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Bradyrhizobium japonicum NnrR, a Denitrification Regulator, Expands the FixLJ-$FixK_2$ Regulatory Cascade

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In *Bradyrhizobium japonicum*, a gene named *nnrR* was identified which encodes a protein with high similarity to FNR/CRP-type transcriptional regulators. Mutant strains carrying an *nnrR* null mutation were unable to grow anaerobically in the presence of nitrate or nitrite, and they lacked both nitrate and nitrite reductase activities. Anaerobic activation of an *nnrR'-'lacZ* fusion required FixLJ and $FixK_2$. In turn, N oxide-mediated induction of *nir* and *nor* genes encoding nitrite and nitric oxide reductase, respectively, depended on NnrR. Thus, NnrR expands the FixLJ-$FixK_2$ regulatory cascade by an additional control level which integrates the N oxide signal required for maximal induction of the denitrification genes.

Denitrification is an alternative form of respiration in which bacteria sequentially reduce nitrate (NO_3^-) or nitrite (NO_2^-) to nitrogen gas (N_2) via the intermediates nitric oxide (NO) and nitrous oxide (N_2O), when oxygen becomes limiting. Reduction of nitrogen oxides is coupled to energy conservation and permits cell growth under microoxic or anoxic conditions. Denitrification is initiated by reduction of nitrate to nitrite catalyzed by the respiratory (dissimilatory) nitrate reductase. Yet this reaction is not unique to denitrification, because it also occurs in dissimilatory and assimilatory ammonification, both of which result in the formation of ammonia. Thus, the defining reaction in denitrification is the reduction of nitrite to the first gaseous intermediate, NO. For reviews see references 6, 28, and 42.

Bradyrhizobium japonicum is a soil bacterium with the capability of reducing NO_3^- simultaneously to NH_4^+ and N_2 when cultured anaerobically with nitrate as the terminal electron acceptor and sole source of nitrogen (34). The recently published genome sequence (20; also see http://www.kazusa.or.jp/rhizobase) indicated that *B. japonicum* lacks genes encoding a membrane-bound nitrate reductase. As with *Pseudomonas* sp. strain G-179 (8), the first step of denitrification in *B. japonicum* depends on the *napEDABC* genes, which specify a periplasmic nitrate reductase (M. J. Delgado, E. J. Bedmar, and P. Müller, personal communication). Subsequent denitrification reactions are catalyzed by the products of *nirK* (37), *norCBQD* (24), and *nosRZDYFLX* (E. J. Bedmar, unpublished data), which encode reductases for nitrite, nitric oxide, and nitrous oxide, respectively.

Microaerobic induction of transcription from the *nir*, *nor*, and *nos* promoter regions depends on the *fixLJ* and *$fixK_2$* genes, whose products form the FixLJ-$FixK_2$ regulatory cascade (2, 24, 25, 37, and E. J. Bedmar, unpublished). Hence, *B. japonicum fixLJ* and *$fixK_2$* mutants are unable to grow anaerobically with nitrate as the terminal electron acceptor.

