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Rhizobium meliloti fixGHI Sequence Predicts Involvement of a Specific Cation Pump in Symbiotic Nitrogen Fixation

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We present genetic and structural analyses of a *fix* operon conserved among rhizobia, *fixGHI* from *Rhizobium meliloti*. The nucleotide sequence of the operon suggests it may contain a fourth gene, *fixS*. Adjacent open reading frames of this operon showed an overlap between TGA stop codons and ATG start codons in the form of an ATGA motif suggestive of translational coupling. All four predicted gene products contained probable transmembrane sequences. FixG contained two cysteine clusters typical of iron-sulfur centers and is predicted to be involved in a redox process. FixI was found to be homologous with P-type ATPases, particularly with K^+ pumps from *Escherichia coli* and *Streptococcus faecalis* but also with eucaryotic Ca^{2+}, Na^+/K^+, H^+/K^+, and H^+ pumps, which implies that FixI is a pump of a specific cation involved in symbiotic nitrogen fixation. Since prototrophic growth of *fixI* mutants appeared to be unimpaired, the predicted FixI cation pump probably has a specifically symbiotic function. We suggest that the four proteins FixG, FixH, FixI, and FixS may participate in a membrane-bound complex coupling the FixI cation pump with a redox process catalyzed by FixG.

Despite their phylogenetic diversity, all diazotrophs examined to date share a common basic enzymatic apparatus, the nitrogenase complex, which reduces N_2 to NH_3. The genetic basis of the nitrogen-fixing ability of *Klebsiella pneumoniae* has been shown to consist of 20 clustered *nif* genes (3, 15), most of which are involved in the biosynthesis, processing, and assembly of the nitrogenase complex. *nifF* and *nifJ*, however, encode specific electron donors required for the reduction of nitrogenase, and *nifL* and *nifA* are *nif*-specific regulatory genes responsible for the coordinate activation of the whole *nif* cluster under appropriate conditions, i.e., anaerobiosis and absence of fixed nitrogen.

Genes homologous to some of the *K. pneumoniae nif* genes have been identified in *Rhizobium meliloti*, the alfalfa symbiont, as well as in other diazotrophs. These are *nifHDK*, the structural genes for nitrogenase (42), *nifB*, *nifE*, and *nifN*, which are necessary for the biosynthesis of the FeMo cofactor of nitrogenase (1, 8), and the regulatory gene *nifA* (50, 53). *fixABCX* were the first characterized genes of *R. meliloti* which were required for nitrogen fixation and were unrelated to *K. pneumoniae nif* genes according to sequence criteria (17). However, (i) *fixABCX* are required for free-living microaerophilic nitrogen fixation in *Bradyrhizobium japonicum* (22) and *Azorhizobium caulinodans* (16), and (ii) genes homologous to *fixABC* are found in the free-living aerobic diazotroph *Azotobacter* sp. (17, 22). Therefore, it has been suggested that they may encode electron donors to nitrogenase (17, 22). In this case, *fixABCX* could be considered functional substitutes of the *K. pneumoniae nifF* and *nifJ* genes.

Rhizobia are remarkable because they fix nitrogen only in symbiotic association with a host plant, in differentiated cells called bacteroids inside the plant nodule. Early biochemical comparisons between undifferentiated bacteria and bacteroids indicated some bacteroid-specific functions: in addition to nitrogenase, bacteroids exhibit different cell wall structures (51) and different cytochromes and oxidases (2). This fact strongly supports the existence in rhizobia, in addition to *nif* functions, of *fix* functions that would be specific of symbiotic nitrogen fixation.

We have previously described (5, 6, 38) a *fix* cluster from *R. meliloti* located 200 kilobases (kb) from the structural genes of nitrogenase on a large plasmid called pSym. Here we present genetic and sequence analyses of part of this cluster, which constitutes a *fix* operon highly conserved among rhizobia. The operon consists of at least three genes, *fixGHI*, and sequence analysis strongly suggests that it contains an additional gene, *fixS*. The four gene products inferred from the DNA sequence are predicted to be membrane bound. *fixGHI* appear unrelated to *K. pneumoniae nif* genes, and sequence comparisons allowed us to predict that FixG is a redox protein and that FixI is a cation pump.

MATERIALS AND METHODS

Microbiological techniques. Strains, plasmids, and bacteriophages used in this study are shown in Table 1. Transposon Tn*5* mutagenesis of pTH2 and marker exchange were performed as described previously (6). For complementation studies, the *recA*::Tn*5-233* allele from *R. meliloti* (14) was transduced into the different *fix* mutants by using phage N3 (35). Tests on plants were done as already described (6).

Transfer of exogenous pSym plasmids into *R. meliloti* GMI955 was performed as follows. Cultures of the donor strains *Rhizobium leguminosarum* T3 (bv. *viciae*) or LPR5035 (bv. *trifolii*) and the recipient *R. meliloti* GMI955 were mixed and filtered on a 0.45-μm-pore-size membrane.

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant characteristics	Source or reference
R. meliloti		
RCR2001		Rothamsted Experimental Station
RCR2011 derivatives		
GMI708	Rif^r	6
GMI955	*fixG181*::Tn*7* Sm^r Spc^r Tp^r	26
RmHG2H	Δ*fixHG2H* deleted of the 3.64-kb *Hin*dIII fragment (see Fig. 1), Rif^r Nm^r	T. Huguet, this laboratory
Rm6026	*recA*::Tn*5-233* Rif^r Gm^r Spc^r	14
R. leguminosarum bv. *phaseoli*		
JI8002		32
JI8400	pSym-cured derivative of JI8002	32
NVRS963A		J. Taylor, Wellesbourne
RCR3618		Rothamsted Experimental Station
R. leguminosarum bv. *viciae*		
128C53		7
B151	pSym-cured derivative of 128C53	7
JI248		25
JI897	Auxotrophic derivative of JI300	25
JI6015	pSym-deleted derivative of JI897	25
RCR1045		Rothamsted Experimental Station
T3	pRL1JI::Tn*5*	25
R. leguminosarum bv. *trifolii*		
LPR5035	pRtr5a::Tn*5*	24
RCR5		Rothamsted Experimental Station
RCR221		Rothamsted Experimental Station
R. fredii		
USDA193		36
USDA194		36
Plasmid		
pDD27	pBR328 derivative, Ap^r Cm^r (see Fig. 1)	12
pIJ1247	2.45-kb *Eco*RI fragment from pRL1JI, containing *nifH* and *nifD*, cloned in pSUP202	A. Downie, John Innes Institute, Norwich
pTH2	pLAFR1 derivative, Tc^r *fixG*$^+$*H*$^+$*I*$^+$	6
Phage N3	Transducing phage of *R. meliloti*	35

Filters were incubated overnight at 30°C, and transconjugants were selected on TY medium (5 g of tryptone and 3 g of yeast extract per liter, with 6 mM $CaCl_2$) supplemented with spectinomycin (100 μg/ml) to select for *R. meliloti* and with neomycin (100 μg/ml) to select for the presence of the Tn*5* transposon. Plasmid content of transconjugants was checked on gels, using the procedure of Eckhardt (18) as modified by Rosenberg et al. (39).

