ISSN 0026-2617, Microbiology, 2011, Vol. 80, No. 1, pp. 117–124. © Pleiades Publishing, Ltd., 2011.

EXPERIMENTAL ARTICLES

# Identification of Nitrogen-Fixing *Paenibacillus* from Different Plant Rhizospheres and a Novel *nifH* Gene Detected in the *P. stellifer*[1]

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Received March 10, 2010

**Abstract**—A total of 534 isolates were selectively obtained from different plant rhizospheres based on their growth on nitrogen-free medium and their resistance to 80°C for 15 min. Of the 534 isolates, 23 isolates had *nifH* gene and exhibited nitrogenase activities. Based on 16S rDNA sequence, G + C content assay and DNA–DNA hybridization, the 23 isolates which divided into four monophyletic clusters were all belonged to the *Paenibacillus* genus. *nifH* gene deduced amino acid alignment aLnalysis revealed that cluster I, including 15 isolates, showed the highest NifH identity with *Paenibacillus* genus; while cluster II identified as *P. stellifer* by DNA–DNA hybridization was consistent with four uncultured bacterial clones. This study suggested that the nitrogen-fixing *Paenibacillus* were distributed in various ecosystems and prevalent in different plant rhizospheres. It was the first demonstration that nitrogen fixation existed in *P. jamilae* and *P. stellifer*. In eight isolates identified as *P. stellifer* species, a novel *nifH* gene was detected in *Paenibacillus*.

*Keywords: Paenibacillus*, isolation, identification, nitrogen-fixing, novel *nifgH* gene.

**DOI:** 10.1134/S0026261711010097

The ability to fix nitrogen is widely distributed among phylogenetical diverse bacteria. With the coexisted format with host and non-host plants, nitrogen-fixing microorganisms are grouped as symbiosis, associative and free-living nitrogen-fixing bacteria. Some leguminous crops such as soybean and *Medicago sativa* generate nodule in which some rhizobia can survive. Inside the legume-root nodule, the bacteria reduce dinitrogen to ammonium which is secreted to the plant in exchange for a carbon and energy source. For these symbiosis, the nitrogen-fixing microorganism specific dependent on the host genotype [1]. At the same time some non-leguminous plants like rice, maize and wheat belong to the poaceae family form staple food for people around the world, so nitrogen fertilizer ranks first among the external inputs to maximize output in agriculture for non-leguminous crops. With the aim to reducing the agricultural input and especially the environmental degradation and human health concern, various alternatives are being harnessed to reduce the dependence upon nitrogen fertilizer for plant nutrients [2]. Under such an environment, our lab devoted many years to exploring the free-living nitrogen-fixing *Paenibacillus*.

As early as 1908, nitrogen-fixing *Bacillus polymyxa* strains were found to be associated with plants [3], then in 1991 the *Bacillus* genus were led to its splitting into three distinct genera. The *Paenibacillus* genus was first modified by [4] on the basis of analysis of the 16S rRNA gene sequences of group 3 bacilli. The *Paenibacillus* was an endospore-forming genus with a resistance to extreme environment and some species consistently showed a great capacity to fix atmospheric nitrogen *Bacillus* Since its creation, continuous transfer of *Paenibacillus* sp. to the genus and descriptions of novel *Paenibacillus* sp. have increased the number of *Paenibacillus* sp. considerably. At the time of writing (February, 2010), the *Paenibacillus* genus comprised more than 100 species. (http://www.bacterio.cict.fr/p/paenibacillus.html), in which 16 type strains had been reported to have nitrogenase activities including *P. polymyxa*, *P. macerans*, *P. peoriae*, *P. graminis*, *P. odo-rifer*, *P. brasilensis*, *P. azotofixans*, *P. borealis*, *P. wynnii*, *P. massiliensis*, *P. sabinae*, *P. zanthoxyli*, *P. donghaensis*, *P. forsythiae*, *P. riograndensis* and *P. sonchi*.

The conventional nitrogenase, the enzyme that catalysed nitrogen fixation, was composed of two proteins: the iron (Fe) protein and the molybdenum iron (MoFe) protein. The *nifH* gene encodes the iron protein while the *nifD* and *nifK* gene encodes the molybdenum iron protein. Evolutionarily conserved amino acid sequences within the *nifH* gene have been exploited to design *PCR* primers to detect the genetic potential for nitrogen fixation in any environment [5].

In this study, strains of nitrogen-fixing *Paenibacillus* from different plant rhizospheres were isolated, then determined by nitrogenase activity assay and by

