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FEMS Microbiology Ecology 25 (1998) 277–286

FEMS MICROBIOLOGY ECOLOGY

Slow-growing and oligotrophic soil bacteria phylogenetically close to *Bradyrhizobium japonicum*

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Received 2 June 1997; revised 1 October 1997; accepted 17 November 1997

Abstract

Eleven isolates of slow-growing oligotrophic bacteria from grassland soil were found to be closely related by partial 16S rRNA sequence similarity and many common taxonomic traits. Analysis of full 16S rRNA gene sequences of four representative isolates and *Agromonas oligotrophica* S58 indicated that they were more closely related to *Bradyrhizobium japonicum*, a symbiotic nitrogen-fixing bacterium, (similarity values: 98.1–98.8%) than other strains such as *Bradyrhizobium elkanii*, *Nitrobacter* spp., *Rhodopseudomonas palustris*, and *Afipia* spp. This result was supported by analysis of phenotypic traits and DNA-DNA hybridization analysis. No strain showed hybridization to *nodD*$_1$*YABC* of *B. japonicum*, and only strain G14130 exhibited hybridization to *nifDK*- and *hupSL*-specific DNA. These latter genotypes are involved in the phenotypes of nodulation and nitrogen fixation under microaerobic conditions. These results suggest that the isolates possess a unique phylogenetic position since they are closely related to *B. japonicum* though they do not have characteristics of symbiotic nitrogen fixation. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: Slow-growing bacteria; Oligotroph; Nitrogen fixation; *Bradyrhizobium japonicum*; *Agromonas oligotrophica*

1. Introduction

Oligotrophic bacteria are widely distributed in terrestrial and aquatic environments [1–3]. Many halo- and organo-sensitive bacteria have been isolated from soils, using 100-fold diluted nutrient broth (DNB) medium [2,4]. These isolates would not grow in full strength nutrient broth (NB), and were designated DNB bacteria [2]. The taxonomic position of such bacteria has not been determined due to their slow growth rates [5] and sensitivity to organic nutrients [4]. Ohta and Hattori [2,6] characterized the physiological, morphological, and chemical features of nitrogen-fixing oligotrophic bacteria from paddy soil, and proposed a new genus, *Agromonas*, with a single species *A. oligotrophica*.

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PII S0168-6496(98)00009-9

Previously, a collection of bacterial isolates from grassland soil was obtained using the colony-forming curves (CFCs) [7], by which the time courses of the colony formation process of soil bacteria on agar plates could be simulated. The bacteria were isolated from colonies that appeared during an incubation period corresponding to each component curve of the CFCs, and were placed in groups designated I, II, III or IV based on the order of colonization [5]. Group IV represented the slowest growers, about half of which were DNB bacteria [5]. The phylogenetic relatedness of the DNB bacteria belonging to group IV has not yet been determined. The objective of this work was to examine the taxonomic position of such slow-growing oligotrophic soil bacteria by analysis of phenotypic traits and molecular phylogeny.

2. Materials and methods

2.1. Bacterial strains and growth media

Eleven strains designated G14003, G14109, G14113, G14115, G14119, G14121, G14123, G14124, G14127, G14130 and G14133 were isolated in September 1987 from grassland soil cultivating orchard grass [5]. The characteristics of the soil were pH (H_2O) 4.5, water content 35%, and organic matter content 9.0% [5]. Growth of all strains was severely suppressed in NB, low-salt NB and low-salt TY, but not in DNB. *A. oligotrophica* S58 (JCM 1494) was used as a well recognized oligotroph from paddy fields [6]. *Bradyrhizobium japonicum* USDA110 was used as a reference strain.

Full strength NB contained 1% (w/v) peptone (Kyokuto Seiyaku Co., Tokyo), 1% (w/v) meat extract (Kyokuto Seiyaku Co., Tokyo) and 1% (w/v) sodium chloride (pH 7.0–7.2). Low-salt NB contained 1% (w/v) peptone (Kyokuto Seiyaku Co., Tokyo), 1% (w/v) meat extract (Kyokuto Seiyaku Co., Tokyo) and 0.01% (w/v) sodium chloride (pH 7.0–7.2). Low-salt TY medium was composed of 1% (w/v) Tryptone (Difco), 0.5% (w/v) yeast extract (Difco) and 0.01% (w/v) sodium chloride. DNB was prepared from a 100-fold dilution of NB with distilled water.

