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# Interspecies homology of nitrogenase genes

**(recombinant DNA/restriction endonuclease mapping/DNA·DNA hybridization)**

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ABSTRACT **Cloned nitrogen fixation (*nif*) genes from *Klebsiella pneumoniae* hybridize to DNA from 19 out of 19 widely divergent nitrogen-fixing bacterial strains but do not hybridize to DNA from 10 different non-nitrogen-fixing species. *K. pneumoniae nif* DNA fragments that hybridize to DNA from other species contain part of the three structural genes that code for nitrogenase polypeptides. We have utilized this homology to clone an *Eco*RI restriction endonuclease fragment from *Rhizobium meliloti* that hybridizes to the *K. pneumoniae nif* structural genes. Some of the species whose DNA hybridizes with *K. pneumoniae nif* DNA have been postulated to have diverged from *K. pneumoniae* $3 \times 10^9$ years ago. Nitrogenase genes are the only known example of such highly conserved prokaryotic translated genes. Nitrogenase genes are either extraordinarily conserved in evolution or have been exchanged between different nitrogen-fixing species relatively recently in evolutionary time.**

The ability to fix atmospheric nitrogen is widely distributed among divergent prokaryotic taxonomic groups, including Azotobacteraceae, Enterobacteriaceae, Rhodospirillaceae, Bacillaceae, Rhizobiaceae, Actinomycetaceae, and Cyanobacteria (1). Although the physiological conditions under which prokaryotes fix nitrogen vary considerably, the enzymatic apparatus involved in nitrogen fixation is remarkably similar. Each nitrogen-fixing species that has been studied produces an enzyme complex called nitrogenase composed of two characteristic components: a molybdenum- iron protein (MoFe protein), which reduces substrate, and an iron protein (Fe protein), which binds MgATP and transfers electrons to the MoFe protein (2). Neither purified component in the absence of the other reduces nitrogen, but it is possible to reconstitute an active nitrogenase complex *in vitro* from purified MoFe and Fe proteins (2).

In some cases, purified MoFe protein from one bacterial species reconstitutes an enzymatically active hybrid nitrogenase with Fe protein from another species (3). In addition, amino acid compositions of nitrogenases from several species are very similar (4). These observations suggest that the structure of nitrogenase has been conserved between evolutionarily distant organisms. We hypothesized, therefore, that the DNA sequences coding for nitrogenase proteins may also have been conserved and that DNA that codes for nitrogenase from one nitrogen-fixing species would hybridize specifically to DNA from other nitrogen-fixing species.

Cannon *et al.* (5, 6) have previously constructed amplifiable plasmids carrying 14 out of 15 *Klebsiella pneumoniae* nitrogen fixation (*nif*) genes, including those that code for the MoFe and Fe proteins of nitrogenase. In the experiments described here, purified DNA fragments containing *K. pneumoniae nif* genes were labeled with $^{32}P$ and hybridized to restriction endonuclease-digested DNAs from various nitrogen-fixing and non-nitrogen-fixing bacteria, using the gel transfer technique of Southern (7). We found that *K. pneumoniae* DNA containing nitrogenase genes hybridized to DNA from 19 out of 19 nitrogen-fixing bacterial strains but did not hybridize to DNA from 10 non-nitrogen-fixing species.

## MATERIALS AND METHODS

**Southern Hybridizations.** Total DNA was isolated from each species by using a simplified Marmur technique (8). Approximately 5 μg of each DNA was digested with 20 units of *Eco*RI purified and used as described (9), and the DNA was electrophoresed in a 13 × 24 × 0.5 cm 0.8% agarose horizontal gel (Dankar plastics) at 2 V/cm for 12–16 hr. The DNA in the gel was transferred to nitrocellulose sheets (Schleicher and Schuell type BA85) by the method of Southern (7), using modifications from Botchan *et al.* (10).

