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The $FixK_2$ Protein Is Involved in Regulation of Symbiotic Hydrogenase Expression in *Bradyrhizobium japonicum*

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The roles of the nitrogen fixation regulatory proteins NifA, $FixK_1$, and $FixK_2$ in the symbiotic regulation of hydrogenase structural gene expression in *Bradyrhizobium japonicum* have been investigated. Bacteroids from FixJ and $FixK_2$ mutants have little or no hydrogenase activity, and extracts from these mutant bacteroids contain no hydrogenase protein. Bacteroids from a $FixK_1$ mutant exhibit wild-type levels of hydrogenase activity. In β-galactosidase transcriptional assays with NifA and $FixK_2$ expression plasmids, the $FixK_2$ protein induces transcription from the *hup* promoter to levels similar to those induced by HoxA, the transcriptional activator of free-living hydrogenase expression. The NifA protein does not activate transcription at the hydrogenase promoter. Therefore, $FixK_2$ is involved in the transcriptional activation of symbiotic hydrogenase expression. By using β-galactosidase transcriptional fusion constructs containing successive truncations of the *hup* promoter, the region of the *hup* promoter required for regulation by $FixK_2$ was determined to be between 29 and 44 bp upstream of the transcription start site.

The slow-growing symbiont of the soybean plant, *Bradyrhizobium japonicum*, expresses a hydrogen uptake hydrogenase that oxidizes hydrogen under both free-living and symbiotic conditions. In the free-living state, the expression of the NiFe hydrogenase is regulated at the transcriptional level by hydrogen, oxygen, and nickel (21). These three signals exert their effects within a 50-bp region of DNA located between 99 and 149 bp upstream of the transcription start site of the hydrogenase structural genes (22). In addition, the hydrogenase promoter is σ^{54} dependent and requires integration host factor for full induction (5).

The *hoxA* gene (32) is located approximately 12 kb downstream of the hydrogenase structural genes, in a region of the hydrogenase gene cluster previously shown to be necessary for free-living hydrogenase activity (23). The *hoxA* gene encodes a protein with extensive homology to transcriptional activators of hydrogenase expression in several other organisms, including HoxA in *Alcaligenes eutrophus* (12), $HupR_1$ in *Rhodobacter capsulatus* (30), and HydG in *Escherichia coli* (31), all of which are members of the NtrC-like family of response regulators (17). Subsequent studies of the role of the HoxA protein in the biosynthesis of hydrogenase by our group (11) and another (33) have confirmed that HoxA is a transcriptional activator of hydrogenase expression under free-living, microaerobic conditions. Its cognate sensor protein is presently unknown. However, bacteroids from nodules formed by *B. japonicum* HoxA mutants exhibit wild-type levels of hydrogenase activity and extracts from Hup^+ bacteroids used in gel retardation assays do not cause a shift of a fragment of the hydrogenase promoter containing the 50-bp regulatory region (11). Because the hydrogenase promoter is σ^{54} dependent, there must be some other, symbiosis-specific, activator of hydrogenase expression that binds the promoter at an alternative site.

The hydrogenase enzyme in the pea symbiont, *Rhizobium leguminosarum*, is expressed solely in symbiotic conditions (28). Interestingly, a defective *hoxA* gene that has been inactivated by several frameshift and deletion mutations has been reported for this organism (6). The hydrogenase structural genes have been shown to be under the transcriptional control of the nitrogen fixation regulatory protein NifA (6). Several groups have suggested that symbiotic hydrogenase expression in *B. japonicum* is also linked to the regulation of nitrogen fixation (11, 33). In addition to NifA, another candidate for a symbiotic regulator is the Fnr-like, DNA binding protein FixK (2). In rhizobial species, FixK is part of an oxygen-responsive regulatory cascade controlled by the FixL-FixJ two-component system (13). *B. japonicum* contains two homologs of the FixK protein (13). $FixK_2$ activates the expression of several genes, including nitrate metabolism genes, the *fixNOQP* operon, and $fixK_1$, in response to low oxygen levels (13). Mutants in this gene are Nif^-. The target or targets for $FixK_1$ are unknown (2). It is possible that one or both of the FixK homologs may be involved in the regulation of other oxygen-responsive genes, including hydrogenase (11, 13).

