The Journal of Microbiology (2011) Vol. 49, No. 4, pp. 525-534
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DOI 10.1007/s12275-011-0457-y

Characterization of Nitrogen-Fixing Bacteria Isolated from Field-Grown Barley, Oat, and Wheat

Anastasia Venieraki, Maria Dimou, Eleni Vezyri, Io Kefalogianni, Nikolaos Argyris, Georgia Liara, Panagiotis Pergalis, Iordanis Chatzipavlidis, and Panagiotis Katinakis*

Laboratory of General and Agricultural Microbiology, Department of Agricultural Biotechnology, Agricultural University of Athens, Iera Odos 75, Votanikos 11855, Athens, Greece

(Received November 10, 2010 / Accepted February 19, 2011)

Diazotrophic bacteria were isolated from the rhizosphere of field-grown *Triticum aestivum*, *Hordeum vulgare*, and *Avena sativa* grown in various regions of Greece. One isolate, with the highest nitrogen-fixation ability from each of the eleven rhizospheres, was selected for further characterisation. Diazotrophic strains were assessed for plant-growth-promoting traits such as indoleacetic acid production and phosphate solubilisation. The phylogenies of 16S rRNA gene of the selected isolates were compared with those based on *dnaK* and *nifH* genes. The constructed trees indicated that the isolates were members of the species *Azospirillum brasilense*, *Azospirillum zeae*, and *Pseudomonas stutzeri.* Furthermore, the *ipdC* gene was detected in all *A. brasilence* and one *A. zeae* isolates. The work presented here provides the first molecular genetic evidence for the presence of culturable nitrogen-fixing *P. stutzeri* and *A. zeae* associated with field-grown *A. sativa* and *H. vulgare* in Greece.

Keywords: *Azospirillum zeae*, *Pseudomonas stutzeri*, diazotrophs, rhizosphere

Diazotrophs are found across the *Archaea* and *Bacteria* domains and show various patterns of living e.g., free-living in soils, waters, and termite guts; association with grasses; and symbioses with legumes (Zehr *et al*., 2003). Diazotrophic bacteria have been isolated from the rhizosphere, rhizoplane, and/or the interior of the roots of wild or cultivated grasses, cereals and food crops (Baldani *et al*., 1997; Francke *et al*., 2009). Plant beneficial properties, documented among the diazotrophic root-associated bacteria and often referred to as plant-growth-promoting bacteria, include associative nitrogen-fixation, production of indole-3-acetic acid (IAA), nitric oxide (NO), and siderophores, deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylase (ACC), phosphate solubilization, and ammonium secretion. Some of these traits (e.g. production of IAA, NO, and ACC) may lead to enhanced root system branching and root elongation, which in turn favour the uptake of soil, water, and minerals (Steenhoudt and Vanderleyden, 2000; Dobbelaere *et al*., 2003; Creus *et al*., 2005; Blaha *et al*., 2006; Rodriguez *et al*., 2006). In addition, contribution to the plant's N budget from plant-associated bacterial nitrogen-fixation has been reported with Brazilian varieties of sugarcane (Boddey *et al*., 2001; Mirza *et al*., 2001), while inoculation with *Azospirillum* has a striking effect on grain yield of cereal crops such as wheat, barley, and oat (Dalla-Santa *et al*., 2004). Furthermore, biological nitrogen-fixation associated with the rhizosphere of barley was found to be considerably higher than that of wheat (Idris *et al*., 2007).

Though cereal crops are cultivated worldwide, little data are available on the genetic diversity of culturable diazotrophic bacteria associated with wheat grown in various countries (Venieraki *et al*., 2011 and references therein) and even less on the genetic diversity of culturable diazotrophic bacteria associated with the roots of barley and oat (Belimov *et al*., 1995; Bashan *et al*., 2004; Sahin *et al*., 2004; Soares *et al*., 2006; Roesch *et al*., 2007).

The aim of the present study was to assess the phylogenetic position of culturable diazotrophs isolated from the rhizosphere of cereal crops (*Triticum aestivum*, *Hordem vulgare*, and *Avena sativa*) grown in various regions of Greece and to test for the occurrence of plant-growth-promoting attributes (nitrogen-fixation, phosphate solubilization, and IAA production).

Material and Methods

Isolation of diazotrophic bacteria

The plants were sampled from different locations of Greece (Table 1). Three plants were collected from each location, carefully removed from the soil and, with adherent soil, placed in a cooling box and transferred to the lab. The root-adhering soil samples were collected from each plant and the isolation of nitrogen-fixing organisms from different varieties of *Triticum aestivum*, *Hordeum vulgare*, and *Avena sativa* was carried out by the enrichment culture technique using semisolid malate N-free medium (NFb) supplemented with 50 mg/L yeast extract (Baldani and Dobereiner, 1980; Baldani *et al.*, 1986). The diazotrophic bacteria count (MPN) in the rhizosphere soil was carried out using semisolid NFb medium. The number of bacteria in the rhizosphere soil of *T. aestivum* was higher (1.1-2.1$\times 10^3$ CFU/g of dry weight) than the values found for *Hordeum vulgare* and *Avena sativa* (0.8-1.2$\times 10^3$ CFU/g of dry weight). The isolates were examined for the characteristic sub-surface pellicle formation and further purified by streaking on Congo Red agar medium (Rodriguez-Caceres, 1982).

