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The symbiotic nitrogen fixation regulatory operon (*fixRnifA*) of *Bradyrhizobium japonicum* is expressed aerobically and is subject to a novel, *nifA*-independent type of activation

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ABSTRACT

The Bradyrhizobium japonicum N_2 fixation regulatory gene, nifA, was sequenced and its transcription start site determined. Between the start of transcription and the nifA gene an open reading frame of 278 codons was found and named fixR. A deletion in fixR which allowed transcription into nifA resulted in a 50% reduced Fix activity. The fixRnifA operon was expressed in soybean root nodules, in cultures grown anaerobically with nitrate as terminal electron acceptor, in microaerobic cultures, and in aerobic cultures. The transcription start site (+1) was preceded by a characteristic nif(-24/-12)-type promoter consensus sequence. Double base-pair exchanges in the -12 but not in the -24 region resulted in a 'promoter-down' phenotype. A promoter-upstream DNA region between -50 and -148 was essential for maximal promoter activity. Expression from the promoter was not dependent on nifA. We conclude that the fixRnifA promoter is positively controlled, and that it requires a newly postulated transcriptional factor in order to become activated.

INTRODUCTION

Expression of nitrogen fixation genes is subject to positive control. It has first been shown in the enterobacterium Klebsiella pneumoniae that the product of the nifA gene is required to activate other nif genes (for review see Refs. 1, 2). The presence of a nifA gene has also been demonstrated in several species of the symbiotic, nitrogen-fixing root and stem nodule bacteria belonging to the genera Rhizobium, Bradyrhizobium and Azorhizobium (3, 4, 5, 6). The NifA protein is believed to bind to a characteristic activator sequence (5'-TGT-N_{10}-ACA-3'; 7, 8) at a position more than 100 bp upstream of the typical nif(ntr,-24/-12) consensus promoter sequence (5'-CTGGCAC-N_5-TTGCA-3'; 9, 10, 11) thereby activating the initiation of nif gene transcription by RNA polymerase, which in turn is dependent on a specific sigma factor (the product of ntrA [rpoN, glnF]; see Ref. 2 for review). [For reasons outlined in the Discussion section we use the term "-24/-12" promoter throughout this paper.] In K. pneumoniae the function of the NifA protein is adversely affected by oxygen and intermediate concentrations of

ammonia via the product of the nifL gene (12, 13) which is located upstream of nifA and forms part of the nifLA operon (1). Evidence for a similar antagonistic effect of a NifL-like protein in other diazotrophs is lacking. In Bradyrhizobium japonicum a nifL-independent, direct response of nifA-mediated nif gene regulation to oxygen has been demonstrated (14).

Of particular interest is the question how the expression of the nifA gene itself is regulated. In K. pneumoniae the nifLA operon is regulated by the global nitrogen control circuitry, which includes that it is activated by the active form of the NtrC protein, whereas it is not expressed under conditions of ammonia excess (2). In Rhizobium meliloti the nifA gene is transcribed from two separate promoters (15, 16). One of them (a -24/-12 promoter) is responsible for the expression of a fixABCXnifA transcript and appears to be activatable by the NtrC and NifA proteins (17). Nevertheless, ntrC mutants of R. meliloti are Fix^+ (17), and this has been explained by the activity of a second promoter located in front of nifA (15, 16). It is not known how transcription from this second promoter is controlled.

The present work deals with the analysis of the transcription of the B. japonicum nifA gene. Since it has recently been shown that the expression of B. japonicum nif and fix genes is controlled by oxygen via a mechanism that involves the NifA protein (5, 14), we wished to know whether or not the nifA gene itself was subject to control by oxygen. We have determined the transcriptional start and the complete nucleotide sequence of the nifA-containing operon and show that it is also expressed aerobically. Expression of this operon does not appear to be dependent on (auto)activation by NifA; however, its promoter carries a -24/-12 consensus sequence and requires an upstream DNA region (between nucleotides -50 and -148 from the transcription start site) for its maximal activity. The presence, in B. japonicum, of an activator protein other than NifA is postulated.

MATERIALS AND METHODS

Bacterial strains, plasmids and M13 phages

These are listed in Table 1. Plasmids constructed in this work are described in the text.

Media, and growth of E. coli and B. japonicum

Growth of E. coli was done routinely in LB medium (18). Selective precultures of E. coli strains used as recipients for M13 phages were grown in minimal-medium as described (19). Antibiotics were added at the following concentrations (μg/ml): ampicillin 200, kanamycin 50, streptomycin 50, tetra-

Table 1. Strains, plasmids and M13 phages

Strains	Relevant genotype or phenotype	Reference
B.japonicum 110spc4	Spc ("wild-type")	(20)
B.japonicum A9	Spc Km nifA::aph	(5)
B.japonicum A14	Spc Km	(5)
B.japonicum ΔR14-1	Spc Km ΔfixR	this work
E.coli MC1061	Δ(lacIPOZYA)X74 hsdR	(48)
E.coli S17-1	hsdR RP4-2 kan::Tn7 tet::Mu	(36)
E.coli DS410	thi minA minB ara lacY xyl malA mtl tonA rpsL Azi λ	(49)
E.coli BMH71-18mutS	Δ(lacproAB) thi supE mutS215::Tn10 F' lacIqZΔM15 proAB+	(19)
E.coli MK30-3	Δ(lacproAB) recA galE strA F' lacIqZΔM15 proAB+	(19)
Plasmids and M13 phages		
pUC18	Ap	(27)
pMC1403	Ap 'lacZYA	(48)
pRK290X	Tc	(8)
pRK2013	Km	(50)
pSUP202	Ap Cm Tc oriT from RP4	(36)
pBR329	Ap Cm Tc	(51)
pRJ1025	nifD'-'lacZ fusion (pRK290X)	(8)
M13mp8/mp9		(52)
M13mp18/mp19		(27)

cycline 10. B. japonicum strains were grown aerobically in PSY medium (20) with the following appropriate antibiotic concentrations (μg/ml): chloramphenicol 10, kanamycin 100, spectinomycin 100, tetracycline 50 (for plates: tetracycline 75). Anaerobic/nitrate growth for nif derepression in free-living bacteria was done in a yeast extract mannitol medium (YEM; 21) plus 10 mM KNO_3. Selection for pRK290X derivatives in this medium was done by adding 50 μg/ml tetracycline. Free-living, microaerobic growth for derepression of the nif genes in B. japonicum has been described (22). Indicator plates for strains carrying lacZ fusions contained Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) at 30 μg/ml.

Recombinant DNA work

Restrictions, modifications and cloning of DNA, and transformation and isolation of plasmids were performed according to standard procedures (23).