2.2. Preparation of total DNA

Bacteria were cultured at 27°C in DNB medium supplemented with 0.01% of yeast extract, and were harvested by centrifugation followed by one wash in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA [pH 8.0]). Cells were then suspended in solution A (0.3 M sucrose, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 0.2 mg ml^{-1} lysozyme, and 3 mg ml^{-1} RNase A) and incubated at 37°C for 30 min. A 1/10 volume of 10% SDS was added and the suspension was further incubated at 50°C for 10 min. This was followed by the addition of a 7/30 volume of 5 M NaCl and an 8/30 volume of 5% (w/v) CTAB in 0.5 M NaCl and incubation at 65°C for 15 min. After phenol and chloroform extractions, DNAs were precipitated by ethanol and sodium acetate, rinsed with 70% ethanol, air dried, and dissolved in TE buffer.

2.3. Sequencing of 16S rRNA gene

Partial 16S rRNA sequencing has been previously described [8]. To determine sequences of extended 16S rRNA gene regions, corresponding to the region of nucleotides 28–1521 in the *Escherichia coli* sequence, polymerase chain reactions (PCR) were carried out using the primers 27f and 1522r as previously described [9]. PCR products were cloned into the pT7blue vector (Novagen), transformed into *E. coli* XL1blue, and sequenced. The determined nucleotide sequences appear in the DDBJ/Genbank/EMBL nucleotide databases under the following accession numbers: D78366 (*A. oligotrophica* S58), AB003458 (G14003), AB003460 (G14109), AB003461 (G14113), AB003462 (G14115), AB003463 (G14119), AB003464 (G14121), AB003465 (G14123), AB003466 (G14124), AB003459 (G14127), AB003457 (G14130), and AB003467 (G14133). A phylogenetic tree was constructed using Clustal W [10] by the neighbor-joining method.

2.4. Cellular morphologies

Exponential phase cells cultured in DNB medium were observed by phase contrast microscopy and scanning electron microscopy (SEM). Samples for

SEM were prepared as follows. Cells were harvested and adsorbed onto a polycarbonate (HTTP02500, Millipore) isopore membrane and washed once with 7 ml of DNB medium by vacuum filtration. Cells were dehydrated gradually in a series of water-ethanol gradient solutions, and then dried by a critical dryer before coating with platinum. Cellular development was observed by use of a microculture method [6].

2.5. Taxonomic characterizations

Unless otherwise indicated, all tests were performed at 27°C. Inocula were prepared by stationary culture in DNB liquid medium without aeration. Cultures were Gram-stained by Huker's modification [11] at both exponential and stationary phases. Motility of cells grown at 20°C was determined by the hanging drop method and flagellar staining by Toda's method [11]. Poly-β-hydroxybutyrate granules were identified by staining with Sudan black B [11]. Catalase activity was determined by gas production by colonies when flooded with a 3% hydrogen peroxide solution [11]. Filter papers for cytochrome oxidase tests (Nissui, Japan) were used to determine oxidase activity. Acid production from glucose was examined after 15 days cultivation on two solid media, Hugh-Leifson medium [12] containing 0.1% yeast extract, and GDNBY medium (0.5% glucose, 0.01% peptone, 0.0 1% beef extract, 0.005% NaCl, 0.03% KH_2PO_4, 0.07% K_2HPO_4, 0.1% yeast extracts, and 1.5% agar, pH 7.0) containing 0.008% bromothymol blue (BTB). As a control, media without glucose were used. The reduction of nitrate was determined by the production of nitrite in DNB semi-solid medium which contained 0.1% KNO_3, 0.1% yeast extract and 0.017% agar (nitrite was detected by Griess-Ilosvay reagent [9]), and denitrification to dinitrogen gas was determined by bubble formation in this semi-solid medium. NaCl requirement was tested by culturing cells on the DNB solid medium containing 0.1% or 20% NaCl.

Nitrogen-fixing activity was examined by acetylene reduction test in DNB semi-solid medium which contained 0.5% glucose and 0.017% agar [9]. After growing for 7 days, 10% acetylene gas was added to tube and the pressure in the tube was adjusted to 1 atm. Ethylene and acetylene contents were analyzed by gas chromatography with Porapack Q-Porapack N column (Shimadzu, Japan). Nodule formation was tested using siratro (*Macroptilium atropurpureum*) as a host plant grown on an agar slant in test tube [9]. The ability to hydrolyze starch, casein, and cellulose was determined by growing organisms on DNB solid media which contained 0.1% starch, 0.05% cellulose powder, or 0.45% milk casein, respectively, and observing the appearance of zones of clearing around colonies after 30 days of inoculation. G+C contents of DNAs were determined by the method of Mesbah et al. [13].