Supercoiled plasmids were prepared by the cleared lysate technique (11). Purified restriction fragments were obtained by gel electrophoresis and eluted by inserting a gel slice containing the desired fragment into a dialysis bag and placing the bag in electrophoresis buffer, followed by electrophoresis at 5 V/cm for 16–24 hr. Intact plasmid DNA and purified restriction fragments were labeled by the nick translation (12, 13) method to a specific activity of $10^7$ to $10^8$ cpm/μg of DNA with thymidine 5′-[$^{32}P$]triphosphate (350 Ci/mmol, Amersham; 1 Ci = $3.7 \times 10^{10}$ becquerels) and DNA polymerase I (Boehringer Mannheim). Hybridizations were performed essentially as described by Botchan *et al.* (10) in a Seal-a-Meal boilable bag (Sears and Roebuck) in a 65°C water bath, with approximately $10^6$ dpm of labeled DNA probe per 24-slot 13 × 24 cm filter. Kodak XR-1 x-ray film was exposed to the dried nitrocellulose filter with an intensifying screen (Du Pont Cronex Lightning Plus) at −70°C for 48–96 hr.

**Melting Temperature Experiments.** Approximately 50 μg of *Rhizobium* DNA was digested with *Eco*RI restriction endonuclease, mixed with 1 μg of *K. pneumoniae* DNA digested with *Eco*RI or *Sal* I, and subjected to electrophoresis in a single 12-cm-long slot on a 13 × 24 × 0.5 cm 1% agarose horizontal gel. The DNA was transferred to nitrocellulose and hybridized to $^{32}P$-labeled *K. pneumoniae nif* DNA as described above. The filter was cut into identical strips, each of which was washed at 65°C in various concentrations of standard saline/citrate (NaCl/Cit: 0.15 M NaCl/0.015 M sodium citrate, pH 7). After washing and drying, the strips were lined up and autoradiographed.

**Cloning of the *Rhizobium meliloti* DNA Fragment Homologous to *K. pneumoniae nif*.** Approximately 10 μg of *R. meliloti* strain M2011 DNA was digested with *Eco*RI and ligated to 5 μg of *Eco*RI-linearized plasmid pBR322 (14) DNA by using phage T4 ligase as described (6). A sample of the li-

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Abbreviations: NaCl/Cit, standard saline/citrate (0.15 M NaCl/0.015 M sodium citrate, pH 7); kb, kilobase pairs; Nif, relating to nitrogen fixation.

gated DNA was used to transform $CaCl_2$-treated *Escherichia coli* strain HB101 cells (15). DNAs from a total of 4000 transformed colonies were hybridized in duplicate to $^{32}P$-labeled *K. pneumoniae nif* DNA by using the colony hybridization technique of Grunstein and Hogness (16) as modified by D. Hanahan (personal communication).

## RESULTS

To test whether *nif* genes from *K. pneumoniae* are homologous to DNA from other nitrogen-fixing species, we labeled *K. pneumoniae nif* DNA with $^{32}P$ and hybridized this labeled DNA to total DNA from a variety of prokaryotic species. Fig. 1*a* shows that a $^{32}P$-labeled *K. pneumoniae nif* fragment carrying the structural genes for nitrogenase (genes *K*, *D*, and *H*; see Fig. 2) hybridized to restriction fragments from 10 different nitrogen-fixing strains. The results of this experiment and other analogous experiments (not shown) are summarized in Table 1. DNA isolated from 19 out of 19 nitrogen-fixing bacterial strains, including a Gram-positive species, an actinomycetous species, and two species of cyanobacteria, hybridized to a *K. pneumoniae* DNA fragment carrying the *nif* structural genes. In contrast, no hybridization was observed when the same $^{32}P$-labeled fragment was used to probe DNA from 10 different species that do not fix nitrogen (data not shown). From these data we conclude that the restriction fragments from the nitrogen-fixing species that hybridize to the *K. pneumoniae nif* DNA fragment carry genes that code for nitrogenase protein (Nif DNA).

