JOURNAL OF BACTERIOLOGY, Mar. 1987, p. 1089–1094
0021-9193/87/031089-06$02.00/0

Vol. 169, No. 3

Tn*5*-Induced Cytochrome Mutants of *Bradyrhizobium japonicum*: Effects of the Mutations on Cells Grown Symbiotically and in Culture

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Received 9 October 1986/Accepted 4 December 1986

Two *Bradyrhizobium japonicum* cytochrome mutants were obtained by Tn*5* mutagenesis of strain LO and were characterized in free-living cultures and in symbiosis in soybean root nodules. One mutant strain, LO501, expressed no cytochrome aa_3 in culture; it had wild-type levels of succinate oxidase activity but could not oxidize NADH or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). The cytochrome content of LO501 root nodule bacteroids was nearly identical to that of the wild type, but the mutant expressed over fourfold more bacteroid cytochrome *c* oxidase activity than was found in strain LO. The Tn*5* insertion of the second mutant, LO505, had a pleiotropic effect; this strain was missing cytochromes *c* and aa_3 in culture and had a diminished amount of cytochrome *b* as well. The oxidations of TMPD, NADH, and succinate by cultured LO505 cells were very similar to those by the cytochrome aa_3 mutant LO501, supporting the conclusion that cytochromes *c* and aa_3 are part of the same branch of the electron transport system. Nodules formed from the symbiosis of strain LO505 with soybean contained no detectable amount of leghemoglobin and had no N_2 fixation activity. LO505 bacteroids were cytochrome deficient but contained nearly wild-type levels of bacteroid cytochrome *c* oxidase activity. The absence of leghemoglobin and the diminished bacterial cytochrome content in nodules from strain LO505 suggest that this mutant may be deficient in some aspect of heme biosynthesis.

Nitrogen fixation by symbiotic microorganisms is an energy-expensive process; 16 to 20 ATP molecules are required to produce 2 molecules of ammonia from dinitrogen (28). N_2 fixation is generally believed to be energy limited; thus, it is of interest to understand the mode of chemical energy production and utilization by these bacteria. The energy for N_2 fixation is derived from oxidative phosphorylation in *Bradyrhizobium japonicum*, and these bacteria express a sophisticated electron transport system which functions with a varying efficiency, depending on the intracellular O_2 concentration (4, 6, 7). Also found in legume nodules is leghemoglobin, which serves to facilitate the flux of O_2 to the vigorously respiring bacteroid cells while maintaining a low free-O_2 concentration (34).

Free-living *B. japonicum* cells express cytochromes aa_3 and *o* as the terminal oxidases (2), but cells isolated from root nodules express a complement of carbon monoxide-reactive heme proteins different from that found in cultured cells (1). In addition, bacteroids express a cytochrome *c* oxidase activity which is apparently due to a flavin-containing metalloprotein (3, 24). Despite the excellent work devoted to the *B. japonicum* electron transport system by the Canberra group (2–4, 6, 7, 11), very little is known about how cytochrome expression is regulated. Oxygen is known to affect cytochrome expression in *B. japonicum* (5, 11, 25), as is true for many bacteria (26), but the molecular basis of regulation by O_2 or other environmental factors is not known.

In the present study, two Tn*5*-induced *B. japonicum* cytochrome mutants were acquired and characterized in the free-living and symbiotic states. The cytochrome mutants described have altered phenotypes under both growth conditions, and they should be useful in providing a molecular approach towards understanding the regulation of cytochrome expression.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were of reagent grade and were obtained from Sigma Chemical Co., St. Louis, Mo., or from the J. T. Baker Chemical Co., Phillipsburg, N.J. *Eco*RI, *Hin*dIII, and proteinase K were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. DNase I, DNA polymerase I, and deoxynucleotides were purchased from Bethesda Research Laboratories, Gaithersburg, Md. SeaKem agarose was purchased from FMC Corp., Marine Colloids Div., Rockland, Maine. [^{32}P]dCTP was purchased from New England Nuclear Corp., Boston, Mass. Nitrocellulose filter paper was obtained from Schleicher & Schuell, Inc., Keene, N.H. All gases were from Arundel Sales and Services Co., Baltimore, Md.

