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Physical Organization of the *Bradyrhizobium japonicum* Nitrogenase Gene Region†

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In *Bradyrhizobium japonicum* USDA 110 the three genes that encode the nitrogenase enzyme complex are separated into two transcription units, *nifH* and *nifDK*. We have physically mapped a 33-kilobase-pair region of the *B. japonicum* genome that contains both *nifH* and *nifDK*. The *nifDK* operon is located transcriptionally upstream from *nifH*, and all three genes are transcribed in the same direction. Within the 20-kilobase-pair region that separates the promoters for these two transcription units, we have identified a region homologous to the *Klebsiella pneumoniae nifA* gene. This *nifA* homology is situated about 6 kilobase pairs upstream from the *nifH* transcriptional initiation signal, in an analogous position to the *nifA*-like locus previously described for *Rhizobium meliloti*. In addition, we have characterized a second distinct *nifA* homology in *B. japonicum* that is not directly linked to the nitrogenase structural gene region.

The biological reduction of dinitrogen is carried out by a number of procaryotic organisms via the nitrogenase enzyme complex. The conservation of DNA and amino acid sequences among nitrogenase genes (*nifH*, *-D*, and *-K*) from a variety of nitrogen-fixing organisms (including *Bradyrhizobium japonicum*) is well documented (7, 14, 41). This interspecies conservation, however, does not extend to include a preservation of *nif* operon structure. Whereas the *nifH*, *-D*, and *-K* genes are transcribed as a single operon (in the order *nifHDK*) in *Klebsiella pneumoniae* (36) and in several fast-growing *Rhizobium* spp. (including *Rhizobium meliloti* [2] and *Rhizobium leguminosarum* [25], they occur as two separate transcription units, *nifHD* and *nifK*, in *Anabaena* 7120 (38) and as *nifH* and *nifDK* in at least some *B. japonicum* strains and cowpea *Rhizobium* spp. (24, 43).

In addition to the genes encoding the three constituent polypeptides of the nitrogenase complex, the synthesis of an active nitrogenase in the free-living nitrogen-fixing bacterium *K. pneumoniae* requires the expression of at least 14 other genes (36, 39, 40). These nitrogen fixation (*nif*) genes are arranged in seven or eight operons clustered within a 24-kilobase-pair (kbp) section of the chromosome. The expression of these *nif* genes is coordinately controlled via the products of the *nifLA* operon (4, 15, 22, 27, 33, 34, 39), which is in turn controlled by the general nitrogen regulatory system (Ntr) common to all enteric bacteria studied (28).

In fast-growing species of *Rhizobium* examined thus far, at least some of the genes responsible for nitrogen fixation (including the *nifHDK* operon) and nodulation of host legumes are closely linked on large plasmids (2, 23). In *R. meliloti*, the *nifHDK* operon and at least two other transcription units essential for nitrogen fixation are found within 15 kbp of one another (5, 9, 10, 42). One of these transcription units encodes a positive regulator of symbiotic nitrogen fixation that is similar in both structure and function to the *K. pneumoniae nifA* gene product (46–47). The arrangement of nitrogen fixation genes in *R. leguminosarum* is somewhat different in that the *nifHDK* operon is separated from other *nif* genes (including a *nifA*-like locus) by a 30-kbp region containing genes that encode nodulation functions (12, 26, 35).

We have begun to characterize the organization and expression of the genes involved in nitrogen fixation by the soybean symbiont *B. japonicum* USDA 110. In this paper we report the determination of the physical arrangement of the *nifH* and *nifDK* transcription units on the *B. japonicum* genome. In addition, we have identified two separate regions of the *B. japonicum* genome that share sequence homology with the *K. pneumoniae nifA* gene. One of these homologies falls between *nifH* and *nifDK* at a similar position with respect to the *nifH* promoter as the *nif* regulatory gene region identified in *R. meliloti*.

