JOURNAL OF BACTERIOLOGY, Apr. 1995, p. 2214–2217 Vol. 177, No. 8
0021-9193/95/$04.00+0

Bradyrhizobium japonicum Cytochrome c_{550} Is Required for Nitrate Respiration but Not for Symbiotic Nitrogen Fixation

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Received 8 December 1994/Accepted 8 February 1995

Bradyrhizobium japonicum **possesses three soluble *c*-type cytochromes, c_{550}, c_{552}, and c_{555}. The genes for cytochromes c_{552} (*cycB*) and c_{555} (*cycC*) were characterized previously. Here we report the cloning, sequencing, and mutational analysis of the cytochrome c_{550} gene (*cycA*). A *B. japonicum* mutant with an insertion in *cycA* failed to synthesize a 12-kDa *c*-type cytochrome. This protein was detectable in the *cycA* mutant complemented with cloned *cycA*, which proves that it is the *cycA* gene product. The *cycA* mutant, a *cycB-cycC* double mutant, and a *cycA-cycB-cycC* triple mutant elicited N_2-fixing root nodules on soybean (Nod^+ Fix^+ phenotype); hence, none of these three cytochromes *c* is essential for respiration supporting symbiotic N_2 fixation. However, cytochrome c_{550}, in contrast to cytochromes c_{552} and c_{555}, was shown to be essential for anaerobic growth of *B. japonicum*, using nitrate as the terminal electron acceptor.**

Several different soluble and membrane-bound *c*-type cytochromes were identified previously in the soybean root nodule bacterium *Bradyrhizobium japonicum*. The cytochrome c_1 and CycM proteins form part of a mitochondria-like electron transport chain in aerobically grown cells (2 [H] $\rightarrow$ Q $\rightarrow$ FeS/bc_1 $\rightarrow$ CycM $\rightarrow$ aa_3 $\rightarrow$ O_2) in which cytochrome c_1 is a subunit of the membrane-bound ubiquinol-cytochrome *c* oxidoreductase (also called cytochrome bc_1 complex) and CycM is a membrane-anchored cytochrome *c* transferring electrons from the bc_1 complex to the aa_3-type terminal oxidase (5, 25). An alternative electron transport pathway branching off at the bc_1 complex has been suggested to operate in the microaerobic, N_2-fixing endosymbiotic bacteroids of soybean root nodules (10, 17, 25). This branch terminates with a novel *cb*-type heme-copper oxidase whose subunits are encoded by the *fixNOQP* operon and in which the FixO and FixP proteins are membrane-anchored mono- and diheme cytochromes *c* (17). Finally, *c*-type cytochromes obviously play a role in denitrification, because *B. japonicum* mutants with lesions in the cytochrome bc_1 genes (*fbcFH*) or in genes for cytochrome *c* biogenesis (*cycVWX* and *cycHJKL*) are unable to grow with nitrate as the terminal electron acceptor (19, 21, 22, 25).

This report is concerned with cytochrome c_{550} which is one of three soluble, low-molecular-weight *c*-type cytochromes isolated previously from cultured cells or from bacteroids of *B. japonicum* (4, 26). Cytochrome c_{550} was characterized as a non-CO-reactive redox protein with a midpoint potential of +0.28 V and an apparent molecular mass of approximately 12 kDa (4, 26). The cytochrome c_{550} structural gene has not been found and characterized thus far, whereas the genes for the other two soluble *c*-type cytochromes, *cycB* for c_{552} and *cycC* for c_{555}, were cloned and sequenced (23, 26). The search for phenotypes caused by mutations in *cycB* and *cycC* did not reveal possible functions of cytochromes c_{552} and c_{555}. The *B. japonicum cycB* mutant was unaffected in anaerobic growth with nitrate (23); the *cycC* mutant was not tested for that phenotype (26). Both mutants and the wild type had identical symbiotic nitrogen fixation activities, suggesting that neither cytochrome c_{552} nor cytochrome c_{555} functions as a component of the bacteroid-specific respiratory chain (23, 26). This left cytochrome c_{550} as a candidate for such a function. The aim of this study was, therefore, to identify the cytochrome c_{550} gene, create a mutation in it, and test the phenotypes of a corresponding mutant. Furthermore, the construction of a triple mutant affecting all three cytochrome *c* genes was of interest, in order to test the previously raised hypothesis (23) that some of the soluble cytochromes *c* might functionally substitute for each other and thereby obscure potential phenotypes in single mutants.