2.6. Quinone analysis

The organisms were cultured in 1 l of DNB liquid medium containing 0.1% yeast extracts, harvested by centrifugation, washed once and suspended in distilled water, and stored at −20°C in plastic tubes. Frozen suspensions were then dried and suspended in a chloroform-methanol solution (2:1, v/v). The suspensions were shaken for 2 h, dried under vacuum at room temperature and the remaining pellets dissolved in 100 ml of acetone. These solutions were then separated by thin-layer chromatography in hexane-diethylether solution (85:15, v/v). After separation, the bands were viewed by UV illumination (254 nm) and recovered by scraping. Isoprenoid quinones were extracted from the silica gel powder with methanol and dried under vacuum. After dissolving the dried materials in 200 ml of acetone, the samples were analyzed by HPLC LC10AD (Shimadzu, Japan) with a Zorpac ODS (Zorpac) column.

2.7. Southern hybridization

Genomic DNAs, digested with the appropriate restriction enzymes, were electrophoresed on TBE agarose gels (0.8%) [14], and transferred and fixed onto nylon membranes [14]. The DNA probes were labelled with the DIG labelling and detection kit (Boehringer Mannheim). DNA hybridization was carried out at 60°C in a hybridization buffer (5×SSC, 1% blocking reagent, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 100 μg ml^{-1} denatured salmon sperm DNA and 15 ng denatured labelled DNA). After hybridization, the membranes were washed twice with 2×SSC-0.1% SDS solution at room tem-

Table 1
Levels of 16S rRNA gene sequence similarities for the DNB bacteria and their phylogenetic neighbors

Strain	Sequence similarity (%)														
	G14003	G12109	G14127	G14130	*B. japonicum*	*B. denitrificans*	*A. oligotrophica*	*R. palustris*	*N. winogradski*	*N. hambrigensis*	*A. felis*	*A. clevelandensis*	*B. elkanii*	*B. indica*	*A. tumefaciens*
G14109	99.8														
G14127	99.7	99.7													
G14130	99.3	99.5	99.2												
Bradyrhizobium japonicum	98.8	98.8	98.8	98.2											
Blastobacter denitrificans	98.7	98.8	98.6	98.2	98.1										
Agromonas oligotrophica	98.6	98.6	98.7	98.1	98.3	99.3									
Rhodopseudomonas palustris	97.3	97.3	97.2	96.8	96.8	96.5	96.7								
Nitrobacter winogradski	97.2	97.2	97.3	96.7	97.2	96.9	97.1	97.0							
Nitrobacter hambrigensis	97.2	97.2	97.3	96.7	97.3	96.7	97.2	97.4	98.6						
Afipia felis	96.6	96.6	96.6	96.5	96.5	96.3	96.6	96.3	96.9	96.5					
Afipia clevelandensis	97.2	97.2	97.2	96.7	96.9	96.9	97.1	96.6	97.2	96.8	98.6				
Bradyrhizobium elkanii	96.4	96.4	96.4	95.9	97.6	96.2	96.6	95.8	95.3	96.1	94.7	95.1			
Beijerinckia indica	90.2	90.2	90.2	90.2	90.0	89.8	89.9	89.7	90.0	89.9	90.5	90.2	89.9		
Agrobacterium tumefaciens	87.3	87.3	87.3	87.1	87.1	87.6	87.5	87.7	87.2	87.0	88.0	87.9	87.0	89.2	

perature and twice at 60°C. A 3.9-kb *Hin*dIII fragment of pRjUT10 [15,16], a 3.5-kb *Bgl*II fragment of pRJ676 [17] and a 2.2-kb *Sst*I fragment of pHU52 [18] were used as probes for *nodD1YABC*, *nifDK* and *hupSL*, respectively. Probes were derived from *B. japonicum* strains USDA110 (*nodD1YABC* and *nifDK*) and USDA122DES (*hupSL*).

2.8. DNA-DNA hybridization

Genomic DNAs were prepared as described above with the addition of proteinase K after the first phenol-chloroform extraction. DNA-DNA hybridization was carried out at 50°C for 8 h in a hybridization buffer (2×SSC, 5×Denhardt solution, 50% formamide, 200 μg ml^{-1} salmon sperm DNA and biotinylated DNA probes) and analyzed fluorometrically in microtiter plates [19–21].

3. Results

3.1. 16S rRNA gene sequencing

In order to determine the phylogenetic position of the 11 DNB bacteria, the partial sequences (436 bp) of 16S rRNA genes (28–518 in *E. coli* numbering) were determined. All isolates were very similar, and seven isolates (G14003, G14109, G14121, G14123, G14124, G14127, and G14133) were perfectly identical. Sequences of the other strains, G14113, G14119, G14115 and G14130, showed 1–4 nucleotide substitutions when compared to the other seven strains. These substitutions were located in different positions, in a total of nine sites (data not shown). All the 16S rRNA partial sequences were very similar to those of *B. japonicum* and *Blastobacter denitrificans*.

