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Detection of Alternative Nitrogenases in Aerobic Gram-Negative Nitrogen-Fixing Bacteria

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Strains of aerobic, microaerobic, nonsymbiotic, and symbiotic dinitrogen-fixing bacteria were screened for the presence of alternative nitrogenase (N_2ase) genes by DNA hybridization between genomic DNA and DNA encoding structural genes for components 1 of three different enzymes. A *nifDK* gene probe was used as a control to test for the presence of the commonly occurring Mo-Fe N_2ase, a *vnfDGK* gene probe was used to show the presence of V-Fe N_2ase, and an *anfDGK* probe was used to detect Fe N_2ase. Hitherto, all three enzymes have been identified in *Azotobacter vinelandii* OP, and all but the Fe N_2ase are present in *Azotobacter chroococcum* ATCC 4412 (MCD1). Mo-Fe N_2ase and V-Fe N_2ase structural genes only were confirmed in this strain and in two other strains of *A. chroococcum* (ATCC 480 and ATCC 9043). A similar pattern was observed with *Azotobacter beijerinckii* ATCC 19360 and *Azotobacter nigricans* ATCC 35009. Genes for all three systems are apparently present in two strains of *Azotobacter paspali* (ATCC 23367 and ATCC 23833) and also in *Azomonas agilis* ATCC 7494. There was no good evidence for the existence of any genes other than Mo-Fe N_2ase structural genes in several *Rhizobium meliloti* strains, cowpea *Rhizobium* strain 32H1, or *Bradyrhizobium japonicum*. Nitrogenase and nitrogenase genes in *Azorhizobium caulinodans* behaved in an intermediate fashion, showing (i) the formation of ethane from acetylene under Mo starvation, a characteristic of alternative nitrogenases, and (ii) a surprising degree of cross-hybridization to the *vnfDGK*, but not the *anfDGK*, probe. *vnfDGK*- and *anfDGK*-like sequences were not detected in two saccharolytic *Pseudomonas* species or *Azospirillum brasilense* Sp7. The occurrence of alternative N_2ases seems restricted to members of the family *Azotobacteraceae* among the aerobic and microaerobic diazotrophs tested, suggesting that an ability to cope with O_2 when fixing N_2 may be an important factor influencing the distribution of alternative nitrogenases.

The biological reduction of dinitrogen (N_2) to ammonia is catalyzed by a family of three nitrogenases (N_2ases). One member of this family is the long-studied molybdenum enzyme (Mo-Fe N_2ase), found in all diazotrophs. The other two alternative enzymes apparently do not require molybdenum and have been identified so far in only two obligately aerobic free-living bacteria in the genus *Azotobacter*. *Azotobacter chroococcum* can synthesize both a Mo-Fe N_2ase and a vanadium nitrogenase (V-Fe N_2ase) (32), while *Azotobacter vinelandii* makes these two enzymes (13, 14) plus a third enzyme type (Fe N_2ase), which seems to require only Fe (8, 26).

The enzymes are structurally similar. Each is composed of a pair of analogous O_2-sensitive component proteins (8, 11, 13, 14, 32). Components 2 (Fe proteins) are homodimers which contain a single 4Fe-4S cluster, bind MgATP, and serve as highly specific electron donors to components 1, which probably contain the site of N_2 binding and reduction. Components 1 are $\alpha_2\beta_2$ tetramers in Mo-Fe N_2ases but are $\alpha_2\beta_2\delta_2$ hexamers in the alternative enzymes. They each contain between 20 and 32 atoms of Fe and two atoms of Mo or V, where applicable. The enzymes are reactive towards the same range of substrates, though some differences in catalytic activities are observed. For example, all three nitrogenases reduce acetylene to ethylene, though only the V-Fe and Fe N_2ases also form approximately 3% ethane. It was therefore suggested that ethane formation from acetylene may be used as a diagnostic test (ethane test) for alternative nitrogenases (9).

