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fixK, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*

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Nitrogen fixation genes are shown to undergo a complex positive and negative regulation in *Rhizobium meliloti*. Activation of *fixN* by *fixLJ* is shown to require a third regulatory gene, *fixK*. As *fixK* is activated by *fixLJ*, we propose a cascade model for *fixN* regulation such that *fixLJ* activates *fixN* via *fixK*. In addition *fixK* negatively regulates expression of the *nif*-specific activator *nifA* as well as its own expression by autoregulation. Thus *nifA* and *fixK* are subject to a mixed regulation, positive (by *fixLJ*) and negative (by *fixK*). The sequence of *fixK* shows homology with the *Escherichia coli* regulators *fnr* and *crp*, which makes *fixK* the third characterized member of this family of prokaryotic regulators.

Key words: Rhizobium meliloti/fixK/nif regulation/Crp/Fnr

Introduction

A remarkable feature of diazotrophs is that they fix molecular nitrogen under very different physiological conditions (Postgate, 1982). For example, *Klebsiella pneumoniae* is an enteric bacterium able to use atmospheric nitrogen for growth in the absence of oxygen and of fixed nitrogen. On the other hand, bacteria of the genus *Rhizobium* fix nitrogen only in non-growing, microaerobic, symbiotic conditions. This suggests a different control of nitrogen fixation (*nif*) genes. Although a similar *nif*-specific activator, *nifA*, exists in *K. pneumoniae* and *Rhizobium meliloti* (Dixon, 1984; Szeto *et al.*, 1984; Weber *et al.*, 1985), the regulation of *nifA* differs. In *K.pneumoniae*, *nifA* is positively controlled by the *ntrBC* operon in response to nitrogen starvation (reviewed by Gussin *et al.*, 1986), while in *R.meliloti nifA* is uncoupled from the *ntrBC* system and the nitrogen status (Szeto *et al.*, 1987; Ditta *et al.*, 1987). Instead *nifA* is activated by *fixLJ* in response to low oxygen concentrations (David *et al.*, 1988). FixL and FixJ belong to a family of prokaryotic regulatory proteins acting by pairs (reviewed by Ronson *et al.*, 1987). FixJ is a transcriptional activator of *nif* and *fix* genes (Hertig *et al.*, 1989) and we have proposed that FixL may act as the sensor of an extracellular signal (oxygen?) and modulate the activity of FixJ (David *et al.*, 1988). Interestingly *fixLJ* also controls the expression of *nifA*-independent *fix* genes such as *fixN*.

In this paper we show that activation of *fixN* by *fixLJ* requires a third regulatory gene, *fixK*, and propose that *fixLJ* activates *fixN* via *fixK*. In addition *fixK* is a negative regulator of itself and of the *nif* regulator *nifA*. The FixK protein is homologous to the regulatory proteins Crp and Fnr from *Escherichia coli*.

Results

A new regulatory gene, fixK, controls fixN expression

We have recently studied the activation of *fixN* by *fixJ*, both in *R.meliloti* and in *E.coli*. Thus a *fixN-lacZ* fusion could be activated by *fixJ* in *E.coli* when the fusion was carried by pTH2, a broad host-range cosmid carrying the entire *fix* cluster except *fixL* (Kahn *et al.*, 1989). However when the same fusion was subcloned on pGMI931, a plasmid containing only the functional *fixN* promoter upstream of the fusion (David *et al.*, 1988), *fixJ* failed to activate *fixN* in *E.coli*. We therefore hypothesized that activation of *fixN* by *fixJ* requires a gene present in pTH2 but absent from pGMI931 (Figure 1). This would be a *fix* gene since it is required to activate *fixN*. Mutations in the *fixGHI* operon do not affect *fixN* expression (David *et al.*, 1988). As a consequence, the putative regulatory gene should map in the central part of the *fix* cluster, which is reiterated on *R.meliloti* pSym (Renalier *et al.*, 1987). Therefore we investigated the genetic organization of this reiteration.

To overcome the difficulty lying in the genetic analysis of genes present in duplicate, we used *R.meliloti* strain GMI5595 (GMI708ΔJB16, Renalier *et al.*, 1987) in which the second copy of the reiteration is deleted entirely. Tn*5* insertions 2.81 and 2.8, which map at the ends of the reiterated region (Figure 1), were recombined into GMI5595. The resulting strains presented a Fix$^-$ phenotype which could be complemented by pTH2. *recA* derivatives of the mutants were constructed and tested for complemen-

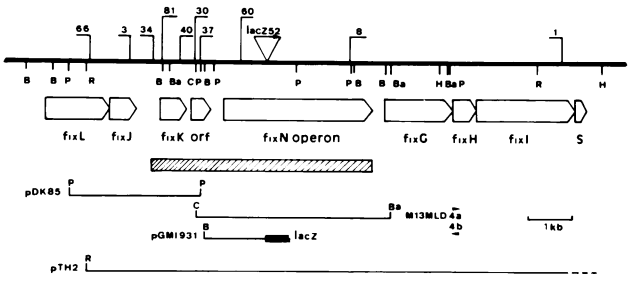

Fig. 1. Physical genetic map of the *fix* cluster. Positions of Tn*5* insertions used in this work are indicated with vertical bars above the physical map and that of the MudII1734 *fixN-lacZ52* insertion is indicated with a triangle. ORFs of *fixLJ* and of *fixGHIS* are as defined by David *et al.* (1988) and Kahn *et al.* (1989) respectively. The hatched box represents the reiterated part of the cluster (Renalier *et al.*, 1987). Restriction sites; B, *Bgl*II; Ba, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI.