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**Table 1.** Nitrogenase activity of isolates and nitrogen-fixing *Paenibacillus* reference strains. Results are means ± standard deviation

| Strains | Nitrogenase activities (nmol $C_2H_4$/(mg protein h) | Origin, place of isolation and reference |
|---|---|---|
| *P. azotofixans* ATCC 35681$^T$ | 1623 ± 89.4 | Wheat roots, Parana state, Brazil |
| *P. zanthoxyli* DSM 18202$^T$ | 4326 ± 127.8 | *Zanthoxylum simulans* rhizosphere, Beijing [6] |
| *P. sabinae* DSM 17841$^T$ | 287 ± 73.5 | *Sabina squamata* rhizosphere, Beijing |
| *P. forsythiae* DSM 17842$^T$ | 268 ± 55.4 | *Forsythia mira* rhizosphere, Beijing [29] |
| *P. odorifer* TOD 45$^T$ | 13.42 ± 1.33 | Wheat roots, Dieulouard, France |
| *P. polymyxa* ATCC 842$^T$ | 8.74 ± 0.84 | Kluyver strain [30] |
| *P. graminis* RSA 19$^T$ | 183.46 ± 33.7 | Maize roots, Ramonville, France |
| Hp7 | 138.9 ± 22.6 | Apple, farmland in Hebei |
| Be17 | 236.86 ± 36.7 | Begonia, plant park in Beijing |
| Bb24 | 82.3 ± 11.4 | Arborvitae, natural forest in Beijing |
| S27 | 232.3 ± 43.1 | *Sophora japonica*, natural forest in Beijing |
| Nz28 | 54.32 ± 6.74 | Chinese Rose, artifical garden in Nanjing |
| Nz29 | 218 ± 43.5 | Japanese Maple, plant park in Nanjing |
| Nz30 | 133.8 ± 25.1 | Coptis Root, plant park in Nanjing |
| Ss35 | 134.8 ± 13.8 | Pomegranate, natural forest in Shandong |
| Bd43 | 657.8 ± 57.4 | Eucommia ulmoides Oliver, mount in Beijing |
| Gb44 | 165.59 ± 20.3 | *Podocarpus nagi*, natural forest in Guangxi |
| Gz45 | 68.91 ± 12.7 | Cape jasmine, artificial garden in Guangdong |
| Gt48 | 95.8 ± 13.1 | *Hibiscus tiliaceus*, plant park in Guangdong |
| Gt49 | 113.8 ± 20.6 | Cycas, artificial garden in Guangdong |
| Sl51 | 85.4 ± 9.47 | Asparagus, farmland in southwest of Shanxi |
| Sx52 | 263.9 ± 35.6 | Siberian Apricot, natural forest in Shanxi |
| Bb54 | 133 ± 21.2 | Baohua Magnolia, artificial park in Beijing |
| Hz55 | 97.8 ± 18.7 | Dateplum Persimmon, natural forest in Hebei |
| By56 | 75.7 ± 13.5 | Cherry, Fenghuang mountain in Beijing |
| Bs57 | 49.30 ± 10.4 | Staphylea, artificial park in Beijing |
| Gc58 | 87.66 ± 12.8 | Roughleaftree, countryside in Gansu |
| Bs63 | 55.96 ± 11.5 | Weeping Willow, artificial park in Beijing |
| Hp91 | 138.7 ± 16.2 | Sequoia, natural forest in Hangzhou |
| Hp92 | 168.9 ± 24.5 | Flowering peach, natural forest in Hangzhou |

sequencing *nifH* gene. This report first demonstrated that nitrogen fixation existed in *P. stellifer* and *P. jamilae*, and first detected novel *nifH* gene in *P. stellifer*. This study suggested that the nitrogen-fixing *Paenibacillus* were distributed in various ecosystems and prevalent in different plant rhizospheres.

## MATERIALS AND METHODS

**Sample collection, isolated procedures and reference strains.** 94 soil samples were collected from different plant rhizospheres in different districts of People's Republic of China. The method dealt with the soil samples were described as Ma et al. [6]. Three times propagation using nitrogen-free agar slants were carried out to minimize the influence of nitrogen from the soils. After 5 d incubation at 30°C, single colony for each possible species of microbe was selected. Because of the severely oligotrophic condition of the nitrogen-free agar slants, isolates were then transferred to the fresh nitrogenase test medium (NTM) which contained 20 $gl^{-1}$ sucrose, 12.06 $gl^{-1}$ $K_2HPO_4$, 3.4 $gl^{-1}$ $KH_2PO_4$, 0.5 $gl^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.01 $gl^{-1}$ NaCl, 0.015 $gl^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.005 $gl^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, 0.05 $gl^{-1}$ yeast extract and 1.3–1.4% ($wv^{-1}$) agar with adjusted pH 7.0 for further study. In this experiment, the preferred incubation temperature was always 30°C. The strains investigated in this study and their sources were listed in Table 1.

Reference type strains *P. polymyxa* ATCC 842$^T$, *P. odorifer* TOD 45$^T$ and *P. graminis* RSA 19$^T$ were kindly provided by Dr. Berge in France; *P. stellifer* DSM 14472$^T$

was generously gifted by Prof Mirja Salkinoja-Salonen in Helsinki University; *P. zanthoxyli* DSM 18202$^T$, *P. forsythiae* DSM 17842$^T$, *P. sabinae* DSM 17841$^T$ and *P. sonchi* LMG 24727$^T$ were deposited in our lab; the strain *P. azotofixans* ATCC 35681$^T$ was ordered from ACCC (Agricultural Culture Collection of China); The strains of *P. durus* DSM 1735$^T$ and *P. riograndensis* CECT 7330$^T$ were ordered from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and CECT (The Spanish Type Culture Collection), respectively.

