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# Isolation of *Rhizobium phaseoli* Tn*5*-Induced Mutants with Altered Expression of Cytochrome Terminal Oxidases *o* and $aa_3$

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**Two *Rhizobium phaseoli* mutants affected in cytochrome expression were obtained by Tn*5-mob* mutagenesis of the wild-type strain (CE3). Mutant strain CFN031 expressed sevenfold less cytochrome *o* in culture, expressed cytochrome $aa_3$ under microaerophilic culture conditions, in contrast to strain CE3, and was affected in its vegetative growth properties and proliferation inside plant host cells. Mutant CFN037 expressed cytochrome $aa_3$ under microaerophilic culture conditions, while bacteroid development and nitrogen fixation occurred earlier than in strain CE3.**

*Rhizobium* respiration is central to nitrogen fixation in the bacteroid-plant symbiosis. Electron transfer to oxygen is believed to represent an oxygen-scavenging mechanism to prevent oxygen damage to nitrogenase (3, 22), while oxidative phosphorylation yields ATP for the nitrogen-fixing reaction (3). Recently we reported the cytochrome composition of the electron transport chain of *Rhizobium phaseoli* (18). Cultured *R. phaseoli* cells express *b*- and *c*-type cytochromes and two cytochrome terminal oxidases: cytochrome *o* and $aa_3$. Both cytochrome oxidases were identified by photodissociation spectra and oxygen binding (18). We also reported the isolation of *R. phaseoli* mutants affected in respiration and symbiotic nitrogen fixation (18). The mutants were isolated by their capacity to oxidize *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). A nitrosoguanidine-induced mutant with increased TMPD oxidase activity when cultured under microaerophilic conditions ($TMPD^{++}$) expressed cytochrome $aa_3$ constitutively and displayed premature symbiotic nitrogen fixation activity (18). In other *Rhizobium* species, cytochrome $aa_3$ is not expressed when the cells are cultured at low $O_2$ tensions (4, 20) or during symbiosis (1).

We report here the isolation and characterization of two Tn*5*-induced mutants of *R. phaseoli* with a $TMPD^{++}$ phenotype. These mutants could provide a molecular approach for the characterization of genes involved in the expression of cytochrome terminal oxidases and symbiotic nitrogen fixation.

A streptomycin-resistant derivative of wild-type *R. phaseoli* CFN42, CE3 (14, 15), was mutagenized with the mobilizable suicide plasmid pSUP5011 carrying Tn*5-mob*, which is present in *Escherichia coli* S17 (17). Kanamycin (30 μg/ml)-resistant transconjugants were selected on complex PY medium (14).

Cells were replica plated to minimal medium plates (14), and $TMPD^{++}$ mutants were detected by their deeper blue color after overlayering of a solution of 9 mM TMPD and 2 mM ascorbate (pH 6.8). Five $TMPD^{++}$ mutants were detected in a screening of about 5,000 $Km^r$ colonies. One of these five $TMPD^{++}$ strains (CFN031) grew poorly when incubated on minimal medium plates. Blot hybridization (16, 19) of *Eco*RI digests of total DNAs from strains CFN031, CFN032, CFN033, CFN036, and CFN037 probed with Tn*5* sequences showed a single band of hybridization at 11.4 kilobases, except for CFN031, which showed a single band of 10.5 kilobases (data not shown). CFN037 and CFN031 were further characterized. Plasmid PJB3 was introduced into the mutants; this plasmid contains the functions necessary to mobilize the *mob* sequence in Tn*5-mob* (6, 17). CFN031 and CFN037 harboring plasmid pJB3 were mated with a rifampin-resistant derivative of CFN42, CE2 (14). Tn*5* was linked to the $TMPD^{++}$ phenotype, since more than 90% of the $Km^r$ transconjugants in each mating were shown to have a $TMPD^{++}$ phenotype.