DNA sequence analysis

Nucleotide sequence data were obtained using both the chemical method (24) and the chain termination method (25). For the chain termination method the following modifications were done to obtain better resolution of DNA

patterns on polyacrylamide gels: deoxy-7-deazaguanosine triphosphate was used instead of dGTP (26) and incubation with the Klenow-fragment was done at 42°C. Oligonucleotides were synthesized in a DNA synthesizer (model 380A; Applied Biosystems, Foster City, CA). Besides the universal M13-specific primer (27), fixRnifA DNA-specific primers were also used.

RNA preparation and transcript mapping

Total RNA from B. japonicum cells was isolated as described (28), except that total RNA used for primer extension experiments with AMV reverse transcriptase was treated in addition with DNaseI (RNase-free), phenol-extracted and isopropanol precipitated. Aerobically grown cells in PSY medium were harvested in the late log phase. Microaerobic, free-living cells grown in derepression medium were harvested after 7 days, when the derepression of the nif-genes could be demonstrated by acetylene reduction. Isolation of bacteroids from nodules for bacteroid RNA extraction was performed as described (29).

Nuclease S1 mapping was performed with a single-stranded [^{32}P]DNA probe synthesized from a M13 template, as described in principle by Burke (30). The 550 bp SmaI-SalI fragment at the 5' end of fixR (see Fig. 1) cloned in M13mp8 was labeled with Klenow-fragment by incorporating [α-^{32}P]dCTP. The labeled, double-stranded DNA was then cut with SalI, and after denaturation, the DNA was directly used for hybridisation with approximately 20 µg of total RNA. DNA-RNA hybridisation was performed for 3 h at 47°C. Nuclease S1 digestion was done as described (31). As reference, the same M13 clone was used for sequencing with the chain termination method, and after primer extension the double stranded DNA was also cut with SalI. Separation of the nuclease S1-protected fragment and the sequencing reactions were done on a 6% polyacrylamide gel.

The transcriptional start was also mapped by primer extension experiments with AMV reverse transcriptase (32). Approximately 20 µg of total RNA was hybridised to ~0.01 pmol 5' end-labeled primer (~5 x 10^6 cpm; 20-mer: 5'-(840)GACCTCGGATCAGATTGTCA(821)-3', cf. Fig. 2) and incubated at 50°C for 2 h. Primer extension was performed as described (32) in the presence of 30 U AMV reverse transcriptase for 1 h at 42°C. As reference, the same non-labeled 20-mer was hybridised to an appropriate M13 clone and used for conventional sequencing with the chain termination method.

Minicell expression

For the expression of fixR, translational fusions with the chloramphenicol acetyltransferase gene (cat) on pBR329 were constructed. Isolation and

^{35}S-methionine labeling of E. coli minicells (strain DS410) have been described previously (33). The analysis of the plasmid-encoded polypeptides was performed by SDS-polyacrylamide gel electrophoresis (34).

Oligonucleotide-directed mutagenesis

For the construction of site-directed point mutations the gapped-duplex DNA approach was used (19) employing E. coli strains BMH71-18mutS and MK30-3. The preparation of M13 DNA (ssDNA) included as addional step the removal of any traces of RNA by incubating the ssDNA with RNaseA (0.5 mg/ml) for 30 min at 37°C. The yield of point mutations with the two mutagenic oligonucleotides was in both cases higher than 42%.

Construction of translational lacZ-fusions and 5'-deletion derivatives

To construct a translational nifA'-'lacZ fusion, the 1.91 kb EcoRI-XhoI fragment carrying fixR and the 5' end of nifA (cf. Fig. 1) was cloned in pMC1403 digested with EcoRI and SmaI. The XhoI site in nifA was blunt-ended with nuclease S1. To construct the translational fixR'-'lacZ fusion, the 0.84 kb EcoRI-AvaII fragment (cf. Fig. 1) was cloned in pMC1403 which has been digested before with EcoRI and BamHI. The AvaII site (position 837 in Fig. 2) was blunt-ended by filling-in, and then a BamHI linker was ligated. The nifA fusion was at the 29th or 6th codon depending on the start of nifA (see Results); the fixR fusion was at the 14th codon. To generate 5'-deletions of the fixR'-'lacZ fusion, the EcoRI-BamHI fragment was first subcloned in a pUC18 vector. Using suitable restriction enzymes discrete 5'-deletions were generated. EcoRI linkers were attached to the shortened fragments which were then recloned into pMC1403 with EcoRI-BamHI. The in-frame nature of all lacZ-fusions was confirmed by sequence analysis. For cloning into pRK290X all pMC1403 derivatives were digested with EcoRI-SalI (SalI cuts after the lacA gene) and ligated with EcoRI-XhoI-linearized pRK290X. All constructs failed to give blue color on Xgal plates in the E. coli host MC1061, but yielded blue colonies in the B. japonicum genetic background, depending on the extent of upstream DNA (see Results).

Mating between E. coli and B. japonicum

The pRK290X derivatives were transferred into B. japonicum by three-parental mating using pRK2013 als helper plasmid (35). The B. japonicum ex-conjugants carrying a pRK290X derivative were streaked on PSY medium containing spectinomycin and tetracycline. Single colonies were then picked and used for selective precultures. The pSUP202 derivatives were transferred to B. japonicum by bi-parental mating using E. coli S17-1 as donor (36). Exconjugants were selected on PSY medium containing kanamycin, streptomycin and,

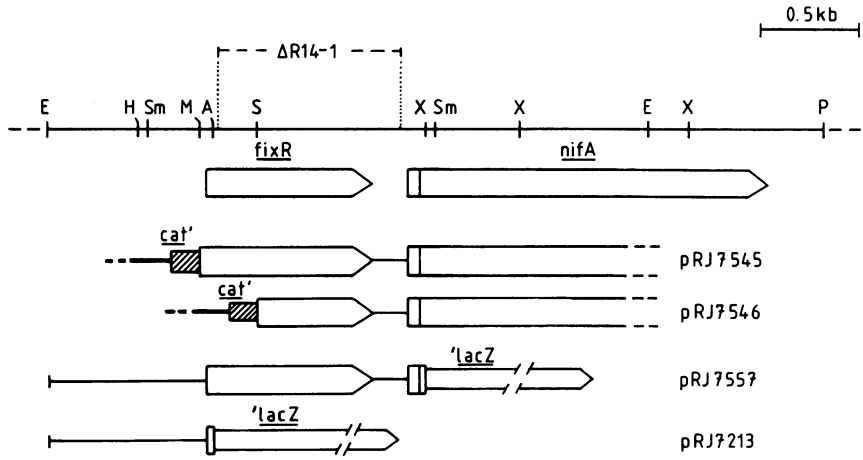

Fig. 1. Physical map of the fixRnifA operon. (Δ) Shows the extent of the chromosomal deletion in strain ΔR14-1. Plasmids pRJ7545 and pRJ7546 are in-frame fusions of the chloramphenicol acetyltransferase gene (cat) to fixR. Plasmids pRJ7557 and pRJ7213 are in-frame fixR'- and nifA'-'lacZ fusions, respectively, cloned in pRK290X. Restriction sites: A (AvaII; only one AvaII site relevant for this work is shown), E (EcoRI), H (HindIII), M (MluI), S (SalI), Sm (SmaI), P (PstI), X (XhoI).

for counterselection against the E. coli donor, chloramphenicol. Integration by a double cross-over event, at the correct genomic position, was checked by Southern hybridisation analysis as described (37).