* For correspondence. E-mail: katp@aua.gr; Tel.: +0030-210-5294342

Table 1. Plant-growth promoting traits of diazotrophic bacterial strains isolated from oat, barley and wheat

Strain	Plant species	Plant variety	Location (Greece)	Type strain (highest match %)	nmoles ethylene/ h/mg protein	IAA μg/mg protein	Phosphate solubility
Gr24	*T. aestivum*	Vergina	Domokos, Fthiotida	*Azospirillum zeae* N7; 98.682	7.3±1.2 (a)	29.8±1.7 (abc)	+
Gr30	*T. aestivum*	Yecora	Larisa, Thessalia	*Azospirillum brasilense* ATCC 49958; 97.500	36.5±7.9 (d)	172.1±16.6 (h)	+
Gr35	*T. aestivum*	Yecora	Domokos, Fthiotida	*Azospirillum zeae* N7; 98.058	6.5±0.8 (a)	111.9±10.2 (f)	+
Gr37	*T. aestivum*	Vergina	Thessaloniki, Macedonia	*Azospirillum brasilense* ATCC 49958; 97.985	7.1±0.2 (a)	194.8±17.1 (i)	+
Gr46	*T. aestivum*	Generosso	Thessaloniki, Macedonia	*Pseudomonas stutzeri* CCUG 11256; 99.725	7.5±1,3 (a)	19.1±3.9 (a)	+
Gr50	*H. vulgare*	Carina	Thessaloniki, Macedonia	*Pseudomonas stutzeri* CCUG 11256; 99.931	4.5±0.2 (a)	44.1±1.7 (c)	+
Gr54	*H. vulgare*	Georgy	Thessaloniki, Macedonia	*Azospirillum brasilense* ATCC 49958; 98.611	44.3±5.7 (e)	18.4±5.5 (a)	+
Gr60	*A. sativa*	local	Skourta, Viotia	*Azospirillum zeae* N7; 99.445	7.6±0.5 (a)	42.7±6.8 (bc)	ND
Gr61	*A. sativa*	local	Thessaloniki, Macedonia	*Azospirillum zeae* N7; 99.307	6.0±0,3 (a)	27.0±5.7 (ab)	ND
Gr65	*H. vulgare*	local	Larisa, Thessalia	*Pseudomonas stutzeri* CCUG 11256; 97.321	28.6±3.7 (cd)	26.1±2.4 (a)	+
Gr66	*A. sativa*	Cassandra	Thessaloniki, Macedonia	*Azospirillum brasilense* ATCC 49958; 98.125	21.0±2.4 (b)	69.8±14.2 (d)	ND

Numbers followed by different letters are significantly different ($P \leq 0.05$), as determined by Duncan's multiple range test.
Nitrogenase activity was measured after growth in NFb semi-solid medium.
IAA was estimated in cultures at the stationary phase.
Bacterial isolates showing a clear halo around the bacterial colonies in both types (Pikovskaya's and Sperber's) of agar plates were considered as positives (+); ND, not detected.

Single colonies (from each rhizosphere) were tested for acetylene reduction (ARA), and ARA-positive colonies were stored in 20% glycerol at -80°C prior to characterization. One isolate with the highest nitrogen-fixation ability from each of the eleven rhizospheres was selected for further characterisation (Table 1). The geographical location, plant species, plant variety, and isolate number are shown in Table 1.

Bacterial strains

Azospirillum brasilense LMG 13127^T and *A. lipoferum* 13128^T were obtained from the Belgian Coordinated Collections of Microorganisms, Laboratory for Microbiology of the Faculty of Sciences of Ghent University (BCCM^{TM}/LMG). *Pseudomonas fluorescens* strain X was kindly provided by Dr. Georgakopoulos.

Acetylene reduction assay

The acetylene reduction assay (ARA) was performed on free-living cultures of each isolate as previously described (Venieraki *et al*., 2011). The amount of ethylene produced by acetylene reduction was measured by a Perkin Elmer F-11 gas chromatograph fitted with a flame ionization detector and a Porapak R 80-100 mesh stainless-steal column (2 mm×2 m). After the ARA of free-living cultures was completed, the cells were collected and broken by sonication. Protein concentration in the resulting mixture was determined by standard method (Bradford, 1976). For the ARA quantification, *A. brasilense* Sp7 was used as control.

Colorimetric estimation of indoleacetic acid production

The nitrogen-fixing isolates were screened for the expression of plant-growth-promoting attributes essentially as described previously (Venieraki *et al*., 2011). In brief, IAA production was estimated by growing the isolates in NFb medium supplemented with NH_4Cl and 100 μg/ml DL-tryptophan at 30°C with shaking for 72 h in the dark. Five milliliter of each culture was centrifuged (20 min, 6,000×g), and indoleacetic acid production was measured as indolic compounds in 2 ml of supernatant by mixing with 2 ml of Salkowski reagent and following the absorbance at 535 nm after 30 min in the dark (Glickman and Dessaux, 1995). A standard curve was used for calibration. For IAA quantitation, *A. brasilense* Sp7 was used as control.

Phosphate solubilization in solid media

The ability of the isolates to solubilize inorganic phosphate was determined by spotting 5 μl (10^8 CFU/ml) of fresh bacterial culture on Pikovskaya's (1948) and Sperber's (1957) agar plates supplemented with 2.1 g/L of $Ca_4(PO_4)_2$. Plates were incubated at 30°C and observed daily for formation of transparent halos around each colony for up to 9 days. Bacterial isolates showing a clear halo around the bacterial colonies in both types of agar plates were considered as positives (Table 1). *P. fluorescens* strain X (Georgakopoulos *et al*., 2002) was used as control.

