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Identification of a Gene Encoding a Thioredoxin-Like Product Necessary for Cytochrome *c* Biosynthesis and Symbiotic Nitrogen Fixation in *Rhizobium leguminosarum*

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A Tn*5*-induced mutant of *Rhizobium leguminosarum* bv. viciae could not form nitrogen-fixing nodules on pea or vetch because of a lesion in electron transport to oxygen. The mutant lacked spectroscopically detectable cytochromes *c* and aa_3. No proteins containing *c*-type cytochromes could be identified in the mutant by heme staining of proteins fractionated on polyacrylamide gels, indicating that the mutant was defective in maturation of all *c*-type cytochromes. The Tn*5* mutation was determined to be located in a gene that was called *cycY*. The *cycY* gene product is homologous to the thioredoxin-like protein HelX involved in the assembly of *c*-type cytochromes in *Rhodobacter capsulatus* and to an open reading frame from a *Bradyrhizobium japonicum* gene cluster containing other genes involved in cytochrome *c* biogenesis. Our observations are consistent with CycY functioning as a thioredoxin that reduces cysteine residues in apocytochromes *c* before heme attachment.

Rhizobium leguminosarum bv. viciae can induce nitrogen-fixing nodules on pea (*Pisum sativum*) and vetch (*Vicia hirsuta*). During free-living, aerobic growth, *R. leguminosarum* bv. viciae possesses cytochrome aa_3 (21) as a terminal oxidase; and when oxygen becomes limiting, cytochrome *d* is induced (21). Photodissociation spectra indicate that cytochrome *o* is also present in the closely related *Rhizobium phaseoli* (40). Therefore, in free-living cultures, *R. leguminosarum* can probably have parallel or branched respiratory pathways terminating with the oxidases cytochromes *d*, *o*, and aa_3. Cytochromes aa_3 and *d* were absent from reduced-minus-oxidized spectra from nitrogen-fixing bacteroids, and the relative amount of absorption attributed to *c*-type cytochromes was significantly increased, indicating an important role for *c* cytochromes during symbiotic nitrogen fixation (21). Similar increases in *c*-type cytochromes are found in *Bradyrhizobium japonicum* bacteroids (2, 3), and mutations affecting cytochrome *c* assembly block nitrogen fixation (35, 36).

The distinguishing feature of these *c*-type cytochromes is a prosthetic heme group with covalent bonds between its two vinyl groups and the two corresponding cysteine side chains of the apocytochrome *c*. This union is specifically catalyzed by the enzyme cytochrome *c* heme lyase. In gram-negative bacteria, the known *c*-type cytochromes are either periplasmic proteins or membrane-anchored polypeptides with the heme group located at the periplasmic surface of the cytoplasmic membrane. Thus, *c*-type cytochromes usually have consensus signal sequences that direct them through the cytoplasmic membrane (1). In *B. japonicum*, the structural genes for the cytochrome c_1 of the cytochrome bc_1 complex (41), for a membrane-anchored cytochrome *c* (8), and for several soluble cytochromes *c* (37, 42) have been characterized. Other genes that encode the different enzymes of the biosynthetic pathway of the prosthetic group heme *c* in *B. japonicum* have been identified (16, 32). This biosynthetic route is common to all types of hemes up to the biosynthesis of protoheme IX (heme *b*) (14, 18). *B. japonicum* (35, 36) and *Rhodobacter capsulatus* (5, 6, 20) mutants specifically affected in the biosynthesis of cytochromes *c* have been isolated and characterized.

MATERIALS AND METHODS

Microbiological techniques. Bacterial strains and plasmids used are described in the text or in Table 1. Media (TY complete medium and Y minimal medium) and general growth conditions were as described by Beringer (7). *Escherichia coli* was grown in L medium (26). Antibiotics were added at the following concentrations (micrograms per milliliter): streptomycin, 400; kanamycin, 20; tetracycline, 5; gentamicin, 10; and ampicillin, 400. Bacterial growth was measured at 600 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. Transduction was carried out with the *R. leguminosarum* phage RL38 (10). Nodulation tests and measurements of acetylene reduction were made with *V. hirsuta* plants (19).

Tn*5* mutagenesis and isolation of respiratory mutants. Tn*5*-induced mutants of *R. leguminosarum* were isolated by conjugal transfer of pSUP2021 (38) into strain 8401, with selection on Y medium containing streptomycin and kanamycin. Respiration-deficient mutants were identified by using the Nadi (cytochrome oxidase) test (28), in which colonies that cannot oxidize *N,N'*-dimethyl-*p*-phenylenediamine remain white or stain very poorly, whereas normal colonies become blue.