β-Galactosidase assay with B. japonicum cultures

Single colonies from B. japonicum strains containing pRK290X derivatives were used to inoculate precultures grown aerobically in PSY medium containing tetracycline. Cultures of 10 ml PSY medium containing tetracycline were then inoculated from the precultures and incubated for 4 days at 28°C. 100 μl of the cultures were taken to assay β-galactosidase (β-gal) activity as described (18). Miller units (18) were calculated from at least 10 independent cultures assayed in duplicate. To measure β-gal activity during anaerobic growth with nitrate, precultures were first grown aerobically in YEM medium plus tetracycline and KNO_3. Cultures of 10 ml YEM medium plus tetracycline and KNO_3 were then inoculated from these precultures and incubated anaerobically for 5 to 6 days at 28°C. β-Gal assays were done as described for the aerobic cells with at least 3 independent cultures. β-Gal assays with microaerobic cultures have been described (8).

RESULTS

Nucleotide sequence of the fixRnifA operon

A restriction map of the previously cloned nifA region (5) is shown in Fig. 1. The depicted 3924 bp EcoRI-PstI fragment was sequenced on both strands and overlapping for all restriction sites. Figure 2 presents the DNA sequence from the HindIII site (near the left end in Fig. 1) to a NruI site located 104 bp before the right-hand PstI site. Two long open reading frames were detected. The first one was called fixR (for reasons given in the Discussion section), the second one is nifA. The most likely start codon for fixR is a GTG at position 801 because it is the only potential translation initiation codon preceded by a perfect Shine-Dalgarno sequence (AGGAG). It should be noted, however, that to the 5' side the fixR open reading frame extends up to position 639 (not shown in Fig. 2). The fixR stop codon (TGA) is at position 1635, which predicts a FixR protein of 278 amino acids and a M_r=29'682. The nifA gene either starts with an ATG at position 1821 or an ATG at position 1890; the latter is preceded by a more reasonable Shine-Dalgarno sequence than the first one (Fig. 2). The nifA gene terminates at position 3636 (TGA) which predicts a NifA protein of 605 or 582 amino acids and a M_r= 65'650 or 63'085, respectively. A more detailed structural and functional analysis of the NifA protein will be presented in a separate paper (Fischer et al., manuscript in preparation).

Expression and mutagenesis of fixR

While it has been possible to weakly express the complete NifA protein in E. coli minicells we failed to obtain expression of the putative FixR protein (data not shown). To confirm the proposed reading frame of fixR, the first 38 codons of the chloramphenicol acetyltransferase gene (cat) of pBR329 were fused in frame to the MluI site (located in the extended fixR frame 30 bp upstream of the GTG start codon), and to the fixR-internal SalI site. This resulted in plasmids pRJ7545 and pRJ7546 (Fig. 1) which in E. coli minicells directed the synthesis of hybrid Cat-FixR proteins with apparent molecular weights of 33'000 and 20'000, respectively (not shown). This is in good agreement with the fixR frame predicted from the nucleotide sequence. As will be shown two paragraphs further below, a translational fixR'-'lacZ fusions yields β-galactosidase activity in B. japonicum which demonstrates that fixR is transcribed and translated in its homologous genetic background.

A mutational analysis presented previously had already shown that insertions of kanamycin resistance cassettes into the B. japonicum region, in

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      HindIII                                                    SmaI
456   AAGCTTGGTACATCAGTTTTATGTTGGGGCGGCTAATGGCCGTGGCCGTTATCCCCGGGCATTTTTCGACTTCGA  530

531   CGAGAGGCGCCGGCCAGCCGCAGGCTCGTACGGTTGTGCGATCGTGAAAGCGAACGCGGGCTGGGTAGCCCTTTC  605

                 NruI
606   ATGAAGCGGGCGAGTGCTCGCGAACTTACGTAGCAGAGTCACCATCGCTGTCAGAAAGCTCACCTCCGCTGTCTT  680

681   GAATGCGCCAAACATTCCGCGTGCGCGACATTAGGACGCAAAACGGAAGCGCATTTACGTACATCGCAGTGGCGC  755

            MluI   +1                SD
756   AAATCCTGCTACGCGTGCGCGGGCGCAAAAGTAGAGGAGGCGGCCGTGGGTCTCGATCTGCCAAATGACAATCTG  830
                                                  (M) G  L  D  L  P  N  D  N  L

831   ATCCGAGGTCCGCTGCCCGAAGCACATTTGGATCGCCTCGTTGACGCTGTTAATGCGCGCGTTGATCGTGGAGAA  905
       I  R  G  P  L  P  E  A  H  L  D  R  L  V  D  A  V  N  A  R  V  D  R  G  E

906   CCGAAGGTGATGTTACTCACCGGCGCATCGCGCGGAATTGGCCATGCCACTGCCAAGCTATTCTCGGAGGCAGGC  980
       P  K  V  M  L  L  T  G  A  S  R  G  I  G  H  A  T  A  K  L  F  S  E  A  G

981   TGGCGCATCATTTCTTGCGCACGCCAACCGTTCGACGGCGAGCGATGCCCCTGGGAGGCTGGGAATGACGATCAT 1055
       W  R  I  I  S  C  A  R  Q  P  F  D  G  E  R  C  P  W  E  A  G  N  D  D  H
            SalI
1056  TTCCAGGTCGACCTCGGCGATCATCGAATGCTGCCGCGCGCAATCACCGAGGTTAAGAAACGCTTGGCCGGTGCG 1130
       F  Q  V  D  L  G  D  H  R  M  L  P  R  A  I  T  E  V  K  K  R  L  A  G  A

1131  CCCTTGCACGCGCTGGTGAATAATGCGGGTGTGTCGCCGAAAACGCCCACAGGCGATCGGATGACATCGTTGACC 1205
       P  L  H  A  L  V  N  N  A  G  V  S  P  K  T  P  T  G  D  R  M  T  S  L  T

1206  ACCTCAACCGATACCTGGATGAGGGTCTTTCATCTTAATCTGGTGGCTCCGATCCTGCTGGCGCAGGGTTTGTTT 1280
       T  S  T  D  T  W  M  R  V  F  H  L  N  L  V  A  P  I  L  L  A  Q  G  L  F

1281  GATGAGCTAAGAGCCGCGTCAGGATCGATCGTGAATGTGACTTCAATCGCGGGCTCGCGGGTGCACCCATTCGCC 1355
       D  E  L  R  A  A  S  G  S  I  V  N  V  T  S  I  A  G  S  R  V  H  P  F  A