Table I. Complementation assays of the Fix phenotype

Complementing plasmid[a]	Relevant background genotype	
	*recA fix*2.81 ΔJB16	*recA fix*2.8 ΔJB16
pTH 2.34	+	+
pTH 2.81	−	+
pTH 2.40	−	+
pTH 2.30	+	+
pTH 2.37	+	+
pMD 52	+	−
pTH 2.60	+	−
pTH 2.8	+	−

[a]Numbers after periods indicate allele numbers of mutations carried by pTH2.

tation of the Fix phenotype by various pTH2::Tn*5* derivatives (Table I). Absence of complementation of the 2.8 mutation by the distal 2.60 insertion indicates that these mutations map in a single operon, the *fixN* operon. On the other hand, the non-complementing mutations 2.81 and 2.40 define a distinct operon because they complement the 2.8 mutation. We named *fixK* the gene carrying the 2.81 insertion. The *fixK* and *fixN* operons are separated by Tn*5* insertion 2.37, which yields a Fix^+ phenotype when recombined into GMI5595.

To determine which locus is responsible for the regulation of *fixN*, we introduced the *fixN−lacZ* fusion plasmid pGMI931 into strains GMI6050 or GMI5595(controls), GMI5630(*fixK*2.81), GMI5685(*fixN*2.60), GMMI6051 (*fix*2.8), GMI5602(pSym2.37) and assayed expression of the fusion under microaerobic conditions which are known to induce *fixN* (David *et al.*, 1988). Expression of *fixN* was unaffected by mutations in the *fixN* operon or by the pSym2.37 insertion (between 970 and 1350 nmol/min/mg), but was abolished (4 nmol/min/mg) by mutation *fixK*2.81 in *trans*. To rule out the possibility of a mutational event in the *fixN−lacZ* fusion itself, pGMI931 was mobilized back from the *fixK* mutant into a wild-type strain and the fusion shown to be still functional. The same regulatory effect was found in symbiosis (1 nmol/min/mg in the *fixK* mutant versus 440 nmol/min/mg in the wild-type). The effect of *fixK* is specific since a *hemA−lacZ* fusion carried by pXLGD4 (Leong *et al.*, 1985) was expressed independently of *fixK* (between 1100 and 1500 nmol/min/mg in microaerobiosis).

We conclude from these experiments that *fixK* encodes an activator required for both microaerobic and symbiotic expression of the *fixN* operon.

Negative regulation of nifA by fixK

Like *fixN*, *nifA* is positively regulated by *fixLJ* (David *et al.*, 1988). It was thus of interest to test whether *nifA* was also regulated by *fixK*. We assayed the expression of the *PnifA−lacZ* fusion plasmid, pCHK57 (Ditta *et al.*, 1987) under the same microaerobic conditions that we used for *fixN*. *nifA* expression was activated under such conditions from 80 to 470 nmol/min/mg in the control strain GMI6050 (Figure 2). We found that *fixK* was not required for activation of *nifA*. Furthermore *fixK* appeared to regulate *PnifA* negatively because microaerobic expression of the fusion was higher in the *fixK* mutant (Figure 2). This was not observed with the control fusion plasmid pXLGD4. Interestingly, in the absence of a functional *fixK* gene, *PnifA* was still regulated by oxygen (Figure 2).

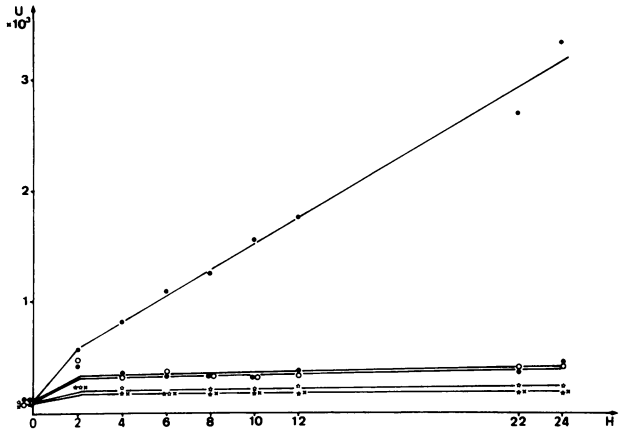

Fig. 2. Kinetics of derepression of the *PnifA−lacZ* fusion plasmid pCHK57 in a *fixK* mutant under microaerobic conditions. β-Galactosidase activity (U, nmol/min/mg) was measured at different times (*H*) after shifting an aerobic culture to microaerobic conditions (*H*=0). ●, GMI6050 (*recA*); *, GMI5630 (*recA fixK*2.81); ○, GMI6051 (*recA fix*2.8); ×, GMI5595; ☆, GM15685 (*fixN*2.60); ★, GMI5602 (*orf151* 2.37).

Nucleotide sequence of fixK

Analysis of the sequence of *fixK* (Figure 3) revealed one major open reading frame (ORF) of 633 bp with a high coding probability and with a rightward polarity. No significant ORF with a homogeneous high coding probability could be identified in the opposite polarity. Using the procedure of Stormo *et al.* (1982) we were able to predict a strong ribosome binding site corresponding to the first ATG of the 633 bp ORF. As mutation 2.81 maps in this ORF, we assigned it to the *fixK* gene. A second ORF with a high coding probability and the same polarity (*orf151*, potentially coding for a 151 amino acid long protein) was found between *fixK* and *fixN*. Surprisingly interruption of *orf151* by insertion 2.37 in the deleted strain GMI5595 led to a Fix^+ phenotype. Neither had this insertion any regulatory effect on *fixN*, *nifA* or *fixK* expression (first section, Figure 2 and Table II). Therefore the function of this sequence, if any, is presently unknown.