Examination of Fig. 1*a* reveals that DNA from all of the nitrogen-fixing species hybridized to approximately the same intensity with the *K. pneumoniae nif* probe (except for the *Rhizobium* species *R. japonicum* strain 110 and *R.* sp. strain 32H1). However, the intensity of hybridization was much lower than that observed for the homologous hybridization to total DNA from *K. pneumoniae*. Fig. 1*b* (lanes A, B, and C) shows that 0.1 μg of *K. pneumoniae* DNA hybridized to the *K. pneumoniae nif* probe with an intensity equal to that of 5 μg of *R. meliloti* DNA.

The 6.0-kb *K. pneumoniae Eco*RI fragment (fragment A, Fig. 2) used to probe the various species listed in Table 1 contains all of *nif* genes *K*, *D*, and *H*, which code for the subunits of nitrogenase, and the $NH_2$-terminal region of *nifE* (17). Fig. 2 shows a restriction endonuclease map of the *K. pneumoniae nif* region and the approximate delineations of 15 *K. pneumoniae nif* genes. The direction of transcription for genes *E*, *K*, *D*, and *H* has been determined to be from right to left in figure 2 (18–20).

A 17.0-kb *Hin*dIII fragment (fragment B, Fig. 2) carries the remainder of the *K. pneumoniae nif* genes (except *nifJ*) (6). This fragment was used as a probe to determine whether *K. pneumoniae nif* genes in addition to *nif E*, *K*, *D*, and *H*, are homologous to the DNAs from other nitrogen-fixing species. DNA fragments in Southern plots similar to the one shown in Fig. 1*a* were hybridized to a $^{32}P$-labeled plasmid carrying *K. pneumoniae nif* fragment B (6). An example is illustrated in Fig. 1*b*, which shows that under conditions in which 0.1 μg of *K. pneumoniae* DNA (lane D) hybridized intensely, no hybridization was observed to 5 μg of *R. meliloti* DNA (lane E). Similar negative results were obtained for all other nitrogen-fixing species tested (Table 1). These data indicate that the region of homology must lie to the right of the *Hin*dIII site in fragment A.

The region of homology between the *K. pneumoniae nif* fragment A and DNAs from the other nitrogen-fixing bacteria was localized more precisely by probing filters similar to those shown in Fig. 1*a* with purified $^{32}P$-labeled restriction fragments A1, A2, and A3 derived from *nif* fragment A (Fig. 2). The results, summarized in Table 1, show that the 3.0-kb fragment A1, which contains the $NH_2$-terminal region of *nifE* and the COOH-terminal region of *nifK*, did not hybridize to DNA