Well-aerated liquid cultures were inoculated with CFN031 and CFN037 to determine their vegetative growth properties. CFN031 could not utilize succinate or glucose as a sole carbon source. In complex liquid media, this strain had the same doubling time as the wild-type strain. CFN037 had the same doubling time as the wild-type strain, regardless of the culture conditions (data not shown).

*R. phaseoli*, like other *Rhizobium* species (2, 7, 11), expresses *b*- and *c*-type cytochromes and two terminal oxidases: cytochromes *o* and $aa_3$ (18). Membrane particles were prepared from cultures grown on PY medium at 30°C and 250 rpm. Cells were collected after 18 h of growth (early stationary phase) and washed with 250 mM Tris hydrochloride (pH 7.4)–5 mM $CaCl_2$–5 mM $MgCl_2$ (TCM buffer). Spheroplasts were prepared by lysozyme-EDTA treatment by the procedure already described for *R. trifolii* (7). After DNase treatment (18), membranes were recovered by centrifugation at 100,000 × *g* for 30 min and suspended in 50% glycerol-containing TCM buffer (18). The cytochrome spectra of membrane particles, cultured cells, or bacteroids were recorded on an SLM Aminco Midan II spectrophotometer as described previously (8). Samples were reduced with dithionite (a few grains) or oxidized with ammonium persulfate. Millimolar extinction coefficients were used for cytochrome quantification as previously described (8, 18).

The dithionite-reduced minus oxidized spectrum of CFN031 membrane particles showed a cytochrome pattern similar to that of the wild-type strain (Fig. 1A). This strain possesses *b*-type cytochromes (peaks at 429 and 560 nM), a *c*-type cytochrome (a shoulder near 550 nm), and cytochrome $aa_3$ (a peak at 603 nm). In contrast, CFN031 showed a very low cytochrome *o* content (a peak at 416 nm,

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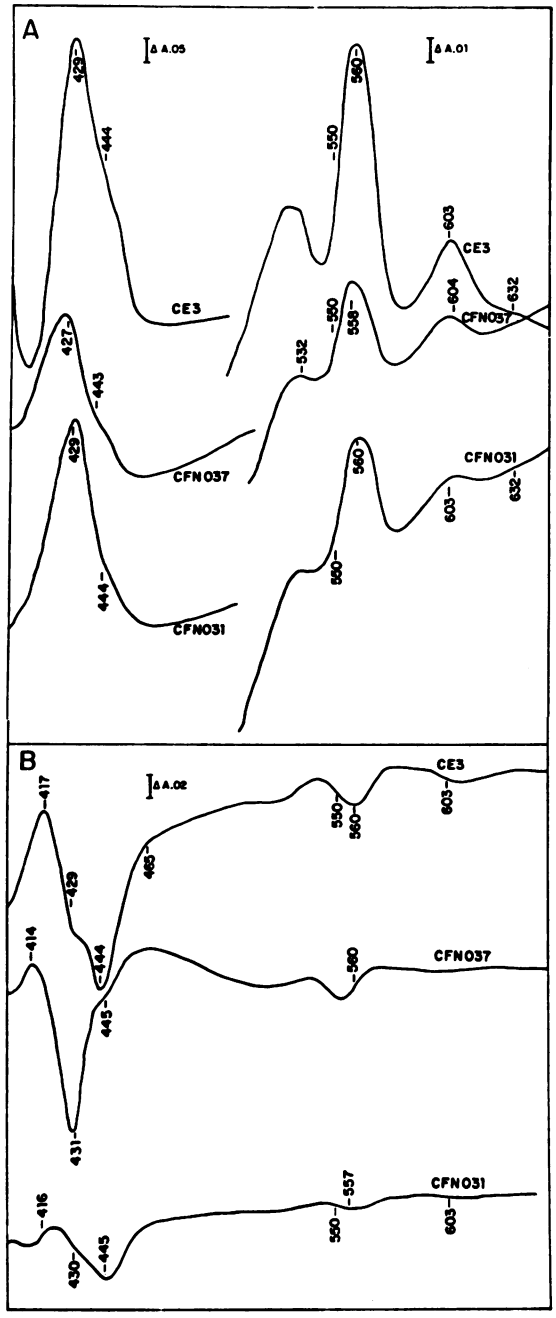