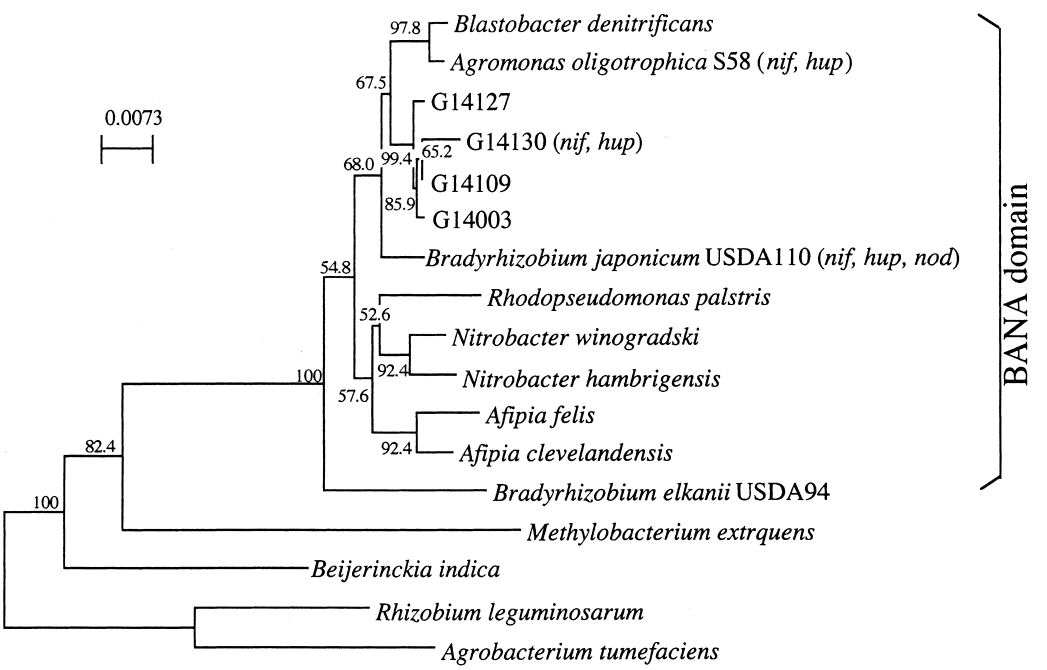

Fig. 1. Phylogenetic relationships of DNB bacteria isolated from glassland soil and *A. oligotrophica* S58 based on near full length sequence similarities of 16S rRNA genes. The phylogenetic tree was constructed using the neighbor-joining method. Bootstrap values are shown at nodes. The scale bar indicates substitutions per site. The name BANA domain has been proposed for the cluster including *Bradyrhizobium, Agromonas, Nitrobacter* and *Afipia* (see text).

1.4-kb regions of 16S rRNA genes were sequenced (28–1522 in *E. coli* numbering) in four representatives of the DNB bacteria (G14003, G14109, G14127 and G14130) and *Agromonas oligotrophica* S58. Table 1 shows the level of 16S rRNA similarity for four strains of DNB bacteria, *A. oligotrophica* S58, and their phylogenetic neighbors. Fig. 1 shows their phylogenetic relationships based on the sequences. It was suggested that the four DNB bacteria and *A. oligotrophica* S58 were closely related to *B. japonicum* and *B. denitrificans*.

3.2. Phenotypic characteristics

Table 2 summarizes morphological, physical and chemical characteristics of the 11 DNB bacteria, *A. oligotrophica*, *B. japonicum* and *B. denitrificans*.

Microscopic observations of the 11 DNB bacteria during the exponential phase indicated that they were irregular rods and rarely branched (Fig. 2). The cellular development of G14127 and G14130 was observed using phase contrast microscopy by a microculture method [6]. Both strains showed polar growth (budding) (data not shown), which was also observed in *A. oligotrophica* [6] and *B. denitrificans* [22].

All DNB bacteria tested were Gram-negative, motile with one subpolar flagella, accumulated poly-β-hydroxybutyrate (PHB) intracellularly, and formed white colonies. They did not produce acid from glucose. All were catalase- and oxidase-positive, and reduced nitrate to nitrite in semi-solid medium, but did not produce bubbles in the medium. These isolates did not hydrolyze starch, casein and cellulose and did not utilize methanol. With respect to dinitrogen fixation, which was tested by the acetylene reduction method under microaerobic conditions, 10 of the 11 DNB bacteria were negative with only one strain G14130 and *A. oligotrophica* S58 exhibiting acetylene reduction. In contrast to *B. japonicum* USDA110 (the other strains were not examined), the four strains tested (G14003, G14109, 14127 and

Table 2
Characteristics of the 11 DNB bacteria, *B. japonicum, A. oligotrophica*, and *B. denitrificans*