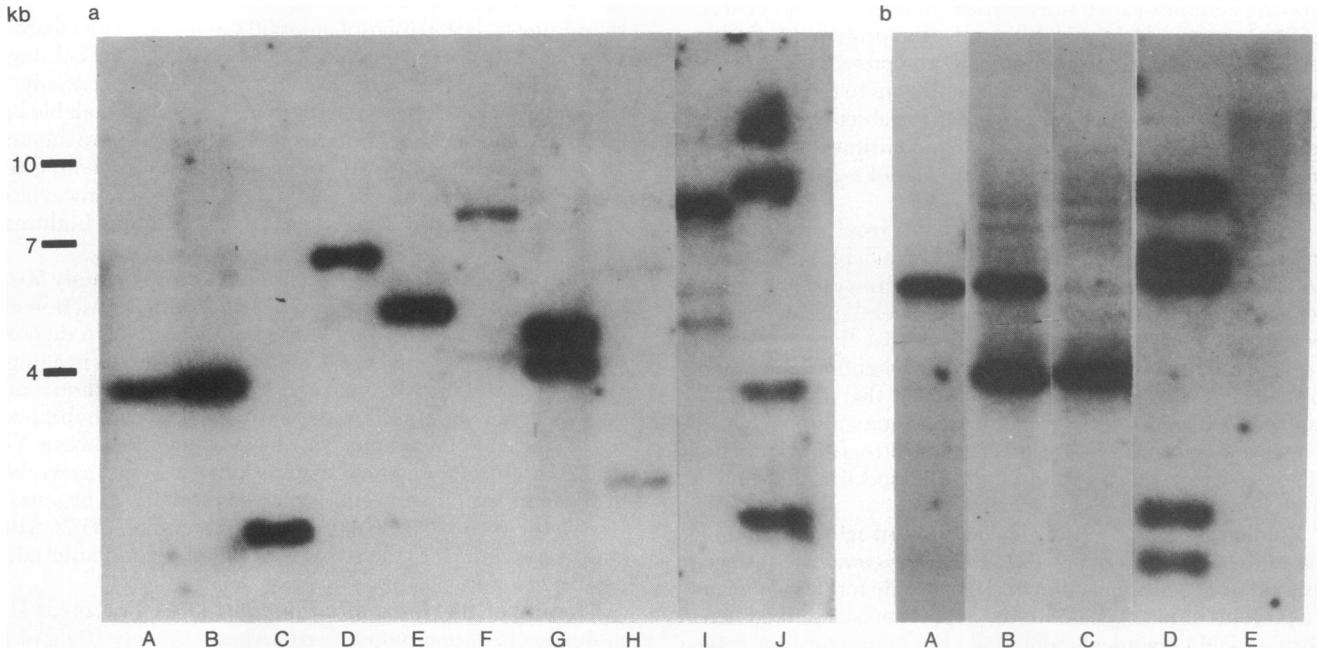


FIG. 1. kb, Kilobase pairs. (*a*) Autoradiogram of $^{32}P$-labeled *K. pneumoniae nif* DNA fragment A hybridized to 5 μg of *Eco*RI-digested DNAs from: A, *Rhizobium meliloti* strain L530; B, *R. meliloti* strain M2011; C, *R. leguminosarum* strain 897; D, *R. leguminosarum* strain 1285; E, *R. trifolii* strain RT1; F, *R.* sp. strain 32H1; G, *R. phaseoli* strain K26; H, *R. japonicum* strain 110; I, *Rhodopseudomonas capsulata* strain B10; J, *Azotobacter vinelandii* strain UW10. (*b*) Autoradiogram of $^{32}P$-labeled *K. pneumoniae nif* fragment A hybridized to: A, 0.1 μg of *K. pneumoniae* DNA digested with *Eco*RI; B, 0.1 μg of *K. pneumoniae* plus 5 μg of *R. meliloti* strain M2011 DNA both digested with *Eco*RI; C, 5 μg of *R. meliloti* strain 2011 DNA digested with *Eco*RI. D and E, $^{32}P$-labeled *K. pneumoniae nif* fragment B hybridized to: D, 0.1 μg of *K. pneumoniae* DNA digested with *Eco*RI; E, 5 μg of *R. meliloti* strain M2011 DNA digested with *Eco*RI.

Table 1. Hybridization of *K. pneumoniae* and *R. meliloti nif* DNA fragments to DNAs from nitrogen-fixing species