DNA sequence analysis. pIJ1980 was constructed by subcloning a 1.3-kb *Eco*RI fragment from pIJ1939 into pUC118. Nested deletions of pIJ1980 were generated by exonuclease III digestion using the Pharmacia double-stranded nested-deletion kit. Double-stranded template DNA was denatured with NaOH and sequenced by the dideoxy chain termination method with 5'-[α^{35}-S]dATP by using the U.S. Biochemical Corp. Sequenase kit.

A 25-nucleotide primer (5'-GAAGTCAGATCCTGGAAA ACGGGAA) was used for PCR amplification and for sequencing the DNA from the end of Tn*5*. The oligonucleotide 5'-GGCGATGGACACCGTCTT, complementary to bases 1283 to 1266 of the 3' end of the sequenced region of *cycY*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Rhizobium strains		
8401	Strain of *R. leguminosarum* lacking a symbiotic plasmid (Str^r)	23
A34	Derivative of 8401 carrying the bv. viciae symbiotic plasmid pRL1JI (previously called 8401/pRL1JI)	15
A313	Derivative of 8401 carrying *cycY21*::Tn*5*	This work
A319	Derivative of 8401 carrying *cycY21*::Tn*5* $Nadi^-$	This work
A257	Derivative of A34 carrying *cycY21*::Tn*5* $Nadi^-$ Fix^-	This work
Plasmids		
pIJ1939	Cosmid clone complementing cytochrome mutant strains A313, A319, and A257	This work
pIJ1983	pML123 (22) containing *cycY* on a 1.3-kb *Eco*RI fragment from pIJ1939	This work
pIJ1980	pUC118 containing *cycY* on a 1.3-kb *Eco*RI fragment from pIJ1939 carrying *cycY*	This work
pRJ2652	*B. japonicum cycW*, ORF263, *cycX*, and ORF132	34

from *R. leguminosarum*, was the other primer. Genomic DNA (200 ng) from strains A257 and A34 was used as a template in 100-μl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 5 mM KCl, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 0.5 μM (each) oligonucleotide primers, 2.5 U of Ampli-Taq (Perkin-Elmer), and 1 mM $MgCl_2$. The reaction was carried out in an MJ Research Inc. PTC-100 programmable thermal controller. Initially, genomic DNA was denatured for 5 min at 94°C, and then 35 cycles of 45 s at 94°C, 45 s at 60°C, and 60 s at 72°C were used. The product was precipitated with ethanol, denatured in 0.2 M NaOH, passed through a Sepharose CL-6B column (Pharmacia) to eliminate oligonucleotides, and sequenced with the Tn*5* primer.

Spectra and respiratory activities. Spectra were determined with an Aminco/SLM DW2 UV/Vis spectrophotometer in the dual wavelength mode. The concentrations of cytochromes were calculated from the reduced-minus-oxidized room temperature spectra by using the following absorption coefficients and wavelength pairs: cytochrome aa_3-$E_{600-615}$, 11.7 mM^{-1} cm^{-1}; cytochrome *b*-$E_{560-575}$, 17.7 mM^{-1} cm^{-1}; cytochrome *c*-$E_{550-535}$, 14.3 mM^{-1} cm^{-1}; and cytochrome *d*-$E_{628-648}$, 18.8 mM^{-1} cm^{-1}.

For spectra, bacteria were cultured in 500 ml of Y minimal medium containing 0.5% (wt/vol) mannitol as the carbon source. After 4 days of growth at 28°C, cells were harvested, washed once with 25 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 7.2), and then suspended in 5 ml of the same buffer.

Spectra of membranes and soluble fractions were determined at a low temperature (77 K). The pellet from 2 liters of cells was washed with 25 mM TES buffer (pH 7.2), resuspended in 10 ml of the same buffer, and passed twice through a French pressure cell. Unbroken cells and cell debris were removed by centrifugation at 5,000 × *g* for 10 min, and membranes were separated from the soluble fraction by ultracentrifugation at 120,000 × *g* for 2 h and resuspended in 25 mM TES (pH 7.2). For low-temperature spectra, glycerol was added to the samples to give a final concentration of 50% (vol/vol).

For photodissociation spectra, the dithionite-reduced cells in 50% (vol/vol) ethylene glycol were bubbled with CO for 1 min, cooled to −20°C for 5 min, and then cooled to −78°C for 10 min in the dark before equilibration at −100°C in the sample compartment of a Johnson Foundation DBS-3 spectrophotometer. The sample was scanned twice to generate a baseline (reduced-plus-CO reading minus reduced-plus-CO reading) and photolysed for 1 min by using a focused 200-W light beam. The spectra shown in Fig. 2 are the difference between the CO-dissociated sample and the reduced-plus-CO sample.