DNA techniques. Interspecies hybridization between the pDD27 probe and blots of plasmid DNAs was carried out under low-stringency conditions. Filters were prehybridized at 34°C overnight in 5× SSC (SSC is 0.15 NaCl plus 0.015 sodium citrate)–50% formamide in the presence of 5× Denhardt solution and 250 μg of calf thymus DNA per ml. The probe was radioactively labeled by nick translation (specific activity, approximately 2×10^7 cpm/μg). Hybridization was allowed to proceed for 24 h at 34°C in the same conditions as prehybrization except that the concentration of calf thymus DNA was lowered at 100 μg/ml. Filters were then washed twice in 5× SSC–0.2% sodium dodecyl sulfate at 65°C, dried briefly, and exposed for 2 weeks at −80°C with two intensifying screens.

DNA sequence analysis. We used three restriction fragments (Fig. 1). The left part of the sequence was obtained as part of the sequence of the 4.4-kb *Cla*I-*Bam*HI fragment. The entire sequence of this fragment was obtained by using the random-deletion approach of Barnes et al. (4). The fragment was excised from pDD5 (12) and cloned between the *Acc*I and *Bam*HI sites of M13mp18 and M13mp19 to yield phages M13MLD4a and M13MLD4b, respectively. Deletions were generated by using DNase I and *Eco*RI or *Hin*dIII (4), and their ends were sequenced by the chain termination method as described elsewhere (43). The overlapping sequences allowed us to determine the sequence on both strands of most of the 4.4-kb *Cla*I-*Bam*HI fragment, inciuding the sequence presented here. The 1.31-kb *Bam*HI fragment was cloned in both orientations from pHB223 into M13mp8, which yielded recombinant phages M13MD1 and M13MD2. Nested deletions were obtained by partial digestion of the 1.31-kb *Bam*HI fragment with *Msp*I and cloning

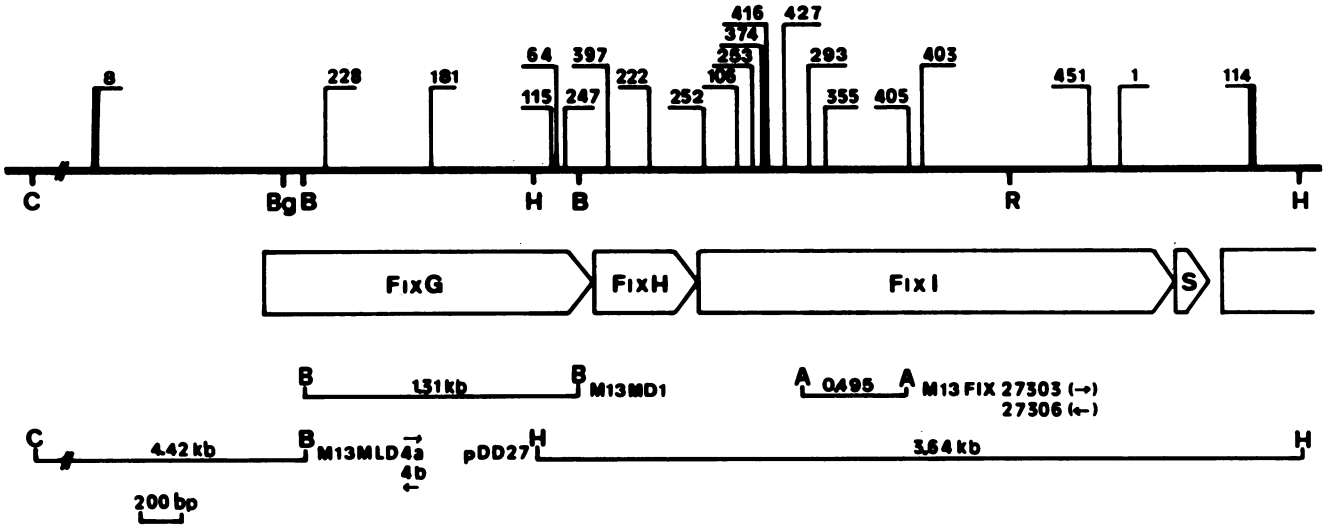


FIG. 1. Genetic-physical map of *fixGHIS*. Transposon insertions yielding a Fix$^-$ phenotype are indicated by thin vertical lines. Thick lines indicate Fix$^+$ Tn*5* insertions. Plasmid and phage constructs are described in the text. Abbreviations: A, *Ava*II; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; R, *Eco*RI; bp, base pairs.

the deleted fragments between the *Bam*HI and *Acc*I sites of M13mp8. The ends of the deletions were sequenced and generated a complete sequence of the fragment on both strands. *The Bam*HI junction between the 4.4-kb *Cla*I-*Bam*HI and the 1.31-kb *Bam*HI fragments was sequenced after cloning of the 1.19-kb *Bgl*II-*Hin*dIII fragment internal to *fixG* (Fig. 1) into M13mp19. The 3.64-kb *Hin*dIII fragment was prepared from pDD27 (12). It was partially digested with *Sau*3A, *Msp*I, or *Taq*I, and the fragments were randomly cloned into suitably (*Bam*HI or *Acc*I) cut M13mp8 or M13mp9. Sequences obtained from these recombinants generated a single contiguity. However, one single region could not be sequenced in the rightward direction by this approach. We therefore purified a 495-base-pair *Ava*II fragment corresponding to this region, filled the cohesive ends with the large fragment of DNA polymerase I, and cloned the fragment into the *Sma*I site of M13mp8. This yielded phages M13FIX27303 and M13FIX27306 (Fig. 1), which permitted completion of the sequencing.

Open reading frames (ORFs) were assessed by (i) codon usage according to the codon usage in *Escherichia coli*, (ii) the presence of possible translation initiation sites (48, 49), and (iii) correlation with the physical maps of Tn*5* insertions of known cytological phenotypes. Comparisons with the NBRF and GENBANK sequence data bases were performed on a DPS8 computer at CITI2 in Paris (20). Significance of an alignment between two sequences was assessed by using program ALIGN of Dayhoff et al. (13), with a gap penalty of 10 and 100 random permutations of both sequences. Multiple sequence alignments were obtained from pairwise alignments, using a multiple-alignment editor written by F. Corpet (Laboratoire de Génétique Cellulaire, INRA, Castanet-Tolosan, France). Potential transmembrane sequences were searched as published elsewhere (19).

RESULTS

The *fix* cluster, 200 kb from the *nif* cluster on *R. meliloti* pSym (6), contains two single-copy *fix* loci on either side of a *fix* region duplicated elsewhere on pSym (38). One locus contains the *fixLJ* operon, which is regulatory and controls the expression of *nif* and *fix* genes (11). The second locus (which is proximal to the *nif* cluster on pSym) is described here. It has been referred to as operon I in a preliminary report (5). Mutants in this region are prototrophic (6) and can therefore be considered as bona fide *fix* mutants.