FixK is homologous to the regulatory proteins Fnr and Crp from E.coli

We looked for proteins homologous to FixK and the putative *orf151* product in the NBRF database (release 16, March 1988) and sequences translated from the GENBANK database (release 54, December 1987). We did not find any sequence homologous to the ORF151 sequence. On the other hand, strong similarity was found between FixK and the regulatory protein Fnr from *E.coli* (Figure 4). Fnr is a regulatory protein which allows *E.coli* to use alternative terminal electron acceptors such as NO_3, NO_2 or fumarate under anaerobic conditions (Chippaux *et al.*, 1981; Griffiths and Cole, 1987; Spiro and Guest, 1987a). The molecular mechanism by which Fnr mediates oxygen regulation of these anaerobic pathways remains to be established. However Fnr is homologous (Shaw *et al.*, 1983) with the cAMP receptor protein from *E.coli* (Crp), which is well characterized at the molecular level. FixK also presents sequence similarity with Crp. A statistical evaluation of the similarity between the three proteins using the program ALIGN (Dayhoff *et al.*, 1983) allowed us to conclude that FixK,

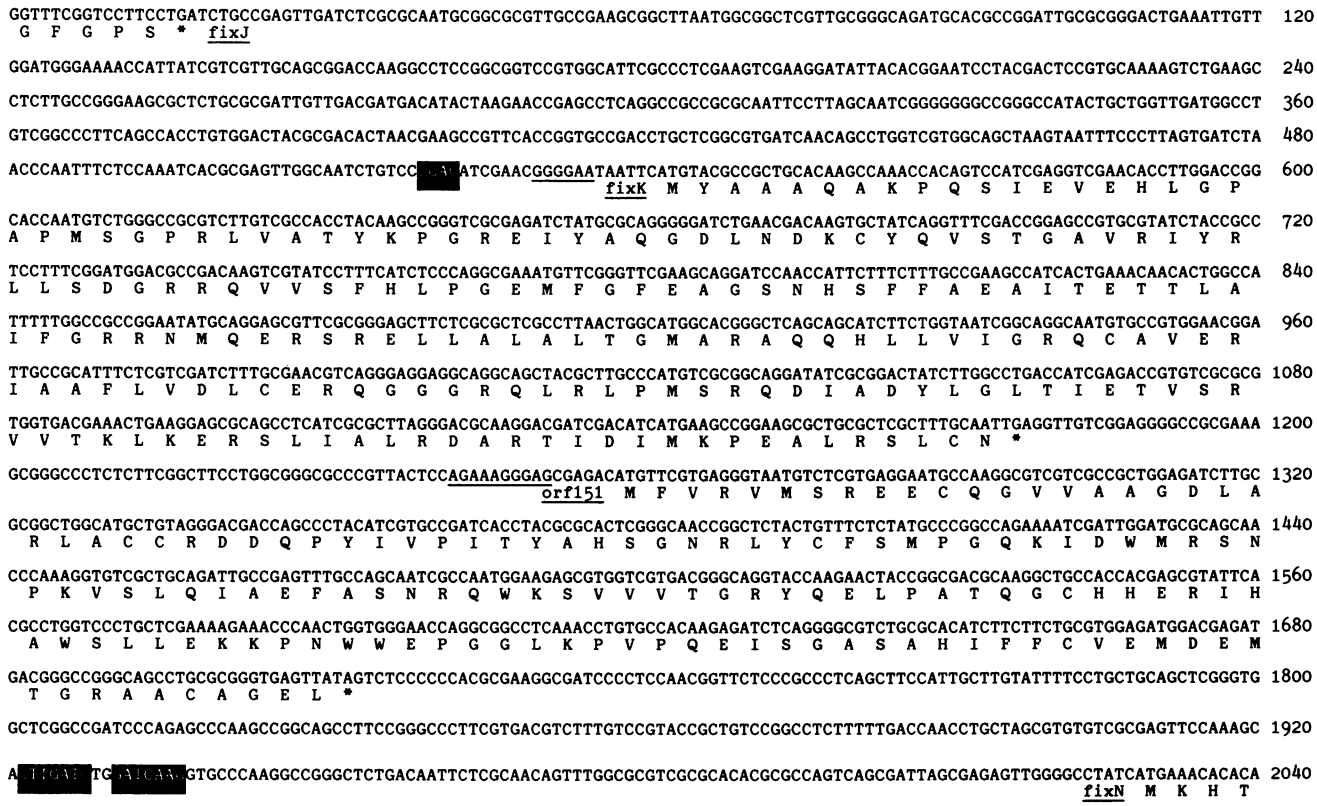

Fig. 3. Sequence of *fixK*. Potential Shine–Dalgarno sequences are underlined. The proposed start of transcription for *fixK* as well as the inverted repeat containing the Fnr-consensus sequence in *PfixN* are indicated in reverse characters.

Fnr and Crp are homologous. In addition, ALIGN scores confirmed a higher similarity between FixK and Fnr (15.8 SD) than between FixK and Crp (5.4 SD) or Fnr and Crp (6.6 SD).

The highest similarity between the three proteins was found in the helix-turn-helix motif directly involved in the specific DNA-binding of Crp (Ebright *et al.*, 1984). A similar motif is found in a large set of prokaryotic DNA-binding regulatory proteins (Pabo and Sauer, 1984). Using Dodd and Egan's (1987) method for evaluating such motifs, we found a score of 1825 for the homologous sequence in FixK (residues 163–182), which can thus be predicted to be a DNA-binding bihelical motif with a high degree of confidence.

Another region of striking similarity is found in the amino-terminal domain between residues 34 and 82 of FixK. In this region there are identical residues or conservative substitutions at 20 positions (41%) when comparing FixK with Crp, and at 26 positions (54%) when comparing FixK with Fnr. The homologous region in Crp is known to constitute the β-strands 2 to 6 which belong to a β-roll structure (Weber and Steitz, 1987). Interestingly, five glycine residues are conserved among the three proteins in this region (enhanced on Figure 4). This feature is important structurally since these glycine residues belong to turns or loops which require positive Φ dihedral angles in the Crp structure. This suggests that FixK and Fnr should have a β-roll structure in their amino-terminal domain as documented for Crp (Weber and Steitz, 1987). This is of interest since this region accommodates the cAMP effector in Crp and is therefore crucial for regulatory properties. However, residues which in Crp bind cAMP (Weber and Steitz, 1987, underlined in Figure 4) are not conserved in FixK nor in Fnr (Shaw *et al.*, 1983). It is worth noting that FixK, as compared to Fnr, lacks 21 amino acids at the N-terminal end. Interestingly, the corresponding region in Fnr was recently shown to be essential for protein activity (Spiro and Guest, 1988) and might be involved in oxygen sensitivity (Unden and Guest, 1985).