FixLJ is a two-component regulatory system consisting of the heme-based sensor kinase FixL and the FixJ response regulator (32 and references therein). In *B. japonicum*, the only known gene that apparently is directly controlled by FixJ is *$fixK_2$*. Its product, $FixK_2$, is a transcriptional activator for a large group of genes involved in anaerobic or microaerobic metabolism (14, 25). $FixK_2$ belongs to the bacterial family of FNR/CRP-type transcriptional regulators. FNR (fumarate and nitrate reductase regulator) of *Escherichia coli* has four domains: (i) the N-terminal redox-sensing domain where three cysteine residues together with a centrally located cysteine coordinate binding of a redox-responsive $[4Fe\text{-}4S]^{2+}$ cluster; (ii) the central β-roll domain that interacts with RNA polymerase; (iii) a long α helix involved in protein dimer formation; and (iv) the C-terminal helix-turn-helix DNA binding motif (for reviews see references 16, 21, and 33). Oxygen-responsive FNR homologs have been identified in a number of other bacteria, e.g., FnrA of *Pseudomonas stutzeri* (9), ANR of *Pseudomonas aeruginosa* (40), FnrL of *Rhodobacter sphaeroides* (41), FnrP of *Paracoccus denitrificans* (35), and $FixK_1$ of *B. japonicum* (3). They all contain the N-terminal cysteine-rich motif. By contrast, $FixK_2$ of *B. japonicum* and its homologs lack that cysteine motif, and there is no evidence that members of this protein class are redox responsive (14). Another category of FNR-like activator proteins comprises *R. sphaeroides* NnrR (22, 31), *P. denitrificans* NNR (36), *P. aeruginosa* DNR (4), and *P. stutzeri* DnrD (39). These regulators also lack the cysteine signature, and they control expression of *nir* and *nor* genes in response to NO or a chemically related species. The precise sensing mechanism is still unknown. The consensus DNA binding site for *E. coli* FNR is TTGAT-N_4-ATCAA (FNR box), which usually is located around position −41.5 relative to the transcription start of FNR-activated promoters (30). A similar motif is found at a comparable position in many target promoters of the FNR-like proteins described above (42). Accordingly, C-terminal amino acid residues predicted to make contact with DNA (Glu209, Ser212, Arg213) (23) are conserved in FNR homologs.

Here we report on the identification and functional characterization of the *B. japonicum nnrR* gene whose product

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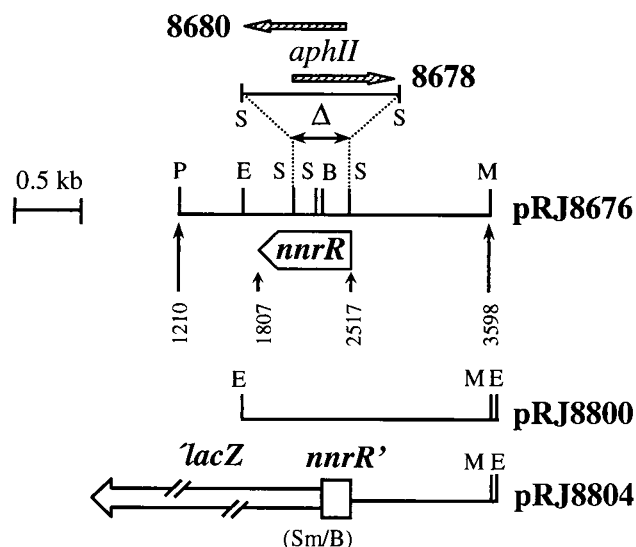

FIG. 1. Physical map of the *B. japonicum nnrR* locus present on plasmid pRJ8676 and genetic constructions relevant to this work. Numbers below vertical arrows refer to nucleotide positions as given in the GenBank database (accession no. AJ311165). The genotype of deletion-insertion mutants 8678 and 8680 is indicated above the physical map, with the orientation of the inserted *aphII* resistance gene cassette emphasized by horizontal arrows and the deleted DNA region indicated by the horizontal double-headed arrow. Below are the insert of plasmid pRJ8800 (cloned in the broad-host-range vector pRK290 [12]), which was used in complementation experiments, and the structure of the translational *nnrR′-′lacZ* fusion on the chromosomally integrated plasmid pRJ8804. Note that the *Eco*RI site at the right end of the plasmid inserts originates from the vector pUCBM20 (Boehringer Mannheim, Mannheim, Germany) that was used for construction of pRJ8676. B, *Bsa*BI; E, *Eco*RI; M, *Mlu*I; P, *Pvu*II; S, *Sal*I; Sm, *Sma*I. Restriction sites in parentheses were destroyed during the cloning procedures.