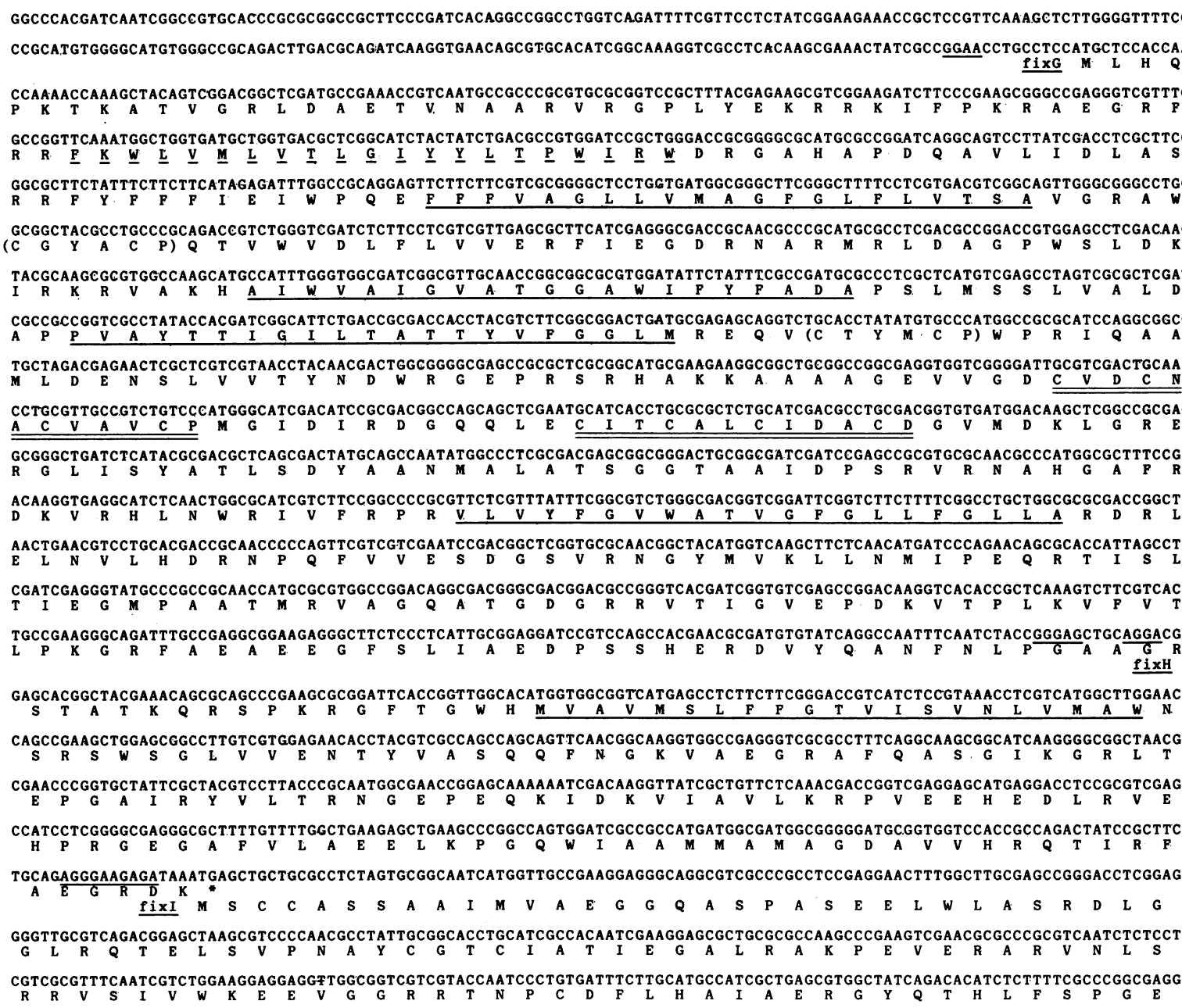
Genetic organization. The genetic organization of the *fix* locus proximal to the *nif* cluster was investigated by mutagenizing the corresponding DNA region with transposon Tn*5*. Two procedures were used. (i) Tn*5* insertions were generated on pTH2 in *E. coli*, and those mapping in the studied region were screened by restriction enzyme analysis. After marker exchange in *R. meliloti* (41), the effect of individual pSym-located Tn*5* insertions on the Fix phenotype was tested on alfalfa (*Medicago sativa*). (ii) Alternatively, Fix$^-$::Tn*5* insertions in the studied region were directly selected by using as a recipient for pTH2::Tn*5* incoming plasmids a Fix$^-$ strain RmHG2H, deleted for most of the region, and screening for Fix$^-$ transconjugants [RmHG2H(pTH2) is Fix$^+$]. These insertions were then recombined into the wild-type *R. meliloti* GM1708 by marker exchange (41). GMI955 is a *fix-181*::Tn*7* mutant generated by regional transposon mutagenesis (26).

To determine whether one or more transcription units were present, complementation experiments between pSym-

TABLE 2. Complementation assays of the Fix phenotype

Complementing plasmid[a]	Relevant background genotype		
	recA fix-181	*recA fix-2.64*	*recA fix-2.1*
pTH2	+	+	+
pTH2.228	−	−	−
pTH2.181	−	−	−
pTH2.64	−	−	+
pTH2.397	−	+	−
pTH2.222	−	+	−
pTH2.106	−	+	−
pTH2.405	−	+	−
pTH2.1	−	+	−