For the determination of TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) oxidase activities, cells were harvested after 48 h of growth at 28°C in Y medium, resuspended in 100 ml of Y minimal medium with no carbon source, and shaken for 48 h to starve the cells. The cells were collected and suspended in 25 mM TES buffer (pH 7.2), and oxygen uptake was measured with a Hansatech oxygen electrode. Whole-cell protein was measured by using cells lysed by sonication.

Cell fractionation, protein gel electrophoresis, and heme staining. *R. leguminosarum* cells were fractionated as described previously (4, 30). Membranes were suspended in 25 mM TES buffer (pH 7.2; Sigma), and the soluble fraction was concentrated with Amicon Centriprep 10 and Centricon 10 filters. Protein concentrations were estimated by using bovine serum albumin as a standard. Membrane and soluble fractions were suspended in loading buffer (124 mM Tris [pH 7.0], 20% glycerol, 4.6% sodium dodecyl sulfate [SDS]) and electrophoresed in SDS-polyacrylamide (10%) gels at room temperature. Proteins were transferred to nitrocellulose filters (9) and stained for heme-dependent peroxidase activity by using chemiluminescence as described previously (43).

Nucleotide sequence accession number. The nucleotide sequence of *R. leguminosarum cycY* has been submitted to the EMBL data base and assigned accession number X79307.

RESULTS

Isolation of a Fix^- respiration-deficient mutant. Approximately 15,000 colonies of Tn*5*-mutagenized *R. leguminosarum* 8401 were screened with the Nadi stain. Several mutants that did not stain (or stained very poorly) were identified. Two mutants, A313 and A319, were quite distinctive in that they grew slowly on both TY and Y media. A cosmid library of *R. leguminosarum* DNA in *E. coli* was transferred into A313 and A319, and transconjugants were screened for complementation of the $Nadi^-$ phenotype. Clones were isolated from five $Nadi^+$ transconjugants of both A313 and A319, and the cloned DNA was analyzed. Identical *Eco*RI fragments were found in each clone; one cosmid (named pIJ1939) was chosen as a representative. DNA from A313 and A319, digested with *Eco*RI, *Hin*dIII, or *Bam*HI, was hybridized with pIJ1939 (or fragments from it). The patterns of hybridizing bands of both mutants were identical and compatible with a single Tn*5* insertion, showing that the two mutants were probably siblings. A319 was selected for further analysis. When pIJ1939 was transferred to other $Nadi^-$ mutants, none was complemented

TABLE 2. TMPD oxidase activities and cytochrome levels in A34 and the mutant A257

Strain	TMPD oxidase activity[a]	Concn[b] of cytochrome:			
		b	*c*	aa_3	*d*
A34	106	0.21	0.31	0.036	0.013
A257	7	0.24	ND[c]	ND	0.070

[a] Activities are reported as nanogram-atoms of oxygen consumed minute^{-1} milligram of protein^{-1}.
[b] Cytochrome concentrations (nanomoles milligram of protein^{-1}) in the membrane fractions were determined from room temperature reduced-minus-oxidized difference spectra.
[c] ND, not detectable.

and therefore mutant A319 was the only representative of this complementation group. The *R. leguminosarum* phage RL38 was plated on A319 and used to transduce A34 (which can normally nodulate legumes because of the presence of the symbiotic plasmid pRLIJI) to kanamycin resistance. All of the transductants inherited the Nadi$^-$ phenotype, showing that Tn*5* had caused the mutation. The pattern of DNA hybridization of one of the transductants (A257) was confirmed to be identical to that of A319. A257 formed nodules that were unable to fix nitrogen (Fix$^-$), as judged from measurements of acetylene reduction (<1% of normal), the small white nodules formed, and the poor growth of the plants. Light microscopy revealed that the nodules contained many infected cells (data not shown), indicating that normal infection had occurred and therefore the mutation had blocked nitrogen fixation by the bacteroids.

Biochemical characterization. The level of cytochrome oxidase activity of the mutant A257 was measured by TMPD oxidation. The O_2 uptake rate was less than 10% of that seen with the control strain A34 (Table 2).

