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Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis

(cytochrome *c*/electron transport/membrane proteins/respiration/root-nodule symbiosis)

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ABSTRACT **We report the discovery of a *Bradyrhizobium japonicum* gene cluster (*fixNOQP*) in which mutations resulted in defective soybean root-nodule bacteroid development and symbiotic nitrogen fixation. The predicted, DNA-derived protein sequences suggested that FixN is a heme *b* and copper-binding oxidase subunit, FixO a monoheme cytochrome *c*, FixQ a polypeptide of 54 amino acids, and FixP a diheme cytochrome *c* and that they are all membrane-bound. The isolation and analysis of membrane proteins from *B. japonicum* wild-type and mutant cells revealed two *c*-type cytochromes of 28 and 32 kDa as the likely products of the *fixO* and *fixP* genes and showed that both were synthesized only under oxygen-limited growth conditions. Furthermore, *fixN* insertion and *fixNO* deletion mutants grown microaerobically or anaerobically (with nitrate) exhibited a strong decrease in whole-cell oxidase activity as compared with the wild type. The data suggest that the *fixNOQP* gene products are induced at low oxygen concentrations and constitute a member of the bacterial heme/copper cytochrome oxidase superfamily. The described features are compatible with the postulate that this oxidase complex is specifically required to support bacterial respiration in endosymbiosis.**

Legume root and stem nodule bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* can live either free in the soil and in laboratory culture or endosymbiotically in infected host cells of the central nodule tissue, where they are capable of fixing N_2. The ambient O_2 concentrations in these habitats may differ by a factor of up to 10^5, ranging from $\approx$250 μM in O_2-saturated environments down to 3–30 nM in endosymbiosis (1, 2). One of the unsolved problems in the biology of this symbiosis is by which mechanism the aerobic rhizobia, while living as so-called bacteroids within the extremely microaerobic nodule compartment, generate sufficient ATP for their own maintenance and particularly for the energy-demanding N_2 fixation process (3). The usual way how aerobic bacteria cope with different O_2 regimes is by induction of different respiratory chains terminating with oxidases that have different affinities for O_2 (4). Whereas mitochondria have only one respiratory chain, aerobic bacteria are often found to have two or more electron transport chains branching off from the quinol pool (4). It is a long-standing hypothesis, therefore, that rhizobial bacteroids possess a symbiosis-specific terminal oxidase with a high affinity for O_2, so that O_2 transfer from oxygenated leghemoglobin in the nodule cell cytoplasm to the respiratory complex in the bacteroid cytoplasmic membrane is facilitated (3, 5–7).

The best-studied rhizobial species, as far as the biochemistry and molecular genetics of respiration are concerned, is *Bradyrhizobium japonicum*, the soybean symbiont. The respiratory chain in this bacterium is composed of at least three branches (8). Under aerobic growth conditions the electrons are channeled from the ubiquinol-10 pool (9) via the Rieske Fe-S protein/cytochrome bc_1 complex and a 20-kDa membrane-bound cytochrome *c* (CycM protein) to the cytochrome aa_3-type terminal oxidase, the site of O_2 reduction (8). The genes for the bc_1 complex (*fbcFH*), the CycM protein (*cycM*), and subunit I of aa_3 (*coxA*) have been identified and characterized (8, 10–12). Aerobic *B. japonicum* cells contain at least one additional oxidase, probably cytochrome *o* (6, 13). Genes for an alternative cytochrome *c* oxidase (*coxMNOP*) were described recently (14), but it is unclear whether they encode the cytochrome *o* complex. Insertion mutations in *cycM*, *coxA*, and *coxN* resulted in *B. japonicum* strains that formed fully effective, N_2-fixing soybean root nodules (Fix^+ phenotype; refs. 8 and 14); hence, the respiratory components encoded by these genes are not essential for symbiosis.

