Microbial Pathogenesis 111 (2017) 225–231

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Molecular and phenotypic characterization of endophytic bacteria isolated from sulla nodules

Hamida Beghalem [a, *], Khadidja Aliliche [b], Ali Chriki [b], Ahmed Landoulsi [a]

[a] Laboratory of Biochemistry and Molecular Biology, Code UR13ES34 Research Unit, Carthage University, Faculty of Sciences of Bizerte, Zarzouna, Bizerte, Tunisia
[b] Laboratory of Genetics, Faculty of Sciences of Bizerte, 7021 Zarzouna, Tunisia

ARTICLE INFO

Article history:
Received 20 April 2017
Received in revised form
18 August 2017
Accepted 30 August 2017
Available online 1 September 2017

Keywords:
Sulla
16Sr RNA
Housekeeping genes
phenotypic characterization
Endophytic bacteria

ABSTRACT

In the current study, bacterial diversity was investigated in root nodules of *Sulla pallida* and *Sulla capitata*. The isolates were analyzed on the basis of their phenotypic and molecular characteristics. The phylogenetic analysis based on *16S rRNA* and housekeeping genes (*recA* and *atpD*) showed that the isolated bacteria related to *Sinorhizobium*, *Neorhizobium*, *Phyllobacterium*, *Arthrobacter*, *Variovorax* and *Pseudomonas* genera. This is the first report of *Neorhizobium* genus associated with *Hedysarum* genus. Phenotypically, all strains tolerate the elevated temperature of 40 °C, and salt stress at a concentration of 2%. In addition, the isolates failed to induce nodulation on their original host; and the symbiotic genes could not be amplified, suggesting that these strains are endophytic bacteria.

1. Introduction

Fabaceae (Leguminosae) is the third largest family of flowering plants with 800 genera and 20,000 species [33]. Within this family, the genus Hedysarum L. comprises about 150 species [42]. It assembled in two main groups, the first group includes the Alpine, Arctic and Asiatic species (2n = 2x = 14 chromosomes) and the second Mediterranean species (2n = 2x = 16) [25]. The systematic of the genus Hedysarum were reviewed and regrouped six Mediterranean species into genus Sulla: *S. coronaria*, *S. capitata*, *S. spinosissima*, *S. pallida*, *S. carnosa*, *S. flexuosa* [7]. They are able to improve soil fertility, and can be exploited in the improvement of degraded areas.

A specific trait of legumes is their ability to establish nitrogen-fixing symbiosis with root-nodule rhizobia. The term bacteria nodulating legume (BNL) was proposed by Ref. [44]. Currently, the described BNL have been classified as belonging to the alpha subgroup of the Proteobacteria, in the Rhizobiaceae family represented by: *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Ensifer* and *Azorhizobium*. Additionally, new genera were found in the α-Proteobacteria class, namely *Devosia*, *Methylobacterium*, *Aminobacter*, *Microvirga*, *Ochrobactrum*, *Shinella*, *Phyllobacterium*, and *Neorhizobium* [18,22,23,34,38].

In addition to rhizobial species, several other bacterial species can co-occupy legume nodules without a clear indication of their role within the host. In the negative nodulation test result, they are considered as endophytic bacteria.

Pseudomonas spp., *Enterobacter* spp., and *Bacillus* spp. are the most abundant endophytic bacteria in legumes [15,45]. Refs. [4,28] reported that endophytes bacteria produce different types of metabolites that play a role in connexion with the plant host. While, the ecological role of endophytic bacteria is not well known, they may have roles in nodulation, plant growth and disease suppression [11].

In this study, bacterial diversity was investigated in root nodules of *S. pallida* (north- African endemic) and *S. capitata* using RFLP analysis of *16S rRNA* gene and sequencing analysis of the *16S rRNA* and housekeeping genes (*recA* and *atpD*). Their symbiotic capacity was checked by the nodulation test and the amplification of *nodC* and *nifH* genes.

2. Materials and methods

2.1. Isolation of bacteria from root nodules

For bacterial isolation, root nodules from *S. pallida* (North

* Corresponding author.
E-mail address: beghalemhamida@hotmail.fr (H. Beghalem).

http://dx.doi.org/10.1016/j.micpath.2017.08.049
0882-4010/

African endemic) and *S. capitata* were collected from Algerian soils (Oran: 35°41′27″ N, 0°38′30″ W; climate: semi-arid; Mascara: 35°23′25″ N, 0°8′58″ E; climate: semiarid), and from Tunisian soils (Siliana: 36°4′55″ N, 9°22′29″ E; climate: semi-arid; Sidi Bouzid (35°2′17″ N, 9°29′5″ E; climate: arid)). 3–5 root samples were collected from each site and were carefully rinsed and stored dried in tubes containing $CaCl_2$.

The nodules were surface sterilized by subsequent immersions in 95% ethanol for 20 s then in 2.5% sodium hypochlorite for 3 min and rinsed eight times with sterile water. The sterilized nodules were crushed in 100 μl of sterile water; the obtained suspension was streaked on yeast-extract mannitol agar (YEMA) plates [39] with Congo red 0.0025% [9,14]. After 10 days of incubation at 28 °C, bacterial colonies were picked on the basis of their absorption of Congo red, their morphologies and number of days of growth. Single chosen colonies were purified several times and stored on 20% glycerol at −20 °C.