Whereas the control strain (A34) had cytochrome absorption peaks at 554 and 603 nm, corresponding to *c*-type cytochromes and cytochrome aa_3, respectively (Fig. 1a), these peaks were absent from A257 (Fig. 1b). Instead, there were a smaller broad peak at 561 nm, which corresponds to *b*-type cytochromes and cytochrome *o*, and a slightly enhanced peak at about 630 nm (followed by a trough at 650 nm) which corresponds to cytochrome *d*. The estimated cytochrome concentrations (Table 2) revealed a level of *b*-type cytochrome content in A257 similar to that of the control, whereas the concentration of cytochrome *d* is higher than that in the control strain. At a low temperature (77 K), membranes from the control strain (Fig. 1c) showed distinct absorption peaks at 551 and 559 nm corresponding to *c*- and *b*-type cytochromes, respectively, a peak at 598 nm corresponding to cytochrome aa_3, a peak-trough system at 626 and 647 nm corresponding to cytochrome *d*, and a peak at 586 nm which is due to the cytochrome *b* component of the cytochrome *bd* complex. With membranes of the mutant A257 (Fig. 1d), the major peak of cytochrome *c* at 551 nm was completely absent, the peak at 559 nm was replaced with a peak at 556 nm and a shoulder at 562 nm, and the cytochrome aa_3 peak at 598 nm was absent. The peak at 592 nm is probably due to the cytochrome *b* component of the cytochrome *bd* complex; and the cytochrome *d* peak-trough system at 626 and 647 nm was normal.

The absence of cytochrome aa_3 from the mutant was confirmed by measuring a CO difference spectrum and a CO photodissociation spectrum. The reduced forms of cytochrome a_3 and cytochrome *o* can bind CO, and the peak at 420 nm and trough at 440 nm in the spectrum of wild-type A34 (Fig. 2a) probably reflect a mixture of cytochromes a_3 and *o*. With the

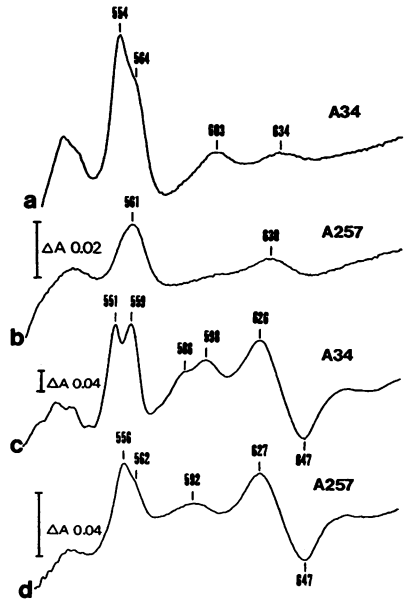

FIG. 1. Reduced (dithionite)-minus-oxidized (ferricyanide) difference spectra of strains A34. Spectra of whole cells of A34 (a) and the mutant A257 (b) were recorded at room temperature. The cell protein concentrations were 16.3 and 7.6 μg/ml for A34 and A257, respectively. Spectra of inner membrane preparations (c and d) were recorded at 77 K. The membrane protein concentrations were 32 mg ml^{-1} with A34 and 12 mg ml^{-1} with A257. The absorption maxima of different peaks are indicated in nanometers, and the absorption sensitivities are shown as optical density units (ΔA).

mutant A257 (Fig. 2b), the peak at 415 nm and trough at 430 nm are characteristic of cytochrome *o*, while the shoulder at 440 nm could be due to cytochrome a_3 or a high-spin cytochrome *b* (e.g., from a cytochrome *bd* complex or a peroxidase). However, whereas the photodissociation spectrum of the wild type shows a peak at 446 nm (Fig. 2c) consistent with the presence of cytochrome a_3, the mutant has a peak at 434 and not at 446 nm (Fig. 2d). This would be consistent with the presence of cytochrome *o* but not cytochrome a_3 in the mutant.

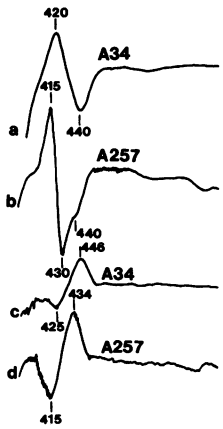

FIG. 2. Carbon monoxide and photodissociation spectra. Reduced (dithionite)-plus-CO minus reduced (dithionite) spectra of cells of A34 (a) and A257 (b) were measured at room temperature. Photodissociation spectra (postphotolysis minus prephotolysis) of A34 (c) and A257 (d) cells were also measured.

