Functional and Regulatory Analysis of the Two Copies of the *fixNOQP* Operon of *Rhizobium leguminosarum* Strain VF39

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Received 11 December 1996. Accepted 21 March 1997.

DNA corresponding to two copies of the *Rhizobium leguminosarum* bv. *viciae* strain VF39 *fixNOQP* operon coding for a putative symbiotic terminal oxidase of the heme-copper oxidase superfamily was cloned, sequenced, and genetically analyzed. The first copy is located upstream of the *fixK-fixL* region on plasmid pRleVF39c, whereas the second copy resides on the nodulation plasmid pRleVF39d. Insertional mutagenesis with antibiotic resistance cassettes confirmed that both copies were functional, and that the presence of at least one functional copy was required for nitrogen fixation. The deduced amino acid sequences of both *fixN* genes are highly similar (95% identity) and contain 15 putative transmembrane helices, suggesting that the *fixN* gene products are integral membrane proteins. Furthermore, six histidine residues predicted to be the ligands for a heme-copper binuclear center and a low-spin heme b are conserved in both *R. leguminosarum* *fixN* proteins. The deduced *fixO* and *fixP* gene products show characteristics of membrane-bound monoheme and diheme cytochrome c, respectively. Upstream of both *fixN* copies putative Fnr-consensus binding sites (anaeroboxes) were found that differ in certain base pairs. As *R. leguminosarum* VF39 possesses two members of the Fnr/FixK regulator family, FnrN and FixK, the possible differential regulation of both *fixN* copies was analyzed with *fixN-gusA* reporter gene fusions. Both *fixN* fusions were induced under free-living microaerobic conditions and in the symbiotic zone of the root nodule. Induction of the expression of *fixNc* and *fixNd* was highly reduced in a *fnrN* mutant background and in a *fixL* mutant background, whereas *fixK* was only marginally involved in *fixN* regulation.

Residual expression of *fixN* was observed in an *fnrN/fixK* double mutant.

Additional keywords: Fnr-like regulators.

Bacteria that form nitrogen-fixing nodules on the roots of leguminous plants (collectively known as rhizobia) have been intensively studied because of their agronomic importance and the inherent biological interest of their complex interactions with their host plants. Use of molecular genetic tools and techniques has revealed that although the rhizobia are phylogenetically diverse, falling into at least four genera (Martinez-Romero and Caballero-Mellado 1996 and references therein), common sets of genes involved in their interactions with plants are quite conserved (for reviews see Fisher and Long 1992; Long and Staskawicz 1993; Fischer 1994; Van Rhijn and Vanderleyden 1995). Nodulation (*nod*) genes specify the synthesis of soluble factors that induce the plant to nodulate, *nif* genes code for the components (shared by all diazotrophs) necessary for synthesis of a functional nitrogenase enzyme, and *fix* genes have been classified as genes essential to the symbiotic, rather than free-living, nitrogen-fixing state. This latter classification is, however, not entirely accurate, as homologues of “*fix*” genes have been found recently in both free-living nitrogen fixers and nondiazotrophs (e.g., Tripathi 1993; Schlüter et al. 1995).

An important cluster of *fix* genes with a variety of functions was first discovered in *Rhizobium meliloti* (Renalier et al. 1987; David et al. 1987). This cluster of genes includes regulatory genes *fixLJ* (David et al. 1988) and *fixK* (Batut et al. 1989), the *fixGHIS* genes coding for a putative cation pump (Kahn et al. 1989), and the *fixN* operon (Batut et al. 1989; Kahn et al. 1993), whose function was unclear, but whose regulation was *fixLJ* and *fixK* dependent. Subsequent studies in *Bradyrhizobium japonicum* (Anthamatten et al. 1992; Fischer 1994; Preisig et al. 1993, 1996a), *Azorhizobium caulinodans* (Kaminski et al. 1991; Mandon et al. 1993, 1994), and *Rhizobium leguminosarum* (D’hooge et al. 1995; Patschkowski et al. 1996) have revealed that elements of this gene cluster are well conserved in the rhizobia, but that there are important differences in gene organization, regulation, and symbiotic relevance of the *fix* genes. The actual physiological

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Nucleotide and/or amino acid sequence data are to be found at EMBL as accession numbers Z80340 and Z80339.

role of the proteins encoded by the *fixNOQP* and *fixGHIS* operons has been demonstrated most clearly in *B. japonicum* (Preisig et al. 1993, 1996a, 1996b; Zufferey et al. 1996). There is compelling evidence that the *fixNOQP* operon codes for an electron transport chain terminating at an oxidase with a high affinity for oxygen (Preisig et al. 1996b; Zufferey et al. 1996) and that genes of the *fixGHIS* operon are necessary for synthesis or assembly of a functional FixNOQP complex (Preisig et al. 1996a).

Rhizobium leguminosarum is the type species of the genus *Rhizobium* (Jordan 1984) and is divided into biovars based on host-specificity. Biovar *viciae* nodulates the plants of the genera *Pisum*, *Lens*, *Lathyrus*, and *Vicia* (peas, lentils, sweet peas, and vetches, respectively), while biovar *trifolii* nodulates clovers (*Trifolium* spp.) and biovar *phaseoli* nodulates common beans (*Phaseolus* spp.). Nodulation and nitrogen fixation by *R. leguminosarum* have been studied in great detail, but when this work was initiated, little information was available on the organization and regulation of *fix* genes analogous to the *R. meliloti* *fix* cluster described above. In particular, since it had been shown that three different plasmids in *R. leguminosarum* bv. *viciae* were involved in formation of functional nitrogen-fixing nodules (Hynes and McGregor 1990), we were interested in determining the location of homologues to the *R. meliloti* *fixL*, *fixJ*, *fixK*, *fixNOQP*, and *fixGHIS* genes, and elucidating their role in symbiosis. Here we describe the identification, localization, isolation, and characterization of two copies of the *fixNOQP* operon in *R. leguminosarum* bv. *viciae*. The importance of the two *fixN* operons for symbiotic nitro-

gen fixation and the influence on expression of both *fixN* copies of the two Fnr-like regulators FnrN and FixK and the putative oxygen-sensor FixL were analyzed. Preliminary reports of this work have been presented at the 13th North American Conference on Symbiotic Nitrogen Fixation, Banff, Alberta, Canada, the 6th International Symposium on Molecular Plant Microbe Interactions, Seattle, WA, and the 9th International Congress on Nitrogen Fixation, Cancun, Mexico.

RESULTS

Identification and cloning of the two *fixNOQP* copies from *R. leguminosarum* bv. *viciae*.

