

Isolation of a *Rhizobium phaseoli* Cytochrome Mutant with Enhanced Respiration and Symbiotic Nitrogen Fixation

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Cultured cells of a *Rhizobium phaseoli* wild-type strain (CE2) possess *b*-type and *c*-type cytochromes and two terminal oxidases: cytochromes *o* and *aa*₃. Cytochrome *aa*₃ was partially expressed when CE2 cells were grown on minimal medium, during symbiosis, and in well-aerated liquid cultures in a complex medium (PY2). Two cytochrome mutants of *R. phaseoli* were obtained and characterized. A Tn5-*mob*-induced mutant, CFN4201, expressed diminished amounts of *b*-type and *c*-type cytochromes, showed an enhanced expression of cytochrome oxidases, and had reduced levels of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, succinate, and NADH oxidase activities. Nodules formed by this strain had no N₂ fixation activity. The other mutant, CFN4205, which was isolated by nitrosoguanidine mutagenesis, had reduced levels of cytochrome *o* and higher succinate oxidase activity but similar NADH and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine oxidase activities when compared with the wild-type strain. Strain CFN4205 expressed a fourfold-higher cytochrome *aa*₃ content when cultured on minimal and complex media and had twofold-higher cytochrome *aa*₃ levels during symbiosis when compared with the wild-type strain. Nodules formed by strain CFN4205 fixed 33% more N₂ than did nodules formed by the wild-type strain, as judged by the total nitrogen content found in plants nodulated by these strains. Finally, low-temperature photodissociation spectra of whole cells from strains CE2 and CFN4205 reveal cytochromes *o* and *aa*₃. Both cytochromes react with O₂ at -180°C to give a light-insensitive compound. These experiments identify cytochromes *o* and *aa*₃ as functional terminal oxidases in *R. phaseoli*.

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, 24), while oxidative phosphorylation yields ATP for the nitrogen-fixing reaction (3).

The cytochrome composition of the electron transport chain of different *Rhizobium* species has been described. Free-living *Rhizobium*-species express *b*-type and *c*-type cytochromes and possibly two terminal cytochrome oxidases: cytochromes *aa*₃ and *o* (2). Both were identified by photodissociation spectra, although the signals from cytochrome *o* were poorly defined and oxygen binding was not demonstrated (2). *Bradyrhizobium japonicum* bacteroids express a complement of carbon monoxide-reactive proteins different from that found on cultured cells (1). It has been reported that *B. japonicum* expresses cytochrome *aa*₃ neither in nonagitated cultures (4) nor during symbiosis (1), probably due to low oxygen tensions (4).

The close relationship between *B. japonicum* respiration and symbiotic nitrogen fixation has been demonstrated by the isolation of mutants affected in respiration and symbiotic nitrogen fixation (10, 16). In both cases mutants were isolated which cannot react with Nadi reagent, which specifically reacts with cytochrome oxidases. Most mutants showed low respiratory activities and low cytochrome *c* and *aa*₃ content (10, 16). These mutants formed ineffective symbiosis. A mutant which lacked cytochrome *aa*₃ and retained cytochrome *c* could still fix nitrogen, implying that cytochrome *aa*₃ is dispensable for symbiosis in *B. japonicum* (16).

In this paper we described the cytochrome composition of the electron transport chain of *Rhizobium phaseoli* and the application to *R. phaseoli* of low-temperature photolysis and ligand exchange techniques, which have proved useful in studying oxidases of the *aa*₃, *o*, and *d* types of other bacteria (18, 19; R. K. Poole, in C. Anthony, ed., *Bacterial Cytochrome Oxidases in Energy Transduction in Bacteria*, in press).

We also described the isolation of two cytochrome mutants, one of which has an altered regulation of cytochrome *aa*₃ expression and has nitrogen fixation activity significantly greater than that of the wild-type strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmid are listed in Table 1.

Media. All media were as described by Noel et al. (15). Two types of complex medium were used: PY1 medium contained 0.5% peptone, 0.3% yeast extract, and 10 mM CaCl₂; the peptone was Peptona de caseina, obtained from Bioxon de México, S.A. de C.V. PY2 medium had the same composition as PY1 medium except for the peptone which was lab M balanced peptone no. 1, obtained from London Analytical and Bacteriological Media. Antibiotics used were the following (in micrograms milliliter⁻¹): kanamycin, 30; rifampin, 25; tetracycline, 10; and streptomycin, 100.

Tn5 mutagenesis. The mobilizable "suicide plasmid" pSUP5011 carrying Tn5-*mob* (23) was mobilized into *R. phaseoli* CE2 (Table 1). Matings were done on PY1 plates overnight at 30°C; the cells were then suspended in sterile water and plated on selective medium (PY1 medium with rifampin and kanamycin).

