INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18F–042/2018/20–7–1672–1676
DOI: 10.17957/IJAB/15.0705
http://www.fspublishers.org

Full Length Article

Biological Diversity of Azotobacteria Isolated from Soils of Mount Emei, China

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Abstract

Analyses were performed of the phenotypic features, ERIC-PCR fingerprints, and 16S rDNA sequences of 58 free-living nitrogen-fixing bacteria (named also azotobacteria) isolated from soils of Mount Emei, Sichuan, China. Four clusters of azotobacteria, defined by 0.32 of Watson's distance, were identified in the numerical analysis: clusters A, B, C, and D, which had 04, 45, 01, and 08 strains, respectively. The results of the ERIC-PCR fingerprints analysis revealed the formation of nine clusters tested strains with a Watson's distance of 0.65Watson's. Using 16S rDNA sequencing analysis, we identified the typical strains in the nine clusters as six genera, *Enterobacter*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, *Serratia*, and *Yersinia*. © 2018 Friends Science Publishers

Keywords: Mount Emei; Azotobacteria; Phenotypic diversity; Numerical taxonomy; Genetic diversity

Introduction

Free-living nitrogen-fixing bacteria, also named azotobacteria, are an important component of plants rhizosphere-promoting bacteria because of their ability for biological nitrogen fixation (Chen *et al*., 2006). The study of azotobacteria began at the end of the 19th century (Chen, 2007). So far, dozens of bacteria with ability for nitrogen autotrophy that are widely distributed in the soil and water have been identified. It has been reported that the annual nitrogen fixation rate of azotobacteria in the soil can reach 60 kg / hm^2 (Fan, 1991). Although nitrogen fixation rate is relatively low compared with the symbiotic nitrogen fixation system, the growth of azotobacteria may have a more important role in natural ecosystems, especially in barren wasteland nitrogen cycle. Increased significance of azotobacteria is due to their many advantages, including the lack of necessity for cooperation with specific plants for their nutrition and growth, as well as their large amounts, wide distribution, and strong adaptability.

Mount Emei is located in Mount Emei City, Sichuan province, China. The region from Qingyin Pavilion to Elephant Bathing Pool is a middle-mountain area with a temperature 4–5°C lower than that of the low-mountain area (e.g., at the Baoguo Temple at Ningbo). However, high-mountain area is spread from Elephant Bathing Pool to the Golden Peak Temple, which is characterized by a temperature that is around 12°C lower than the one of the low-mountain area. These complex specific climatic conditions inevitably affect the ecological distribution of azotobacteria in the soil of Mount Emei.

Enterobacterial Repetitive Intergenic Consensus (abbreviated as ERIC) is a reverse-repeat sequence in the genome of some Gram-negative bacteria, presented by multiple copies with an initial length of 126 bp in multiple copies (Sharples and Lloyd, 1990). Hulton *et al.* (1991) also found the same highly conserved repetitive sequence in their experiment; this sequence was predominantly present in the Enterobacteriaceae family. Enterobacterial repetitive intergenic consensus-polymerase chain reaction (abbreviated as ERIC-PCR) is an important method of molecular biology typing, in which a set of primers are designed for PCR amplification based on reverse-repeats in the repeating consensus sequence of Enterobacteriaceae. The unique conserved position of the primers allow this PCR to produce a variety of unique amplification products, and distinction of specific bacterial strains (types) can be performed based on the electrophoretic bands of the amplification products (Gao *et al*., 2003; Luan, 2007; Yao, 2007).

In recent years, 58 strains of azotobacteria were isolated from the soil in Mount Emei. In this research, the phenotypic diversity, genetic diversity and phylogeny of 58 strains were studied by using culture characteristics, physiological and biochemical characteristics, ERIC-PCR and 16S rDNA full sequence sequencing method.

