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Nitrogen Fixation Genes in an Endosymbiotic *Burkholderia* Strain

DANIELA MINERDI,[1] RENATO FANI,[2] ROMINA GALLO,[2] ALESSANDRA BOARINO,[1] AND PAOLA BONFANTE[1]*

Dipartimento di Biologia Vegetale dell'Università di Torino and Centro di Studio sulla Micologia del Terreno del Consiglio Nazionale delle Ricerche, 10125 Turin,[1] and Dipartimento di Biologia Animale e Genetica, Università degli Studi di Firenze, 50125 Florence,[2] Italy

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In this paper we report the identification and characterization of a DNA region containing putative *nif* genes and belonging to a *Burkholderia* endosymbiont of the arbuscular mycorrhizal fungus *Gigaspora margarita*. A genomic library of total DNA extracted from the fungal spores was also representative of the bacterial genome and was used to investigate the prokaryotic genome. Screening of the library with *Azospirillum brasilense nifHDK* genes as the prokaryotic probes led to the identification of a 6,413-bp region. Analysis revealed three open reading frames encoding putative proteins with a very high degree of sequence similarity with the two subunits (NifD and NifK) of the component I and with component II (NifH) of nitrogenase from different diazotrophs. The three genes were arranged in an operon similar to that shown by most archaeal and bacterial diazotrophs. PCR experiments with primers designed on the *Burkholderia nifHDK* genes and Southern blot analysis demonstrate that they actually belong to the genome of the *G. margarita* endosymbiont. They offer, therefore, the first sequence for the *nif* operon described for *Burkholderia*. Reverse transcriptase PCR experiments with primers designed on the *Burkholderia nifH* and *nifD* genes and performed on total RNA extracted from spores demonstrate that the gene expression was limited to the germination phase. A phylogenetic analysis performed on the available *nifK* sequences placed the endosymbiotic *Burkholderia* close to *A. brasilense*.

Arbuscular mycorrhizal (AM) fungi are one of the most widespread microbial communities of the rhizosphere, since they establish symbiotic associations with the roots of about 80% of plant species (38). They are of very ancient origin. Fossil and molecular data demonstrate that they first appeared 400 to 350 million years ago, suggesting that the origin of land plants may be closely correlated to their symbiosis with AM fungi (36, 41).

The success of mycorrhizas in evolution is mainly due to the central role their fungi play in the capture of nutrients from the soil in almost all ecosystems (43). As a consequence, AM fungi are crucial determinants of plant biodiversity, ecosystem variability, and the productivity of plant communities (46).

It has been demonstrated that the AM fungus *Gigaspora margarita* (BEG isolate 34) harbors a homogeneous population of endobacteria in its cytoplasm throughout its life cycle (9). The high number of bacterial cells (about 250,000 per spore) and their presence in successive generations (V. Bianciotto, unpublished data) suggest that they are an integral part of the fungal system. On the basis of 16S rDNA sequence analysis, they have been assigned to the genus *Burkholderia* (9), an extremely heterogeneous group which includes soil bacteria, plant growth promoting rhizobacteria, and human and plant pathogens (6, 43). Strains of *Burkholderia cepacia* can survive within vacuoles in different isolates of the protozoa *Acanthamoeba* (27). In addition, *Burkholderia* isolates from rhizosphere have been proposed but not definitively shown to fix nitrogen (18).

Our objectives were to determine whether the symbiotic *Burkholderia* spp. possess the nitrogen fixation genes and whether these genes are expressed. Nitrogenase, the enzyme responsible for fixation, consists of two components, component I (also called FeMo protein or dinitrogenase), an $\alpha_2\beta_2$ tetramer encoded by the *nifD* and *nifK* genes, and component II (dinitrogenase reductase or Fe protein), a homodimer encoded by the *nifH* gene. Component I contains two unusual, rare metal clusters, one of which is the iron molybdenum cofactor (FeMo-co), regarded as the site of dinitrogen reduction (26). The two components are conserved in structure, function, and amino acid sequence through diazotrophs (15, 32). For this reason we wished to detect the *nifH*, *nifD*, and *nifK* genes. Since *Burkholderia* cannot be maintained on a cell-free medium, we took advantage of a genomic library constructed from *G. margarita* spores (BEG 34) that also contained the bacterial genome (45). As a probe we used two *Azospirillum brasilense* DNA fragments (16, 32; R. Fani, unpublished data) harboring the *nifH* and *nifDK* genes, respectively. The second fragment has been previously reported to hybridize strongly with the DNA of *Burkholderia* nitrogen-fixing strains (40). A DNA region from an endosymbiotic *Burkholderia* strain harboring the −24 to −12 promoter region and the *nifHDK* operon was identified, isolated, and molecularly characterized. Reverse transcriptase PCR (RT-PCR) experiments showed the expression of the *nif* genes in germinated spores. In addition, in order to compare these genes with those from free-living *Burkholderia*, a partial sequence of the *nifK* gene from *Burkholderia vietnamiensis* TVV75 was obtained.

* Corresponding author. Mailing address: Dipartimento di Biologia Vegetale dell'Università di Torino, Viale P. A. Mattioli 25, 10125 Turin, Italy. Phone: 390116502927. Fax: 390116707459. E-mail: p.bonfante@csmt.to.cnr.it.

MATERIALS AND METHODS

DNA extraction. Two fungal isolates were used: *G. margarita* Becker et Hall (New Zealand isolate BEG 34) and *Gigaspora rosea* Nicolson et Schenck (BEG 9), which have been demonstrated to possess or not possess bacteria in their cytoplasm (10). About 50 spores of both of the AM fungal species were recovered from pot cultures of *Trifolium repens* L. by wet sieving (17). They were rinsed five times with sterile, filtered, distilled water; as a further step they were surface sterilized with 4% chloramine-T and 300 ppm of streptomycin for 30 min, treated with 14 U of DNase (Promega) at 37°C for 30 min, sonicated five times, and then rinsed seven times for 1 h total with sterile, filtered distilled water. The intact spores were incubated with the Live/Dead *Bac*Light bacteria viability kit (Molecular Probes Europe BV) at room temperature in the dark for 15 min to visualize contaminant bacteria, according to the manufacturer's instructions. This kit contains a proprietary mixture of nucleic acid stains that distinguishes live bacteria from dead bacteria (9).