The different nitrogenase systems are encoded by three distinct sets of genes. Among *Azotobacter* species, structural genes for the Mo-Fe N_2ase are organized into a single operon cotranscribed in the order *nifH* for component 2 and *nifD* and *nifK* for the α and β subunits of component 1 (5, 19). Structural genes for V-Fe N_2ase in *Azotobacter chroococcum* and *Azotobacter vinelandii* are arranged into two operons (17, 28, 34, 35). One operon encodes *vnfH* for the Fe protein associated with this system, and a closely linked operon encodes *vnfD*, *vnfG*, and *vnfK* for the α, δ, and β subunits of component 1. In *Azotobacter vinelandii*, the Fe N_2ase structural genes are organized into a single operon in the order *anfH*, *anfD*, *anfG*, and *anfK* and encode the Fe protein and the α, δ, and β subunits, respectively, of component 1 (16).

At present, there is little information about the distribution of alternative nitrogenases, although there is circumstantial evidence for V-Fe N_2ases in *Anabaena variabilis* (20), *Methanosarcina barkeri* (37), and *Clostridium* species (9, 15) based on the stimulation of diazotrophy by vanadium and positive responses in the ethane test. Probes made from Mo-Fe N_2ase structural genes (*nifHDK*) have proved useful in the detection of homologous sequences in a wide range of diazotrophs (36) because structural genes for this enzyme type are highly conserved. In this study, we have investi-

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gated the use of structural gene probes for both alternative nitrogenases to survey for the presence of comparable enzymes in nonsymbiotic and symbiotic N_2-fixing aerobic bacteria.

MATERIALS AND METHODS

Growth and maintenance of bacteria. *Azotobacter* and *Azomonas* strains, with the exception of *Azotobacter paspali*, were maintained and grown aerobically at 30°C on liquid or solidified Rich medium, a modified Burk's medium (38) containing Difco nutrient broth at 0.8 g liter^{-1} (31). *Azotobacter paspali* strains were grown in Burk's medium. *Rhizobium* strains were grown and maintained in YEM, which contained the following (per liter of distilled H_2O): KH_2PO_4, 0.5 g; NaCl, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; yeast extract, 1 g; and mannitol, 10 g; *Pseudomonas* strains were grown and maintained on Difco nutrient broth, tryptic soy broth, or tryptic soy agar. *Azorhizobium caulinodans* ORS 571 was maintained on TYC medium, which contained the following (per liter of distilled H_2O): tryptone, 5 g; yeast extract, 3 g; and $CaCl \cdot 2H_2O$, 0.88 g. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium containing ampicillin at 100 μg ml^{-1} when required.

Molecular biology techniques. Genomic DNAs from *Azotobacter*, *Pseudomonas*, and *Azospirillum* strains were prepared as described elsewhere (31). Briefly, the procedure involved the lysis of washed cells in sodium dodecyl sulfate (SDS) and the precipitation of DNAs in ethanol. Further purification involved protease K treatment and phenol extraction prior to reprecipitation with ethanol. *Rhizobium* strains grown to mid-exponential phase were treated with lysozyme and EDTA to facilitate lysis. Cells were washed in 3% NaCl, resuspended in 5 ml of TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], and treated with lysozyme (5 mg ml^{-1}) at 37°C for 10 min. Na_2EDTA was added to 50 mM, and the incubation was continued for a further 10 min. Cells were lysed by the addition of SDS to 2% (wt/vol). Thereafter, the DNAs were purified as described for *Azotobacter* genomic DNA (31). To prepare Southern blots, genomic DNAs were first digested with the appropriate restriction endonuclease and subjected to electrophoresis in agarose (0.8% [wt/vol] in TAE). After the DNAs had been denatured and neutralized, they were electroblotted to GeneScreen membrane (Dupont). Plasmids were prepared by alkaline lysis (4) and purified either by ultracentrifugation in $CsCl_2$-ethidium bromide gradients or by using Quiagen columns according to the manufacturer's instructions. Radioactive DNA probes were made from specific restriction fragments purified by agarose electrophoresis and recovered from agarose by the freeze squeeze method (40). One-hundred-nanogram amounts of DNA were radioactively labeled by nick translation (30) with [^{32}P]dCTP (3,000 Ci/mmol). Southern blots were prehybridized at 42°C for 1 h in 25 ml of Bovine Lacto Transfer Technique Optimizer (BLOTTO) (18) containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.6), 66 mg of nonfat milk, 10% (wt/vol) dextran sulfate, and deionized formamide at between 25 and 50% (vol/vol), depending on the stringency required. The probe was added to the prehybridization solution, and hybridization was carried out for a further 18 h. Blots were washed twice at room temperature in 100-ml amounts of 1× SSC–0.1% (wt/vol) SDS and twice at 65°C in 100-ml amounts of 0.5× SSC containing 0.1% (wt/vol) SDS before exposure to Kodak XAR5 X-ray film for 24 h. In some cases, blots were stripped by boiling in 0.1× SSC and reused after removal of radioactivity had been checked by autoradiography.