Characteristic	The 11 DNB bacteria	*B. japonicum*[a]	*A. oligotrophica*[b]	*B. denitrificans*[c]
Gram staining	−	−	−	−
Cell shape	rods	rods	rods	rods
Budding	+[d]		+	+
Motility	+	+	+	+
Flagellar arrangement	subpolar	polar	polar	subpolar
Colony color	white	white	white	white
Fluorescent pigment	−	−	−	
Catalase	+	+	+	+
Oxidase	+	+	+	+
Acid from glucose	−	−	−	+
Poly-β-hydroxybutyrate accumulation	+	+	+	
Growth at 60°C or higher	−	−	−	
Reduction from nitrate to nitrite	+	+	+	+
Denitrification	−	−	−	+
Requirement of at least 0.1% NaCl	−	−	−	
Growth with 20% or more NaCl	−	−	−	
Methanol utilization	−		−	+
Nodulation	−[e]	+	−	
Fixation of N_2 gas in vitro	One strain G14130: + The other 10 strains: −	+ or −	+	
Major quinone	Q10[f]	Q10	Q10	
GC content (mol%)	62.7–63.5		65.8	64.5

[a][30]; [b][6]; [c][22].
[d]G14127 and G14130 were tested.
[e]G14003, G14109, G14127 and G14130 were tested.
[f]G14003, G14121, G14127 and G14130 were tested.

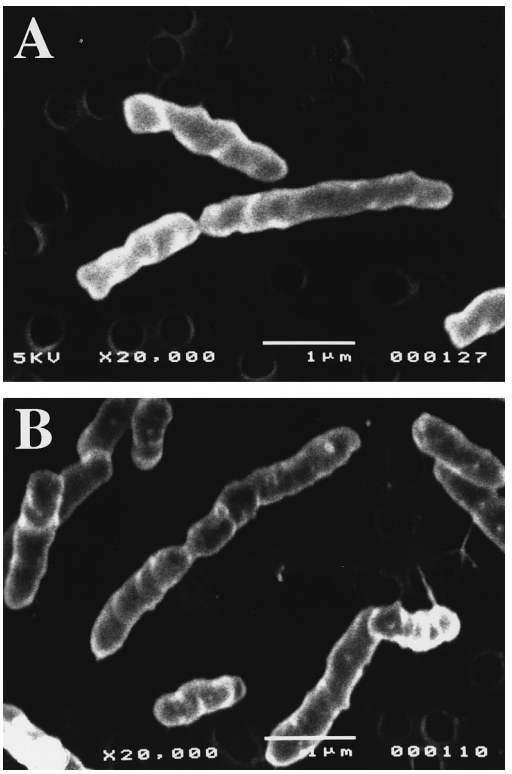

Fig. 2. Cellular morphologies of DNB bacteria G14127 (A) and *B. japonicum* USDA110 (B) as shown by scanning electron microscopy.

G14130) did not nodulate siratro. Four strains, G14003, G14121, G14127 and G14130, had ubiquinone Q10 as the major quinone. The mol% G+C contents of the 11 strains were between 62.7 and 63.5.

From the above results, the 11 DNB bacteria and *A. oligotrophica* were found to be generally similar to *B. japonicum*, but different from *B. denitrificans* in terms of acid production from glucose, denitrification and methanol utilization. If nitrogen fixation and nodulation are scored as phenotypic traits for genus identification, strain G14130 fell into the genus *Agromonas*, while the remaining DNB bacteria belonged to neither *Bradyrhizobium* nor *Agromonas*.

3.3. Hybridization to $nodD_1YABC$, *nifDK*, and *hupSL*

Total DNA digests of the DNB bacteria and *A. oligotrophica* S58 were analyzed by Southern hybridization with a DNA fragment containing $nodD_1YABC$, *nifDK*, or *hupSL* from *B. japonicum* (Fig. 3). The *nodD* gene encodes a transcriptional activator of nodulation genes, and the *nodABC* genes are involved in the synthesis of *N*-acylated chitooligosaccharides, core structures of Nod factor molecules. These genes have been found in all rhizobia