Materials and Methods

Soil Samples

Experimental sites (Altitude: Golden Summit Temple 3071

To cite this paper: Hu, X., Q. Guan, P. Yin, M. Gong and Y. Chen, 2018. Biological diversity of azotobacteria isolated from soils of Mount Emei, China. *Int. J. Agric. Biol*., 20: 1672–1676

m, Leidongping 2430 m, Xixinsuo Temple 1575 m, Zero kilometer 1380 m, Wannian Temple 1029 m, Qingyin pavilion 750 m, Baoguo Temple 551 m) were randomly selected at different altitudes. Each experimental site was divided into three 3 m × 3 m sample areas (sample spacing 5 m). After the surface litter and gravel were removed, eight tubes with soil samples were collected using the diagonal cross-sampling method. A soil sampler with a diameter of 2.5 cm was used at a depth of 15 cm to collect the samples. Five repeats of specimens were taken in each area investigated, mixed evenly into sterile self-styled bags, and transported to the laboratory for isolation of azotobacteria.

Isolation of Azotobacteria

Azotobacteria were isolated by the dilution coating plate method (Sheng *et al.,* 2006) using Ashby's nitrogen-free culture medium (Chen *et al.,* 2006). Upon appearance of bacterial growth, rounded colorless transparent sticky individual colonies were selected from the culture medium.

Phenotypic Characterization and Phenotypic Diversity Analysis of 58 Strains Azotobacteria

Based on the WHITE medium, a total of 81 physiological and biochemical characteristics of the tested strains were determined (He *et al*., 2004; Gong *et al*., 2005). The specific experimental methods and media preparation referred to the method described by Wei *et al*. 2003). There were 15 carbon sources, including maltose, sodium pyruvate, D-fructose, D-xylose, L-rhamnose, D-galactose, L-arabinose, D-sorbitol, D-sorbitol, crystalline sodium acetate, sodium uranate, inositol, L-sorbose, L-malic acid, and sucrose. The glucose in the WHITE medium was replaced by the corresponding tested carbon source (1 g L^{-1}). NA medium was used as a positive control and the WHITE medium without a carbon source as a negative control (Gong *et al*., 2005). There were 19 nitrogen sources, including L-methionine, L-isoleucine, L-alanine, L-serine, L-cystine, L-cystine, L-threonine, L-threonine, L-alanine, L-lysine, L-tryptophan, L-lysine, L-leucine, L-cysteine, L-tryptophan, glycine, urea, and histidine, replace $NaNO_3$ in the WHITE medium as a corresponding test nitrogen source (10 g L^{-1}) and added glucose (10 g L^{-1}) as a carbon source. There were 20 antibiotic treatments, including 5 kinds of antibiotics (spectinomycin, tetracycline hydrochloride, streptomycin sulfate, carboxy penicillin, and gentamicin sulfate) and 4 concentrations (5 μg mL^{-1}, 50 μg mL^{-1}, 100 Mg mL^{-1} and 300 μg mL^{-1}). There were 10 dye resistance, including 5 kinds of dye (gentian violet, crystal violet, methyl red, malachite green, and Congo red) and 2 concentrations (1 g L^{-1} and 2 g L^{-1}). In addition, the characteristics of salt tolerance, acid and alkali resistance, initial pH value, optimum growth temperature, oxidase and catalase were measured (Gong *et al*., 2005). Each trait test was repeated three times, except for the ones in which the carbon and nitrogen sources were tested with WHITE medium, other properties were measured using NA medium.

Phenotypic characterization test results were recorded as "1" for positive response and negative for "0", after encoding into computer (Gong *et al*., 2009b), 0–1 system clustering analysis with the class average chained clustering method (UPGMA) was analyzed by DPS software (Guan *et al*., 1997; Tang and Feng, 2007) with Watson distance to obtain phenotypic diversity clustering tree.