Since previous studies had shown that two plasmids of *R. leguminosarum* strain VF39, in addition to the nodulation plasmid, were required for formation of a functional nitrogen-fixing nodule (Hynes and McGregor 1990), we were interested in exploring the possibility that known *fix* genes might reside on these plasmids. As, at the time this work was initiated, the location of the genes corresponding to the *R. meliloti* *fixL-J-K-NOQP-GHIS* cluster had not been firmly established, we set out to use DNA fragments from this cluster as probes. To identify *fixN* homologous genes in *R. leguminosarum* bv. *viciae*, a *R. meliloti* *fixN* probe was hybridized to total DNA of strain VF39. The *fixN* probe from plasmid pLZC4 (a 1.7-kb *Pst*I fragment carrying the 5' part of *R. meliloti* *fixN*) hybridized to an 11-kb *Eco*RI fragment and a 6.8-kb *Eco*RI fragment. The 6.8-kb fragment was absent in strain LRS39401 cured of pRleVF39d (the nodulation plasmid) whereas the 11-kb fragment was no longer present in strain LRS39301 cured of pRleVF39c. Strain LRS393401, missing both plasmids pRleVF39c and pRleVF39d, showed no homology to the *fixN* probe (Fig. 1). Therefore, it could be concluded that *R. leguminosarum* bv. *viciae* contains two copies of a *fixN* homologous region present on plasmids pRleVF39c and pRleVF39d. The 6.8-kb *Eco*RI fragment carrying one copy of the *R. leguminosarum* *fixN* homologous region was cloned from a partial library of VF39 DNA generated by cloning DNA from plasmids pRleVF39c and pRleVF39d purified from Eckhardt gels (see Material and Methods). One recombinant clone (pLZC6) carrying the 6.8-kb *Eco*RI was identified by hybridization to the *R. meliloti* *fixN* probe. Plasmid pLZC6 (*fixN* homologous region of plasmid pRleVF39d) was used as a probe for colony hybridization experiments to screen a cosmid library of VF39 total DNA in order to isolate the second *fixN* homologous region. Two overlapping cosmid clones (pLBS3 and pLBS4) were isolated that contained DNA that originated from pRleVF39c and hybridized to pLZC6 and the *R. meliloti* *fixN* probe. An 11-kb *Eco*RI fragment hybridizing to the *fixN* probe was subcloned from cosmid pLBS3 into pBluescript-IKS, resulting in plasmid pFG92. As well, two overlapping cosmids carrying the pRleVF39d *fixN* copy (pLBS1 and pLBS2) were isolated. Further hybridizations to these cosmids revealed that all contained, immediately adjacent to the *fixN* homologous region, DNA homologous to probes based on the *R. meliloti* *fixGHIS* operon. The characterization of these reiterated *fixGHIS* genes will be presented elsewhere (M. J. Mitsch, P. Rochepeau, J. Quandt, and M. F. Hynes, *unpublished*). *fixL* homologous regions were also detected upstream from the *fixN* operon on pRleVF39c, but not pRleVF39d (see

Fig. 1. Hybridization of total DNA digested with *Eco*RI from *Rhizobium leguminosarum* derivatives to *R. meliloti* *fixN* probe from pLZC4. Lane A: LRS39301; lane B: VF39SM; lane C: LRS393401; lane D: LRS39401; lane E: LRS39301; lane F: LRS39501.

also Patschkowski et al. 1996), although use of probes from *R. meliloti* corresponding to this gene had not revealed any homology at the total DNA level. A genetic map of the regions of pRleVF39c and pRleVF39d carrying the *fix* genes, based on restriction analysis, hybridization, and DNA sequencing (see below), is shown in Figure 2.

Sequence analysis of the two *R. leguminosarum* bv. *viciae* *fixN* homologous regions.

To localize the *fixN* homologous region on the 11-kb *Eco*RI fragment originating from pRleVF39c and present on plasmid pFG92, hybridization experiments to subclones with the *R. meliloti* *fixN* probe were performed. In this way, the *fixN* homologous region could be delimited to a 2.6-kb *Hind*III/*Bam*HI subfragment of plasmid pFG92. The complete nucleotide sequence of this fragment was established on both strands (part of this sequence has been published previously by Patschkowski et al. 1996). In addition, a 3-kb *Xho*I fragment overlapping the 2.6-kb *Hind*III/*Bam*HI fragment by 147 bp was used for sequencing of the complete *fixNOQP* operon. The sequence of 1,651 bp of this *Xho*I fragment was determined on both strands. An open reading frame (ORF) of 1,617 bp coding for a protein of 539 amino acids (aa) with a calculated molecular weight of 60.9 was identified (see Figure 3);

the deduced protein sequence is homologous to FixN and CcoN proteins of different species (94.8 to 63% identity; see Table 1). A putative ribosomal binding site is located 7 bp upstream of the *fixNc* ATG start codon (c denotes that the *fixN* operon is present on plasmid pRleVF39c), and a DNA sequence motif (TTGAT-N4-ATCAA) that is identical to the Fnr-consensus binding site was found 77 bp upstream of the *fixNc* start codon. Downstream of *fixNc*, three other ORFs of 732 bp (244 aa, 27.2 kDa), 150 bp (50 aa, 5.5 kDa), and 861 bp (287 aa, 30.8 kDa) were found, the deduced gene products of which are homologous to rhizobial FixO, FixQ, and FixP proteins and therefore were designated accordingly. All three genes were preceded by convincing ribosomal binding sites 6 to 8 bp upstream of the ATG start codon, respectively. Hybridization analysis revealed that the *fixN* homologous region present on plasmid pLZC6 (*fixNd*) could be delimited to a 2-kb *Xho*I fragment (see Figure 2B). One long ORF of 1,620 bp was detected after sequencing of the 1,997-bp *Xho*I fragment. The corresponding gene product of 540 aa with a calculated molecular weight of 61 shows 94.8% similarity to the *R. leguminosarum* VF39 *fixNc* gene product originating from plasmid pRleVF39c and 81.3% similarity to FixN of *R. meliloti*. A putative ribosomal binding site is located 7 bp upstream of the ATG start codon and a DNA sequence motif

Fig. 2. Physical and genetic maps of the two *Rhizobium leguminosarum* VF39 *fixN* regions. One *fixN* copy is located upstream of the *fixK* gene on plasmid pRleVF39c (A); the second *fixN* copy resides on plasmid pRleVF39d (B). Open reading frames (ORFs) homologous to rhizobial *fixK*, *fixL*, and *fixNOQP* are indicated. Black lines indicate DNA regions sequenced on both strands. ORFs homologous to rhizobial *fixO* and *fixP* on plasmid pRleVF39d are represented by broken lines because they were deduced from partial DNA sequence analysis. Black squares upstream of both *fixN* copies represent DNA motifs highly similar to the Fnr-consensus binding site (anaerobox). Insertions of two (*Nm^r*, neomycin resistance, and *Sp^r*, spectinomycin resistance) of the resistance gene cassettes used for mutational analysis are shown below restriction map. Other constructs described in text and in Quandt and Hynes (1993). Plasmids pTP111 and pMK5 carry *fixNc-gusA* and *fixNd-gusA* transcriptional reporter gene fusions, respectively. Location of restriction sites: B, *Bam*HI; Bc, *Bcl*I; E, *Eco*RI; H, *Hind*III; S, *Sal*I; and X, *Xho*I; given for both *fixN* regions (only those *Bcl*I sites relevant for the construction of *gusA* reporter gene fusions are shown).

(TTGAC-N4-ATCAA) that is very similar to the Fnr-consensus binding site was found 77 bp upstream of the *fixNd* start codon. Nine base pairs downstream of *fixNd* there is the beginning of an incompletely sequenced ORF that shows homology to rhizobial *fixO* genes. A possible *fixO* ribosomal binding site overlaps the *fixN* stop codon by 2 bp. Furthermore, partial DNA sequence analysis revealed the presence of a gene homologous to *fixP* downstream of *fixO*. Sequencing of the *fixGHISd* operon of VF39 also revealed the 3' end of an

intact ORF with high homology to *fixP* genes (M. J. Mitsch, P. Rochepeau, J. Quandt, and M. F. Hynes, *unpublished*). The presence of a *fixQ*-like gene downstream of *fixO* remains speculative since no sequence information is currently available on that region. No genes with similarity to rhizobial *fixL*, *fixJ*, or *fixK* could be detected upstream of *fixNd* by partial DNA sequence analysis. Comparison of the two *R. leguminosarum* bv. *viciae* *fixN* gene products revealed that they are highly similar to each other (only 28 aa substitutions over a