Isolation of mutants. CE2 cells were mutagenized with

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
<i>Rhizobium phaseoli</i>		
CFN42	Wild type	Quinto et al. (22)
CE3	Sm ^r derivative of CFN42	Noel et al. (15)
CE2	Rif ^r Cm ^r derivative of CFN42	Noel et al. (15)
CFN4201	Mutant isolated after transfer of pSUP5011 to strain CE2	This work
CFN4205	Mutant isolated after nitroso-guanidine mutagenesis of strain CE2	This work
<i>Escherichia coli</i>		
HB101	<i>recA hsdR hasM Sm^r pro leu</i>	Boyer and Roulland-Dussoix (7)
Plasmid		
PJB3	R68.45 Km ^s Cm ^r Ap ^r	Brewin et al. (8)
pSUP5011	pBR325 Tn5- <i>mob</i> (<i>mob</i> inserted into Tn5)	Simon et al. (23)

nitrosoguanidine as described previously (10) or with Tn5-*mob* and plated on minimal medium (MM) plates with 10 mM succinate and 10 mM NH₄Cl as carbon and nitrogen sources, respectively. After 5 days of growth at 30°C, TMPD⁻ mutants were screened by overlaying a solution of 9 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and 0.4 mM ascorbate. Colonies unable to oxidize TMPD, identified by their white color, were purified on PY1-kanamycin plates. TMPD⁺⁺ mutants were screened as TMPD⁻ mutants, but 4 mM ascorbate was used in the overlaying solution instead of 0.4 mM since under these conditions the staining capacity of the cells is significantly lowered. Colonies with increased staining were purified until a stable TMPD⁺⁺ phenotype was established. The mutant strains reported in Table 2 retained the antibiotic-resistant markers of the parental strain.

TABLE 2. Cytochrome levels in membrane particles of *R. phaseoli* strains

Strain	Concn ^a of cytochrome:			
	<i>b</i>	<i>c</i>	<i>aa</i> ₃	<i>o</i> -CO
CE2	0.310	0.166	0.077	0.069
CFN4201	0.156	0.06	0.122	0.112
CFN4205	0.134	0.160	0.089	0.031

^a Cytochrome concentrations (nanomoles milligram of protein⁻¹) in the membrane fraction were determined from the difference spectra at room temperature (not shown). *b*-type and *aa*₃-type cytochromes were determined from a dithionite-reduced minus -oxidized preparation, the *c*-type cytochrome concentration was determined from ascorbate-TMPD-reduced amino -oxidized spectra, and the *o*-type cytochrome concentration was determined from dithionite plus CO minus dithionite-reduced spectra.

Membrane preparations. A biofermentor containing 20 liters of PY1 medium (adjusted at 30°C, 8 liters of air min⁻¹, and agitation at 250 rpm) was inoculated with 1 liter of an active culture in PY1 medium. Cells were collected after 18 h of growth (early stationary phase), washed with 50 mM Tris hydrochloride (pH 7.4)–5 mM CaCl₂–5 mM MgCl₂ (TCM buffer), and suspended in 500 ml of Tris hydrochloride (pH 7.4) buffer containing 40 mM EDTA and 0.5 M sucrose (TES buffer). Spheroplasts were made by lysozyme-EDTA treatment, and membrane particles were prepared therefrom by the procedure described for *Rhizobium trifolii* (9). The homogenate was incubated at room temperature for 15 min with a few crystals of DNase and centrifuged at 8,000 × *g* for 10 min; the pellet, which contained nondisrupted cells, was discarded. Membranes were recovered by centrifugation at 100,000 × *g* for 30 min and stored under liquid nitrogen until used.

Respiratory activities. NADH and succinate oxidase activities were determined at 30°C in a model 52 oxygen meter (Yellow Springs Instrument Co.). The reaction vessel contained 3 ml of 50 mM potassium phosphate (pH 7.4) and membranes (1.5 mg of protein); the reaction was started by the addition of 40 mM succinate or 5.5 mM NADH (final concentrations). TMPD oxidase was determined under the same conditions except that the pH was 6.8 and 10 mM ascorbate (pH 6.8) and 0.1 mM TMPD were used as electron donors.

Spectral analysis of cytochrome. Cytochrome spectra of membrane particles, cultured cells, or bacteroids were recorded on an SLM Aminco Midan II spectrophotometer as described previously (11). Samples were suspended in TCM buffer and reduced with dithionite (few grains) or with 10 mM ascorbate and 0.1 mM TMPD. Membrane particles, whole cells, or bacteroids were oxidized with ammonium persulfate. Spectra were obtained at room temperature (1.0-cm-light path cuvettes) or under liquid nitrogen (2.0-mm-path cuvettes). For the quantification of cytochromes, the following wavelength pair and millimolar extinction coefficients were used (11): cytochrome *aa*₃—*E*_{603–630}, 24 mM⁻¹ cm⁻¹; cytochrome *b*—*E*_{560–575}, 22 mM⁻¹ cm⁻¹; cytochrome *c*—*E*_{554–544}, 23 mM⁻¹ cm⁻¹; and cytochrome *o*-CO—*E*_{415–430}, 160 mM⁻¹ cm⁻¹. For low-temperature, ligand exchange studies, cells were grown in 500 ml of PY2 medium for 24 h as a starter culture and used to inoculate 10 liters of PY2 medium in a 12-liter Biostat V fermentor (FT Scientific; at 30°C, aerated at 4 liters of air min⁻¹ and with stirring at 250 rpm). Cells were collected after 18 h of growth (early stationary phase). Cells were washed and suspended to about 30% (packed by volume, =12 mg of protein ml⁻¹) in 46 mM potassium phosphate buffer (pH 7.4); ethylene glycol was added to give a final concentration of 30% (vol/vol). The cell suspension was reduced with sodium succinate (10 mM final concentration) for 30 min, and CO was bubbled into the cuvette for 5 min. The cuvette (2.0-mm path) was cooled in an ethanol-solid CO₂ bath to –23°C and allowed to equilibrate for 5 min in the dark. Where indicated, O₂ was added by vigorously stirring the sample with vertical strokes of a closely fitting coiled wire for 30 s. The anoxic or O₂-supplemented sample was then quickly frozen in an ethanol-dry CO₂ bath at –78°C, where it was maintained for at least 5 min in the dark before equilibration (10 to 20 min) at the temperature of the experiment in the sample compartment of the spectrophotometer. Difference spectra were recorded using a Johnson Foundation DBS-3 dual-wavelength scanning spectrophotometer described previously (26). Temperature control (±1°C) was achieved by blowing a stream of N₂