**DNA extraction.** The genomic DNA for PCR amplification was extracted according to standard methods [7]. The purity was estimated by A260/A280/A230 extinction ratios using UV spectrophotometer (LabTech UV-1000 USA).

**Amplification, cloning and sequencing of *nifH* gene.** *nifH* gene fragment was amplified using the degenerate primers: forward 5'-GGCTGCGATCC(CGA)AAGGCCGA(CT)TC(CGA)ACCCG-3' and reverse 5'-CTG(GCA)GCCTTGTT(CT)TCGCGGAT(CG)GGCATGGC-3' as described by Ding et al. [8]. The *nifH* PCR product was purified according to the protocol of Nucleic acid Recycle and Purification Kid (Tiangen Biotech CO.LTD., P. R. China). Purified nucleotide sequences and plasmid vector pMD18-T (Takara, Japan) were ligated at 16°C for 16 h. Recombined plasmids were transformed into *Escherichia coli* DH5α using electroporation and subsequently selected by blue/white screening procedure. Plasmids containing *nifH* sequences were extracted and purified [7]. Purified plasmids were then sequenced using the M13F and M13R primers on an ABI377 automatic sequencer (Applied Biosystems, USA).

**Nitrogenase activity assay**. Nitrogenase activities was estimated by using the acetylene reduction assay (**ARA**) on *NTM* medium under air. Strains were incubated under liquid *NTM*, then centrifuged the culture to collect the strain and adjust the $OD_{600}$ value to 1000, after intimidated under 10% ($vv^{-1}$) acetylene/air for 2 h and ethylene production was analyzed by *GC* (HP6890 USA). Then strains were collected and the protein quantity was tested as described by [9].

**Amplification and sequencing of 16S rRNA**. A nearly complete sequence of the 16S rRNA gene was amplified by using the universal forward primer P1 and the universal reverse primer P6. Primer P1 (5'-AGAGTTTGATCCTGGTCAGAACGCT-3') corresponds to positions 8–37 and primer P6 (5'-TACGGCTACCTTGTTACGACTTCACCCC-3') corresponds to positions 1479–1506 in the *E. coli* 16S rRNA gene [10]. The purification and sequencing steps were same as the described above for the *nifH* sequences.

**Sequence analysis and construction of the phylogenetic trees**. The *nifH* and 16S rDNA gene sequences of isolated strains were compared using Basic Local Alignment Search Tool (**BLAST**) with sequences held in DDBJ/EMBL/GenBank. The phylogenetic trees were constructed from evolutionary distance matrices by the neighbour-joining method using software package MEGA3.1 [11]. All 16S rDNA sequences of the type strains belonging to *Paenibacillus* genus were obtained from the website (http://www.bacterio.cict.fr/p/paenibacillus.html).

**Estimation of G + C content and DNA–DNA hybridization values.** Total genomic DNA was extracted and purified according to the method described by [12]. The purity was assessed from the A260/A280/A230 extinction ratios by UV spectrophotometer (LabTech UV-1000 USA). DNA base composition was determined by using the thermal melting protocol [13] and using *E. coli* K12 as a standard. DNA–DNA hybridization was performed according to the method of [14].

## RESULTS

**Isolation and identification of by *nifH* gene sequences and nitrogenase activities.** In this study, 94 soil samples were collected from the different plant rhizospheres and 534 stains were selectively obtained based on their growth on nitrogen-free medium and their resistance to 80°C for 15 min. PCR amplification using two degenerate primers for nitrogenase Fe protein gene was performed with chromosomal DNA extracted from the 534 isolates. The results suggested that a *nifH* amplification of 320 nucleotides was detected in 23 isolates (Table 1) and most of the isolates could secrete exopolysaccharides outside the cells.

The *nifH* gene segments amplified from the 23 isolates were sequenced and their GenBank accession numbers were indicated after the bacterial names in Fig. 1. The deduced amino acid sequences of the *nifH* gene products from the 23 isolates were aligned with the NifH sequences from other proteobacteria and the alignments were used to generate a phylogenetic tree which branched into two major clusters I and II (Fig. 1). The cluster I was all clustered together with *Paenibacillus* sp., and the NifH sequence identity of this clusters and other *Paenibacillus* sp. were 88.01%. The cluster II (with 99.0–99.54% NifH sequence identity among each other) which displayed 63.55% *nifH* sequence identity with cluster I shared 90 to 99% NifH sequence identity to four uncultured bacterial clones.

The 23 isolates with *nifH* genes were assayed for their nitrogenase activities, in comparison with nitrogen-fixing reference strains of *Paenibacillus*. As described in Table 1, it was obvious that all of the isolates in this study showed nitrogenase activities.

