kb *Pst*I fragment; *fixN-lacZ* gene fusion	This work

[a] Str, streptomycin; Tc, tetracycline.

were used at the following concentrations: rifampin, 50 mg/liter; tetracycline, 5 mg/liter; kanamycin, 30 mg/liter; and streptomycin, 100 mg/liter.

DNA manipulations. Cloning, restriction mapping, transformation, plasmid isolation, and β-galactosidase measurements were done as described (12). *Sal*I and *Pst*I clones from cGD101 were subcloned into pBluescript SK(+) vector and sequenced (4,832 bp; GenBank accession no. U76906) at the automated DNA sequencing facility at the Molecular Genetics Core Facility in the Department of Microbiology and Molecular Genetics, University of Texas—Houston Medical School. Computer-assisted sequence analysis and comparisons with the GenBank sequence were done using the Gene Works 2.6 program from Intelligenetics, Inc.

Spectral and electrophoretic analysis of cytochromes. Cells were grown overnight on PY medium. Cells were washed and diluted 50-fold on fresh YS medium and grown on a rotary shaker (200 rpm) at 30°C for 36 h. Cells were harvested by centrifugation, washed, and suspended to 30% (wt/vol) in 50 mM Tris hydrochloride (pH 7.4)–5 mM $CaCl_2$–5 mM $MgCl_2$. Cytochrome spectra of whole cells or membrane preparations in an SLM Aminco Midan II spectrophotometer were recorded. Samples were reduced with dithionite (a few grains) or oxidized with ammonium persulfate. Carbon monoxide difference spectra were obtained by bubbling with CO (2 min) to reduce a cell sample, and spectra were recorded against a reduced sample. Spectra were obtained at room temperature with 1.0-cm light path cuvettes. *c*-type proteins were analyzed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of membrane samples, which were prepared mechanically as previously described (23). Protein blotting and heme peroxidase detection were done as reported (26), except that in Western blotting the detection reagents used for peroxidase detection were obtained from Pierce (SuperSignal Substrate; Rockford, Ill.); the use of these chemicals greatly enhanced the sensitivity of this technique. Protein was determined as described (13).

Nitrogen fixation determination. For acetylene reduction measurements, *Phaseolus vulgaris* cv. negro jamapa was surface sterilized in hypochlorite and germinated on moist sterile filter paper. Three-day-old seedlings were transferred to plastic growth pots, inoculated with a bacterial suspension in PY medium, and grown with nitrogen-free salts in a greenhouse (23). Nitrogenase was determined by measuring the acetylene reduction of nodulated plant roots transferred to tubes with rubber seal stoppers by injecting acetylene to a final concentration of 10% of the gas phase. Samples were incubated 40 min at room temperature, and ethylene production was determined by gas chromatography in a Packard model 430 chromatograph (23). For each strain, the total nitrogen of 60-day-old nodulated plants was determined for three plants in four pots each (total, 12 plants) using an Antek 720 nitrogen detector as previously described (4).

Bacteroid preparation. Nodules were harvested 30 days after inoculation. Bacteroids were isolated by layering a nodule extract on a sucrose gradient as previously reported (23).

RESULTS

Cytochrome production in an *ntrC* mutant of *R. etli*. Cytochrome production was analyzed in cells cultured aerobically to the stationary phase of growth and in bacteroids of the wild-type (CE3) and the *ntrC* mutant (CFN2012) strains. Figure 1A shows difference spectra (spectra for reduced cells minus spectra for oxidized cells) of free-living cells, showing that CE3 produced *c*-type cytochromes (peak at 553 nm) and