FIG. 1. Cytochrome spectra of membranes of strains CE3 (10.8 mg of protein $ml^{-1}$), CFN037 (5.8 mg of protein $ml^{-1}$), and CFN031 (5.9 mg of protein $ml^{-1}$). (A) Dithionite-reduced minus ammonium persulfate-oxidized spectrum recorded at room temperature. (B) CO difference spectrum (dithionite-reduced membranes).

a shoulder at 430 nm, and features near 560 nm) in carbon monoxide difference spectra (Fig. 1B). CFN037 showed a cytochrome pattern similar to that of the wild-type strain in both the reduced minus the oxidized and the CO difference spectra (Fig. 1A and B). Table 1 shows the cytochrome concentrations found in the mutants. CFN031 had at least a sevenfold lower cytochrome *o* content than did the wild-type strain. CFN037 showed almost a twofold lower *b*-type cytochrome content and similar concentrations of the other cytochromes compared with the wild-type strain (Table 1).

TABLE 1. Cytochrome contents and respiratory activities in membrane particles of *R. phaseoli* strains

| Strain | Cytochrome concn[a] | | | | Oxidase activity[b] | | |
|---|---|---|---|---|---|---|---|
| | *b* | *c* | $aa_3$ | *o*-CO | Succinate | NADH | Ascorbate-TMPD |
| CE3 | 0.310 | 0.166 | 0.077 | 0.069 | 65.2 | 217.3 | 190.0 |
| CFN031 | 0.200 | 0.162 | 0.052 | 0.010 | 87.0 | 255.4 | 229.0 |
| CFN037 | 0.176 | 0.177 | 0.054 | 0.063 | 68.2 | 86.1 | 141.3 |

[a] Cytochrome concentrations (nanomoles milligram of protein$^{-1}$) (12) in membrane fraction were determined on the basis of difference spectra at room temperature (Fig. 1). Representative results of three experiments with a variation of less than 15% are shown.

[b] Activities are reported as nanogram-atoms of oxygen consumed minute$^{-1}$ milligram of protein$^{-1}$. Representative results of three experiments with a variation of less than 15% are shown.

Table 1 also shows the respiratory activities obtained with membrane particles from the different strains. NADH, ascorbate-TMPD, and succinate oxidase activities were determined at 30°C in a model 52 oxygen meter (Yellows Springs Instrument Co.) as previously described (8, 18). Strain CFN031 had 20% higher ascorbate-TMPD oxidase activity and similar succinate oxidase and NADH oxidase activities compared with strain CE3, whereas CFN037 had more than twofold lower NADH oxidase activity and succinate oxidase and TMPD oxidase activities similar to those of CE3.

The $TMPD^{++}$ phenotype of a previously described $TMPD^{+}$ *R. phaseoli* mutant was due to a higher ascorbate-TMPD oxidase activity of cells cultured on plates because of a higher cytochrome $aa_3$ content (18). In *Bradyrhizobium japonicum*, cytochrome $aa_3$ is not expressed when the bacterium is grown in static cultures (4) or during symbiosis (1). To determine whether the $TMPD^{++}$ phenotype of strains CFN031 and CFN037 was due to altered regulation in cytochrome $aa_3$ expression, these strains and the wild type were incubated under microaerophilic conditions. Microaerophilic cultures were achieved by diluting an active inoculum 10 times in PY medium and incubating it for 48 h at 30°C with no agitation. Aerated cultures were achieved by diluting an active inoculum 30 times and incubating it for 18 h at 30°C and 250 rpm. Cytochrome $aa_3$ concentrations and ascorbate-TMPD oxidase activities of whole cells were determined. Aerated cultures of CE3, CFN031, and CFN037 had a similar cytochrome $aa_3$ contents and similar ascorbate-