**Sequencing and phylogeny of 16S rDNA.** The nearly complete 16S rDNA gene sequences of the 23 novel strains were compared with the sequences held in DDBJ/EMBL/GenBank. The comparison indicated that all of these novel strains were *Paenibacillus*. The GenBank accession numbers of the strains

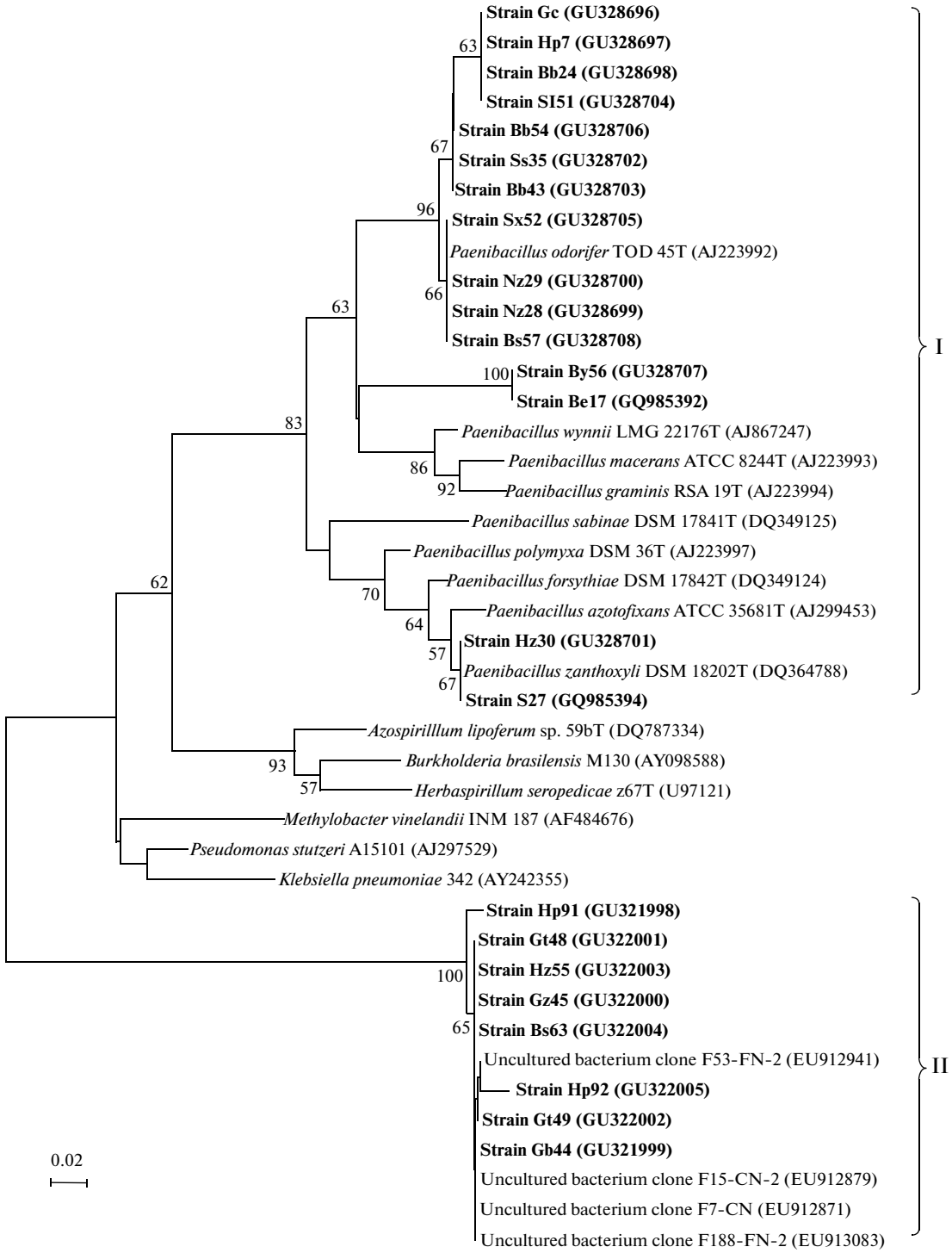


**Fig. 1.** Phylogenetic tree based on partial NifH sequences comparison obtained using the neighbour-joining method, showing the position of isolated strains. Numbers at branching points represent bootstrap values from 1000 replicates. Only values greater than 50% are shown. Bar, 0.02 substitutions per nucleotide position.

were indicated after the bacterial names in Fig. 2. A phylogenetic tree derived from the distance data which branched into four clusters was described as in Fig. 2. The cluster I including eight isolates clustered with *P. stellifer* exhibiting 99.6 to 99.9% 16S rDNA sequence similarities with *P. stellifer*, Cluster II harbored strains of Be 17 which had highest 16S rDNA sequence similarity with *P. graminis* (98.9%) and By56 with a 16S rDNA sequence similarity of 99.9% to *P. sonchi*; the cluster III which consisted of two strains

S27 and Nz30 formed a monophyletic cluster with *P. zanthoxyli* and *P. durus*. The strain S27 showed the highest 16S rDNA sequence similarity with *P. durus* (98.1%), while the strain Nz30 displayed the highest similarity with *P. zanthoxyli* (99.8%); Cluster IV totally containing 11 isolates all had the highest 16S rDNA sequence similarity with *P. jamilae*, ranging from 99.8 to 100%. The high 16S rDNA sequence similarity (≥99%) was observed between each other despite the fact that these strains originated from different geographic regions.