| Species | Strain | Size of hybridizing *Eco*RI fragment,* kb | Hybridization of other *nif* probes to *Eco*RI fragments† | | | | |
|---|---|---|---|---|---|---|---|
| | | | A1 | A2 | A3 | B | pRmR1 |
| Gram-negative bacteria | | | | | | | |
| *Klebsiella pneumoniae* | M5a1 | 6.0 | + | + | + | + | + |
| *Azotobacter vinelandii* | UW10 | 12.5 | − | − | + | − | − |
| | | 8.1 | − | − | + | − | − |
| | | 3.5 | − | − | + | − | − |
| | | 2.2 | − | + | − | − | − |
| | | 0.8 | − | + | + | − | − |
| *Rhizobium meliloti* | M2011 | 3.6 | − | + | + | − | + |
| | F51 | 3.6 | − | + | + | − | + |
| | L530 | 3.6 | − | + | + | − | + |
| *Rhizobium leguminosarum* | 897 | 1.6 | − | + | + | NT | + |
| | 1285 | 6.2 | − | + | + | − | + |
| *Rhizobium trifolii* | RT1 | 4.8 | − | + | + | − | + |
| | 16257 | 4.8 | NT | + | + | NT | NT |
| *Rhizobium phaseoli* | K26 | 4.2 | − | + | + | − | + |
| | | 4.1 | − | + | + | − | + |
| | | 3.8 | − | + | + | − | + |
| *Rhizobium japonicum* | 110 | 6.0 | − | + | + | − | + |
| | | 2.3 | − | − | + | − | + |
| | | 1.0 | − | NT | NT | − | + |
| *Rhizobium* sp. | 32H1 | 7.6 | NT | + | + | − | + |
| | | 4.2 | NT | + | + | − | + |
| *Rhodopseudomonas capsulata* | B10 | 7.6 | − | + | + | − | − |
| | | 4.3 | − | + | − | − | − |
| *Rhodospirillum rubrum* | 1.1.1 | 1.7 | NT | NT | NT | NT | NT |
| | | 1.4 | NT | NT | NT | NT | NT |
| | | 1.0 | NT | NT | NT | NT | NT |
| | | 0.9 | NT | NT | NT | NT | NT |
| Gram-positive bacteria | | | | | | | |
| *Clostridium pasteurianum* | | 6.2 | NT | + | − | NT | NT |
| Actinomycetes | | | | | | | |
| *Frankia* | CPI1 | 7.4 | NT | NT | NT | NT | NT |
| | | 5.2 | NT | NT | NT | NT | NT |
| Cyanobacteria | | | | | | | |
| *Anabaena* | 7120 | 20.0 | NT | NT | NT | NT | NT |
| | | 18.0 | NT | NT | NT | NT | NT |
| | | 10.0 | NT | NT | NT | NT | NT |
| *Anabaena variabilis* (Kütz) | ATCC 29413 | 10.5 | NT | NT | NT | NT | NT |
| | | 4.9 | NT | NT | NT | NT | NT |
| *Plectonema boryanum* | 594 | 3.6 (*Bgl* II) | NT | NT | NT | NT | NT |

The DNA from the following species which are not known to fix nitrogen failed to hybridize with *Klebsiella pneumoniae nif* DNA: *K. pneumoniae nif* deletion Kp107 (F. Cannon), *Agrobacterium tumefaciens* strains B653 and C58 (M. Hanson), *Serratia marcescens* (J. Pero), *Salmonella typhimurium* strain DB (T. Poteete), *Escherichia coli* strains JC5466 (F. Cannon) and HB101 (H. Boyer), *Beneckea harveyi* strain MB20AR1 (J. W. Hastings), *Clostridium perfringens* (Sigma), *Bacillus subtilis* strain CB20 (C. Hanson), *Saccharomyces cerevisiae* (H. Greer), *Petunia* chloroplasts (M. Hanson and B. deLorimier). Sources of bacterial species which fix nitrogen: *A. vinelandii* (W. Brill), *R. meliloti* strain L530 (J. Denarie), *R. meliloti* strains M2011 and F51 (H. Meade), *R. leguminosarum* strain 897 (J. Beringer), *R. leguminosarum* strain 1285 and *R. trifolii* strains RT1 and 16257 (P. Albersheim), *R. phaseoli* strain K26 and *R. japonicum* strain 110 (E. Signer), *R.* sp. strain 32H1 (B. Ludwig), *Rhodopseudomonas capsulata* and *Rhodospirillum rubrum* (H. Gest), *C. pasteurianum* (L. Mortenson), and *Frankia* CPI1 (D. Baker and J. Torrey). The data presented in this table on *Anabaena* and *Plectonema* were obtained by Mazur *et al.* (21). The hybridizations with *Frankia* were performed by R. Riedel. *Plectonema* DNA would not digest with *Eco*RI and the data presented are for a *Bgl* II digest.

* Fragments hybridized with *K. pneumoniae nif* fragment A.