TABLE 2. Cytochrome $aa_3$ contents and ascorbate-TMPD oxidase activities in whole cells of *R. phaseoli* strains

| Strain | Cytochrome $aa_3$ concn[a] | | Ascorbate-TMPD oxidase activity[b] | |
|---|---|---|---|---|
| | Aereated cultures | Static cultures | Aereated cultures | Static cultures |
| CE3 | 0.045 | 0.010 | 184.42 | 61.24 |
| CFN031 | 0.048 | 0.046 | 155.68 | 176.03 |
| CFN037 | 0.049 | 0.043 | 193.2 | 168.20 |

[a] Cytochrome $aa_3$ concentrations (nanomoles milligram of protein$^{-1}$) were determined on the basis of spectra of whole cells. Representative results with a variation of less than 10% are shown.

[b] A 30% (wet weight per volume) cell suspension was sonicated six times (for 30 s each time) with 30-s intervals for cooling with an MSE Sonicator. Activity is expressed as nanogram-atoms of oxygen consumed minute$^{-1}$ milligram of protein$^{-1}$. Representative results of three experiments with a variation of less than 20% are shown.

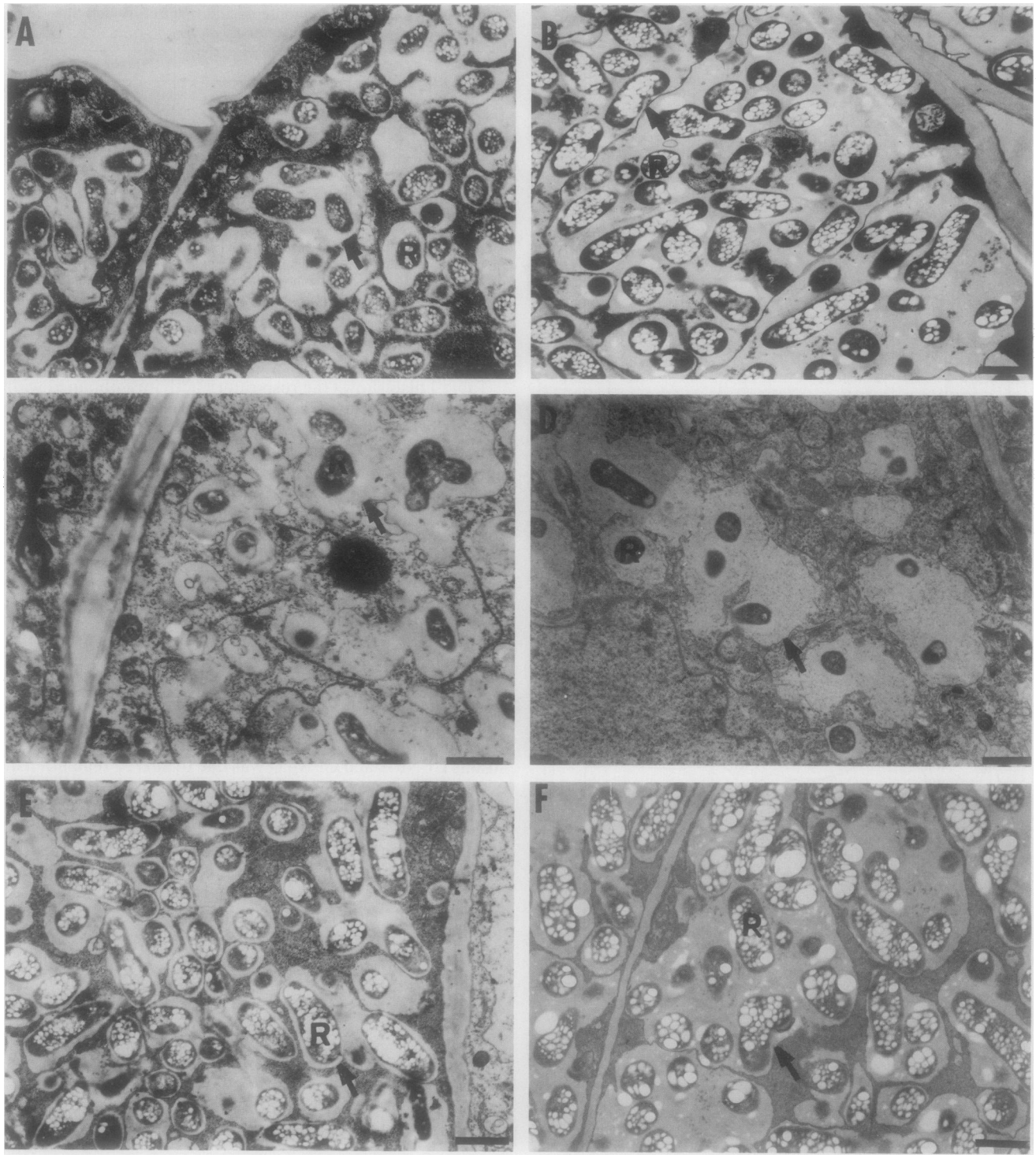


FIG. 2. Electron micrographs of thin sections of nodules from *P. vulgaris* beans 15 (A, C, and E) and 22 days (B, D, and F) after inoculation with *R. phaseoli* strains CE3 (A and B), CFN031 (C and D), and CFN037 (E and F). Note the bacteroids (R) and the peribacteroid membrane (arrows). Bars represent 1.5 μm, except for that in panel F, which represents 1.2 μm.

TMPD oxidase activities (Table 2). In contrast, static cultures of these strains showed that CE3 had at least a fourfold lower $aa_3$ content and threefold lower ascorbate-TMPD oxidase activity than CFN031 and CFN037, showing that the mutations present in CFN031 and