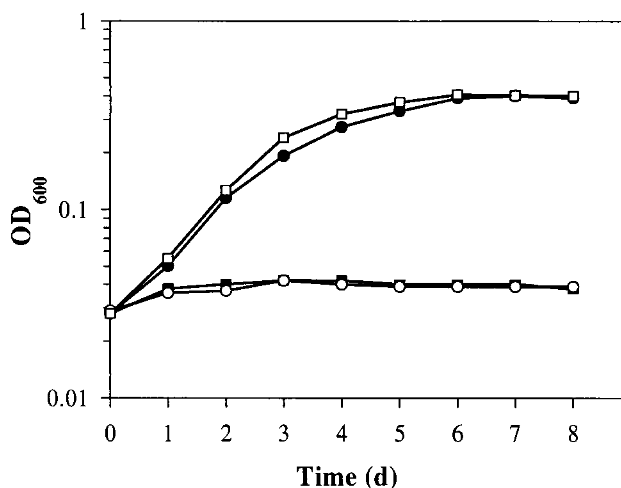

FIG. 2. Anaerobic growth of wild-type *B. japonicum* (●), *nnrR* mutant strain 8678 (■), strain 8678 harboring the vector pRK290 (control) (○), and the *nnrR*-containing plasmid pRJ8800 (□). Cells were inoculated into YEM medium plus 10 mM KNO_3, and anaerobic growth was followed by monitoring the optical density at 600 nm (OD_{600}).

(NnrR) mediates control of denitrification genes in response to N oxide. We provide evidence that *nnrR* itself is a target gene for the FixLJ-$FixK_2$ regulatory cascade, thereby extending it by an additional control level.

Identification and mutational analysis of *B. japonicum nnrR*. This gene was originally identified as *orf236* in the course of analyzing a gene cluster which encodes a heme uptake system (26; position numbers 1807 to 2517 on the complementary strand of the sequence were deposited under GenBank accession no. AJ311165). The *nnrR* gene is 710 bp in length, has a G+C content of 68.5 mol%, and encodes a protein of 236 amino acids with a calculated molecular mass of 25,870 Da. The *B. japonicum* NnrR protein shares significant similarity with NnrR of *R. sphaeroides* strains (47 to 49% identity) (AAB69132, AAC44402, AAD27624) (22, 31) and with an uncharacterized, predicted protein from *Rhodopseudomonas palustris* (74% identity) (ZP_00008933). The N terminus of *B. japonicum* NnrR lacks the cysteine motif that is characteristic for redox-responsive FNR-like proteins. Near the C terminus it contains a predicted helix-turn-helix motif likely to be involved in DNA binding (F_{177}PISRQDIAQMTGTTLHTVSRILSG WEQQGLV$_{208}$; MotifScan; http://www.expasy.org/prosite/).

A 2.39-kb *Pvu*II/*Mlu*I fragment harboring *nnrR* was subcloned in plasmid pRJ8676 (Fig. 1). For functional analysis, two *nnrR*-internal *Sal*I fragments comprising 425 bp were deleted and replaced by a kanamycin resistance gene cassette (*aphII*) on a 1.22-kb *Sal*I fragment from pBSL86 (Fig. 1) (1). Conventional marker exchange mutagenesis (17, 18) resulted in mutant strains 8678 and 8680 (Fig. 1).

In contrast to the wild type, cells of *B. japonicum* 8678 did not grow when cultured anaerobically in yeast extract-mannitol (YEM) medium (10) supplemented with 10 mM KNO_3 (Fig. 2). The same results were obtained with mutant 8680 or when nitrate was replaced by KNO_2 (100 μM) as the final electron acceptor (data not shown). Complementation of mutant strain 8678 with plasmid pRJ8800 containing the wild-type *nnrR* gene (Fig. 1) restored growth under these conditions (Fig. 2). For the determination of nitrate and nitrite reductase activities, cells were first grown aerobically in YEM medium, collected by centrifugation, washed twice with YEM, and then incubated anaerobically for 96 h in the same medium supplemented with 10 mM KNO_3 in completely filled, rubber-stoppered serum bottles. Enzyme activities were assayed as described previously (11). Unlike wild-type cells, *nnrR* mutant cells lacked both nitrate and nitrite reductase activity. Both *nnrR* mutants were also used in soybean plant infection tests (15, 17) and were found to have a wild-type phenotype with regard to nodulation and nitrogen fixation (acetylene reduction) (data not shown).