† *Eco*RI fragments that hybridized to *K. pneumoniae nif* fragment A were tested for hybridization with *K. pneumoniae nif* probes A1, A2, A3, and B, and with *R. meliloti* Nif probe pRmR1. NT, not tested.

from any of the species tested, whereas *nif* fragments A2 and A3 hybridized to DNA from all species that hybridized to fragment A. Because both *nif* fragments A2 and A3 hybridized, the region of homology cannot be limited to only *nifK* or only *nifH*. Three possibilities that remain are: (*i*) the $NH_2$-terminal region of *nifK* and at least the part of *nifD* that lies to the right of the *Bam*HI site; (*ii*) *nifH* and at least the part of *nifD* that lies to the left of the *Bam*HI site; or (*iii*) part or all of *nifD* flanking the *Bam*HI site.

We determined an upper limit of the length of *R. meliloti* DNA homologous to *K. pneumoniae nif* DNA by constructing a restriction map of the *R. meliloti* chromosomal region homologous to the *K. pneumoniae nif* probe (see Fig. 2). This was accomplished by digesting total DNA from *R. meliloti* with various restriction enzymes and combinations of two enzymes, and hybridizing Southern blots of these digests to $^{32}P$-labeled *K. pneumoniae* fragment A. A 14-kb *Xho* I restriction fragment and a 3.6-kb *Eco*RI restriction fragment were homologous to

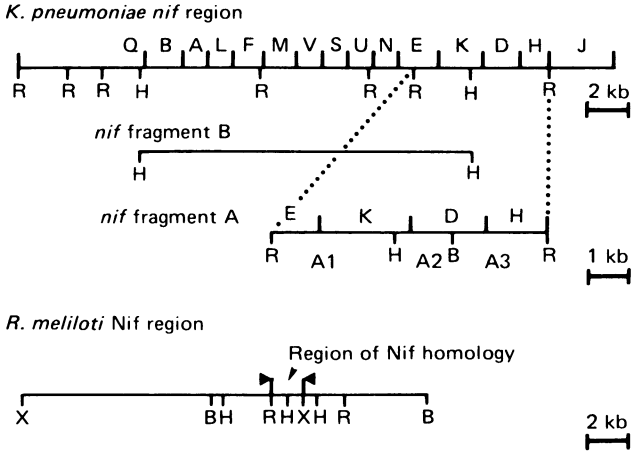


FIG. 2: Restriction maps of Nif regions from *K. pneumoniae* and *R. meliloti*. Endonuclease cleavage sites are indicated below each map by: X, *Xho* I; R, *Eco*RI; H, *Hin*dIII; and B, *Bam*HI.

the *K. pneumoniae nif* probe. However, when *R. meliloti* DNA was digested with both *Eco*RI and *Xho* I, a single hybridization band of 1.6 kb was observed, suggesting that, within our limits of detection, all of the homology to the *K. pneumoniae nif* region was on this fragment. On the basis of heteroduplex mapping between *K. pneumoniae nif* fragment A and a cloned homologous DNA fragment from *Anabaena* 7120, Mazur *et al.* (21) have found the region of homology to be 2.0 kb in length.

**Measurement of Degree of Homology Between the Nif Genes of *Rhizobium* and *K. pneumoniae*.** To determine the extent of divergence between the *R. meliloti* and *K. pneumoniae* Nif DNA sequences, the melting temperature ($t_m$) of the heterologous *K. pneumoniae–R. meliloti* Nif DNA hybrid was compared to that of the homologous *K. pneumoniae–K. pneumoniae* Nif DNA duplex. We probed identical Southern blots of a mixture of *K. pneumoniae* and *R. meliloti* DNAs digested with *Eco*RI with $^{32}$P-labeled *K. pneumoniae nif* fragment A and washed each nitrocellulose strip in buffers of different ionic strengths.