	I	II	
R.l. <i>FixNc</i>	MNYTTETMVI A V A A F L A L L G A A F A H -- D H L F A V H M G I L C F C L A V G A A L		46
R.l. <i>FixNd</i>	MNYTTETMVI A V A A F L A L L L A A F A H -- D H L F A V H M G I L C F C L A A G A A V		46
A.t. <i>FixN</i>	MNYTLETADRALGAFPALLGAAFAH--DSLFAAHMWLFFFTLVVSTLL		46
R.m. <i>FixN</i>	MKHTVEMVVL SVGAFLALVGAGLAQ--DRLFGAHMWLFFALLAGTLV		46
R.c. <i>CcoN</i>	MWDYVKLVALGVVAAIAAAYASQAR--DLPYVMNMVEVALAAVIALIW		46
P.d. <i>CcoN</i>	MLDTIKLIALGTIAVLAAIAANYARPDDLAYLVNALIIMLAAGIMFLR		48
B.j. <i>FixN</i>	MSQPSISKSMITIGESGLAVVFAATAFLCVIAAAKAL--DAPFAFHAALSAAASVAAVFC		57
A.c. <i>FixN</i>	MSIVQTPAKRMTGGELGLILVFAALGFFSIVVAAKAY--TPEYAFHAYLFAAASIATVVFV		58
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	III		
R.l. <i>FixNc</i>	LVRNVDFS PQ-PTAESR--YISYFDEVIRYGLIATVFWGVVGVFLVGVVIALQLAYPDLNI		103
R.l. <i>FixNd</i>	LIRRVDFS PAGQRKVD--ISGYFDEVIRYGLIATVFWGVVGVFLVGVVIALQLAFPDLNI		104
A.t. <i>FixN</i>	LLRRVSFLPPVAGPPCR--RTEYFDEVVKYGVMAVFWGVVGVFLVGVVVALQLAFPDLNI		104
R.m. <i>FixN</i>	LMRRVDFRPAVAGHPGR--RREYFDEVVKYGVVATVFWGVVGVFLVGVVVALQLAFPDLNI		104
R.c. <i>CcoN</i>	VLRTMGDPKPSKD-----EYFDGVI RAGVIATTFWGI VGVFLVAVVIAFQLAFPALNL		98
P.d. <i>CcoN</i>	VLRQMGNEQPALEPHPE---TQYMDDVVRAGVIATAFWGVVGVFLVGVVIAFQLAFPALNL		105
B.j. <i>FixN</i>	IVNRYFERPAALPPAEINGRPNYNGMPIKFSSFMAMFWGIAGFLVGLIIASQLAWPALNF		117
A.c. <i>FixN</i>	IGNRYMDRPAELPQPTIDGKPNYNMAPVKVGTLLAVFWGIAGFLIGVIIALQMAYPLNF		118
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	IV	V	
R.l. <i>FixNc</i>	APY----LNFGRRLRPVHTSAVIFAFGGNALIMTSFYVVQRTCRARLFGGSLA-WFVFWGY		158
R.l. <i>FixNd</i>	APY----LNFGRRLRPVHTSAVIFAFGGNALIMTSFYVVQRTCRARLFGGSLA-WFVFWGY		159
A.t. <i>FixN</i>	APY----FNFGRMRPLHTSAVIFAFGGNALIATSFYVVQRTCRARLFGGSLA-WFVFWGY		159
R.m. <i>FixN</i>	EPW----FNFGRVRPLHTSAVIFAFGGNALIATSFYVVQRTSRARLFGGDLG-WFVFWGY		159
R.c. <i>CcoN</i>	EFG-NGMLNFGRLRPLHTSAVIFAFGGNALIASAFYVVQRTSARLFGGTLGWFVFWGW		157
P.d. <i>CcoN</i>	SDITMGYTNFGKLRPLHTSAVIFAFGGNLIATSFYVVQRTSARLWGGNAA-WFVFWGY		164
B.j. <i>FixN</i>	DLP---WISFGRRLRPLHTSAVIFAFGGNVLIATSFYVVQKSCRVLRLAGDLAP-WFVFWGY		173
A.c. <i>FixN</i>	DLP---WISFGRRLRPLHTSAVIFAFGGNVLIATSFYVVQRTSHARLAGYLAP-WFVFWGY		174
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	VI		
R.l. <i>FixNc</i>	QLFIVMAATGYVLGITQAREYAEPEWYVDLWLTIVVWVAYLAVYLGTLTKRKEPHIYVANW		218
R.l. <i>FixNd</i>	QLFIVMAATGYVLGITQAREYAEPEWYVDLWLTIVVWVAYLAVYLGTLTKRKEPHIYVANW		219
A.t. <i>FixN</i>	NLFII MAATGYLLGITQGREYAEPEWYVDLWLTIVVWVAYLATFLGTILTRKEPHISVANW		219
R.m. <i>FixN</i>	QLFIVLAASGYLLGITQGREYAEPEWYVDLWLTIVVWVAYLAVLGTILTRKEPHIYVANW		219
R.c. <i>CcoN</i>	QLIIVTAATSYLLGSGQKEYAELNWHLDILVAVVWVAYLIAFLGTIFKRKEPHIYVANW		217
P.d. <i>CcoN</i>	QLFIVLAATGYILGATQKEYAELNWHLDILVAVVWVAYLIAFLGTILTRKEPHIYVANW		224
B.j. <i>FixN</i>	NFFILVAGTGYLLGVTQKEYAELNWHLDILVAVVWVAYLIAFLGTILTRKEPHIYVANW		233
A.c. <i>FixN</i>	NFFIVIAGTGYLLGITQKEYAELNWHLDILVAVVWVAYLIAFLGTILTRKEPHIYVANW		234
	* * * * *		
	VII	VIII	
R.l. <i>FixNc</i>	FYLSFIVTIAMHLVNNLAVPASFLGSKSYSAFSGVQDALTQWWYGHNAVGFLLTAGFLG		278
R.l. <i>FixNd</i>	FYLA FIVTIAMHLVNNLAVPVSLGSKSYSAFSGVQDALTQWWYGHNAVGFLLTAGFLG		279
A.t. <i>FixN</i>	FYLSFIVTIAMHLVNNLAVPVSLGSKSYSAFSGVQDALTQWWYGHNAVGFLLTAGFLG		279
R.m. <i>FixN</i>	FYLA FIVTIAMHLVNNLAVPVSLGSKSYSAFSGVQDALTQWWYGHNAVGFLLTAGFLA		279
R.c. <i>CcoN</i>	FYLSFIVTIAMHLVNNLAVPVSLGSKSYSAFSGVQDALTQWWYGHNAVGFLLTAGFLG		277
P.d. <i>CcoN</i>	FYLSFIVTIAMHLVNNLAVPVSLGSKSYSAFSGVQDALTQWWYGHNAVGFLLTAGFLG		284
B.j. <i>FixN</i>	FYLA FIVTIAMHLVNNLAVPVSLGSKSYSAFSGVQDALTQWWYGHNAVGFLLTAGFLA		293
A.c. <i>FixN</i>	FYLA FIVTIAMHLVNNLAVPVSLGSKSYSAFSGVQDALTQWWYGHNAVGFLLTAGFLA		294
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Fig. 3. Amino acid sequence alignment of the *Rhizobium leguminosarum* VF39 (R.l.) *fixNc* and *fixNd* gene products to the homologous proteins of *Agrobacterium tumefaciens* (At), (Schlüter et al. 1995), *Rhizobium meliloti* (Rm), (Kahn et al. 1993), *Rhodobacter capsulatus* (R.c.), (Thöny-Meyer et al. 1994) *Paracoccus denitrificans* (P.d.), (de Gier et al. 1996), *Bradyrhizobium japonicum* (Bj), (Preisig et al. 1993), and *A. caulinodans* (Ac), (Mandon et al. 1994). Amino acid residues identical in all eight sequences are marked by asterisks. Conservative substitutions are indicated by points. Histidine residues suggested to be involved in heme and copper binding are marked (+). The histidine residue marked by (•) has been proposed to be the second axial ligand of the low-spin heme b. Putative transmembrane helices are underlined. *Continued on next page.*

length of 540 aa; 94.8% identity). The most striking difference is located in a region from amino acid position 55 to 67 where 9 aa residues are different. All characteristics assigned to the rhizobial *fixNOQP* gene products are also valid for the corresponding *R. leguminosarum* proteins: (i) *fixNc* and *fixNd* are membrane proteins that contain 15 putative transmembrane helices; (ii) six conserved histidine residues suggested to serve as ligands for the CuB-heme binuclear center and the low-spin heme b are conserved in both *R. leguminosarum fixN* proteins; (iii) FixOc and FixOd are most probably membrane-anchored cytochrome c proteins that contain a heme c binding motif; (iv) FixQc is similar to rhizobial FixQ proteins (83.6 to 64.5% identity) and displays a hydrophobic character; and (v) FixPc

Phenotype of *R. leguminosarum* bv. *viciae* strains mutated in *fixNc*, *fixNd*, or both.