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


GGCCCACGATCAATCGGCCGTGCACCCGCGCGGCCGCTTCCCGATCACAGGCCGGCCTGGTCAGATTTTCGTTCCTCTATCGGAAGAAACCGCTCCGTTCAAAGCTCTTGGGGTTTTCCA 120
CCGCATGTGGGGCATGTGGGCCGCAGACTTGACGCAGATCAAGGTGAACAGCGTGCACATCGGCAAAGGTCGCCTCACAAGCGAAACTATCGCCGGAACCTGCCTCCATGCTCCACCAAC 240
fixG M L H Q
CCAAAACCAAAGCTACAGTCGGACGGCTCGATGCCGAAACCGTCAATGCCGCCCGCGTGCGCGGTCCGCTTTACGAGAAGCGTCGGAAGATCTTCCCGAAGCGGGCCGAGGGTCGTTTCC 360
P K T K A T V G R L D A E T V N A A R V R G P L Y E K R R K I F P K R A E G R F
GCCGGTTCAAATGGCTGGTGATGCTGGTGACGCTCGGCATCTACTATCTGACGCCGTGGATCCGCTGGGACCGCGGGGCGCATGCGCCGGATCAGGCAGTCCTTATCGACCTCGCTTCCC 480
R R F K W L V M L V T L G I Y Y L T P W I R W D R G A H A P D Q A V L I D L A S
GGCGCTTCTATTTCTTCTTCATAGAGATTTGGCCGCAGGAGTTCTTCTTCGTCGCGGGGCTCCTGGTGATGGCGGGCTTCGGGCTTTTCCTCGTGACGTCGGCAGTTGGGCGGGCCTGGT 600
R R F Y F P F F I E I W P Q E F F F V A G L L V M A G F G L F L V T S A V G R A W
GCGGCTACGCCTGCCCGCAGACCGTCTGGGTCGATCTCTTCCTCGTCGTTGAGCGCTTCATCGAGGGCGACCGCAACGCCCGCATGCGCCTCGACGCCGGACCGTGGAGCCTCGACAAGA 720
(C G Y A C P) Q T V W V D L F L V V E R F I E G D R N A R M R L D A G P W S L D K
TACGCAAGCGCGTGGCCAAGCATGCCATTTGGGTGGCGATCGGCGTTGCAACCGGCGGCGCGTGGATATTCTATTTCGCCGATGCGCCCTCGCTCATGTCGAGCCTAGTCGCGCTCGATG 840
I R K R V A K H A I W V A I G V A T G G A W I F Y F A D A P S L M S S L V A L D
CGCCGCCGGTCGCCTATACCACGATCGGCATTCTGACCGCGACCACCTACGTCTTCGGCGGACTGATGCGAGAGCAGGTCTGCACCTATATGTGCCCATGGCCGCGCATCCAGGCGGCCA 960
A P P V A Y T T I G I L T A T T Y V F G G L M R E Q V (C T Y M C P) W P R I Q A A
TGCTAGACGAGAACTCGCTCGTCGTAACCTACAACGACTGGCGGGGCGAGCCGCGCTCGCGGCATGCGAAGAAGGCGGCTGCGGCCGGCGAGGTGGTCGGGGATTGCGTCGACTGCAACG 1080
M L D E N S L V V T Y N D W R G E P R S R H A K K A A A A G E V V G D C V D C N
CCTGCGTTGCCGTCTGTCCCATGGGCATCGACATCCGCGACGGCCAGCAGCTCGAATGCATCACCTGCGCGCTCTGCATCGACGCCTGCGACGGTGTGATGGACAAGCTCGGCCGCGAGC 1200
A C V A V C P M G I D I R D G Q Q L E C I T C A L C I D A C D G V M D K L G R E
GCGGGCTGATCTCATACGCGACGCTCAGCGACTATGCAGCCAATATGGCCCTCGCGACGAGCGGCGGGACTGCGGCGATCGATCCGAGCCGCGTGCGCAACGCCCATGGCGCTTTCCGTG 1320
R G L I S Y A T L S D Y A A A N M A L A T S G G T A A I D P S R V R N A H G A F R
ACAAGGTGAGGCATCTCAACTGGCGCATCGTCTTCCGGCCCCGCGTTCTCGTTTATTTCGGCGTCTGGGCGACGGTCGGATTCGGTCTTCTTTTCGGCCTGCTGGCGCGCGACCGGCTAG 1440
D K V R H L N W R I V F R P R V L V Y F G V W A T V G F G L L F G L L A R D R L
AACTGAACGTCCTGCACGACCGCAACCCCCAGTTCGTCGTCGAATCCGACGGCTCGGTGCGCAACGGCTACATGGTCAAGCTTCTCAACATGATCCCAGAACAGCGCACCATTAGCCTGA 1560
E L N V L H D R N P Q F V V E S D G S V R N G Y M V K L L N M I P E Q R T I S L
CGATCGAGGGTATGCCCGCCGCAACCATGCGCGTGGCCGGACAGGCGACGGGCGACGGACGCCGGGTCACGATCGGTGTCGAGCCGGACAAGGTCACACCGCTCAAAGTCTTCGTCACGC 1680
T I E G M P A A T M R V A G Q A T G D G R R V T I G V E P D K V T P L K V F V T
TGCCGAAGGGCAGATTTGCCGAGGCGGAAGAGGGCTTCTCCCTCATTGCGGAGGATCCGTCCAGCCACGAACGCGATGTGTATCAGGCCAATTTCAATCTACCGGGAGCTGCAGGACGAT 1800
L P K G R F A E A E E G F S L I A E D P S S H E R D V Y Q A N F N L P G A A G R *
fixH M
GAGCACGGCTACGAAACAGCGCAGCCCGAAGCGCGGATTCACCGGTTGGCACATGGTGGCGGTCATGAGCCTCTTCTTCGGGACCGTCATCTCCGTAAACCTCGTCATGGCTTGGAACGC 1920
S T A T K Q R S P K R G F T G W H M V A V M S L F F G T V I S V N L V M A W N A
CAGCCGAAGCTGGAGCGGCCTTGTCGTGGAGAACACCTACGTCGCCAGCCAGCAGTTCAACGGCAAGGTGGCCGAGGGTCGCGCCTTTCAGGCAAGCGGCATCAAGGGGCGGCTAACGAC 2040
S R S W S G L V V E N T Y V A S Q Q F N G K V A E G R A F Q A S G I K G R L T T
CGAACCCGGTGCTATTCGCTACGTCCTTACCCGCAATGGCGAACCGGAGCAAAAAATCGACAAGGTTATCGCTGTTCTCAAACGACCGGTCGAGGAGCATGAGGACCTCCGCGTCGAGCT 2160
E P G A I R Y V L T R N G E P E Q K I D K V I A V L K R P V E E H E D L R V E L
CCATCCTCGGGGCGAGGGCGCTTTTGTTTTGGCTGAAGAGCTGAAGCCCGGCCAGTGGATCGCCGCCATGATGGCGATGGCGGGGGATGCGGTGGTCCACCGCCAGACTATCCGCTTCAT 2280
H P R G E G A F V L A E E L K P G Q W I A A M M A M A G D A V V H R Q T I R F I
TGCAGAGGGAAGAGATAAATGAGCTGCTGCGCCTCTAGTGCGGCAATCATGGTTGCCGAAGGAGGGCAGGCGTCGCCCGCCTCCGAGGAACTTTGGCTTGCGAGCCGGGACCTCGGAGGC 2400
A E G R D K *
fixI M S C C A S S A A I M V A E G G Q A S P A S E E L W L A S R D L G G
GGGTTGCGTCAGACGGAGCTAAGCGTCCCCAACGCCTATTGCGGCACCTGCATCGCCACAATCGAAGGAGCGCTGCGCGCCAAGCCCGAAGTCGAACGCGCCCGCGTCAATCTCTCCTCC 2520
G L R Q T E L S V P N A Y C G T C I A T I E G A L R A K P E V E R A R V N L S S
CGTCGCGTTTCAATCGTCTGGAAGGAGGAGGTTGGCGGTCGTCGTACCAATCCCTGTGATTTCTTGCATGCCATCGCTGAGCGTGGCTATCAGACACATCTCTTTTCGCCCGGCGAGGAG 2640
R R V S I V W K E E V G G R R T N P C D F L H A I A E R G Y Q T H L F S P G E E