**DNA–DNA hybridization and G + C content.** DNA–DNA hybridization was carried out to study the taxonomic relationships based on the sequences and phylogeny of 16S rDNA from the 23 isolates. The strains which showed 16S rDNA sequence similarity of more than 97% with type strains were chosen to perform DNA hybridization. The value of DNA–DNA hybridization and G + C content were shown in Table 2. According to the recommendations of the ad hoc committee [15] and the description of [16], it was concluded that cluster I was identified as the different strains in the *P. stellifer* species; the isolates S27 and Bel7 were two new type strains in this genus; the strain Nz30 belonged to *P. zanthoxyli* species and By56 was identified as *P. sonchi*; in Cluster IV, four isolates which were Bb24, Ss35, S151 and Bb54 displaying 100% 16S rDNA sequence similarities with *P. jamilae*, were identified as *P. jamilae* species. Other seven isolates had the 99.8–99.9% 16S rDNA similarity with *P. jamilae*.

**Novel *nifH* gene in *Paenibacillus stellifer*.** The *nifH* gene sequences in cluster II (Fig. 1) were compared using Basic Local Alignment Search Tool (BLAST) with sequences held in DDBJ/EMBL/GenBank. It was found that the sequences all had the high identity with sequences belonged to uncultured bacterial clone and the highest similarities with four uncultured bacterial clone were described in Fig. 1. It was obvious that the *nifH* gene in this cluster had a distant evolutionary distance with other strains in *Paenibacillus*. There were two assumptions for this phenomenon. One was that there existed horizontal gene transfer in this species. With this phenomenon, [17] had specifically discussed the nitrogen fixation gene cluster horizontal transfer in cyanobacterium. In the report, it stated that the gene and protein sequences of the *Microcoleus chthonoplastes* nif-cluster showed highest identity with the family of *Desulfovibrionceae* which is not only had a close living circumstance association with the *M. chthonoplastes* but also had the capability of transducing DNA fragments through phages. So there was low possibility for existence of horizontal gene transfer in all of the eight strains which belonged to *P. stellifer* in this research. The reason was that there were not any signals to qualify these situations. At the same time, many researches had reported that there existed diversity *nifH* gene sequences in *Paenibacillus* [18–20]. So the most likely explanation was that there might be a novel *nifH* gene existed in the *P. stellifer*.

**Table 2.** The G + C content and the value of DNA-DNA hybridization among some isolates and their phylogenetic closest type strains

| Strains | S27[1] | Nz30 | Be17[1] | By56 | ClusterII[2] |
|---|---|---|---|---|---|
| *P. durus* DSM 1735$^T$ | 37.64% | 50.3% | | | |
| *P. zanthoxyli* DSM 18202$^T$ | 34.99% | 77.8% | | | |
| *P. sabinae* DSM 17841$^T$ | 23.12% | 24.37% | | | |
| *P. forsythiae* DSM 17842$^T$ | 25.6% | 45.9% | | | |
| *P. stellifer* DSM 14472$^T$ | | | | | 78–99% |
| *P. graminis* RSA19$^T$ | | | 47.9% | 51.3% | |
| *P. riograndensis* CECT 7330$^T$ | | | 34.5% | | |
| *P. sonchi* LMG 24727$^T$ | | | 38.7% | 73.17% | |
| Content of G + C (mol %) | 46.0 | 50.14 | 52.86 | 49.74 | 52.65–53.9 |

Notes: 1. Which will be specifically discussed in the unpublished result.

2. Which included the strains Gb44, Gz45, Gt48, Gt49, Hz55, Bs63, Hp91and Hp92, they all had been hybridized with type strain *P. stellifer* DSM 14472T and had the DNA hybridization values between 78–99%.

## DISCUSSION

In this study, a total of 534 isolates were selectively obtained from different plant rhizospheres. However, only 23 of 534 isolates had *nifH* gene segments amplified by *PCR* (Fig. 1). Based on 16S rDNA sequence similarities, NifH sequences, G + C content and DNA–DNA hybridization, these strains were identified. It was demonstrated here for the first time that nitrogen fixation abilities and *nifH* gene sequences existed in the type strains *P. stellifer* and *P. jamilae*, and in the *P. stellifer* there existed a novel *nifH* gene which might be firstly detected in *Paenibacillus*. Two new type strains S27 and Be 17 in *Paenibacillus* were obtained (unpublished results). Except all of these, some important facts could be concluded in this study.

Firstly, after following the method of dealing with the soil sample in hot water at 80°C for 15 min, most of the bacteria were killed and almost only endospore-forming *Paenibacillus* could be obtained [21]. The reasons may be that the strains in this genus form a thick outer spore coat which is 30–60% of their weight, and these endospore coats can helps them to survive for many years in a dormant, dry form [22]. The