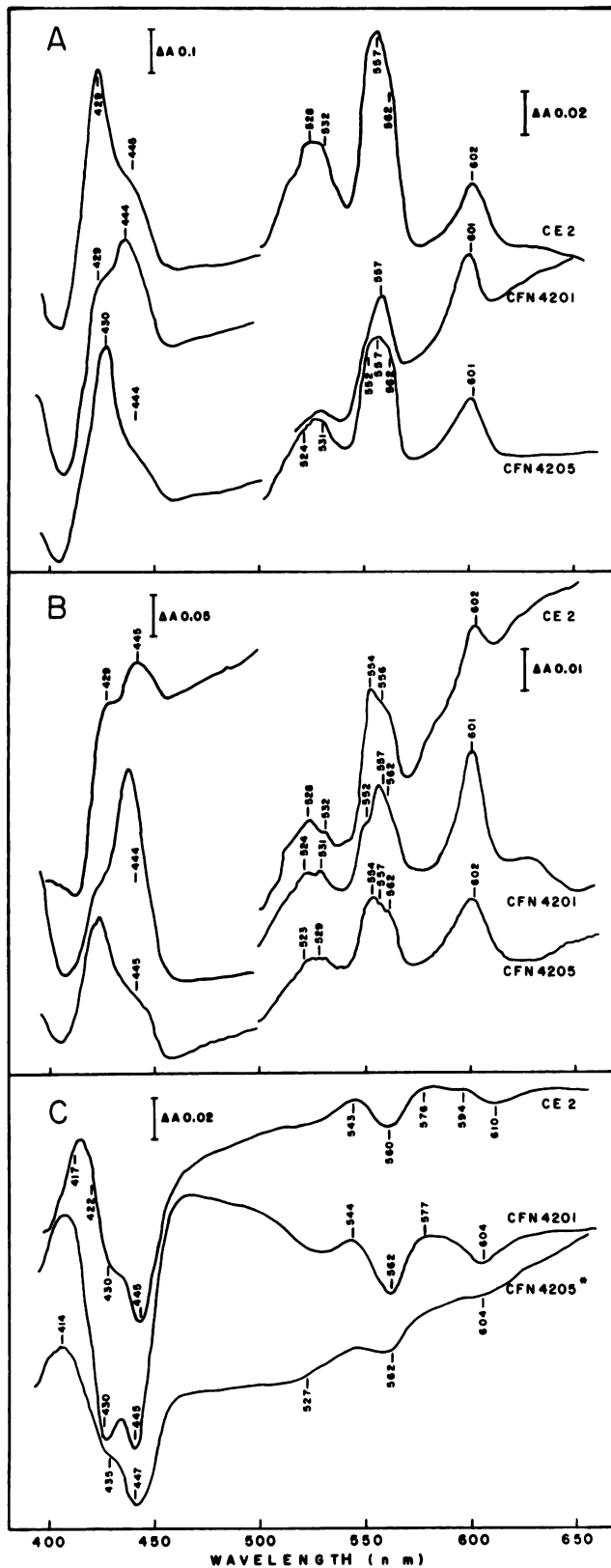


FIG. 1. Spectra of cytochrome in membranes of strains CE2 (5.4 mg of protein ml⁻¹), CFN4201 (5.8 mg of protein ml⁻¹), and CFN4205 (4.8 mg of protein ml⁻¹). (A) Dithionite-reduced minus ammonium persulfate-oxidized spectrum recorded at 77 K; (B) ascorbate-TMPD (10 mM, 0.1 mM)-reduced minus ammonium per-

(cooled by circulation through liquid N₂) over the base of the cuvette coupled with a small heater and temperature regulation circuit. Illumination by the measuring and reference beams was avoided during temperature equilibration. The sample was then scanned twice, generating a reduced plus CO minus reduced plus CO base line and then photolysed. Photolysis by white light was achieved using a 60-s exposure to the light from a 150-W projector lamp which was focused by a lens onto one afferent limb of a bifurcated light guide leading to the cuvette, the other limb transmitting reference and measuring beams.

Nitrogen fixation determination. For nodulation tests and 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 PY1 medium, and grown with nitrogen-free salts (25) in a greenhouse. After 25 days of inoculation, nodulation was scored and 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. Ethylene production was determined by gas chromatography in a Packard model 430 chromatograph. The percentage of plant nitrogen content was estimated in total shoots and leaves which were dried and homogenized. A sample (100 mg) of homogenate was digested by heating in the presence of 1 g of selenium reagent mixture and 3 ml of 7 M H₂SO₄. The digest mixture was distilled in the presence of 0.1 mg of phenolphthalein and 25% NaOH. The distillation products were recovered in 4% boric acid and a pH indicator (usually bromocresol green). The nitrogen content was estimated by titration with a 0.1 N HCl solution as described previously (6).

Bacteroid preparation. Nodules were harvested 32 to 35 days after planting. Bacteroids were isolated by layering a nodule extract on a sucrose gradient as reported by Awnaike et al. (5).

Protein determination. Protein was measured by the method of Markwell et al. (14), using serum albumin as the standard.