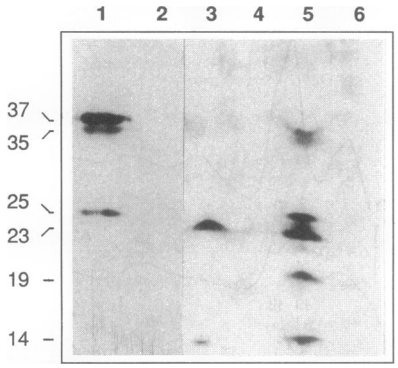

FIG. 3. Heme stain. Proteins from strains A34 (lanes 1, 3, and 5) or A257 (lanes 2, 4, and 6) were used. Lanes 1 and 2 contain 250 μg of membrane proteins, lanes 3 and 4 contain 400 μg of periplasmic proteins, and lanes 5 and 6 contain 400 μg of the cytoplasmic-plus-periplasmic fraction. Covalently bound heme-containing proteins were detected by chemiluminescence techniques (43).

In this case, the high-spin cytochrome *b* component does not contribute to the spectrum since the CO-cytochrome *b* complex does not photodissociate.

The reduced-minus-oxidized absorption peaks from the soluble fractions of A34 and A257 were also compared. Whereas there was a single absorption peak at 555 nm in the soluble fraction of A34, the corresponding *c*-type cytochrome was completely absent from A257. It appears that A257 lacks all *c*-type cytochrome absorption peaks, and the simultaneous loss of cytochrome aa_3 indicated that the entire branch of the electron transport chain including cytochromes bc_1, *c*, and aa_3 appears to be absent.

Mutant A257 lacks all *c*-type cytochromes. The covalently bound heme proteins were analyzed by heme staining of protein samples from the mutant A257 and the control strain A34. Spheroplasts were prepared and pelleted by centrifugation, the supernatant from this step containing primarily periplasmic proteins. The spheroplasts were sonicated, an inner membrane preparation was pelleted by centrifugation, and the supernatant (containing a mixture of cytoplasmic and periplasmic proteins) was collected. Proteins from the different fractions, separated on an SDS-polyacrylamide gel, were transferred to nitrocellulose and stained for heme proteins. Several different heme staining bands were identified in the fractions of the control strain A34 (Fig. 3). They correspond to proteins with covalently bound heme groups, since under the conditions used, noncovalently bound *a*, *b*, and *d* heme moieties would have been lost (43). No staining was seen with any fractions from A257 (Fig. 3). Therefore, although from these observations we cannot be sure of the identities of all the stained proteins, it is clear that they are all absent from A257, indicating that the mutation in A257 does not affect a specific

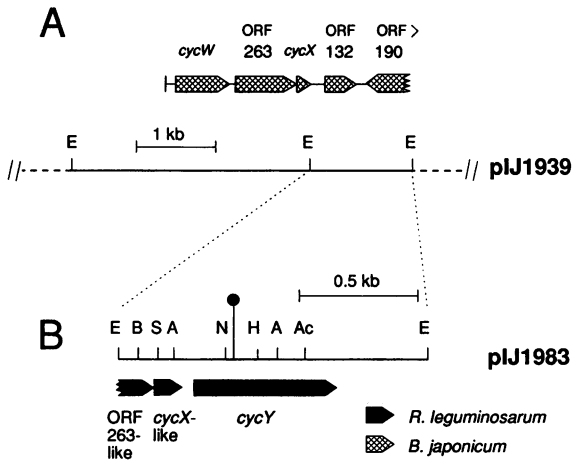

FIG. 4. Physical and genetic map of the *R. leguminosarum cycY* gene region. The gene sizes and orientations based on the DNA sequence and the *B. japonicum* genes described previously (35) are indicated (arrows), and homologous genes are present in *R. capsulatus* (5, 6). (A) Two *Eco*RI fragments of 3.0 and 1.3 kb from pIJ1939, both of which hybridize to probes carrying the *B. japonicum cyc* gene cluster (data not shown). (B) Open reading frames found in the 1.3-kb *Eco*RI fragment. The location of the Tn*5* insertion in mutant A257 is indicated (●). The restriction endonuclease sites are *Eco*RI (E), *Bgl*II (B), *Sph*I (S), *Ava*II (A), *Nco*I (N), *Hin*cII (H), and *Acc*I (Ac).

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GAATTCCTGCGCCCGCTGTTCATCATGGCGATCGCCTTCACCCTGCTTTTCTTCACGCTG 60
E F L R P L F I M A I A F T L L F F T L