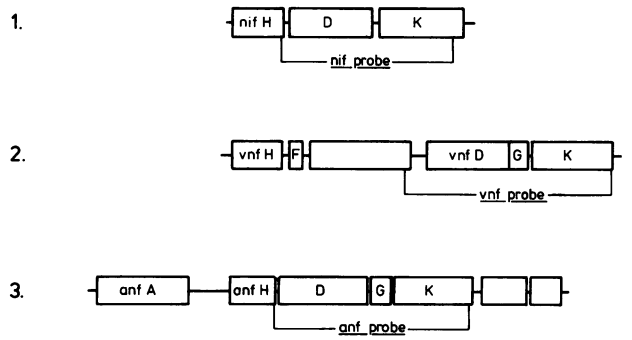

FIG. 1. DNA fragments used to prepare the various hybridization probes. Row 1 (*nifDK* probe), 3-kb *Sal*I-*Sac*I fragment from pER4, containing *nifD* and *nifK* from *Azotobacter chroococcum* (18a, 19); row 2 (*vnfDGK* probe), 4.1-kb *Asp* 718-*Bam*HI fragment from pSEQ10, containing *vnfD*, *vnfG*, and *vnfK* from *Azotobacter chroococcum* (35); and row 3 (*anfDGK* probe), 3.1-kb *Eco*RI fragment from pJWD3, containing *anfD*, *anfG*, and *anfK* from *Azotobacter vinelandii* (16).

Figure 1 shows DNA fragments used to synthesize radioactive probes used in these hybridization studies. We used DNA encoding only structural genes for components 1 (*nifDK*, *vnfDGK*, and *anfDGK*) and excluded DNA encoding components 2 (*nifH*, *vnfH*, and *anfH*) for the following reasons. Several organisms possess apparent reiterations of *nifH* without implying the presence of alternative nitrogenases. For example, in *Clostridium pasteurianum*, five *nifH*-like genes have been identified, and only one is known to be involved in N_2 fixation (41). Similarly, *nifH*-like sequences apparently unrelated to N_2 fixation are located in a photosynthetic gene cluster in *Rhodobacter capsulatus* (42) and the chloroplast genome of *Marchantia polymorpha* (25). Secondly, the relatively high degree of similarity among genes for this family of proteins (e.g., 89% identity between *nifH* and *vnfH*) does not promise the required specificity. The use of *vnfG* or *anfG* as a specific probe was also rejected because it is not clear whether their gene products (δ subunits of components 1 of the alternative nitrogenases) are essential or could be present in comparable enzymes in other genera.

Assay of C_2H_2 reduction. Nitrogenase activity in *Azorhizobium caulinodans* was assayed by C_2H_2 reduction. Organisms were grown aerobically to mid-exponential phase in modified Burk's medium containing 20 mM succinate as the sole carbon source and supplemented with 0.1% (wt/vol) NH_4Cl. Cells were washed in N-free medium and derepressed for 18 h under 1% O_2 in N_2. To start the assay, 10% of the headspace was replaced with C_2H_2. C_2H_4 and C_2H_6 formed from C_2H_2 were detected by flame ionization after chromatography on a stainless steel column (3 mm by 183 cm) packed with Durapak phenyl isocyanate-Poracil C (Waters Associates, Milford, Mass.) operated at 35°C with N_2 (30 ml min^{-1}) as the carrier.