RESULTS

Cytochrome composition of the electron transport chain of *R. phaseoli*. A rifampin-resistant derivative (strain CE2) of *R. phaseoli* CFN42 was used in this study and has been described previously (15). Membrane particles were obtained from cultured cells of strain CE2 in order to determine its cytochrome composition. Figure 1A shows the dithionite-reduced minus -oxidized difference spectrum. This strain contains *b*-type (peaks at 429 and 557 nm) and *aa*₃-type (shoulder at 445 nm and peak at 602 nm) cytochromes. No cytochrome *c* was clearly distinguishable at 417 to 420 nm, but a shoulder near 550 nm was observed. O'Brien et al. (16, 17) showed that in *B. japonicum* ascorbate-TMPD is oxidized by the cytochrome *c-aa*₃ branch since a mutant which lacks both cytochromes or one lacking just cytochrome *aa*₃ could not oxidize ascorbate-TMPD. Figure 1B shows the ascorbate-TMPD-reduced minus -oxidized difference spec-

sulfate-oxidized spectrum at 77 K; (C) CO difference spectrum (dithionite-reduced membranes) recorded at room temperature. *, Spectra recorded at an absorbance (A) of 0.01.

TABLE 3. Respiratory activities in membrane particles and cytochrome *aa*₃ levels in whole cells of *R. phaseoli* strains

Strain	Oxidases activity ^a			Cytochrome <i>aa</i> ₃ concn ^b			
	Succinate	NADH	Ascorbate-TMPD	PY1 liquid	PY2 liquid	MM plates	Symbiosis
CE2	65.2	217.3	190.0	0.0452	0.0097	0.0105	0.0118
CFN4201	16.0	16.8	33.5	ND	ND	ND	ND
CFN4205	185.1	227.1	172.0	0.0434	0.0321	0.0480	0.0251

^a 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 10% are shown.

^b The cytochrome *aa*₃ concentration (nanomoles milligrams of protein⁻¹) was determined from spectra of whole cells on MM plates, in plants, and in liquid PY medium. Representative results of three experiments with a typical variation of less than 10% are shown. ND, Not determined.

tra of CE2 membrane particles. Cytochrome *b* is partially reduced by ascorbate-TMPD, as shown by the 429-nm absorption peak; two components in the α region were resolved, with maxima of 554 nm ("c₅₅₄") and a shoulder at 562 nm ("b₅₆₂"). A similar cytochrome *b* has been reported to be present in *B. japonicum* and *R. trifolii* (2, 9).

Putative terminal oxidases were identified in carbon monoxide difference spectra (Fig. 1C). Membranes showed two CO-reactive cytochromes, cytochrome *o* (peak at 417 nm, shoulder at 430 nm, and features near 560 nm) and cytochrome *aa*₃ (troughs at 445 and 610 nm and shoulder at 594 nm). Both CO-reactive cytochromes were identified as terminal oxidases by photodissociation spectra and oxygen binding (see below). The cytochrome composition of the *R. phaseoli* respiratory chain thus resembles that of the respiratory chains proposed for other *Rhizobium* species (2, 9, 12).

Isolation and characterization of *R. phaseoli* mutants. Mutants with altered respiration capacities were screened by using the TMPD overlay procedure described in Materials and Methods. Colonies with functional cytochromes stained a blue color within a few minutes.

CE2 cells were mutagenized with Tn5-*mob* or with nitrosoguanidine as described previously (10, 23), and two types of mutants were screened, negative color mutants (TMPD⁻) and mutants with increased staining capacities (TMPD⁺⁺) (see Materials and Methods for details). About 4,000 Km^r mutant colonies were screened for putative cytochrome mutants. One TMPD⁻ mutant was identified and further characterized; no TMPD⁺⁺ mutants were detected after Tn5 mutagenesis.

About 7,000 colonies were screened from nitrosoguanidine mutagenesis; two TMPD⁻ mutants and one TMPD⁺⁺ mutant were identified. Further analyses were performed with the TMPD⁻ Tn5-induced mutant (CFN4201) and with the TMPD⁺⁺ mutant strain (CFN4205).

Strain CFN4201 has a single Tn5 insertion, as proven by blot hybridization against Tn5 sequences (data not shown). The Tn5 insertion in strain CFN4201 was shown to be genetically linked to the TMPD⁻ phenotype. Plasmid PJB3 was introduced into strain CFN4201; this plasmid contains the functions necessary to mobilize the *mob* sequences present in Tn5 (8, 23). Strain CFN4201(PJB3) was mated with a streptomycin-resistant derivative of CFN42 (CE3). The Km^r phenotype was mobilized at a frequency of 10⁻⁴, and all of the transconjugants were found to be TMPD⁻, indicating that this phenotype was due to the Tn5 insertion.

Strain CFN4201 and CFN4205 had the same doubling time (2.5 h) in complex liquid cultures. In MM with succinate as the sole carbon source, strain CFN4201 showed a lag phase (4 h) but grew with the same doubling time as strains CE2 and CFN4205 (3 h). Strain CFN4201, unlike CE2 and CFN4205, could not utilize glucose as the sole carbon

source. Strain CFN4205 reached a lower culture protein content (48 μ g ml⁻¹) when compared with strain CE2 (77 μ g ml⁻¹) with glucose as the sole carbon source.