DNA was extracted with a slightly modified cetyltrimethylammonium bromide method, according to Henrion et al. (22). Extreme care was taken to avoid any contamination. All solutions were filter sterilized, and sterile procedures were used throughout the DNA extraction.

RNA extraction. About 100 spores of *G. margarita*, treated as reported above, germinated in filter-sterilized distilled water at 28°C for 6 days. RNA was extracted using the Qiagen RNeasy mini kit (Promega) according to the manufacturer's instructions from either germinated or nongerminated spores of *G. margarita*.

Bacterial lysates. *B. vietnamiensis* strain TVV75 (40) was grown on tryptic soy agar medium (Sigma) at 37°C. Bacterial lysates were prepared by placing the samples in filter-sterilized distilled water at 92°C for 5 min and on ice for 2 min. Centrifugation at 14,000 × *g* followed, and the supernatant was collected and used in PCRs.

Screening of the genomic library. A genomic library was constructed into the *Bam*HI site of Lambda DASH II starting from total DNA extracted from *G. margarita* spores (including the *Burkholderia* endosymbiont DNA) and was partially digested with the restriction endonuclease *Sau*3AI (45). It was first established whether this library was representative of the bacterial genome. Calculations with Clarke and Carbon's equation (13) indicated that 2,000 primary recombinants are needed to represent the *Burkholderia* genome with 99% probability. To calculate this value it was considered that in each spore of *G. margarita* there are 2,000 to 3,000 fungal nuclei (7) of about 10^8 bp each (23) and 250,000 bacteria with a genome of approximately 10^6 bp (9). Therefore, about 30% of recombinant clones in the library should be prokaryotic. Since the number of total clones in the library was 65,000 (45), the number of prokaryotic clones should be about 22,000, thus making this library largely representative of the *Burkholderia* genome.

PCR products were separated by electrophoresis on 1.2% agarose gel in 1× Tris-borate-EDTA (TBE) running buffer. Approximately 4×10^4 Lambda DASH II (Stratagene) bacteriophage particles were plated onto NZY agar medium (per liter: 5 g of NaCl, 2 g of $MgSO_4$, 5 g of yeast extract, 10 g of casein hydrolysate [pH 7.5], 15 g of agar) using *Escherichia coli* XL1-Blue MRA (P2) as the host (Stratagene). After incubation for 6 to 8 h at 38°C, the plates were overlaid for 2 min at room temperature with Hybond N^+ nylon membranes (Amersham) (4 min in the case of duplicate filters). DNA was denatured and fixed by autoclaving the filters for 5 min at 120°C. The filters were then hybridized with a *Pst*I-*Sma*I fragment of 1.7 kbp from plasmid pAFG1 (16) containing the entire *nifD* and the 5′-proximal region of the *nifK* genes of *A. brasilense* strain Sp7 (15, 30) and with an *Eco*RI-*Pst*I fragment of about 1.5 kbp from plasmid pAFT4 (R. Fani, unpublished data) containing the entire *nifH* gene of *A. brasilense* strain Sp7. The probes were labeled using a chemiluminescence system (ECL Direct DNA labelling and detection system, Amersham). The filters were prehybridized with hybridization solution (0.5 M NaCl, 5% blocking reagent in ECL hybridization buffer) at 42°C for 1 h. Hybridization was performed overnight at 42°C. Then filters were washed twice for 20 min each at 42°C with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.4% sodium dodecyl sulfate, 1 M urea, and finally, twice for 5 min each with 2× SSC at room temperature. An X-ray film (Hyperfilm ECL; Amersham) was exposed to the membranes for 3 h and developed as recommended by the manufacturer. Single positive plaques were identified after secondary and tertiary screening of positive areas using both probes. Positive plaques obtained using the *nifDK* probe were cut out from the agar plates and amplified. The DNA from one was purified with a Qiagen Lambda mini kit (Promega). The DNA was treated with 30 U of each of the restriction endonucleases *Eco*RI, *Sal*I, *Not*I, and *Xba*I (Promega) which cleaved close to the *Bam*HI site at 37°C for 3 h. The reaction products were analyzed by 0.8% agarose gel electrophoresis in TBE (4.5 mM Tris-borate, 1 mM EDTA) buffer.

Southern blotting analysis. DNA fragments were transferred onto Hybond N^+ membranes (Amersham) using standard procedures (33), and hybridizations were performed with the chemiluminescence detection system.

Subcloning and DNA sequencing. Two *Sal*I fragments of 800 bp and 1,500 bp giving a positive signal when hybridized to the *nifDK* probe were purified from agarose gel using the Qiaex II gel extraction kit (Promega) following the manufacturer's instructions. They were subcloned into the *Xho*I site of pZErO-1 plasmid vector (Invitrogene) using the Zero Background Cloning kit (Invitrogen) according to the manufacturer's instructions.

DNA sequencing was carried out by dideoxy sequencing (34) with the vector primers M13f and M13r, using the Sequenase kit (Pharmacia, Bridgewater, Mass.). Subsequent sequencing was done automatically using fluorescent dye-linked internal primers on the phage DNA and an Applied Biosystems model 370A DNA sequencer (Genome Express Society, Grenoble, France) and an Applied Biosystems model 373 stretch with XL upgrade DNA sequencer (Service de Sequencage, Université de Laval, Quebec, Canada). The sequence analysis was performed using the McVector program (IBI, New Haven, Conn.). (See "Nucleotide sequence accession numbers" below.)