In this work, we show that mutants in the *fixLJ-fixK* nitrogen fixation regulatory cascade are deficient in symbiotic hydrogenase activity. Unlike in *R. leguminosarum*, NifA does not seem to be involved in the symbiotic transcriptional activation of *B. japonicum* hydrogenase expression. Instead, the results here are consistent with $FixK_2$ acting as a symbiotic transcriptional activator. A possible $FixK_2$ binding site is centered at 40 bp from the transcription start site.

Bacterial strains and plasmids. All bacterial strains and plasmids used in this work are listed in Table 1. *B. japonicum* JH (16) is a derivative of USDA I-110 and is considered the wild type. JHΔA (11) is derived from strain JH and contains an 886-bp in-frame genomic deletion that removes most of the *hoxA* gene. Strains 7360 (1), 7454 (2), and 9043 (13) are all derived from *B. japonicum* 110spc (29). Strains 7360 and 7454 contain an insertion of the kanamycin resistance gene that disrupts the *fixJ* and $fixK_1$ genes, respectively. In strain 9043, the $fixK_2$ gene is replaced by the spectinomycin resistance gene. *Escherichia coli* ET8000 (24) is a *lac* mutant strain that is used as a background strain in β-galactosidase transcriptional assays. Plasmid pRJ9044 (unpublished data; a gift of H. Fischer) contains the *B. japonicum* $fixK_2$ gene on a 1.85-kb *Bam*HI-*Sal*I fragment cloned into pBluescript SK under control of the *lac*

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TABLE 1. Bacterial strains and plasmids

Bacterial strains or plasmids	Genotype or relevant features	Reference or source
Strains		
B. japonicum		
JH	Wild-type derivative of USDA I-110	16
JHΔA	In-frame deletion of 886 bp of *hoxA* gene	11
7360	*fixJ*::*aphII* (Spc^r Kan^r)	1
7454	*fixK*$_1$::*aphII* (Spc^r Kan^r)	2
9043	*fixK*$_2$::Ω (Spc^r Str^r)	Unpublished data; see also reference 13
E. coli ET8000	*rbs lacZ*::IS*1 gyrA hutC*$_K$	24
Plasmids		
pRJ9044	*B. japonicum fixK*$_2$ cloned into pSK under control of the *lac* promoter	13a
pMC71A	*K. pneumoniae nifA* cloned into pACYC184 under control of the Tc^r gene promoter	8
pSKA	*B. japonicum hoxA* cloned into pSK in the same orientation as the *lac* promoter	This study
pSY7	*hup-lacZ* fusion; −681 to +1649	21
pGHh1	*hup-lacZ* fusion; −171 to +39	21
pGHp1	*hup-lacZ* fusion; −149 to +171	21
pGR1	*hup-lacZ* fusion; −99 to +171	21
pGHf1	*hup-lacZ* fusion; −44 to +171	22
pGBs3	*hup-lacZ* fusion; −29 to +171	22
pGNSdB	*hup-lacZ* fusion; −220 to +162 with region −64 to −29 deleted	22

promoter. Plasmid pMC71A (7) contains the *Klebsiella pneumoniae nifA* gene cloned into the multicopy vector pACYC184 (9) under control of the promoter of the tetracycline resistance gene. Plasmid pSKA contains the *B. japonicum hoxA* gene on a 1.5-kb *Kpn*I-*Spe*I fragment cloned into pBluescript SK in the same orientation as the vector *lac* promoter. Plasmid pSY7 (21) is a *hup-lacZ* transcriptional fusion construct derived from pGD499 (10) and contains a 2.4-kb *Bam*HI-*Pst*I fragment of the hydrogenase structural genes including 680 bp of the promoter region. The remaining plasmids, pGHh1, pGHp1, pGR1, pGHf1, pGBs3, and pGNSdB (21, 22), are all derived from plasmid pSY7 and contain successive deletions of the *hup* promoter region (listed in Table 1) fused to a promoterless *lacZ* gene.