DNA extraction, PCR amplification, and sequencing of 16S rRNA, *nifH*, *ipdC*, and *dnaK* gene fragments

Genomic DNA from bacterial cultures of the isolates was extracted using the GenElute^{TM} Bacterial Genomic DNA kit according to the manufacturer's instructions (Sigma-Aldrich, USA). DNA concentration and purity was assessed by the Nanodrop ND-1000 spectrophotometer. The *ipdC* amplicons were amplified using the primer set *ipdC*2F (5′-TCCCAGCGCAAGATCGACCTG-3′) and *ipdC*2R (5′-CTTAGC

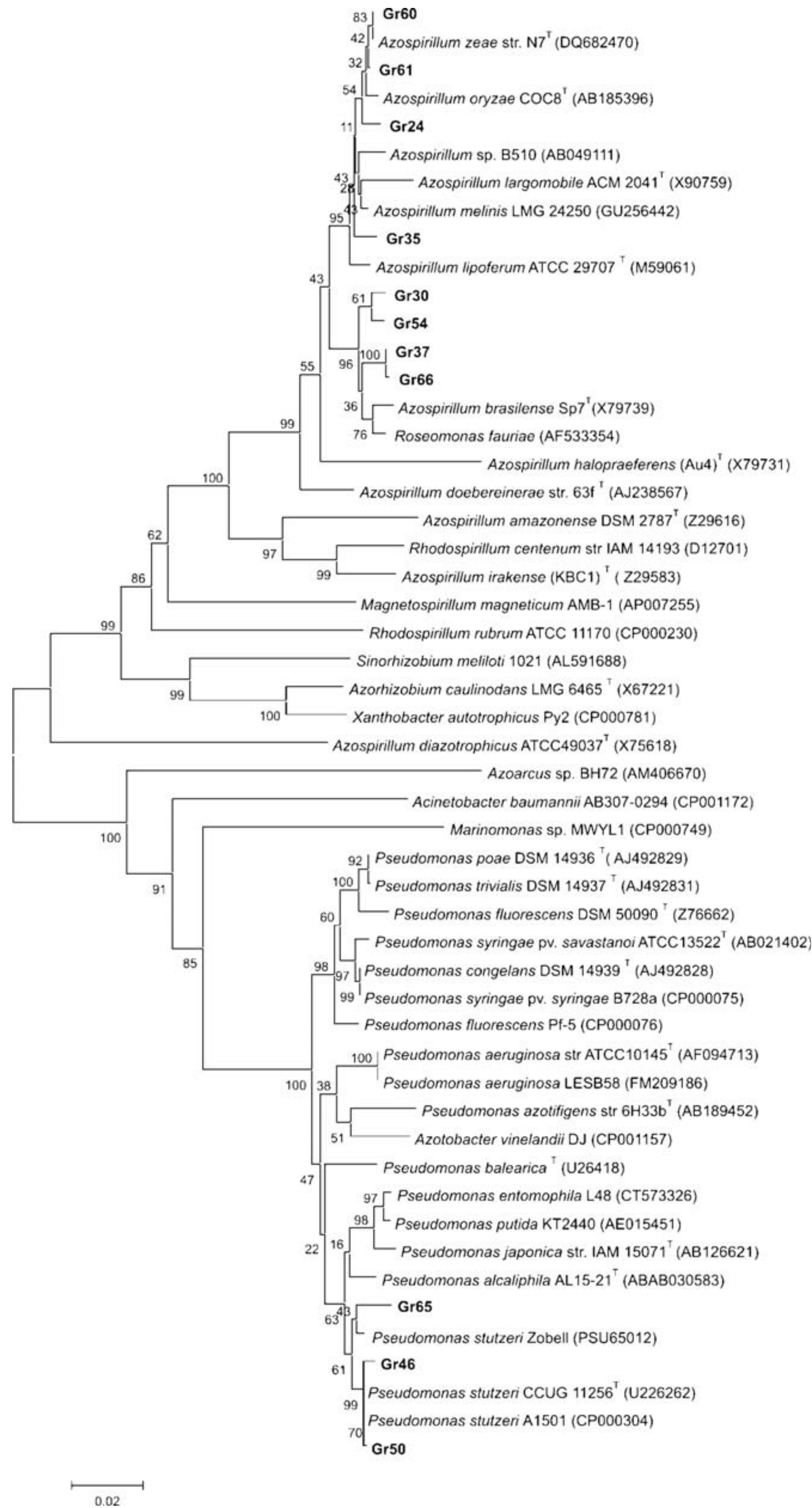

Fig. 1. Neighbor-joining phylogenetic trees of 16S rRNA constructed using the partial nucleotide sequence from the 11 isolates obtained from rhizosphere soil of oat, barley, and wheat and related sequences obtained from NCBI. Numbers shown at nodes indicate bootstrap values (percentage of 1,000 replicates). The bar scale indicates the rates of substitution per nucleotide position. Sequence accession numbers are given in parentheses. ^{T}=type strain.

CGGAAGTTGCTGGACCTGC-3′). The PCR amplification mixture contained two U DyNAzyme-EXT DNA polymerase (Finnzymes, Finland); 10× DyNAzyme-EXT buffer; 0.25 μmol $MgCI_2$; 50 pmol of each oligonucleotide and 40 ng of DNA template. A final volume of 50 μl was adjusted with distilled water. PCR reactions were cycled in a Bio-Rad (Italy) thermocycler (MJ mini) with a hot start step at 94°C for 5 min, followed by 35 cycles of amplification at 94°C for 1 min; at 66°C for 45 sec; and at 72°C for 1.5 min; and extended at 72°C for 10 min at the final step. The 16S rRNA, *dnaK* and *nifH* gene fragment were amplified as described by Venieraki *et al*. (2011).