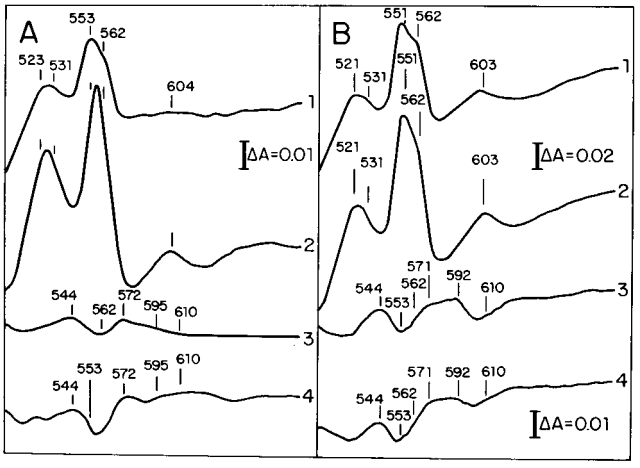

FIG. 1. Difference spectra (spectra for reduced cells minus those for oxidized cells) (spectra 1 and 2 in panels A and B) and CO difference spectra (spectra 3 and 4 in panels A and B) of *R. etli* whole cells. (A) Spectra 1 and 3 are for CE3 cells cultured for 36 h (18.7 mg of protein ml^{-1}), and spectra 2 and 4 are for CFN2012 cells cultured for 36 h (23.5 mg of protein ml^{-1}). (B) Spectra of bacteroids of CE3 (17.7 mg of protein ml^{-1}) (spectra 1 and 3) and CFN2012 (25.5 mg of protein ml^{-1}) (spectra 2 and 4). Free-living cells were cultured aerobically in YS medium.

b-type cytochromes (shoulder at 562 nm) but no aa_3 cytochromes (peak at 603 nm) under this culture condition. CFN2012 produced *c*-type, *b*-type, and aa_3 cytochromes (Fig. 1A). Carbon monoxide (CO) difference spectra were obtained since CO reacts specifically with cytochrome terminal oxidases. CO difference spectra showed that the CE3 cells produced only cytochrome terminal oxidase *o* (peaks at 544 and 572 nm and trough at 562 nm) and no cytochrome aa_3 (trough at 610 nm). In contrast, CFN2012 produced a different CO-reactive cytochrome with a spectrum signal showing a characteristic trough at 553 nm and no cytochrome aa_3 (Fig. 1A). The lack of evidence for cytochrome aa_3 in CO difference spectra of CFN2012 membranes suggests that the absorption peak at 603 nm found in difference spectra (spectra for reduced cells minus those for oxidized cells) of CFN2012 could be due to a different heme-containing protein that absorbs near 600 nm (e.g., catalase).

Bacteroids of the CE3 and CFN2012 strains had similar cytochrome production patterns, producing *c*-type (peak at 551 nm), *b*-type (shoulder at 562 nm), and aa_3 (peak at 603 nm) cytochromes (Fig. 1B). Figure 1B also shows that bacteroids of these strains produced a CO-reactive cytochrome with a spectrum with a trough at 553 nm, very similar to the CO-reactive cytochrome produced by CFN2012 in free-living cultures, and also cytochrome aa_3 (trough at 610 nm).

***c*-type cytochrome production by CFN2012.** The *c*-type cytochromes produced by CE3 and CFN2012 strains cultured aerobically in YS medium to the stationary growth phase were analyzed. CE3 and CFN2012 cells harboring plasmid pMSfix^c, which contains the *B. japonicum fixNOQP* operon fused to the *nptII* promoter (24), were included as controls for the production of FixO (27 kDa) and FixP (32 kDa) *c*-type cytochrome subunits of terminal oxidase cbb_3. Figure 2 shows that CE3 cell membranes contained detectable levels of a 32-kDa *c*-type cytochrome, as was previously reported (23); in contrast, CFN2012 produced higher levels of the 32-kDa protein and produced an additional *c*-type cytochrome of 27 kDa (Fig. 2). CE3/pMSfix^c produced both the 32- and 27-kDa proteins, while CFN2012/pMSfix^c showed very high production levels of