Strains and media. *B. japonicum* LO, a Nal^r derivative of strain USDA DES122, was the wild-type strain used in the present study. LO501 and LO505, Tn*5* mutants of strain LO, are Nal^r Kan^r Str^r strains. *Escherichia coli* SM10 is a Km^r strain which contains the plasmid RP4 gene coding for transfer functions integrated into the chromosome (30). pSUP1011 (pSUP101::Tn*5*) carries Cm- and Km-resistance genes (30). *B. japonicum* was grown on modified Bergersen's medium (BM [23]), or MSY medium (19) at 29°C, and *E. coli* was grown at 37°C on LB medium (20) with the appropriate antibiotic.

Tn*5* mutagenesis of strain LO. Tn*5* was introduced into *B. japonicum* cells by conjugation with *E. coli* SM10-(pSUP1011) as described previously (30). pSUP1011 contains Tn*5*, as well as the *mob* site of plasmid RP4 which allows it to be mobilized from strain SM10 (30). The cells were mated for 3 days and then plated onto MSY medium containing kanamycin and streptomycin (each at 75 μg/ml).

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Because pSUP1011 harbored in SM10 can mobilize into, but cannot replicate in, *B. japonicum*, the antibiotic resistances coded for by Tn*5* are only expressed when Tn*5* has transposed into the *B. japonicum* genome. Furthermore, streptomycin counterselects for *E. coli* since Tn*5* only confers Sm^r in *B. japonicum* (15). Km^r Sm^r *B. japonicum* colonies arose from the mating at a frequency of 2×10^{-6} per recipient.

Selection of cytochrome mutants. About 7,000 Km^r Sm^r mutant colonies were treated with a 1:1 mixture of 1% α-naphthol in ethanol and 1% *N,N,*-dimethyl-*p*-phenylenediamine) in water (Nadi reagent) as described previously (21). In the presence of O_2, *B. japonicum* and other bacteria (12, 21) catalyze the formation of an indophenol blue compound from α-naphthol and *N,N'*-dimethyl-*p*-phenylenediamine due to cytochrome oxidase activity. Colonies which failed to turn blue were potential cytochrome mutants and were restreaked onto fresh media for further analysis. Nadi-negative mutants were grown in 2-liter cultures of BM containing kanamycin and streptomycin (each at 50 μg/ml) to an optical density (at 540 nm) of 0.15 to 0.2, and cell extracts of these cultures were analyzed spectrophotometrically for cytochrome content. The cells were harvested at a low optical density because many of the mutants did not grow well at higher cell concentration.

DNA isolations. Genomic DNA from *B. japonicum* was isolated as previously described (17), except that 500 μg of RNase A per ml was included during cell breakage. Plasmid pSUP1011 was isolated from *E. coli* SM10 by CsCl gradient centrifugation as previously described (20).

Southern hybridization analyses. Genomic or plasmid DNA was cut with the appropriate restriction endonuclease and separated on the basis of size by agarose gel electrophoresis. DNA was transferred from the gel to a nitrocellulose filter by published procedures (31). The filter was hybridized with ^{32}P-labeled pSUP1011 to detect the presence of Tn*5* by the method of Maniatis et al. (20); hybridization occurred for 20 h at 42°C and in the presence of 50% formamide. The filter was then exposed to Kodak XAR-5 X-ray film to detect radioactivity. pSUP1011 was labeled by nick translation of the plasmid in the presence of [^{32}P]dCTP as described previously (20).

Preparation of cell extracts. Culture cells or bacteroids were ruptured by two passages through a French pressure cell at 1,440 kg/cm^2 and centrifugation to separate out the unbroken cells and other debris. All manipulations were performed at 4°C, and the protease inhibitor phenylmethylsulfonyl fluoride was added to the cells to a concentration of 1 mM immediately before breakage.