Our conclusions are (i) the predicted product of *fixK* is

Table II. β-Galactosidase activities of the *PfixK–lacZ* fusion plasmid pJJ5 in *R.meliloti*

Strain	Relevant bcakground	β-Galactosidase activity (nmol/min/mg)		
		Aerobic	Microaerobic	Symbiotic
GMI5600	*lac*	145	1150	920
GMI5601	*lac nifAZ*239	150	1300	1350
GMI5703	*lac fixI*2.1	170	750	750
GMI5704	*lac fixJ*2.3	17	25	10
GMI5705	*lac fixL*2.66	35	67	6
GMI6050	*recA* ΔJB16	45	2000	1600 (48%)
GMI5630	*recA* ΔJB16 *fixK*2.81	140	10300	2200 (20%)
GMI6051	*recA* ΔJB16 *fix*2.8	49	1450	1150 (30%)
GMI5595	ΔJB16	25	1450	NT
GMI5685	ΔJB16 *fixN*2.60	62	2100	NT
GMI5602	ΔJB15 *orf151* 2.37	33	2100	NT

Figures in parentheses indicate the stability of pJJ5 in the corresponding experiments. Background β-galactosidase of Lac$^+$ strains was inactivated as described previously (David *et al.*, 1988). NT, not tested.

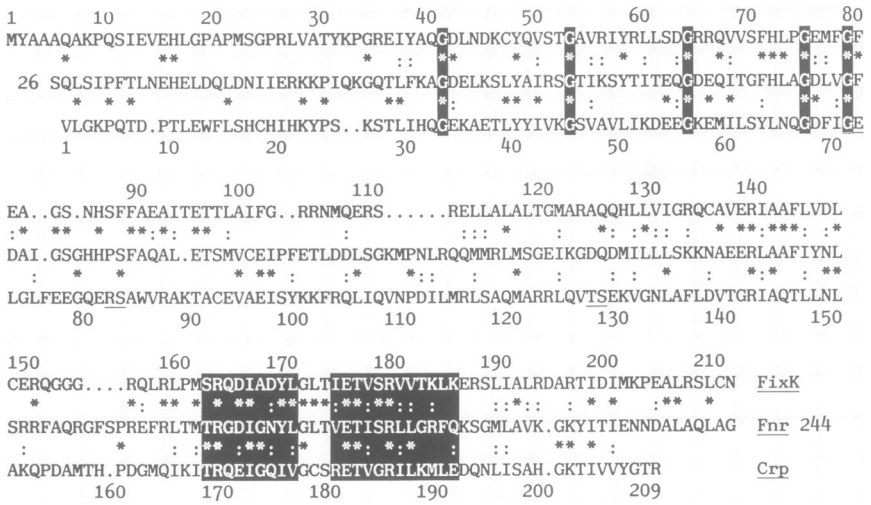

Fig. 4. Sequence alignment between FixK, Fnr and Crp. Residues that are conserved (*) or that belong (:) to the same group (ILVM, DENQ, KR, FY) between adjacent sequences are shown. The conserved glycine residues in the N-terminal part of the proteins and the helix-turn-helix motif in the C-terminal part are shown enhanced. Residues of Crp which bind cAMP are underlined (Weber and Steitz, 1987).

homologous to both Crp and Fnr, (ii) FixK can be predicted to be a DNA-binding protein, and (iii) FixK is more closely related evolutionarily to Fnr than to Crp.

The functions of fixK and fnr are not equivalent

The homology between FixK and Fnr could suggest that *fixK* is the *R.meliloti* equivalent of the *fnr* gene from *E.coli*. We therefore tested whether *fixK* controls dissimilatory nitrate reduction in *R.meliloti* like *fnr* does in *E.coli*. Kiss *et al.* (1979) described two types of nitrate reductase activities in *R.meliloti*. One is an assimilatory nitrate reductase induced under aerobic conditions in the presence of nitrate as sole nitrogen source. The second activity resembles the *fnr*-dependent respiratory nitrate reductase of *E.coli* since it is induced under poorly aerated conditions.

Utilization of nitrate as sole nitrogen source was unaffected in the *fixJ*2.3 mutant and in the *fixK*2.81 Δ*fixK*JB16 double mutant (data not shown). The 'respiratory' nitrate reductase activity could also be induced at wild-type levels in both regulatory mutants under microaerobic conditions. From these experiments we conclude that *fixK* is not a functional equivalent of the *fnr* gene as defined in *E.coli*.

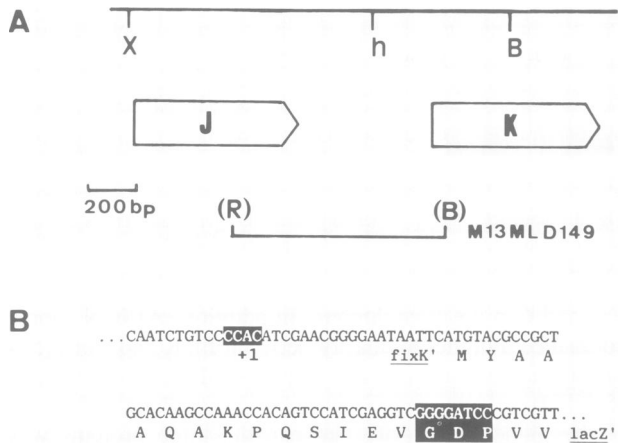

Fig. 5. Structure of the *PfixK*–*lacZ* fusion in pJJ5. **Panel A**: open boxes represent the *fixJ* and *fixK* ORFs. Restriction sites: X, *Xba*I; h, *Hin*cII; B, *Bam*HI; R, *Eco*RI. Sites in parentheses belong to the M13mp8 polylinker. **Panel B**: sequence of the *fixK*–*lacZ* fusion in pJJ5. The transcription start site and the fusion junction are indicated in reverse characters.