Little is known so far about the enigmatic bacteroid oxidase. Spectroscopic measurements of bacteroid extracts, however useful, have not led to a precise description of this oxidase (6, 13, 15, 16). It is clear that it differs from cytochromes aa_3 and *o*, because the latter two oxidases are not present in bacteroids of most *B. japonicum* strains (6, 13). *B. japonicum* mutants defective in the cytochrome bc_1 complex ($fbcF^-$ and $fbcH^-$) had a Fix^- phenotype (10). This implied that a symbiosis-specific electron transport chain branches off at the site of the bc_1 complex. By analogy with bc_1-dependent respiratory chains in other bacteria, this branch ought to consist of a *c*-type cytochrome and a heme/copper-containing cytochrome oxidase. Three soluble *c*-type cytochromes (c_{550}, c_{552}, c_{555}) that were previously thought to be symbiotically relevant (17) are probably not part of this branch, because mutations in their respective genes (*cycA*, *cycB*, *cycC*) yielded strains with a Fix^+ phenotype (refs. 18 and 19; M. Bott and H.H., unpublished data).

In this paper we present evidence that the symbiotically essential *fixNOQP* gene cluster of *B. japonicum* encodes a membrane-bound, cytochrome *c*-containing heme/copper oxidase which is specifically induced upon O_2 limitation. We postulate that this might be the bacteroid oxidase.†

MATERIALS AND METHODS

***B. japonicum* Strains and Growth.** *B. japonicum* strain 110*spc*4 (20) is called the wild type throughout this paper. Mutant derivatives of it were created by marker-replacement

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; ORF, open reading frame.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L07487).

mutagenesis using Tn*5* or *aphII* cassette insertions (21, 22). Cells were grown aerobically in peptone/salts/yeast extract (PSY) medium at 28°C (20). The same medium was used for microaerobic growth in closed 1-liter flasks with half the volume as culture and the other half as N_2 gas phase containing 0.5% O_2 that was replaced every 24 hr. Anaerobic growth occurred in yeast extract/mannitol (YEM) medium (23) plus 10 mM KNO_3. Cells were harvested in the exponential growth phase.

Standard Techniques. The following routine techniques used in our laboratory were described in the corresponding references: DNA isolation, recombinant DNA work, and DNA sequencing (14, 21, 24); computer-assisted sequence analysis (14, 25); sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis, heme staining, and Western blotting with rabbit anti-cytochrome c_1 serum (10, 26); *in vivo* difference spectroscopy (8, 13); nodulation of soybean (*Glycine max* L. Merr. cv. Williams) and symbiotic N_2 fixation assay (21, 22).

Determination of Oxidase Activity. *In vivo* oxidation of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) was measured amperometrically in a magnetically stirred 2-ml chamber of an O_2 electrode (Rank Brothers, Cambridge, England) at 28°C. Cells grown under aerobic, microaerobic, and anaerobic conditions were washed and resuspended in 100 mM sodium phosphate buffer, pH 7.0. A small volume of the cell concentrate was added to 2 ml of the same, air-saturated (250 μM O_2) buffer so that the suspension in the chamber had an OD_{550} in the range between 2.0 and 12.0 (1 OD_{550} unit corresponded to $\approx$200 μg of protein per ml). The oxygen consumption rates were quantified before and after addition of 10 mM sodium ascorbate and 0.2 mM TMPD. The difference between these two rates was calculated as the TMPD oxidase activity in nanomoles of O_2 consumed per minute per milligram of protein. As a control, the rate of autoxidation of TMPD plus ascorbate was tested, but no significant autoxidation was observed.