2.2. Phenotypic characterization and nodulation test

Divers phenotypic characteristics of isolated bacteria such as the ability of isolates to use carbohydrates (1%: glucose, lactose, maltose saccharose, glycogen and glycerol), and amino acids (0.1%: L-glycine, L-histidine, L-phenylalanine, and L-cysteine) as a sole carbon and nitrogen sources were respectively tested. Acid and alkali production were corroborated YMA with 0.0025% bromothymol blue [9,14]. Tolerance to NaCl (0.5, 1, 2, 3 and 4%); temperatures (4, 28, 37, 40 and 42 °C) and pH (5, 6, 7, 8, 9, 10 and 12). Resistance to heavy metals was tested on solid YEM medium containing the following filter-sterilization heavy metals: in (μg ml^{-1}), $CdCl_2$ (50), $CoCl_2$ (50), $HgCl_2$ (50), $AlCl_3$ (400), and antibiotic resistance: Streptomycin: 60 and 100 μg ml^{-1}, Ampicillin: 60 and 100 μg ml^{-1}, Kanamycin: 60 and 100 μg ml^{-1} and oxalycin: (50 and 100 μg ml^{-1}) and colimycin (50 and 100 μg ml^{-1}). Plates were incubated at 28 °C for 7 days.

To realize the nodulation test, the collected seeds were scarified and surface sterilized in 98% sulfuric acid for 30 min. After acid treatment, the seeds were washed thoroughly with sterile water and germinated in Petri dishes in the dark at 25 °C. In order to evaluate the infectivity of isolates, the germinated seedlings were aseptically transferred to tubes containing Fahraëus seedling agar [39]. Seedlings were inoculated with individual strains by adding 1 ml of the exponentially growing bacterial cultures. Uninoculated seedlings were used as negative controls. Fifteen isolates were further tested for symbiotic efficiency on their origin host, three replicates were included. Plants were watered twice a week with a sterilized nitrogen free nutrient solution. After 2 months, the roots were checked for nodulation.

2.3. PCR amplification of 16S rRNA gene

DNA was extracted according to the method of Ref. [6]. Primers fD1 and rD1 [41] were used for PCR amplification of the almost complete *16S rRNA* gene. Polymerase chain reaction (PCR) amplification was carried out in a 50 μL volume mixing 1 μL of template DNA with polymerase reaction buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 100 μM dNTPs, 0.5 μM of each primer, and 1.25U of Taq DNA polymerase (Vivantis Technologies). PCR amplifications were performed in a thermal cycler PCR Express instrument (Techgene France). PCR products were examined by electrophoresis on a Tris–Borate–EDTA (TBE) 1% agarose gel (100 V) with 1 μL aliquots of PCR product.

The aliquot of the PCR products was digested separately with restriction endonucleases *Hin*fI, *Hae*III, and *Msp*I according to the production guide. The restricted fragments were separated by electrophoresis in 2. 5% (w/v) agarose gels, and then were combined for clustering analysis using the unweighted pair grouping with mathematics average (UPGMA) with the Gelcompar II software package. The isolates with identical RFLP patterns were designated as an rDNA type. The 16S rRNA of the representative strains were sequenced using the method of direct sequencing from PCR.

2.4. PCR amplifications of recA, atpD, nodC and nifH genes

For *recA* gene amplification, the primers 63F and 504R were used; the *atpD* gene was amplified using the primers 273F and 771R. The conditions and program of PCR amplification were as

Table 1
Results of phenotypic characteristics of *S. pallida* and *S. capitata* isolated bacteria.

Characteristics	*S. pallida*	*S. capitata*
No of isolates	38	24
Growth at pH		
5	–	–
6	+	+
7	+	+
8	+	+
9	–(4)	–(10)
10	–(15)	–(18)
12		
Growth at temperature		
4	–	–
28	+	+
37	+	+
40	+	+
42	–	–
Source of carbohydrates (1%)		
Glucose	+(2)	+(9)
Lactose	+(25)	+(18)
Maltose	+(27)	+
Saccharose	+(25)	+(15)
Glycogen	–	–
Glycerol	+	+
Source of amino acids (0.1%)		
L-glycine	–(13)	–(10)
L-histidine	+	+
L-phenylalanine	+	+
L-cysteine	–(3)	–(2)
NaCl tolerance (%)		
0.5	+	+
1	+	+
2	+	+
3	–	–
4		
Heavy metals resistance (μg/ml)		
CdCl2 (50)	–	–
CoCl2 (50)	–(4)	–(5)
HgCl2 (50)	–(12)	–(14)
AlCl3 (400)	+	+
Antibiotic resistance (μg/ml)		
Streptomycin		
60	+(6)	+(7)
100	–	–
Ampicillin		
60	+(9)	+(6)
100	–	–
Kanamycin		
60	–	–
100	–	–
Oxalycin		
50	–(5)	–(2)
100	–	–
Colimycin		
50	–(8)	–(7)
100	–	–

Note: –, no growth/absent; +, positive growth/present. The number in parentheses indicates the number of isolates from the whole number of isolates.