1356  GGTAGCGCCTATGCGACCTCGAAAGCTGCGCTTGCGAGCCTCACACGCGAATTGGCCCACGACTATGCGCCGCAT 1430
       G  S  A  Y  A  T  S  K  A  A  L  A  S  L  T  R  E  L  A  H  D  Y  A  P  H

1431  GGCATTCGCGTCAATGCGATCGCGCCCGGCGAAATCAGGACCGACATGCTGTCGCCCGACGCGGAAGCGCGCGTC 1505
       G  I  R  V  N  A  I  A  P  G  E  I  R  T  D  M  L  S  P  D  A  E  A  R  V

1506  GTGGCAAGTATCCCGCTGCGCAGAGTGGGTACTCCGGACGAAGTGGCCAAGGTCATCTTCTTCCTATGCTCGGAT 1580
       V  A  S  I  P  L  R  R  V  G  T  P  D  E  V  A  K  V  I  F  F  L  C  S  D

1581  GCGGCGAGCTATGTTACGGGTGCCGAGGTGCCGATCAACGGTGGCCAGCATCTGTGAAGCGGCCGGCCCGGCGGC 1655
       A  A  S  Y  V  T  G  A  E  V  P  I  N  G  G  Q  H  L  *

1656  GAAGCTGACTTGAGCTTCCCAACGGACTCACGTTCTCCGCTGACCACGCGCATACAATCAACAGAAAATGAATCG 1730

1731  AAGGCACACGGCAAGTTTACTGTCGTGCCATGAAAAAGTGTCACGGTTCGATGTCATCTCGTCAACACTTGGCGC 1805

          SD                                                              SD
1806  TAGATCGAGAGTGACATGCAAAACGAACCTCGCCAATCCGGACATGGGTCCAGGGAGGATGATCAAGGAAACGGA 1880
                     M  Q  N  E  P  R  Q  S  G  H  G  S  R  E  D  D  Q  G  N  G
                              XhoI                                          SmaI
1881  GACAGGCTTATGCTGCATATCCCCTCCTCGAGCGAAAGACCTGCCTCGCAGCCGGAACCGGAGCGTGCCCCCCG 1955
       D  R  L  M  L  H  I  P  S  S  S  E  R  P  A  S  Q  P  E  P  E  R  A  P  P

1956  GGGGAGCCGTCGCATGAGAGCGCGCTGGCCGGCATCTACGAAATATCGAAAATACTAAATGCTCCCGGCCGGCTC 2030
       G  E  P  S  H  E  S  A  L  A  G  I  Y  E  I  S  K  I  L  N  A  P  G  R  L

2031  GAAGTCACCTTGGCCAACGTTCTCGGTCTTCTGCAATCGTTTGTGCAGATGCGACACGGCCTCGTCTCGCTGTTC 2105
       E  V  T  L  A  N  V  L  G  L  L  Q  S  F  V  Q  M  R  H  G  L  V  S  L  F

2106  AACGATGACGGCGTTCCAGAACTTACAGTTGGCGCCGGCTGGAGCGAAGGCACTGACGAACGTTACCGGACGTGC 2180
       N  D  D  G  V  P  E  L  T  V  G  A  G  W  S  E  G  T  D  E  R  Y  R  T  C

2181  GTGCCGCAGAAAGCAATCCACGAGATCGTAGCGACAGGCAGGTCTCTTATGGTCGAGAATGTAGCCGCCGAGACG 2255
       V  P  Q  K  A  I  H  E  I  V  A  T  G  R  S  L  M  V  E  N  V  A  A  E  T

2256  GCCTTCAGCGCTGCCGACCGGGAGGTTCTCGGCGCCTCTGATAGTATACCGGTAGCGTTCATCGGGGTCCCGATT 2330
       A  F  S  A  A  D  R  E  V  L  G  A  S  D  S  I  P  V  A  F  I  G  V  P  I
                                                                        XhoI
2331  CGCGTTGATTCGACGGTCGTTGGTACGCTGACGATCGACCGTATCCCGGAAGGCAGTTCAAGTCTTCTCGAGTAC 2405
       R  V  D  S  T  V  V  G  T  L  T  I  D  R  I  P  E  G  S  S  S  L  L  E  Y
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                                NruI
2406 GATGCGCGGCTGCTCGCCATGGTCGCGAACGTGATAGGACAAACGATCAAGTTACATCGCTTGTTCGCCGGCGAT 2480
      D  A  R  L  L  A  M  V  A  N  V  I  G  Q  T  I  K  L  H  R  L  F  A  G  D
    NruI                                                                  NruI
2481 CGCGAACAATCGTTGGTGGACAAGGACCGGCTAGAGAAACAGACAGTTGATCGTGGGCCGCCCGCTCGCGAGCGC 2555
      R  E  Q  S  L  V  D  K  D  R  L  E  K  Q  T  V  D  R  G  P  P  A  R  E  R

2556 AACGAGCTTCAGGCACACGGGATCATTGGCGACAGCCCGGCGCTGAGCGCACTGCTTGAGAAGATTGTCGTTGT  2630
      N  E  L  Q  A  H  G  I  I  G  D  S  P  A  L  S  A  L  L  E  K  I  V  V  V
                                          Asp718
2631 GCCAGATCAAACAGCACGGTTCTGCTGCGTGGCGAATCCGGTACCGGGAAGGAGCTGGTAGCCAAGGCCATTCAC 2705
      A  R  S  N  S  T  V  L  L  R  G  E  S  G  T  G  K  E  L  V  A  K  A  I  H

2706 GAGTCGTCCGTTCGTGCTAAGCGGCCGTTCGTTAAGCTGAATTGCGCGGCGCTCCCCGAGACGGTCCTGGAATCG 2780
      E  S  S  V  R  A  K  R  P  F  V  K  L  N  C  A  A  L  P  E  T  V  L  E  S

2781 GAATTGTTTGGCCATGAGAAAGGTGCCTTTACCGGTGCTGTCAGCGCCCGCAAGGGGCGCTTCGAGCTTGCTGAC 2855
      E  L  F  G  H  E  K  G  A  F  T  G  A  V  S  A  R  K  G  R  F  E  L  A  D

2856 AAAGGGACGCTATTTCTTGACGAGATCGGAGAGATCTCACCTCCGTTCCAGGCGAAGTTGCTGCGAGTTCTGCAA 2930
      K  G  T  L  F  L  D  E  I  G  E  I  S  P  P  F  Q  A  K  L  L  R  V  L  Q

2931 GAGCAGGAGTTCGAGCGCGTCGGCAGCAATCACACGATTAAAGTCGATGTTCGGGTGATAGCTGCGACCAACAGG 3005
      E  Q  E  F  E  R  V  G  S  N  H  T  I  K  V  D  V  R  V  I  A  A  T  N  R