MATERIALS AND METHODS

Bacterial strains and media. The *Escherichia coli* K-12 strain ED8654 (*gal met hsdR$_k$ supE supF*) was used for general plasmid cloning and maintenance as well as for maintenance of the *B. japonicum* genomic DNA library cloned into the broad-host-range cosmid cloning vehicle pLAFR1 (17). For colony hybridization to the *K. pneumoniae nifA* gene, an identical cosmid library was maintained in the glutamine auxotroph, *E. coli* ET8051 Δ(*rha-glnA*) *hutC$_k$ rbs* nal^r, which contains a deletion of the entire *glnALG* operon (16). *B. japonicum* USDA 110 was grown in YEX medium (0.04% yeast extract, 0.3% xylose, 3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl).

DNA techniques. Genomic DNA from *B. japonicum* was purified by phenol extraction (31). Relaxed replicon plasmid DNA was isolated by the method of Clewell and Helinski (8). Isolation of plasmid DNA from the low-copy-number cosmid clone bank was modified as described by Friedman et al. (17). Isolation of DNA restriction endonuclease fragments for use as probes was as described previously (1). All other restriction nuclease mapping and enzymatic cloning techniques were standard (29).

Construction and maintenance of a *B. japonicum* cosmid gene bank. Total DNA from *B. japonicum* USDA 110 was partially digested with *Eco*RI and subjected to centrifugation

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for 8 hr at 4°C and 155,000 × g_{av} through a 12-ml 5 to 20% (wt/vol) sucrose gradient dissolved in 1 M sodium acetate–10 mM Tris-hydrochloride–1 mM EDTA–0.01% sodium lauroyl sarcosinate (pH 8). Fractions from this gradient that contained DNA molecules greater than 15 kbp in size were pooled and used for construction of a gene bank in the broad-host-range cosmid vector pLAFRI (17). *Eco*RI-cleaved, phosphatase-treated vector DNA was ligated to the partially digested *B. japonicum* DNA at a molar ratio of 5:1 (vector/insert) as described by Maniatis et al. (29). After ligation, DNA was packaged as described previously (29) and used to transduce *E. coli* K-12 ED8654 (or ET8051) to tetracycline resistance. Transductants were replicated in an ordered array to both agar stabs and liquid medium in polycarbonate microtiter dishes. For screening, the cosmid-containing isolates were transferred with the aid of a steel prong replicator to sheets of cellulose nitrate lying on a dish of agar-solidified medium and then allowed to grow to colonies.

Hybridization procedures. *E. coli* ED8654 (or ET8051) colonies harboring the ordered cosmid library were lysed, and their DNA was bound to nitrocellulose filters as described by Grunstein and Hogness (19). Before hybridization, filters were incubated for 1 h at 65°C in 5× Denhardt solution (11)–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$ [pH 7.7], 1 mM EDTA)–200 μg of sheared and denatured salmon sperm DNA per ml. ^{32}P-labeled hybridization probes were prepared by nick translation (30). Hybridization reactions were carried out for 12 to 24 h at 65°C in 5× SSPE–1.5× Denhardt solution–100 μg of salmon sperm DNA per ml. After hybridization, filters were washed twice for 15 min each at room temperature in 2× SSPE–0.1% sodium dodecyl sulfate followed by two washes in 0.1× SSPE–0.1% sodium dodecyl sulfate. Hybridization signals were detected by autoradiography.