Respiration assays. Substrate oxidation by *B. japonicum* was performed with cell extracts of culture cells by using NADH, succinate, or ascorbate–*N,N,N',N'*,-tetramethyl-*p*-phenylenediamine (TMPD) as the substrate. In the remainder of the text, the ascorbate-TMPD mixture is referred to as simply TMPD since it is this compound that is being oxidized by the electron transport system. The oxidation of substrates by cell extracts was monitored amperometrically with an O_2 electrode as previously described (23). For the inhibitor studies, the O_2 electrode chamber containing the sample was titrated with cyanide or azide before the addition of substrate to determine the inhibitor concentration required to inhibit respiration by 50%.

Growth of soybeans and harvesting of bacteroids from root nodules. Soybeans (*Glycine max* cv. Essex) were grown in a greenhouse in vermiculite containing N-free supplemental nutrients as described by Vincent (33). The plants were grown under natural light in Baltimore, Md., during the summer of 1986. Germinated seedlings were each inoculated with about 10^8 cells of *B. japonicum* at the time of planting, and nodules were harvested 32 to 36 days after planting and were either used immediately or stored at −70°C for future experiments. Bacteroids were isolated from nodules as described previously (24). Bacteroids restreaked onto agar media retained the antibiotic resistance markers of the inoculum, showing that Tn*5* was stable symbiotically and that no bacterial cross-contamination occurred.

Cytochrome spectra. Cytochrome spectra of cell extracts of cultured cells or bacteroids were performed with a Hitachi-Perkin Elmer model 557 spectrophotometer as described previously (23). Samples were reduced with dithionite and oxidized with potassium ferricyanide.

Leghemoglobin assays. The leghemoglobin concentration of nodule cytosol was determined by measuring the absorption difference of the pyridine hemochromogen at 556 and 539 nm by the method of Bisseling et al. (8).

Nitrogen fixation assays. Nitrogen fixation was measured as C_2H_2 reduction to C_2H_4 by excised nodule-bearing soybean root segments. Acetylene was added to a concentration of 5% to 70-ml stoppered vials containing the root samples, and the C_2H_4 formed after 30 min was measured with a Shimadzu flame ionization gas chromatograph.

Cytochrome *c* oxidase assays. Cytochrome *c* oxidase activity of bacteroid cell extracts was determined as described previously (24). Horse heart cytochrome *c* (2 mM) was reduced with 40 mM ascorbate and then passed through a small G-25 Sephadex column to remove the ascorbate. Cytochrome *c* oxidation was measured as the change in absorption at 550 nm minus 540 nm by using the empirically derived E_{mM} of 12.8.

Protein determination. The protein concentration of cell extracts was determined by the dye-binding method of Bradford (9).

RESULTS

B. japonicum LO was mutagenized with Tn*5* as described by Simon et al. (30), and the resulting kanamycin- and streptomycin-resistant colonies were treated with the Nadi reagent to screen for putative cytochrome mutants (see Materials and Methods). Eleven Nadi-negative colonies were further analyzed for cytochrome content in free-living culture by recording dithionite-reduced minus ferricyanide-oxidized absorption spectra of cell extracts. All of the mutants examined had no, or diminished amounts of, cytochrome aa_3, and many had alterations in the amount of *b*- and *c*-type cytochrome as well. None of the mutants were totally deficient in *b*-type cytochrome, implying that such a mutant would be lethal or that the Nadi reagent does not specifically select for cytochrome *b*-related mutations.