CFN037 affected cytochrome $aa_3$ expression. These data explain the $TMPD^{++}$ phenotype of strains CFN031 and CFN037. Bacteroids of strains CE3 and CFN037 prepared by layering nodule extracts from 21-day-old plants on a sucrose gradient as previously described (5) had similar cytochrome $aa_3$ contents (0.019 and 0.022 nmol mg of $protein^{-1}$). The cytochrome $aa_3$ content of CFN031 bacteroids could not be

determined, as very few bacteroids were recovered from nodules induced by this strain (see below).

*P. vulgaris* beans grown under nitrogen-free conditions were inoculated with strain CE3, CFN031, or CFN037 to determine their symbiotic phenotypes. *P. vulgaris* cv. negro jamapa seeds were 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 (23) in a greenhouse. Acetylene reduction activity was determined by gas chromatography in a Packard 430 chromatograph (18). The total nitrogen of 34-day-old plants was determined from total dry shoots and leaf homogenates with an Antek 720 nitrogen detector (18).

The mutant strains were able to nodulate *P. vulgaris*, but nodules formed by strain CFN031 were green rather than pink and smaller than those formed by CE3. CFN031-inoculated plants contained 58% (1.52 ± 0.28 [standard deviation {SD} of three determinations] mg of nitrogen 100 mg [dry weight]$^{-1}$) of the nitrogen found in wild-type-inoculated plants (2.61 ± 0.27 [SD of three determinations] mg of nitrogen 100 mg [dry weight]$^{-1}$), whereas CFN037-inoculated plants had 22% (3.16 ± 0.21 [SD of three determinations] mg of nitrogen 100 mg [dry weight]$^{-1}$) more nitrogen than did wild-type-inoculated plants.