RESULTS

Identification of *nif*-specific cosmid clones. To determine whether *B. japonicum* USDA 110 *nif* genes are clustered in a manner similar to that seen for other nitrogen-fixing organisms, we have used DNA sequences from the *nifH* and *nifD* genes of *B. japonicum* as well as the *nifA* gene of *K. pneumoniae* (see Fig. 1 for restriction maps and references) as hybridization probes to screen a library of *B. japonicum* genomic DNA cloned into the broad-host-range cosmid vector pLAFR1 (17). This library is maintained as an ordered array of 1,426 individual transformants. The average insert size per recombinant cosmid is 24 kbp, therefore, with a genome size of 10,000 kbp (T. Casper and B. K. Chelm, unpublished data), any genomic sequence will have a 97% probability of being represented. The six cosmids identified in this manner can be separated into three classes on the basis of DNA homology (Table 1). The first class contains two cosmids that hybridize to both the *nifH* and the *nifA* probes. The single class II cosmid also hybridizes to the *K. pneumoniae nifA* gene. This clone, however, shows no homology to the *nifH* gene probe, but instead hybridizes strongly to the *B. japonicum nifD* gene and can thus be explained if a *nifA* homology falls between the *nifH* and *nifDK* transcription units. Alternatively, *nifH* and *nifDK* of *B. japonicum* may each be linked to a separate *nifA* homology, but not to each other. Finally, the third class of cosmid clones contains three members, all of which share homology with only the *Klebsiella nifA* gene. This group of cosmids can fit into either of the two models for *nif* gene arrangement described above. This third hybridization class can also be explained by a region of *nifA* homology in the *B. japonicum* genome that is not closely linked to either of the nitrogenase structural gene transcription units.

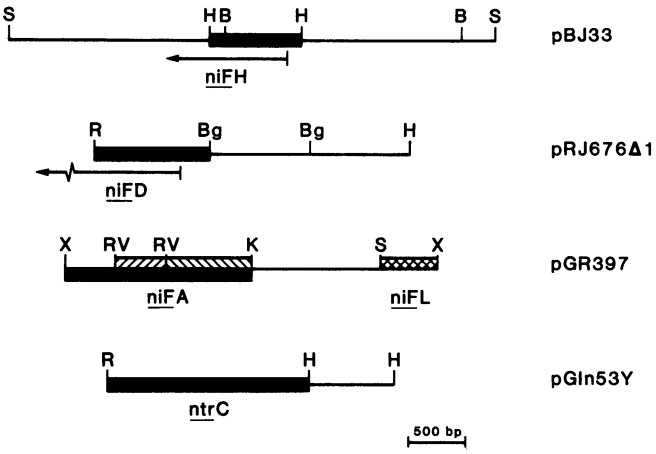

FIG. 1. Summary of hybridization probes. Boxed regions indicate restriction endonuclease fragments isolated for use as hybridization probes. The positions of transcriptional initiation sites for the *B. japonicum nifH* and *nifD* genes within the probe fragments isolated from pBJ33 and pRJ676Δ1 (21), respectively, are indicated by the arrows. The plasmid pBJ33 is a subclone of the *Sal*I fragment containing the *nifH* gene from λNH-1 (1). The 1.6-kbp *Kpn*I-*Xma*I fragment from pGR397 (37) was used as a general *nifA* hybridization probe. The two internal *Eco*RV sites were used to divide this region into a 750-bp *Kpn*I-*Eco*RV 5′ *nifA* probe and a 450-bp *Eco*RV-*Eco*RV 3′ *nifA* probe. In addition, a 500-bp *Xma*I-*Sal*I fragment internal to the *K. pneumoniae nifL* gene was also obtained from pGR397. The entire *E. coli ntrC* gene was isolated as a 1.7-kbp *Hin*dIII-*Eco*RI fragment from pGln53Y (6). Restriction endonuclease sites are *Sal*I (S), *Hin*dIII (H); *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (R), *Xho*I (X), *Eco*RV (RV), and *Kpn*I (K).

In addition to the homologies discussed above and summarized in Table 1, there is a fourth group of cosmid clones worthy of mention. These cosmids hybridize to the *nifD* probe, although much more weakly than does pRJcos2-63. We have detected 33 different cosmid clones with this property. Southern hybridization to *Eco*RI digests of these cosmids indicates that the *B. japonicum* genome carries at least 16 distinct regions of homology to the *nifD* probe described in Fig. 1 (data not shown). Since the probe used in this experiment contains the *nifD* promoter as well as the 5′ end of the *nifD* gene, it is likely that these cosmids hybridize on the basis of homology to the *nifD* regulatory region. Similar homologies have been observed in *R. meliloti* when probed with the promoter region of the *R. meliloti nifHDK* operon (3). If this is indeed the case, it is interesting that the *nifH* probe used in these experiments, which also contains the *nifH* regulatory region, did not hybridize to any of the cosmids in this group. Since we have found that the *nifH* and *nifD* promoters share only 30 base pairs (bp) of 70% homolo-