Two of the Tn*5*-induced cytochrome mutants described above were particularly interesting and were studied further. Mutant strain LO501 grown in culture was completely missing cytochrome aa_3, as discerned by the lack of a peak at 602 nm of the dithionite-reduced minus ferricyanide-oxidized absorption spectrum (Fig. 1A). Cytochrome aa_3 is a terminal oxidase (2) and is only expressed in free-living *B. japonicum* cells. Strain LO501 is the first such mutant described in a *Rhizobium* or *Bradyrhizobium* species. The second mutant, LO505, showed no absorption peaks at 551 or 602 nm (Fig. 1A), indicating that this mutant does not express any *c*-type cytochrome or cytochrome aa_3 respectively. Furthermore, strain LO505 expressed less than one-half the amount of *b*-type cytochrome than did the wild type, as evident in the

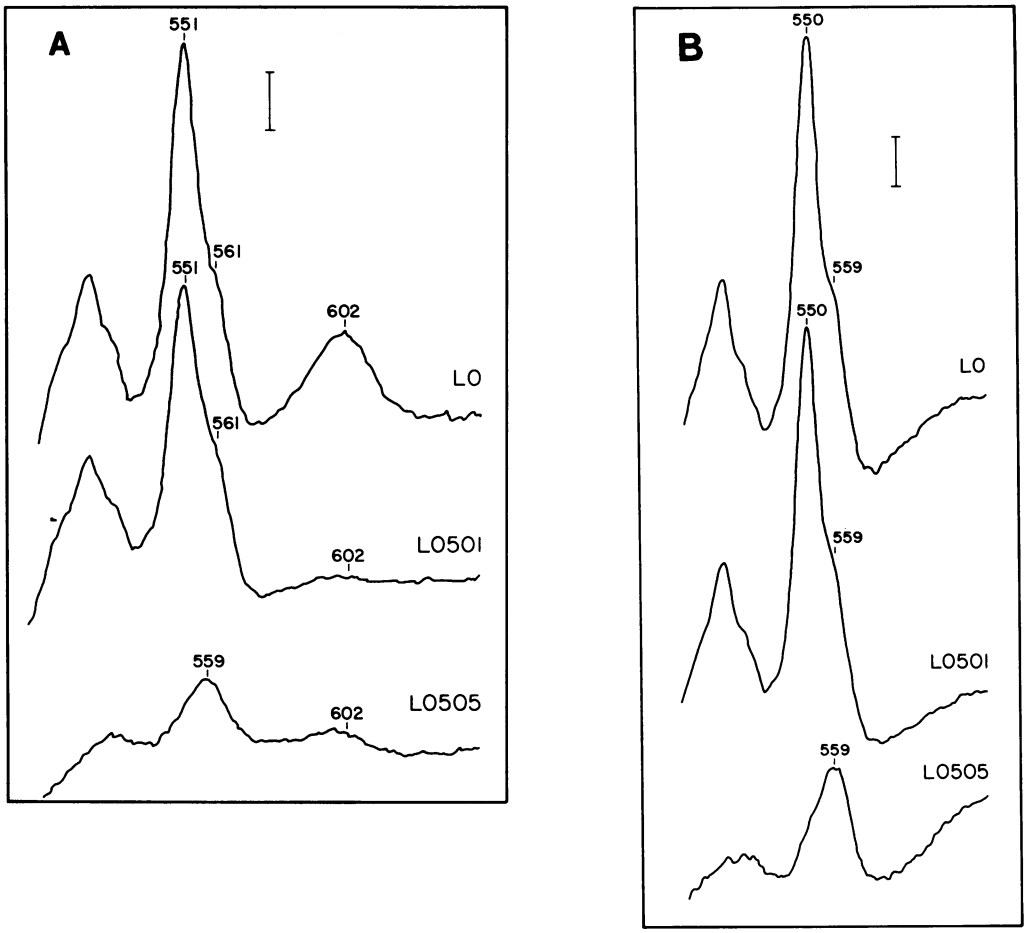

FIG. 1. Cytochrome spectra of cell extracts of strains LO, LO501, and LO505 grown symbiotically and in culture. Dithionite-reduced minus ferricyanide-oxidized absorption spectra of cell extracts from cultured cells (6.2 mg of protein ml^{-1}) (A) and from root nodule bacteroids (3.1 mg of protein ml^{-1}) (B) were performed as described in the text. The vertical bars represent a change in absorbance of 0.005.

diminished peak at 559 nm. Carbon monoxide spectra of strain LO505 indicated the presence of CO-reactive cytochrome *b* (data not shown), which was probably cytochrome *o*. Mutants similar to LO505 with respect to their cytochrome content have been obtained in *B. japonicum* treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (12). Strains LO501 and LO505 grew slowly in minimal media, with doubling times of about 22 and 40 h, respectively. The doubling time for strain LO was about 10 h.