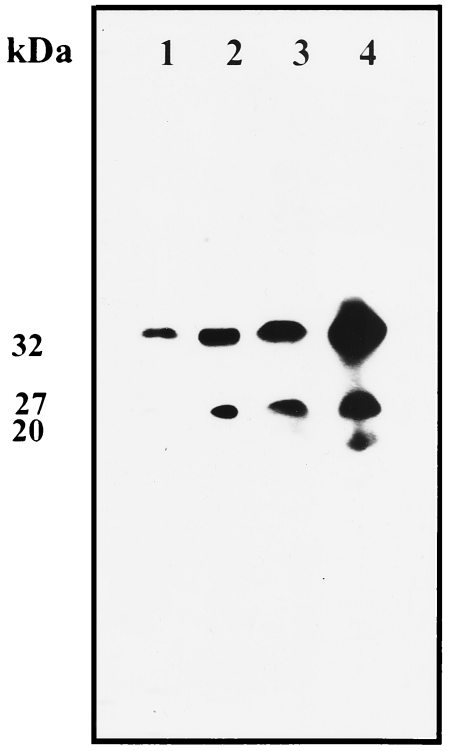

FIG. 2. Proteins containing *c*-type heme from membrane particles. Each lane contains 50 μg of protein from membrane particles from CE3 cells (lane 1), CFN2012 cells (lane 2), CE3/pMSfix^c cells (lane 3), or CFN2012/pMSfix^c cells (lane 4) grown aerobically in YS medium for 36 h.

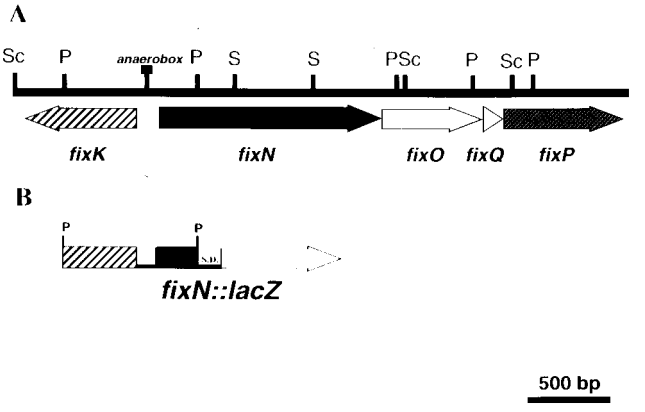

FIG. 3. Physical-genetic map of the *fixK-fixNOQP* sequenced region. (A) Physical-genetic map of the *R. etli fixK-fixNOQP* genetic region. The restriction sites used in subcloning for sequencing are shown. Sc, *Sac*I; P, *Pst*I; S, *Sal*I. The position of the anaerobox sequence is shown. (B) Diagram of *fixN-lacZ* transcriptional gene fusion construction. S.D., Shine-Dalgarno sequence. Arrows indicate the directions of transcription.

both *c*-type cytochromes and also detectable levels of a 20-kDa *c*-type cytochrome (Fig. 2). The production of the two *c*-type cytochromes by the *ntrC* mutant, as well as the results of the CO difference spectral analysis (Fig. 1A), suggests that in this strain a terminal oxidase similar to a cbb_3 terminal oxidase was produced in aerobic cultures of free-living stationary-phase cells. We quantified the production levels of cbb_3 terminal oxidase by measuring the 32- and 27-kDa *c*-type proteins produced when *fixNOQP* was transcribed from the *nptII* promoter. This analysis showed that CFN2012 produced eightfold-higher levels of this oxidase than CE3.

Expression of *R. etli fixNOQP*. In order to further analyze the free-living cell production of the cbb_3 terminal oxidase in CFN2012, we decided to study the expression of the *R. etli fixNOQP* operon. A cosmid clone (cGD101) of the *R. etli* symbiotic plasmid pd (9), which hybridized against heterologous gene probes from *fixK* (from *Sinorhizobium meliloti*) (1) and *fixN* (from *B. japonicum*) (20), was identified (data not shown). In order to localize the *fixNOQP* operon, 4,832 bp were sequenced (GenBank accession no. U76906).