TABLE 1. *nifA*,-*H*, and-*D* cosmid classes

Class	Clone No.	nifH	nifD	nifA(*B*)
I	pRJcos1-62	+	−	+
	pRJcos14-58	+	−	+
II	pRJcos2-63	−	+	+
III	pRJcos2-43	−	−	+
	pRJcos7-3	−	−	+
	pRJcos12-29	−	−	+

gy (1), it is not surprising that these two promoter regions would show no cross-hybridization.

Characterization of *nif* cosmid classes. To distinguish between the alternative explanations for the *nifH*, *nifD*, and *nifA* hybridization data discussed above, DNA was isolated from each of the cosmids and digested with the restriction endonuclease *Eco*RI. The ethidium bromide-stained banding pattern of these cosmids is shown in Fig. 2a. The first three lanes contain the class III cosmids, which hybridize only to the *K. pneumoniae nifA* probe. The *Eco*RI digestion patterns of all three cosmids in this group appear to share a number of fragments, suggesting a common genomic origin for these clones. On the other hand, the *Eco*RI restriction digest patterns for class I and class II cosmids (lanes 4 and 5, respectively) share no common bands with the class III clones, but do have at least three *Eco*RI fragments in common. In addition, Southern hybridization (44) of these cosmid digests to the *K. pneumoniae nifA* gene indicated that all of the class III cosmids have an 11-kbp *Eco*RI fragment that contains the *nifA* homology, whereas the class I and class II cosmids both contain *nifA* homology on a 4.9-kb *Eco*RI fragment. This, coupled with additional restriction endonuclease mapping described below, leads to the conclusion that the *B. japonicum* genome contains two separate regions that hybridize to the *K. pneumoniae nifA* gene. One of these homologies is located between the *nifA* and *nifDK* transcription units, whereas the other is not directly linked to this region.

Characterization of the *nifH-nifDK* genomic region. The extent of the overlap between class I and class II cosmids was determined by hybridizing radiolabeled pRJcos2-63 (class II) to various restriction endonuclease digests of pRJcos1-62 (class I) (Fig. 3). Since these recombinant cosmids were constructed by the insertion of *Eco*RI partial digestion products (see above), the full extent of the overlapping region can most easily be seen in the *Eco*RI digest of pRJcos1-62 (Fig. 3a and b, lanes 4). There are four *Eco*RI fragments totalling 13 kbp in length in pRJcos1-62 that hybridize to pRJcos2-63. This overlapping region includes the 4.9-kbp *Eco*RI fragment, which shares homology with the *K. pneumoniae nifA* gene (Fig. 2, lanes 4 and 5), confirming that the class I and class II *nifA* homologies are identical.