Cloning, sequencing, and phylogenetic analysis

The PCR products were separated by electrophoresis in 1.5% (w/v) agarose gel (Invitrogen, UK); the band was excised and purified using a QIAquick® Gel Extraction kit (QIAGEN, Germany). The recovered DNA was cloned into the pGEM-T Easy Vector (Promega, USA) according to the manufacturer's protocol. Four white colonies were chosen at random, transferred to a new plate, and incubated overnight. Small aliquots from four white colonies from each transformation experiment were chosen and transferred to the PCR mixture containing the vector primers T7 and SP6. Clones containing the desired insert

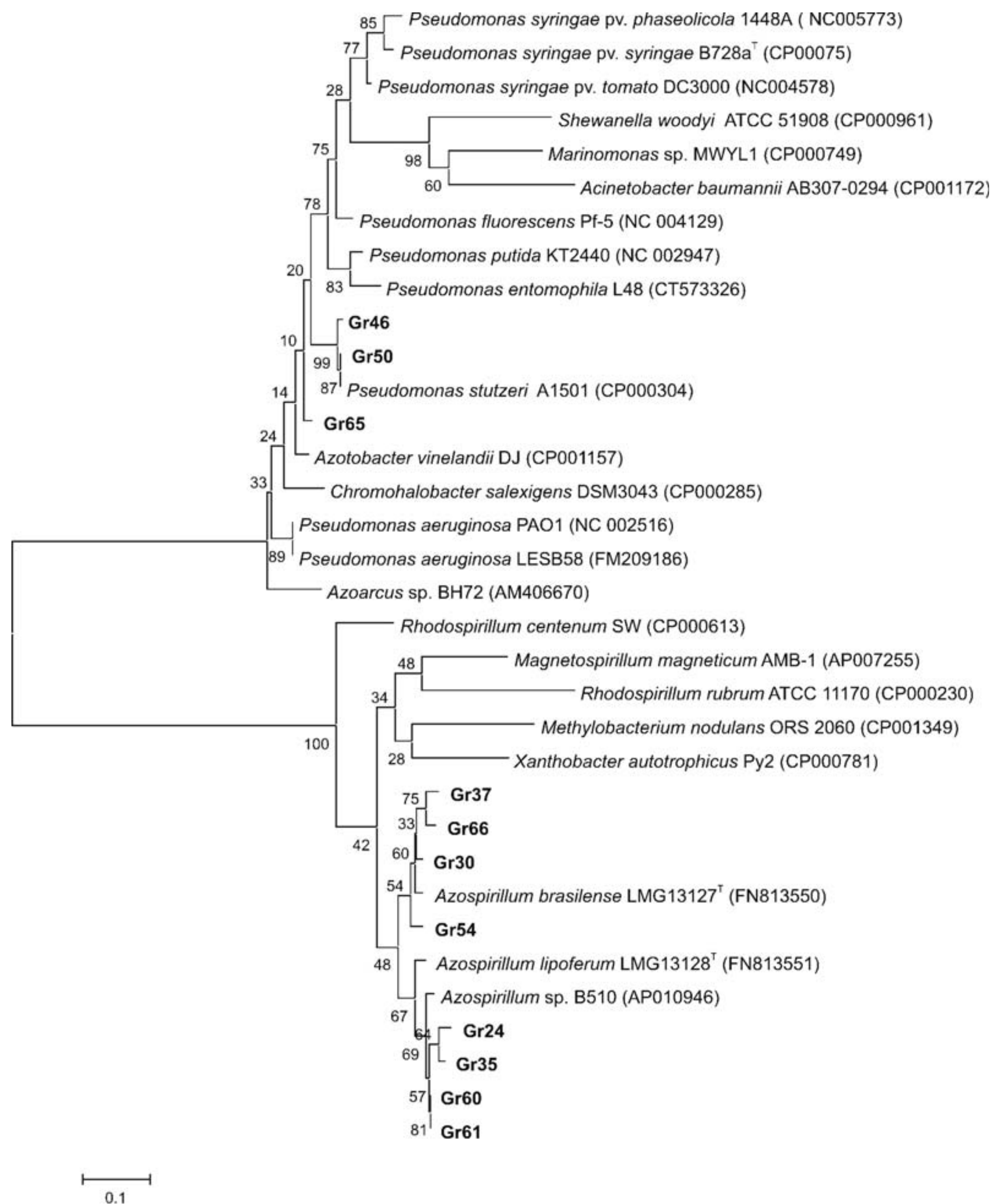

Fig. 2. Neighbor-joining phylogenetic trees *dnaK* constructed using the partial nucleotide sequence obtained from the 11 isolates from the rhizosphere soil of oat, barley, and wheat and related sequences obtained from NCBI. Numbers shown at nodes indicate bootstrap values (percentage of 1,000 replicates). The bar scale indicates the rates of substitution per nucleotide position. Sequence accession numbers are given in parentheses. ^{T}=type strain.

