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Different Methods for Isolation and Preliminary Identification of *Azotobacter*

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are known to influence plant growth by various direct or indirect mechanisms. Thirty–two strains were isolated from 15 soils sampled in central Iran, by using and comparing three different methods. The screening of soil samples by means of soil paste–plate method combined with isolation on mannitol agar proved to be the best strategy in terms of reliability and selectivity. These test isolates were biochemically characterized. These isolates were screened in vitro and identified by using BIBI(Bioinformatics Bacterial Identification Tool). BIBI was designed to automate DNA sequence analysis for bacterial identification in the different fields. BIBI relies on the use of BLAST and CLUSTAL W programs applied to different subsets of sequences extracted from GenBank. These sequences are filtered and stored in a new database, which is adapted to bacterial identification.

Keywords: PGPR, BIBI, CLUTAL W, BLAST.

INTROBUCTION

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and or indirectly. In last few decades a large array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligens*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* have reported to enhance plant growth (Kloepper *et al*., 1989; Okon & Labandera-Gonzalez, 1994; Glick, 1995). The direct promotion by PGPR entails either providing the plant with a plant growth promoting substances that is synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effect of one or more phytopathogenic microorganisms.

The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of plant growth regulators like indoleacetic acid, gibberellic acid, cytokinins and ethylene (Arshad & Frankenberger, 1993; Glick, 1995), (ii) asymbiotic N2 fixation (Boddey & Dobereiner, 1995), (iii) antagonism against phytopathogenic microorganisms by production of siderophores (Scher & Baker, 1982), antibiotics (Shanahan *et al*., 1992) and cyanide (Flaishman *et al*., 1996), (iv) solubilization of mineral phosphates and other nutrients (De Freitas *et al*., 1997; Gaur, 1990). Most popular bacteria studied and exploited as biocontrol agent includes the species of fluorescent Pseudomonas and Bacillus. Some PGPR may promote plant growth indirectly by affecting symbiotic N2 fixation, nodulation or nodule occupancy (Fuhrmann & Wollum, 1989). However, role of cyanide production is contradictory as it may be associated with deleterious as well as beneficial rhizobacteria (Bakker & Schippers, 1987; Alstrom & Burns, 1989).

In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil (Cattelan *et al*., 1999). Unfortunately, the interaction between associative PGPR and plants can be unstable. The good results obtained in vitro cannot always be dependably reproduced under field conditions (Chanway & Holl, 1993; Zhender *et al*., 1999). The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effect on the plant. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil. To achieve the maximum growth promoting interaction between PGPR and nursery seedlings it is important to discover how the rhizobacteria exerting their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms (Bent *et al*., 2001).

MATERIALS AND METHODS

SOIL SAMPLING

Soil samples were collected during Spring in different regions of central Iran from cultivated soils. Samples were withdrawn at a depth of 10–15 cm below the surface, collected into sterile vials as described by Kole & Altosaar (1988), sieved through a 4–mm–mesh sieve, and stored at 4°C.

ISOLATION

Three different isolation methods were used: (a) streaking of serial soil dilutions on plates containing Ashby medium containing (per 1l): 20 g mannitol, 0.2 g K_2HPO_4, 0.2 g $MgSO_4$–$7H_2O$, 0.2 g NaCl, 0.1 g K_2SO_4, 5 g $CaCO_3$, 15 g agar (Brown *et al*., 1962; Knowles, 1982); (b) enrichment in Winogradsky solution for 7–14 days (Augier, 1956; Pochon & Tardieux, 1962) followed by streaking onto Ashby medium; (c) a combination of the soil paste (Becking, 1981) and the direct sowing of

single soil grains (Pochon, 1954) methods realised as follows: about 30–50 g of each soil sample were accurately mixed with 20% (v/w) of sterile water with 0.5–1.0 g of mannitol, 0.5 g of $CaCO_3$, 0.12 ml of 10% aqueous K_2HPO_4 solution, 0.12 ml of 10% aqueous $MgSO_4$ solution. The soil paste, prepared in a porcelain mortar, was transferred and pressed inside a petri dish with a sterile spatula to obtain a smooth and levelled surface. After 3–7 days incubation at 27–30°C, the soil paste–plates presenting growth of Azotobacter were revealed by the appearance of slimy, glistening colonies, turning brown with aging if produced by the species A. chroococcum. Subsequently, in order to carry out isolation, soil samples resulted positive for the presence of these free–living nitrogen–fixing bacteria were subjected to sowing of single grains on the Mannitol-agar medium proposed by Pochon (1954), containing (per 1l): 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$.$7H_2O$, 0.1 g NaCl, 1.0 g yeast extract, 3.0 g $CaCO_3$, 20 g agar (Becking, 1981).

All the isolates were purified by streaking on NA plates. Long–term storage of the purified isolates was at –80°C in the LG broth medium with 50% (w/v) glycerol.