Respiratory properties of the mutants. Membrane particles were obtained from liquid cultured cells in order to determine cytochrome composition and respiratory activities. Strain CFN4201 showed low levels of *b*-type cytochromes since the absorption peaks at 429 and 57 nm were clearly lower than those in strain CE2 (Fig. 1A). Strain CFN4201 also showed reduced levels of *c*-type cytochrome. Figure 1B shows the ascorbate-TMPD-reduced minus -oxidized difference spectra; clearly, the 552-nm absorption peak is lower than that in the parent strain. Strain CFN4201 retained both CO-reactive cytochromes (Fig. 1C). Strain CFN4205 showed a cytochrome pattern similar to that of the CE2 strain, although it showed reduced levels of cytochrome *b* (peak at 557 nm) (Fig. 1A) and of cytochrome *o* (Fig. 1C). Table 2 shows the cytochrome concentration found in the three strains from spectra obtained at room temperature (not shown). Strain CFN4201 had threefold-lower *c*-type cytochrome and twofold-lower *b*-type cytochrome. Strain CFN4205 had twofold-lower *b*-type cytochrome and almost twofold-lower cytochrome *o* than did the wild-type strain.

Table 3 shows the respiratory activities obtained with membrane particles from the different strains. Strain CFN4201 had fourfold-lower succinate, 13-fold-lower NADH, and 5.6-fold-lower ascorbate-TMPD oxidase activities than did CE2, whereas CFN4205 had 2.6-fold-higher succinate oxidase activity than did CE2 and NADH and ascorbate-TMPD oxidase activities similar to those of CE2. Ascorbate-TMPD oxidase activity was determined in whole cells cultured on plates. Strains CFN4205 and CFN4201 showed similar ascorbate-TMPD oxidase activity, regardless of culture conditions; nevertheless, strain CE2 showed a lower ascorbate-TMPD oxidase activity in cells cultured on plates (93.33 ng-atom of O₂ min⁻¹ mg of protein⁻¹) than in cells cultured in liquid medium. The different ascorbate-TMPD oxidase activity of strains CFN4205 and CE2 in cells cultured on plates explains the TMPD⁺⁺ phenotype shown by strain CFN4205.

Effect of growth conditions on cytochrome complement of *R. phaseoli* strains. Since strain CE2, but not CFN4205, showed a different ascorbate-TMPD oxidase activity when cultured under different conditions (see above), the cytochrome composition of these strains was analyzed in cells grown under various conditions (spectra not shown). The major difference observed was in the cytochrome *aa*₃ content of these strains (Table 3). Strains CE2 and CFN4205 showed a similar cytochrome *aa*₃ content when cultured in PY1 medium; nevertheless, strain CE2 showed a fourfold-less cytochrome *aa*₃ content when cultured on MM plates, PY2 medium, or during symbiosis, where strain CFN4205 showed a higher cytochrome *aa*₃ content (Table 3).