Analysis of sequence data. Amino acid and nucleotide sequences were retrieved from the GenBank, EMBL, and PIR databases. The ClustalW program (1) was used for pairwise and multiple alignments using default gap penalties. The structure and organization of the *nifH*, *nifD*, and *nifK* genes were deduced from the data available from the microorganisms available in data banks. BLAST (1) probing of the DNA and protein databases was performed with the tBLASTn, BLASTn, and BLASTP programs.

PCR and RT-PCRs. PCRs were carried out on DNA extracted from the spores of *G. margarita* and *G. rosea* and on the washing solution of the spores, in a final volume of 20 μl containing 2 μl of DNA preparation, 250 mM each deoxynucleoside triphosphate, 2 μl of 10× buffer (Dynazyme; Celbio), 0.7 mM each primer, and 2.5 U of Dynazyme *Taq* polymerase (Celbio). The following amplification program was run in a Hybaid thermal cycler: 1.5 min at 95°C (1 cycle); 1.5 min at 95°C, 1 min at 62°C, and 2 min at 72°C (30 cycles); and a final elongation at 72°C for 10 min. PCR products were analyzed by electrophoresis on 1.2% agarose gel run in 1× TBE buffer (33). The following primers each amplified a 760-, 630-, and 1,080-bp fragment: nifHf (5′-GGCAAGGGCGGTATCGGCA AGTC-3′), nifHr (5′-CCATCGTGATCGGGTCGGGATG-3′), nifDf (5′-TGC ACTTCACCTCGGATTTCCAGG-3′), nifDr (5′-CCTTGATGCTGTCGTCG AACAGAGC-3′), nifKf (5′-AAGGCTGCGTCGCCTATTACCG-3′), and nifKr (5′-GTCGCGCTCCAGATATTTGCC-3′).

PCR also was carried out on the washing solution of the spores using bacterial universal primers 704f and 1495r (9). Amplification of the *B. vietnamiensis* strain TVV75 *nifK* partial sequence was carried out by using primers nifKf and nifKr and by changing the annealing temperature (55°C instead of 62°C). The nucleotide sequence was determined via direct sequencing (Genome Express Society). (See "Nucleotide sequence accession numbers" below.)

RT-PCR experiments were carried out on RNA extracted from germinated and nongerminated spores of *G. margarita* using the Qiagen OneStep RT-PCR kit (Promega), according to the manufacturer's instructions, in a final volume of 25 μl containing 2 μl of RNA preparation, 10 mM each deoxynucleoside triphosphate, 5 μl of 5× OneStep RT-PCR buffer, 0.7 mM each primer, and 1 μl of OneStep RT-PCR enzyme mix. The amplification program was the same as described above, and the primers used were nifHf, nifHr, nifDf, nifDr, and the two additional primers BLOf and BLOr, which were specifically designed to amplify the 16S rDNA region of the endosymbiotic *Burkholderia* (9). Samples without reverse transcriptase were used as negative controls. RT-PCR products were analyzed by agarose gel electrophoresis in a 1× TBE running buffer (33). The amplified products were purified from agarose gel using the QIAEXII gel extraction kit (Promega) following the manufacturer's instructions. The nucleotide sequence was determined via direct sequencing (Genome Express Society).

Phylogenetic analysis. Preliminary multiple alignments of amino acid sequences were generated with the program ClustalW (42) using default gap penalties. The selection of characters eligible for the construction of phylogenetic trees was optimized by comparing all sections of all the available NifK alignments with comprehensive inventories of significant binary alignments obtained by BLAST probing of the DNA and protein databases with representative archaeal and bacterial sequences (19). Phylogenetic trees were constructed using the distance matrix (DM) and maximum-parsimony (MP) methods. MP analysis used the PROTPARS program of the Phylogeny Inference Package (PHYLIP), version 3.57c (Department of Genetics, University of Washington, Seattle, Wash.); the PHYLIP programs SEQBOOT, PROTPARS, and CONSENSE were used to derive an MP tree which has replicated in 100 bootstraps. Evolu-

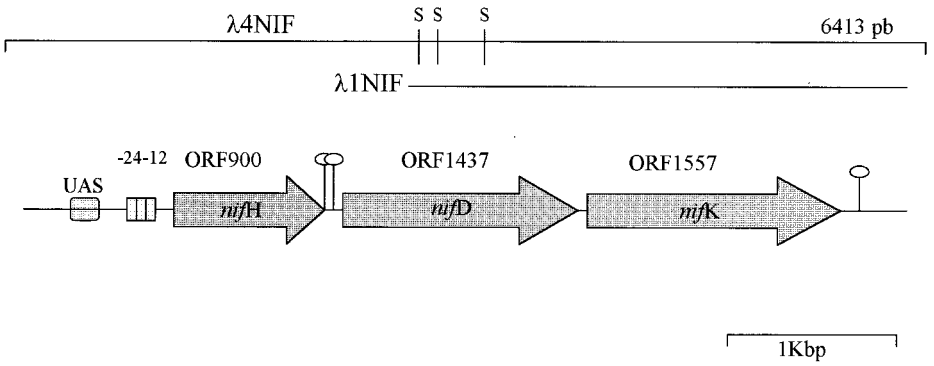

FIG. 1. Schematic representation of the DNA region containing the putative *Burkholderia nifHDK* operon. Symbols: stem-loops, putative transcription terminators; UAS, upstream activator sequence; S, recognition site for *Sal*I; −24−12 is the putative promoter recognized by σ^{54}. The two lines represent the λ4NIF DNA insert and the overlapping λ1NIF sequence.

tionary distances between all pairs of taxa (DM analysis) were calculated with the program PROTDIST of the PHYLIP 3.57c package, which estimates the number of expected amino acid replacements per position using a substitution model based on the Dayhoff 120 matrix; the resultant distant matrix was then used to construct a neighbor-joining tree with the program NEIGHBOR. The PHYLIP programs SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE were used (in that order) to derive a consensus tree based on 100 bootstrap replications of the original alignment.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been assigned GenBank accession numbers AF194084 (see "Subcloning and DNA sequencing" above) and AF175210 (see "PCR and RT-PCRs" above).