The *hup* phenotype of *fixJ*, *fixK*$_1$, and *fixK*$_2$ regulatory mutants. Free-living *B. japonicum* strains were grown in modified Bergerson's medium (3) and derepressed for hydrogenase activity by incubation for 18 to 20 h in no-carbon medium (26) under standard conditions (4, 11) of 5 μM nickel and an atmosphere of 84% nitrogen, 10% hydrogen, 5% carbon dioxide, and 1% oxygen. Whole bacteroids were prepared as previously reported (11, 20) by crushing nodules harvested from soybean plants inoculated with each *B. japonicum* strain and grown for 5 to 6 weeks as described previously (20, 25).

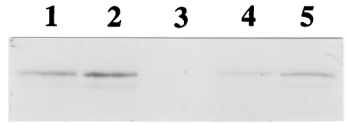
As shown in Table 2, all three of the nitrogen fixation regulatory mutants (*fixJ*, *fixK*$_1$, and *fixK*$_2$) are not affected in hydrogenase activity under free-living, microaerobic conditions. The FixK$_1$ mutant is also Hup$^+$ in symbiosis (Fig. 1, lane 5). However, bacteroids from the FixJ and FixK$_2$ mutants exhibit little or no hydrogenase activity and extracts from these mutant bacteroids contain little or no hydrogenase protein (Fig. 1, lanes 3 and 4) as detected by immunoblotting with antibody to the large subunit of hydrogenase (15). NifA mutants are severely affected in the ability to form an effective symbiosis and in nodule morphology (14). Therefore, a NifA mutant could not be assayed for symbiotic hydrogenase activity. The HoxA mutant JHΔA was assayed for hydrogenase activity as a control and was Hup$^-$ in free-living conditions (Table 2) and Hup$^+$ in symbiosis (Fig. 1, lane 2) as expected.

Since the FixJ and FixK$_2$ mutants are defective in nitrogen fixation, the possibility exists that the Hup$^-$ phenotype observed in bacteroids from these strains is due to an indirect effect of a lack of hydrogen (a known requirement for *hup* transcription) produced by the nitrogenase enzyme rather than the absence of either FixJ or FixK$_2$. To investigate this possibility, bacteroids from a *B. japonicum* mutant strain harboring a Tn*5* insertion in the nitrogenase structural gene *nifD* were assayed for hydrogenase activity. Bacteroids from this *nif* mutant are also Hup$^-$ (data not shown). However, the data do not

TABLE 2. Hydrogenase activities of free-living *B. japonicum* strains

Strain	Hydrogenase activity (nmol of H_2 oxidized/10^8 cells/min)[a]
JH	281 ± 14
JHΔA	0
9043 (*fixK*$_2$)	203 ± 55
7360 (*fixJ*)	215 ± 73
7454 (*fixK*$_1$)	165 ± 71

[a] Activities were measured amperometrically in samples of whole cells. Activities are averages ± standard deviations of nine separate experiments.



Hydrogenase activity (nmol H_2/min/mg protein) 30±4 25±3 0 4±2 19±2

FIG. 1. Immunoblotting and hydrogenase activities of wild-type and mutant bacteroid samples. The hydrogenase activity of whole bacteroids was measured amperometrically (18, 34). Activities are the averages ± standard deviations of six separate determinations. Western blots of bacteroid extracts prepared as previously described (11) were probed with antibody against the large subunit of *B. japonicum* hydrogenase. Lane 1, JH; lane 2, JHΔA; lane 3, 9043; lane 4, 7360; lane 5, 7454.

TABLE 3. Transcriptional activity of the *hup* promoter measured in a *lac* mutant *E. coli* strain (ET8000) carrying various plasmids[a]

Plasmid	β-Galactosidase activity[b]
pSY7 (*hup-lacZ*)	11 ± 0
pKS	25 ± 1
pSY7 + pKS	26 ± 1
pSY7 + pSKA (HoxA)	364 ± 11
pSY7 + pRJ9044 ($FixK_2$)	145 ± 3
pSY7 + pMC71A (NifA)	5 ± 0

[a] The following strains were also assayed and found to have fewer than 6 Miller units of activity: ET8000 and ET8000 carrying plasmid pMC71A, pRJ9044, or pSKA.