RESULTS AND DISCUSSION

Discovery of a Symbiotically Important Gene Region in *B. japonicum*. The *B. japonicum* DNA region upstream of the previously reported genes for the oxygen-responsive two-component regulatory FixLJ system (22) was cloned, sequenced, and subjected to mutational analysis (Fig. 1). Six complete open reading frames (ORFs) that complied best with the typical *B. japonicum* codon preference and G+C bias (25) were identified. They read in divergent orientation to the *fixLJ* genes. At the right end of the sequenced region we located the 5′ end of another ORF (Fig. 1) for which a homologous counterpart (36% identity) was found in sequence database searches: the *Rhizobium meliloti fixG* gene (27). About 5 kb of DNA separate *fixLJ* and *fixG* both in *B. japonicum* and in *R. meliloti* (ref. 28; Fig. 1). *R. meliloti* harbors the so-called "*fixN* region" in this DNA section (28), and we could show by interspecies hybridization that this was also true for *B. japonicum* (for experimental details see ref. 29). The *R. meliloti fixN* region apparently consists of four genes organized in an operon, *fixNOQP* (see ref. 30 for a preliminary account). Neither the exact position on the restriction map (28) nor the nucleotide sequence of these genes has been reported; however, D. Kahn (Institut National de la Recherche Agronomique, Castanet-Tolosan, France) kindly provided us with the unpublished *R. meliloti* FixP amino acid sequence, which shared 49% identity with the predicted product of the rightmost complete ORF (Fig. 1), now also called *fixP*, of *B. japonicum*. Based on this similarity, the preceding ORFs were named *fixN*, *fixO*, and *fixQ*, as in *R. meliloti*, whereas the two other putative genes between *fixL* and *fixN* were named ORF277 and ORF141 (Fig. 1). The latter two ORFs shared no sequence similarity with known genes in databases.

A *B. japonicum* mutant deleted for ORF141, *fixN*, and part of *fixO* (strain Bj9027) and a *fixN*::Tn*5* insertion mutant (Bj3613) were defective in symbiotic N_2 fixation (Fix$^-$, with 2.5–5% residual Fix activity as compared with the wild type). Soybean nodules induced by the mutants were white or greenish, and the infected plant cells contained only a few bacteroids as judged by electron microscopy (results not shown). Wild type and mutants grew alike both aerobically in PSY medium and anaerobically in YEM medium with nitrate as the terminal electron acceptor. A Tn*5* insertion located 152 nucleotides upstream of the *fixN* start codon (strain Bj3611) produced no detectable phenotypes. This suggested that the *fixN* promoter is on the right of the insertion in strain Bj3611 (Fig. 1) and that ORF277 and ORF141 are not part of the *fixN* transcription unit. The closely adjacent arrangement of the *fixN*, *fixO*, *fixQ*, and *fixP* genes suggests that the four genes form an operon, *fixNOQP*. This assumption is further corroborated (*i*) by the presence of a putative regulatory DNA sequence with dyad symmetry upstream of *fixN*, 5′-TTGATTNNAATCAA-3′ (Fig. 1), a potential binding site for an Fnr/FixK-like protein (31); (*ii*) by a probable factor-independent transcription terminator [inverted repeat with a $\Delta G°$ at 25°C of −27.2 kcal (32)] following *fixP* (Fig. 1); and (*iii*) by the polar effect of the *fixN* mutation on the expression of the *fixP* gene product (see Fig. 4).

Amino Acid Sequences Predict a Cytochrome Oxidase Complex as the Product of *fixNOQP*. The amino acid sequences derived from the nucleotide sequences of *fixN*, *fixO*, *fixQ*, and *fixP* were inspected for recognizable sequence motifs and for similarities to known proteins compiled in databases (European Molecular Biology Laboratory, Release 33.0; SwissProt, Release 24.0; Max-Planck-Institute Protein Se-