Identification of Tn*5* in the genomes of LO501 and LO505. Because *B. japonicum* Tn*5* mutants with multiple transposon insertions have been reported (29), and because strain LO505 is deficient in several cytochromes, it was important to demonstrate that LO501 and LO505 each had a single Tn*5* insertion in their genomes. Total DNA of LO501 or LO505 cut with *Eco*RI and electrophoresed on an agarose gel yielded a single DNA fragment which hybridized to a ^{32}P-labeled Tn*5* probe (Fig. 2, lanes 1 and 6, respectively). This result is expected for a single Tn*5* insertion since *Eco*RI does not cut into Tn*5* itself (16); therefore, the entire transposon insert should be within a single *Eco*RI fragment. The Tn*5*-containing *Eco*RI fragment of LO501 was about 8 kilobases (kb) in size; that of LO505 was about 19 kb in size. The hybridization pattern of LO501 or LO505 DNA cut with *Hin*dIII was also consistent with the presence of a single Tn*5* insertion (Fig. 2, lanes 2 and 7, respectively). A single 3.3-kb fragment resulting from the cleavage at the two *Hin*dIII sites within Tn*5* was observed, along with two other *Hin*dIII fragments, each composed of Tn*5* DNA and *B. japonicum* DNA which flanked the transposon. DNA from the parent strain LO yielded no fragments which hybridized with the Tn*5* probe (Fig. 2, lanes 3 and 4).

Substrate oxidation by LO, LO501, and LO505. The oxidations of several substrates by cell extracts of LO, LO501, and LO505 grown in culture were measured to determine the effects of the cytochrome mutations on respiration. The oxidation rate of TMPD or NADH by LO501 and LO505 was drastically less than was found for LO (Table 1), showing that the bulk of electron flux from these substrates was allocated to cytochrome aa_3 rather than to another oxidase. El Mokadem and Keister (12) also found that *B. japonicum* mutants which lack cytochromes *c* and aa_3 are deficient in NADH oxidase activity. Bacteria capable of oxidizing TMPD generally express cytochrome *c* and either cytochrome aa_3 or another oxidase (32); thus, the failure of strains LO501 and LO505 to oxidize TMPD is not surprising. The oxidation of succinate by LO501 or LO505, however, was no less than was found for LO (Table 1), indicating that cytochromes *c* and aa_3 are not responsible for the observed succinate-dependent respiration. The fact that succinate reduces cytochromes *c* and aa_3, as discerned spectrophotometrically (2, 23), does not conflict with the present findings