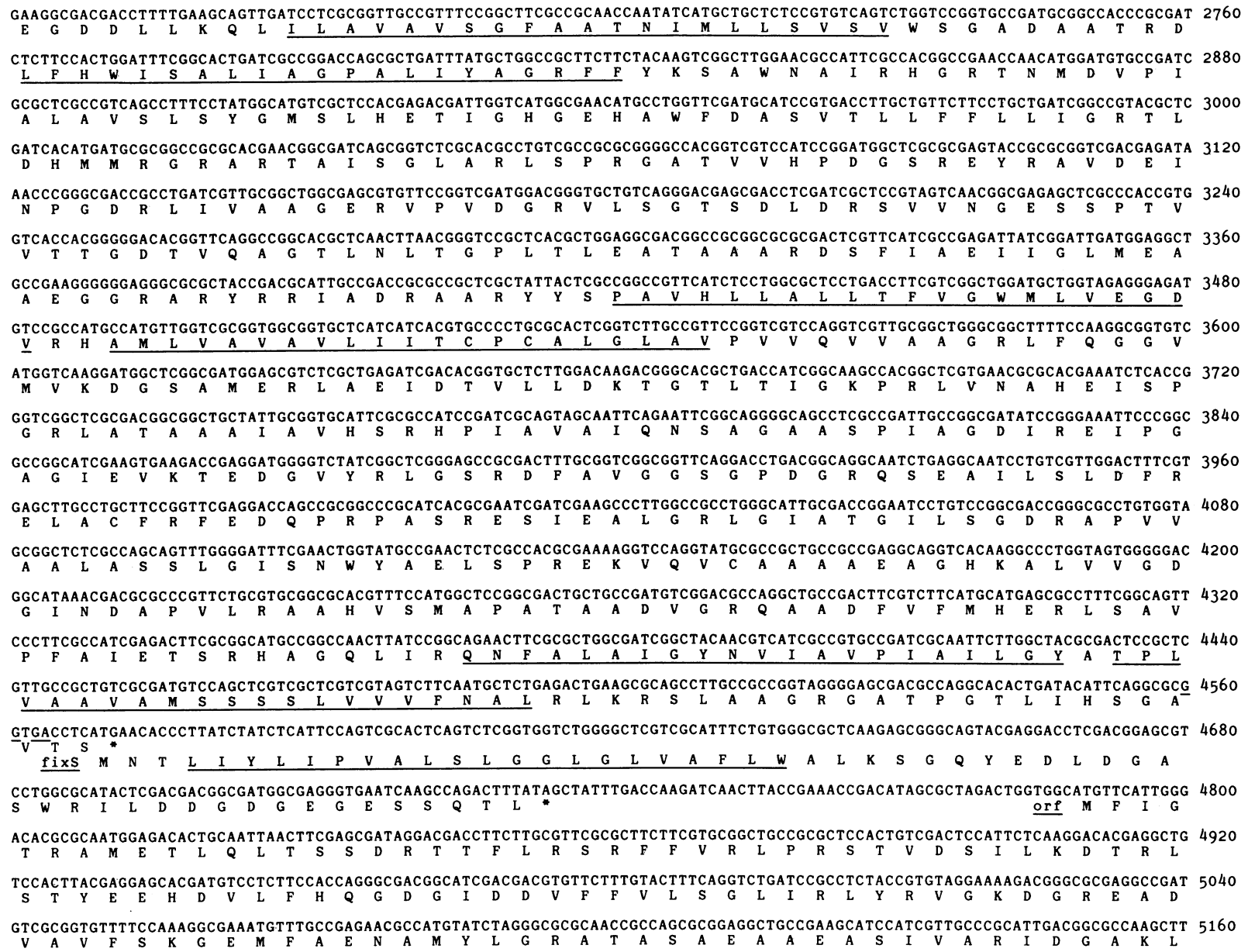


FIG. 2. Sequence of *fixGHIS*. Purine-rich stretches upstream of proposed translation initiation sites are underlined, as are potential transmembrane sequences. The first potential transmembrane sequence of FixG, predicted as globular by the hydrophobic moment plot (19),is indicated by dashes. Cysteine clusters of FixG are underlined twice, and potential half-clusters are indicated in parentheses.

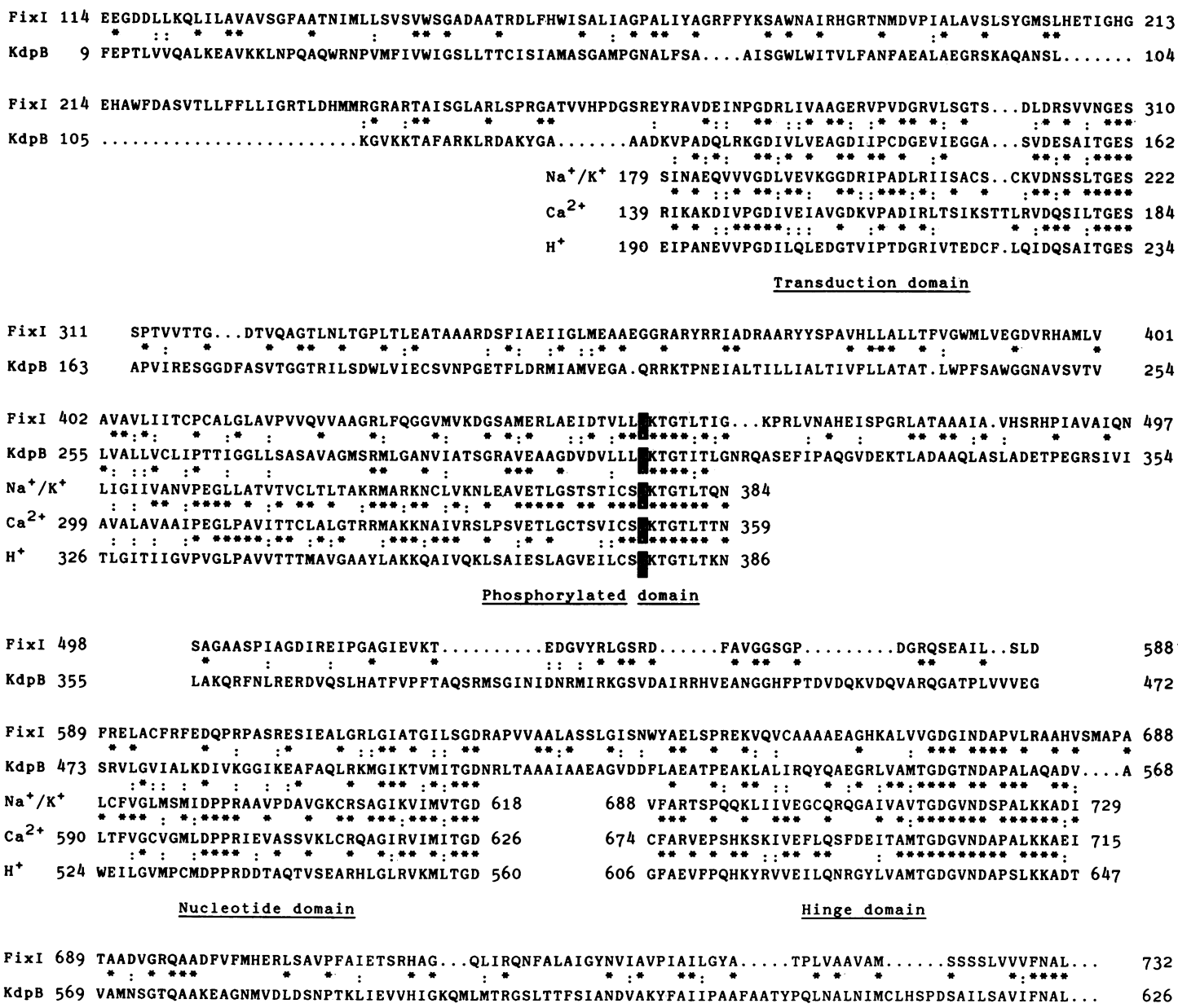


FIG. 3. Alignment of FixI with P-type ATPases. Sequences of FixI and KdpB (23) are aligned with the sequences of eucaryotic Na^+/K^+(28), Ca^{2+} (33), and H^+ (44) ATPases in the most conserved regions. The conserved phosphorylated aspartate residue is highlighted.

and pTH2-located transposon insertions were conducted in a Rec$^-$ genetic background, and merodiploids were tested for nitrogen-fixing ability on alfalfa. Absence of complementation between distal transposon insertions (Fig. 1 and Table 2) indicated that this region constitutes a single transcription unit. The positive complementation between mutations *2.397*, *2.222*, *2.106*, *2.405*, and *2.1* on the one hand and *2.64* on the other demonstrated that the Tn*5*-*2.64* insertion affects a different gene and can be explained by assuming that this particular Tn*5* insertion is nonpolar or weakly polar on downstream genes. The direction of transcription thus implied is the same as the one predicted from the sequence data presented below.