ERIC-PCR Genetic Diversity of 58 Strains Azotobacteria

The total DNA of azotobacteria was extracted by the modified SDS method (Chen *et al*., 2009), specific primers, total reaction system and ERIC-PCR reaction procedure based on the method described by Ma *et al*. (2008). After the end of the reaction, PCR tubes were removed and 1% agarose gel electrophoresis was performed for 3.5 h, Next, the samples were transferred into a gel imaging system. After the gel image was scanned by the computer, the tape at the same position was recorded as "1", and the lack of a tape at the same position was recorded as "0". In the DPS data processing system (V7.05) (Tang and Feng, 2007), we followed the average chained clustering method (UPGMA) based on the Watson's distance to classify the 0–1 system cluster, obtaining an ERIC-PCR genetic diversity tree (Zhang, 2006).

Determination of 16S rDNA Sequences and Phylogenetic Analysis of Azotobacter

16S rDNA amplification primers (P1 and P6) (Chen *et al*., 2009) were synthesized by Shanghai Biotech Biotechnology Engineering Service Co., Ltd, then the amplified products sequencing of the tested strains was also undertake by the company. The prepared sequences were identified in the public database such as GenBank (http://www.ncbi.nlm.nih.gov/), and the sequence of 16S rDNA gene of the relevant strain was clustered with clustal X. System evolution matrix, which was estimated by Kimura model. MEGA4.0 (Molecular Evolutionary Genetics Analysis) software was used to perform neighbor-joining cluster analysis, based on the results of which, a phylogenetic tree was constructed (Gong *et al.*, 2009a; Yang *et al.*, 1999).

Results

Isolation of Azotobacter in Mount Emei Soil

A total of 58 strains of azotobacter were isolated from the soil in Mount Emei, which were numbered from N1 to N58.

Phenotypic Diversity Analysis of 58 Strains Azotobacteria

The numerical classification tree showed that all tested strains were clustered together at a Watson distance of 0.39 and divided into four clusters at the Watson distance of 0.32, at which, N49 appeared as an independent group (apparent group C) as shown in Fig. 1. Cluster A, B and D consisted of 4 (N9, N9, N9 and N28), 45 (the remaining 45 strains) and 8 (N50, N51, N52, N53, N54, N55, N56, and N58) strains, respectively. Cluster B was further divided into 12 subgroups at a Watson distance of 0.16.

ERIC-PCR Genetic Diversity of 58 Strains Azotobacteria

By the ERIC-PCR amplification map (shown in Fig. 2), all tested strains were clustered at a genetic distance of 0.83 and further classified into 9 ERIC groups at the genetic distance of 0.65, respectively named A, B, C, D, E, F, G, H, and I (in Fig. 3).

Phylogenetic Analysis of Azotobacter

By 16S rDNA sequencing analysis, 23 tested strains and related known strains with high similarity can be divided into four branches (*Enterobacter*, *Bacillus*, *Acinetobacter* and *Pseudomonas*), including 6 genera (*Enterobacter*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, *Yersinia* and *Serratia*) (in Fig. 4).

Eleven strains (N11, N29, N12, N40, N40, N30, N34, N43, N50, N57, and N1) together constituted the developmental branch of *Bacillus*, in which four strains (N11, N29, N12, and N40) were closely related to *Bacillus pumilus*, strain N57 was closely related to *Bacillus subtilis*, three strains (N30, N34, and N43) of them were closely related to *Bacillus atrophaeus*, and the remaining three strains (N14, N50 and N1) were closely related to *Bacillus cereus*. Four strains (N5, N18, N48, and N8) constituted the branch of *Enterobacter*; Other four strains (N36, N16, N20, and N24) constituted the branch of *Acinetobacter*. Additionally, strains N52 and N49 constituted the branch of *Pseudomonas*; Strain N55 was closely related to *Serratia marcescens*; Strain N9 was closely related to *Yersinia massiliensis.*

Discussion

In this study, 58 strains of azotobacteria were isolated from soils in Mount Emei, China. The analysis results of 81 phenotypic characteristics of the 58 strains showed significant differences among the different strains, which was a result of their long-term adaptation to different environmental conditions (Gong *et al.*, 2009b). The exposure of these strains to long-term saline-alkali, drought, and other selective pressures at different growth