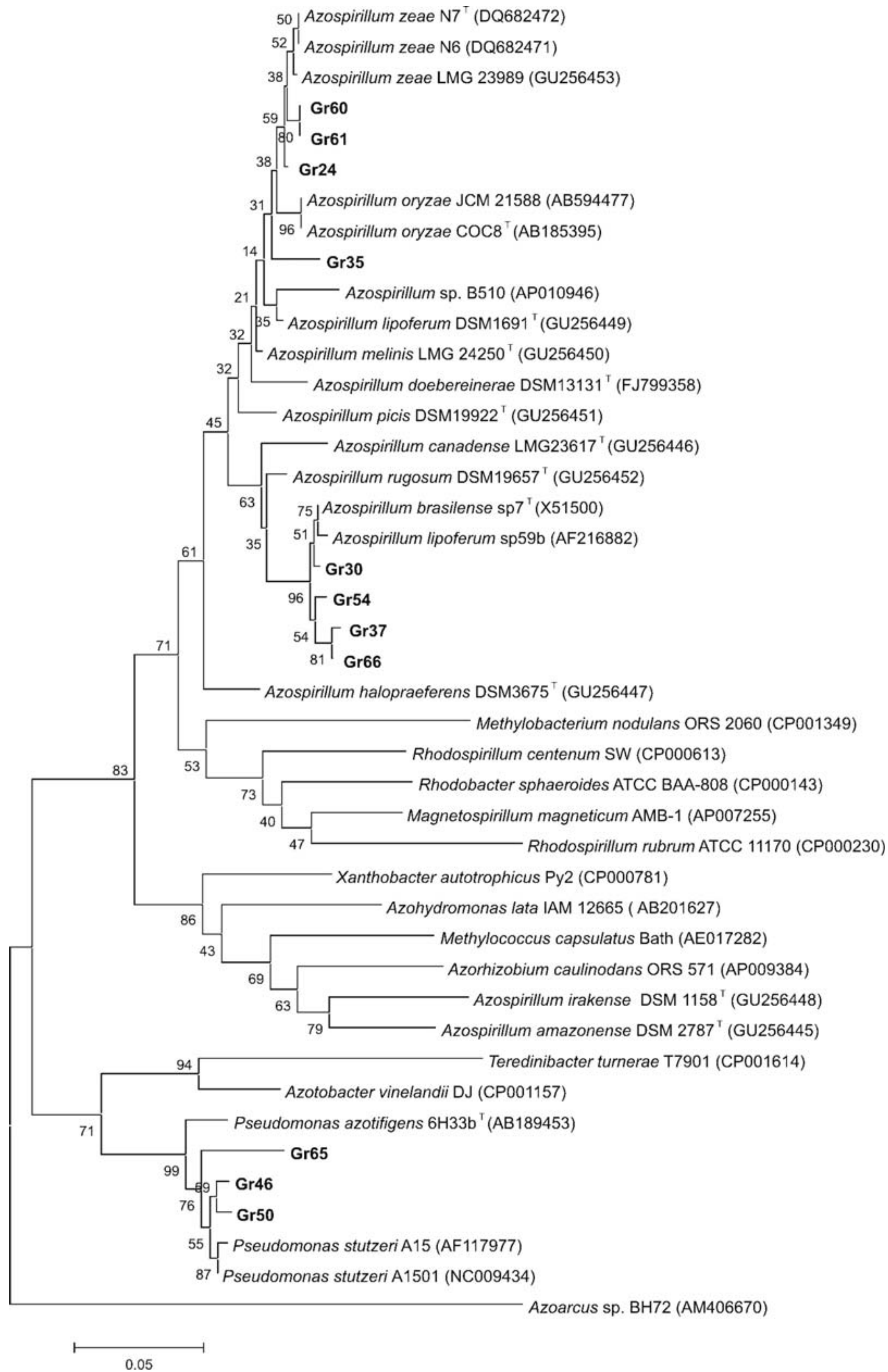

Fig. 3. Neighbor-joining phylogenetic trees of *nifH* constructed using the partial nucleotide sequence from the 11 isolates obtained from rhizosphere soil of oat, barley, and wheat and related sequences obtained from NCBI. Numbers shown at nodes indicate bootstrap values (percentage of 1,000 replicates). The bar scale indicates the rates of substitution per nucleotide position. Sequence accession numbers are given in parentheses. ^{T}=type strain.

DNA were identified by agarose electrophoresis. Plasmids containing the appropriate inserts were isolated from 2 ml of bacterial cultures using the QIAprep® Spin Miniprep kit, according to the manufacturer's instructions (QIAGEN). Purified plasmids were commercially sequenced (Eurofin MWG, Germany) in both directions. The nucleotide identity of all the sequenced clones were compared to the GenBank database (http://blast.ncbi.nih.gov) online to obtain the representative that related most closely to 16S rRNA, *nifH*, *ipdC*, and *dnaK* genes in the database. Phylogenetic trees based on nucleotide sequences of the 16S rRNA, *nifH*, *ipdC*, and *dnaK* gene fragments were constructed with Molecular Evolutionary Genetics Analysis software version 4.0 using the neighbor-joining algorithm (1,000 bootstrap replication) (Tamura *et al.*, 2007). The pairwise 16S rRNA gene sequence was calculated using the EzTaxon gene sequence server (Chun *et al.*, 2007).

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the GenBank database under the following accession numbers: 16S rRNA (FR667882, FR667883, FR667885, FR667886, FR667893, FR667896, FR667897, FR667899, FR667890, FR667891, FR667898), *dnaK* (FR669258, FR669259, FR669261, FR775383, FR669491, FR669492, FR669493, FR669496, FR669498, FR669499, FR669500), *nifH* (FR669132, FR669134, FR669135, FR669136, FR669140, FR 669141, FR669143, FR669146, FR669147, FR669148, FR669149), *ipdC* (FR670776, FR670777, FR670778, FR670780, FR670782).