RESULTS AND DISCUSSION

Evidence for alternative nitrogenases among members of the *Azotobacteraceae*. Several members of the *Azotobacteraceae*

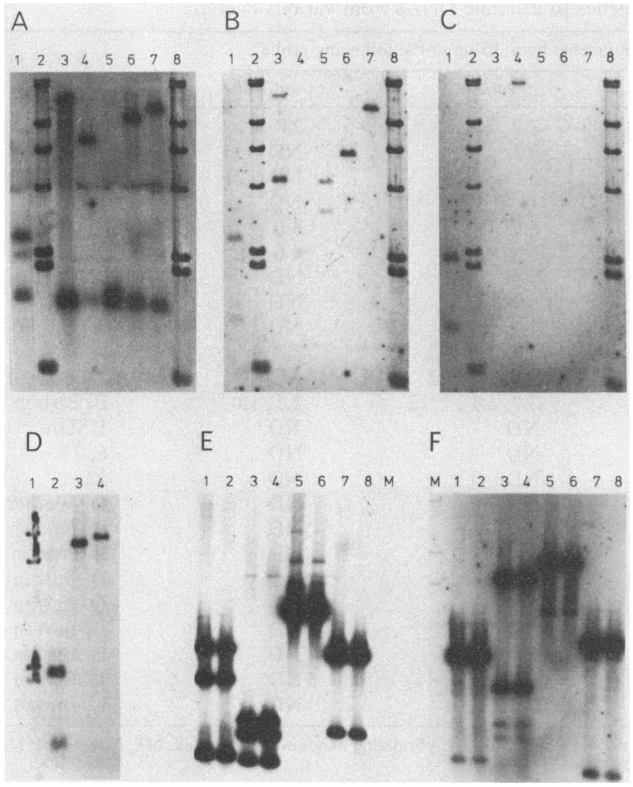

FIG. 2. Hybridization of *nifDK*, *vnfDGK*, and *anfDGK* probes to genomic DNAs from various members of the *Azotobacteraceae*. All hybridizations were performed under stringent conditions (42°C, 50% formamide) except where otherwise stated. For panels A (*nifDK* probe), B (*vnfDGK* probe), and C (*anfDGK* probe), the same Southern blot was used but was stripped between different probing experiments (see Materials and Methods). Genomic DNA was digested with *Eco*RI in each case. Lanes: 1, *Azotobacter vinelandii* CA (OP); 2 and 8, ^{35}S-labeled *Hin*dIII restriction fragments of bacteriophage λ; 3, *Azotobacter chroococcum* ATCC 9043; 4, *Azomonas agilis* ATCC 7494; 5, *Azotobacter nigricans* ATCC 35009; 6, *Azotobacter chroococcum* ATCC 480; and 7, *Azotobacter beijerinckii* ATCC 19360. (D) Hybridization at 42°C and 45% formamide of *anfDGK* to *Eco*RI-digested genomic DNA from *Azotobacter chroococcum* MCD1 (lane 3) and a derivative, MCD1301 (lane 4), deleted in both *nifHDK* and *vnfDGK*. Lane 1, radioactive marker DNA fragments as in panel A. Lane 2 contained an *Eco*RI digest of genomic DNA from *Azotobacter vinelandii*. (E and F) Hybridization of *vnfDGK* and *anfDGK* probes, respectively, to genomic DNA from *Azotobacter paspali*. Odd-numbered lanes contained DNA from ATCC 23367, and even-numbered lanes contained DNA from ATCC 23833. DNAs were digested with restriction enzymes as follows: lanes 1 and 3, *Eco*RI; lanes 3 and 4, *Sal*I; lanes 5 and 6, *Xho*I; and lanes 7 and 8, *Pst*I, lanes M, radioactively labeled *Hin*dIII restriction fragments from bacteriophage lambda.

were surveyed for the presence of the different nitrogenase systems. The existence of Mo-Fe and V-Fe N_2ases in *Azotobacter chroococcum* MCD1, a derivative of ATCC 4412, was demonstrated earlier (32). Though strains deleted in both sets of nitrogenase structural genes were incapable of fixing N_2, the possibility of a third nitrogenase system could not be entirely excluded. Indeed, the *anfDGK* probe hybridized to a single 8-kb *Eco*RI genomic DNA fragment in MCD1 (Fig. 2D). However, the hybridizing *Eco*RI fragment shifted to 8.6 kb in *Azotobacter chroococcum* MCD1301, a derivative of MCD1, in which a 1.4-kb *Bgl*II fragment within the *vnfDGK* cluster in the chromosome was replaced with a 2-kb spectinomycin resistance gene cassette (35). These results indicate that the *anfDGK* probe hybridized to the *vnfDGK* analogs only and confirms that *Azotobacter chroococcum* MCD1 does not possess the *anf*-encoded system.