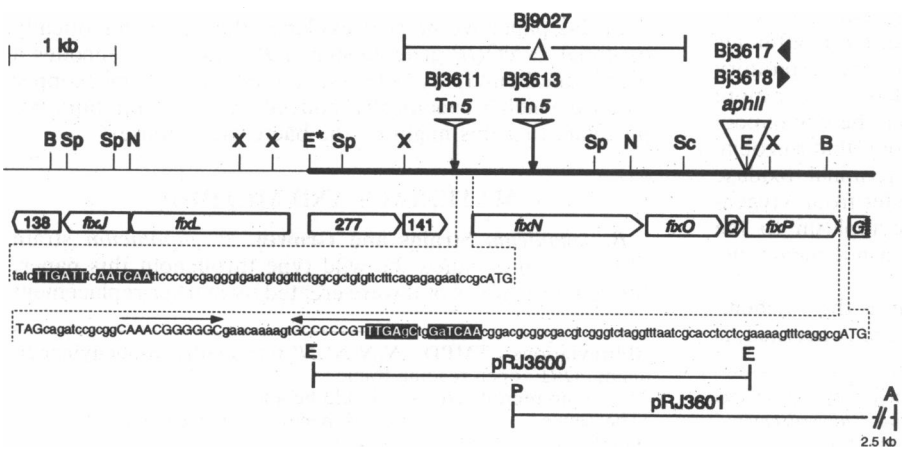

FIG. 1. *B. japonicum fixLJ–fixNOQP* region. Bold line indicates DNA sequenced in this work. The DNA deleted in Bj9027 (Δ) and the Tn*5* and *aphII* cassette insertions in other mutants are shown (filled arrowheads denote orientation of *aphII* cassette). Plasmid subclones and nucleotide sequences immediately before and after *fixNOQP* are shown below the map; capital letters emphasize start and stop codons, potential Fnr/FixK binding sites (white-on-black characters), and a probable transcription terminator (horizontal arrows). A, *Apa* I; B, *Bam*HI; E, *Eco*RI, E*, *Eco*RI site cut only in cloned DNA from *Escherichia coli* but not in DNA from *B. japonicum*; N, *Nsi* I; P, *Pvu* II; Sc, *Sca* I; Sp, *Sph* I; X, *Xho* I.

quence, Release 35). Several notable features emerged (Figs. 2 and 3).

FixN (549 amino acids; calculated M_r 61,272 for apoprotein) shares a low but significant sequence similarity with subunit I of heme/copper oxidases from mitochondria and many different aerobic bacteria. For example, a computer-assisted comparison with the *Escherichia coli* CyoB protein (33) or the *Paracoccus denitrificans* CtaDI protein (34) revealed a 52% similarity and a 20–21% identity in both cases. Two cytochrome oxidase genes were previously identified in *B. japonicum, coxA* for subunit I of cytochrome aa_3 (8, 12) and *coxN* for subunit I of an alternative oxidase (14), which are similar not only to each other (42% identity) but also to the FixN protein (21–22% identity in both cases) (see Fig. 2 for an alignment of FixN, CoxA, and CoxN). The presence of up to 13 potential membrane-spanning domains, some of which are roughly at equivalent positions as those in subunit I of other heme/copper oxidases (ref. 35; Fig. 2), strongly suggests a membrane location for FixN. Four essential, absolutely conserved histidine residues were previously implicated in forming the ligands to the high-spin heme-Cu_B binuclear center in oxidases (36, 37), and, in fact, all four histidines are also present in FixN (His-280, His-330, His-331, and His-418; Fig. 2). We interpret this to mean that FixN belongs to the superfamily of heme/copper-containing oxidases. However, while the known subunits I of this oxidase family possess two further, conserved histidines as axial ligands for a low-spin heme (36, 37), the FixN protein differs by having conserved only one of the two—namely, His-420