The intensity of the heterologous *K. pneumoniae–R. meliloti* Nif hybridization band at 3.6 kb relative to the homologous *K. pneumoniae–K. pneumoniae* hybridization band at 6.9 kb was unchanged in 0.5× and 0.4× NaCl/Cit but began to decrease in 0.2× NaCl/Cit and decreased dramatically in the 0.1× NaCl/Cit wash (data not shown). The homologous *K. pneumoniae–K. pneumoniae* hybridization band intensity decreased in the 0.05× NaCl/Cit wash. From the formula $t_m = 81.5 + 16.6 \log[Na^+] + 0.5$ (% G+C) (22), this corresponds to a difference of from 5°C to 13°C between the $t_m$ of the exactly homologous *K. pneumoniae–K. pneumoniae nif* hybridization and the heterologous *R. meliloti–K. pneumoniae nif* hybridization. A similar result was found for a *R. trifolii–K. pneumoniae* Nif hybridization. To rule out the possibility that the more stable homologous *K. pneumoniae–K. pneumoniae* Nif hybridization was simply due to a larger region of homology, we digested *K. pneumoniae* DNA with restriction endonuclease *Sal* I, which generates 1.5-kb regions of homology, and probed this DNA with the *K. pneumoniae nif* probe. We found that these duplexes were stable until washed with 0.05× NaCl/Cit.

**Cloning of *Rhizobium meliloti* Nif Homologous DNA.** We have screened a gene library of *R. meliloti* for clones containing the Nif homologous DNA fragment. Out of 4000 tetracycline-resistant colonies screened, 7 hybridized to the *K. pneumoniae nif* fragment A probe, of which one, upon isolation of plasmid DNA (pRmRl) and digestion with *Eco*RI, showed a 3.6-kb fragment that also hybridized to the *K. pneumoniae nif* fragment A probe.

When $^{32}$P-labeled pRmR1 was then used as a hybridization probe to Southern blots analogous to those in Fig. 1*a*, it was found to be homologous to many but not all of the DNAs that hybridized to the *K. pneumoniae nif* probe (Table 1). Two DNAs that did not hybridize to the pRmRl probe were from *A. vinelandii* and *Rhodopseudomonas capsulata*. DNAs from all "fast-growing" rhizobial species, *phaseolus, leguminosarum,* and *trifolii,* hybridized with equivalent intensity to the pRmRl and *K. pneumoniae nif* probes A2 and A3. In addition, DNAs from the "slow-growing" *R.* sp. 32H1 and *R. japonicum* 110, which hybridized poorly to the *K. pneumoniae nif* probe, also hybridized poorly to the pRmRl probe.

## DISCUSSION

We report here that DNA from 19 out of 19 bacterial strains of nitrogen-fixing prokaryotes hybridized to a *K. pneumoniae* DNA fragment carrying the structural genes for nitrogenase. In contrast, DNA from a total of 10 non-nitrogen-fixing species did not hybridize. We conclude that the hybridization observed with DNAs from the nitrogen-fixing species is due to the presence of nitrogen-fixing genes that share sequence homology with *K. pneumoniae nif* genes. This interspecies *nif* gene homology appears to be limited to the structural genes for nitrogenase because a *K. pneumoniae* DNA fragment that carries at least 12 *nif* genes, but not the structural genes for nitrogenase, failed to hybridize to DNA from the other nitrogen-fixing species. Our finding that the majority of the *K. pneumoniae nifK* gene is not homologous to DNA from other nitrogen-fixing species suggests that most if not all of the primary sequence of this gene product is not under the high level of selection we observe for the other *nif* structural genes.

It is not known how much DNA sequence homology is necessary to allow detection of evolutionarily related genes when the Southern blotting and hybridization technique is used. The intensity of hybridization observed depends, in part, on homology length, length and distribution of mismatched regions, G+C content, and genome complexity. In our experiments, DNAs from several species hybridized to the *K. pneumoniae nif* probe at nearly equivalent but lower intensities than did *K. pneumoniae* DNA (Fig. 1). The genome complexities of all prokaryotes so far tested varies only from 2000 to 12,000 kb (23), suggesting that the difference in Nif hybridization intensities between *K. pneumoniae* and the other species is not due simply to lower complexity of the *K. pneumoniae* genome. We estimate that the DNA sequence divergence between *K. pneumoniae* and *R. meliloti* Nif genes is from 8 to 20%, assuming a 1°C decrease in $t_m$ per 1.5% mismatch (24). Sequence divergence calculations of this sort are not precise because they do not take into account lengths and distributions of regions of homology and mismatch; however, similar estimates have recently been confirmed by determination of DNA nucleotide sequences (25, 26).