RESULTS

Isolation of DNA fragments harboring the endosymbiont *Burkholderia nifHDK* genes. Low-stringency screening of 40,000 plaques of the genomic library of *G. margarita* with the *A. brasilense nifDK* and *nifH* probes gave 30 and 40 positive areas, respectively. Two single positive plaques were isolated following secondary and tertiary screening. They were designated λ1NIF and λ4NIF, respectively. The phage DNA of λ1NIF was purified and digested with four restriction enzymes (*Eco*RI, *Sal*I, *Not*I, *Xba*I) that cleaved close to the cloning site. A Southern blotting experiment using the *A. brasilense nifDK* genes as a probe revealed the presence of strong hybridization signals (data not shown), suggesting that the recombinant phage harbored *Burkholderia* sequences homologous to the *A. brasilense nifDK* genes. Two *Sal*I DNA fragments of about 800 and 1,500 bp, which gave strong hybridization signals with the *nifDK* probe, were purified and subcloned into the pZErO plasmid vector, and their nucleotide sequences were determined (not shown). A primer-walking strategy was adopted to obtain the nucleotide sequence of the flanking regions of the two *Sal*I fragments.

The same primers were also used to determine the nucleotide sequences of the DNA of λ4NIF that gave a strong hybridization signal with the *A. brasilense nifH* probe. Results showed that the DNA inserts of λ1NIF and λ4NIF partially overlapped, as shown in Fig. 1. In this way the nucleotide sequence of a 6,413-bp region was obtained (accession number AF194084).

The analysis of the entire nucleotide sequence revealed the presence of three open reading frames (ORFs) of 900, 1,437, and 1,557 bp (hereinafter ORF900, ORF1437, and ORF1557). After tBLASTn and BLASTP searches, the putative amino acid sequences showed very low E values with the NifH and NifD-NifK Fe-Mo proteins from archaeal and bacterial diazotrophs (see below). These data confirmed that the DNA inserts harbored by λ1NIF and λ4NIF actually contained sequences encoding putative NifH, NifD, and NifK proteins, in agreement with hybridization data. The codon usage was in agreement with the GC contents of the three ORFs (63.0, 59.9, and 60.7%, respectively), similar to the GC content of the entire region (60.1%), with most codons ending with G or C.

The analysis of the region immediately upstream of the three ORFs revealed the presence of Shine and Dalgarno sequences (AGGAG, AGGAGG, and AAGGAA, respect ively) located 9 bp from the ATG codon of each ORF. A sequence (CTGGCAC-N_5-GTGCA) showing consensus with the −24 to −12 *nif* promoters (20) was found 80 nucleotides upstream of the *nifH* (ORF900) ATG codon. Moreover, two overlapping regions, identical to the upstream activated sequences of NifA-regulated genes (12) and characterized by the consensus sequence 5′-TGT-N_{10}-ACA-3′, were found upstream of the putative *Burkholderia nifH* promoter region (Fig. 1). This suggested that the *Burkholderia nifHDK* genes might be subject to a regulation similar to that of other diazotrophs.

In the 192-bp *nifH-nifD* intergenic region, two repeated and inverted sequences that could act as putative Rho-dependent transcription terminators were found at positions 2092 and 2158, whereas no −24 to −12 sequences were found (Fig. 1). On the contrary, in the 34-bp *nifD-nifK* intergenic region, no sequence resembling transcription terminators was found. Finally, a putative strong Rho-independent transcription terminator (ΔG at 25°C of −90.5 kcal/mol) was found 67 bp downstream from the end of ORF1557 (*nifK*).

From these data we suggest that the three ORFs are arranged in an operon and that their transcription starts at the −24 to −12 promoter upstream from the *nifH* gene. A BLASTn analysis revealed that the nucleotide sequence of the three ORFs exhibited the highest degree of sequence identity to the corresponding *A. brasilense* genes (accession number M64344). On the contrary, the 1,000 bp downstream from ORF1557 (*nifK*) did not reveal the presence of ORFs (Fig. 1) and did not display a significant degree of identity with any sequence available in databases.