Sequence of *fixGHIS*. The sequence of the operon (Fig. 2) exhibited three large ORFs in the rightward polarity. No extensive ORF existed in the reverse polarity. Analysis of coding probability indicated that the three ORFs are very likely coding. Moreover, J. Vasse, S. Camut, F. de Billy, and G. Truchet (manuscript in preparation) have studied the cytological phenotypes of mutants in this region and classified them into three phenotypes. These phenotypes correlate with the ORFs in which the Tn*5*s are inserted. These results allowed us to define three genes, *fixG*, *-H*, and *-I*, which encode products of 524, 167, and 757 amino acids, respectively. Between adjacent genes in the operon we found an interesting ATGA motif such that the (A)TGA stop codons of upstream genes overlap the ATG(A) start codons of downstream genes, with a −1 frameshift. This motif is very suggestive of translational coupling between adjacent genes. The presumptive initiation codons of *fixG*, *fixH*, and *fixI* are preceded by purine-rich stretches, indicative of potential ribosome-binding sites (48). However, analysis of the sequence for procaryotic translational initiation sites by the method of Stormo et al. (49) surprisingly revealed a single highly probable initiator codon at position 4569, at the end of *fixI*. This potential initiator is followed by a short ORF (55 amino acids), which is likely to code for a protein as judged by its codon usage. Furthermore, the same ATGA motif as above was found between *fixI* and the short ORF downstream, again suggestive of potential translational coupling. Although we have no Tn*5* insertion in this ORF to demonstrate that it corresponds to a gene, we have tentatively named it *fixS* (in preliminary reports this potential gene had been named *fixX* [27] and then *fixY* [D. Kahn, J. Batut, M.-L. Daveran, M. David, and P. Boistard, *in* F. O'Gara, S. S. Manian, and J. J. Drevon (ed.), *The Physiological Limitations and the Genetic Improvement of Symbiotic Nitrogen Fixation*, in press], but it was renamed after other *fix* genes had been given these names [17, 29]).

FixG, a membrane-located iron-sulfur protein. The sequences of the predicted FixG, FixH, FixI, and FixS proteins were analyzed for potential transmembrane helices (19) and found to contain numerous such sequences (underlined in Fig. 2). Therefore, we propose that the four proteins are transmembrane proteins. The four proteins were compared with sequences from the National Biomedical Research Foundation data base (release 16, March 1988) or translated from the GENBANK data base (release 54, December 1987).

FixG contains two cysteine clusters of the type found in bacterial ferredoxins: CysxxCysxxCysxxxCys(Pro) (twice underlined in Fig. 2). Two other half-motifs, CysxxxCysPro, are also apparent (bracketed in Fig. 2). In ferredoxins, CysxxCysxxCysxxxCys(Pro) motifs are known to coordinate iron-sulfur centers (21). These motifs are also found in other redox proteins such as fumarate reductase (9) and succinate dehydrogenase (10), but no extensive homology has been detected between FixG and these oxidoreductases. From this analysis, we predict that FixG is involved in a redox process important for symbiotic nitrogen fixation.

TABLE 3. ALIGN scores of functional domains of P-type ATPases[a]

Domain (residues)	K^+	KdpB	Na^+/K^+	Ca^{2+}	H^+
Transduction					
FixI (262–351)	12.7	13.4	6.9	4.1	6.6
K^+ (88–176)		7.9	4.6	3.6	8.3
KdpB (114–206)			6.6	3.6	9.0
Na^+/K^+ (174–273)				14.5	11.2
Ca^{2+} (132–238)					10.6
H^+ (185–275)					
Phosphorylated					
FixI (432–502)	10.7	7.5	4.0	(0.7)[b]	6.1
K^+ (256–352)		6.3	3.3	(1.7)	4.4
KdpB (286–386)			(1.3)	3.1	4.6
Na^+/K^+ (355–498)				11.3	7.6
Ca^{2+} (330–505)					7.4
H^+ (357–465)					
Nucleotide					
FixI (502–612)	7.2	5.9	(0.8)	(1.4)	3.0
K^+ (352–454)		8.0	3.0	3.4	(2.1)
KdpB (386–496)			5.9	4.7	5.5
Na^+/K^+ (498–694)				13.5	7.9
Ca^{2+} (505–680)					7.8
H^+ (465–612)					
Hinge					
FixI (612–672)	11.0	6.6	10.8	11.0	11.5
K^+ (454–514)		13.4	12.1	16.4	10.0
KdpB (496–556)			15.1	12.0	11.2
Na^+/K^+ (694–755)				19.9	16.2
Ca^{2+} (680–740)					16.7
H^+ (612–672)					

[a] Shown are the number of standard deviations between the score of the alignment of the real sequences and the mean score of 100 alignments obtained with randomly permuted sequences (13). Functional domains have been defined by MacLennan et al. (33) for the Ca^{2+} ATPase. Sequences used in this analysis are the K^+ ATPase from *S. faecalis* (47), the KdpB ATPase from *E. coli* (23), the Na^+/K^+ ATPase from *Torpedo californica* (28), the rabbit sarcoplasmic Ca^{2+} ATPase (33), and the yeast plasma membrane H^+ ATPase (44).

[b] Figures in parentheses indicate statistically nonsignificant alignments.

Predicted function of FixI: a cation pump. FixI was found to be homologous to the KdpB ATPase from *E. coli* (23) (Fig. 3). This protein is the catalytic subunit of a K^+ pump encoded by the *kdpABC* operon (31). It belongs to ATPases of the P type (37), which also include the K^+ ATPase from *Streptococcus faecalis* (47) and eucaryotic cation pumps such as the Ca^{2+} ATPase (33), the Na^+/K^+ ATPase (28, 46), the H^+/K^+ ATPase (45), and the plasma membrane H^+ ATPase (44). This class of cation ATPases is remarkable because they contain an aspartate residue phosphorylated during enzyme turnover (37). This residue is crucial for activity and is highly conserved in all of these ATPases and in FixI (highlighted in Fig. 3). The best-characterized P-type ATPase is the sarcoplasmic Ca^{2+} ATPase (33). It is composed of 10 transmembrane helices which anchor the protein into the membrane. The five helices in the amino-terminal part of the protein protrude into the cytoplasm and constitute a stalk which connects the globular cytoplasmic domains to the membrane. Four major cytoplasmic domains have been described (33): (i) a transduction domain, (ii) the phosphorylated domain, (iii) the nucleotide-binding domain, and (iv) a hinge domain. All four domains from FixI showed