Mixed regulation of fixK

In order to define the relationship between the regulatory gene *fixK* and *fixLJ*, we constructed an in-frame translational fusion of *fixK* with the *lacZ* gene from *E.coli*. A 780 bp insert, including the first 14 codons of *fixK* and the whole DNA region between *fixJ* and *fixK* (Figure 5) and therefore presumably containing all the *cis*-acting regulatory sequences upstream of *fixK*, was cloned upstream of a truncated *lacZ* gene on the broad host-range *lacZ* fusion vector pIJ1363 (Rossen *et al.*, 1985). The insert was recombined into pIJ1363 in both orientations to yield pJJ5 (in-frame orientation) and pJJ7 (reverse orientation).

When conjugated into *R.meliloti*, pJJ5 and pJJ7 yielded blue and white colonies respectively on plates containing Xgal, which indicated that pJJ5 indeed contains a functional *lacZ* gene fusion. The *PfixK*–*lacZ* fusion of pJJ5 was expressed at a low level in free-living aerated cultures and strongly induced during symbiosis (Table II). Moreover, like *fixN*, *PfixK* could be induced in free-living cultures in microaerobiosis. Expression of *PfixK* depended strictly on *fixL* and *fixJ*, but not on *nifA* or *fixI*, both in free-living microaerobic cultures and in symbiosis (Table II). These results are consistent with previous data showing that symbiotic transcription of *fixK* DNA depends on *fixLJ* but not on *nifA* (David *et al.*, 1987, 1988).

Since both *crp* and *fnr* are negatively autoregulated (Aiba, 1983; Cossart and Gicquel-Sanzey, 1985; Pascal *et al.*, 1986; Spiro and Guest, 1987b), we asked whether *fixK* is autoregulated too. We found strong negative autoregulation of *fixK* in the free-living state (Table II). Therefore *fixK* is subject to a mixed regulation, positive (by *fixLJ*) and negative (by itself). As already noted for *PnifA*, *PfixK* expression still depended on microaerobiosis in a *fixK* mutant (Table II).

The fixK promoter

The regulation of *fixK* was further studied at the promoter level. In a preliminary experiment, the 1.40 kb *Xba*I–

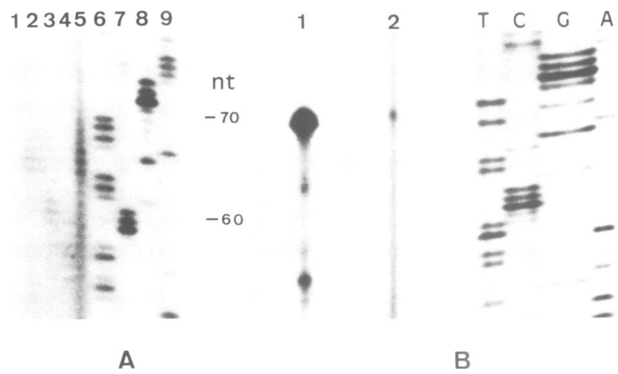

Fig. 6. Mapping of *PfixK*. **Panel A**: S1 mapping, **Lane 1**: no endosymbiotic RNA, 100 U S1; **lane 2**: 100 U S1, 5 μg RNA; **lane 3**: 300 U S1, 5 μg RNA; **lane 4**: 1000 U S1, 5 μg RNA; **lane 5**: 100 U S1, 10 μg RNA; **lanes 6−9**: sequence ladder. **Panel B**: primer extension mapping. **Lane 1**: RNA from microaerobically induced GM16043 (wild-type); **lane 2**: endosymbiotic RNA; T,C,G,A: dideoxy sequence of *PfixK*.

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                -60       -50       -40       -30       -20       -10
PFixK   CAGCTAAGTAATTTCCCTTAGTGATCTAACCCAATTTCTCCAAATCACGCGAGTTGGCAA
          ***     * *  *  **   **    ** *** *        *  *  * *    **
PNifA   TCGCTCTCCGACTGTCAATACGCATACCTCCTAATATTAAGCGGGCGAGAAAATGACTAA

                 +1       10        20        30        40        50
PFixK   TCTGTCCCCACATCGAACGGGGAATAATTCATGTACGCCGCTGCACAAGCCAAACCACAG
          **  ****   * *   *   *       *     **  *    *     *   * **
PNifA   GGTGCTCCCATCGCAACTCGTTCAGGGGAGTTAGTGCCCTGTCTGTACCTTCACAAAGAG
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Fig. 7. Comparison of *PfixK* and *PnifA*. Stars indicate conserved residues. Reverse characters indicate the positions of the transcription start sites in *PfixK* (this work) and *PnifA* as determined by Buikema *et al.* (1985) and Virts *et al.* (1988). The −45,−62 sequence which is essential for *PnifA* activity (Virts *et al.*, 1988) is underlined.

*Bam*HI fragment (Figure 5) was 5′ end-labeled and used as a probe in an S1 nuclease protection experiment with *R. meliloti* endosymbiotic RNA. We obtained strong protection of a fragment of ~0.35 kb. To determine which 5′ end was protected the labeled fragment was subjected to a secondary cut with *Hinc*II. Only the *Hinc*II−*Bam*HI probe yielded the protected fragment (data not shown), which showed that the corresponding promoter indeed directs transcription of *fixK*. We next used the M13mp8 recombinant M13MLD149 (Figure 5) to determine the precise location of the *fixK* promoter. We found *PfixK* between 21 and 23 bp upstream of the *fixK* initiator codon (C<u>CC</u>ACA, Figure 6A). The same transcription start (CCC<u>AC</u>A) was determined by the primer extension procedure (Figure 6B, lane 2). Furthermore, we found that the same promoter is used under symbiotic and free-living microaerobic conditions (Figure 6B, lane 1).

Since *PfixK* and *PnifA* have similar patterns of regulation it was of interest to compare their structures. A direct comparison of *PfixK* and *PnifA* after alignment of the transcription start sites showed two regions with some similarity (Figure 7). One lies around the transcription start site and the second one is in the −35 region. It should be noted that a region between −45 and −62 which is essential for *nifA* activation (underlined in Figure 7, Virts *et al.*, 1988) is not conserved at the same location in *PfixK* and *PnifA*.