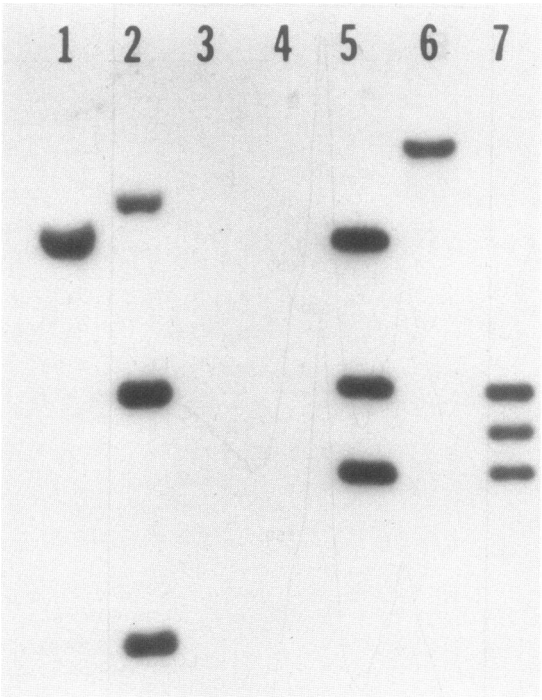

FIG. 2. Southern hybridization analysis of total genomic DNA of strains LO, LO501, and LO505. Genomic DNA was tested for the presence of Tn*5* with ^{32}P-labeled pSUP1011 as described in the text. Lanes: 1 and 2, LO501 DNA cut with *Eco*RI and *Hin*dIII, respectively; 3 and 4, LO DNA cut with *Eco*RI and *Hin*dIII, respectively; 6 and 7, LO505 DNA cut with *Eco*RI and *Hin*dIII, respectively; 5, pSUP1011 cut with *Hin*dIII, yielding fragments 7.0, 3.3, and 2.4 kb in size.

since the cytochrome spectra give no indication of the rate of electron flux through the cytochromes.

To support the observation that TMPD and NADH are oxidized by a different oxidase than is succinate in free-living cells, the sensitivity of these substrates to oxidase inhibitors was examined. The oxidations of TMPD and NADH by cell extracts of strain LO were inhibited 50% by 1.1 and 1.2 μM cyanide, respectively, whereas it took approximately fivefold more cyanide to inhibit succinate oxidation to the same extent. These data suggest that succinate respiration involves a terminal oxidase less sensitive to cyanide than the oxidase involved in TMPD and NADH respiration. Similar results were obtained with azide as the inhibitor; succinate-dependent respiration of strain LO was about eightfold less sensitive to azide inhibition ($I_{0.5}$ = 533 μM) than was NADH or TMPD oxidation. The inhibitor data, along with the respiration data of the mutants, indicate that cytochrome aa_3 is the oxidase more reactive with cyanide and azide. The less sensitive oxidase could be cytochrome *o*, which is known to be an oxidase in cultured *B. japonicum* cells (2), or it could be another heme protein found in those cells (2).

TABLE 1. Respiratory activity of strains LO, LO501, and LO505 grown in culture

Strain	Activity on substrate[a]		
	TMPD	NADH	Succinate
LO	163 ± 4	101 ± 5	7 ± 0.8
LO501	14 ± 1	8 ± 0.8	8 ± 0.3
LO505	2 ± 0.7	6 ± 0.6	9 ± 0.4

[a] Activity of cell extracts is expressed as nanomoles of O_2 oxidized min^{-1} mg of $protein^{-1}$ ± the standard deviation of four trials. The concentrations of the substrates were 10 mM succinate, 0.2 mM NADH, and 10 mM ascorbate plus 150 μM TMPD.

TABLE 2. Some symbiotic properties of strains LO, LO501, and LO505

Strain	Acetylene reduction activity[a]	Leghemoglobin concn[b]	Cytochrome *c* oxidase activity[c]
LO	9.3 ± 0.8	148 ± 9	268 ± 38
LO501	10.0 ± 1.3	145 ± 5	1186 ± 105
LO505	<0.2	<5	221 ± 33

[a] Micromoles of C_2H_4 formed h^{-1} g (fresh weight)$^{-1}$ ± the standard deviation of nine samples.

[b] Nanomoles of leghemoglobin g of nodule (fresh weight)$^{-1}$ ± the standard deviation of four samples.

[c] Nanomoles of cytochrome *c* oxidized min^{-1} mg of $protein^{-1}$ by bacteroid cell extracts ± the standard deviation of six measurements. To start the reaction, 80 μM reduced cytochrome *c* was added.

Symbiotic properties associated with LO, LO501, and LO505. Soybeans grown under nitrogen-free conditions were inoculated with strain LO, LO501, or LO505 to determine the effects of the cytochrome mutations on symbiotically grown cells and on the plant host. Thirty-day-old plants inoculated with strain LO505 produced yellow leaves and sparser foliage and were shorter than plants inoculated with strain LO (Fig. 3). However, plants inoculated with strain LO501 were healthy and were indistinguishable from plants inoculated with wild-type *B. japonicum* cells. Root nodules were formed on plants inoculated with strains LO, LO501, and LO505, but the nodules formed by LO505 were green, rather than pink, and were smaller than those formed by strain LO. Nodules formed by LO and LO501 had good N_2 fixation activity, but those formed by LO505 had no discernible activity (Table 2).