CACATCATGGCGATGAGGAACGAGATCTGGCGCCGCCGCATTGCCGCCCAGCGCCGTCTT 120
H I M A M R N E I W R R R I A A Q R R L

GCCGCCCGCATGGCGAGCCGGGAGGAATAGCCGTGACGCATGCCTTCTACGCCTACGCCT 180
A A R M A S R E E *CycX V T H A F Y A Y A S

CCTATGGCTTCGCGGCCCTGGTGACGATCGCCGTCACCGTCTGGACCTGGGCCGACGCGC 240
  Y G F A A L V T I A V T V W T W A D A R

GCCGTCGCCGGGAACTTGCAGCCCTTGAGGCCGCCGGTATCCGCCGCGCTCGGCCGCGCG 300
  R R R E L A A L E A A G I R R A R P R A

CAAGGGACGGCGAATGAACGACACACCGGAAAAGACGACCGACAAACCGCGGGCTGGGTC 360
  R D G E *                CycY L G R

GCTACACCTTGGCGCTTCTGCCGCTGATCGTCTTCGGCGGCATTGCGCACGGCGCAAAAA 420
  Y T L A L L P L I V F G G I A H G A K M

TGCTCTACGACCAGGATTTCCATGGCAAGAACATTGCCGAAATTCCCTCCGCCCTGAGTC 480
  L Y D Q D F H G K N I A E I P S A L S H

ACCAAGGCGCCGACGCTGAACCTGCCGCCGCTCGACGCGCCACACTGCCGGCGCTGACCG 540
  Q G A D A E P A A A R R A T L P A L T D

ACGCGGCAATCAAGGGCAAGCTGACCCTCGTCAACGTCTTCGCCTCATGGTGTCTCCCCT 600
  A A I K G K L T L V N V F A S W C L P C

GCCGGGACGAACATCCGGTGTTGAAGGAACTGGCGAAAGACGGACGGCTGAACATCGTCG 660
  R D E H P V L K E L A K D G R L N I V A

CCATCAATTACAAGGACCAGAGCGACAACGCCCTGCGTTTCCTCGGCGAACTCGGCAACC 720
  I N Y K D Q S D N A L R F L G E L G N P

CCTATCAGGCGATCGGCATCGATCCGAACGGCAAGGCGGCAATCGACTGGGGCGTCTACG 780
  Y Q A I G I D P N G K A A I D W G V Y G

GCATTCCGGAAAGCTATCTGGTCGGCGCAGACGGCACGATCCTCTACAAGCGCGTCGGCC 840
  I P E S Y L V G A D G T I L Y K R V G P

CCTCGACGAATATCAGCCTGAAGGAAGGGCTGGTTCCAGCGATGGAAAAGGCGCTCGGCA 900
  S T N I S L K E G L V P A M E K A L G K

AGCCGGTTTCGTAGCGATTACTCCACAAAGCCCCCTCCCCAACCCCTCCACAAGGGGGAT 960
  P V S *

GGACTAACTCGTGGCCCGCATCGAGCAAATATCAGCCTCTCTGCGGATGAAACGATGAAA 1020
TGAAAAGCGTCGGAGCGGCATCTAAGCCCTCCCCTTGTGAGAGGGGTCGGGGAGGGTTTA 1080
TCGGGACAAAGAAGCGCTAAAGCCCGCTCTGCCAGACCTTGATCGCCTCCACCGGCCAGA 1140
TCAGCATGACAACGTTGAGCGTCAGGTTGTCGCGGATCATATAGCCGGTGAAAATCTCGA 1200
AGAAGATGCGATCGCCACCGTCAGTGCCACCGGGCCGCCGCGCGAAGAAGAAGCCGATGC 1260
ACATGAAGACGGTGTCCATCGCCGAATTC 1289
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FIG. 5. Nucleotide sequence of the 1,289-bp *Eco*RI fragment carrying *cycY*. The DNA and predicted amino acid sequences of *cycY* are shown. The translated sequence from nucleotides 1 to 150 is homologous to ORF263 (35) from *B. japonicum* and to HelC (6) from *R. capsulatus*. The protein sequence encoded by nucleotides 153 to 314 is homologous to CycX from *B. japonicum* and to HelD from *R. capsulatus* and is indicated as CycX. The predicted protein sequence (CycY) encoded by nucleotides 353 to 911 is homologous to ORF132 from *B. japonicum* (35) and to HelX from *R. capsulatus* (5). It contains a potential N-terminal transit sequence followed by a possible cleavage site (open arrow). The underlined sequence is complementary to the 18-nucleotide oligomer used in the PCR to identify the Tn*5*-insertion site (solid arrow).