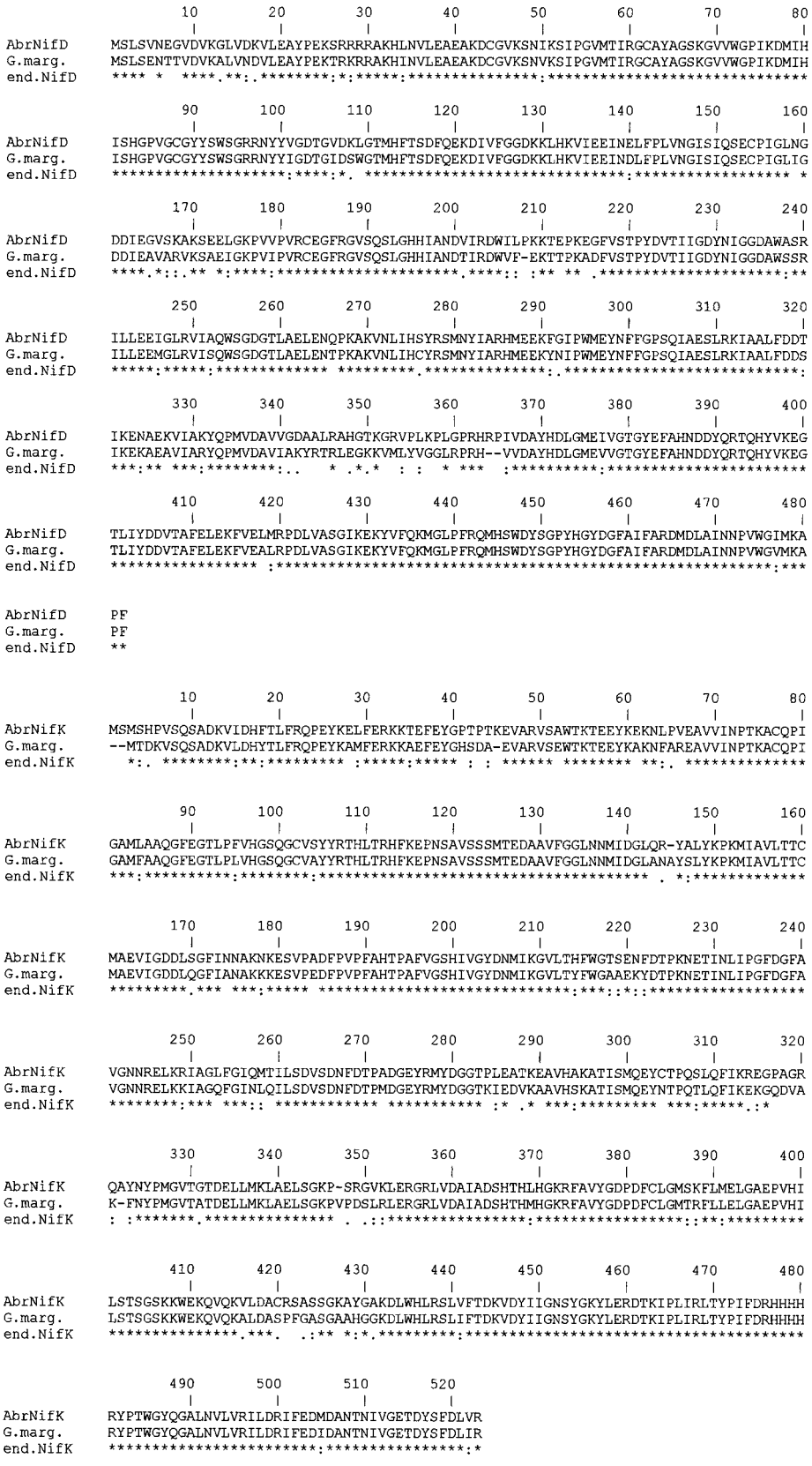

FIG. 2. ClustalW alignment of the amino acid sequences deduced from the *Burkholderia nifH*, *nifD*, and *nifK* genes and from the *A. brasilense* orthologous genes. The amino acids are indicated by single-letter codes. Gaps were introduced for optimal alignment. Identical residues between two sequences are indicated by an asterisk (*); similar residues are indicated by one dot (low similarity) or two dots (high similarity).

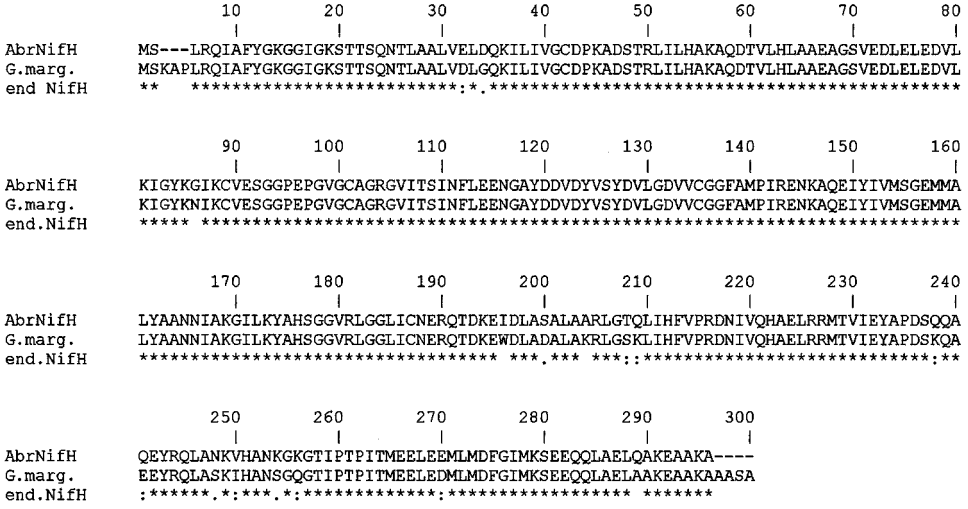

FIG. 2—*Continued*.

Analysis of the amino acid sequence of the *Burkholderia* NifH, NifD, and NifK proteins. The three ORFs encoded putative proteins of 300, 479, and 518 amino acids, with molecular masses of 32,330, 53,938, and 58,012 Da, respectively. Amino acid sequences of the three proteins were then compared with those available in databases. A BlastP search revealed that the proteins encoded by ORF900, ORF1437, and ORF1557 exhibited sequence similarity (data not shown) to all the available archaeal and bacterial NifH, NifD, and NifK protein sequences, respectively.

A ClustalW pairwise comparison of the *Burkholderia* NifH, NifD, and NifK amino acid sequences with available orthologous archaeal and bacterial sequences determined their degrees of sequence identity and similarity. Results showed that the *Burkholderia* NifH, NifD, and NifK proteins exhibited the highest degree of sequence similarity to the *A. brasilense* NifH, NifD, and NifK (Fig. 2).