		IX	++	
R.l. FixNc		MMYYFRPKQANRPVYSYRLSIIHF	WALIFMDIWAGPHHLHYTALPDWAQTGLMVFSIMLW	338
R.l. FixNd		MMYYFVPKQANRPVYSYRLSIIHF	WALIFMYIWAGPHHLHYTALPDWAQTGLMVFSIMLW	339
A.t. FixN		MMYYFI PKQVNRPVYSYRLSIIHF	WALIFMYIWAGPHHLHYTALPDWAQTGLMVFSIMLW	339
R.m. FixN		MMYYFI PKQVNRPVYSYRLSIIHF	WALIFMYIWAGPHHLHYTALPDWAQTGLMVFSIMLW	339
R.c. CcoN		MMYYFVPKQAEPRVYSYKLSIVHF	WALIFLYIWAGPHHLHYTALPDWASTGLMVMVSILW	337
P.d. CcoN		MMYYFI PKQAERPVYSYKLSIIHF	WALIFLYIWAGPHHLHYTALPDWASTGLMVFSIILW	344
B.j. FixN		IMYYFI PKRAEPIYSYRLSIIHF	WALIFLYIWAGPHHLHYTALPDWTQTGLMTFSIMLW	353
A.c. FixN		LMYYFI PKRADKPVYSYRLSIVHF	WALIFLYIWAGPHHLHYTALPDWAQTGLMTFSIMLW	354
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		X	XI	395
R.l. FixNc		MPSWGGMINGLMTLSGAWDKIRTDPI	IRMMIVAIAFYGMSTFEFGPMMSVKTVNSLSHYTE	398
R.l. FixNd		MPSWGGMINGLMTLSGAWDKIRTDPI	IRMMIVAIAFYGMSTFEFGPMMSVKTVNSLSHYTE	399
A.t. FixN		MPSWGGMINGLMTLSGAWDKIRTDPI	VRMMVMVAFAFYGMATFEFGPMMSIKAVNSLSHYTD	399
R.m. FixN		MPSWGGMINGLMTLSGAWDKIRTDPI	VRMMVMVAFAFYGMATFEFGPMMSIKTVNSLSHYTD	399
R.c. CcoN		MPSWGGMINGLMTLSGAWDKLRDTPV	IRMVVVISIGFYGMSTFEFGPMMSIKAVNSLSHYTD	397
P.d. CcoN		MPSWGGMINGLMTLSGAWDKLRDTPV	IRMLVVAVAFGYMATFEFGPMMSIKAVNFVSHYTD	404
B.j. FixN		MPSWGGMINGLMTLSGAWDKLRDTPV	LRMLVVSAFYGMSTFEFGPMMSIKVNSLSHYTD	413
A.c. FixN		MPSWGGMINGLMTLSGAWDKLRDTPV	IRMMVVAVAFYGMATFEFGPMMSVKSVNSLSHYTE	414
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		+ + XII	XIII	
R.l. FixNc		WTIGHVHSGALGWVGMITFGAIYYLT	PKLWGRERLYSPRMVNWHFWLCNLGIVVIYA AVLW	458
R.l. FixNd		WTIGHVHSGALGWVGMITFGAIYYLT	PKLWGRERLYSLRMVNWHFWLATLGIVVYA AVLW	459
A.t. FixN		WTIGHVHSGALGWNGMITFGAIYYLT	PKLWGRDRLYSLQLVNWHFWLATLGIVVYA AVLW	459
R.m. FixN		WTIGHVHSGALGWNGLITFGAIYYLT	VPKLWNRERLYSVRMVNWHFWLATLGIVVYA AVLW	459
R.c. CcoN		WTIGHVHSGALGWNGMITFGMLYFLT	PRLWGRSGLYSLKLVS WHFWLATIGIVLYASAMW	457
P.d. CcoN		WTIGHVHSGALGWNGMITFGALYYLV	PRLWGRERLYSTGLVSWHFWLATIGIVLYAASMW	464
B.j. FixN		WTIGHVHSGALGWGVFSFGALYCLVP	WAWNKRGLYSLKLVNWHFWLATLGIVLYISAMW	473
A.c. FixN		WGIGHVHSGALGWVAYISFGAIYCLIP	WLWNKREMYSMKAIEWHFWVSTLGIVLYICAMW	474
		* ..* ..* ..* ..* ..* ..* ..* ..* ..*	*****.*****.* ** .****.	
		XIV	XV	
R.l. FixNc		VAGIQQLMWREYN SQFLVYSFAETVAAM	FPPYYVVRVGGTYLAGLVMAWN VFMTIR	518
R.l. FixNd		VAGIQQLMWREYN SQFLVYSFAETVAAM	FPPYYVLRVGGTYLAGLVMAWN VFMTIR	519
A.t. FixN		VAGIQQLMWREYDSQFLVYSFAESVAAL	FPPYYVMRALGGLMPLSGALIMAYNV TMTIL	519
R.m. FixN		VAGIQQLMWREYDDQFLVYSFAETVAAM	FPPYYVMRAAGGALFLAGALIMAFNV TMTIL	519
R.c. CcoN		VTGIMEGLMWREVDAQFLVNADFVAAKF	PMNVVRVGGVLYLLGGLIMAYNLWATVA	517
P.d. CcoN		VSGIMEGLMWREVDAQFLVNADFVAAKF	PMNVVRVGGVLYLLGGLIMCYNLWATVA	524
B.j. FixN		VSGILLQGLMWRYATSLGFLEYSFIETVEAM	HPFY IIRAAGGGLFLIGALIMAYNLWMTVR	533
A.c. FixN		VAGILLQGLMWRYATSLGFLEYSFIETVEAM	HPLVYIRAIGGILFLAGSLIMAWN VFMTIT	534
		*** *** ..* ..* ..* ..* ..* ..*	*****.*****.* ** .****.	
		GRLRDEAAIPTTFVPQAQPAE		539
R.l. FixNd		GHLRDEAAIPTTFAPQLQPAE		540
A.t. FixN		GHOREEGASKG-AAPSLQPAE		539
R.m. FixN		GRVRDEEPIFG-AAPLPAPAE		539
R.c. CcoN		KQP--KTANLAVAVPAE		532
P.d. CcoN		KQP--KTQSTAAAVPAE		539
B.j. FixN		VGAEAVQMP-VALQPAE		549
A.c. FixN		RAETVSPQSGAALAPAE		555

Table 1. Comparison of the *Rhizobium leguminosarum* bv. *viciae* *fixN* gene products with related proteins of other species

Protein	Amino acid identity (%)							
	<i>R.l. FixNc</i>	<i>R.l. FixNd</i>	<i>A.t. FixN</i>	<i>R.m. FixN</i>	<i>R.c. CcoN</i>	<i>P.d. CcoN</i>	<i>B.j. FixN</i>	<i>A.c. FixN</i>
<i>R.l. FixNc</i>	100	...	...	...	...	...	...	...
<i>R.l. FixNd</i>	94.8	100	...	...	...	...	...	...
<i>A.t. FixN</i>	81.1	82.0	100	...	...	...	...	...
<i>R.m. FixN</i>	80.0	81.3	85.0	100	...	...	...	...
<i>R.c. CcoN</i>	65.9	67.4	66.5	66.4	100	...	...	...
<i>P.d. CcoN</i>	68.6	69.5	70.7	70.0	78.7	100	...	...
<i>B.j. FixN</i>	63.1	63.8	63.6	65.4	61.4	62.9	100	...
<i>A.c. FixN</i>	63.0	64.1	63.7	64.4	59.2	61.8	75.9	100

^z Comparison of gene products was performed with the align program of the FASTA package (version 1.6c; Pearson 1990). Abbreviations: *R.l.* = *Rhizobium leguminosarum*; *A.t.* = *Agrobacterium tumefaciens*; *R.m.* = *Rhizobium meliloti*; *R.c.* = *Rhodobacter capsulatus*; *P.d.* = *Paracoccus denitrificans*; *B.j.* = *Bradyrhizobium japonicum*; *A.c.* = *Azorhizobium caulinodans*.