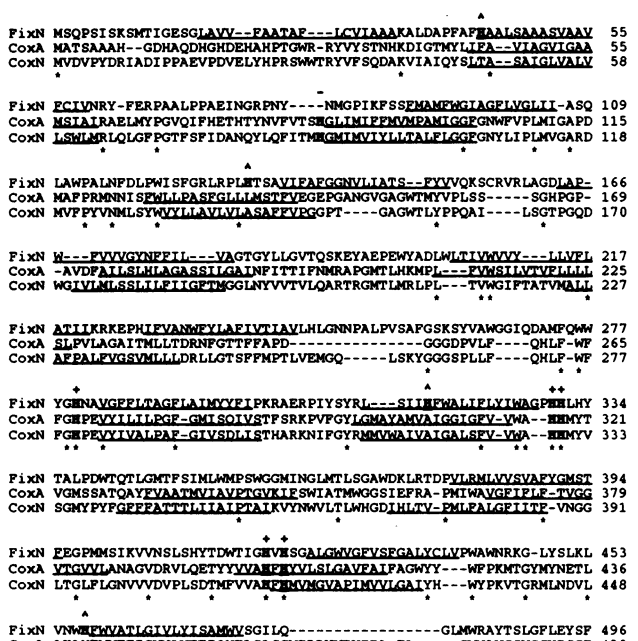

FIG. 2. Amino acid sequence alignment of the *B. japonicum fixN, coxA*, and *coxN* gene products. The CoxA and CoxN sequences are from refs. 8 and 14. Amino acids that are identical in all three sequences are marked by stars below the CoxN sequence. Potential transmembrane helices are underlined. Certain histidines discussed in the text are printed in boldface letters and are marked above the FixN sequence by the following symbols: +, histidines that are strictly conserved in all known subunit I sequences of heme-copper oxidases; −, histidine present in all known subunit I sequences but absent in FixN; ∧, histidines in FixN which are candidates to serve as ligands for heme binding, preferentially heme *b*.

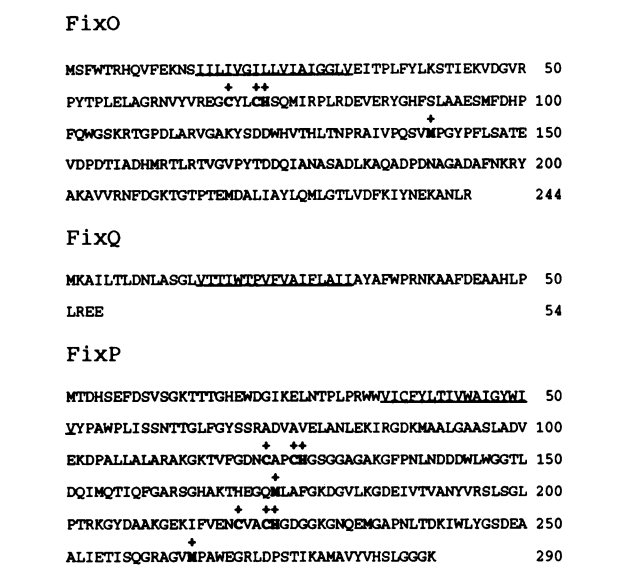

FIG. 3. Amino acid sequences of the *B. japonicum fixO, fixQ*, and *fixP* gene products. Membrane-spanning domains are underlined. Plus signs mark amino acids that are probably involved in the binding of heme *c*.

(Fig. 2). Hence, in addition to His-420, one of several other histidines (e.g., His-43, His-131, His-316, or His-457) might fulfill the task as a ligand for a second six-coordinate heme in FixN. Interestingly, all of the aforementioned histidines are located within or close to membrane-spanning helices, and their flanking amino acids comply well with the rules of Esposti (38), according to which these histidines may be ligands for *b*-type hemes. Incidentally, the eight amino acids surrounding His-316 (four on each side) show the highest score, with seven of them being diagnostic of a heme *b* binding domain (38).

FixO (244 amino acids; calculated M_r 27,352 for apoprotein) is very likely a membrane protein, too, as it has one perfect transmembrane helix near its N terminus (Fig. 3). The amino acid sequence at positions 68–72 (Cys-Tyr-Leu-Cys-His) and the Met-Pro motif at 140–141 are indicative of a heme *c* binding site, in which Cys-68 and Cys-71 provide the thioether bonds to the vinyl side chains of protoheme IX, and His-72 and Met-140 serve as the fifth and sixth coordinates of the heme iron (39, 40). Therefore, we predict that FixO is a membrane-anchored cytochrome *c*, even though the overall amino acid sequence shows hardly any similarity to known *c*-type cytochromes (39).