Five open reading frames were identified as the *R. etli fixK* and *fixNOQP* operon (Fig. 3). The predicted FixK protein is comprised of 239 residues. FixK is a transcriptional activator involved in *fixNOQP* induction in *S. meliloti* (1). This protein has 63% identity with FixK of *Rhizobium leguminosarum* bv. viciae (19) (GenBank accession no. Z70305) and lacks the cysteine-amino-terminal domain present in Fnr. The predicted FixN protein is comprised of 540 residues. *R. etli* FixN has significant homology with FixN from different organisms, i.e., 78% identity with FixN of *S. meliloti* (GenBank accession no. Z21854) and 92% identity with both copies of *fixN* gene products in *R. leguminosarum* (GenBank accession no. Z80339 and Z80340). FixN contains the six conserved histidine residues proposed to bind the low-spin *b*-type heme (H117 and H406), CuB (H266, H136, and H317), and the high-spin *b*-type heme (H404). FixO, comprised of 244 residues, has 86% identity with FixO of *R. leguminosarum* and 75% identity with FixO of *S. meliloti*. FixO contains two cysteine residues, at positions 69 and 72, which are likely to be involved in the attachment of *c*-type heme. FixQ, comprised of 50 residues, has 88% identity with FixQ from *R. leguminosarum* and 66% identity with FixQ from *S. meliloti*. Finally, FixP, comprised of 287 residues, has identities of 81 and 61% with FixP of *R. leguminosarum* and of *S. meliloti*, respectively, and contains the four conserved cysteine residues involved in the attachment of two *c*-type hemes (C121, C124, C216, and C219).

Similar to *R. leguminosarum* bv. viciae (19), in *R. etli fixK* and *fixNOQP* are transcribed in opposite directions with a spanning DNA region of 230 bp (Fig. 3). These data suggest that the promoters of both genes are contained in this DNA region. The "anaerobox" sequence (TTGATGTAGATCAA) is located 88 bp in front of the beginning of *fixN* (Fig. 3).

In order to generate transcriptional *lacZ* gene fusions of *fixN* (plasmid pOLfix10) promoter, a 1.1-kb *Pst*I fragment containing the amino-terminal part of FixN and FixK was cloned in plasmid pMP220, which carries the *E. coli lacZ* gene (25) (Fig. 3B).

The expression of *fixNOQP* in CE3 and CFN2012 strains cultured aerobically or microaerobically was studied. The expression of *fixN* was induced 15-fold in cells cultured microaerobically, as has been shown in several other *Rhizobium* species, showing that in *R. etli* as well, oxygen is the most important metabolic signal triggering *fixNOQP* expression. The level of expression of *fixN* was slightly lower in the wild-type than in the CFN2012 strain under stationary-phase aerobic (60%) and microaerobic (70%) conditions. This analysis revealed that the production of cbb_3 terminal oxidase by CFN2012 was not due to the enhanced expression of *fixNOQP*, suggesting that NtrC modulates cbb_3 production at a level different from *fixNOQP* transcription. When cells were grown on YS medium under atmospheric O_2 tension for 8 h, under atmospheric O_2 tension for 36 h, and under 2 kPa O_2 tension for 8 h, the CE3 strain carrying the *fixN-lacZ*(pOLfix10) re-