[b] Miller units per 10^8 cells. Values are averages ± standard deviations of six separate determinations.

rule out a role for $FixK_2$ in the symbiotic regulation of hydrogenase genes. It is possible and, in fact, probable that, as in free-living conditions, symbiotic hydrogenase expression is regulated by multiple signals. Presumably, hydrogen acts as an environmental signal in addition to oxygen and the positive signals for *hup* transcription are passed on by as-yet-unidentified components to the oxygen-responsive FixLJ-FixK regulatory cascade, which then acts on the hydrogenase genes.

Transcriptional control of the *hup* promoter by $FixK_2$. To demonstrate a role for the $FixK_2$ protein in the symbiotic regulation of hydrogenase biosynthesis and to rule out the NifA protein as a symbiotic transcriptional activator, β-galactosidase transcriptional assays (27) were done in the heterologous background of the *lac* mutant *E. coli* strain ET8000 (24). Plasmid pSY7, containing the *hup* promoter fused to a promoterless *lacZ* gene, was cotransformed into ET8000 with each of the following plasmids: pRJ9044 and pSKA, which constitutively express *B. japonicum* $FixK_2$ and HoxA, respectively, from the *lac* promoter of pBluescript SK, and pMC71A, which constitutively expresses *K. pneumoniae* NifA from the tetracycline resistance gene promoter of pACYC184.

Both the HoxA and $FixK_2$ proteins induce expression from the hydrogenase promoter to levels 6- to 14-fold above the background levels represented by plasmid pSY7 and plasmid pSY7 cotransformed with pBluescript KS (Table 3). Expression of the *hup* promoter was not activated by the NifA protein. In addition, the *B. japonicum* hydrogenase promoter was provided on a multicopy plasmid in a *K. pneumoniae* wild-type strain and the activity of the nitrogenase enzyme was measured by acetylene reduction (19). It has been shown that multiple copies of a NifA binding sequence will reduce nitrogenase activity as measured by acetylene reduction due to the titration of NifA (6, 8). The *hup* promoter plasmid reduced levels of acetylene reduction to the same extent as a control vector with no insert, while a plasmid containing the *B. japonicum nifH* promoter region, which is known to bind NifA, eliminated acetylene reduction activity (data not shown). These data indicate that $FixK_2$, and not NifA, has a role in the transcriptional regulation of the *B. japonicum* hydrogenase structural genes. This is in contrast to the situation in *R. leguminosarum*, in which NifA has been shown to bind and regulate the hydrogenase promoter (6).

Localization of a potential $FixK_2$ binding site in the hydrogenase promoter region. A visual inspection of the hydrogenase promoter region revealed the presence of two potential binding sites for $FixK_2$ (Fig. 2A). Both sites are about 50% identical to the FixK consensus binding sequence (Fig. 2B) (13). The sites are located between 213 and 228 bp and between 32 and 47 bp upstream of the transcription start site.