SCREENING OF ISOLATES

The bacterial isolates were characterized by their cultural conditions, morphological and biochemical characteristics (utilization of glucose, fructose, maltose, raffinose, trehalose, growth at diffrerent temperatures, catalase, oxidase, Gram-stain reaction) using standard methods (Cappuccino & Sherman, 1992).

MOLECULAR ANALYSIS

For molecular analysis, one isolete was grown for 2–3 days on LG. Crude template DNA was extracted using alkaline lysis method(Rademaker & de Bruijin, 1997). The 16S rRNA gene was amplified by means of universal primers 27f and 1495r (Weisburg *et al*., 1991). The PCR reaction was run for 35 cycles as follows: denaturation at 94°C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 2 min. An initial denaturation step at 95 °C for 4 min and a final extension step at 72 °C for 15 min was also performed and the PCR product was sequenced by biobasic company(Canada).

BIOINFORMATICS ANALYSIS

For bioinformatic analysis, we used a specific bioinformatics tool dedicated to bacterial identification (BIBI, for Bioinformatics Bacterial Identification) in order to simplify sequences analysis within a bacterial identification framework. BIBI fully automates and speeds up different operations for the treatment of sequences. BIBI, which can be accessed at http://pbil.univ –lyon1.fr/bibi/, enables the identification of a microorganism from a gene fragment sequence of previously described cultured bacteria.

The program implements a chaining of two well-known tools: BLAST (Altschul *et al*., 1997) and CLUSTAL W (Thompson *et al*., 1994). CLUSTAL W runs are accelerated by the use of prealigned BLAST results. BIBI is written in standard ANSIC language, and the interface is implemented in HTML–PHP. Analysis of an unknown sequence proceeds in four phases: search for matching sequences, sequence extraction and parsing, sequence alignment, and display of results.

RESULTS AND DISCUSSION

SOIL SAMOLING

The sampling strategy described in this work was chosen taking into account the different parameters influencing the presence of azotobacteria in soil. Since distribution of Azotobacter in the rhizosphere is not dependent on the type of plant (Kole & Altosaar, 1988) soil samples were indifferently collected from the rhizosphere of gramineum.

ISOLATION METHODS

The three methods utilised in the present work were described by different authors as feasible for Azotobacter isolation. Method ‘a’ allowed the direct isolation of Azotobacter like colonies on selective Ashby medium from 18 out of 15 soil samples utilised. All members of genus Azotobacter produced slimy, glistening, smooth, whitish, weakly convex, colonies.

Method ‘b’ was tested on 15 soil samples. As expected, growth of Azotobacter strains on Winogradsky solution was revealed by an increase of turbidity and the appearance of a thin pellicle on the liquid surface. Moreover, growth of most Azotobacter was accompanied by the production of diffusible pigments. Although, the results observed with the strains in Winogradsky solution were unambiguous, those obtained after inoculation with soil sample dilutions were not so easily understandable. Indeed, growth of different microorganisms (e.g. aerobic and microaerophilic species in the nearby of the liquid surface) led to the production of a milky and creamy pellicle, browning with aging, and to a significant increase in turbidity which rendered the observation of diffusible pigments not possible. Consequently, due to the impossibility to individuate the positive tubes for Azotobacter, pellicles coming from all the tubes showing growth were streaked onto Ashby medium. In this way, two objectives were contemporary pursued: the individuation of Azotobacter like colonies and the achievement of pure cultures. As a result, 14 Azotobacter like colonies were isolated from 15 soil samples screened.

According to method ‘c’, soil samples to be employed in the isolation step were selected by means of the soil paste–plate technique, thanks to the appearing of slimy and glistening colonies. upon the smoothed soil paste surface. 21 out of 15 soil samples screened were therefore selected and utilised for Azotobacter isolation onto mannitol medium, through the direct sow of single soil grains. The utilisation of this combined method led to the isolation of 15 Azobacter–like cultures.

SCREENING OF THE ISOLATES

On the basis of cultural, morphological and biochemical characteristics a total of 32 soil isolates were grouped into Azotobacter (Table 1) as described in Bergey’s Manual of Determinative Bacteriology (Holt *et al*., 1994). Also, Results obtained of sequencing indicated that strain Az_1 belonging to genus *Azotobacter*. Phylogenetic tree are displayed by Java applet: Jalview (version 1.7) [http://www2.ebi.ac.uk/_michele/jalview/]) (Fig. 1). The tree revealed that Az_1 is similar with strains *A. chroococcum* ISSD- 356, *A. chroococcum* ISSD- 859, *A. chroococcum* ISSD- 863, *A. chroococcum* ISSD- 347, *A. chroococcum* ISSD- 865, *A. chroococcum* ISSD- 86, *A. chroococcum* ISSD- 397, *A. chroococcum* ISSD- 10006.