3006 AACCTTGAAGAGGCTGTGGCAAGGAGCGAATTCCGCGCGGACCTCTACTATCGTATTAGCGTAGTTCCCTTGTTG 3080
      N  L  E  E  A  V  A  R  S  E  F  R  A  D  L  Y  Y  R  I  S  V  V  P  L  L
                NruI
3081 TTGCCGCCGCTTCGCGAAAGACGCAGTGATATTCCGCTGCTCGCAAGAGAGTTCCTCAGAAAGTTTAACAGCGAG 3155
      L  P  P  L  R  E  R  R  S  D  I  P  L  L  A  R  E  F  L  R  K  F  N  S  E

3156 AACGGCCGCTCTCTTACTCTGGAGGCGAGTGCGATCGATGTACTGATGAGCTGTAAATTTCCGGGAAATGTCCGC 3230
      N  G  R  S  L  T  L  E  A  S  A  I  D  V  L  M  S  C  K  F  P  G  N  V  R
        XhoI                                                              SphI
3231 GAACTCGAGAACTGCATCGAGCGGACCGCGACACTCAGTGCCGGAACATCGATTGTAAGAAGTGACTTTGCATGC 3305
      E  L  E  N  C  I  E  R  T  A  T  L  S  A  G  T  S  I  V  R  S  D  F  A  C

3306 AGCCAAGGCCAATGCCTTTCCACGACGCTTTGGAAAAGCACATCGTATGGCAAGACAGACCCGGCGGCACCGATG 3380
      S  Q  G  Q  C  L  S  T  T  L  W  K  S  T  S  Y  G  K  T  D  P  A  A  P  M

3381 CAACCGGTGCCTGCAAAGTCCATCATCCCGCTGGCTGAAACGGCCCCTCCGCCGCAGGCTGTCTGCGAACCGGGC 3455
      Q  P  V  P  A  K  S  I  I  P  L  A  E  T  A  P  P  P  Q  A  V  C  E  P  G

3456 TCATTGGCACCTTCCGGCACAGTTCTCGTTAGTGGTGCGAGAATGGCTGATCGCGAGCGGGTCGTTGCGGCTATG 3530
      S  L  A  P  S  G  T  V  L  V  S  G  A  R  M  A  D  R  E  R  V  V  A  A  M

3531 GAAAAGTCTGGCTGGGTGCAGGCCAAGGCAGCGCGCCTACTCGGGCTAACCCCGCGCCAAGTCGGCTACGCGCTG 3605
      E  K  S  G  W  V  Q  A  K  A  A  R  L  L  G  L  T  P  R  Q  V  G  Y  A  L

3606 AGGAAATACGGAATCGAGATAAAGCGGTTCTGAGCGCCCCGCATCGGCCTGGACTGATTCCGCCAAATCGATAAG 3680
      R  K  Y  G  I  E  I  K  R  F  *