On the basis of ribosomal RNA sequence data, Fox *et al.* (27) have proposed that the evolutionary divergence among Gram-negative species such as *K. pneumoniae,* Gram-positive species such as *Clostridium pasteurianum,* and cyanobacteria such as *Anabaena variabilis* occurred as long ago as $3 \times 10^9$ years. The conservation of Nif DNA sequences that we observe in these species appears to be quite unusual for translated genes.

DNA sequence conservation of translated genes comparable

to *nif* gene conservation has been observed only for the eukaryotic chromosomal protein histone H4. The amino acid sequence of H4 differs by only two residues out of 102 between cow and pea, species estimated to have diverged 1 to $2 \times 10^9$ years ago (28, 29). RNA transcribed from cloned histone genes H1 through H4 from sea urchin hybridized to DNAs from *Drosophila melanogaster*, but the $t_m$ of the hybrid was 19°C lower than that of the same RNAs hybridized to sea urchin DNA (30). This suggests approximately 28% DNA sequence divergence over the estimated $6 \times 10^8$ years since these species diverged, assuming a 1°C decrease in $t_m$ per 1.5% mismatch.

In general, DNA sequences of homologous translated genes have not been conserved between distantly related species. For example, in the case of prokaryotes, radioactive *trp* mRNA isolated from *Shigella dysenteriae*, *Salmonella typhimurium*, and *Serratia marascens*, all enterobacteriaceae, hybridized to *trp* DNA from closely related *E. coli*, but *trp* mRNA from *Bacillus subtilis*, a Gram-positive bacterium, did not hybridize (31). In addition, we have found that a DNA fragment carrying the *K. pneumoniae hisD* and *hisG* genes hybridized only to DNAs from other enterobacteriaceae, even though that DNA was capable of complementing *hisD* mutations in *R. meliloti* (unpublished results).

If the Nif DNA sequence homology that we observe is due to descent from a common ancestral Nif gene over a period of $3 \times 10^9$ years, a level of selection on amino acid sequence comparable to that on histone H4 is demanded. Amino acid compositions of the MoFe and Fe protein from three species of nitrogen-fixing bacteria (4) are sufficiently different to preclude total amino acid homology but do not rule out the possibility of relatively long runs of exact amino acid homology that could result in the levels of DNA·DNA hybridizations that we observe. Selection could also exist on the DNA sequence directly if secondary structures of RNA or DNA were important, as appears to be the case in the enterobacterial *trp* attenuator region (25, 26), or if codon use were restricted (32). Nucleotide sequencing of Nif genes from different species will allow us to distinguish between selection on amino acid sequence or on nucleotide sequence.

An alternative explanation for the conservation of Nif DNA sequences is relatively recent radiation of Nif genes to many bacterial species, followed by "normal" divergence of both protein and DNA sequences. This would explain the similarity in DNA sequences between such divergent species without demanding extreme natural selection on protein primary sequence. It is widely known that there is a flow of genetic information between widely divergent bacterial species mediated by promiscuous resistance transfer factors (33, 34), which can transfer genes from one bacterial species to other unrelated species via chromosome mobilization, replicon fusion, or transposition of genes onto plasmids. Once transfer has occurred, there would be a selective advantage in maintaining the Nif genes in an ecosystem limited by fixed nitrogen (1).

If transposed ancestral Nif genes were radiated to many species by conjugative plasmids, one might expect to find Nif genes on plasmids in some species. In support of this hypothesis, F. Cannon (personal communication) has found DNA homologous to *K. pneumoniae* fragment A located on large indigenous plasmids in *R. leguminosarum* 897 and we have found similar homology to a large (150-kb) plasmid in *R. trifolii* RT1 (unpublished results).

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