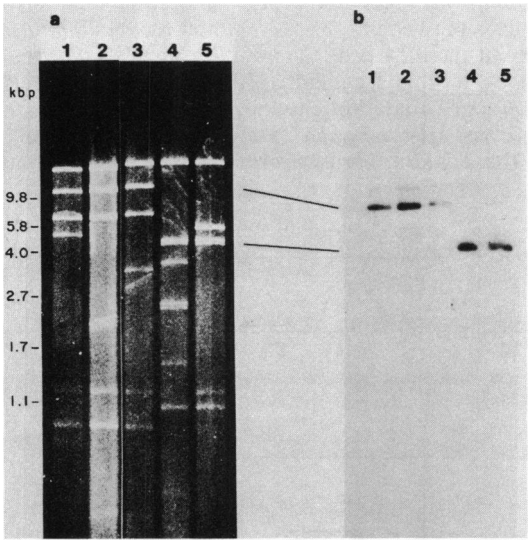

FIG. 2. Hybridization of the *K. pneumoniae nifA* gene to *Eco*RI-digested recombinant cosmids. (a) Ethidium bromide-stained agarose gel of *Eco*RI-digested cosmid clones. Lanes: 1, pRJcos2-43; 2, pRJcos7-3; 3, pRJcos14-58; 4, pRJcos1-62; 5, pRJcos2-63. (b) The *Kpn*I-*Xma*I fragment of pGR397 (36), which contains the *K. pneumoniae nifA* gene, was nick translated and hybridized to a cellulose nitrate transfer of the gel shown in a. Lane designations are the same as in a.

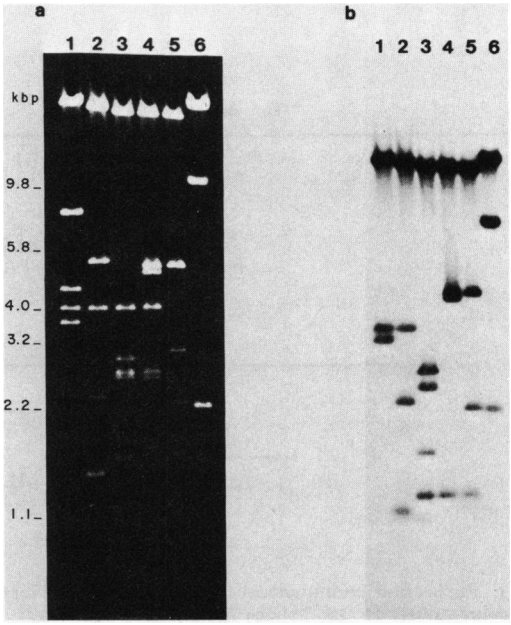

FIG. 3. Hybridization of nick-translated pRJcos2-63 to restriction endonuclease digests of pRJcos1-62. (a) Ethidium bromide-stained agarose gel of restriction endonuclease-digested pRJcos1-62. Lanes: 1 *Hin*dIII; 2, *Hin*dIII plus *Bam*HI; 3, *Hin*dIII plus *Eco*RI; 4, *Eco*RI; 5, *Eco*RI plus *Bam*HI; 6, *Bam*HI. (b) Hybridization of nick-translated pRJcos2-63 to a cellulose nitrate transfer of the gel in a. Lane designations are the same as in a. The top band in each lane contains the vector DNA, pLAFR1. All other bands represent *B. japonicum* sequences.

Cosmids pRJcos1-62 and pRJcos2-63 were used to construct a restriction endonuclease map of approximately 33 kbp of *B. japonicum* DNA containing the *nif* gene cluster (Fig. 4). The second class I cosmid clone (pRJcos14-58) contains an *Eco*RI restriction pattern that is nearly identical to that pRJcos1-62 (data not shown) and thus has not been further analyzed. The positions and directions of the *nifD* and *nifK* genes and the small gene region of unknown function marked (?) were defined previously within the 8.5-kbp *Hin*dIII fragment by using the plasmid clone pRJ676 (18, 21). The *nifH* gene was previously isolated as a recombinant lambda phage, λNH-1 (1), and its position within this region is shown. The approximate position of the *nifA*-like region as determined by Southern hybridization is illustrated.

To verify that these cosmid clones accurately represent the true genomic arrangement of the *nif* gene cluster in *B. japonicum*, total genomic DNA was digested with the restriction enzymes used to map this region, separated on agarose gels, and blotted to cellulose nitrate. The resulting blots were hybridized to either the 1.2-kbp *Xho*I fragment from pBJ86 (Fig. 5a) or to pBJ87 (Fig. 5b), each of which had been radiolabeled by nick translation (see Fig. 4 for map positions of probes). In each case the hybridization pattern predicted from the map shown in Fig. 4 was in agreement with the results. This, coupled with the fact that *B. japonicum* DNA regions that were independently cloned in λNH-1 and pRJ676 are unaltered in these cosmid clones, indicates