Results

ARA ability and plant-growth-promoting traits of the isolates

ARA assay was carried out to examine the nitrogen-fixing ability of the 11 diazotrophic isolates under laboratory conditions. The data revealed considerable variability in the nitrogen-fixing ability among the studied strains that ranged from 4.5±0.2 to 44.3±5.7 nmoles ethylene/h/mg protein (Table 1). The 11 isolates were further tested for their ability to solubilize insoluble mineral phosphate and produce IAA. All 11 isolates grown in the minimal medium supplemented with tryptophan exhibited considerable production of IAA with amounts ranging from 18.4±5.4 to 194.8±17.1 μg IAA/mg protein. *Azospirillum* Gr37 isolate showed the greatest IAA production (Table 1). In addition to nitrogen-fixing ability and IAA production, several of the diazotrophic isolates mediated solubilization of tricalcium phosphate in both Pikovskaya's and Spreber's solid agar medium (Table 1). All the *Pseudomonas* isolates solubilized insoluble inorganic phosphate while two of four *A. zeae* and three of four *A. brasilense* isolates formed a halo of phosphate solubilization (Table 1).

Phylogenetic position of the diazotrophic isolates

PCR amplification and cloning of the 16S rRNA gene allowed the nucleotide sequence of about 1,450 bp of the 16S rRNA gene from each of the 11 studied strains to be determined. A BLASTN search of the 16S rRNA sequences database of type strains implemented by the EzTaxon server (Chun *et al.*, 2007) revealed the closest type strain affiliated to each of the studied strains (Table 1). The 16S rRNA gene nucleotide sequences of two isolates (Gr46 and Gr50) showed high similarity (99.931%) to the 16S rRNA gene sequences of type strain *Pseudomonas stutzeri* CCUG 11256, while isolate Gr65 showed relative less similarity (97.331%) (Table 1). Four strains (Gr24, Gr35, Gr60, and Gr61) showed high similarity (98.058-99.445%) to the 16S rRNA gene sequences of type strain *A. zeae* N7, and four strains (Gr30, Gr37, Gr54, and Gr66) showed similarity (97.500-98.611%) to the 16S rRNA gene sequences of type strain *A. brasilence* ATCC 49958. The phylogenetic position based on the 16S rRNA and *dnaK* gene sequences of the isolates is shown in Figs. 1 and 2. The topology of *dnaK* gene nucleotide sequence in the phylogenetic tree closely resembled the respective 16S rRNA gene sequences phylogenetic tree (Figs. 1 and 2).

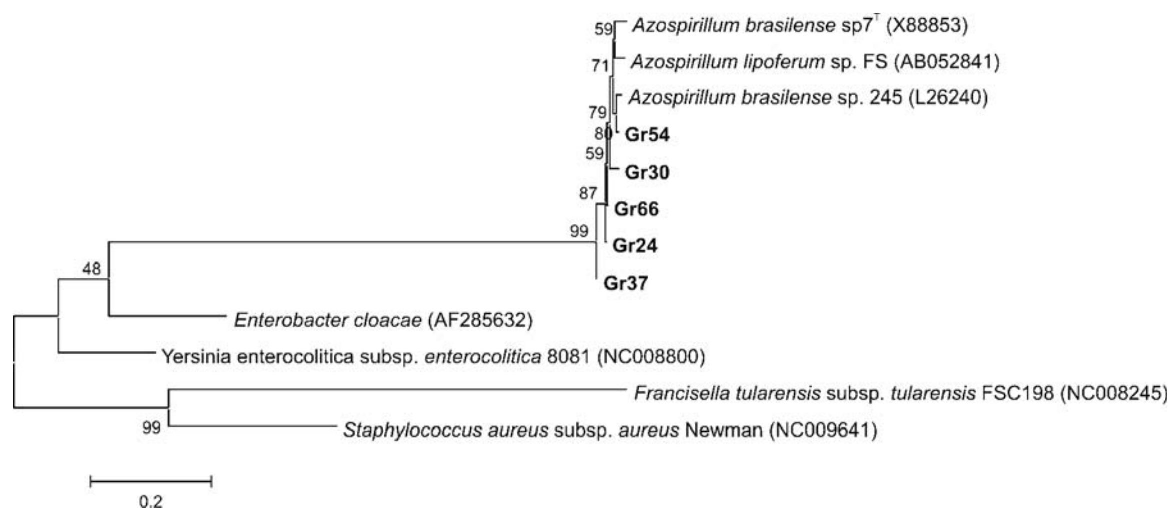

Fig. 4. Neighbor-joining phylogenetic trees of *ipdC* constructed using the partial nucleotide sequence from 5 isolates obtained from rhizosphere soil of oat, barley, and wheat and related sequences obtained from NCBI. Numbers shown at nodes indicate bootstrap values (percentage of 1,000 replicates). The bar scale indicates the rates of substitution per nucleotide position. Sequence accession numbers are given in parentheses. ᵀ=type strain.

Phylogenetic analysis of the phytobeneficial *nifH* and *ipdC* gene

The primers utilized for the amplification of a *nifH* gene fragment generated a 360 bp amplicon in all 11 isolates. A phylogenetic tree constructed based on *nifH* gene nucleotides revealed that *nifH* gene sequences from isolates belonging to *P. stutzeri* species clustered with *P. stutzeri* A1501 (Fig. 3), while the phylogenetic arrangement of the isolates belonging to *Azospirilla* clustered with either *A. zeae* or *A. brasilense* species similar to that of the 16S rRNA gene nucleotide sequences (Fig. 1).