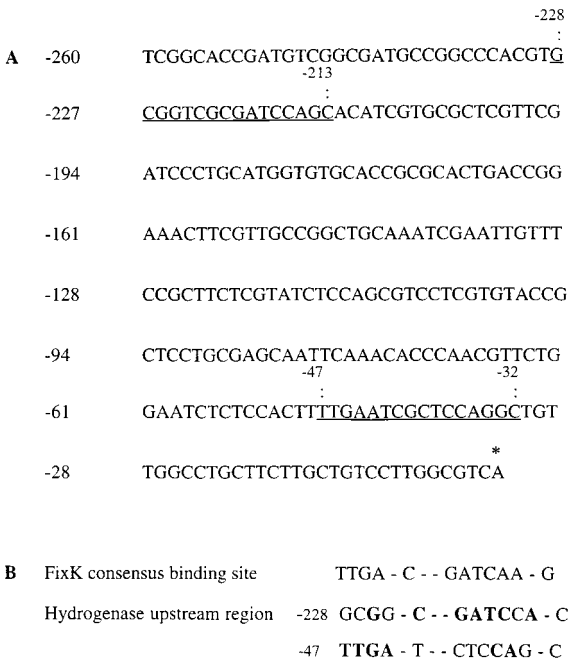


FIG. 2. Sequence of the upstream region of the hydrogenase structural gene operon and FixK consensus binding sequence. (A) Two potential FixK binding sites extending from −213 to −228 and from −32 to −47 bp upstream of the transcription start site of the *hupSL* operon (marked with an asterisk) are underlined. (B) Comparison of the hydrogenase promoter region with the FixK consensus binding sequence. Identical residues in each potential binding site are in boldface type.

When compared to the placement of FixK binding sites upstream of genes known to be regulated by FixK (13), either site upstream of the hydrogenase structural genes is equally likely to be the actual binding area.

To determine the region of the hydrogenase promoter necessary for regulation by $FixK_2$, plasmid pRJ9044 was cotransformed into strain ET8000 with various plasmids containing successive truncations of the hydrogenase promoter region fused to a promoterless *lacZ* gene (Table 1) (21, 22) and β-galactosidase activity was measured (see Table 4). Induction of the hydrogenase promoter by the $FixK_2$ protein is unaffected when the promoter region is truncated to 99 bp upstream of the transcription start site as in plasmid pGR1 but is reduced to just twice background levels when the promoter region is truncated to 44 bp upstream of the hydrogenase structural genes as in plasmid pGHf1 (Table 4). Hydrogenase promoter activity is reduced even further, to background levels, when the upstream region is truncated to 29 bp (i.e., plasmid pGBs3). A deletion of the promoter region between 29 and 64 bp upstream of the transcription start site in plasmid pGNSdB also abolishes induction of the *hup* promoter by $FixK_2$ (Table 4). In plasmids pGBs3 and pGNSdB, the integration host factor binding site shown to be necessary for full induction of the hydrogenase promoter under free-living conditions (5) is not present. However, in previous studies with pGNSdB, *hup* promoter activity was only reduced to 50% of the activity observed with full-length promoter constructs (5). No *hup* promoter activity was measured with either plasmid pGBs3 or pGNSdB in our experiment. Therefore, the reduction in *hup* promoter activity is due to a lack of binding of a factor ($FixK_2$) other than integration host factor. The partial reduction of hydrogenase promoter activity with a 44-bp upstream region indicates that

TABLE 4. $FixK_2$ induction of the hydrogenase promoter

Fusion construct[a]	β-Galactosidase activity[b]
pSY7 (alone)	22 ± 3
pSY7	311 ± 52
pGHh1	304 ± 37
pGHp1	213 ± 55
pGR1	409 ± 33
pGHf1	50 ± 9
pGBs3	30 ± 5
pGNSdB	28 ± 6

[a] The exact end points of each fusion construct are described in Table 1. Except where noted, each strain contains the fusion construct indicated and the $FixK_2$ expression plasmid pRJ9044.

[b] Activities are the averages ± standard deviations of nine representative measurements. Units are Miller units per 10^8 cells.

sequences within the putative FixK binding site closest to the transcription start site are important for activation of *hup* transcription by $FixK_2$.

Taken together, these data show that in contrast to *R. leguminosarum*, NifA is not a regulator of symbiotic hydrogenase expression in *B. japonicum*. Instead, the Fnr-like DNA binding protein $FixK_2$ is involved in the symbiotic transcriptional activation of the hydrogenase genes in this organism. The possibility exists that $FixK_2$ exerts its effect on hydrogenase expression in an indirect fashion, through some as-yet-unidentified component(s). However, the β-galactosidase transcriptional assays with the $FixK_2$ expression plasmid provide strong evidence for the $FixK_2$ protein being at least one point at which the regulation of symbiotic hydrogenase expression and the regulation of nitrogen fixation merge.

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