Discussion

So far, four genes have been shown to regulate nitrogen fixation in *R.meliloti*: *nifA* (Szeto *et al.*, 1984), *fixL*, *fixJ* (David *et al.*, 1988) and *fixK*. Whereas *fixLJ* are positive regulators of *nifA*, *fixK* and *fixN* (David *et al.*, 1988, and this work), *fixK* has a dual function since it is both an activator of *fixN* and a negative regulator of *fixK* and *nifA*.

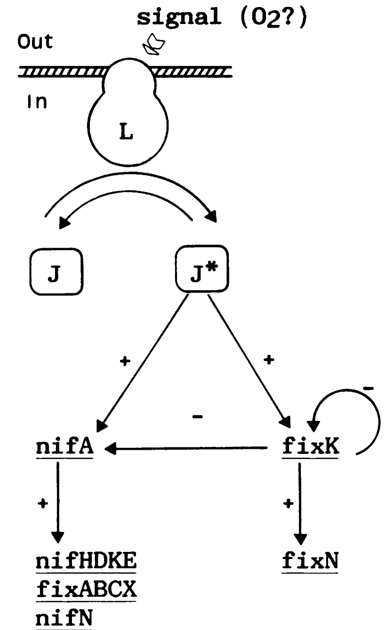

Fig. 8. Current model for the regulation of *nif* and *fix* genes in *R.meliloti*.

Cascade regulation of fixN

The question is raised of how *fixL*, *fixJ* and *fixK* participate in the regulatory pathway. The relationship between *fixL* and *fixJ* has been recently documented by David *et al.* (1988). From the homology of FixLJ with other prokaryotic two-component regulatory systems, FixL was predicted to be the sensor of an extracellular physiological signal which would modulate the activity of FixJ. FixJ on the other hand was predicted to be a transcriptional activator. This was confirmed recently by the demonstration of FixJ-dependent transcription activation of both *PnifA* and *PfixK* in *E.coli* in the absence of any other *Rhizobium* gene (Hertig *et al.*, 1989). In this paper, we have shown that *fixK* is required to activate *fixN* expression. Since *fixK* itself is positively controlled by *fixLJ*, the simplest mechanism we can propose to accommodate these results is a cascade mechanism in which *fixLJ* activates *fixK* which in turn activates *fixN*. This cascade parallels the regulatory cascade of *nif* genes via *nifA* (Figure 8). However, presently we cannot rule out the possibility that both FixK and FixJ may be required simultaneously to activate the *fixN* promoter.

fixK and *nifA* are both under the direct control of FixJ. However direct comparison of *PfixK* and *PnifA* does not reveal salient features that would be characterisetic of the regulation by FixJ. In particular, the −62,−45 region required for activity of *PnifA* (Virts *et al.*, 1988) does not seem conserved in *PfixK*. Understanding the mechanism of *PfixK* activation will require a functional analysis and would of course be facilitated by the identification of other *fixJ*-dependent promoters.

Negative control of nif genes

We assayed the expression of the *nifA*−*lacZ* fusion plasmid pCHK57 in bubbled cultures with a constant 2% oxygen

input (David *et al.*, 1988) and in the stoppered tube assay system set up by Ditta *et al.* (1987) to study *nifA* expression. We did not find a significant difference for *nifA* expression under these two conditions (data not shown). However *nifA* expression under microaerobic conditions was weaker (about one half) in the *R.meliloti* GMI708 derivatives used throughout this paper than in the *R.meliloti* strain GMI5600 used in a previous study (David *et al.*, 1988). The reason for this difference is unknown. It could be due to the Rif^R mutation carried by GMI708 which may affect the ability of RNA polymerase to interact with the FixJ transcriptional activator protein.

fixK is not required to activate *nifA*, which is in agreement with the finding that *fixJ* is sufficient to activate *nifA* in *E.coli* (Hertig *et al.*, 1989). However *fixK* regulates *nifA* negatively, so that *nifA* undergoes a mixed regulation, positive by *fixLJ* and negative by *fixK*. Similarly *fixK* is regulated positively by *fixLJ* and negatively by itself. This autoregulation can be considered as a simple homeostatic mechanism for controlling *fixK* expression.

Up to now we have been able to demonstrate negative regulation of *nifA* and *fixK* only in free-living and not in symbiotic conditions. The reason for this is unclear. One distinct possibility could be that negative control by *fixK* operates in *planta* only under particular physiological conditions that are not met in our experiments. Whatever the relevant physiological parameter, the discovery of negative regulation of *nif* genes in *R.meliloti* should allow the rational manipulation of *Rhizobium* to increase nitrogen fixation. Indeed it should be possible to affect the negative control of *nifA* specifically without altering positive control.

FixK is predicted to bind DNA

The homology between FixK, Fnr and Crp allows us to suggest some probable features of FixK action. The FixK sequence carries a very typical sequence with all the features of a DNA-binding helix-turn-helix motif. This sequence is homologous with the proven DNA-binding sequence of Crp. Therefore we predict that FixK binds to the DNA of target promoters. Because of the high similarity between the DNA-binding bihelical motifs of FixK and Fnr, it is tempting to speculate that these proteins may recognize similar DNA sequences. In agreement with this hypothesis is the presence in *PfixN* of a consensus sequence found in *fnr*-dependent promoters (enhanced in Figure 3) and which has been proposed to be the Fnr-binding site (Spiro and Guest, 1987b; Jayaraman *et al.*, 1988). An attractive hypothesis is therefore that FixK may bind to this sequence in the *fixN* promoter.