Role of *nnrR* in the regulation of *nirK* and *norCBQD* genes. Chromosomally integrated, transcriptional *lacZ* fusions to the promoters of *nirK* and *norCBQD* were used in regulatory studies. For construction of the *nirK-lacZ* fusion, an 878-bp *Eco*RI/*Pst*I fragment from pNIRLZ (37) was cloned into the pSUP202-based (29) *lacZ* fusion vector pSUP3535 (H. M. Fischer, unpublished data), resulting in plasmid pRJ2498. The *norC-lacZ* fusion was constructed by cloning an *Eco*RI fragment of about 4.3 kb from pNORLZ (24) into vector pSUP3535, yielding plasmid pRJ2499. The fusion plasmids pRJ2498 and pRJ2499 were integrated by homologous recom-

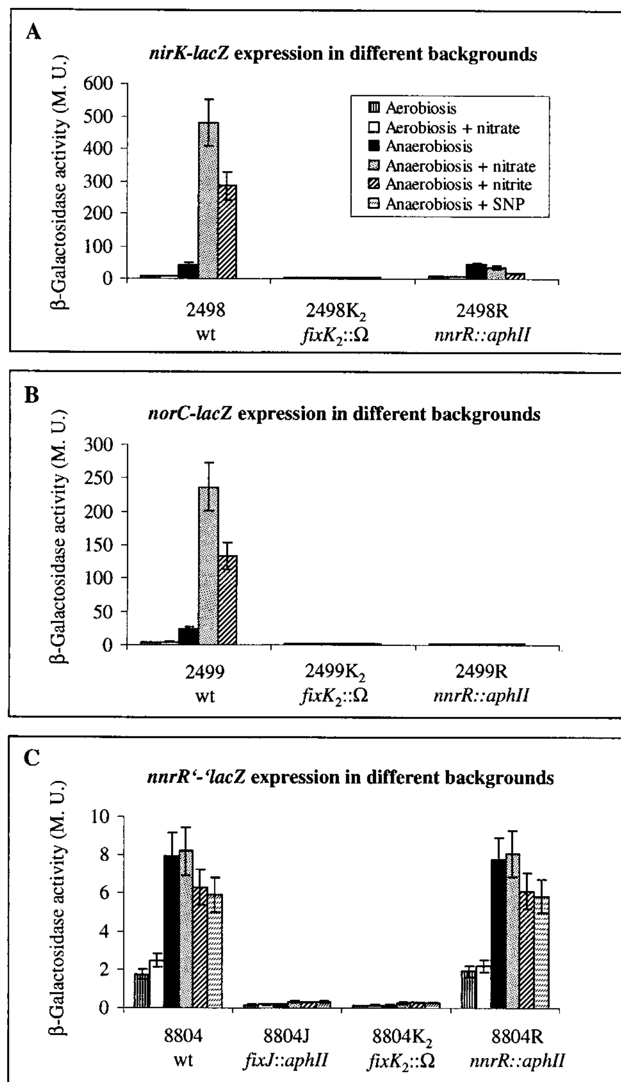

FIG. 3. β-Galactosidase activities resulting from expression of an *nirK-lacZ* (A), *norC-lacZ* (B), or *nnrR'-'lacZ* (C) fusion chromosomally integrated in different *B. japonicum* backgrounds. The relevant genotype of host strains is indicated below strain designations (for details see the text). Cells were first grown aerobically in YEM medium, collected by centrifugation, and then incubated aerobically or anaerobically (filled rubber-stoppered tubes) for 96 h in the same medium supplemented with 10 mM KNO_3, 10 mM KNO_2 (periodically added to 100 μM in regular 24-h intervals), or 10 μM SNP. The legend shown in panel A is also valid for panels B and C. Values are means ± standard errors from at least two independent experiments with two cultures assayed in duplicate. β-Galactosidase activity is expressed in Miller units (M. U.). wt, wild type.