A ClustalW multialignment of all the available NifH, NifD, and NifK sequences was used to identify conserved regions and to detect the degrees of sequence similarity and identity between the *Burkholderia* NifH, NifD, and NifK proteins and the available orthologous proteins. The *Burkholderia* NifH protein contained four cysteine residues located at positions 39, 86, 98, and 132 and found at the corresponding positions in all known NifH proteins (39). Comparison of the α subunit sequences of component I of nitrogenase showed that the *Burkholderia* NifD protein contained five conserved cysteine residues at positions 62, 88, 154, 183, and 275 (12). As previously shown for other NifK proteins, the *Burkholderia* NifK protein contained three conserved cysteine residues at positions 75, 100, and 158 (5).

Amplification and sequencing of *B. vietnamiensis* TVV75 *nifK* partial sequence. Primers nifKf and nifKr designed on the endosymbiont *Burkholderia nifK* sequence were used to amplify the corresponding region of *B. vietnamiensis* strain TVV75. Analysis of the 1,032-bp amplification fragment revealed that it encodes a putative polypeptide showing a very high degree of sequence similarity with the available NifK proteins (data not shown). ClustalW pairwise comparison of this partial sequence with the orthologous gene products revealed that the highest degrees of sequence identity and similarity were shown with the *Bradyrhizobium japonicum* NifK protein (68.9% identity; 86.6% considering amino acids with similar characteristics) rather than with the endosymbiont *Burkholderia* NifK (58.4% identity and 78.8% similarity).

Phylogenetic analysis. A ClustalW multialignment followed by visual inspection and manual editing of the NifK sequences reduced the complete sequences to a 322-amino-acid length where regions of ambiguous sequence alignment were excluded. Phylogenetic trees of the NifK amino acid sequences were inferred from the selected sequences by the DM and MP methods. Both the DM and MP analyses gave the same results; as shown in Fig. 3, both endosymbiont *Burkholderia* and *B. vietnamiensis* TVV75 belonged to a clearly separated group, which also includes *A. brasilense, Parasponia rhizobium, B. japonicum*, and *Thiobacillus ferrooxidans*. In agreement with the ClustalW results, endosymbiont *Burkholderia* was clearly placed closer to *A. brasilense* than to *B. vietnamiensis* TVV75, which is closer to *B. japonicum*.

Amplification of *Burkholderia nif* genes from *Gigaspora* spores. The sequences encoding the putative NifH, NifD, and NifK proteins were used to design three sets of primers for PCR experiments performed on DNA extracted from the spores of *G. margarita* and *G. rosea*. Unlike *G. margarita* isolates, *G. rosea* does not contain bacteria in its cytoplasm and therefore represents a negative control (10). The expected amplified fragments of about 760 bp (Fig. 4a), 630 bp (Fig. 4b), and 1,080 bp (Fig. 4c) were obtained from the *G. margarita* DNA. The results were further confirmed by Southern blotting experiments using as a probe the DNA of λ1NIF and λ4NIF amplified with the primers designed on the *Burkholderia nifH*, *nifD*, and *nifK* genes. By contrast no amplification product was obtained with DNA extracted from *G. rosea* (Fig. 4a to c).

To exclude the presence of contaminating bacteria on the sporal surface at the end of the sterilization procedures, the intact spores of *G. margarita* and *G. rosea* were stained with the Live/Dead *Bac*Light kit and observed in confocal microscopy as described by Bianciotto et al. (9). In both cases, the resulting spore surface was free from any bacterial contamination (data

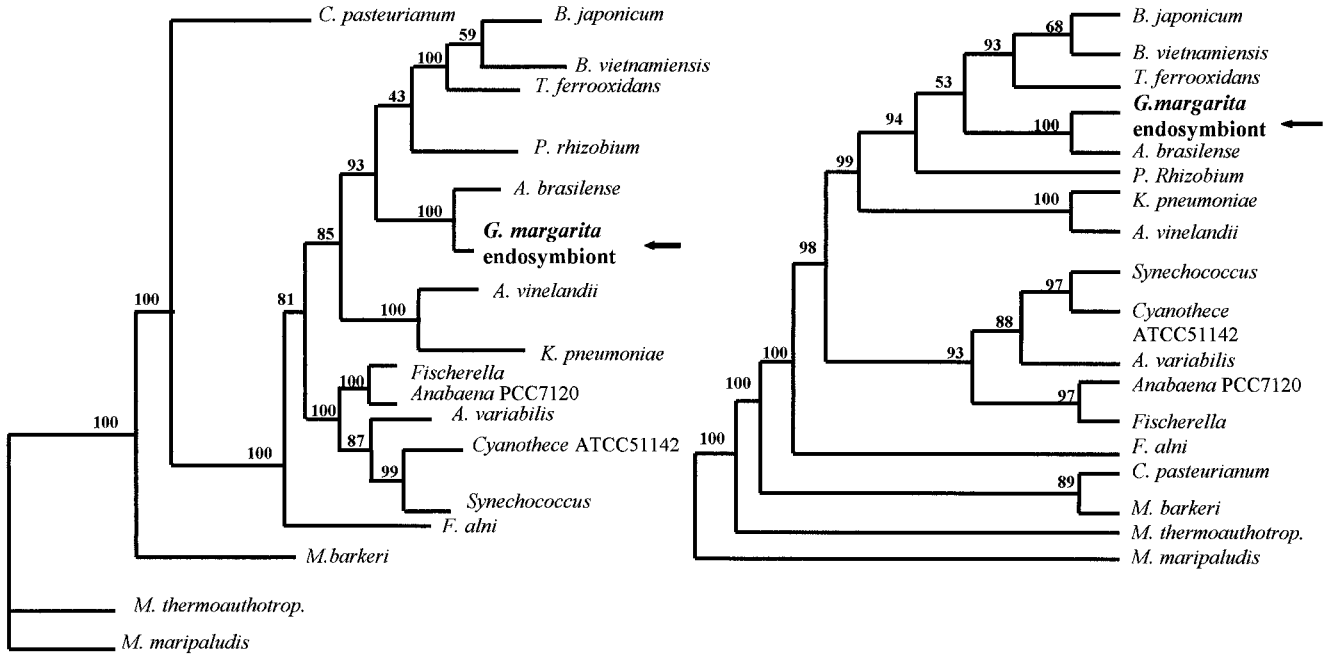

FIG. 3. Neighbor-joining (left) and maximum-parsimony (right) bootstrap consensus trees generated from the NifK alignment.