The leghemoglobin concentrations in nodules formed by all three strains were determined (Table 2); LO505 nodules contained no detectable amount of this heme protein. On the other hand, strain LO501 nodules contained essentially the same amount of leghemoglobin as did strain LO nodules. The diminished amount of cytochrome synthesized by LO505 culture-grown cells and bacteroids (see below) and the lack of leghemoglobin in nodules from plants infected

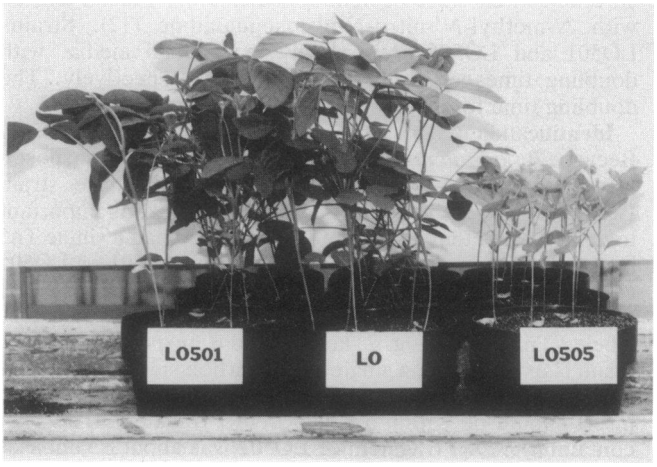

FIG. 3. Soybean plants inoculated with LO, LO501, or LO505. Soybeans were grown for 30 days as described in the text. Germinated seeds were inoculated with the *B. japonicum* strain at the time of planting.

with strain LO505 suggest that LO505 may be deficient in some aspect of heme biosynthesis.

Cytochrome spectra of bacteroids. Cell extracts of bacteroids isolated from root nodules were examined for cytochrome content (Fig. 1B). Strain LO501 expressed the same amount of *b*- and *c*-type cytochrome as did strain LO, showing that the mutation in LO501 did not seem to affect cytochrome expression symbiotically. Whereas this result is not surprising since cytochrome aa_3 is not normally expressed in bacteroids, it would be erroneous to assume that the cytochrome aa_3 mutation is of no consequence symbiotically. Strain LO505 expressed no *c*-type cytochrome and a diminished amount of cytochrome *b* (Fig. 1B); this result is similar to what was found in cultured cells of this mutant. Carbon monoxide spectra of LO505 showed that this mutant did not synthesize cytochrome P-450, which is found in the wild type, and that the only feature of this spectrum was a broad peak at 422 nm (data not shown).

Cytochrome *c* oxidase activity in LO, LO501, and LO505. Appleby (3) described a cytochrome *c* oxidase activity in *B. japonicum* bacteroids that is inhibited by cyanide and other metal chelators but not by CO, indicating that this oxidase is a metalloprotein but not a cytochrome. Furthermore, inhibitor and spectral data suggest that this bacteroid oxidase is a flavoprotein (3, 24). In the present study, it was found that cell extracts of LO505 bacteroids had good cytochrome *c* oxidase activity (Table 2), ranging from between 82 and 89% to that of the wild type in three experiments. This observation supports the conclusion that the cytochrome *c* oxidase of *B. japonicum* is not a cytochrome. Even more intriguing is the finding that strain LO501 had about 4.5-fold-greater cytochrome *c* oxidase activity than was found in the wild type (Table 2). The reason for this increased oxidase activity is not clear, but it is extremely interesting that a lesion in a gene involved in the expression of an enzyme unique to the free-living state exerts an effect on the phenotype of bacteroids.