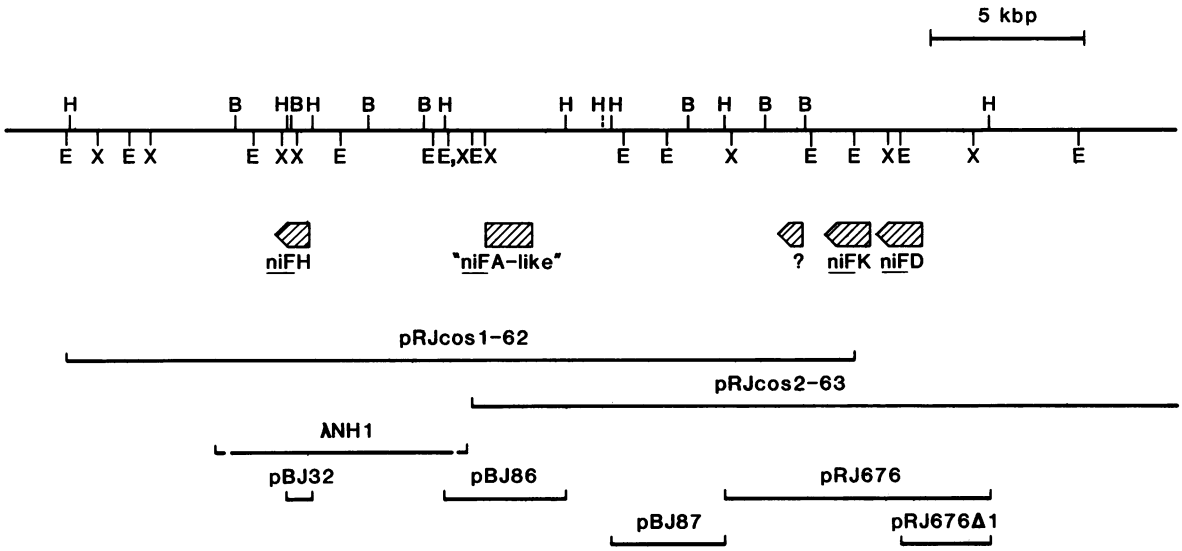

FIG. 4. Restriction endonuclease map of the *B. japonicum nif* gene cluster. The positions and transcriptional directions of *nif* genes described previously (1, 18, 21) are indicated by the hatched arrows. The position of the *nifA*-like region is indicated by the hatched box. Recombinant DNA clones of this region that were used in this work and are discussed in the text are illustrated at the bottom. Restriction endonuclease sites are *Eco*RI (E), *Hin*dIII (H), *Xho*I (X), and *Bam*HI (B).

that pRJcos1-62 and pRJcos2-63 accurately reflect the physical structure of this region of the *B. japonicum* genome.

Characterization of the *B. japonicum nifA*-like regions. The DNA regions containing *nifA* homologies contained pRJcos2-43 (class III) and pRJcos1-62 class I and II) were subcloned into standard plasmid vectors for further characterization. Restriction endonuclease maps of the appropriate regions have been determined (Fig. 6). We have localized the regions that hybridize to the *K. pneumoniae nifA* gene (designated Hna-1 and Hna-2 for homology to *nifA* in Fig. 6) with the probes described in Fig. 1.

The *nifA* homology that falls within the *nif* gene cluster Hna-1 is contained within a 1.2-kbp *Xho*I-*Sal*I fragment that is located approximately 6.0 kbp upstream from the *nifH* promoter and 13 kbp downstream from the site of transcriptional initiation for the *nifDK* operon. This same fragment hybridizes to probes specific to the 5′ or 3′ end of the *K. pneumoniae nifA* gene (Fig. 1), suggesting that the DNA homologies between *B. japonicum* and *K. pneumoniae* exist throughout the *nifA* gene. In addition, the *E. coli ntrC* gene hybridizes to this fragment, although not as strongly as *Klebsiella nifA* (data not shown).