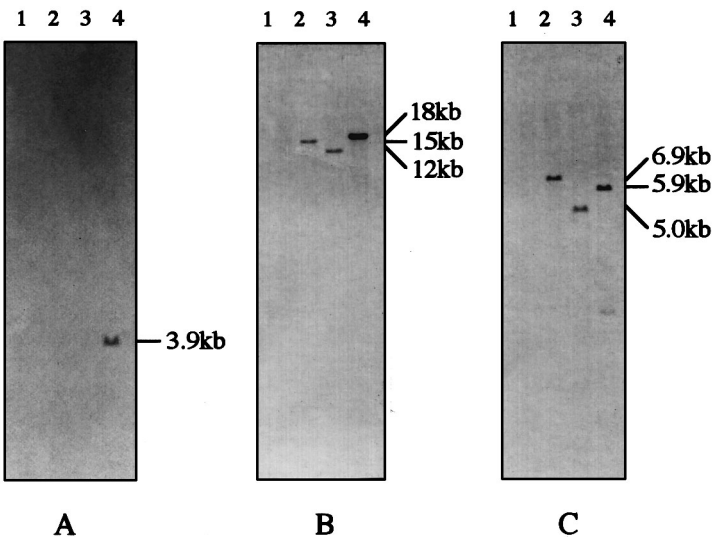

Fig. 3. Southern hybridization of total DNA of G14127 (lane 1), G14130 (lane 2), *A. oligotrophica* S58 (lane 3) and *B. japonicum* USDA110 (lane 4) digested with *Hin*dIII (A and C) and *Bam*HI (B) to $nodD_1YABC$ (A), *nifDK* (B) and *hupSL* (C) genes from *B. japonicum*.

studied so far and are called common *nod* genes [23]. The *nifD* and *nifK* genes encode α and β subunits of the FeMo protein of nitrogenase, the enzyme responsible for the reduction of atmospheric dinitrogen to ammonia [24]. The *hupS* and *hupL* genes encode the small and large subunits of an uptake hydrogenase, which recycles electrons that would otherwise be lost in the production of hydrogen in the nitrogenase-catalyzed reaction [25]. The 11 DNB bacteria and *A. oligotrophica* did hybridize to $nodD_1YABC$ while *B. japonicum* USDA110 showed a positive signal. One strain, G14130, and *A. oligotrophica* S58 showed *nifDK*- and *hupSL*-specific hybridization signals, while the rest of the DNB bacteria did not. These positive signals coincided with the phenotype of nitrogen fixation. Strain G14130 exclusively reduced acetylene under microaerobic conditions (Table 2), and *A. oligotrophica* showed nitrogen-fixing activity under 1% O_2 [6].

3.4. DNA-DNA hybridization

Levels of similarities based on total DNA-DNA hybridization are shown in Table 3. When total DNA of G14127 was used as probe, similarity values to G14109, G14121, G14124 and G14133 were more than 70%. When the DNA of G14130 was used as a probe, similarity values to the other 10 DNB bacteria ranged from 30% to 40%.

Using the total DNAs of *B. japonicum* USDA110 and *A. oligotrophica* S58 as probes, similarity values to the 11 DNB bacteria ranged from 26% to 40% and from 11% to 20%, respectively. These were relatively lower than expected from the comparisons of the 16S rRNA sequences and the phenotypic traits. Nevertheless, DNA-DNA hybridization analysis demonstrated that the DNB bacteria tested were more closely related to *B. japonicum* USDA110 than *A. oligotrophica* S58.

4. Discussion

In this study, we polyphasically characterized the 11 DNB bacteria isolated from grassland soil and *A. oligotrophica* S58 by 16S rRNA gene sequence similarity, DNA-DNA hybridization and other phenotypic traits. Sequence analysis of the 16S rRNA gene demonstrated that the DNB bacteria and *A. oligotrophica* S58 showed high similarities (98.3–99.3%, Table 1) to each other. Moreover, they clustered with *B. japonicum* and *B. denitrificans*. Young [26] demonstrated that the phylogenetic cluster formed by all bradyrhizobia, surprisingly, includes a number of bacteria that are not rhizobia: *Rhodopseudomonas*, *Nitrobacter*, and *Afipia*. Recently, Hattori [27] proposed the name BANA (the first letters of *Bradyrhizobium, Agromonas, Nitrobacter* and *Afipia*) domain for this cluster (Fig. 1), since the BANA domain includes important bacteria of terrestrial origin. The soil DNB bacteria tested in this study were more closely related to *B. japonicum* than those three genera. This phylogenetic relatedness was supported by various phenotypic traits (Table 2).

The close phylogenetic relationship among *B. japonicum*, *A. oligotrophica*, *B. denitrificans* and the 11

Table 3
Levels of DNA similarity for the 11 DNB bacteria, *B. japonicum* USDA110 and *A. oligotrophica* S58

Source of biotin-labelled DNA	Similarity (%) to unlabelled DNA from:												
	G14003	G14109	G14113	G14115	G14119	G14121	G14123	G14124	G14127	G14130	G14133	*B. japonicum* USDA110	*A. oligotrophica* S58
G14127	54	80	53	37	51	120	60	92	100	61	99	46	20
G14130	30	30	44	31	41	46	49	30	32	100	35	38	9
Bradyrhizobium japonicum USDA110	26	26	37	36	33	38	35	36	34	40	35	100	2
Agromonas oligotrophica S58	16	11	14	16	14	20	16	16	16	20	18	19	100

DNB bacteria in the BANA domain was also supported by common features such as oligotrophy and slow growth rates. Crist et al. [28] demonstrated that *B. japonicum* cells remained viable in purified water for 1 year or longer, and Ozawa and Doi [29] reported that the competitive nodulation ability of *B. japonicum* was increased by oligotrophic growth in purified water. *Blastobacter* spp. have been isolated from an oligotrophic environment, freshwater habitats [22,30].