bination into the chromosome of wild-type *B. japonicum*, *fixK*$_2$ mutant 9043 (25), and *nnrR* mutant 8678 (this work), resulting in strains 2498, 2498K$_2$, 2498R, 2499, 2499K$_2$, and 2499R, respectively. Expression of the *lacZ* reporter gene was determined in β-galactosidase activity assays with cells grown under different conditions (Fig. 3A and B).

Only background expression levels were observed with both fusions in aerobically grown cells, regardless of the presence or absence of nitrate (10 mM KNO_3). Maximal induction was observed in wild-type cells that were incubated anaerobically in the presence of nitrate. Nitrate (or an N oxide derived from it) is crucial for maximal induction, because expression levels were only about 10% of the maximal values when no nitrate was added to the anaerobic cultures. Supplementation with nitrite (repeated addition of KNO_2 to 100 μM in regular 24-h intervals) (Fig. 3A and B) or 10 μM sodium nitroprusside (SNP), an NO^+-generating agent (reference 7 and data not shown), instead of nitrate resulted in half-maximal expression of the reporter fusions. No significant expression was observed in the *fixK*$_2$ mutant background under all conditions tested, confirming our previous finding that FixK$_2$ is absolutely required for activation of both the *nirK* and the *norCBQD* promoter (Fig. 3A, strain 2498K$_2$, and B, strain 2499K$_2$) (24, 37). Induction of the *norCBQD* promoter was completely abolished in the absence of a functional *nnrR* gene (Fig. 3B, strain 2499R). By contrast, anaerobic induction of the *nirK* promoter was retained in the *nnrR* mutant background (Fig. 3A, strain 2498R), implying that the *nirK* and the *norCBQD* promoter exhibit slight differences with regard to their dependence on FixK$_2$ in the absence of NnrR.

Regulation of *nnrR* expression. To evaluate a potential regulatory link between FixLJ-FixK$_2$ and NnrR, we studied expression of *nnrR* in different backgrounds. For this purpose, a translational fusion of *nnrR* (87th codon) to *lacZ* was constructed by cloning a 1,356-bp *Eco*RI-*Bsa*BI fragment from pRJ8676 into pSUP482 (H. M. Fischer, unpublished) to yield plasmid pRJ8804 (Fig. 1). This plasmid was integrated by homologous recombination into the chromosome of wild-type *B. japonicum*, *fixJ* mutant 7360 (2), *fixK*$_2$ mutant 9043 (25), and *nnrR* mutant 8678 (this work), resulting in strains 8804, 8804J, 8804K$_2$, and 8804R, respectively. In strain 8804R, the *nnrR*::*aphII* null mutation was retained after integration of the *nnrR'-'lacZ* fusion. Cells of these strains were grown under different conditions, and *nnrR* expression was determined in β-galactosidase activity assays (Fig. 3C).

Expression of *nnrR'-'lacZ* was quite low (<10 Miller units) under all growth conditions. In anaerobically incubated cells, expression was significantly enhanced three- to fourfold compared to that for aerobically cultivated cells, regardless of the absence or presence of KNO_3, KNO_2, or SNP. The same expression pattern was observed in the *nnrR* mutant background, indicating that *nnrR* does not control its own expression. By contrast, only very low *nnrR'-'lacZ* expression was detected in cells of strains 8804J and 8804K$_2$, regardless of the incubation conditions. These findings are compatible with a model that places NnrR in the FixLJ-FixK$_2$ cascade downstream of FixK$_2$ (Fig. 4). The presence of a putative, albeit rather poorly conserved, FixK$_2$ box upstream of *nnrR* further supports this model (TTGCG-N$_4$-CGCAA$_{53}$, with the underlined nucleotides matching the consensus FNR/FixK box [the nucleotide position number refers to the translational start of *nnrR*]) (14, 42).