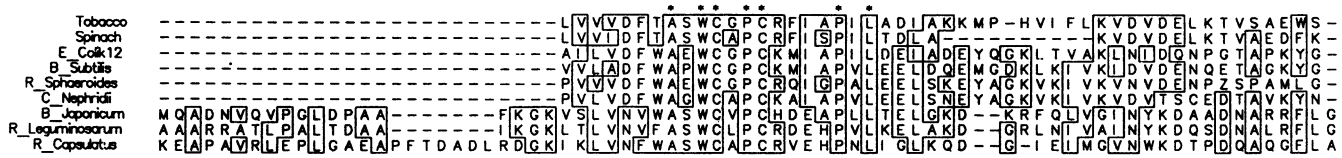

FIG. 6. Sequence comparison of part of the deduced amino acid sequence of CycY with *B. japonicum* ORF132, *R. capsulatus* HelX, and bacterial and eukaryotic thioredoxins. The sequences (with the amino acid residue numbers shown in parentheses) are as follows: thioredoxin from tobacco (residues 36 to 84) (29), thioredoxin *h* from spinach (residues 1 to 40) (27), TrxA from *E. coli* K-12 (residues 22 to 71) (24), ThrA from *Bacillus subtilis* (residues 19 to 65) (11), thioredoxin from *Rhodobacter sphaeroides* Y (20 to 69) (12), thioredoxin C-2 from *Corynebacterium nephridii* (23 to 72) (31), ORF312 from *B. japonicum* (residues 1 to 68) (35), CycY from *R. leguminosarum* (residues 51 to 118), and HelX from *R. capsulatus* (residues 39 to 112) (5). Conserved identical residues are boxed, and the asterisks indicate the identical residues present in all the sequences. The overall identities of the thioredoxins to CycY are estimated to be tobacco, 21%; spinach, 37%; *E. coli*, 31%; *B. subtilis*, 37%; *R. sphaeroides*, 33%; *C. nephridii*, 28%; *B. japonicum*, 58%; and *R. capsulatus*, 40%.

c-type cytochrome but appears to block the maturation of all of the *c*-type cytochromes.

DNA sequence of the gene mutated in A257. DNA hybridization revealed that the Tn*5* in A257 was inserted into an *Eco*RI fragment of 1.3 kb (Fig. 4). This fragment (in pIJ1983) could complement the Nadi$^-$ phenotype of A257. The DNA sequence of the fragment and the predicted open reading frames are shown in Fig. 5. An oligonucleotide primer (Fig. 5) complementary to the 3′ end of the sequence was used together with a primer from the end of Tn*5* to generate an amplified fragment by the PCR by using genomic DNA from the mutant A257. A fragment of 0.8 kb was generated, and the DNA sequence of the junction with Tn*5* revealed that the Tn*5* in mutant A257 is inserted at nucleotide 476 (solid arrow in Fig. 5), within the largest of the three open reading frames found. The predicted protein sequence of this open reading frame was used to search a translation of the EMBL sequence data base. Strong homology was found to several proteins, and partial protein alignments are shown (Fig. 6) for some of the similar proteins. The strongest homology was to ORF132, a predicted protein encoded by a gene within a *B. japonicum* gene cluster containing other genes (Fig. 4), including *cycV*, *cycW*, and *cycX*, shown to be involved in cytochrome *c* biogenesis (35). Following the nomenclature used with *B. japonicum*, we have called this gene *cycY* (Fig. 4 and 5). There was also very strong similarity between CycY and the predicted product of the *R. capsulatus* gene *helX*, which is also involved in cytochrome *c* biogenesis. HelX contains an N-terminal transit signal sequence and is located in the periplasm (5). A good potential transit sequence is also present at the predicted N terminus of the *R. leguminosarum cycY* gene product; the probable signal cleavage site is shown with an open arrow in Fig. 5. The *B. japonicum* ORF132 sequence is significantly shorter than CycY or HelX, but upstream of ORF132 there is an alternative potential upstream translation start (35) which aligns close to the potential start for CycY used here.

The short reading frame encoded by nucleotides 153 to 314 shows significant homology to CycX (36% identity) from *B. japonicum* (35) and to HelD (28% identity) from *R. capsulatus* (5, 6). On the basis of the sequence homology and following the nomenclature used with *B. japonicum*, we have called this gene *cycX* (Fig. 4 and 5). The protein sequence encoded by the partial open reading frame extending from nucleotides 1 to 147 is homologous to the C-terminal regions of HelC (34% identity) from *R. capsulatus* and ORF263 (63% identity) in the *cyc* gene cluster from *B. japonicum*.

The *helA*, *helB*, *helC*, *helD*, and *helX* genes are all involved in cytochrome *c* biogenesis in *R. capsulatus* (5, 6), and a similar gene cluster encoding CycV, CycW, ORF263, CycX, and ORF132 is present in *B. japonicum* (35). Mutations in *cycV*, *cycW*, and *cycX* affect cytochrome *c* biogenesis in *B. japonicum* (35), but no mutation in ORF132 was identified. It is clear that mutation of CycY, the *R. leguminosarum* homolog of ORF132, blocks cytochrome *c* biogenesis, and it appears that a secondary effect is the loss of spectroscopically detectable cytochromes aa_3. It is evident that at least two of the genes upstream of *cycY* are homologous to equivalently positioned genes in both *B. japonicum* (Fig. 4) and *R. capsulatus* (5, 6).