not shown). In addition, PCR experiments were carried out on the washing solution of *G. margarita* and *G. rosea* spores, using both the *Burkholderia nif* primers and the universal bacterial primers 704f and 1495r (9). As expected, universal bacterial primers amplified a DNA fragment of the expected size of about 790 bp, confirming the presence of contaminant bacteria on the surface of both of the fungal species prior to the spore sterilization protocol. No amplification product was obtained using the primers designed on the *Burkholderia nif* genes (data not shown).

RT-PCR experiments. RT-PCR experiments were performed on the RNA extracted from nongerminated and germinated spores of *G. margarita*. Using the two sets of primers nifHf/nifHr and nifDf/nifDr, an amplified product of the expected size was obtained on RNA extracted from germinating spores. No amplified product was obtained from the RNA of nongerminated spores (data not shown) or from the RT negative controls. An amplified fragment of the expected size was also obtained using the primers BLOf and BLOr, specifically designed on the endosymbiont *Burkholderia* 16S rDNA sequence (9), on the RNA of germinated and nongerminated spores (Fig. 5).

The amplified products obtained with the *nifHD* genes and 16S rDNA primers were purified and sequenced. A nucleotide sequence of about 200 bp was obtained for all PCR products, showing a 100% sequence identity to the *nifH*, *nifD*, and 16S ribosomal genes of the endosymbiont *Burkholderia*.

DISCUSSION

The present paper demonstrates that the unculturable endosymbiotic *Burkholderia* harbored by *G. margarita*, an obligate mycorrhizal fungus, contains the *nifHDK* operon in its genome. To our knowledge, this is the first *nif* operon described for a bacterium of the genus *Burkholderia*. At least the *nifH* and *nifD* genes are expressed in the germinated spores of the fungal host. This is the first evidence in favor of the hypothesis that a fungus which improves P uptake (21) might also benefit from nitrogen fixation through a symbiosis with a diazotroph.

Identification and characterization of the *nif* genes in *Burkholderia*. Unculturable bacteria may be successfully identified by using 16S rDNA isolated directly from natural samples (2, 28). Genes different from those encoding 16S or 23S rRNA, however, have rarely been investigated in unculturable microorganisms. Nevertheless, highly conserved genes, such as those encoding the nitrogenase complex (*nifH*, *nifD*, and *nifK*), can be investigated from unculturable bacteria by using total DNA extracted from a given environment and by amplifying them via PCR. Following this approach and starting from a mixed DNA preparation, Ueda et al. (44) detected 23 *nifH* sequences belonging to different bacteria.

In our experiments, *Burkholderia nif* genes were identified and characterized by using a genomic library constructed from *G. margarita* spores (45) and representative of the endosymbiont *Burkholderia* genome. Bacterium-free genomic libraries can be constructed only by following special procedures, as demonstrated by Hosny et al. (24). We were able to sequence the *nifHDK* operon from the fungal-bacterial genomic library. Analysis of a 6,413-bp DNA region revealed that it harbors genes coding for putative proteins showing a very high degree of sequence similarity with three proteins of the nitrogenase complex, NifH, NifD, and NifK. The three proteins exhibited the highest degree of sequence similarity to FeMo NifD and NifK and to Fe NifH, suggesting that the *Burkholderia* nitrogenase should require a FeMo cofactor for its function. Moreover, the presence of a strong Rho-independent transcription terminator downstream from *nifK* and the absence of se-