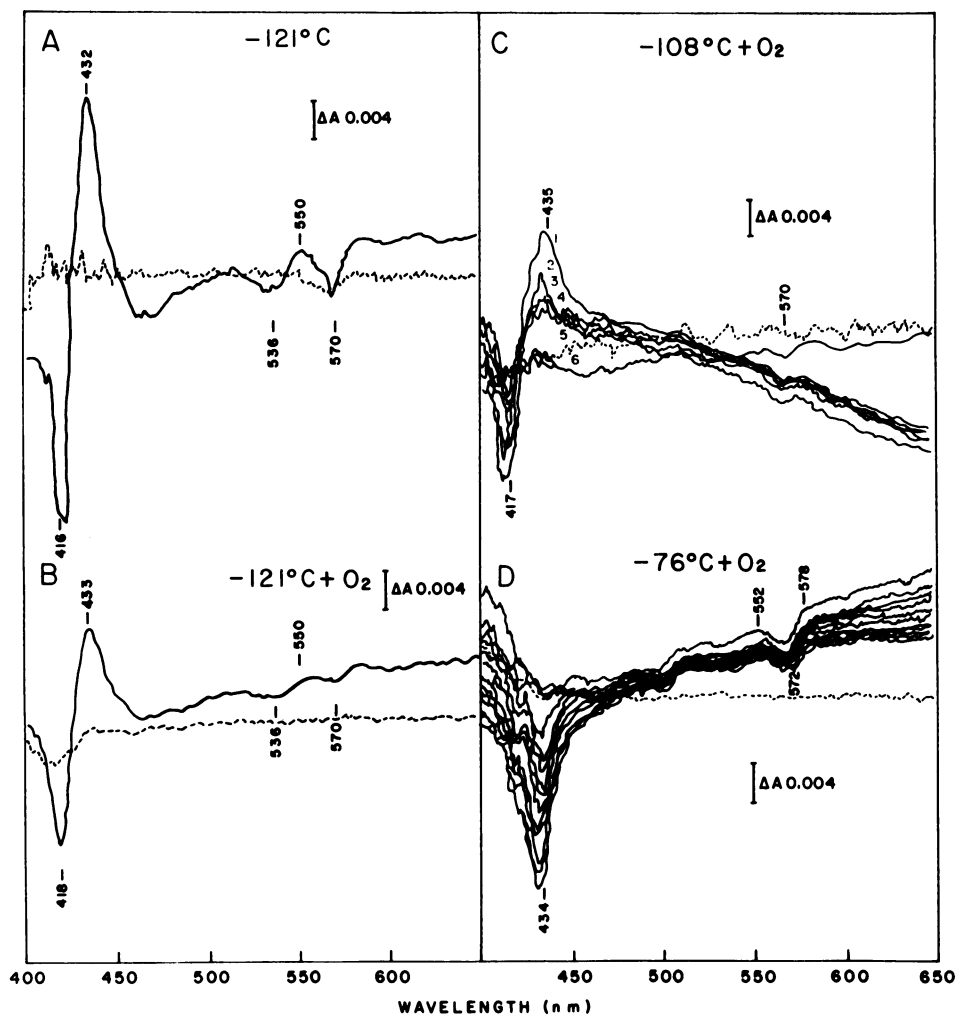


FIG. 2. Photolysis and initial stages of O_2 binding to cytochrome o in a whole-cell suspension of strain CE2. The spectra of CO-liganded, succinate-reduced cells were scanned and stored in a digital memory of a dual-wavelength spectrophotometer. The first scan, before photolysis, yielded a reduced plus CO minus reduced plus CO base line (----). (A) Spectra obtained after scanning a CE2 cell suspension after 60 s of exposure to white light at -121°C in the absence of O_2 . (B) Spectra of a similar cell suspension at -121°C in the presence of O_2 . (C) Spectra of a similar cell suspension at -108°C in the presence of O_2 . Spectra were initiated 2 min (1), 6 min (2), 9 min (3), 17 min (4), and 30 min (5) later, after photolysis; (6) reflashing sample after last scan. (D) Spectra of a similar cell suspension at -76°C ; last scan recorded 40 min after photolysis. Spectra were scanned at 2.86 nm s^{-1} with 500 nm as a reference wavelength and a spectral band width of 8 nm .

Photolysis of reduced, CO-liganded cytochromes in intact cells of CE2 and CFN4205. The fact that CE2 and CFN4205 had different CO-binding cytochromes when cultured in PY2 medium (Table 3; see below) allowed us to determine carbon monoxide and oxygen binding to cytochromes o and aa_3 independently. Thus, CO and O_2 binding to cytochrome o was analyzed in CE2 cells, while CO and O_2 binding to cytochrome aa_3 was analyzed in CFN4205 cells grown on PY2 medium.

As a prerequisite to studying the reaction of cytochrome oxidases with O_2 , attempts were made to photodissociate CO from cytochromes o and aa_3 . Reduced, CO-saturated whole cells of CE2 were photolyzed for 60 s with white light at -121°C . The resulting (postphotolysis-minus-prephotolysis) photodissociation spectrum showed the features of a pure cytochrome o spectrum (Fig. 2; see reference 21). There were absorption maxima at 432 and about 552 nm, due to the generation by photolysis of reduced cytochrome o , and minima at 416, 536, and 570 nm, due to a loss of CO-liganded cytochrome o .

Repetitive scanning of this sample for 12 min showed no change in the spectrum, suggesting no CO recombination (data not shown). When a similar experiment was done in the presence of O_2 , the difference spectrum was similar to that founded in the absence of O_2 , although the signals were smaller, probably due to some oxidation before freezing (Fig. 2B). Nevertheless, repetitive scanning of this sample showed no change in the spectrum, suggesting no further O_2 recombination at this temperature (data not shown). Oxygen recombination with cytochrome o was analyzed in CE2 cells after photolysis for 60 s with white light at -108°C . The first scan, which was measured 1 min after photolysis, showed a trough at 417 nm and a maximum at 435 nm attributable to cytochrome o . The α -region signals (minima at 536 and 570 nm) were weak (Fig. 2C). Repetitive scanning at this temperature showed a progressive loss of the 417- and 435-nm signals due to O_2 recombination, since reflashing of the sample did not restore the spectral signals (Fig. 2C).

The identification of the component responsible for the electron transfer to cytochrome oxidases could be estab-