Physiological function of fixK

The homology between the three regulatory proteins FixK, Fnr and Crp also raises the question of the regulation of FixK activity by physiological conditions. Crp responds to glucose availability by interacting with the cAMP effector. However the residues interacting with cAMP in Crp are not conserved in FixK (nor in Fnr), and we have been unable to detect any effect of cAMP on expression of *fixN* in *R.meliloti*. Therefore cAMP is unlikely to be an effector of FixK. Fnr senses the anaerobic status of the cell but the effector molecule for Fnr, if any, is unknown. The close evolutionary relationship between FixK and Fnr at first suggested to us that FixK might respond to oxygen, like Fnr in enteric bacteria. However, the results presented in this paper clearly show that *fixK does not* play a *primary* role in oxygen regulation since expression of both *nifA* and *fixK* responds to microaerobiosis in the absence of a functional *fixK* gene. This does not rule out the possibility that *fixK* may have a *secondary* role in oxygen regulation. This could make sense if expression of *fixN* was more stringently regulated by oxygen than *fixK* or *nifA* expression. This, however, does not seem to be the case. Alternatively FixK might respond to another physiological signal, the nature of which still remains elusive.

Materials and methods

Microbiological techniques

Bacterial strains, plasmids and phages used are listed in Table III. Microbiological techniques, complementation assays and plant tests were as described previously (Renalier *et al.*, 1987). To obtain mutants affected in both copies of the reiterated *fix* genes, pSym-located Tn*5* insertions were transduced into GMI5595 using phage N3 (Martin and Long, 1984), and selected on streptomycin (400 μg/ml). The position of Tn*5* was verified by recombining Tn*5* on pTH2 followed by restriction enzyme analysis of the pTH2−Tn*5* derivatives (Batut *et al.*, 1985). *recA* derivatives were constructed by transduction of a *recA*::Tn*5*−*233* mutation (De Vos *et al.*, 1986).

Activity of nif−lacZ and fix−lacZ fusions

Microaerobic induction of *fixK* or *nifA* was obtained by bubbling a bacterial culture in M9 synthetic medium (containing 10 μg/ml tetracycline) with 2% oxygen−98% nitrogen at 28°C for 2 h (or more as in Figure 2) as described for *fixN* (David *et al.*, 1988). Symbiotic expression of the fusions was determined 3 weeks after inoculation of Nod^+ strains, and 5 weeks after inoculation of GMI5595 derivatives because nodulation by these strains is delayed by 2 weeks (Nod^d phenotype, Renalier *et al.*, 1987). Indigenous β-galactosidase was thermally inactivated (15 min at 50°C) in some experiments (David *et al.*, 1988). This treatment did not affect significantly β-galactosidase activities of the fusions carried by pGMI931, pCHK57, pJJ5 or pXLGD4. β-Galactosidase activity was expressed in nmol of *o*-nitrophenyl-β-D-galactoside hydrolysed per min and per mg of bacterial protein (calculated as in David *et al.*, 1988).

Detection of R.meliloti dissimilatory nitrate reductase

Strains were grown overnight at 28°C in complete TY medium in well-aerated flasks. Cultures were shifted to microaerobic conditions before adding 7 mM KNO_3. After 9 h incubation the nitrite accumulated in the medium was determined spectrophotometrically at 540 nm, 10 min after addition of Griess's reagent (Prolabo).

DNA sequence analysis

The sequence of the *fixK* region was obtained from the sequence of pDK85 (David *et al.*, 1988) and of the 4.4 kb *ClaI*−*Bam*HI fragment cloned in M13MLD4a and M13MLD4b (Kahn *et al.*, 1989) (see Figure 1). The sequence was read entirely on both strands and analysed as previously described (David *et al.*, 1988). Homology between the proteins FixK, Fnr and Crp was assessed using the program ALIGN of Dayhoff *et al.* (1983) with a gap penalty of 10 and 100 random permutations of each sequence. The program calculates the number of standard deviations (SD) between the alignment score of the real sequences and the mean alignment score of 100 randomly permutated sequences.

Construction of the PfixK−lacZ fusion plasmid pJJ5

We used the broad host-range vector pIJ1363 (Rossen *et al.*, 1985), which contains a transcription terminator from phage fd upstream of a truncated *lacZ* gene. Translational fusions can be constructed in this vector by recombining fragments containing a promoter and translation initiation signal into the *Bam*HI site of *lacZ*. The recombinant phage M13MLD149, obtained during the sequencing of pDK85, was particularly well suited for the construction of a *PfixK*−*lacZ* fusion: (i) it contains the first 14 codons of *fixK*, (ii) the M13mp8 polylinker *Bam*HI site allows the construction of an in-frame *fixK*−*lacZ* fusion (Figure 5) and (iii) its 780 nt insert includes the whole region between *fixJ* and *fixK* and therefore presumably contains all the *cis*-acting regulatory elements upstream from *fixK*. The replicative form of M13MLD149 was linearized with *Eco*RI and ligated with an *Eco*RI to *Bam*HI adaptor (Amersham) (before ligation, the adaptor was phosphorylated with T4 polynucleotide kinase). The ligation mixture was digested with *Bam*HI and the 0.78 kb *Bam*HI fragment was purified on an agarose gel