Table 2. Symbiotic nitrogen-fixation phenotype of *Rhizobium leguminosarum* bv. *viciae* *fixNc* and *fixNd* deletion/insertion mutants^x

<i>R. leguminosarum</i> strain	Relevant genotype	Dry weight (g per plant) ^z			Total N (mg per plant) ^z
		A. r. ^y			
VF39SM	Wild type	+	0.598 a		0.886 a
VF39-JQ12	<i>fixNc</i> :: Ω Sp	+	0.322 ab		0.384 bc
VF39-JQ302	Δ <i>fixNc</i> - <i>fixG</i> :: Ω Nm	+	0.417 ab		0.486 b
VF39-JQ11	<i>fixNd</i> :: Ω Sp	+	0.448 ab		0.563 ab
VF39-JQ321	<i>fixNd</i> :: Ω Sp, <i>fixNc</i> - <i>fixG</i> :: Ω Nm	–	0.235 b		0.269 c
Uninoculated control		–	0.255 b		0.282 c

^x The host plant *Pisum sativum* (cv. Trapper) was inoculated with the *R. leguminosarum* *fixN* mutants.

^y Acetylene reduction. Analyzed 32 days after inoculation.

^z Dry weights and total N values represent the mean values of six plants. Values followed by the same letter are not significantly different at *P* = 0.05 (least significant difference). Similar results were observed in three independent experiments.

bp *SalI* fragment by a *Sp*^r interposon cassette (Prentki and Krisch 1984; Fellay et al. 1987). This mutation was transferred into the VF39 genome via a double cross-over event resulting in mutant VF39-JQ11. *fixNc* mutants were constructed by replacement of a *fixNc* internal 575-bp *SalI* fragment by *Nm*^r and *Sp*^r cassettes, as well as by creating a larger deletion encompassing most of the *fixNOQP* operon and part of *fixG* (see Materials and Methods). These mutations were transferred into *R. leguminosarum* VF39SM (wt) resulting in mutant strains VF39-JQ10, JQ12, and JQ302, and into strain VF39-JQ11, resulting in the *fixN/fixNd* double mutants VF39-JQ1011 and VF39-JQ321. Nodules induced by the *fixNc* mutants on *Pisum sativum* plants showed a *Fix*⁺ phenotype, although shoot dry weight and total nitrogen content of the plants inoculated with this mutant were less than those of plants inoculated with the parent strain, VF39SM (Table 2). The *fixNd* mutant (VF39-JQ11) showed a similar phenotype. Nodules induced by the *fixNc/fixNd* double mutants (VF39-JQ321 and VF39-JQ1011) showed a distinct *Fix*[–] phenotype, demonstrating that both VF39 *fixN* copies are functional and that at least one functional copy is essential for nitrogen fixation. Plant tests with *Vicia hirsuta* as a host gave results for mutants similar to those for tests done on *P. sativum* although the statistical significance was less pronounced due to smaller sample and plant sizes.

Analysis of *fixNc* and *fixNd* expression under free-living microaerobic conditions

The presence of putative Fnr-consensus binding sites up-

Table 3. Expression of *Rhizobium leguminosarum* VF39 *fixNc*-*gusA* and *fixNd*-*gusA* reporter gene fusions in different mutant backgrounds

<i>R. leguminosarum</i> bv. <i>viciae</i> strain	Relevant genotype	β -glucuronidase activity (units) ^z	
		Aerobic	Micro-aerobic
VF39SM	Wild type	21.4	18.3
VF39 (pTP111)	Wild type, <i>fixNc</i> - <i>gusA</i>	19.3	159.0
VF39-TP4 (pTP111)	<i>fixL</i> , <i>fixNc</i> - <i>gusA</i>	14.8	44.6
VF39-TP6 (pTP111)	<i>fnrN</i> , <i>fixNc</i> - <i>gusA</i>	16.5	34.5
VF39-TP11 (pTP111)	<i>fixK</i> , <i>fixNc</i> - <i>gusA</i>	27.2	243.0
VF39-TP12 (pTP111)	<i>fnrN/fixK</i> , <i>fixNc</i> - <i>gusA</i>	20.5	38.1
VF39 (pMK5)	Wild type, <i>fixNd</i> - <i>gusA</i>	19.1	115.7
VF39-TP4 (pMK5)	<i>fixL</i> , <i>fixNd</i> - <i>gusA</i>	17.7	45.9
VF39-TP6 (pMK5)	<i>fnrN</i> , <i>fixNd</i> - <i>gusA</i>	17.7	38.1
VF39-TP11 (pMK5)	<i>fixK</i> , <i>fixNd</i> - <i>gusA</i>	20.4	200.4
VF39-TP12 (pMK5)	<i>fnrN/fixK</i> , <i>fixNd</i> - <i>gusA</i>	16.3	33.2

^z β -glucuronidase units were calculated according to Sambrook et al. (1989) and represent the mean values of at least three independent measurements. Standard deviations were less than 23 % in every case.

stream of both *fixN* copies, together with the model for *fixN* regulation in *R. meliloti* (Batut et al. 1989), suggested that transcription of the *fixN* operons is regulated by an Fnr-like regulatory protein. The Fnr-consensus binding sites located upstream of *fixNc* and *fixNd* differ in certain base pairs (*fixNc*: TTGATGTAGATCAA versus *fixNd*: TTGACGCAGATCAA) and VF39 possesses two known Fnr-like proteins, FnrN and FixK (Schlüter et al. 1992; Patschkowski et al. 1996), so it was possible that the two copies are differentially regulated. Therefore, the influence of these regulatory components on the expression of the *fixN* operons was analyzed. In addition, *fixN* expression was studied in a *fixL* mutant background since *fixL* codes for a putative oxygen-sensing regulatory component. To analyze *fixN* expression, *gusA* transcriptional reporter gene fusions of both *fixN* copies were constructed. A *BclI* restriction site is conserved 75 bp downstream of the ATG start codon in *fixNc* and *fixNd* and was used for insertion of a promoterless *gusA* cassette. The *fixNc*-*gusA* and *fixNd*-*gusA* fusions, present on the mobilizable plasmids pTP111 and pMK5, respectively, were transferred to *R. leguminosarum* strains VF39SM (wt), VF39-TP4 (*fixL*), VF39-TP6 (*fnrN*), VF39-TP11 (*fixK*), and VF39-TP12 (*fnrN/fixK*) and integrated into the *fixN* regions via homologous recombination. Correct integration of the fusions into the corresponding *fixN* operons (*fixNc* or *fixNd*, respectively) was verified by hybridization analysis with labeled *fixNc* and *fixNd* probes, respectively.

Both *fixN*-*gusA* fusions were induced under microaerobic

Fig. 4. Symbiotic expression of *Rhizobium leguminosarum* VF39 *fixNc-gusA* and *fixNd-gusA* fusions in *Vicia hirsuta* root nodules 25 days after inoculation. Sections of root nodules (thickness approx. 100 μ m) that were induced by the strains (A) VF39SM (wild type; wt) (pTP111, *fixNc-gusA*); (B) VF39SM (pMK5, *fixNd-gusA*); (C) VF39-TP6 (*fnrN*) (pMK5, *fixNd-gusA*); (D) VF39-TP11 (*fixK* Δ) (pMK5, *fixNd-gusA*); (E) VF39-TP12 (*fnrN/fixK* Δ) (pMK5, *fixNd-gusA*); (F) VF39-TP4 (*fixL*) (pMK5, *fixNd-gusA*) were stained with X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) for β -glucuronidase activity.

conditions by a factor of 8 for *fixNc* and 6 for *fixNd* (Table 3). Induction of *fixN* expression was impaired in *fixL* and *fnrN* mutants (induction factor: 2 to 3) whereas *fixN* expression was even higher in a *fixK* mutant background, compared with the wild type (induction factor: 9 to 10). A mutation in *fixK* and *fnrN* (double mutant VF39-TP12) had nearly the same effect on *fixN* expression as a single mutation in *fnrN* (VF39-TP6). Differential responses of *fixNc* and *fixNd* to the regulatory components FnrN, FixK, and FixL could not be observed. These results document that FixL and FnrN are involved in positive regulation of both *fixN* operons under free-living microaerobic conditions, whereas FixK obviously is dispensable for *fixN* expression.