Detection of *ipdC* gene in *Azospirillum* and *Pseudomonas* isolates

All four *A. brasilense* isolates and one of four of the isolates belonging to the *A. zeae* species generated a 540 bp *ipdC* gene fragment. Purified amplicons of the *ipdC* gene fragments were cloned and their nucleotide sequence determined. BLASTN and BLASTP searches revealed that the *ipdC* gene sequences of the nine strains showed maximum similarity (>97%) at the nucleotide and amino acid sequence level with the sequences of *A. brasilense*. The topology of the tree constructed based on *ipdC* gene sequences is shown in Fig. 4.

Discussion

Analysis of a large number of prokaryotic genomes has shown that evolutionary relationships among prokaryotes are generally represented by 16S rRNA gene sequences (Stackebrandt *et al*., 2002). In addition, *dnaK* gene sequences have been proven, in some cases, to provide higher resolution than the 16S rRNA (Strepkowski *et al*., 2003; Huang *et al*., 2010). In the present study, the diazotrophic isolates were classified based on phylogenetic position of their 16S rRNA and *dnaK* gene sequences, as members of *A. brasilense*, *A. zeae*, and *P. stutzeri* species. The presence of *nifH* genes and their ability for nitrogen-fixation, as judged by the ARA assay, confirmed the grouping of all the isolates as diazotrophs. All 11 isolates produced IAA, whereas eight (Gr24, Gr30, Gr35, Gr37, Gr46, Gr50, Gr54, and Gr65) exhibited the phosphate solubilisation phenotype.

Our present study showed that the 16S rRNA gene nucleotide sequences of isolates Gr46 and Gr50 exhibit a striking similarity (>99%) to the 16S rRNA gene nucleotide sequences of the *P. stutzeri* CCUG11256 and *P. stutzeri* A1501, while the 16S rRNA gene nucleotide sequences of the isolate Gr65 exhibit a divergence close to the threshold (97-98.7% identity) for assigning two organisms to the same species (Stackebrandt *et al*., 2002; Stackebrandt and Ebers, 2006). The constructed phylogenetic tree of 16S rRNA gene clearly shows that the 16S rRNA gene of the isolate Gr65 clusters in a separate branch (Fig. 1). The phylogenetic trees of *nifH* and *dnaK* gene nucleotide sequences are congruent with those of 16S rRNA gene (Figs. 1, 2, and 3). Taken together, these data suggest that isolate Gr65 may represent a new species of the genus *Pseudomonas*, adding one more to the increasing the list of nitrogen-fixing *Pseudomonas* species (Kulakov *et al*., 2002; Hatayama *et al*., 2005; Mirza *et al*., 2006; Muthukumarasamy *et al*., 2006; Doty *et al*., 2009; Mehnaz *et al*., 2009; Yim *et al*., 2009). *P. balearica* was thought to be a member of the *P. stutzeri* group, but Multiple-Locus Sequence Analysis confirmed *P. stutzeri* and *P. balearica* as two closely related but different species (Bennasar *et al*., 1996; Mullet *et al*., 2010).

Interestingly, most nitrogen-fixing *Pseudomonas* strains isolated thus far have been found in the rhizosphere of field-grown wheat, rice, sorghum, and sugarcane (Krotzky and Werner, 1987; You *et al*., 1991; Vermeiren *et al*., 1999; Mehnaz *et al*., 2009; Venieraki *et al*., 2011), suggesting that the root system of gramineae plants may provide the appropriate ecological niche for survival and growth of these bacterial species. In favour of this suggestion is the observation that *P. stutzeri* A15, a strain that harbours nitrogen-fixation genes on a putative genomic island (Yan *et al*., 2008), survived well over a period of 60 days in flooded soil planted with rice (Lin *et al*., 2000), and that *P. stutzeri* A15, a strain that harbours nitrogen fixation genes on a putative genomic island (Yan *et al*., 2008), survived well over a period of 60 days in flooded soil planted with rice (Lin *et al*., 2000), and that *P. stutzeri* A15 exhibited nearly equal colonisation ability and survival index upon inoculation to wheat and rice (Rediers *et al*., 2009). Thus, it would be of interest to investigate whether the recruitment of nitrogen-fixation genes is a general trend amongst the numerous *P. stutzeri* strains available in the various collections, since there is evidence that *P. stutzeri* JM300 is able to fix atmospheric nitrogen (Gauthier *et al*., 2000).

Our findings reveal that wheat, barley, and oat rhizosphere soils are also colonised by *A. brasilense* and *A. zeae*. The association of indigenous *Azospirillum* spp. with cereals is well-established (Reis *et al*., 2000), and *Azospirillum* spp. are amongst the most abundant bacteria found in oat roots (Soares *et al*., 2006). However, the association of members of *A. zeae* and *P. stutzeri* species with the roots of *H. vulgaris* and/or *A. sativa* is reported for the first time in this work. The *A. zeae*-type strain was originally isolated from roots of maize in Canada (Mehnaz *et al*., 2007), and to our knowledge, no similar species have been detected in association with cereals either by culturable or non-culturable approaches.