Table 1: Biochemical and morphologic characterization of the test isolates

Biochemical and morphologic characters	*Azotobacter*
Number of isolates	32
Gram reaction	-
Catalase	+
Oxidase	+
Carbohydrate utilization:	
Glucose	+
Fructose	+
Maltose	+
Raffinose	+
Trehalose	+
Growth in different temperatures	+

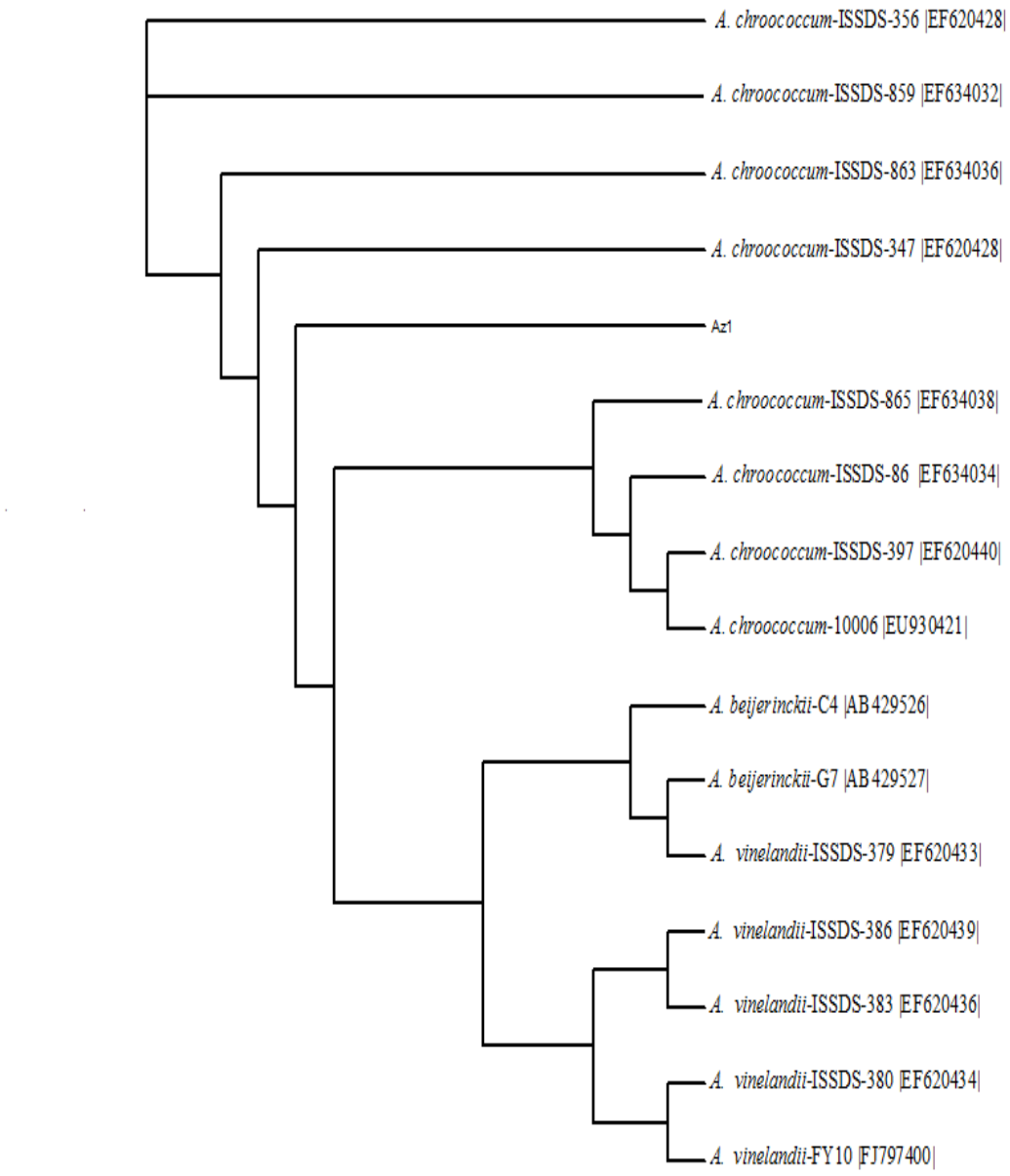

Fig. 1: phylogenetic tree of strain Az_1

CONCLUSION

This research work firstly aimed to compare three different methods reported in literature for the isolation of free N-fixing bacteria from soil samples, in order to individuate the most effective one. In second instance, it aimed to verify whether LG medium, described up to now as a selective substrate for the isolation of Azotobacter can be successfully employed to screen soil isolates for a presumptive recognition of microorganisms belonging to the genus of interest.

In conclusion, our results showed that the most reliable strategy for the isolation and preliminary identification of *Azotobacter* is given by the combination of paste–plate and soil grains sowing methods followed by screening on LG.

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