DISCUSSION

Two *B. japonicum* cytochrome mutants, each arising from a single Tn*5* insertion, were isolated and characterized in the free-living and the symbiotic states. Mutant strain LO501 was missing the terminal oxidase cytochrome aa_3 when grown in culture and could not oxidize TMPD or NADH. Nodules formed on soybean inoculated with strain LO501 were nearly identical to the wild type with respect to N_2 fixation ability and leghemoglobin content, and the plants harboring these nodules grew well under nitrogen-free conditions. The cytochrome content of LO501 bacteroids was also very similar to that of strain LO; this result is not surprising since cytochrome aa_3 is not normally expressed symbiotically in *B. japonicum*. The only symbiotic property examined in which strains LO and LO501 differed was the cytochrome *c* oxidase activity of the two strains; LO501 had much more activity than was found in the wild type. It has been postulated that the bacteroid cytochrome *c* oxidase is involved in "protective respiration," in that it helps to prevent O_2 damage to nitrogenase by maintaining a low intracellular O_2 concentration (3); the reason for the elevated expression of this activity in mutant strain LO501 is not clear. It is noteworthy that cytochrome aa_3 and the bacteroid cytochrome *c* oxidase share some functional similarities. Both oxidases are quite sensitive to cyanide and azide (3, 24; this study), each may oxidize cytochrome *c* in situ (2, 3, 24; this study), and both oxidases seem to have a low affinity for O_2 compared with the other oxidases in their respective systems (3, 5, 6, 11, 25). However, the fact that cytochrome aa_3 is not normally expressed symbiotically makes it difficult to explain the observed cytochrome *c* oxidase activity of LO501 as compensation for the cytochrome aa_3 lesion in that mutant. Nevertheless, there may be a less obvious relationship between the two enzymes which would explain the observed phenomenon, and obtaining the gene mutated in strain LO501 may provide a tool for understanding how the expression of these oxidases is regulated.

The other cytochrome mutant, strain LO505, did not express any cytochrome *c* or aa_3 in culture, and it synthesized less *b*-type cytochrome than did the wild type. Despite this cytochrome deficiency, strain LO505, as well as strain LO501, showed wild-type levels of succinate oxidase activity. The observation that the cytochrome aa_3 mutant (LO501) and the cytochrome *c* and aa_3 mutant (LO505) had oxidation rates very similar to each other when three different substrates were used supports the notion that the cytochromes *c* and aa_3 are part of the same branch of the electron transport system (2, 23). It is interesting that the oxidations of TMPD and NADH by strain LO were much faster than was succinate oxidation, and it is the two former substrates which are affected by the cytochrome aa_3 mutation. It is possible that the participation of different oxidases in the oxidation of different substrates is a kinetic phenomenon, with electrons being directed toward cytochrome aa_3 when the flux is high.

Soybean plants inoculated with strain LO505 grew poorly in nitrogen-free soil. This mutant was infective but produced small nodules which had no nitrogen fixation activity. The cytochrome pattern of bacteroid cell extracts was similar to that of cultured cells in that some *b*-type cytochrome was present but no other heme proteins were detected. In addition, nodules from plants infected with LO505 contained no detectable amount of leghemoglobin as determined by the pyridine hemochromogen assay. The deficiency of bacteroid heme protein and of leghemoglobin in nodules from strain LO505 suggests that this strain may be mutated in a gene affecting heme biosynthesis. There is a considerable body of literature suggesting that the heme moiety of leghemoglobin is bacterial in origin (10, 13, 18, 22, 27). However, Guerinot and Chelm (14) reported that a *B. japonicum* strain with a mutation in the δ-aminolevulinic acid synthase gene (*hemA*) produces root nodules containing 60% of the leghemoglobin found in the wild type. Interestingly, a mutation in the *Rhizobium meliloti hemA* gene results in nodules which contain no leghemoglobin (18). It will be useful to determine the nature of the gene mutated in strain LO505 which causes the failure of cytochrome and leghemoglobin expression.

ACKNOWLEDGMENTS

This work was supported by a research grant from Allied Corporation.

We thank Patricia Novak for carefully reading and evaluating this manuscript.

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