FixQ (54 amino acids; calculated M_r 6031) appears to be a small membrane protein, owing to the presence of one characteristic membrane-spanning domain (Fig. 3). We found no other obvious sequence motifs in it.

FixP (290 amino acids; calculated M_r 31,023 for apoprotein) is quite obviously a membrane-anchored diheme cytochrome *c* (Fig. 3). As in FixO, there is a transmembrane domain near the N terminus. We found two heme *c* binding motifs in FixP (Cys-Ala-Pro-Cys-His plus perhaps Met-173, and Cys-Val-Ala-Cys-His plus Met-264; Fig. 3). Interestingly, there is considerable internal homology (51% similarity, 32% identity) between two amino acid stretches, positions 96–190 and positions 196–288 (data not shown). The *fixP* gene, therefore, may have evolved by tandem duplication of an ancestral gene for a low-molecular-weight monoheme cytochrome *c*. Each of the two homologous stretches shows sequence similarity to the cytochrome c_6 ("c_{553}") class (39) of cyanobacteria and algal chloroplasts (data not shown).

In conclusion, it is tempting to speculate from all of these predicted properties that the *fixNOQP* gene products form a

membrane-bound four-subunit terminal oxidase complex containing two *c*-type cytochromes and a heme/copper-binding protein. Biochemical data in support of this notion will be presented next.

***fixN* Mutations Affect Microaerobically Induced Oxidase Function.** O_2 reduction in whole cells of the *B. japonicum* wild type and the *fixNO* deletion strain (Bj9027) that were grown under different conditions was measured with an O_2 electrode (Table 1). Ascorbate-reduced TMPD was used as an artificial electron donor. No significant difference in oxidase activity was observed in cells grown aerobically, whereas there was a substantial decrease in oxidase activity when cells had been grown anaerobically with nitrate as terminal electron acceptor (35% residual activity) or under microaerobic conditions (20% residual activity) (Table 1). Similar results as with Bj9027 were also obtained with strain Bj3613 (Fig. 1), which carries a Tn*5* insertion in the *fixN* gene. Moreover, we observed a 70% decrease in oxidase activity when we compared isolated wild-type and mutant membranes *in vitro* by using reduced horse heart cytochrome *c* as electron donor (D. Ritz and O.P., unpublished results). It thus appears as if the major amount of oxidase activity produced during anaerobiosis or O_2 limitation is contributed by the *fixNOQP* gene products.

A comparison between the *in vivo* difference spectra of wild type and strain Bj9027 grown anaerobically in YEM medium plus nitrate was also made (all data not shown). Difference spectra with dithionite-reduced minus air-oxidized samples (3.5 mg of protein per ml) revealed the presence in both strains of two peaks at 522 nm and 551/552 nm (plus shoulders) reflecting *b*- and *c*-type cytochromes, whereas the characteristic 602-nm peak from heme *a*-containing cytochromes, which normally abound in aerobically grown cells (6, 8), was absent. A noteworthy feature was a 40% loss of *c*-type hemoproteins in the mutant. A similar decrease was also observed in CO difference spectra (smaller trough at 551/552 nm in Bj9027). Furthermore, a wild-type peak at 566 nm in the CO difference spectrum was shifted to 561 nm in the mutant, reflecting a possible effect on a *b* hemoprotein. While all of the spectra are probably masked by the omnipresent cytochrome bc_1 complex and the anaerobically induced denitrification enzymes, they may allow the tentative conclusion that the *fixNOQP*-encoded oxidase is not an *a*-type oxidase but more likely a heme *b*- and *c*-containing oxidase.