The class III-type *nifA* homology has been localized within the 1.3-kbp *Xho*I-*Bam*HI fragment as illustrated in

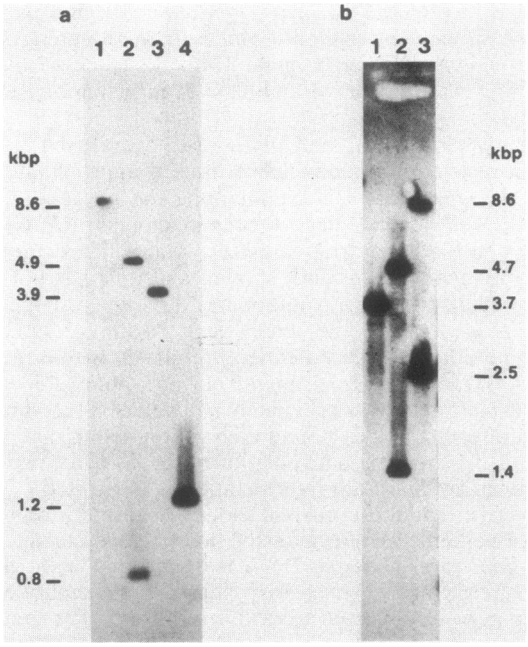

FIG. 5. Verification of the *B. japonicum nif* genomic structure by Southern hybridization to restriction endonuclease digests of total genomic DNA. Genomic DNA from *B. japonicum* was digested with restriction endonucleases and separated electrophoretically on 1% agarose gels. Cellulose nitrate transfers of these gels were then hybridized to radioactively labeled (a) pBJ87 or (b) the 1.2-kbp *Xho*I fragment from pBJ86 (Fig. 4). (a) Lanes: 1, *Bam*HI; 2, *Eco*RI; 3, *Hin*dIII. (b) Lanes: 1, *Xho*I; 2, *Hin*dIII; 3, *Eco*RI; 4, *Bam*HI.

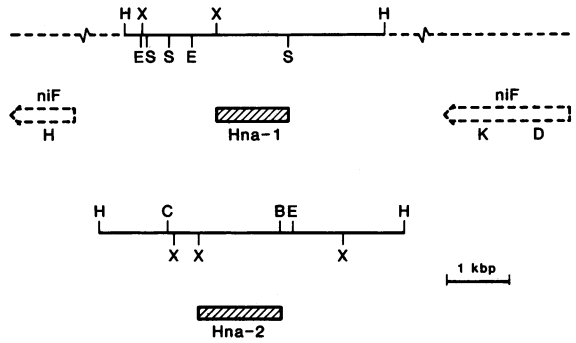

FIG. 6. Restriction endonuclease maps of the *B. japonicum nifA*-like regions. The approximate positions of the *B. japonicum nifA*-like regions as determined by hybridization to the *K. pneumoniae nifA* gene are indicated by the hatched boxes labeled Hna-1 and Hna-2. The organization of the *nifH*, *-D*, and *-K* genes relative to Hna-1 are indicated by the arrows. Restriction endonuclease sites are *Hin*dIII (H), *Eco*Ri (E), *Xho*I (X), *Sal*I (S), and *Cla*I (C).

Fig. 6 (Hna-2). This homology seems to be specific to the 3′ end of the *K. pneumoniae nifA* gene, since the 5′-specific probe shows no detectable hybridization in this region (data not shown). In addition to the lack of *nifA* 5′ homology seen with this clone, Hna-2 differs from Hna-1 in that no hybridization to the *E. coli ntrC* gene is observed.

In *K. pneumoniae* the *nifA* gene is part of an operon that also includes the repressor of *nif*-specific transcription, *nifL* (22). To see whether a similar gene arrangement exists in *B. japonicum*, we used a 500-bp *Xma*I-*Sal*I restriction endonuclease fragment that contains the 5′ portion of the *nifL* gene (Fig. 1) to probe Southern blots of cosmids containing Hna-1 and Hna-2 (data not shown). No hybridization was observed in either region. Similarly, no homology was observed between these cosmids and the *ntrC*-linked *ntrB* and *glnA* genes. These experiments do not, however, eliminate the possibility that functionally analogous genes are linked to either Hna-1 or Hna-2.