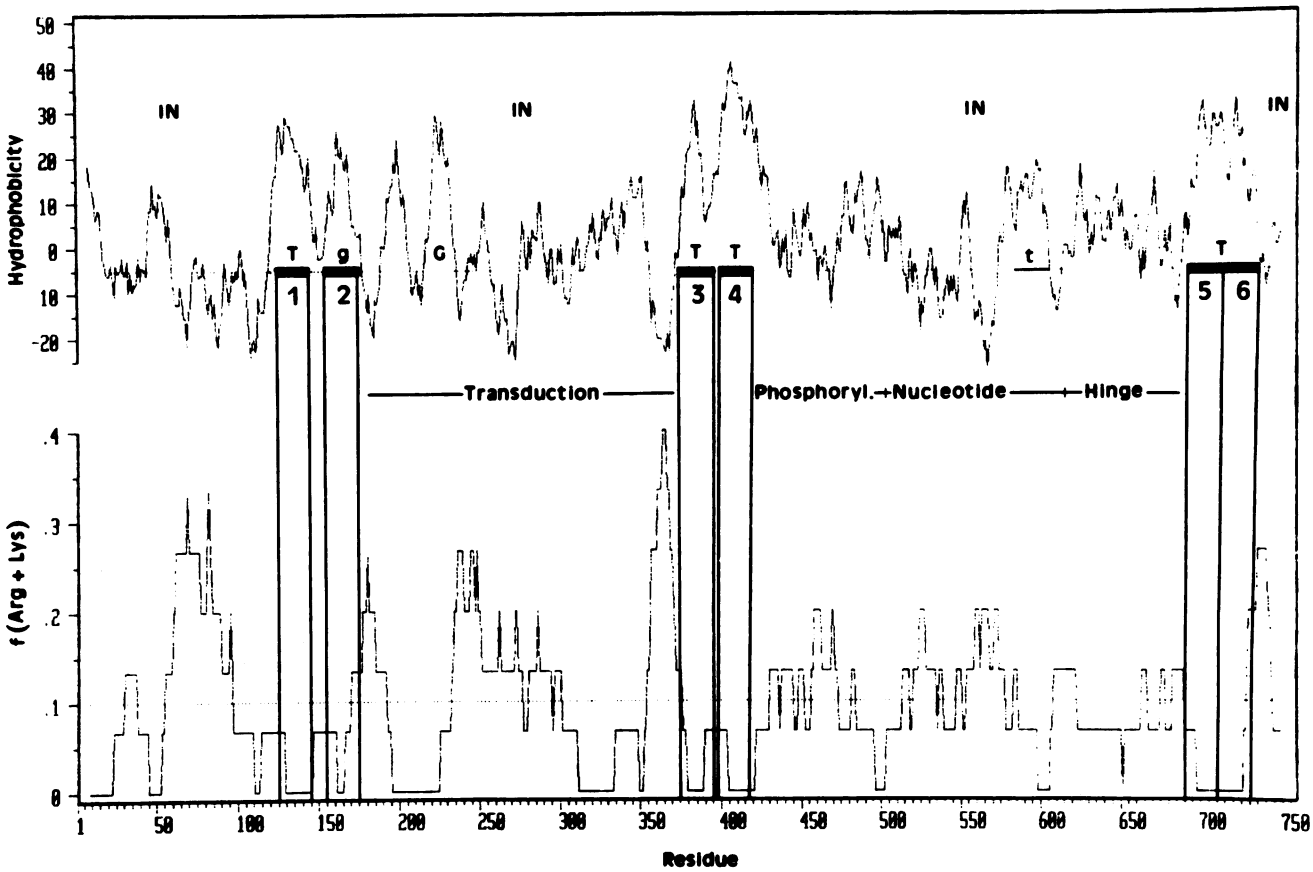


FIG. 4. Proposed transmembrane topology of FixI. Hydrophobicity (top [30]) and Arg-plus-Lys content (bottom [52]) of FixI are plotted, using a window of 15 residues. Proposed transmembrane sequences are numbered 1 to 6. Hydrophobic sequences predicted to be transmembrane or globular by the method of Eisenberg et al. (19) are indicated by T or G, respectively, when the prediction agrees with the proposed topology and by t or g when it disagrees (see text for discussion). Locations of the transduction, phosphorylation, nucleotide, and hinge domains are derived from the analysis of mammalian Ca^{2+} ATPase (33).

significant homology with the corresponding domains of other P-type ATPases (Table 3). The stalk and the transmembrane helices, however, were not highly conserved.

The homology between FixI and procaryotic K^+ pumps prompted us to test the potassium requirements of a *fixI2.1* mutant. The mutant grew as well as the wild type in minimal medium containing as little as 1 μM K^+. We conclude that *fixI* is not equivalent to *E. coli kdpB* in that it is not required for growth in low potassium. It therefore appears very likely that the function of the FixI cation pump is specific for the symbiotic state.

Transmembrane topology of the FixI ATPase. We took advantage of the homology between FixI and P-type ATPases to propose the transmembrane topology of FixI shown in Fig. 4. First, possible transmembrane sequences of FixI and of the Ca^{2+}, Na^+/K^+, H^+, and K^+ ATPases were tentatively assigned to globular, transmembrane, or surface domains, using the hydrophobic moment plot procedure of Eisenberg et al. (19). Second, alignment of these sequences allowed us to derive a consensus core topology, which consists of six transmembrane helices organized in three transmembrane hairpins. This topology is consistent with the currently accepted structure of P-type ATPases. Our analysis highlighted the fact, already stressed by Eisenberg et al. (19), that the hydrophobic moment plot alone does not allow a firm assessment of all potential transmembrane helices, mainly because boundaries of regions of the hydrophobic moment plot are empirical and not clearly defined (19). For instance, analysis of the FixI sequence by using the hydrophobic moment plot led to four correctly predicted transmembrane sequences (labeled T in Fig. 4) but predicted transmembrane sequence 2 as globular and an additional transmembrane sequence in what appears to be a cytoplasmic domain of P-type ATPases (labeled t in Fig. 4). Similar disagreements between predictions using the hydrophobic moment plot and the consensus structure of P-type ATPases were found with the Ca^{2+} (33), H^+ (44), and K^+ (47) ATPases.

Another approach to study the topology of bacterial inner membrane proteins has been proposed by von Heijne (52), who noticed that the frequency of arginine-plus-lysine residues is significantly higher in cytoplasmic loops than in periplasmic loops. This analysis applies only to relatively short loops (less than 65 residues long) and therefore does not apply to the large cytoplasmic domains of FixI. However, the plot of f(Arg+Lys) for FixI (Fig. 4) shows marked peaks on the cytoplasmic sides of transmembrane sequences 2, 3, and 6. Although it is not the purpose of this paper to assess the significance of such features, it is tempting to speculate that arginine and lysine residues may tend to cluster at the cytoplasmic sides of transmembrane sequences and lock the topology of inner membrane proteins.

Conservation of *fixGHI* among *Rhizobium* sp. To determine whether sequences homologous to the *fixGHI* operon could