Identification of Two Microaerobically Induced Membrane-Bound *c*-Type Cytochromes, Probable Products of *fixO* and *fixP*. We isolated membrane fractions from *B. japonicum* wild-type and mutant cells and analyzed them for the presence of *c*-type cytochromes by SDS/polyacrylamide gel electrophoresis and subsequent staining of covalently bound heme *c*. It was shown previously that aerobically grown wild-type cells had two membrane-bound *c*-type cytochromes, the 28-kDa cytochrome c_1 and the 20-kDa CycM protein (8, 10, 11). The same proteins were also detected in membranes from anaerobically and microaerobically grown

Table 1. Oxidase activity in intact *B. japonicum* cells grown under various conditions, with TMPD as the electron donor

B. japonicum strain	TMPD oxidase activity, nmol of O_2 reduced per min per mg		
	Aerobic growth	Microaerobic growth	Anaerobic growth (+ NO_3^-)
Wild type	53.2 ± 1.4	112.1 ± 6.3	40.4 ± 2.5
Mutant 9027 (Δ*fixNO*)	57.1 ± 16.9	23.4 ± 5.2	14.2 ± 6.1

At least three measurements were made with cultures grown to an OD_{550} of 0.2–0.4. Values are means ± SD.

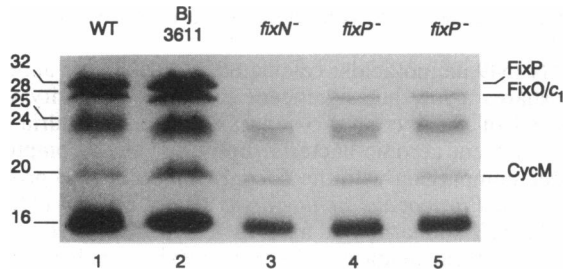

FIG. 4. Analysis of *c*-type cytochromes (heme stain) in membranes of *B. japonicum* cells grown anaerobically in YEM medium with nitrate. Approximately 200 μg of protein of each sample was separated by SDS/polyacrylamide gel electrophoresis. The membranes were isolated from the following strains: lane 1, wild type (WT); lane 2, mutant Bj3611 with a Tn*5* insertion that does not affect the *fixNOQP* gene cluster (see Fig. 1); lane 3, mutant Bj3613 (*fixN*::Tn*5*); lane 4, mutant Bj3617 (*fixP*::*aphII*); lane 5, mutant Bj3618 (*fixP*::*aphII*). The apparent molecular masses of the proteins (kDa) are shown at left.

wild-type cells, but these membranes contained at least four additional *c*-type cytochromes (Figs. 4 and 5, lane 1), with apparent molecular masses of 32, 25, 24, and 16 kDa. As detailed below, there is probably a fifth additional protein of 28 kDa comigrating with cytochrome c_1. Two of these proteins are missing in *fixN*::Tn*5* and Δ*fixNO* mutant strains: the 32-kDa protein and the other 28-kDa protein, which is not cytochrome c_1 (Fig. 4, lane 3; Fig. 5, lane 2).

The most easily interpretable case is the 32-kDa protein. It is completely absent from two *fixP*::*aphII* insertion mutants carrying the kanamycin-resistance cassette in both possible orientations (Fig. 4, lanes 4 and 5). Since *fixP* is the last gene in the *fixNOQP* cluster (Fig. 1), it seems compelling that the 32-kDa protein is the *fixP* gene product and that its absence in the *fixN*::Tn*5* and Δ*fixNO* mutants is caused by a polar effect of the mutations on the expression of the downstream *fixP* gene. A membrane-bound *c*-type cytochrome as the product of *fixP* is fully consistent with the predictions made from the FixP amino acid sequence (Fig. 3), and the apparent molecular mass determined by gel electrophoresis (32 kDa) accords well with the molecular mass deduced from the sequence (32,323 Da for apoprotein plus two protohemes IX).