With NnrR we have added to the FixLJ-FixK$_2$ cascade an additional control level which integrates the N oxide signal, presumably either NO_2^- or NO or both, that is critical for maximal induction of the *B. japonicum* denitrification genes. Our data indicate that N oxide control of *B. japonicum* denitrification genes occurs at the *nnrR* posttranscriptional level, yet the identity of the signaling molecule and the sensing mech-

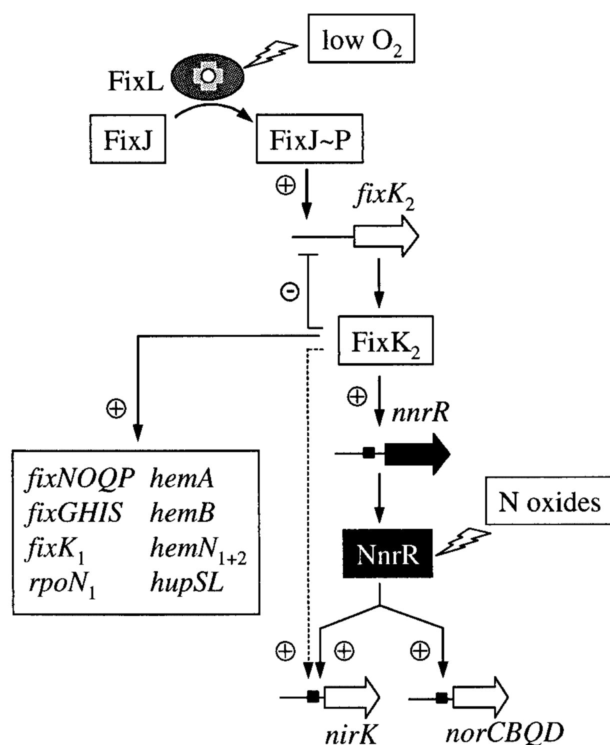

FIG. 4. Location and function of NnrR in the FixLJ-FixK_2 regulatory cascade of *B. japonicum*. Details are explained in the text. Note that even though the model suggests a direct hierarchical organization of FixLJ, FixK_2, NnrR, and denitrification genes, additional control levels situated in between cannot be excluded. Similarly, N oxide signaling to NnrR may be direct or indirect. The dashed arrow refers to anaerobic activation of *nirK* (but not of *norCBQD*) by FixK_2, which is retained in an *nnrR* mutant background. Filled boxes symbolize putative binding sites for FixK_2 and NnrR which are similar to that of the consensus FNR box (TTGAT-N_4-ATCAA).