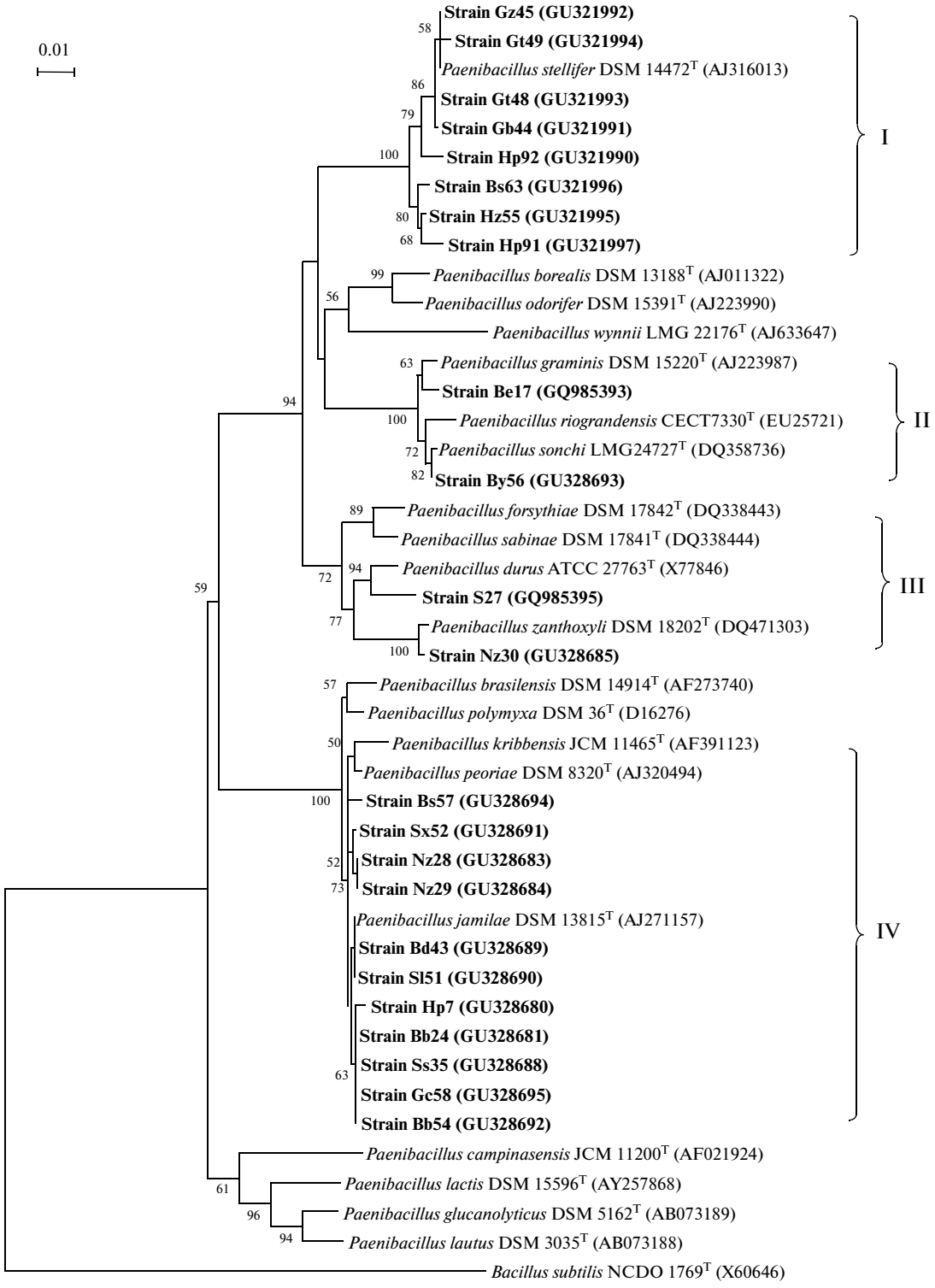


**Fig. 2.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of isolated strains with the nearest species of the genus *Paenibacillus*. *Bacillus subtilis* NCDO 1769$^T$ was used as an out group. Bootstrap percentage values as obtained from 1 000 resamplings of the date set are given at the nodes of the tree. Only values greater than 50% are shown. Bar, 0.01 substitutions per nucleotide position.

endospore is also highly resistant to heat, ultraviolet light radiation, some toxic chemicals, extreme desiccation and oxidizing agents, so these free-living endospore forming *Paenibacillus* has an unexampled positive effect, together with its nitrogen fixing ability as a biofertilizer for non-legumes plants.

Secondly, some of the isolates obtained in this research grew much better on the *NTM* than on *LD* medium, they could secrete much more exopolysaccharides outside the cells on an oligotrophic environment. These root-associated bacteria always have some mechanism to enhance their interaction with host plants, and the exopolysaccharides had been explored as signals between microbe and plant [23–24]. In this research, all of the soil samples were collected from plant rhizosphere, so these exopolysaccharides might play an important role in the recognition and signaling between the bacteria and the host plant [25]. The surface polysaccharides of these strains were also considered to be involved in the capability of symbiotic partners to achieve selection recognition [26]. If the molecular regulated mechanisms of these interaction procedures are revealed, we can not only enhance the interaction between the rhizosphere microorganism and host plants but also expand the usage spectrum of these free-living nitrogen-fixing bacteria.