The close relationship among the soil DNB bacteria and *B. japonicum* prompted us to examine whether the soil DNB bacteria harbored genes responsible for symbiotic nitrogen fixation similar to *B. japonicum*. No strains hybridized to the *nodD1YABC* genes from *B. japonicum*. Most of the DNB bacteria did not exhibit the *nifDK*- and *hupSL*-specific hybridization signals, while one DNB strain, G14130, and *A. oligotrophica* S58 did hybridize to the *nifDK* and *hupSL* genes from *B. japonicum*.

Recently, the presence of non-symbiotic rhizobia and the transfer of chromosomal symbiotic genes has been demonstrated to occur in the environment. Ronson et al. [31] isolated genetically diverse bacteria in nodules of *Lotus corniculatus* at a field site devoid of natural rhizobia. These bacteria had regions of DNA identical to those encoding the symbiotic functions of *Rhizobium loti* ICMP3153, the inoculant strain which was used at the site. Furthermore, they isolated non-symbiotic rhizobial strains [32]. The diverse nodulating rhizobial strains and the non-symbiotic rhizobial strains exhibited 27–39% total DNA similarity to the inoculant strain. Consequently, they suggested that the nodulation genes which were located on the chromosome might be laterally transferred to their phylogenetic neighbors defective in symbiotic genes in the field environment [29,31].

The 11 DNB bacteria exhibited total DNA similarities of 26–40% with *B. japonicum* USDA110 (Table 3), the levels of which corresponded to the similarities among diverse symbiotic and non-symbiotic *R. loti* isolates [31,32], and also ranged within the natural diversity of the genus *Bradyrhizobium* [33]. When 16S rRNA phylogenetic trees were constructed from all published data of *Bradyrhizobium* and the four DNB bacteria (G14003, G14109, G14127, and G14130), the *Bradyrhizobium* cluster included the four DNB bacteria (data not shown). Provided that chromosomal symbiotic genes of *B. japonicum* are horizontally transferred, it is possible that the soil DNB bacteria might become its recipients as non-symbiotic neighbors in the environment.

References

[1] Hirsh, P., Bernhard, M., Cohen, S.S., Ensign, J.C., Jannasch, H.W., Koch, A.L., Marshall, K.C., Matin, A., Poindexter, J.S., Rittenberg, S.C., Smith, D.C. and Veldkamp, H. (1979) Life under conditions of low nutrient concentrations, Group report. In: Strategies of Microbial Life in Extreme Environments (Shilo, M., Ed.), pp. 357–372. Verlag Chemie, Weinheim.

[2] Ohta, H. and Hattori, T. (1980) Bacteria sensitive to nutrient broth medium in terrestrial environments. Soil Sci. Plant Nutr. 26, 99–107.

[3] Ishida, Y. and Kodota, H. (1981) Growth patterns and substrate requirements of naturally occurring obligate oligotrophs. Microb. Ecol. 7, 123–130.

[4] Hattori, R. and Hattori, T. (1980) Sensitivity to salts and organic compounds of soil bacteria isolated on diluted media. J. Gen. Appl. Microbiol. 26, 1–14.

[5] Kasahara, Y. and Hattori, T. (1991) Analysis of bacterial populations in a grassland soil according to rates of development on solid media. FEMS Microbiol. Ecol. 86, 95–102.

[6] Ohta, H. and Hattori, T. (1983) *Agromonas oligotrophica* gen. nov. sp. nov. a nitrogen-fixing oligotrophic bacterium. Antonie van Leewenhoek 49, 429–446.

[7] Hashimoto, T. and Hattori, T. (1989) Grouping of soil bacteria by analysis of colony formation on agar plates. Biol. Fertil. Soils 7, 198–2018.

[8] Mitsui, H., Gorlach, K., Lee, H., Hattori, R. and Hattori, T. (1997) Incubation time and media requirements of culturable bacteria from different phylogenetic groups. J. Microbiol. Methods 30, 103–110.

[9] Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R. (1994) Methods for General and Molecular Bacteriology. American Society of Microbiology, Washington, DC.

[10] Saitoh, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol.4, 406–425.

[11] Hasegawa, T. (1985) Classification and Identification of Micro-organisms (in Japanese). Gakkai-syuppan-senta, Japan.