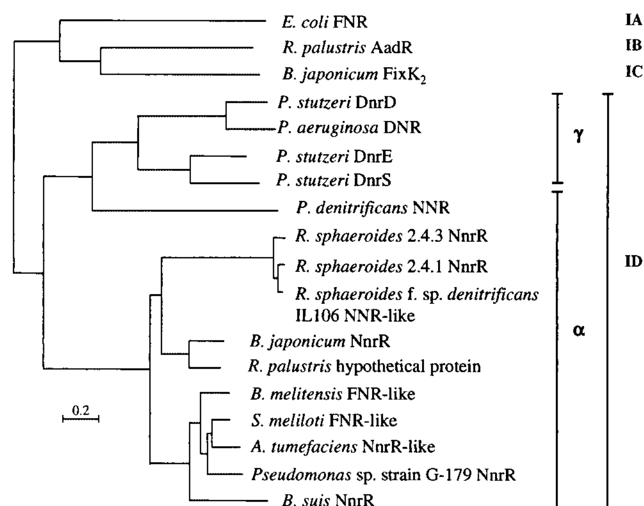

FIG. 5. Phylogenetic tree of 15 FNR-like transcriptional activator proteins belonging to subgroup ID (14, 16, 39). For comparison, one representative reference protein of each subgroup IA, IB, and IC is included in the tree. Amino acid sequences were aligned with the program T-COFFEE (27; http://www.ch.embnet.org/software/TCoffee.html), and phylogenetic analyses were performed with the phylogeny inference package PHYLIP (13; http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html). The scale bar represents a distance of 0.2 substitutions per position. Accession numbers of individual protein sequences are the following: *E. coli* FNR, AAC74416; *R. palustris* AAdR, Q01980; *B. japonicum* FixK_2, CAA06287; *P. stutzeri* DnrD, CAC14591; *P. aeruginosa* DNR, BAA08744; *P. stutzeri* DnrE, CAB40906; *P. stutzeri* DnrS, CAB40908; *P. denitrificans* NNR, AAA69977; *R. sphaeroides* 2.4.3 NnrR, AAC44402; *R. sphaeroides* 2.4.1 NnrR, AAB69132; *R. sphaeroides* f. sp. *denitrificans* IL-106 NNR-like, AAD27624; *B. japonicum* NnrR, CAC38738; *R. palustris* NnrR-like, ZP_00008933; *Brucella suis* NnrR, AAN33482; *Brucella melitensis* FNR-like, NP_541964; *Pseudomonas* sp. strain G-179 NnrR, AAB96771; *Sinorhizobium meliloti* FNR-like, NP_435925; *Agrobacterium tumefaciens* NnrR-like, NP_534858.

anism remain unknown. Nitric oxide (NO) has been suggested or shown to act as a signal molecule for NnrR of *R. sphaeroides* (22, 31), NNR of *P. denitrificans* (19, 36), DNR of *P. aeruginosa* (5), and DnrD of *P. stutzeri* (38). It seems likely that NnrR and its homologues can switch from an inactive to an active form in response to NO.

NnrR-like proteins constitute a distinct cluster within the ID subgroup of the FNR family of transcriptional regulators. Based on overall amino acid sequence similarity and distinct structural features, the family of FNR-like proteins has been divided into four subgroups, with *E. coli* FNR (IA), *R. palustris* AadR (IB), rhizobial FixK proteins (IC), and *P. aeruginosa* DNR (ID) as representative reference proteins of each group (14, 16, 39). We determined the phylogenetic relationship of 15 members of subgroup ID (including *B. japonicum* NnrR) and compared it with those of the other subgroups of the protein family (Fig. 5). The proteins of subgroup ID can be further divided into two subclusters that largely follow the classification of the proteobacterial subgroups of the respective bacterial species. One subcluster is formed by DNR-like proteins from γ-proteobacterial *Pseudomonas* species, while the other branch comprises NnrR-like proteins from various α-proteobacterial species. The NNR protein of the α-proteobacterium *P. denitrificans* represents an exception in that it clusters with the γ-proteobacterial DNR-like proteins. The position of the NnrR-like protein of *Pseudomonas* sp. strain G-179 within the group of α-proteobacterial homologues is not surprising, because this species is most likely a rhizobial strain as deduced from 16S rRNA analyses (8). Zumft and coworkers noted characteristic distinctions between DNR- and NnrR-like proteins which may eventually lead to their classification in different subgroups (39, 43): (i) in contrast to the EXXSR motif in DNR-like proteins, the recognition helix for DNA binding of NnrR-like regulators includes a conserved HXXSR motif; (ii) DNR- and NnrR-like proteins seem to correlate with the regulation of cytochrome cd_1- and Cu-containing nitrite reductase enzymes, respectively. The NnrR protein of *B. japonicum* perfectly matches these criteria.

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