Two other strains of *Azotobacter chroococcum* were also examined for the presence of all three systems (Fig. 2A through C). *nifDK* and *vnfDGK* probes both hybridized strongly to different sets of restriction fragments in genomic DNAs from ATCC 480 and ATCC 9043 (Table 1). However, there was no evidence for *anfDGK* analogs in either strain. Therefore, all three *Azotobacter chroococcum* strains tested contained only Mo-Fe and V-Fe N_2ases.

Two other strains, *Azotobacter beijerinckii* ATCC 19360 (type strain) and *Azotobacter nigricans* ATCC 35009, also apparently contained only the Mo-Fe N_2ase and V-Fe N_2ase genes (Fig. 2A through C). In *Azotobacter beijerinckii*, apparently identical 13.5-kb *Eco*RI fragments (Table 1) were identified by both probes. This could be explained by an unusual degree of cross-hybridization between the *vnfDK* probe and the *nifDK* genes. Alternatively, it could be explained if *nifDK* and *vnfDGK* are closely linked in this organism. In both *Azotobacter chroococcum* MCD1 and *Azotobacter vinelandii* OP, there is no evidence for close linkage between these gene clusters.

Type strains of two species, *Azotobacter paspali* and *Azomonas agilis*, appear similar to *Azotobacter vinelandii* in apparently containing all three systems (Fig. 2A through C, E and F). Both strains of *Azotobacter paspali* tested showed identical hybridizing fragments for all three probes (Fig. 2E and F) and therefore appear closely related, if not identical.

Of members of the *Azotobacteraceae* tested in this study, all apparently contain both V-Fe N_2ase and Mo-Fe N_2ase, suggesting that both systems are common in this family of organisms. This agrees with an early study (3) showing that vanadium stimulated diazotrophy in 7 of 10 strains of *Azotobacter chroococcum* and in 19 of 20 strains of *Azotobacter vinelandii*. However, we found no evidence for the presence of genes for the Fe N_2ase in *Azotobacter chroococcum*, *Azotobacter beijerinckii*, or *Azotobacter nigricans*, which, together with *Azotobacter armeniacus*, form a subfamily of closely related species (39) which we suggest may inhabit environments where possession of a nitrogenase system requiring neither molybdenum nor vanadium is of no selective value.

Three distinctly different species, *Azotobacter vinelandii*, *Azotobacter paspali*, and *Azotobacter agilis*, appear to possess all three systems. All of these species, but none of the previous group, are also capable of synthesizing the yellow-green family of fluorescent siderophores under Fe- or Mo-limited conditions (39) and therefore may be adapted for environments in which molybdenum and vanadium are limiting and in which the sequestration of iron is crucial. Becking (3) found evidence for stimulation of N_2 fixation by vanadium in only 1 of 20 isolates of *Azomonas* (*Azotobacter*) *agilis*. Our data show that the type strain, at least, is likely to be versatile in N_2 fixation.

Members of the family *Rhizobiaceae*. Members of the family *Rhizobiaceae* constitute a large group of agronomically important, symbiotic N_2-fixing bacteria estimated to contribute 70% of biological N_2 fixation. In these strains, the possession of alternative nitrogenases could potentially spare molybdenum and/or vanadium for other roles in the plant. Many of these organisms also do not express nitroge-

TABLE 1. Hybridization of nitrogenase structural genes to genomic DNAs from various bacteria