Cytochrome c_{550} was purified from soybean root nodule bacteroids of *B. japonicum* CC705 (4). When subjected to Edman degradation in a protein sequencer (Applied Biosystems model 471A), the amino terminus was found to be blocked. Therefore, tryptic fragments were isolated and analyzed, yielding the following four peptide sequences: peptide 1, KSGTVEGYSYTDANK (a peptide without the N-terminal Lys was also obtained); peptide 2, NSGITWDEAVFK; peptide 3, MAFAGIK; and peptide 4, NETEIXXLXAYVAD FDK. A search for homologous sequences in the protein sequence database revealed a substantial degree of similarity between all four peptides and corresponding stretches in cytochrome c_{550} of *Nitrobacter winogradskyi* (24), a bacterium incidentally known to be a close phylogenetic relative of *B. japonicum* (11). An alignment of the peptides to the latter protein (not shown) allowed us to tentatively conclude that peptides 1 and 2 as well as peptides 3 and 4 are adjacent in *B. japonicum* cytochrome c_{550}: (1 + 2) KSGTVEGYSYTD ANKNSGITWDEAVFK and (3 + 4) MAFAGIKNETEIXX LXAYVADFDK. The underlined amino acids were found to be suitable for the design of two degenerate oligonucleotides

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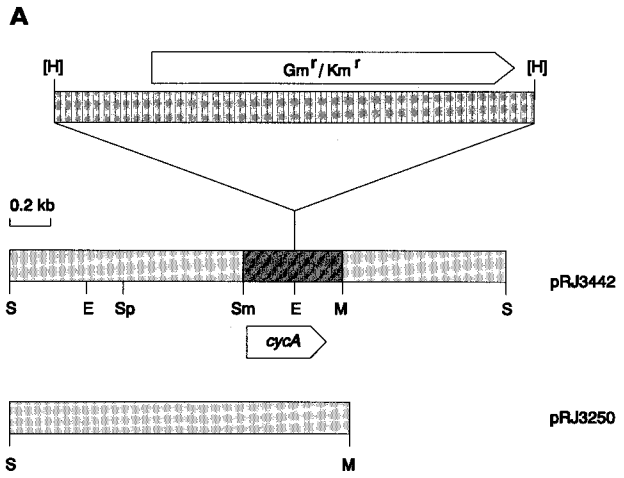
(a 27-mer and a 39-mer) by taking into account the characteristic *B. japonicum* codon usage (18): 5′-ATCACG(T/C)GG GAC(G/T)AGGCG(G/C)TG(T/C)TCAAG-3′ and 5′-AA GATGGC(G/C)TTCGC(G/C)GG(C/I)ATCAAGAA(C/ T)GAGAC(G/C)GAGATC-3′. The radioactively labelled 27-mer and 39-mer oligonucleotides produced a similar pattern of hybridizing bands when used as probes in two separate Southern blot hybridizations with restriction enzyme-digested *B. japonicum* 110 DNA (data not shown). We used strain 110 as the DNA source rather than strain CC705 (the original source of cytochrome c_{550}) because strain 110 is now the generally accepted reference strain in genetic work with *B. japonicum*. *Hin*dIII-digested DNA of the hybridizing 6.0-kb region was isolated from a preparative agarose gel, and the fragments were cloned in *Escherichia coli*, using vector pUC19. White colonies on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates were screened by colony hybridization, again using the 27-mer and 39-mer oligonucleotides as probes in two separate experiments. Three colonies that hybridized with both probes were found, and plasmid DNA isolated from these clones had an identical restriction pattern (not shown). An internal 2.5-kb *Sal*I fragment thereof was then cloned in vector pKS+, resulting in plasmid pRJ3442 (Fig. 1A).

The nucleotide sequence between the *Sma*I and *Mlu*I sites (Fig. 1A) was established on both DNA strands. We found an open reading frame of 136 codons and named it *cycA*. The derived *cycA* gene product (Fig. 1B) showed all of the typical characteristics of a cytochrome *c* pre-apoprotein (16): it had a hydrophobic, N-terminal 28-amino-acid signal sequence with a putative signal peptidase recognition site (Ala-Met-Ala) between positions −3 and −1; a 108-amino-acid cytochrome *c* apoprotein with the conserved protoheme IX binding site (Cys-Leu-Ala-Cys-His) between positions 13 and 17; and a methionine, the sixth ligand for the heme iron, at position 79. The predicted molecular weight of the CycA apoprotein is 11,703, and that of the holoprotein (including covalently bound ferroprotoheme IX) is 12,319.5. The following positional amino acid sequence identities between the CycA protein and soluble cytochromes *c* of other organisms were found: *N. winogradskyi* cytochrome c_{550} (24), 72%; *Rhodopseudomonas viridis* cytochrome c_2 (9), 65%; *Rhodopseudomonas acidophila* cytochrome c_2 (1), 61%; and mitochondrial cytochrome *c* (15), 50 to 55%. Figure 1B also shows a comparison between the *B. japonicum* 110 CycA sequence and the four peptides isolated and sequenced from cytochrome c_{550} of *B. japonicum* CC705. Of the 51 sequenced amino acids of strain CC705 cytochrome c_{550}, 9 were different in strain 110 CycA (~17% difference). A similar difference (~16%) was observed previously between cytochrome c_{552} of strain CC705 and the corresponding CycB protein of strain 110 (23). Most likely this reflects the fact that the two strains belong to two phylogenetically quite divergent *B. japonicum* homology groups (12, 13). All in all, the sequence analyses show that *cycA* is the c_{550} structural gene, even more so as it shares little similarity with the other two *B. japonicum* genes, *cycB* and *cycC*, encoding soluble *c*-type cytochromes (23, 26).