In situ expression analysis of *fixNc-gusA* and *fixNd-gusA* fusions in *V. hirsuta* root nodules.

To analyze *fixN* expression in planta, *V. hirsuta* seedlings were inoculated with *R. leguminosarum* strains VF39SM (wild type, wt), VF39-TP4 (*fixL*), VF39-TP6 (*fnrN*), VF39-TP11 (*fixK*), and VF39-TP12 (*fnrN/fixK*) carrying either the integrated *fixNc-gusA* or the *fixNd-gusA* reporter gene fusion. Histochemical analysis of β -glucuronidase (GUS) expression in the root nodule tissue was done by the GUS-specific staining technique with X-Gluc in longitudinal sections (thickness of about three cell layers) of the nodule. Both *fixN-gusA* fusions were expressed in the symbiotic zone of the root nodule (Fig. 4). Expression of *fixNc* and *fixNd* was highly reduced in *fnrN* and *fixL* mutant backgrounds. However, some residual *fixN* expression could be detected in these mutants. No significant difference could be observed when comparing *fixN* expression in the *fnrN* mutant (VF39-TP6) and in the *fnrN/fixK* double mutant (VF39-TP12). A mutation in *fixK* had no effect on the expression of either *fixN* fusion. Differential expression of the *fixN* copies in response to the regulatory components FixL, FnrN, and FixK could not be observed, and it is for this reason that only the pMK5 derivatives are shown in Figure 4. These results demonstrate that FixL and FnrN are involved in positive symbiotic regulation of *fixN* expression whereas FixK is dispensable for *fixN* expression.

DISCUSSION

The *fixNOQP* genes and their homologues have now been identified and characterized in four different rhizobia, as well as *Agrobacterium*, *Rhodobacter*, and *Paracoccus* (see references in Figure 3). When the structure of these genes and the flanking sequences in the different rhizobia are analyzed, it can be seen that there are several differences. Both *R. meliloti* (Renalier et al. 1987; David et al. 1988) and *R. leguminosarum* have two copies of the operon, but in *R. meliloti* they are on the same megaplasmid, whereas in *R. leguminosarum* they occur on different plasmids. This reiteration of *fixNOQP* on two plasmids is not restricted to strain VF39; we have shown by hybridization that most other strains we have examined have a similar arrangement (data not shown). *Bradyrhizobium japonicum* and *Azorhizobium caulinodans* have only one copy of this operon (Preisig et al. 1993; Mandon et al. 1994). All the rhizobia studied so far have *fixGHIS* genes located downstream from the *fixNOQP* operon (Kahn et al. 1989; Mandon et al. 1994; Preisig et al. 1996a), although in *R. meliloti* this is true for only one copy of the *fixNOQP* operon. It is also common for some or all of the *fixL*, *fixJ*, and *fixK*

genes to be located upstream of *fixNOQP*, and this is also true for one copy (the pRleVF39c copy) in VF39 (Patschkowski et al. 1996). However, the orientation of these genes differs in different rhizobia. The arrangement of these *fix* genes in *R. etli*, the bean symbiont, is remarkably similar to that described in this paper, as there are two copies of *fixNOQP* on different plasmids, and it is the copy on the plasmid other than the nodulation plasmid that is preceded by *fixL* (but not *fixJ*) (Maria de Lourdes Girard, *personal communication*).

Just as there are differences in arrangement of these genes, there appear to be significant differences in their importance to symbiosis among the different rhizobia, and in the regulation of the genes. Mutants of *R. meliloti* (Renalier et al. 1987) and *B. japonicum* (Preisig et al. 1993) lacking *fixNOQP* activity are unable to fix nitrogen symbiotically, whereas *A. caulinodans* mutants retain considerable nitrogen-fixing ability (Mandon et al. 1994). We have shown here that both copies of *fixNOQP* in VF39 are functional, and that at least one intact copy is required for nitrogen fixation. Single mutants fixed less nitrogen (sometimes significantly so) than wild-type VF39SM in our growth chamber experiments; in fact, if statistical significance was taken at $P = 0.1$ instead of $P = 0.05$, our results always showed a difference between single mutants and wild type. Therefore, it appears possible that, under field conditions, both copies are required intact for optimal nitrogen fixation.

The data presented here on regulation of both *fixN* operons under free-living and symbiotic conditions reveal several novel features not seen in other rhizobia studied so far. In *R. meliloti*, FixL, FixJ, and FixK are all required for *fixN* expression (Batut et al. 1989; David et al. 1988), and this is also the case in both *B. japonicum* (Preisig et al. 1993) and *A. caulinodans* (Mandon et al. 1994). In contrast, we have shown here that expression of *fixN* is largely independent of FixK, and that there is still residual induction of expression of both copies of the *fixN* operon in the absence of FixL or FnrN. These data are compatible with the fact that although FnrN and FixL do play a role in N fixation (Colonna-Romano et al. 1990; Patschkowski et al. 1996), they themselves are not absolutely required for N fixation. The evidence strongly suggests that there is a further regulatory protein that participates in activation of *fixN* promoters in *R. leguminosarum*, as D'hooge et al. (1995) have also shown that *fixL* mutants of biovar *phaseoli* are still able to fix nitrogen. There is evidence from analysis of *hup* gene expression in strain UPM791 of *R. leguminosarum* bv. *viciae* that there may be a further copy of an *fnr*-like gene (Hernando et al. 1995). Alternatively, other, as yet unidentified, proteins may interact with the *fixN* promoters.

The function of the *fixNOQP* genes has been most widely studied in *B. japonicum*, where it was first demonstrated that the deduced gene products have characteristics typical of subunits of terminal oxidases. The *fixN* gene product possesses six conserved histidine residues that serve as ligands for a heme-copper binuclear center and a low-spin heme group. FixO and FixP were predicted to be membrane-anchored cytochrome c proteins (Preisig et al. 1993). Extensive biochemical FixP constitute a cbb3-type oxidase (Preisig et al. 1996b; Zufferey et al. 1996). The *fixQ* gene product that is also encoded by the *fixNOQP* operon is dispensable for the function of the cbb3-oxidase. Biochemical analyses of the *B. japonicum* FixNOP oxidase complex also led to the conclusion that it sup-

ports microaerobic respiration of bacteroids in the root nodule (Preisig et al. 1996b), and it has now been shown that the genes of the *fixGHIS* operon are necessary for synthesis or assembly of FixNOP proteins, and probably encode a copper transport system (Preisig et al. 1996a). It has long been postulated that an alternative terminal oxidase with a high affinity for oxygen is expressed by rhizobia in the bacteroid state (Appleby 1984; Hunt and Layzell 1993), as this would be a prerequisite for energy generation by the rhizobia, most of which are strict aerobes. It now appears quite certain that in all of the rhizobia, the FixN, FixO, and FixP proteins fulfill

this function, although *A. caulinodans* appears to contain a system that can at least partially complement for the absence of FixNOP. Analysis of the results of the present study give further confirmation to this model in that (i) the deduced amino acid sequences of the *fixNOQP* gene products show all the features of a *cbb3*-type oxidase system pointed out for *B. japonicum*, (ii) a functional *fixNOQP* operon is required for nitrogen fixation, and (iii) expression of both copies of the operon is induced under low-oxygen conditions. Very recently, two symbiosis-specific c-type cytochromes and a putative oxidase were identified at the protein level in bacteroids of *R.*