DISCUSSION

A number of similarities have been observed between the nucleotide sequences of the *nifH* and *nifDK* promoters in *B. japonicum* and those of *nif*-specific operons in *K. pneumoniae* and *R. meliloti* (1). This suggests that similar mechanisms for transcriptional control of *nif* gene expression may function in all three organisms. With this in mind, we have used the *K. pneumoniae nifA* gene, the product of which activates *nif*-specific transcription, as a heterologous probe to screen a library of *B. japonicum* USDA 110 genomic DNA maintained in the broad-host-range cosmid vector pLAFR1. Two distinct *K. pneumoniae nifA* homologies, Hna-1 and Hna-2 (Fig. 6), have been identified in *B. japonicum*.

One of these *nifA* homologous regions (Hna-1) falls within a cluster of *B. japonicum nif* genes (Fig. 4). This region contains the two nitrogenase structural gene operons (*nifH* and *nifDK*) as well as Hna-1. The initiation sites for *nifH* and *nifDK* transcription units, which have been mapped previously (1), are separated by approximately 20 kbp, and all three nitrogenase genes are transcribed in the same direction. The *nifDK* operon is transcriptionally upstream from the *nifH* gene (Fig. 4). The nitrogenase gene order is thus modified from that observed in most other nitrogen-fixing bacteria (2, 25, 36, 38). Despite this alteration in the *nifH*, *-D*, and *-K* gene order, the *nifA* homologous region (Hna-1) is in an identical position with respect to the *nifH* promoter as is the *nifA*-like locus described in *R. meliloti* (45–47). With this in mind, we suggest that the differences observed between *nif* operon structures in *B. japonicum* and *R. meliloti* could be explained by a simple transposition event changing the *nifHDK* genomic arrangement while leaving the *nifA*-to-*nifH* region unaltered. The *nifA*-like gene has also been identified in *R. leguminosarum* (12), where it is located about 30 kbp upstream of *nifHDK*, indicating that a different set of rearrangements may have occurred in the evolution of this species.

Whereas Hna-1 is located between the nitrogenase structural gene operons, Hna-2 is found elsewhere in the *B. japonicum* genome. Mapping data of the Hna-1-containing *nif* gene cluster and of the Hna-2-containing cosmid clones indicates that the second *B. japonicum nifA* homologous region (Hna-2) must be located at least 20 kbp away from either the *nifH* or the *nifDK* operon. The *B. japonicum* strain with which this analysis has been carried out (USDA 110) contains no detectable plasmids (20), so all of these genes must be assumed to be located on the chromosome.

The observation that *B. japonicum* contains two regions of homology to the *K. pneumoniae nifA* gene is not surprising in light of the *nifA-ntrC* homology in *K. pneumoniae* (13, 32, 34). There are, in fact, many interesting differences between Hna-1 and Hna-2 with respect to *nifA* and *ntrC* hybridization patterns, suggesting that these regions represent unique genomic sequences rather than a simple duplication. As with the *nifA*-like region of *R. meliloti* (46, 47), Hna-1 hybridizes to both the *K. pneumoniae nifA* gene and to the *E. coli ntrC* gene. Hna-2 hybridization, however, is limited to the 3′ end of the *K. pneumoniae nifA* gene. These differences in DNA sequence homology may reflect functional differences in these two putative regulatory genes. Alternatively, one or both of these regions may simply represent pseudo-*nifA* genes.

We are in the process of constructing *B. japonicum* strains that contain mutations in the Hna-1 and Hna-2 genomic regions. If these *nifA* homologous regions indeed encode *nifA*- or *ntrC*-like products, these mutants should prove invaluable in developing an understanding of the regulatory circuitry of nitrogen control in *B. japonicum*.

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