The 11 isolates recovered in this study are capable of IAA production (Table 1). The capacity to synthesize IAA is widespread among soil plant-growth-promoting bacteria such as *Azospirillum* spp., *Klebsiella* sp., and *Enterobacter cloacae* (Spaepen *et al*., 2007). However, our experimental approach revealed the presence of the *ipdC* gene in only one (isolate Gr24) of four (isolates Gr24, Gr35, Gr60, and Gr61) *A. zeae* recovered in this study, while all four *A. brasilence* isolates harboured the *ipdC* gene sequence. None of the nitrogen-fixing *P. stutzeri* isolates yielded a PCR-amplified product with the primers used for *ipdC* gene fragment amplification. Genomic analysis of *Azospirillum* sp. B510 and *P. stutzeri* A1501 revealed the presence of genes involved in indole-3-acetamide pathway (*IaaH* and *IaaM*) and absence of those involved in indole-3-pyruvate pathway (*ipdC*). However, the presence of genes involved in the indole-3-pyruvate pathway is well-documented for *A. brasilense* strains (Spaepen *et al*., 2007). Searches within the *P. stutzeri* A1501 genome revealed the presence of putative *iaaM* and *iaaH* genes, while genes homologous to *ipdC* gene appear to be absent. Since biosynthesis of IAA from tryptophan occurs via three alternatives pathways (Spaepen *et al*., 2007), the amounts of IAA produced by *P. stutzeri* (isolates Gr46, Gr50, and Gr65) and the three *A. zeae* isolates (Gr24,

Gr60, and Gr61) recovered in this study could be attributed to alternative pathways (e.g., indole-3-acetamide pathway or the tryptamine oxidase). The presence of the tryptamine pathway in *Azospirillum* has also been reported (Hartmann *et al*., 1983), and the presence of the alternative IAA biosynthetic has also been demonstrated in *A. brasilense* (Spaepen *et al.,* 2007; Malhorta and Srivastava, 2008). Furthermore, production of IAA has also been documented in members of the *P. stutzeri* group including strain A15 (Liba *et al*., 2006; Pedraza *et al*., 2006; Mehnaz *et al*., 2009). Of note is that *P. stutzeri* A1501 was reisolated from rice roots inoculated with strain A15 (Lalucat *et al*., 2006). However, we cannot exclude the possibility that our *Pseudomonas* and the three *A. zeae* isolates contain a highly divergent gene coding for IPyA decarboxylase (IPDC) that is not identified by the method employed. A southern blot analysis of DNA isolated from the *P. stutzeri* isolates did not reveal the presence of any hybridisation signal using the *A. brasilense ipdC* gene as a probe.

The phosphate solubilization phenotype is widespread among the members of *Pseudomonas* species including *P. stutzeri* (Peix *et al*., 2003; Naz and Bano, 2010; Sashidar and Podile, 2010) and *Azospirillum* species (Seshardi *et al*., 2000; Rodriguez *et al*., 2004; El-Komy, 2005; Venieraki *et al*., 2011). Our data revealed that all the *P. stutzeri* strains exhibited the capacity for solubilization of insoluble mineral phosphate while three (Gr60, Gr61, and Gr66) of eight *Azospirillum* isolates showed no such property. The phosphate solubilizing phenotype of soil bacteria has been linked to the production and exudation of organic acids, mainly gluconic acid. The other strains studied either did not produce organic acids and/or gluconic acid or production of these organic acids was too weak, which rendered the phosphate solubilization assay employed problematic. The gluconic acid production results from the extracellular oxidation of glucose via the pyrroloquinone-quinone (PQQ)-dependent glucose dehydrogenase (Sashidar and Podile, 2010). The gluconic acid production results from the extracellular oxidation of glucose via the pyrroloquinone-quinone (PQQ)-dependent glucose dehydrogenase (Sashidar and Podile, 2010). Searches of the *P. stutzeri* A1501 genome (Yan *et al*., 2008) revealed the presence of both putative pyrroloquinone-quinone (*pqqA*,*B*,*C*,*D*,*E* and *F*) and glucose dehydrogenase (*gdh*) biosynthetic genes, while *Azospirillum* sp. 510 genome (Kaneko *et al*., 2010) appears to lack only the *pqqA*. Genes with homologue sequences to pyrroloquinone-quinone biosynthetic genes and PQQ-depended glucose dehydrogenase encoding genes were also found in the incomplete genome of *A. brasilense* Sp245 (http://genome.ornl.gov/microbial/abra/). Fragments of the gene coding for *gdh* and *pqqE* were detected by a PCR approach in the *P. stutzeri* Gr46 and Gr50 and *A. brasilense* Gr30, and Gr37 isolates (Venieraki *et al*., unpublished observations).

In the present study, we established that members of the *A. brasilense*, *A. zeae*, and *P. stutzeri* species predominate in the culturable nitrogen-fixing bacterial community associated with the roots of field-grown wheat, barley and oat in Greece. This study, in combination with our previous studies (Venieraki *et al*., 2011) emphasizes on the wide dispersal of nitrogen-fixing *Pseudomonas* in the rhizosphere of field-grown cereals. The relative abundance of nitrogen-fixing *Pseudomonas* and *Azospirillum* in the rhizosphere of field-grown cereals has yet to be investigated using non-culturable experimental approaches. Furthermore, the increasing number of reports documenting that *P. stutzeri* strains harbor nitrogen-fixing genes (Vermeiren *et al*., 1999; Gauthier *et al*., 2000; Eckford *et al*., 2002; Venieraki *et al*., 2011) combined with the observations that members of *P. stutzeri* species are naturally transformable either under laboratory conditions or in soil by homologous or heterologous DNA (Sikorski *et al*., 1998; Lorenz and Sikorski, 2000; Sikorski *et al*., 2002), raises questions as to whether the organization of nitrogen-fixation genes present in our *P. stutzeri* isolates is similar to the nitrogen-fixation island identified in *P. stutzeri* strain A1501 (Yan *et al*., 2008).

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