Organism	Size (kb) of *Eco*RI fragments hybridizing to the following probe[a]:			Source or reference[b]
	nifDK	*vnfDGK*	*anfDGK*	
Azomonas agilis ATCC 7494[c]	7.5, 1.4	2.2, 2.0	23	ATCC
Azorhizobium caulinodans ORS 571[d]	9.2	9.2	ND	C. Elmerich
Azospirillum brasilense Sp7[d]	6.7	ND	ND	C. Elmerich
Azotobacter beijerinckii ATCC 19360[d]	13.5, 1.4	13.5	ND	ATCC
Azotobacter chroococcum MCD1[e]	14, 1.4	8.0	8.0	31
Azotobacter chroococcum MCD1301[f]	NT	8.6	8.6	35
Azotobacter chroococcum ATCC 480[c]	11.5, 1.4, 1.3	6.4	ND	ATCC
Azotobacter chroococcum ATCC 9043[c]	16.0, 1.4, 1.3	6.4	ND	ATCC
Azotobacter nigricans ATCC 35009[c]	1.5, 1.4, 1.3	4.5, 3.4	ND	ATCC
Azotobacter paspali ATCC 23367[c]	2.5, 1.6	3.6, 2.6, 1.2	3.5	ATCC
Azotobacter paspali ATCC 23833[c]	NT	3.6, 2.6, 1.2	3.5	ATCC
Azotobacter vinelandii CA (OP)[c]	2.6, 2.2, 1.4	2.7, 1.1	2.1, 1.0	P. Bishop
Bradyrhizobium japonicum 61A76[g]	5.6, 5.0	ND	ND	USDA
Pseudomonas strain 4B (ATCC 43038)[g]	2.0, 1.6	ND	ND	6, 7
Pseudomonas strain DC[g]	2.6, 1.6	ND	ND	12
Pseudomonas stutzeri CMT.9.A[g]	ND	ND	ND	D. Werner
Pseudomonas stutzeri JM300[g]	ND	ND	ND	R. Knowles
Cowpea *Rhizobium* strain 32H1[g]	4.6, 1.8	ND	ND	L. Nelson
Rhizobium meliloti SU47[g]	4.1	ND	ND	L. Barran
Rhizobium meliloti 3-373[g]	4.1	ND	ND	L. Barran
Rhizobium meliloti 5-1218[g]	6.5, 4.2, 2.1	ND	ND	L. Barran
Rhizobium meliloti 17-27[g]	4.1, 2.6	ND	ND	L. Barran
Rhizobium meliloti 32-811[g]	4.1, 2.6, 1.5	ND	ND	L. Barran
Rhizobium meliloti 70-674[g]	4.2, 2.7, 1.6	ND	ND	L. Barran

[a] Hybridizations were carried out at 42°C with various concentrations of formamide. Only significantly hybridizing bands were recorded. NT, Not tested; ND, none detected.
[b] ATCC, American Type Culture Collection; USDA, U.S. Department of Agriculture.
[c] 50% formamide.
[d] *nifDK* and *anfDGK*, 40% formamide; *vnfDGK*, 45% formamide.
[e] *nifDK* and *vnfDGK*, 50% formamide; *anfDGK*, 45% formamide.
[f] 45% formamide.
[g] 40% formamide.

nase activity ex planta, and DNA hybridization could be an important diagnostic tool for the detection of potential alternative nitrogenases.

Several species were screened for sequences homologous to the *nif*, *vnf*, or *anf* probe. Use of the *nifDK*-specific probe both provided an internal control and enabled us to identify possible cross-hybridization between *nif* sequences and the *vnf* and *anf* probes. The *Azotobacter nifDK* probe showed good homology in all examples (Fig. 3C and E), and our hybridization data agreed with available published data (Table 1). There was no convincing evidence for the possession of *vnfDGK*- or *anfDGK*-like sequences in reference strains of the root-nodulating species, including the slowly growing *Bradyrhizobium japonicum* 61A76 and cowpea *Rhizobium* strain 32H1, which can fix N_2 ex planta (29), and the fast-growing *Rhizobium meliloti* SU47. To determine whether the occurrence of these enzymes may be sporadic, we examined a further five strains of *Rhizobium meliloti* that seemed to be potential candidates for possessing alternative nitrogenases because they contain apparent reiterations of *nifHD*-like DNA (1), as confirmed here with the *nifDK* probe (Table 1). Again, only strong hybridization to *nifDK* was observed in each case. When the stringency of the hybridization was lowered, many genomic fragments were labeled, some more conspicuously than others. For example, four of six *Rhizobium meliloti* strains tested exhibited a conspicuous band at 9 kb in *Xho*I digests (Fig. 3D), which did not coincide with any of the fragments picked out by the *nifDK* probe. However, when the blot was subjected to washes of increasing stringency, the hybridizing bands did not persist. We conclude that the hybridization observed was nonspecific and that this group of rhizobia in all probability possess only the Mo-Fe N_2ase system; however, if they contain other systems, these are unlikely to be closely related to those characterized so far in *Azotobacter* strains.