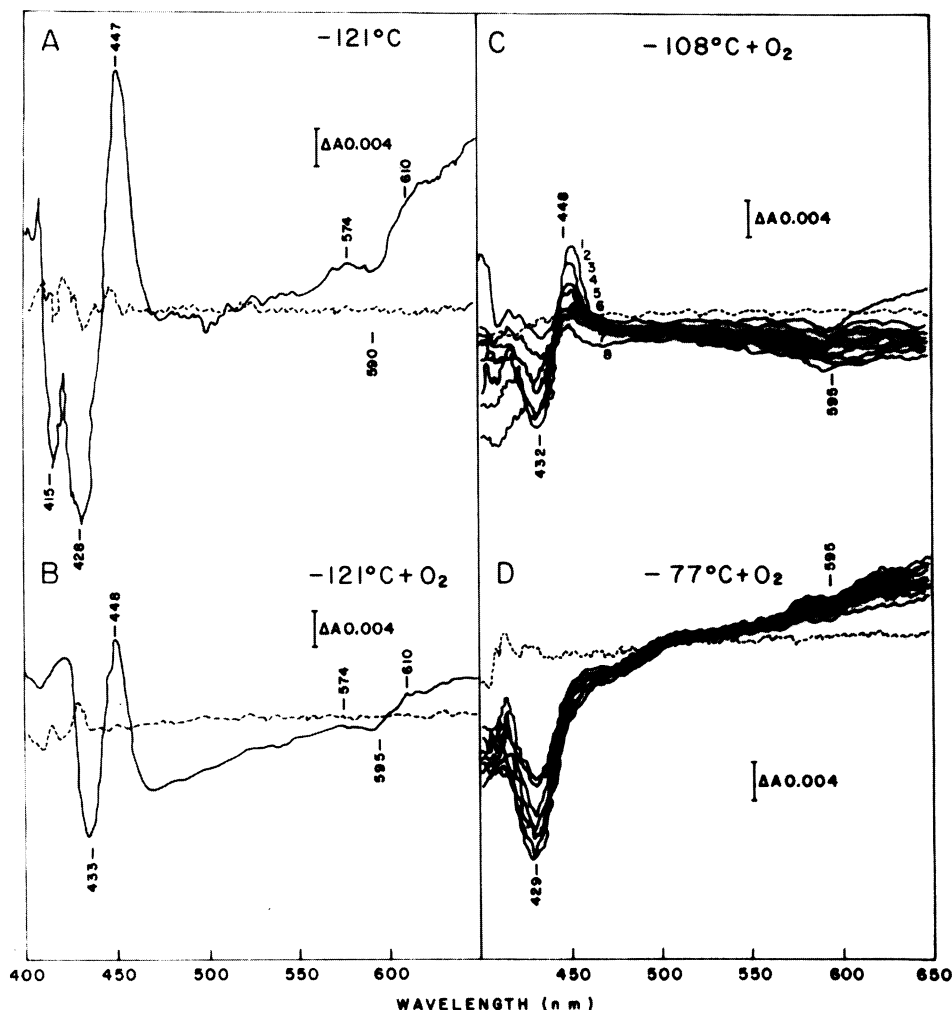


FIG. 3. Photolysis and initial stages of O_2 binding to cytochrome a_3 in a whole-cell suspension of strain CFN4205. The spectra of CO-liganded, succinate-reduced cells were scanned and stored in a digital memory of a dual-wavelength spectrophotometer. The first scan, before photolysis, yielded a reduced plus CO minus reduced plus CO base line (----). (A) Spectra obtained after scanning a CFN4205 cell suspension after 60 s of exposure to white light at -121°C . (B) Spectra of a similar cell suspension at -121°C in the presence of O_2 . (C) Spectra of a similar cell suspension at -108°C in the presence of O_2 . Spectra were initiated 1 min (1), 4 min (2), 7 min (3), 9.5 min (4), 14.25 min (5), 22 min (6), and 33.25 min (7) after photolysis; (8) reflashing sample after last scan. (D) Spectra of a similar cell suspension at -76°C ; last scan recorded 40 min after photolysis. Spectra were scanned as stated in the legend to Fig. 2.

lished by obtaining a photodissociation spectrum at warmer temperatures in the presence of O_2 , where the oxidation of the cytochrome oxidase is achieved more rapidly. Thus, any change in the spectra after repetitive scanning is due to the oxidation (by electron transfer to the cytochrome oxidase) of the immediate component of the electron transport chain.

To identify the component responsible for the electron transfer to cytochrome o , a photodissociation spectrum was obtained from a similar CE2 cell suspension in the presence of O_2 at -76°C . The first scan showed no signal that could be assigned to cytochrome o in the Soret region, due to complete ligand binding to cytochrome o at this temperature (Fig. 2D). Repetitive scanning showed the progressive development of a trough (relative to the CO-liganded form) at 434 nm, attributed to the oxidation of a b -type cytochrome (Fig. 2D).

A suspension of CFN4205 cells was photolyzed for 60 s at -121°C as described above for CE2 cells. The resulting (postphotolysis-minus-prephotolysis) photodissociation spectrum showed the features of a pure cytochrome a_3 photodis-

sociation spectrum (Fig. 3A; see references 13 and 20) and little cytochrome o (trough at 415 nm). The absorption maximum at 447 nm was due to the appearance of reduced cytochrome a_3 . Repetitive scanning of this sample for 20 min after photolysis showed no change in the spectrum, suggesting no CO recombination (data not shown). When a similar experiment was done in the presence of O_2 , no trace of cytochrome o was found, probably due either to rapid O_2 binding after photolysis or to CO displacement by O_2 before freezing (trough at 415 nm). A pure cytochrome aa_3 photodissociation spectrum was obtained (Fig. 3B). Repetitive scanning of this sample showed no change in the spectrum, suggesting no O_2 recombination at this temperature (data not shown). Oxygen recombination with cytochrome a_3 was analyzed in a suspension of CFN4205 cells, in the presence of O_2 , which was photolyzed for 60 s with white light at -108°C . The first scan, which was recorded 1 min after photolysis, showed a minimum at 432 nm and a maximum at 448 nm; the α -region signal at 595 nm was very weak (Fig. 3C). Repetitive scanings at this temperature showed a

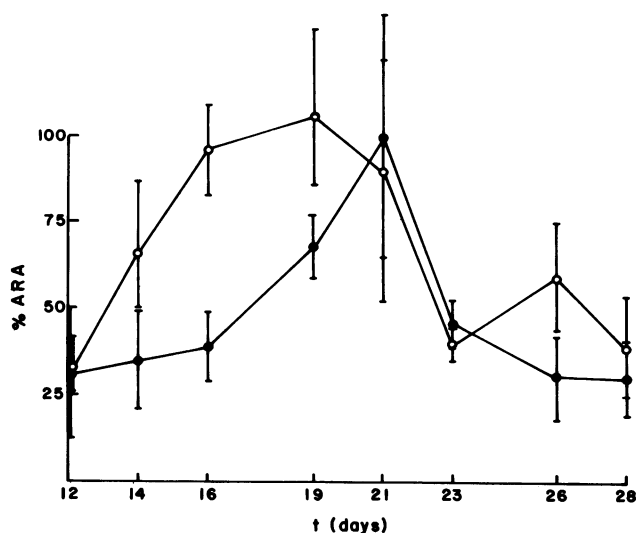


FIG. 4. Acetylene reduction activity (ARA) in plants inoculated with strains CE2 (●) and CFN4205 (○). Ten pots with three plants each were inoculated with the two strains. Acetylene reduction activity was determined in one pot on the specified days. One hundred percent represents an activity of 565 nm of C_2H_4 nodule⁻¹ h⁻¹. Vertical bars represent SD of three determinations.

progressive loss of 432- and 445-nm signals due to O_2 recombination, since reflashing of the sample did not restore the spectrum signals (Fig. 3C). The first scan after photolysis at $-76^\circ C$ showed a minimum at 429 nm but no maximum at 448 nm, due to a complete ligand combination to cytochrome a_3 at this temperature (Fig. 3D). Repetitive scanning showed the progressive development of a trough (relative to the CO-liganded form) at 429 nm, attributed to the oxidation of a *b*-type cytochrome, although different from that found to be oxidized by cytochrome *o* (Fig. 2D and 3D).