3681 TGAAGACGGTCCGCGTAGTTAATCTAGCAATCTGAGGGGAATGGACCGCCGGGGGCGTCGGCTTGCCACGACCCC 3755

                                                                    NruI
3756 GAGAAGGGCTCAACTATTCAGCGACAGATGGGGCGGAGAGCCTCACTTGCGAGTCGCACAATCGCGAACCGCCGG 3830
```

Fig. 2. Nucleotide sequence of the fixRnifA operon. The translation of the two open reading frames of fixR and nifA is also shown. The sequence begins with the left HindIII site and ends at a NruI site located 104 bp before the PstI site (see Fig. 1). The -24/-12 consensus promoter, and possible translation initiation codons are underlined. The transcriptional start site is indicated with '+1'. (SD) Presumptive ribosome binding sites on the mRNA level.

which we now have identified fixR, had no severe effect on nitrogen fixation (Fix^+) provided that these insertions were not polar on nifA expression (5). A novel B. japonicum mutant, ΔR14-1 (Fig. 1), was constructed by marker exchange mutagenesis (37) using a kanamycin resistance gene insertion in the

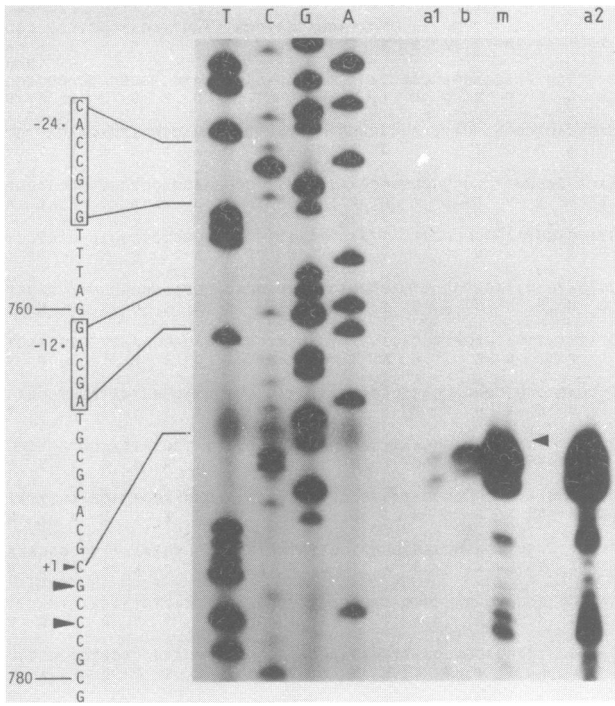

Fig. 3. Autoradiograph of a transcription start-site mapping experiment of the fixRnifA operon by primer extension. Total RNA used for primer extension was from the following B. japonicum cells: (lane a1) strain 110spc4 grown aerobically in PSY medium; (lane b) 110spc4 bacteroids from soybean root nodules; (lane m) 110spc4 grown microaerobically in derepression medium; (lane a2) 110spc4 carrying pRJ7213 (fixR'-'lacZ) grown aerobically in PSY medium plus tetracycline. The left margin shows the nucleotide sequence of the promoter (coding strand) and the transcription start points (arrowheads). The numbering refers to Fig. 2. The C is the first transcribed nucleotide (+1).

HindIII site upstream of fixR as selectable marker. The deletion was created by removing a 920 bp HindII fragment between nucleotide numbers 873 and 1793 (cf. Fig. 2). Mutant ΔR14-1 was thus deleted for almost the entire fixR gene plus most of the fixR-nifA intergenic region while leaving the putative Shine-Dalgarno sequence(s) of nifA intact (Fig. 1); its phenotype was Nif^+ in free-living, microaerobic culture, and it had approximately 50% Fix activity (measured by acetylene reduction) in soybean root-nodules as compared to the non-deleted, but otherwise isogenic, control strain A14. This mutant shows (i) that fixR is not strictly required for N_2 fixation, and (ii) that nifA expression is not severely affected by the deletion.

Mapping of the fixRnifA promoter

The mutational analysis described previously (5) and in the preceding paragraph suggested that fixR and nifA were both transcribed from a promoter located somewhere between the HindIII site and the beginning of fixR (Fig. 1). To confirm this assumption, low resolution S1 protection experiments were first performed using the M13 template approach (30). Different, radioactively labeled DNA fragments (coding strand) from within the 2.4 kb region between the left EcoRI site (Fig. 1) and the XhoI site at position 2397 in nifA (Fig. 2) were annealed to root-nodule bacteroid RNA and subjected to nuclease S1 digestion. A signal indicating partial protection was detected only with the 551 bp SmaI-SalI fragment covering the fixR 5' end (data not shown).

The exact transcription start site was determined by primer extension (32) using an oligonucleotide (see Methods) that was complementary to the mRNA region corresponding to position 821 to 840 (cf. Fig. 2). Reversely transcribed DNA was resolved on a sequencing gel (Fig. 3) next to a sequencing ladder generated by using the same oligonucleotide as primer for the chain termination sequencing technique. Bands of similar size were obtained using RNA from aerobic (lanes a1, a2) and microaerobic B. japonicum cultures (lane m) as well as from bacteroids (lane b). The longest extended fragment (lane m) within a group of at least 2 to 3 major bands pointed to a C which corresponded to the complementary G at position 774 of the non-coding DNA upstream of fixR (compare Figs. 2 and 3). The more intense signal in lane a2 as compared to lane a1 was probably due to the higher fixR copy number (see Legend to Fig. 3). With regard to the other lanes, it is difficult to draw conclusions about the mRNA content in microaerobic and bacteroid cells, because stability and recovery of the mRNA may be influenced by the physiological state of the cells. Taken together, the fixRnifA transcript is initiated at the same start site under all physiological conditions tested. The sequence (5'-GTGGCGC-N_5-CTGCT-3') between 25 and 9 nucleotides upstream of the transcription start site (i.e., between positions 749 and 765 in Fig. 2) closely resembles the -24/-12 consensus promoters associated with many other nif and fix genes of rhizobia and bradyrhizobia.

Expression of fixR'-'lacZ and nifA'-'lacZ fusions in B. japonicum wild-type and nifA mutant backgrounds

To confirm and extend the findings obtained with the transcript mapping experiments we studied the expression of translational fixR'-'lacZ and nifA'-'lacZ fusions (Fig. 1) cloned into the wide host-range plasmid pRK290X (8). For comparison, a nifD'-'lacZ fusion plasmid was also tested. Expression of

Table 2. Activities of extrachromosomal nifD'-'lacZ, fixR'-'lacZ and nifA'-'lacZ fusions in B.japonicum under aerobic and anaerobic (nif-derepressing) growth conditions

Host	Presence of plasmids	Relevant genotype	β-Galactosidase activity	
			aerobic cultures[b]	anaerobic cultures[c]
110spc4 (wild-type)	-	-	12	7
	pRJ1025	nifD'-'lacZ	101	1525
	pRJ7213[a]	fixR'-'lacZ	3184	1237
	pRJ7557[a]	nifA'-'lacZ	448	134
A9(nifA⁻)	-	-	7	11
	pRJ1025	nifD'-'lacZ	105	75
	pRJ7213[a]	fixR'-'lacZ	2157	1039
	pRJ7557[a]	nifA'-'lacZ	500	212

[a] The structure of these plasmids is shown in Fig. 1
[b] Growth in PSY medium; selection for fusion plasmid with tetracycline
[c] Growth in YEM medium plus KNO_3; selection for fusion plasmid with tetracycline

β-gal activity from all plasmids was measured in the B. japonicum wild-type, and in the nifA mutant, A9, carrying a nifA-internal deletion of 837 bp (5). The results are shown in Table 2. Cells were cultivated (i) aerobically, (ii) anaerobically with nitrate as terminal electron acceptor, and (iii) microaerobically with <0.2% O_2 in the gasphase (β-gal values not shown). Selection for plasmid maintenance by tetracycline was possible only in the first two cases, as the cells did not grow microaerobically in the presence of tetracycline. Table 2 shows that the nifD promoter (control) was expressed in the wild-type only under anaerobic conditions, but not under aerobiosis, and that its expression was dependent on nifA, which is consistent with previous data (5, 14). In contrast, the promoter of the fixRnifA operon was expressed both aerobically as well as anaerobically, and the nifA⁻ background did not lead to a significant reduction of the β-gal values seen in the wild-type. β-Gal values of the nifA'-'lacZ fusion were usually 5 to 10 times lower than with the fixR'-'lacZ fusion (see Discussion). In microaerobic cultures (data not shown) all lacZ fusions were expressed (except pRJ1025 in strain A9) but the β-gal values were generally lower because of the instability of the plasmids in the absence of tetracycline selection.

We also tested the expression of the nifA'-'lacZ fusion in soybean root-nodule bacteroids. For this purpose a B. japonicum strain was constructed into which the nifA'-'lacZ fusion was integrated between the non-essential