In the case of the stem-nodulating species *Azorhizobium caulinodans* ORS 571, the situation is unclear because the *vnfDGK* probe detected unique hybridizing fragments under moderately high stringency (Fig. 3G through I). However, the *nifDK* probe hybridized strongly to apparently identical fragments (Fig. 3F) consistent in size with those known to contain the *nifD* and *nifK* genes (10, 23). Nevertheless, there appears to be a degree of cross-hybridization between the *vnfDGK* probe (but not the *anfDGK* probe) and *nifDK* genes in this organism which we did not observe with *Azotobacter* strains. This organism also formed ethane in the acetylene reduction assay, which is a characteristic of alternative nitrogenase systems. When the organism was grown under N_2-fixing conditions without added Mo or W, the proportion of ethane to ethylene formed was 0.2%. When sodium tungstate was added to the medium at between 0.1 and 5 mM, the proportion of ethane formed increased to 5%. This level was lowered to 0.1% when 10 μM Mo was also added. These data suggest that this organism may possess an alternative nitrogenase by the ethane test criterion. However, we found no evidence for an additional set of func-

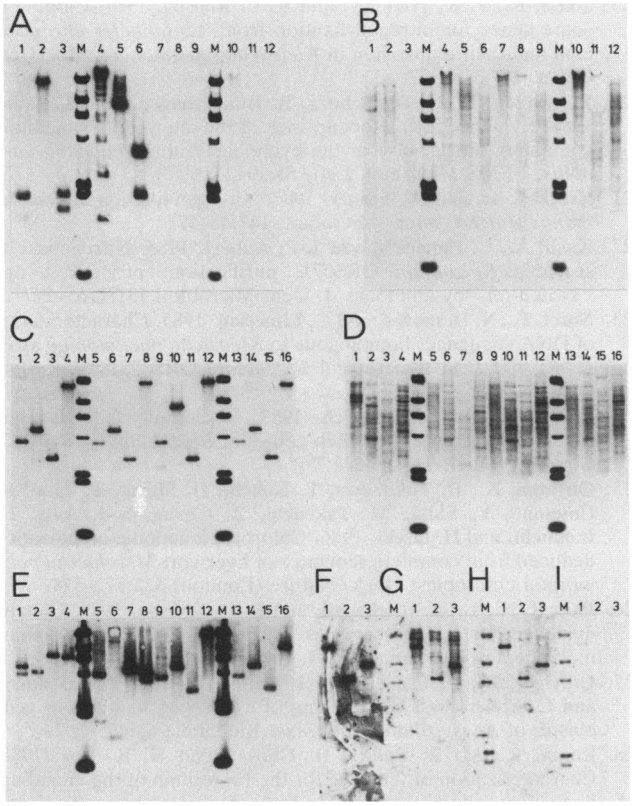

FIG. 3. Hybridization of *nifDK*, *vnfDGK*, and *anfDGK* probes to genomic DNAs from *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* species. (A and B) Hybridization of *nifDK* and *vnfDGK* probes, respectively, to genomic DNAs from *Pseudomonas* species. Lanes in both panels contained restriction digests of genomic DNAs from the following strains: lanes 1 through 3, *Pseudomonas* strain 4B; lanes 4 through 6, *Pseudomonas* strain DC; lanes 7 through 9, *Pseudomonas stutzeri* CMT.9.A; and lanes 10 through 12, *Pseudomonas stutzeri* JM300. DNAs were digested as follows: lanes 1, 4, 7, and 10, *Eco*RI; lanes 2, 5, 8, and 11, *Pst*I; and lanes 3, 6, 9, and 12, *Sal*I. Hybridizations were carried out at 42°C and with 40% formamide. (C and D) Hybridization of *nifDK* and *vnfDGK* probes, respectively, to genomic DNAs from *Rhizobium meliloti* strains. Lanes in both panels contained restriction digests from the following strains: lanes 1 through 4, strain 5-1218; lanes 5 through 8, strain 17-27; lanes 9 through 12, strain 32-811; and lanes 13 through 16, strain 70-674. DNAs were digested as follows: lanes 1, 5, 9, and 13, *Eco*RI; lanes 2, 6, 10, and 14, *Pst*I; lanes 3, 7, 11, and 15, *Sal*I; and lanes 4, 8, 12, and 16, *Xho*I. Hybridizations were carried out at 42°C and with 40% formamide. (E) Hybridizations of the *nifDK* probe to restriction digests of genomic DNAs from the following strains: lanes 1 through 4, *Bradyrhizobium japonicum* 61A76; lanes 5 through 8, cowpea *Rhizobium* strain 32H1; lanes 9 through 12, *Rhizobium meliloti* SU47; and lanes 13 through 16, *Rhizobium meliloti* 3-373. Genomic DNAs were digested as follows: lanes 1, 5, 9, and 13, *Eco*RI; lanes 2, 6, 10, and 14, *Pst*I; lanes 3, 7, 11, and 15, *Sal*I; and lanes 4, 8, 12, and 16, *Xho*I. Hybridizations were carried out at 42°C and with 40% formamide. (F through I) Hybridizations of *nifDK* (F) and *vnfDGK* (G through I) probes to genomic DNA from *Azorhizobium caulinodans* ORS 571. In each panel, the restriction digests were as follows: lane 1, *Bam*HI; lane 2, *Bgl*II; and lane 3, *Eco*RI. In all cases hybridizations were carried out at 42°C with 40% (F and G), 45% (H), or 50% (I) formamide. Lanes M, Radioactively labeled *Hin*dIII restriction fragments of bacteriophage lambda.