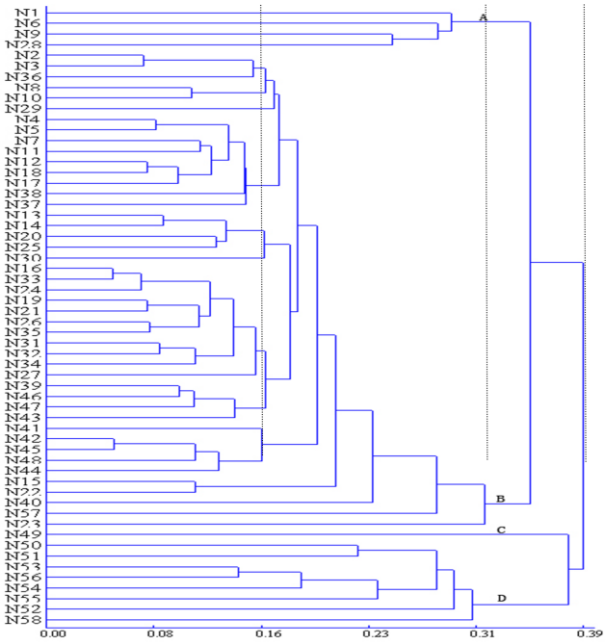

Fig. 1: Numerical Classification Tree of Phenotypic Characteristics of 58 strains azotobacteria

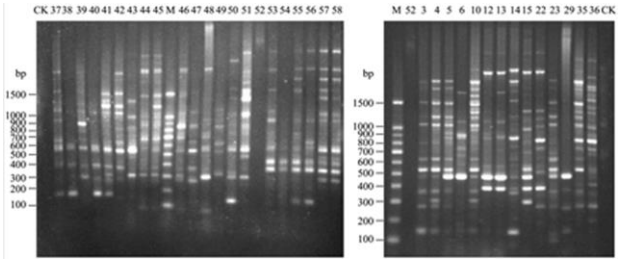

Fig. 2: ERIC-PCR fingerprints of different azotobacteria. CK means Negative control; M means 100 bp ladder marker

periods, as well as to the influence of other factors, led to the acquisition of the above characteristics. Generally, salt-resistant strains exhibited also a strong drought resistance. The rich genetic resources contained in these salt-resistant strains were of substantial significance in the desertification control in high temperature saline-alkali areas.

Based on the analysis of 81 phenotypic characteristics, 58 strains could be divided into four clusters at the Watson distance of 0.32. Some of them had unique physiological and biochemical characteristics. For example, three strains (N1, N6, and N28) did not use L-aspartic acid as their sole nitrogen source. Moreover, strain N49 used only maltose and sucrose as carbon sources. Additionally, strains N1 and N6 displayed tolerance to methyl blue in concentrations of 1 g L^{-1} and 2 g L^{-1}, respectively. Strains N23 possessed tolerance to all the antibiotics except for tetracycline hydrochloride.