To construct a *B. japonicum cycA* null mutant, the *cycA* gene cloned in pRJ3442 was first disrupted by insertion of a gentamycin-kanamycin resistance cassette from Tn*5-233* (7) into the *cycA*-internal *Eco*RI site involving fill-in reactions and blunt-end ligation (Fig. 1), and the mutated gene was then introduced into the *B. japonicum* wild type for marker replacement resulting in the *cycA* mutant strain 3447. *B. japonicum* mutants with insertions in *cycB* (strain C3505) and *cycC* (strain BJ1004) were available from previous work (23, 26). A *cycB-cycC* double mutant was constructed by introducing the original



B

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-28          MTKLTFGALVALAMTAAASTAMSSKAMA          -1
                  *  **
  1  QDAAAGKTSFNKCLACHAIGEGAKNKVGPELNGLNG          36

 37  RKSGTAPDYSYSDANKNSGITWDEATFKEYIKDPKA          72
       KSGTVEGYSYTDANK(1)
                     NSGITWDEAVFK(2)
           *
 73  KIPGTKMAFAGIKNETEINNLWTFVSQFDKDGKIKQ  108
         MAFAGIK(3)
                NETEIXXLXAYVADFDK(4)
```

FIG. 1. The *cycA* gene and its product. (A) Restriction map of the *cycA* region cloned in plasmid pRJ3442. Dark shading indicates the segment that was sequenced. The insertion cassette used to disrupt the *cycA* gene at the *Eco*RI site is shown on top. The DNA fragment cloned in pRJ3250 (bottom) was used for complementation of the *cycA* mutation. Restriction sites: E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I. Gm^r/Km^r, gentamycin and kanamycin resistance. (B) Amino acid sequence of the CycA pre-apoprotein derived from the nucleotide sequence of the *cycA* open reading frame of *B. japonicum* 110 (boldface letters). The putative, N-terminal signal sequence is in italics. Amino acids involved in heme binding are marked by asterisks. The peptides (*1*) to (*4*) were obtained after tryptic digestion of cytochrome c_{550} from *B. japonicum* CC705.

cycC::*aph* mutation (from plasmid pRET51 [26]) into the *cycB* mutant strain C3505. This resulted in strain C3524 (*cycB*::Ω *cycC*::*aph*). Strain C3524 finally served as the recipient for the introduction of the aforementioned *cycA* mutation, which resulted in a *cycA-cycB-cycC* triple mutant (strain 3448). All mutants elicited fully developed, nitrogen-fixing root nodules on soybean plants (Nod^+ Fix^+ phenotype); hence, none of the soluble *c*-type cytochromes of *B. japonicum* is essential for symbiotic nitrogen fixation. This result has important implications regarding the nature of the electron donor for the symbiotically essential *cb*-type cytochrome oxidase encoded by the *fixNOQP* operon (17). It appears possible that this oxidase complex receives the electrons directly from the cytochrome bc_1 complex in vivo, in that one of the two membrane-bound *c*-type cytochromes, probably the diheme FixP protein, might transfer the electrons from the bc_1 complex to the core complex (FixNO) of the oxidase (27). However, the formal possibility that a new, hitherto unidentified membrane-bound cytochrome *c* fulfills the role of a mediator between the two complexes cannot be ruled out at present.

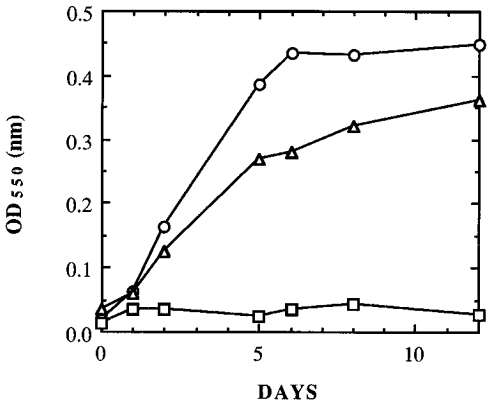

FIG. 2. Anaerobic growth with nitrate. Cells were grown in yeast extract-mannitol medium containing 10 mM KNO_3 (6). ○, *B. japonicum* wild type (strain 110*spc*4); □, *cycA* mutant (strain 3447); △, *cycA* mutant complemented with plasmid pRJ3250. OD_{550} (nm), optical density at 550 nm.

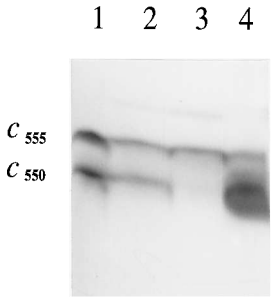