tional nitrogenase genes in *Azorhizobium caulinodans* because strain 57186, which carried a deletion in the *nifHDK* cluster (11a), was incapable of fixing N_2 irrespective of the metal status of the culture medium. Therefore, nitrogenase in this organism exhibits some characteristics reminiscent of alternative nitrogenases, though the enzyme is known to contain molybdenum (22). The *nifHDK* cluster and a closely linked second copy of *nifH* have been cloned (10, 23), and nucleotide sequence data are available for both *nifH* gene copies (24). It will be interesting to examine *nifD* and *nifK* genes in this organism for similarity to *vnfD* and *vnfK* homologs. However, if this organism possesses only a typical Mo-Fe N_2ase, then the ethane test may not be an unequivocal indicator of alternative nitrogenase systems.

Pseudomonads and azospirilla. Three species of N_2-fixing pseudomonads were examined for the potential presence of alternative nitrogenase genes. *nifDK* was again used as a control. Genomic DNAs from two saccharolytic species (*Pseudomonas* strain 4B [ATCC 43038] and *Pseudomonas* strain DC) both showed good homology to the *nifDK* probe (Fig. 3A and Table 1) but showed no homology to either the *vnfDGK* or the *anfDGK* probe, even under low stringency. Surprisingly, two previously accredited N_2-fixing isolates of *Pseudomonas stutzeri*, CMT.9.A and JM300 (2, 21), showed no homology to the *nifDK* probe (Fig. 3B). With no prior reports of the use of *nifHDK* gene probes with this species, it seemed possible that these strains contain only alternative nitrogenases. However, neither the *vnfDGK* nor the *anfDGK* probe hybridized to DNA from these strains (Fig. 3B). Krotzky and Werner (21) suggested that *nif* genes may be plasmid borne in this species, and hence it is possible that the strains have been cured for this plasmid. Alternatively, these organisms may possess a nitrogenase which is not closely genetically related to the enzymes from azotobacters.

In *Azospirillum brasilense* Sp7, we detected good homology to the expected genomic fragments by using the *nifDK* probe (27) but observed no homology to either the *vnfDGK* or the *anfDGK* probe (Table 1).

In summary, alternative nitrogenases appear common among members of the *Azotobacteraceae* but do not appear widely distributed among nonsymbiotic microaerobic or symbiotic microaerobic N_2-fixing bacteria. It is possible that alternative nitrogenases have evolved in a recent ancestor of the family *Azotobacteraceae*. However, studies on the phylogeny of genes for α and β subunits from all three enzymes suggest that the alternative enzymes arose from an ancient gene duplication event and therefore could be widely distributed (11b). The distribution of alternative nitrogenases is likely to be governed by the metal status of particular environments. The ability of any diazotroph to cope with O_2 when fixing N_2 (33) may also be important since the alternative nitrogenases are especially O_2 sensitive. The O_2 relationship could explain why alternative nitrogenases may persist in obligate anaerobes such as clostridia (9, 15) and *Methanosarcina barkeri* (37) or in *Anabaena variabilis* (20) and members of the *Azotobacteraceae*, which have elaborate O_2 protection mechanisms (33).

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