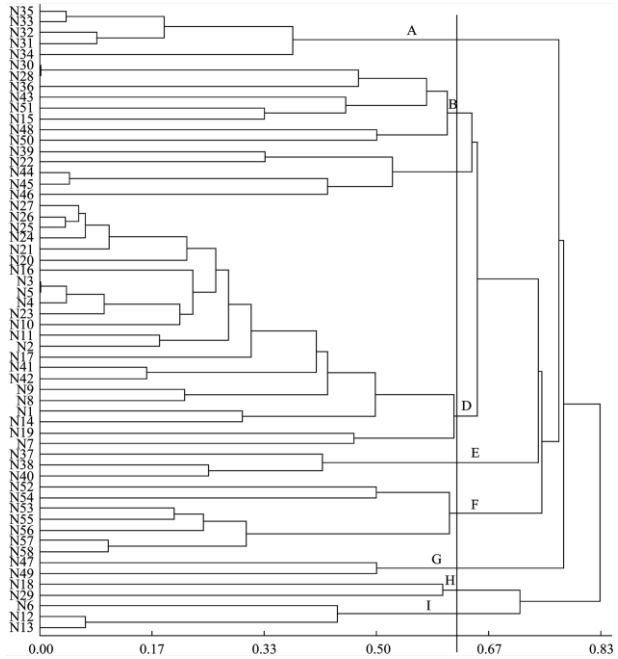

Fig. 3: ERIC-PCR clustering analysis tree of 58 strains azotobacteria. N1-N58: tested strains; A-I: ERIC group number; 0.01: genetic distance

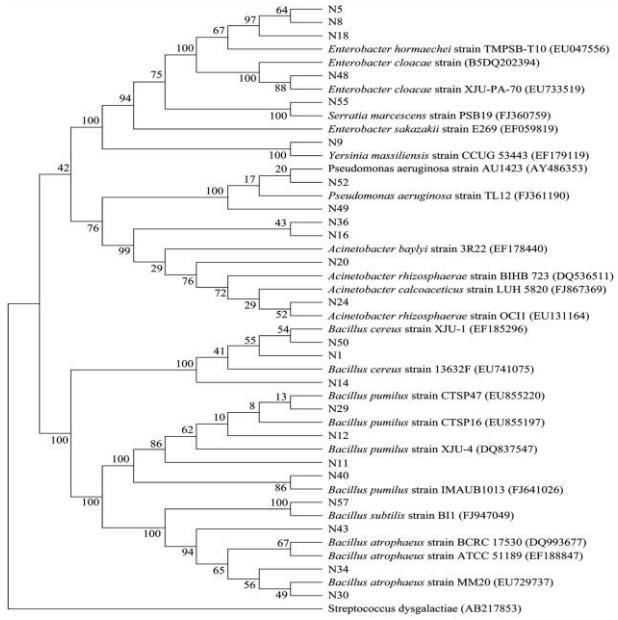

Fig. 4: 16S rDNA full sequence phylogenetic tree of 23 strains azotobacteria. The number on the branch in the figure indicates the tree confidence, and the accession number of the strain in the gene bank was shown in the brackets

The number of azotobacteria in soil of Mount Emei varied greatly with the change of climatic conditions. Does this change law relevant to the nitrogen fixation of azotobacteria in the special micro-ecological environment of Mount Emei? What types of azotobacteria in the different soils of Mount Emei can play an important role on nitrogen fixation of the micro-habitat in Mount Emei? The answers to these questions are important to find for the successful establishment of a healthy and balanced soil micro-ecosystem in the area of Mount Emei.

Conclusion

Cluster analysis showed that the tested azotobacteria formed 4 clusters defined by 0.32 of Watson distance, clusters A, B, C and D had 4, 45, 1 and 8 strains, respectively. Nine clusters of tested strains were formed at a Watson's distance of about 0.65 based on the results of the ERIC-PCR fingerprints analysis. The typical strains of 9 clusters were identified as *Enterobacter*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, *Serratia*, *Yersinia* by 16S rDNA sequencing analysis.

The 58 strains of azobacteria in the different soils of Mount Emei were divided into nine ERIC-PCR groups, respectively belonging to *Enterobacter*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, *Yersinia* and *Serratia.* .Our findings indicate that the genetic diversity of azotobacteria in the soil of Mount Emei is abundant. The exact classification status of these azotobacteria also needs to be confirmed by combining the physiological and biochemical characteristics of the strain with the hybridization of the known strain.

Acknowledgements

This work was supported by the project of the Science and Technology Department in Sichuan province (2018NFP0107), the Natural Science Foundation of China (31500346) and the scientific research fund of Sichuan Provincial Education Department (15TD0026, 16ZA0306).

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(Received 22 March 2018; Accepted 02 April 2018)