Symbiotic phenotype of the mutants. The symbiotic phenotype of the mutant strains was determined by inoculating three pots with three plants of *P. vulgaris* cv. negro jamapa each with the different strains. Nitrogen fixation was estimated by the total nitrogen content determined in the three pots independently. The mutant strains were able to nodulate *P. vulgaris*, but the nodules formed by CFN4201 were green rather than pink and smaller than those formed by strain CE2. CFN4205-nodulated plants had 22% (4.80 ± 0.23 [standard deviation {SD}] of three determinations) mg of nitrogen 100 mg [dry weight]⁻¹) more nitrogen content than did the wild type (3.92 ± 0.35 [SD of three determinations] mg of nitrogen 100 mg [dry weight]⁻¹), whereas CFN4201-inoculated plants had 40% (1.58 ± 0.11 [SD of three determinations] mg of nitrogen 100 mg [dry weight]⁻¹) of the nitrogen content found in plants inoculated with the wild-type strain. Subtracting the nitrogen content found in plants which were not inoculated with bacteria (1.30 ± 0.10 [SD of three determinations] mg of nitrogen 100 mg [dry weight]⁻¹) showed that plants inoculated with strain CFN4205 had 33% more nitrogen content than did the wild-type-inoculated plants and plants inoculated with strain CFN4201 had only 10% of the nitrogen content found in wild-type inoculated plants.

The nitrogen content differences found between plants inoculated with the wild-type strain and those inoculated with CFN4205 are the result of nitrogen accumulation during the period of nitrogen fixation (Fig. 4). Nitrogen fixation was

measured during a period of 14 days by the acetylene reduction assay. The first determination was done 12 days after inoculation since this is the 1st day that nitrogenase activity can be determined. CFN4201-inoculated plants showed no detectable activity. Figure 4 shows that plants inoculated with strains CFN4205 and CE2 reached a similar nitrogenase activity. However, CFN4205-inoculated plants reached the optimum activity 5 days before plants inoculated with the wild-type strain (Fig. 4).

DISCUSSION

Two *R. phaseoli* cytochrome mutants were isolated and characterized in the free-living and symbiotic states. Mutant strain CFN4201 had diminished amounts of *b*-type and *c*-type cytochromes in culture and forms ineffective symbiosis. This mutant has a phenotype similar to those of mutants previously isolated in *B. japonicum* (10, 16). Mutant CFN4205 showed higher levels of cytochrome aa_3 when cells were incubated on MM plates, PY2 medium, or during symbiosis than did the wild-type strain. Plants inoculated with strain CFN4205 had a nitrogen fixation capacity higher than that of wild-type-inoculated plants. The reason for this enhanced nitrogenase activity could be due to an enhanced supply of ATP or reducing equivalents to support nitrogenase or to respiratory protection of nitrogenase from O_2 damage or both. The fact that the enhanced nitrogenase activity is only apparent in young nodules could reflect the participation of the respiratory chain in the protection of nitrogenase from oxygen, as has been suggested by Appleby: "perhaps it is only in very young nodules or in the vicinity or air tubules that local O_2 concentration might rise above L_b -saturating levels and provoke protective respiration" (3). The only cytochrome content difference between CFN4205 and CE2 bacteroids was twofold-higher cytochrome aa_3 of strain CFN4205. Cytochrome aa_3 was shown to be genetically dispensable during symbiosis in *B. japonicum* (16). Nevertheless, the cytochrome aa_3 mutant characterized in that study showed an enhanced expression of an alternative cytochrome *c* oxidase activity which could compensate for the absence of cytochrome aa_3 (16).

When the wild-type strain is cultured on MM plates or during symbiosis, the cytochrome aa_3 content is fourfold lower than in cells grown on a complex medium (PY1). It has been reported that *B. japonicum* expresses cytochrome aa_3 neither in nonagitated cultures (4) nor during symbiosis (1), probably due to low oxygen tensions (4). In other bacterial species O_2 deprivation tends to cause the replacement of cytochrome aa_3 by cytochrome *o* (Poole, in press). The low content of cytochrome aa_3 of cells cultured on MM plates may also be due to low oxygen tension, since cells grown in well-aerated liquid cultures in this medium expressed a higher cytochrome aa_3 content (data not shown).

The fact that CE2 and CFN4205 cells had different CO-binding cytochromes when cultured in PY2 medium allowed us to determine CO and O_2 binding to cytochromes *o* and aa_3 independently. Photodissociation spectra of cultured cells showed that both cytochrome *o* and cytochrome aa_3 function as terminal cytochrome oxidases. Such spectra also showed that a *b*-type (434-nm) cytochrome is oxidized by cytochrome *o*. The oxidation of cytochrome *b* (429 nm) by cytochrome aa_3 suggests an electron transfer from *b* to aa_3 , although different from the *b*-type cytochrome oxidized by cytochrome *o*. In other bacterial species cytochrome aa_3 is reduced preferentially by cytochrome *c* (18; Poole, in press).

We have shown here that there is a correlation between bacterial respiration and symbiotic nitrogen fixation in *R.*

phaseoli. We have also established a method which resulted in the isolation of a mutant with an increased nitrogen fixation capacity. The agricultural benefits of the improvement of nitrogen fixation could be important but remains to be determined.

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