Table 4. Bacterial strains and plasmids

Strains	Relevant characteristics	Source or reference
<i>Escherichia coli</i>		
S17-1	Modified RP4 plasmid integrated into the genome of <i>E. coli</i> 294 <i>recA</i>	Simon et al. (1983)
JM83	$\Delta(lac\ pro)$, <i>thi</i> , $\Phi 80d$, <i>lacZ</i> , $\Delta M15$, <i>rpsL</i>	Vieira and Messing (1982)
XL1-Blue	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>hsdR17</i> , <i>supE44</i> , <i>lac</i> , [<i>F'</i> <i>proAB</i> , <i>lacIq</i> <i>lacZ</i> $\Delta M15$, <i>Tn10</i> (Tc ^r), <i>thi</i>]	Bullock et al. 1987
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>		
VF39	Wild type	Hynes et al. (1988)
VF39SM	Spontaneous Sm ^r derivative of wild-type VF39	Priefer (1989)
VF39-TP4	VF39SM <i>fixL</i> ::Gm ^r mutant	Patschkowski et al. (1996)
VF39-TP6	VF39SM <i>furN</i> ::Gm ^r mutant	Patschkowski et al. (1996)
VF39-TP11	VF39SM <i>fixK</i> deletion mutant	Patschkowski et al. (1996)
VF39-TP12	VF39SM <i>furN</i> / <i>fixK</i> double mutant	Patschkowski et al. (1996)
LRS39301	VF39SM, pRleVF39c plasmid cured, Fix ⁻	Hynes and McGregor (1990)
LRS39401	VF39SM, pRleVF39d plasmid cured, Nod ⁻	Hynes and McGregor (1990)
LRS393401	VF39SM, pRleVF39c and d cured, Nod ⁻	This work
LRS39501	VF39SM, pRleVF39e plasmid cured, Fix ⁻	Hynes and McGregor (1990)
VF39-JQ10	VF39SM, <i>fixNc</i> :: Ω Nm, Fix ⁺	This work
VF39-JQ11	VF39SM, <i>fixNd</i> :: Ω Sp, Fix ⁺	This work
VF39-JQ12	VF39SM, <i>fixNc</i> :: Ω Sp, Fix ⁺	This work
VF39-JQ302	VF39SM, <i>fixNc</i> - <i>fixG</i> :: Ω Nm, Fix ⁺	This work
VF39-JQ1011	VF39SM, <i>fixNc</i> :: Ω Nm, <i>fixNd</i> :: Ω Sp, Fix ⁻	This work
VF39-JQ321	VF39SM, <i>fixNd</i> :: Ω Sp, <i>fixNc</i> - <i>fixG</i> :: Ω Nm, Fix ⁻	This work
Plasmids		
pBluescript-II	Sequencing vector, Ap ^r	Stratagene (La Jolla, CA)
pSVB28	Sequencing vector, Ap ^r	Arnold and Pühler (1988)
pUC18	sequencing vector, Ap ^r	Yannish-Perron et al. (1985)
pSUP202	pBR325 derivative, mob, Tc ^r , Ap ^r , Cm ^r	Simon et al. (1983)
pK18-mob	pK18 derivative, mob, Km ^r	Schäfer et al. (1994)
pJQ200KS	pACYC184 derivative, mob Gm ^r , <i>sacB</i> , pBluescript-II KS mcs	Quandt and Hynes (1993)
pRK7813	Cosmid vector	Jones and Guttererson (1987)
pHP45W	pBR322 derivative (Ap ^r) carrying a Sm ^r /Sp ^r interposon	Prentki and Krisch (1984)
pBI221.1	pUC19-derivative, <i>gusA</i> cassette	Jefferson (1987)
pLZC4	pBluescript-II KS ⁺ derivative, 1.7-kb <i>PstI</i> fragment carrying <i>R. meliloti</i> <i>fixN'</i>	This work
pLZC6	pBluescript-II KS ⁺ derivative, 6.8-kb <i>EcoRI</i> fragment carrying the VF39 <i>fixNd</i> region	This work
pLBS1/pLBS2	pRK7813 cosmid derivatives carrying the VF39 <i>fixNd</i> region	This work
pLBS3/pLBS4	pRK7813 cosmid derivatives carrying the VF39 <i>fixNc</i> region	This work
pFG92	pBluescript-II KS ⁺ derivative, 11-kb <i>EcoRI</i> fragment carrying the VF39 <i>fixNc</i> region	This work
pTP86	pBluescript-II KS ⁺ derivative carrying a promoterless <i>gusA</i> cassette	Patschkowski et al. (1996)
pTP93	pBluescript-II KS ⁺ derivative, 3-kb <i>XhoI</i> fragment carrying the VF39 <i>fixOQP</i> region	This work
pTP94	pBluescript-II KS ⁺ derivative, 2.6-kb <i>HindIII</i> / <i>BamHI</i> fragment carrying VF39 <i>fixK'</i> - <i>fixNO'</i>	This work
pSWS1	pSVB28 derivative, 2-kb <i>XhoI</i> fragment carrying the VF39 <i>fixNd</i> gene	This work
pJQ245	pJQ200KS derivative, <i>fixNd</i> :: Ω Sp	Quandt and Hynes (1993)
pJQ302	pJQ200SK, <i>fixNc</i> - <i>fixG</i> :: Ω Nm	Quandt and Hynes (1993)
pJQ291	pJQ200SK, 3.3-kb <i>NotI</i> - <i>XhoI</i> fragment carrying <i>fixL'</i> - <i>fixK</i> - <i>fixNc</i>	This work
pJQ292	pJQ200SK, <i>fixNc</i> :: Ω Nm	This work
pJQ292Sp	pJQ200SK, <i>fixNc</i> :: Ω Sp	This work
pTP111	pSUP202 derivative, <i>fixNc</i> - <i>gusA</i> fusion	This work
pMK5	pK18-mob derivative, <i>fixNd</i> - <i>gusA</i> fusion	This work

^z Abbreviations for antibiotic resistances: Ap = ampicillin; Cm = chloramphenicol; Gm = gentamicin; Km = kanamycin; Nm = neomycin; Sp = spectinomycin; Sm = streptomycin; Tc = tetracycline.

leguminosarum bv. *viciae* (Vargas et al. 1996), and it now appears quite likely that these proteins correspond to the products of the genes of the *fixNOQP* operons.

It is still unclear why *R. meliloti* and *R. leguminosarum* have two copies of the *fixNOQP* operon, whereas *B. japonicum* and *A. caulinodans* have only one. Our study showed no evidence of differential regulation of the two copies, and this question has not yet been addressed in *R. meliloti*. However, our results from measurements of total nitrogen in plants inoculated with mutants in single copies of *fixN* do suggest that both copies are required for optimal fixation of nitrogen. Measurements of acetylene reduction did not reveal this difference but this is not surprising, as acetylene reduction measures fixation at only one point in time, and not over the growth cycle of a plant. It is thus important to use other parameters to assess symbiotic performance, as we have done in this study. Since it appears to be the case that a single copy of *fixNOQP* does not give optimal symbiotic performance, it is interesting to speculate that increased expression of this operon might be a means to enhance nitrogen fixation in genetically engineered strains of *R. leguminosarum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Bacterial strains and plasmids are listed in Table 4. *Escherichia coli* was grown at 37°C in Antibiotic Medium No. 3 (Difco, Detroit, MI). Antibiotics, where needed, were used at concentrations as described by Patschkowski et al. (1996). *Rhizobium* strains were grown in TY medium (Beringer 1974) at 30°C. When needed, antibiotics were added at concentrations as outlined by Patschkowski et al. (1996). Cultivation of *Rhizobium* strains under aerobic and microaerobic conditions was done as described elsewhere (Patschkowski et al. 1996). Indicator medium for strains expressing β -galactosidase activity was supplemented with 40 g of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) per ml (final concentration). GUS activity was detected on agar medium containing 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) at a final concentration of 40 g/ml.

Genetic methods.

The protocols for mating experiments and mobilization of plasmids between *E. coli* and *Rhizobium* strains were as described previously (Simon 1984). Strain LRS393401, cured of both plasmids pRleVF39c and pRleVF39d, was constructed by labeling the plasmids of LRS39401 with Tn5B12S (Hynes et al. 1989) and isolating an insert in pRleVF39c, and subsequently curing the plasmid with *sacB* selection, as described by Hynes et al. (1989).