[12] Hugh, R. and Leifson, E. (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J. Bacteriol. 66, 22–26.

[13] Mesbah, M., Premachandran, U. and Whiteman, W.B. (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. Int. J. Syst. Bacteriol. 39, 159–167.

[14] Irvin, N., Ford, N., Nojan, C., Ferguson, M. and Ockler, M.

(1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[15] Russel, P., Schell, M.G., Nelson, K.K., Halverson, L.J., Sirotkin, K.M. and Stacey, G. (1985) Isolation and characterization of the DNA region encode nodulation functions in *Bradyrhizobium japonicum*. J. Bacteriol. 163, 1301–1308.

[16] Nieuwkoop, A.J., Banfalvi, Z., Deshate, N., Gerhold, D., Schell, M., Sirotkin, K. and Stacey, G. (1987) A locus encoding host range is linked to the common nodulation genes of *Bradyrhizobium japonicum*. J. Bacteriol. 169, 2631–2638.

[17] Hennecke, H. (1981) Recombinant plasmids carrying nitrogen fixation genes from *Rhizobium japonicum*. Nature 291, 354–355.

[18] Sayavedra-Soto, L.A., Powell, G.K., Evans, H.J. and Morris, R.O. (1988) Nucleotide sequence of genetic loci encoding subunits of *Bradyrhizobium japonicum* uptake hydrogenase. Proc. Natl. Acad. Sci. USA 85, 8395–8399.

[19] Ezaki, T., Hashimoto, Y. and Yabuuti, E. (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. 39, 224–229.

[20] Ezaki, T., Hashimoto, Y., Yamamoto, H., Lucida, M.L., Liu, S.L., Kusunoki, S., Asano, K. and Yabuuti, E. (1990) Evaluation of the microplate hybridization method for rapid identification of *Legionella* species. Eur. J. Clin. Microbiol. Infect. Dis. 9, 213–217.

[21] Forster, A.C., McInnes, J.L., Skingle, D.C. and Symons, R.H. (1985) Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. Nucleic Acids Res. 13, 745–761.

[22] Hirsh, P. and Muller, M. (1985) *Blastobacter aggregatus* sp. nov., *Blastobacter capsulatus* sp. nov., and *Blastobacter denitrificans* sp. nov., new budding bacteria from freshwater habitats. Syst. Appl. Microbiol. 6, 281–286.

[23] Rhijn, P. and Vanderleyden, J. (1995) The Rhizobium-plant symbiosis. Microbiol. Rev. 59, 124–142.

[24] Fischer, H.-M. (1994) Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. 58, 352–386.

[25] Evans, H.J., Harker, A.R., Papen, H., Russell, S.A., Hanus, F.J. and Zuber, M. (1987) Physiology, biochemistry, and genetics of the uptake hydrogenase in rhizobia. Annu. Rev. Microbiol. 41, 335–361.

[26] Young, J.P.W. (1996) Phylogeny and taxonomy of rhizobia. Plant Soil 186, 45–52.

[27] Hattori, T. (1996) Advance in soil microbial ecology and biodiversity. In: Proceedings of the Eighth International Congress for Culture Collections (Samson, R.A., Stalper, J.A., Mei, D. Stouthamer, A.H., Eds.), pp. 118–122.

[28] Crist, D.K., Wyza, R.E., Mills, K.K., Bauer, W.D. and Evans, W.R. (1984) Preservation of *Rhizobium* viability and symbiotic infectivity by suspension in water. Appl. Environ. Microbiol. 47, 895–900.

[29] Ozawa, T. and Doi, R. (1996) Increase in the competitive nodulation ability of *Bradyrhizobium japonicum* strains grown in purified water. Microbes Environ. 11, 87–90.

[30] Hugenholtz, P., Stackebrandt, E. and Fuerst, J.A. (1994) A phylogenetic analysis of the genus *Blastobacter* with a view to its future reclassification. Syst. Appl. Microbiol. 17, 51–57.

[31] Sullivan, J.T., Patrick, H.N., Lowther, W.L., Scott, D.B. and Ronson, C.W. (1995) Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. Proc. Natl. Acad. Sci. USA 92, 8985–8989.

[32] Sullivan, J.T., Eardly, B.D., Berkum, P.V. and Ronson, C.W. (1996) Four unnamed species of nonsymbiotic rhizobia isolated from rhizosphere of *Lotus corniculatus*. Appl. Environ. Microbiol. 62, 2818–2825.

[33] Hollis, A.B., Kloos, W.E. and Elkan, G.H. (1981) DNA: DNA hybridization studies of *Rhizobium japonicum* and related Rhizobiaceae. J. Gen. Microbiol. 123, 215–222.