DISCUSSION

An interesting question about the synthesis of cytochromes *c* in prokaryotes is how the heme is inserted. In *Paracoccus denitrificans*, the polypeptides for two *c*-type cytochromes, c_{550} and cd_1, occur in the periplasm of cells in which heme incorporation is blocked by either mutation or inhibition of heme synthesis (33). *R. capsulatus hel* mutants also transported apocytochrome c_2 without the heme attached (6). These findings are consistent with the idea that the polypeptide can be translocated to the periplasm before the incorporation of the heme.

The products encoded by *B. japonicum* ORF132 (35), *R. capsulatus helX* (5), and *R. leguminosarum cycY* (this study) are homologous to bacterial and eukaryotic thioredoxins, and the strongest conservation is found around the active site region C-X-P-C (Fig. 6). Thioredoxins are small ubiquitous proteins containing an active site with a redox-active disulfide. They function in electron transfer via the reversible oxidation of two vicinal protein-SH groups to a disulfide bridge. They are hydrogen donors for various reductive enzymes, such as ribonucleotide reductase, protein disulfide oxidoreductases, photosynthetic regulatory factors, one subunit of bacteriophage T7 DNA polymerase, essential components for the assembly of small viruses, and possibly protein disulfide isomerases (17). On the basis of these similarities, we infer that the CycY protein is probably involved in reduction of thiol groups necessary either for heme *c* synthesis or for its insertion into cytochrome *c* apoprotein(s). However CycY, HelX, and ORF132 appear to be a distinct group of thioredoxin-like proteins, since they are more homologous to each other than to the other thioredoxins. In addition, CycY and HelX show extended homology at the N terminus, including a typical membrane-transit signal sequence, and a possible signal sequence may be present in the translation of the DNA sequence upstream of ORF132 in *B. japonicum* (35). Thus, it is likely that these rhizobial gene products are both, like HelX (5), periplasmically located thioredoxin-like proteins involved in cytochrome *c* biogenesis. A different thioredoxin-like protein is

involved in the biogenesis of cytochrome aa_3 in *B. japonicum* (25).

The precise role of the *cycY* gene product in the biogenesis of *c*-type cytochromes is not known. The heme group is not attached to the apocytochrome in the mutant A257. At least two separate steps in cytochrome *c* biosynthesis could involve a thioredoxin function. The first could be the heme attachment. The sulfhydryl groups of the apoprotein must be reduced for heme attachment (13). Thus, CycY could maintain the SH groups of apocytochrome(s) *c* in the reduced state.

Data from *B. japonicum* (34, 35), *R. capsulatus* (5, 6, 20), and *R. leguminosarum* (this study) suggest that, at least among gram-negative bacteria, there is a very well-conserved system involved in the biosynthesis of *c*-type cytochromes. The common feature of all *cyc* or *hel* mutants is the absolute lack of all holocytochromes *c*. Interestingly, the electron transport pathways requiring *c*-type cytochromes are essential for specialized functions during part of the life cycle of these bacteria: *R. capsulatus hel* mutants cannot grow anerobically in the light (20), *B. japonicum cyc* mutants have defects in symbiotic nitrogen fixation and H_2 oxidation (34), and the *R. leguminosarum* mutant described here is affected in its aerobic growth and is also Fix^-. The inability to fix nitrogen does not result from abnormal infection, since bacteroids were present, although interestingly, a cytochrome *c*-deficient mutant of *R. phaseoli* formed empty nodules on *Phaseolus* beans (39).

The inability of the mutant A257 to fix nitrogen during symbiosis indicates that *c*-type cytochromes are necessary for electron transport during symbiotic nitrogen fixation, even though other electron pathways to oxygen via cytochromes *o* and *d* are functional. It has been proposed that a special cytochrome oxidase with high affinity for oxygen is required by bacteroids (2). Thus, A257 may be unable to form a *c*-type cytochrome (or cytochromes) that participates in such a pathway. In *B. japonicum* (41) and *R. leguminosarum* (data not shown), mutations affecting the cytochrome bc_1 complex also cause a Fix^- phenotype. Thus, the cytochrome bc_1 complex is essential for the symbiosis of *R. leguminosarum* and therefore we conclude that the Fix^- phenotype of mutant A257 results from the lack of holocytochrome c_1 and hence of the bc_1 complex.

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