Since we predicted from the amino acid sequence (Fig. 3) that the *fixO* gene product could also be a membrane-bound *c*-type cytochrome (28,002 Da for apoprotein plus one protoheme IX), we suspected that the 28-kDa heme-stainable band seen on gels was in reality composed of two tightly comigrating proteins: cytochrome c_1 and perhaps the FixO protein. Evidence for the validity of this assumption came from the fact that a cytochrome bc_1-defective mutant (strain

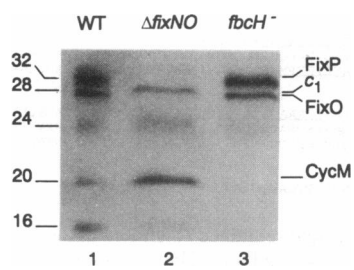

FIG. 5. Analysis of *c*-type cytochromes (heme stain) in membranes of *B. japonicum* cells grown microaerobically in PSY medium. Approximately 100 μg of protein of each sample was loaded on the SDS/polyacrylamide gel. The membranes were from the following strains: lane 1, wild type (WT); lane 2, strain Bj9027 (Δ*fixNO*); lane 3, strain 2800 (Tn*5* insertion in *fbcH*, the structural gene for cytochromes *b* and c_1; refs. 10 and 26). The apparent molecular masses of the proteins (kDa) are shown at left.

2800, *fbcH*::Tn*5*) which neither synthesizes the c_1 protein nor the CycM protein (8, 10, 11) still produced a 28-kDa *c*-type cytochrome (Fig. 5, lane 3). As expected, this 28-kDa protein did not crossreact with anti-c_1 immunoglobulins in Western blots, whereas the 28-kDa protein seen in the *fixN*::Tn*5* and Δ*fixNO* strains (Fig. 4, lane 3; Fig. 5, lane 2) clearly crossreacted with the anti-c_1 serum (results not shown). Although antibodies specific for FixO are not available to prove the case, we infer from these experiments that the 28-kDa protein seen in lane 3 of Fig. 5 is most likely the *fixO* gene product. It should be emphasized here again that this protein, just like the 32-kDa FixP protein, was found only in membranes of cells that were grown anaerobically or microaerobically.

Conclusions. It was gratifying to observe that the results obtained here from selected biochemical tests concurred with the predictions made from DNA-derived amino acid sequences: (*i*) the membrane-bound nature and identification as *c*-type hemoproteins of FixP and probably also FixO confirmed the predicted transmembrane helices and heme *c* binding motifs; (*ii*) the fact that ≈75% of the oxidase activity detectable in anaerobically grown cells was due to *fixNOQP* paralleled the prediction that FixN is an oxidase subunit with characteristic histidines as heme/copper ligands; (*iii*) the *in vivo* difference spectrum reflecting the absence of *a*-type hemoproteins in anaerobically grown cells was consistent with the predictions that FixN might be a *b* heme-binding oxidase subunit rather than a subunit of an aa_3-type oxidase.

An oxidase complex consisting solely of cytochromes *c* and a heme *b*/copper-binding subunit would clearly be a novelty, but such a composition is not entirely without precedent. An *o*-type oxidase was isolated from *Pseudomonas aeruginosa* that consisted of four subunits, of which two were *c*-type hemoproteins of 29 and 21 kDa (41). Very recently, we learned of the existence of a *bc*-type cytochrome *c* oxidase in *Rhodobacter sphaeroides* that had three subunits of 40, 35, and 25 kDa, of which the smaller two contained heme *c* (42). It will be exciting to see whether or not the FixNOQP-oxidase is a prototype of a subfamily within the heme/copper oxidase superfamily.

Finally, an attractive line of future research will aim at proving that the *fixNOQP*-encoded proteins are constituents of the enigmatic bacteroid oxidase operating at extremely low free O_2 tension in root nodule endosymbiosis. At present we can state only that the oxidase described here is an ideal candidate: (*i*) it is important for bacteroid development and symbiotic N_2 fixation; (*ii*) it is induced microaerobically; and (*iii*) it meets all of the requirements to fit into a cytochrome bc_1-dependent respiratory branch (presence of cytochromes *c* and a heme/copper subunit; see Introduction). The goals must now be to purify this oxidase and document that it is capable of reducing O_2 delivered by oxygenated leghemoglobin (5, 6).

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