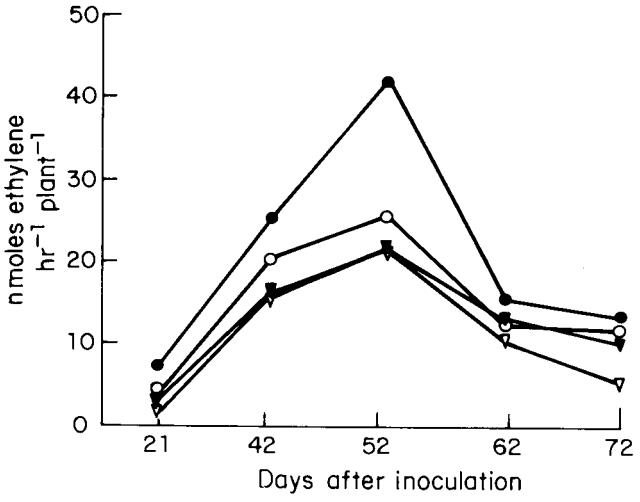

FIG. 4. Acetylene reduction activity in plants inoculated with strains CE3 (▽), CE3/pMSfix^c (▼), CFN2012 (○), and CFN2012/pMSfix^c (●). Acetylene reduction activity for four plants in each of two pots was determined on the specified days. Data are means for two pots (eight plants); variations were 30% or less.

porter gene fusion showed β-galactosidase activities of 474, 342, and 6,199 U/mg of protein, respectively, and the CFN2012 strain carrying the same fusion showed activities of 526, 557, and 8,719 U/mg of protein, respectively (values are expressed after subtraction of activities of strain without any plasmid [range, 50 to 90 U/mg of protein]).

Symbiotic nitrogen fixation of plants inoculated with different *R. etli* strains. *P. vulgaris* cv. negro jamapa plants were inoculated with CE3 and CFN2012 strains and the same strains harboring pMSfix^c, and nitrogenase activity was determined at different days after inoculation. Figure 4 shows that for plants inoculated with CE3 the highest nitrogenase activity (23 nmol of ethylene produced h^{-1} per plant) was reached at 52 days after inoculation and decreased gradually afterwards. No effect of plasmid pMSfix^c was found in this strain. Plants inoculated with CFN2012 demonstrated nitrogenase activity more rapidly than those inoculated with CE3, but similar nitrogenase activities were observed. CFN2012 strain harboring pMSfix^c induced nitrogenase activity more rapidly and achieved a twofold-higher nitrogenase activity than the CE3 strain (Fig. 4). In addition, nitrogen fixation was estimated by the total nitrogen content of plants 60 days after inoculation with the different strains. CE3/pMSfix^c-inoculated plants contained 90% (3.51 ± 0.092 mg of nitrogen [100 mg dry weight]$^{-1}$; $n = 4$) of the nitrogen found in CE3-inoculated plants (3.90 ± 0.031 mg of nitrogen [100 mg dry weight]$^{-1}$; $n = 4$), whereas CFN2012- and CFN2012/pMSfix^c-inoculated plants contained 8% (4.25 ± 0.102 mg of nitrogen [100 mg dry weight]$^{-1}$; $n = 4$) and 18% (4.57 ± 0.065 mg of nitrogen [100 mg dry weight]$^{-1}$; $n = 4$) more nitrogen, respectively, than CE3-inoculated plants.

DISCUSSION

Symbiotic nitrogen fixation is an energy-consuming process requiring up to 20 ATP molecules to reduce one molecule of N_2. Also, symbiotic nitrogen fixation occurs at very low oxygen tensions (2). Therefore, a high-affinity oxidase, of the cbb_3 type, efficiently coupled to ATP production is produced during symbiosis. During free life, *Rhizobium* species do not produce the cbb_3 terminal oxidase, mainly due to a low level of expression of the *fixNOQP* operon in the presence of O_2 (2).