Table III. Bacterial strains, plasmids and phages

Strain, plasmid or phage	Relevant characteristics	Source or reference
R.meliloti 2011 derivatives		
GMI708	Rif^r	Batut *et al.* (1985)
GMI5595 (GMI708ΔJB16)	Δ(*nod fix*) JB16 Rif^r Nm^r Bleo^r Nod^d Fix$^+$	Renalier *et al.* (1987)
GMI5600	*lac* pSym20::Tn*5* Sm^r Nm^r Bleo^r Fix$^+$	David *et al.* (1988)
GMI5601	*lac nifA*Z239::Tn*5* Sm^r Nm^r Bleo^r	David *et al.* (1988)
GMI5602	Δ(*nod fix*)JB16 *orf151* 2.37::Tn*5* Rif^r Nm^r Bleo^r Sm^r Nod^d Fix$^+$GMI5595 derivative	This study
GMI5630	Δ(*nod fix*)JB16 *fixK* 2.81::Tn*5* *recA*::Tn*5*−*233* Rif^r Nm^r Bleo^r Sm^r Gm^r Spc^r Nod^d GMI5595 derivative	This study
GMI5685	Δ(*nod fix*)JB16 *fixN* 2.60::Tn*5* Rif^r Nm^r Bleo^r Sm^r Nod^d GMI5595 derivative	This study
GMI5703	*lac fixI*2.1::Tn*5* Sm^r Nm^r Bleo^r	David *et al.* (1988)
GMI5704	*lac fixJ*2.3::Tn*5* Sm^r Nm^r Bleo^r	David *et al.* (1988)
GMI5705	*lac fixL*2.66::Tn*5* Sm^r Nm^r Bleo^r	David *et al.* (1988)
GMI6043	*recA*::Tn*5*−*233* Rif^r Gm^r Spc^r GMI708 derivative	David *et al.* (1988)
GMI6050	Δ(*nod fix*)JB16 *recA*:: Tn*5*−*233* Rif^r Nm^r Bleo^r Gm^r Nod^d Fix$^+$ GMI5595 derivative	This study
GMI6051	Δ(*nod fix*)JB16 *fix*2.8:: Tn*5* *recA*::Tn*5*−*233* Rif^r Nm^r Bleo^r Sm^r Gm^r Spc^r Nod^d GMI5595 derivative	This study
Plasmids		
pCHK57	pGD926 derivative *PnifA*−*lacZ* Tc^r	Ditta *et al.* (1987)
pDK85	pBR322 derivative Tc^r	David *et al.* (1987)
pGMI931	pIJ1363 derivative *PfixN*−*lacZ* Tc^r	David *et al.* (1988)
pIJ1363	pRK290 derived *lacZ* fusion vector Contains a terminator from phage fd upstream *lacZ* Tc^r	Rossen *et al.* (1985)
pJJ5	pIJ1363 derivative *PfixK*−*lacZ* Tc^r	This study
pMD52	pTH2 *fixN*−*lacZ*52 *fixK*$^+$ *fixN*$^-$ Tc^r Nm^r Bleo^r	David *et al.* (1988)
pTH2	pLAFR1 derivative *fixK*$^+$ *fixN*$^+$ Tc^r	Batut *et al.* (1985)
pXLGD4	pGD499 derivative *hemA*−*lacZ* Tc^r	Leong *et al.* (1985)
Phages		
N3	Transducing phage of *R.meliloti*	Martin and Long (1984)

and recombined into *Bam*HI-cut pIJ1363. After transformation of *E.coli*, recombinants were screened by colony hybridization using the purified *Eco*RI−*Bam*HI-digested M13MLD149 insert as a probe. Two clones containing the insert in the in-frame (pJJ5) and reverse (pJJ7) orientation were chosen for further studies.

S1 nuclease mapping of PfixK

Endosymbiotic bacteroid RNA was extracted as previously described (David *et al.*, 1987). The 1.4-kb *Xba*I−*Bam*HI fragment (0.2 pmol) was 5′ end-labelled using the [γ-^{32}P]ATP phosphate exchange reaction catalysed by T4 polynucleotide kinase in the presence of ADP (Maniatis *et al.*, 1982). After one phenol−chloroform and two chloroform extractions, the DNA was precipitated with ethanol and the resulting pellet was rinsed in 70% ethanol and dried *in vacuo*. The DNA probe (25% of the total) and the RNA (0 or 10 μg) were dissolved and combined in 20 μl hybridization buffer (40 mM Pipes, pH 6.4, 1 mM EDTA, 0.4 M NaCl and 80% formamide) and brought to 80°C for 10 min. Annealing occurred in a 65°C water bath which was slowly cooled down to 45°C. After hybridization, 6 μg of carrier DNA was added and the volume adjusted to 300 μl with S1 buffer (50 mM sodium acetate, pH 4.6, 0.3 M NaCl and 4.5 mM $ZnSO_4$). Hybrids were digested for 30 min at 37°C in the presence of 1000 units S1 nuclease (Boehringer), extracted with phenol−chloroform, precipitated with ethanol and electrophoresed on an agarose gel in the presence of methylmercury (Maniatis *et al.*, 1982) before autoradiography.

For high resolution S1 mapping, the probe was synthesized from 50 ng M13MLD149 single-stranded DNA using a universal primer (17mer), the large fragment of DNA-polymerase, 40 pmol [α-^{32}P]dCTP (600 Ci/mmol) and 1 nmol dATP, dGTP and dTTP. The resulting duplex was digested with *Eco*RI and the 780 nt single-stranded probe purified on a 4% acrylamide sequencing gel. The probe was recovered in 400 μl 0.5 M ammonium acetate, extracted three times with 800 μl 1-butanol and precipitated in ethanol. The DNA probe (20% of the total) and endosymbiotic RNA (0, 5 or 10 μg) were dissolved and combined in 20 μl of hybridization buffer without formamide, and annealed at 65°C overnight. The resulting hybrids were digested as above with varying amounts of S1 nuclease in S1 buffer containing 1 μg yeast carrier RNA. After one phenol−chloroform extraction and ethanol precipitation, protected fragments were visualized on a 6% acrylamide sequencing gel.

Primer extension mapping of PfixK

Endosymbiotic RNA and RNA from microaerobically induced bacteria were prepared according to David *et al.* (1988) and Ditta *et al.* (1987) respectively. Primer extension mapping was performed as described by Ausubel *et al.* (1987). A 21mer oligonucleotide 5′-AGACATTGGTGCCGGTCCAAG (complementary to *fixK* between coordinates 591 and 611 in Figure 3) was 5′ end-labelled by [γ-^{32}P]ATP using T4 polynucleotide kinase. The labelled primer was allowed to hybridize overnight at 30°C with 50 μg RNA. Extension by AMV reverse transcriptase (20 U, Pharmacia) took place at 30°C for 15 min then 1 h at 40°C. Samples were treated with RNase A before loading on a sequencing gel. A dideoxy sequencing reaction of the M13mp8 derivative FIX8536 (which extends between *Sau*3A sites located at positions 432 and 650 in Figure 3) was run in parallel using the same oligonucleotide as a primer.

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