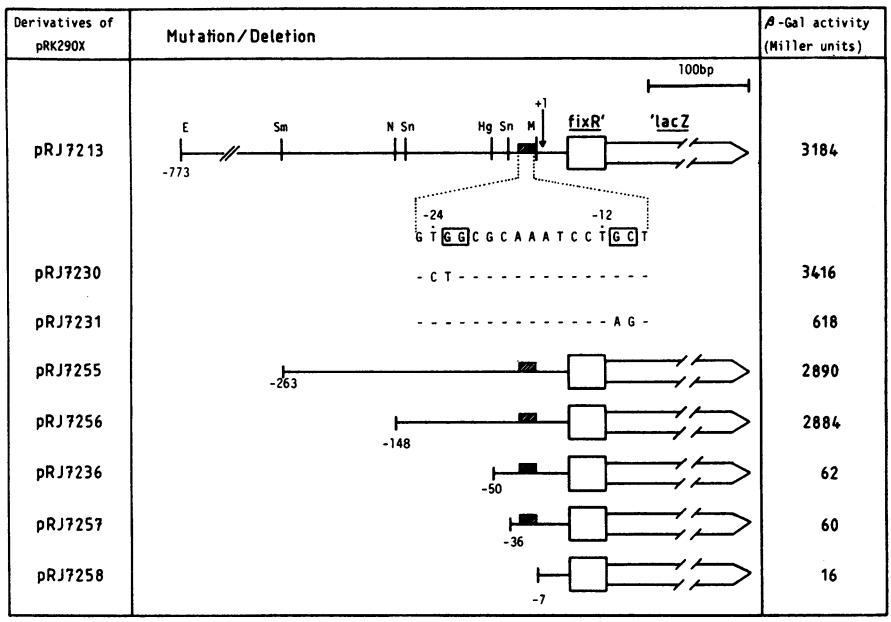

Fig. 4. Maps and β-galactosidase activities of the fixR'-'lacZ fusion plasmids containing promoter mutations and promoter-upstream deletion mutations. All fusions are cloned in pRK290X. The mutated nucleotides in pRJ7230 and pRJ7231 are shown below the wild-type fixR promoter sequence. In the deletion plasmids the numbers at the right deletion end points correspond to the distance (upstream) from the transcription start (arrow; +1). For determination of β-gal activity (right margin) B. japonicum strain 110spc4 carrying the respective plasmid was grown aerobically in PSY medium with tetracycline. Restriction sites: E (EcoRI), Hg (HgaI), M (MluI), N (NruI), Sm (SmaI), Sn (SnaBI).

RSα9-RSβ3 genomic region using a recently designed integration vector, pRJ1035 (64). Soybean nodules infected by this strain accumulated 342 units of β-gal activity at day 16 after inoculation. This value was about 50 times lower than the value obtained with a strain containing an integrated nifD'-'lacZ fusion (64). The data show that the nifA gene is expressed in root-nodule bacteroids at much lower level than the nitrogenase genes which is not surprising in view of the nifA product being a regulatory protein.

In another series of experiments we attempted to express the fixR promoter in E. coli MC1061 by providing constitutively expressed K. pneumoniae or B. japonicum NifA and K. pneumoniae NtrC proteins in trans (5, 11): no β-gal activity was obtained (data not shown).

Promoter mutations and promoter-upstream deletion mutations

Since we have not encountered conditions showing strict regulation of the fixRnifA promoter, while all other -24/-12 promoters known to date are regulated, we wished to obtain functional evidence for the -24/-12 promoter located in front of the transcriptional start site of fixR. Unfortunately, it was not possible to test the dependence of this promoter on the NtrA protein, because a B. japonicum ntrA mutant was not available, and in E. coli the fixR'- and nifA'-'lacZ fusions were not expressed at all. We therefore created defined base-pair exchanges (see Fig. 4) in the "-24" region (a TG to CT exchange: plasmid pRJ7230), and in the "-12" region (a GC to AG exchange: plasmid pRJ7231). Both double mutations affected the invariable GGs (-24 region) and GCs (-12 region) which are present at a distance of exactly 10 nucleotides in all -24/-12 consensus promoters. The strength of the mutant promoters was measured as β-gal activity of the corresponding fixR'-'lacZ fusions in aerobically grown B. japonicum cells (Fig. 4). The mutations in the -12 region resulted in a 81% reduction of wild-type β-gal activity. A similar reduction (80%) was seen in cells grown anaerobically with nitrate. A somewhat unexpected result was that the mutation in the -24 region did not negatively affect the fixR promoter activity. The implications of this finding will be discussed below.

Maximal expression from all -24/-12 consensus promoters known to date is not only dependent on the presence of the NtrA protein as specific σ factor, but also on a positive regulatory protein such as NifA, NtrC, or DctD (38). It is believed that these activator proteins bind to specific DNA regions upstream of the promoters (7, 8, 39). It was of interest, therefore, to test whether DNA sequences upstream of the fixRnifA promoter were required for its maximal function. Unidirectional deletions were constructed by removing increasing amounts of DNA between the EcoRI site (constant left deletion end point) and the beginning of fixR (see Fig. 4). Deletions up to position -148 (in pRJ7256) did not affect fixR promoter activity. However, removal of DNA between -148 and -50 (pRJ7236) resulted in 98% loss of promoter activity. The residual 60 units of β-gal activity (= 2%) were contributed by the fixR promoter alone (pRJ7236 and pRJ7257). The deletion up to position -7 (pRJ7258) destroyed fixR promoter activity completely (the 16 units β-gal activity correspond to background without any lacZ fusion plasmid). These results show that fixR promoter activity can be enhanced ca. 50-fold by an upstream activator sequence located between nucleotides -50 and -148 from the transcription start site.

DISCUSSION

The fixR and nifA genes are on one operon

DNA sequencing around the B. japonicum nifA gene has revealed the presence of two open reading frames, fixR and nifA. Although the two genes are separated by a fairly large intergenic region of 182 bp (Fig. 2) there is ample evidence that both are part of the same transcript. First, only one transcriptional start signal in front of fixR, but not in front of nifA, was detected by the techniques of S1 mapping and primer extension. Second, several insertion mutations upstream of nifA have been described as being polar on the expression of nifA (5). Third, the use of fixR'-'lacZ and nifA'-'lacZ fusions has shown that, under all growth conditions tested, these fusions were expressed coordinately (Table 2). It was noticed, however, that β-gal values obtained with the fixR'-'lacZ fusion were always at least 5 times higher than with the nifA'-'lacZ fusion. Several explanations for this observation are possible: (i) the N-terminal amino acids derived from the NifA protein may have a negative influence on β-gal activity; (ii) fixR may be more efficiently translated than nifA; (iii) transcription may partly terminate in the fixR-nifA intercistronic region; (iv) the fixR part may destabilize the mRNA from the nifA'-'lacZ fusion. We have no data that would distinguish between these possibilities.

The function of the fixR gene product remains unknown

In contrast to previously constructed mutations (5) the fixR mutation in strain ΔR14-1 had the advantage of having more than 90% of the fixR gene deleted while the nifA gene was still expressed from its natural fixR promoter. However, it can still not be decided whether the 50% reduced Fix activity observed with strain ΔR14-1 was due to the deletion of fixR or due to a negative interference with nifA expression because of the closer proximity of the fixR promoter. At any rate, it is clear that, under the assay and culture conditions applied in our laboratory, the fixR gene does not play an absolutely essential role in symbiotic or microaerobic nitrogen fixation. Apart from the reduced Fix activity in strain ΔR14-1 we believe that fixR is a gene and deserves its name because (i) it is the promoter-proximal cistron of an operon containing the regulatory gene nifA, (ii) its transcription is controlled by a -24/-12 promoter and, hence, is coregulated with nifA, and (iii) it is an actively translated gene in B. japonicum as shown by the expression of the translational fixR'-'lacZ fusion. In fast-growing rhizobia such as R. meliloti the nifA gene is preceded by the fixABCX operon (40); the latter genes have also been identified in B. japonicum in which they are located

elsewhere (41, 42, T. Zürcher, personal communication). In K. pneumoniae the nifL gene is located on the 5'-flanking side of nifA. FixR, however, does not share sequence homology with nifL (43), and, unlike in K. pneumoniae nifL mutants, nif gene expression in a B. japonicum fixR mutant (strain A11) does not escape from repression by oxygen (5). Also, fixR does not appear to influence its own expression since the β-gal activity of the fixR'-'lacZ fusion was the same in mutant ΔR14-1 and in wild-type backgrounds (data not shown). The computer-assisted search of a protein data bank (NBRF Protein Data Library, Release 12.0, March 1987) did not uncover significantly homologous proteins. In conclusion, the biochemical function of the putative FixR protein has yet to be clarified.