In this work we present data showing that, in *R. etli*, in addition to oxygen regulation, NtrC represses the free-living cell production of the cbb_3 symbiotic terminal oxidase. The analysis of cytochrome production revealed that in aerobic cultures an *ntrC* mutant produced a terminal oxidase very similar to that produced in bacteroids, in contrast with the wild-type strain. This suggests that nitrogen availability could modulate symbiotic cytochrome production in *R. etli*. The molecular mechanism by which NtrC represses cbb_3 production is still unclear. However, two results indicate that this regulation is exerted after transcription of the structural *fixNOQP* genes: (i) no difference was found in *fixNOQP* expression between CFN2012 and CE3; and (ii) under conditions where *fixNOQP* was expressed under a strong promoter, CFN2012 produced at least eightfold-higher levels of cbb_3 than CE3, suggesting that a posttranscriptional step involved in cbb_3 biogenesis was negatively regulated by NtrC. In several *Rhizobium* species, including *R. etli*, several genes that participate in the correct assembly of the cbb_3 terminal oxidase have been identified. These include genes necessary for the covalent attachment of heme to *c*-type apoproteins (5) and genes (*fixGHIS*) that are probably involved in the transport of copper ions, which are essential for oxygen reduction by cbb_3 (5, 22).

The *ntrC* mutation in CFN2012 had a positive effect on nitrogenase activity only when the *fixNOQP* genes were expressed from a strong promoter; also, CFN2012/pMSfix^c produced an eightfold-higher level of cbb_3 terminal oxidase than CE3/pMSfix^c in culture. These data suggest that increased cbb_3 terminal production correlates with enhanced nitrogen fixation. However, because *ntrC* is known to be global in its regulatory role, it cannot be ruled out that other effects of *ntrC* mutation, in addition to the elevated levels of the terminal oxidase, could also participate in the enhanced nitrogen fixation capacity of this strain. In fact plants inoculated with CFN2012 accumulated more nitrogen than CE3-inoculated plants. It is important to study the respiratory physiology of *ntrC* mutant-derived bacteroids.

Total nitrogen determinations of plants inoculated with the different strains correlated roughly with nitrogenase activities. However, plasmid pMSfix^c had a negative effect on nitrogen accumulation for plants inoculated with the CE3 strain even though plants inoculated with these strains showed very similar nitrogenase activities. Therefore, we cannot rule out the possibility that in CFN2012/pMSfix^c-inoculated plants nitrogen accumulation was also affected by the presence of this plasmid. At this moment we have no explanation for the negative effect of this construct, although the process of replication of the plasmid or enhanced transcription could compete for ATP with nitrogen fixation. It is important to introduce the *nptII-fixNOQP* construct into the genome to determine the reason for the negative effect of plasmid pMSfix^c on plant nitrogen accumulation.

Two possible reasons for the enhanced symbiotic nitrogen fixation observed for CFN2012/pMSfix^c can be proposed: one is a higher nitrogenase activity due to enhanced supply of ATP to nitrogenase, and the other is a more-efficient performance during infection and bacteroid development. *R. etli* bacteroids repress *ntrC* expression, and NtrC protein could not be detected (18). This explains the similar cytochrome production patterns found in bacteroids of CE3 and CFN2012 strains. However, it was shown that bacteria inside the infection thread and young bacteroids could express *ntrC* (18). Since NtrC is not produced in well-developed bacteroids, it seems possible that the difference in nitrogenase activity between CE3/pMS-

fix^c and CFN2012/pMSfix^c could be due to a better performance of CFN2012 during infection. This could suggest that the repression of cbb_3 terminal oxidase production by NtrC could be relevant during the infection process whereas another terminal oxidase could support growth. Alternatively, the negative regulation of the production of the symbiotic terminal oxidase by NtrC could be an additional control point for the repression of symbiosis when combined nitrogen is available.

ACKNOWLEDGMENTS

We thank Alejandra Bravo for revising the manuscript, Guadalupe Espín for providing us the CFN2012 strain, Yolanda Mora for total nitrogen determinations, and Jose Luis Zitlalpopoca for technical assistance.

This work was partially supported by the European Communities through the International Scientific Co-operation Program, contract CI1*-CT94-0042, by CONACyT contract no. 3372-N9309, and by DGAPA contract no. IN204697.

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