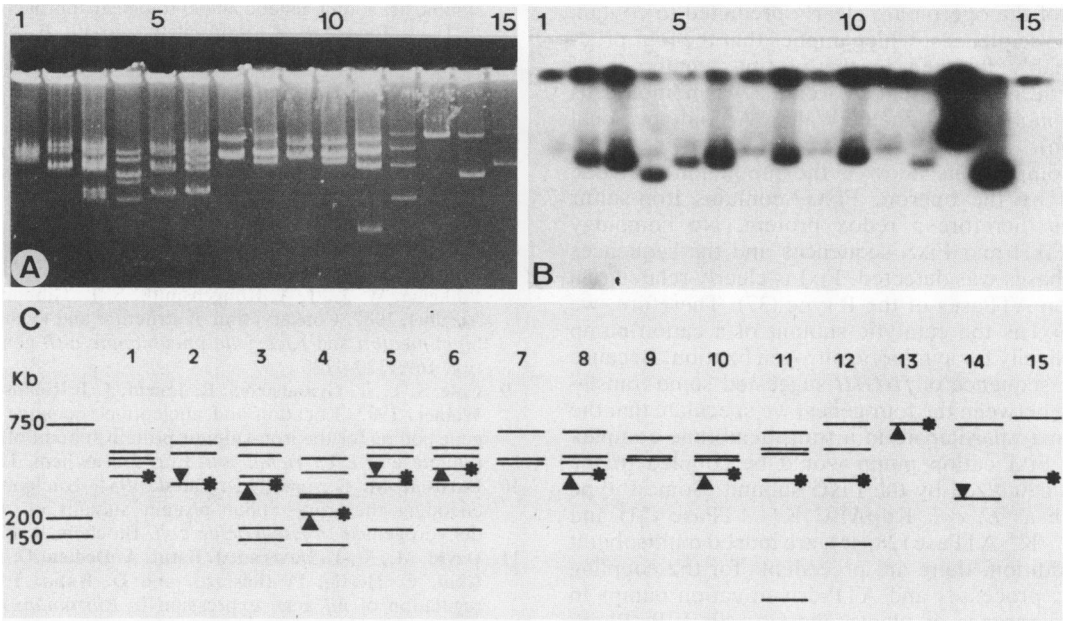


FIG. 5. Conservation of *fixGHI* among *Rhizobium* sp. (A) Agarose gel electrophoresis of lysates from *R. leguminosarum* (bv. *trifolii*) RCR221 (1), RCR5 (2) (bv. *viciae*), RCR1045 (3), JI248 (4), JI6015 (pSym-deleted derivative of JI897) (5), JI897 (6), B151 (pSym-cured derivative of 128C53) (7), 128C53 (8) (bv. *phaseoli*), JI8400 (pSym-cured derivative of JI8002) (9), JI8002 (10), NVRS963A (11), and RCR3618 (12), from *R. meliloti* RCR2001 (13), and from *R. fredii* USDA194 (14) and USDA193 (15). (B) Autoradiogram after hybridization with pDD27. (C) Schematic representation showing pSyms (*) and plasmids with homology to *fixGHI* (▲, ▼). The location of pSyms (except in lane 5) was determined by hybridization with the *nif*-containing plasmid pIJ1247.

be found in other *Rhizobium* sp., pDD27, which carries part of *fixG* and the entire *fixH* and *fixI* genes (Fig. 1), was used to probe plasmid DNAs from *R. leguminosarum* bv. *viciae*, bv. *phaseoli*, and bv. *trifolii* and *Rhizobium fredii* separated on agarose gel by the procedure of Eckhardt (18) (Fig. 5A). Strains cured of or deleted in the pSym plasmids were introduced as controls. To identify pSym plasmids unambiguously, the same blot was hybridized with pIJ1247, which carries the *nifHD* genes from *R. leguminosarum* (data not shown). The pDD27 probe hybridized strongly with the pSym of *R. meliloti* RCR2001 and of *R. leguminosarum* RCR1045, JI248, JI897, 128C53 (bv. *viciae*), RCR5 (bv. *trifolii*), and JI8002 (bv. *phaseoli*) and with a non-Sym plasmid of *R. fredii* USDA194 (Fig. 5B and C). The same pattern was obtained with the purified 2.25-kb *Hin*dIII-*Eco*RI fragment internal to the *fixGHI* operon as a probe (Fig. 1). *R. fredii* USDA193 did not appear to contain any plasmid with homology to *fixGHI* (Fig. 5, lane 15). However, hybridizing bands were detected when total genomic DNA was probed (data not shown). This result could indicate a chromosomal location of *fixGHI* homologous sequences in this strain. On the other hand, *R. fredii* USDA194 had a plasmid with very strong homology to *fixGHI* (Fig. 5, lane 14), whereas *nif* and *nod* genes are located on the chromosome of this strain (36).

To investigate whether the sequences homologous to *fixGHI* in other *Rhizobium* species were functional, we tested the ability of two of them to complement *R. meliloti fixGHI* mutants. Two Tn*5*-tagged self-transmissible pSym plasmids, pRL1JI from *R. leguminosarum* bv. *viciae* JI248 (25) and pRtr5a from *R. leguminosarum* bv. *trifolii* RCR5 (24), which bear homology with *fixGHI* (Fig. 5, lanes 4 and 2, respectively), were introduced into the *R. meliloti fixG181* mutant GMI955. Both *R. meliloti* GMI955(pRL1JI) and GMI955(pRtr5a) transconjugants were Fix$^+$ on alfalfa.

These results show that genes homologous to the *R. meliloti fixGHI* genes exist and are functional in other *Rhizobium* spp. In preliminary experiments, we detected sequences homologous to the *fixGHI*-specific probe in various members of the genus *Bradyrhizobium* and in *A. caulinodans* ORS571 (data not shown). This finding suggests that the *fixGHI* genes could be further widespread among symbiotic nitrogen fixers.

DISCUSSION

The *fixGHI* operon is remarkably conserved among rhizobia as judged from DNA hybridization and interspecies complementation experiments. The implication is that *fixGHI* probably are essential nitrogen fixation genes in other rhizobia. In addition, pDD27 DNA, which contains part of *fixG* and the entire *fixH* and *fixI* genes, did not hybridize significantly with genomic DNA from *Azotobacter chroococcum* (R. Robson and D. Kahn, unpublished data). Therefore, *fixGHI* may be absent from a typical aerobic nitrogen-fixing organism such as *A. chroococcum*. This would argue in favor of a specifically symbiotic function of *fixGHI* instead of a function essential for free-living aerobic nitrogen fixation. This hypothesis could be tested after identification of genes homologous to *R. meliloti fixGHI* in a *Bradyrhizobium* species capable of both symbiotic and free-living microaerophilic nitrogen fixation.

Sequence analysis of *fixGHI* revealed some interesting features. First, the operon appears to be tightly packed, which is quite common among procaryotes, and the overlap between stop and start codons in the form of an ATGA motif suggests that the genes of this operon undergo some translational coupling. Second, the sequence data suggest the existence of a fourth gene in the operon. This putative gene, *fixS*, would also be translationally coupled to *fixI*. Third, all

four products of the operon are clearly predicted to contain transmembrane sequences, which implies that the four products are probably inserted in the plasma membrane of rhizobia. Fourth, none of the four proteins was homologous to *K. pneumoniae nif* products (Walter Arnold, personal communication).

Sequence comparisons allowed the prediction that one protein coded by the operon, FixG, contains iron-sulfur centers and is therefore a redox protein. No homology between the FixH and FixS sequences and the sequences from the data bases was detected. FixI is clearly related to a family of cation ATPases of the P type (37). Therefore, we predict that FixI is the catalytic subunit of a cation pump required specifically for symbiotic nitrogen fixation. Because the nucleotide sequence of *fixGHI* suggested some translational coupling between the four genes, we speculate that the four proteins may participate in a transmembrane complex such that the FixI cation pump would be coupled to the redox process catalyzed by the FixG subunit. Some P-type ATPases, such as *E. coli* KdpABC K^+ ATPase (31) and eucaryotic Na^+/K^+ ATPase (28, 46), are indeed multisubunit proteins. In addition, there are precedents for the coupling between redox processes and ATP-driven cation pumps in the plasma membranes of plant cells (34, 40; J.-P. Blein, Ph.D. thesis, Université de Paris-Sud, Orsay, France, 1986). It would be of interest to test whether a similar coupling exists in the plasma membrane of *Rhizobium* bacteroids. These hypotheses can be investigated by using constructs expressing *fixGHI* in free-living cultures, which should also allow the biochemical relationships between FixGHI and the nitrogen fixation apparatus of *R. meliloti* to be defined.

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