The nitrogen content differences shown by CFN037 could be due to higher nitrogenase activity in young nodules, as in the previously described *R. phaseoli* TMPD$^{++}$ mutant (18). Therefore, we measured acetylene reduction activity in plants nodulated by the different strains 19 and 24 days after inoculation. CFN031-nodulated roots had no acetylene reduction activity. Nineteen days after inoculation, strain CFN037 showed twofold higher acetylene reduction activity (150 ± 41% of CE3 [SD of three determinations] of ethylene produced min$^{-1}$ mg of nodule dry weight$^{-1}$) than CE3-inoculated plants (78 ± 8.4 [SD of three experiments] min$^{-1}$ mg$^{-1}$) but acetylene reduction activity (91.2 ± 41 [SD of three experiments] min$^{-1}$ mg$^{-1}$) 24 days postinoculation similar to that of CE3 (100 ± 35.4 [SD of three experiments] min$^{-1}$ mg$^{-1}$).

Since CFN031-induced nodules showed morphological abnormalities and CFN037-induced nodules had higher nitrogenase activity, which was apparent only in young nodules, *P. vulgaris* beans were inoculated to determine the ultrastructure of nodules harvested at 15 and 22 days postinoculation.

Slices of nodules were fixed with glutaraldehyde and $OsO_4$ treated as previously described (13, 21). After dehydration in increasing dilutions of ethanol, samples were embedded in Epon 81.2. Thin sections (0.9 μm) were stained with 2% uranyl acetate and 5% leaf citrate and observed under a JEOL 100-B transmission electron microscope operated at 60 kV.

Fifteen days after inoculation, two or three bacteroids were found per enveloping peribacteroid membrane in CE3-induced nodules (Fig. 2A). These bacteroids were not fully developed, since they were quite small and had only a few deposits of poly-β-hydroxybutyrate (Fig. 2). Electron microscopy of CFN031 showed that one or two bacteroids were found per enveloping membrane and bacteroids were small and showed no poly-β-hydroxybutyrate deposits (Fig. 2C); this phenotype is similar to the one reported for *R. trifolii* $C_4$ dicarboxylate transport mutants (9). Fifteen-day-old nodules induced by CFN037 had two or three bacteroids per enveloping membrane (Fig. 2E). In contrast to the wild-type strain, CFN037 bacteroids were completely developed, they were bigger than CE3 bacteroids, and they showed pronounced deposits of poly-β-hydroxybutyrate (Fig. 2E). Although the relationship between nitrogenase activity and accumulation of poly-β-hydroxybutyrate is still uncertain (10, 24), in *B. japonicum* bacteroids, 80% of the final poly-β-hydroxybutyrate content is accumulated during nitrogen fixation (10).

Examination of the ultrastructure of 22-day-old nodules induced by CE3 showed that *Rhizobium* cells had proliferated, since 15 to 20 bacteroids were found per enveloping membrane (Fig. 2B). Bacteroid development was completed, since the bacteroids were bigger and had pronounced deposits of poly-β-hydroxybutyrate (Fig. 2B). In 22-day-old nodules induced by CFN031, bacteroids were still few and small and premature degradation and lysis was evident. At 22 days after inoculation, electron microscopy of nodules induced by CFN037 and CE3 revealed no significant difference in bacteroid content or development (Fig. 2F).

We have identified two different *R. phaseoli* mutants which express cytochrome $aa_3$ under microaerophilic culture conditions and which have altered symbiotic phenotypes. These mutants differed in the localization of the Tn*5* insertion, in that CFN031 expressed very low levels of cytochrome *o* and had altered vegetative growth properties. CFN031 was also severely affected in the proliferation of rhizobia in plant cells. This phenotype suggests that cytochrome *o* could play an important role in plant cell infection; nevertheless, we cannot rule out the possibility of pleiotropic effects of the Tn*5* mutation. The DNA sequence of the mutated gene in CFN031 could be a way to identify the gene involved in cytochrome *o* expression. CFN037 showed higher symbiotic nitrogen fixation, which was related to premature bacteroid development; this phenotype is similar to the one shown by a previously identified TMPD$^{++}$ *R. phaseoli* mutant (18). The characterization of the gene mutated in CFN037 will be important for the comprehension of cytochrome terminal oxidase expression in rhizobia.

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