Standard DNA techniques.

Isolation of total and plasmid DNA was performed as described previously (Priefer 1984; Priefer et al. 1984). Plasmid DNA and DNA fragments were purified with a Qiagen Plasmid Kit (Qiagen, Hilden, Germany) or a Prep-a-Gene Kit (BioRad, Hercules, CA) containing a glass powder suspension according to the manufacturer's instructions. Restriction enzyme digestion, alkaline phosphatase treatment, agarose gel electrophoresis, DNA cloning, and transformations were carried out according to Sambrook et al. (1989). Eckhardt gel

analysis was done as outlined by Hynes and McGregor (1990). Southern blotting and hybridization experiments were done by alkaline blotting with Zeta Probe membranes (BioRad) and digoxigenin-labeled probes (Boehringer Mannheim, Laval, Canada), following the protocols specified by the manufacturers.

Cloning of the *R. leguminosarum* VF39 *fixN* homologous regions.

The clone pLZC6 carrying the *R. leguminosarum* bv. *viciae* *fixN* copy from plasmid pRleVF39d was obtained by purifying DNA from plasmids pRleVF39c and pRleVF39d from Eckhardt gels run as described by Hynes and McGregor (1990) by a freeze/squeeze technique, digesting this DNA with *EcoRI*, and ligating it into dephosphorylated *EcoRI*-cut pBluescript-IKS. Clones containing a *fixN* homologous region were identified by isolating plasmid DNA from transformants, digesting the DNA with *EcoRI*, and Southern hybridization with the *R. meliloti* *fixN'*, present on plasmid pLZC4, as a probe. pLZC4 carries a 1.7-kb *PstI* fragment from pTH2 (Renalier et al. 1987) that includes the N terminal part of the *fixN* operon (Batut et al. 1989; David et al. 1988). To clone the second *fixN* copy, a cosmid library of VF39 total DNA in pRK7813 (Jones and Gutterson 1987) was prepared by the procedure outlined by Hynes et al. (1986) with the following modifications: DNA was partially digested with *BamHI*, and vector DNA was dephosphorylated with a column containing immobilized alkaline phosphatase (purchased from BioCan Scientific, Toronto). Colony hybridization experiments with the *fixN* region present on plasmid pLZC6 as a probe led to the identification of cosmid clones pLBS3 and pLBS4 carrying the second *R. leguminosarum* VF39 *fixN* homologous region.

Sequence analysis.

An 11-kb *EcoRI* fragment hybridizing to the *fixN* probe (pLZC6) was subcloned from cosmid pLBS3 into pBluescript-IKS, resulting in plasmid pFG92. The *fixN* homologous region could be delimited to a 2.6-kb *HindIII/BamHI* subfragment of plasmid pFG92. This subfragment was cloned into pBluescript-IKS, resulting in plasmid pTP94, and subjected to nested deletion formation with a Nested Deletion Kit (Pharmacia, Uppsala, Sweden). Sequencing of the resulting subclones on both strands was done on an A.L.F. DNA Sequencer (Pharmacia) following the instructions of an AutoRead sequencing kit (Pharmacia). In addition, a 3-kb *XhoI* fragment overlapping the 2.6-kb *HindIII/BamHI* fragment by 147 bp was used for sequencing the whole *fixN* operon. Sequencing of the 3-kb *XhoI* fragment (present on plasmid pTP93) was achieved as outlined above. The *fixN* homologous region present on plasmid pLZC6 could be delimited to a 2-kb *XhoI* fragment by hybridization analysis. This *XhoI* fragment was cloned into the sequencing vector pSVB28, resulting in plasmid pSWS1, and subjected to nested deletion formation. Sequencing of the fragment was done as described above. Sequence analysis and determination of the coding probability was done with the computer programs of Staden (1986). The deduced amino acid sequences were compared with the protein data base NCBI Blast E-Mail Server (Altschul et al. 1990; Gish and States 1993). Hydrophobicity analyses were done with the Profile-graph program.

Construction of *R. leguminosarum* VF39 *fixN* mutants.

Mutation of the *fixNd* copy originating from plasmid pRleVF39d was achieved by cloning the 2-kb *XhoI* fragment carrying *fixNd* into the *SalI* site of pJQ200KS and then replacing a *fixNd* internal 0.575-kb *SalI* fragment with a spectinomycin (Sp) resistance gene cassette (the Ω interposon described by Prentki and Krisch [1984] and Fellay et al. [1987]). The resulting plasmid (pJQ245) was used for homogenization of the *fixNd* mutation into the plasmid pRleVF39d (as described by Quandt and Hynes [1993]), giving strain VF39-JQ11. Construction of *fixNc* mutants was carried out in the same fashion by inserting a Ω Sp cassette and a Ω Nm cassette in place of the *SalI* fragment in pJQ291, to give pJQ292Sp and pJQ292, respectively. A further mutant was created by replacing a large (ca. 4.8 kb) *SalI* fragment that takes in most of the *fixN* operon and the 5' part of *fixG* with a Ω Nm cassette to give pJQ302 (Quandt and Hynes 1993). Double mutants were created by introducing this latter mutation into strain VF39-JQ11 to give VF39-JQ321. A second double mutant, with *fixGc* unaffected, was created by recombining pJQ292 into strain VF39-JQ11 to give VF39-JQ1011. All of these mutant strains were verified by the presence of the correct antibiotic resistance markers as well as by Southern hybridization.

Construction of a *R. leguminosarum* VF39 *fixNc-gusA* fusion.

A 522-bp *BclI* fragment from plasmid pTP94, containing the 3' part of *fixK* and the 5' end of *fixNc*, was cloned into the *BamHI* site of pUC18. A *XbaI/PstI* *gusA* cassette originating from plasmid pTP86 was joined to the 5' part of *fixNc*, thus creating a *fixNc-gusA* transcriptional fusion. An *EcoRI* fragment carrying the *fixNc-gusA* fusion was subsequently cloned into the mobilizable plasmid pSUP202, resulting in plasmid pTP111.

Construction of a *R. leguminosarum* VF39 *fixNd-gusA* fusion.

A 1,155-bp *HindIII/BclI* fragment carrying the 5' end of *fixNd* (see Figure 2) was cloned into the mobilizable plasmid pK18-mob (Km^r). An *SmaI/EcoRI* *gusA* cassette originating from plasmid pBI221.1 was ligated to the 5' part of the *fixNd* gene, thus creating a *fixNd-gusA* transcriptional fusion. The resulting plasmid was designated pMK5.

Enzyme tests.

GUS activity was measured with the substrate p-nitrophenyl- β -D-glucuronide, as described by Jefferson (1987). GUS units were calculated according to the Miller method as described by Sambrook et al. (1989).

GUS-specific staining of nodule sections.

Nodules were excised from the roots of *V. hirsuta* plants and embedded in 6% agarose. Sections of about 100- μ m thickness were cut with a manual microtome. Nodule sections were stained for 30 min in GUS-staining buffer containing 2 mM potassium-ferricyanide, 2 mM potassium-ferrocyanide, and 2 mM X-Gluc. Stained sections were rinsed in water and transferred onto slides. Sections were photographed with an Olympus OM-4 camera mounted onto an Olympus BX50 microscope.

Plant tests.

Nodulation and nitrogen fixation tests with *P. sativum* and *V. hirsuta* as host plants were performed as described by Hynes et al. (1988), with both dry weights and total nitrogen concentrations being measured. As a confirmatory test, acetylene reduction assays for determination of the presence of nitrogenase activity were carried out as described by Priefer (1989).

ACKNOWLEDGEMENTS

We are indebted to Neil Mattatal for technical assistance, and to Pierre Boistard for providing us with *R. meliloti* clones from the *fix* region. The financial support of an NSERC research grant and a National Biotechnology strategy fund grant to M.F.H., and grants from the Deutsche Forschungsgemeinschaft (Pr 279/3-1) and the European Community (BIOT-CT90-0166 and CHRX-CT94-0699) to U.B.P., are gratefully acknowledged.

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