Thirdly, it was first detected that nitrogen fixation ability and *nifH* gene existed in *P. stellifer* and *P. jamilae*. The *P. stellifer*, proposed as a new species by [27], was initially isolated from paperboard as the mainly contamination; while the speices *P. jamilae* which was also obtained in this study, initially described as a new species by [28], isolated from olive-mill wastewater could secrete exopolysaccharides. In this study, it was found that all of these two species, isolated from different plant rhizosphere in different district of China, showed nitrogen fixation ability and a novel *nifH* gene could be detected in *P. stellifer*. Meanwhile, when *nifH* gene was amplified from type strain *P. stellifer* DSM 14472$^T$ using same condition mentioned above, no specific fragment could be obtained. So it was concluded that even the organism with highly identity phylogenetic position, they still might exhibit different physiological or biochemical function.

In this study, the free-living nitrogen-fixing *Paenibacillus* were isolated from different plant rhizospheres, which suggested that the nitrogen-fixing *Paenibacillus* were abundant in diverse ecosystem with its self endospore-forming properties and resistance to many disadvantage environments. We have confidence that it will definitely closer to developing an eco-friendly nutrient source with the research of reinoculating these nitrogen-fixing microorganisms to different cereal crops. Given that the research succeeds, the significance is far more than reducing the cost for poor farmers but also making a positive contribution to mitigate the more global environment concern from the use of nitrogenous fertilizer [2].

## ACKNOWLEDGMENTS

We thank Dr. Odile Berge in France and Prof Mirja Salkinoja–Salonen in Helsinki University for generous gift the reference type strain. We also thanks for Prof Jose E. Ruiz–Sainz in University of Seville for his help in posting the reference strain from Spain to us. This work was supported by the State Key Basic Research and Development Plan of China (Grant no. 2010CB126504).

## REFERENCES

1. Adams, P. and Kloepper, J., Effect of Host Genotype on Indigenous Bacterial Endophytes of Cotton (*Gossypium hirsutum* L.), *Plant and Soil*, 2002, vol. 240, no. 1, pp. 181–189.
2. Bhattacharjee, R.B., Singh, A., and Mukhopadhyay, S.N., Use of Nitrogen-Fixing Bacteria as Biofertiliser for Non-Legumes: Prospects and Challenges, *Appl. Microbiol. Biotechnol.*, 2008, vol. 80, no. 2, pp. 199–209.
3. Von Bredemann, G., Untersuchungen uber die Variation und das Stickstoffbindungsvermogen des *Bacillus asterosporus* A. M., Ausgefuhrt an 27 Stammen Verschiedener Herkunft. *Zbl. Bakteriol. Parasitenkd. Infektionskr. Hyg.*, 1908, no. 2, pp. 44–89.
4. Ash, C., Priest, F.G. and Collins, M.D., Molecular Identification of rRNA Group 3 Bacilli (Ash, Farrow, Wallbanks and Collins) Using a PCR Probe Test, *Antonie van Leeuwenhoek*, 1993, vol. 64, no. 3–4, pp. 253–260.
5. Auman, A.J., Speake, C.C., and Lidstrom, M.E., *nifH* Sequences and Nitrogen Fixation in Type I and Type II Methanotrophs, *Appl. Environ. Microbiol.*, 2001, vol. 67, no. 9, pp. 4009–4016.
6. Ma, Y., Zhang, J., and Chen, S., *Paenibacillus zanthoxyli* sp. nov., a Novel Nitrogen-Fixing Species Isolated from the Rhizosphere of *Zanthoxylum simulans*, *Int. J. Syst. Evol. Microbiol.*, 2007, vol. 57, no. Pt 4, pp. 873–877.
7. Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning: A Laboratory Manual, *Cold Harbor*, 1989.
8. Ding, Y., Wang, J., Liu, Y., and Chen, S., Isolation and Identification of Nitrogen-Fixing Bacilli From Plant Rhizospheres in Beijing Region, *J. Appl. Microbiol.*, 2005, vol. 99, no. 5, pp. 1271–1281.
9. Bradford, M.M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye Binding, *Anal. Biochem.*, 1976, vol. 72, no. 1–2, pp. 248–254.
10. Yanagi, M. and Yamasato, K., Phylogenetic Analysis of the Family *Rhizobiaceae* and Related Bacteria by Sequencing of 16S rRNA Gene Using PCR and DNA Sequencer, *FEMS Microbiol. Lett.*, 1993, vol. 107, no. 1, pp. 115–120.
11. Kumar, S., Tamura, K., and Nei, M., MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment, *Brief. Bioinform.*, 2004, vol. 5, no. 2, pp. 150–163.
12. Yoon, J.H., Kim, H., Kim, S.B., Kim, H.J., Kim, W.Y., Lee, S.T., Goodfellow, M., and Park, Y.H., Identification of *Saccharomonospora* Strains by the Use of Ge-

nomic DNA Fragments and rRNA Gene Probes, *Int. J. Syst. Bacteriol.*, 1996, vol. 46, no. 2, pp. 502–505.