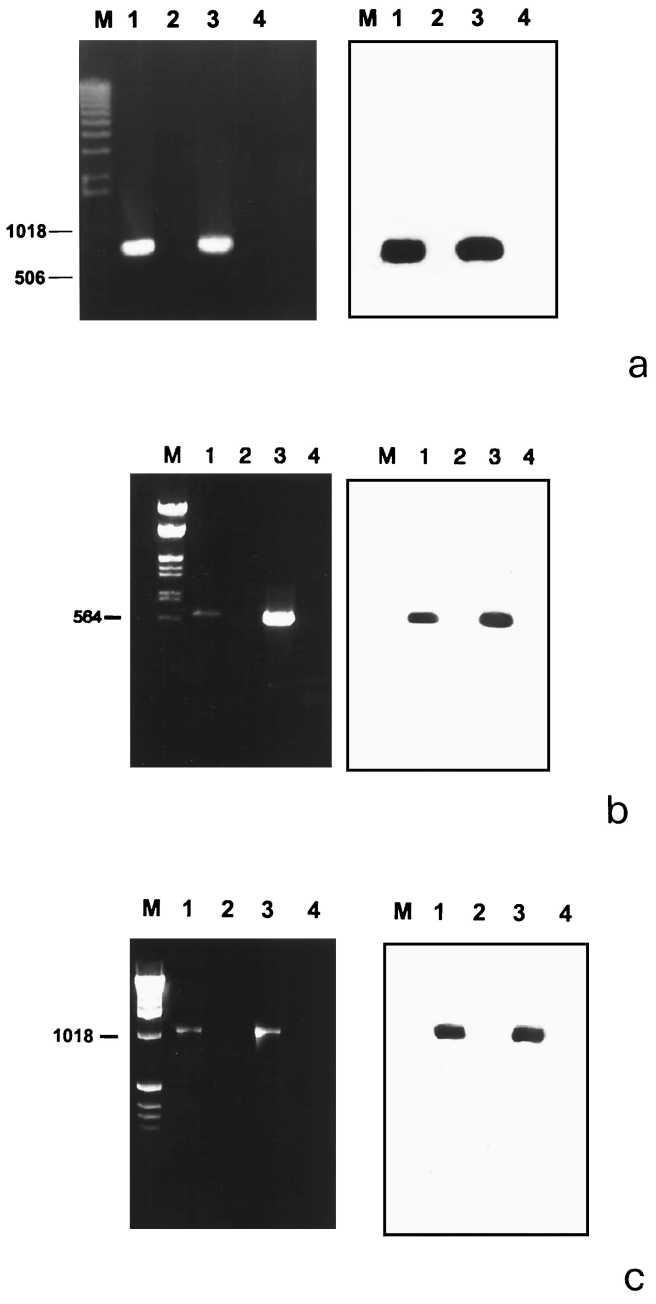

FIG. 4. Agarose gel (left) and corresponding Southern blot (right) of PCR products amplified by using primers specific for the *nifH* (a), *nifD* (b), and *nifK* (c) genes. Lane 1, DNA from the spores of *G. margarita*; lane 2, DNA from the spores of *G. rosea*; lane 3, DNA from positive Lambda DASH phage; lane 4, no DNA. DNA markers (sizes are in base pairs) are shown on the left.

quences resembling a −24 to −12 promoter in the short (34-bp) intergenic region suggested that they belong to the same transcriptional unit. The presence of two putative termination stem-loops located in the *nifH-nifD* intergenic region could be the signal for the production of *nifH*, *nifDK*, and *nifHDK* transcripts, suggesting a nitrogenase expression regulated at the level of initiation and termination of transcription (16).

The symbiont *Burkholderia* NifH, NifD, and NifK proteins

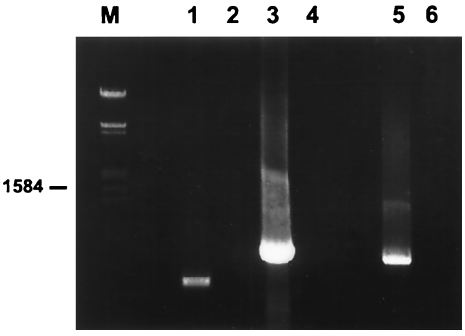

FIG. 5. Agarose gel of RT-PCR products amplified by using primers specific for the endosymbiont *Burkholderia* 16S ribosomal gene and the *nifH* and *nifD* genes. Lane 1, RNA from the germinated spores of *G. margarita* amplified with primers BLOf and BLOr; lane 2, no RT; lane 3, RNA from the germinated spores of *G. margarita* amplified with primers nifHf and nifHr; lane 4, no RT; lane 5, RNA from the germinated spores of *G. margarita* amplified with primers nifDf and nifDr; lane 6, no RT. A DNA marker (size in bp) is shown on the left.

showed the highest degree of sequence similarity to *A. brasilense*, a nitrogen-fixing rhizosphere bacterium often associated with maize (16, 35) and with diazotrophs involved in symbiotic relationships with legume plants, whereas the lowest degree of sequence similarity was shared with free-living bacterial and archaeal diazotrophs (31). The endosymbiont *Burkholderia* is more related to *A. brasilense* than to *B. vietnamiensis*. The apparent paradox of *nif* gene sequence similarity between unrelated species may be explained by horizontal gene transfer. Hurek et al. (25) found a high divergence of the *nifH* gene sequence in the genus *Azoarcus*. This situation is responsible for the deviation from the 16S ribosomal gene-based phylogeny. The divergence between the two *Burkholderia* NifK sequences might also be the consequence of a horizontal gene transfer which occurred between microorganisms belonging to distant taxa, as already demonstrated (4, 37).

***nifHDK* genes belong to the genome of the endosymbiont *Burkholderia*.** Southern blotting and PCR experiments on spores harboring (*G. margarita*) or not harboring (*G. rosea*) bacteria using specific primers designed on the *Burkholderia nifHDK* sequences provide evidence that the *nifHDK* genes characterized in this work actually belong to the genome of the *G. margarita* endosymbiont. The presence or absence of endosymbiotic bacteria in the two species was confirmed by morphological observations as well as by PCR experiments with universal and specific primers (9, 10).

It is known that rhizobacteria colonize the rhizosphere of AM plants (3) and may adhere to the surface of AM fungi during their extraradical phase (8). An obvious concern is that the DNA sequences may belong to such bacteria. However, these bacteria isolated from the rhizosphere, irrespective of their origin (endogenous or inoculated), have never been demonstrated to colonize the cytoplasm of living spores and hyphae. They are very different from the intracellular *Burkholderia* (29). In addition, they are easily detached from the sporal surface according to our cleaning procedures. By contrast, the presence of *Burkholderia* in successive fungal generations in vitro, as well as in situ hybridization experiments with the specific intracellular *Burkholderia* primers (10), confirm that they are an integral part of the fungal system.

Even if our results do not show directly that the sequences are derived from endosymbiotic bacteria, they surely show that bacteria are internal to the spores and that related fungi that lack bacteria lack the sequences. Taken as a whole, the results, coupled with the fact that *nif* genes are unknown for eukaryotes, strongly suggest that the *nifHDK* genes belong to the genome of the endosymbiotic *Burkholderia*.

Conclusion. This is the first demonstration that an AM fungus contains symbiotic intracellular bacteria possessing *nif* genes which are expressed at least during the germination of the spores. The significance of this finding (the potential capacity of a mycorrhizal fungus to fix nitrogen through a specific bacterial population) lies in the interest of such a combination for sustainable agriculture. The natural system consisting of mycorrhizal fungi and nitrogen-fixing bacteria may be an excellent "biofertilizer" with which to expand crop production and minimize the negative impact of chemical fertilizers.

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