The fixRnifA operon is positively controlled

It was shown that fixRnifA expression occurred aerobically (Table 2). The β-gal values of fixR'-'lacZ or nifA'-'lacZ fusions were usually even higher under aerobiosis than under microaerobic growth conditions or during anaerobic growth with nitrate as terminal electron acceptor. On the other hand, S1 mapping and primer extension experiments usually gave a weaker signal with RNA from aerobic culture (Fig. 3), but quantitative measurements on RNA recovery using an internal standard have not been done. Since the fixR promoter was of the -24/-12 type, the possibility existed that it might be activated by the NifA protein. However, under microaerobic and anaerobic growth conditions similar β-gal values were expressed in the B. japonicum wild-type and in the nifA mutant (strain A9). This argues against (auto)-activation of the fixRnifA promoter by the NifA protein. Under aerobic growth conditions we could not expect, and also did not see, an effect of the nifA background because it has recently been shown that the in vivo activation of nif genes by the B. japonicum NifA protein was sensitive to oxygen (14).

In the light of a presumptive oxygen-reactive NifA protein (14), the strategy of the cell to express the fixRnifA operon aerobically seems wasteful. Indeed, it cannot be excluded that B. japonicum cells afford to express the fixRnifA operon aerobically, perhaps at low level, just to have the NifA protein available when it is needed (i.e., in the early steps of nodule formation; 5). Such a seemingly wasteful mechanism is not without precedence: K. pneumoniae synthesizes the NifL protein in anaerobic cells, in which it is "unemployed", to be used just in case rapid repression of nif genes by oxygen or ammonia is required (1). The more likely mechanism in B. japonicum, however, is that the fixRnifA operon is regulated by some physiological parameters other than oxygen and by positive control involving an activator pro-

Table 3. A list of genes carrying the -24/-12 promoter, and additional requirements (if known) for their activation.

Bacterium	Genes or operons containing the -24/-12 promoter[a]	Requirement for UAS[b] shown	Activating protein identified	Known environmental stimulus	Reference
Pseudomonas sp. RS16	carboxypeptidase G2 gene			growth on folate	(53)
P.putida	xylCAB, xylS		XylR	m-methyl-benzyl alcohol, m-xylene	(54,55)
P.aeruginosa	pilin genes				(56)
Neisseria gonorrhoeae	pilin gene				(57)
Caulobacter crescentus	fla genes	+		cell cycle control	(63)
Enterobacteria[c] (E.c, K.p, S.t)	fdhF	+		O_2 limitation	(58,59)
	argTr		NtrC	N limitation	(60)
	glnAntrBC	+	NtrC	N limitation	(39,61)
	nifLA	+	NtrC	N limitation	(1,2)
	other nif genes	+	NifA	N and O_2 limitation, T<37°C	(1,2,62)
R.leguminosarum	dctA		DctD	growth on C4 dicarboxylates	(38,45)
R.meliloti	nif and fix genes		NifA/ NtrC	symbiosis, N limitation	(2,17)
B.japonicum	fixRnifA	+			(this work)
	other nif and fix genes	+	NifA	O_2 limitation	(5,8,14)

[a] Only genes are listed for which the transcription start site has been determined.

[b] UAS=upstream activator sequence

[c] E.c=E.coli, K.p=K.pneumoniae, S.t=Salmonella typhimurium

tein other than NifA. The strongest support for this assumption comes from the facts that (i) a fixR promoter-upstream DNA sequence is required to drastically enhance the expression of a fixR'-'lacZ fusion (Fig. 4), and that (ii) the fixR promoter is of the -24/-12 type which implies that it needs to be positively controlled similar to many other, probably all, promoters of this kind (Table 3).

The essential nucleotides within the fixR promoter-upstream region (UAS) are not known. Typical nifA- or ntrC-dependent consensus UASs (7, 8, 39) are

missing in this region, but close homologues of them are present (for example, a TGT-N_{10}-ACC sequence between positions 654 and 669 in Fig. 2). It remains to be determined whether these, or any other sequences, are responsible for acting as a UAS. The double base exchange in the -12 region of the fixR promoter has led to a strong 'promoter-down' phenotype (Fig. 4) which demonstrates that these nucleotides, as expected, are essential for maximal nif promoter activity (2). Unexpectedly, however, the mutations in the -24 region were not promoter-down mutations, in contrast to the effect of a transversion of one of the 'invariable' G residues to a T in the nifA-dependent B. japonicum nifH promoter (44). Since it is currently believed (2) that not only the UAS but also the -24/-12 promoter consensus sequence are important for the promoter to become activated by NifA or NtrC, it is possible that the recognition specificity of the newly postulated activator protein for the fixR promoter is such that it does not strictly require those nucleotides in the -24 region that have been altered. It must also be kept in mind that the measurements concerning promoter strength were done with multicopy plasmids (pRK290 derivatives), so it cannot be ruled out that any weak promoter-down phenotype has been obscured.

As shown in this paper the NifA protein was ruled out as a candidate for being the (auto)activator of the fixRnifA operon. It is also unlikely that it is the NtrC protein because (i) we failed to obtain activation of the fixR promoter in E. coli by providing the K. pneumoniae NtrC protein in trans (data not shown), and (ii) because ntrC mutants of Azorhizobium sesbaniae and R. meliloti were shown to have a Fix^+ phenotype (6, 17). As the list in Table 3 shows, another activator, DctD, is known in R. leguminosarum to activate the -24/-12 promoter-containing dctA gene involved in dicarboxylic acid transport (38, 45). It seems worth to test the possibility that DctD activates the fixR promoter. All three proteins, NifA, NtrC and DctD, belong to a class of homologous regulatory proteins for -24/-12 promoters (46, Table 3). In this context it is also of interest that the B. japonicum genome has been shown to contain 5 to 6 DNA fragments hybridizing with a nifA probe (47). One of these fragments might encode the activator needed for the fixR promoter. Whichever the activator is, our results have shown that activation occurred under all media and growth conditions tested, i.e., the environmental stimulus (if it exists) for the activation of the fixR promoter has always been present.

The information available to date on genes carrying the -24/-12 promoter is compiled in Table 3. It shows that the fixRnifA promoter belongs to a group of promoters which are neither subject to nitrogen control nor to

activation by the NifA protein. It is for this reason that we have abandoned the "nif" or "ntr" promoter terminology. Rather, we propose to use the term "-24/-12" promoter. Furthermore, since all these promoters are probably recognized by a RNA polymerase holoenzyme containing the same type of sigma factor (38), the existing designations for the gene of that alternate sigma factor (ntrA/glnF/rpoN, N stands for nitrogen control) will have to be revised. In keeping with the arguments raised by Ronson et al. (38) we suggest to rename it rpoE, while rpoD remains the accepted name for the gene encoding the sigma factor (σ^{70}) that is specific for the -35/-10 E. coli consensus promoter.

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