13. De Ley, J., Reexamination of the Association Between Melting Point, Buoyant Density, and Chemical Base Composition of Deoxyribonucleic acid, *J. Bacteriol.*, 1970, vol. 101, no. 3, pp. 738–754.
14. Ziemke, F., Hofle, M.G., Lalucat, J., and Rossello-Mora, R., Reclassification of *Shewanella putrefaciens* Owen's Genomic Group II as *Shewanella baltica* sp. nov, *Int. J. Syst. Bacteriol.*, 1998, vol. 48, no. Pt 1, pp. 179–186.
15. Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P., and Trüper, H.G., Report of the Ad-Hoc-Committee on Reconciliation of Approaches to Bacterial Systematics, *Int. J. Syst. Bacteriol.*, 1987, vol. 37, no. 4, pp. 463–464.
16. Stackebrandt, E. and Goebel, B.M., Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology, *Int. J. Syst. Bacteriol.*, 1994, vol. 44, no. 4, pp. 846–849.
17. Bolhuis, H., Severin, I., Confurius-Guns, V., Wollenzien, U.I., and Stal, L.J., Horizontal Transfer of the Nitrogen Fixation Gene Cluster in the Cyanobacterium *Microcoleus chthonoplastes*, *ISME J.*, 2010, vol. 4, no. 1, pp. 121–130.
18. Rosado, A.S., Duarte G.F., Seldin L., Elsas J.D. van, and Van Elsas J.D., Genetic Diversity of *nifH* Gene Sequences in *Paenibacillus azotofixans* Strains and Soil Samples Analyzed by Denaturing Gradient Gel Electrophoresis of PCR-Amplified Gene Fragments, *Appl. Environ. Microbiol.*, 1998, vol. 64, no. 8, pp. 2770–2779.
19. Rosado, A.S., de Azevedo F.S., da Cruz D.W., van Elsas J.D., and Seldin L., Phenotypic and Genetic Diversity of *Paenibacillus azotofixans* Strains Isolated From the Rhizoplane or Rhizosphere Soil of Different Grasses, *J. Appl. Microbiol.*, 1998, vol. 84, no. 2, pp. 216–226.
20. Xie, G.H., Cui, Z., Yu, J., Yan, J., Hai, W., and Steinberger Y., Identification of *nifH* Genes in $N_2$-Fixing Bacterial Strains Isolated from Rice Fields Along the Yangtze River Plain, *J. Basic. Microbiol.*, 2006, vol. 46, no. 1, pp. 56–63.
21. Bendt, H.T., How to Control Bacteria Growth in Food-Grade Paperboard Production, *Pulp. Pap.*, 1985, vol. 59, pp. 119–121.
22. Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P., Resistance of *Bacillus* Endospores to Extreme Terrestrial and Extraterrestrial Environments, *Microbiol. Mol. Biol. Rev.*, 2000, vol. 64, no. 3, pp. 548–572.
23. Van Workum, W.A.T., Cremers, H., Wijfjes, A.H.M., vanderKolk, C., Wijffelman, C.A., and Kijne, J.W., Role of Exopolysaccharides of *Rhizobium leguminosarum* bv. *viciae* as Host Plant-Specific Molecules Required for Infection Thread Formation During Nodulation of *Vicia sativa*, *Mol. Plant Microb. In.*, 1998, vol. 11, no. 12, pp. 1233–1241.
24. Djordjevic, S.P., Chen, H., and Batley, M., Nitrogen Fixation Ability of Exopolysaccharide Synthesis Mutants of *Rhizobium* sp. Strain NGR234 and *Rhizobium trifolii* is Restored by the Addition of Homologous Exopolysaccharides, *J. Bacteriol.*, 1987, vol. 169, no. 1, pp. 53–60.
25. Albersheim, P. and Anderson-Prouty, A.J., Carbohydrates, Proteins, Cell Surfaces, and the Biochemistry of Pathogenesis, *Annu. Rev. Plant. Physiol.*, 1975, vol. 26, no. 1, pp. 31–52.
26. Gaskins, M.H., Albrecht, S.L., and Hubbell, D.H., Rhizosphere Bacteria and Their Use to Increase Plant Productivity: A Review, *Agr. Ecosyst. Environ.*, 1985, vol. 12, no. 2, pp. 99–116.
27. Suominen, I., Sproer, C., Kampfer, P., Rainey, F.A., Lounatmaa, K., and Salkinoja-Salonen, M., *Paenibacillus stellifer* sp. nov., a Cyclodextrin-Producing Species Isolated From Paperboard, *Int. J. Syst. Evol. Microbiol.*, 2003, vol. 53, no. Pt 5, pp. 1369–1374.
28. Aguilera, M., Monteoliva-Sanchez, M., Suarez, A., Guerra, V., Lizama, C., Bennasar, A., and Ramos-Cormenzana, A., *Paenibacillus jamilae* sp. nov., an Exopolysaccharide-Producing Bacterium Able to Grow in Olive-Mill Wastewater, *Int. J. Syst. Evol. Microbiol.*, 2001, vol. 51, no. 5, pp. 1687–1692.
29. Ma, Y.C. and Chen, S.F., *Paenibacillus forsythiae* sp. nov., a Nitrogen-Fixing Species Isolated from Rhizosphere Soil of *Forsythia mira*, *Int. J. Syst. Evol. Microbiol.*, 2008, vol. 58, no. 2, pp. 319–323.
30. Smith, N.R., Gibson, T., and Gordon, R.E., and Sneath, P.H.A., Type Cultures and Proposed Neotype Cultures of Some Species in the Genus *Bacillus*, *J. Gen. Microbiol.*, 1964, vol. 34, pp. 269–272.