FIG. 3. Electrophoretic separation of soluble *c*-type cytochromes. The low-molecular-weight range of a sodium dodecyl sulfate-polyacrylamide gel stained with *o*-dianisidine for covalently bound heme (8) is shown. Lane 1 contains purified cytochromes c_{555} and c_{550} from bacteroids (4), used as a reference. The other tracks were loaded with 1 mg each of soluble crude-extract protein from aerobically grown wild type (lane 2), *cycA* mutant strain 3447 (lane 3), and *cycA* mutant complemented with pRJ3250 (lane 4). Cytochrome c_{552} is poorly expressed under aerobic growth conditions and is, therefore, not visible here.

The only phenotype observed with the *cycA* mutant was a defect in anaerobic growth, with nitrate as the electron acceptor (Fig. 2). Logically, the *cycA-cycB-cycC* triple mutant had the same phenotype. By contrast, the *cycB-cycC* double mutant did grow anaerobically with nitrate (not shown). The cytochrome c_{550}, therefore, seems to play a specific role in anaerobic respiration with nitrate, a function that apparently cannot be replaced by cytochrome c_{552} or cytochrome c_{555} in the *cycA* mutant. While an involvement of *c*-type cytochromes in nitrate respiration by *B. japonicum* has been suggested previously (19, 20, 25), the individual redox proteins in the various denitrification steps have not been characterized, so that a more specific assignment of the biochemical function of cytochrome c_{550} is currently not possible. Also, questions concerning the biochemical nature and the number of respiratory nitrate reductases present in *B. japonicum* (i.e., membrane-bound versus periplasmic forms) have not been addressed until very recently (14). Our attempts to determine whether the *cycA* mutant was able to grow anaerobically with nitrite or nitrous oxide instead of nitrate as the terminal electron acceptor failed because even the *B. japonicum* wild type could not be cultivated under these conditions.

An important control in the assessment of the phenotype caused by the *cycA* mutation was to exclude any possible polar effects of the insertion on genes potentially located immediately downstream of *cycA*. For this purpose we constructed a complementing plasmid (pRJ3250) (Fig. 1A) that carried a *cycA*-containing *Sal*I-*Mlu*I fragment with very little downstream DNA (77 bp after the *cycA* stop codon). Successful complementation of the *cycA* mutation in strain 3447 with pRJ3250 was shown (i) by the restoration of nitrate respiration (Fig. 2), and (ii) by the reappearance, and even overproduction, of a 12-kDa *c*-type cytochrome (Fig. 3, lane 4) that is normally present in smaller amounts in the wild type (Fig. 3, lane 2) and absent in the *cycA* mutant (Fig. 3, lane 3). These results confirm the notion that the nitrate respiration defect in strain 3447 is specifically due to the absence of the 12-kDa cytochrome c_{550} and that *cycA* is indeed the structural gene for that protein.

In conclusion, this study has eliminated a previous hypothesis, according to which at least one of the soluble cytochromes *c* of *B. japonicum* was thought to play a role in bacteroid respiration under conditions of oxygen limitation in soybean root nodules as a requirement for symbiotic nitrogen fixation (2, 3). While the functions of cytochrome c_{552} (23) and cytochrome c_{555} (26) still remain enigmatic, we present evidence for a role of cytochrome c_{550} in the anaerobic respiration of *B. japonicum* with nitrate. In this context it is somewhat puzzling, however, that cytochrome c_{550} is synthesized also in aerobically grown *B. japonicum* cells (4, 26) (Fig. 3).

Nucleotide sequence accession number. The nucleotide sequence for *cycA* was deposited in the GenBank database under accession number L39642.

We thank P. Kast for the synthesis of oligonucleotides and N. F. Totty for help with the amino acid sequencing.

This work was supported by a grant from the Swiss National Foundation for Scientific Research to H.H.

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