Genetic and Biochemical Characterization of Some Egyptian Rhizobia Isolates Nodulating Faba Bean

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Abstract

Ten Rhizobial isolates were isolated from root nodules of Faba bean plants (Vicia faba L.) grown in different soil types and representing different geographic locations in Egypt. The isolates were characterized biochemically for their production of each IAA and catalase. The tested isolates differed in their IAA production. The maximum production of IAA, catalase, and nitrogen fixation (nif and fix) were carried on a plasmid called the symbiotic plasmid or pSym [19-21]. Although, strains can lose some of their traits due to loss or partial deletion of a plasmid, Plasmid profiling is a common means of strain identification [16]. In general, the symbiosis between Rhizobium and legumes species is very important and accounts for 50% of 175 million tons of total biological nitrogen fixation used in agriculture [6]. The Rhizobium leguminosarum biovar. Viciae (Rlv) is among fast-growing rhizobia and able to nodulate Vicia faba and Pisum sativum [7]. Faba bean (Vicia faba L.) is a major leguminous crop grown around the world and is most intensively cultivated in the North East Africa [8]. In Egypt, Faba bean is considered as one of the most important food legumes and plays a major role in the Egyptian diets as a source of protein [9,10]. Although the great importance of Rlv and its host Faba bean in Egypt, few molecular studies concentrated on the biodiversity of Rlv [11]. The previous studies done in other countries around the world showed the diversity and the wide distribution of Rlv strains [12] characterized 625 isolates of Rlv, and concluded that Faba bean-nodulating strain formed a distinct phylogenetic subgroup of Rlv nodulation genotypes. In addition, [13] found a great genetic diversity among 75 Rhizobial isolates associated with Faba bean from China and most of these isolates were Rlv. Moreover, [14] characterized 27 isolates collected from Italy and the RFLP analysis indicated that the majority of strains were consistent with Rlv.

Plasmids are important genetic elements for their roles in divergence and adaptation of microbial populations against different stresses. Since, symbiosis-related genes of Rlv exist on plasmids [15], a study of plasmid profiling is a common means of strain identification of Rlv [16]. In general, Rlv contains from 1 to 10 plasmids which vary in size [17,18]. Most of the genes required for nodulation formation (nod) and nitrogen fixation (nif and fix) are carried on a plasmid called the symbiotic plasmid or pSym [19-21]. Although, strains can lose some of their traits due to loss or partial deletion of a plasmid, Plasmid profiles can be considered as a stable character in rhizobia [22,23]. The SDS-PAGE analysis of whole cell proteins helps in identifying of the rhizobial strains [24,25] and is very useful in the differentiation among the isolates within the same group [26,27]. The objectives of this study were to evaluate the diversity between ten isolates of Rlv from nodules of Faba bean plants representing different geographic locations in Egypt based on molecular and biochemical levels.

Materials and Methods

Collection and isolation of Rhizobium isolates

Ten isolates of Rlv were isolated from nodules of Faba bean plants (Vicia faba L.) representing different soil types and geographic locations in Egypt (Table 1) according to the methods [28]. The samples collection area were planned to cover approximately the cultivated governorates in Egypt and with soil types varied from sand, loam to clay (Table 1).

| Isolate code | Soil type | Location       |
|--------------|-----------|----------------|
| RLP          | Clay      | Quesna City    |
| RLS          | Sand      | Sadat City     |
| RLG          | Clay      | Kaha City      |
| RLI          | Sand      | El-Ismailla City|
| RLT          | loam      | El-tor City    |
| RLZ          | Clay      | Zefta City     |
| RLA          | loam      | El-Arish City  |
| RLB          | Clay      | Benisuef City  |
| RLM          | loam      | El-Menia City  |

Table 1: Name of isolates and its locations.

Keywords: Rhizobia; Faba bean; Catalase; IAA; Plasmid profiles; SDS-PAGE analysis

Introduction

Rhizobia are diverse group of gram-negative unicellular soil bacteria which have been widely used in agricultural systems for enhancing the ability of legumes to fix atmospheric nitrogen [1]. Nitrogen is well known to be an essential nutrient for plant growth and development [2,3]. Rhizobiumis able to form a nitrogen-fixing root nodules as result of symbiosis with legumes and this permit plant growth in the absence of exogenous N, fertilizers [4,5]. In general, the symbiosis between Rhizobium and legumes species is very important and accounts for 50% of 175 million tons of total biological nitrogen fixation used in agriculture [6]. The Rhizobium leguminosarum biovar. Viciae (Rlv) is among fast-growing rhizobia and able to nodulate Vicia faba and Pisum sativum [7].

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| RLT          | loam      | El-Arish City  |
| RLB          | Clay      | Benisuef City  |
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Biochemical characterization of \( rlv \) isolates

**IAA production test:** All the isolates were tested for IAA production in Yeast Extract Mannitol (YEM) broth [28] supplemented with 100 µg/ml L-tryptophan. The test tubes were covered with brown paper and incubated at 28°C for 5 days on a rotary shaker. The broth was centrifuged at 10,000 rpm for 15 minutes. 2 ml of supernatant was collected and 2-3 drops of o-phosphoric acid were added. The aliquots were shaken, added 4 ml of reagent (1 ml of 0.5 M FeCl\(_3\) in 49 ml of 35% perchloric acid (HClO\(_4\)) and vortexed thoroughly. The samples were incubated at room temperature for 25 minutes and their absorbance was read at 530 nm. Auxin quantification value was recorded by extrapolating calibration curve made by using IAA as standard (10–100 µg/ml) [29,30].

**Catalase test:** Catalase activity of \( rlv \) isolates was tested according to [31] with modifications. Two drops of hydrogen peroxide (H\(_2\)O\(_2\)) were added to 72 h old isolates growing on YEM plates and checked for formation of oxygen gas bubbles.

**Plasmid isolation and digestion**

Plasmid DNA was isolated by the method of [32]. The extracted plasmids were digested with EcoRI and MSPI enzymes according to Thermo scientific fermentas kit (http://www.thermoscientificbio.com/fermentas/).

**Protein banding patterns of rhizobial isolates**

The cultures of \( rlv \) isolates growing on Broth YEM medium were pelleted and resuspended in 40 µl of Laemmli Sample Buffer, 5 µl of 10% SDS and 5 µl of β-mercaptoethanol. The mixture was then boiling for 5 min and centrifugation to obtain the supernatant which contains proteinfractions. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed [33].

**Statistical Analysis**

Data obtained were statistically analyzed using SPSS analysis program (version 11.5) using SPSS Statistical Package with Duncan’s multiple-range test at 5% level of Significance. The NTSYS-pc version 2.11 W (Numerical Taxonomy System) program was used to perform cluster analysis based on Jaccard’s similarity coefficient. Dendrogram was constructed according to the Unweighted Pair-Group Method with Arithmetical average (UPGMA) clustering method.

**Results and Discussion**

**Biochemical characterization of \( rlv \) isolates**

**A. Catalase activity:** Active oxygen such as H\(_2\)O\(_2\) is well known to damage the proteins, lipids and DNA components. The previous studies have shown that catalase plays an important role in the defense of cells against these toxic forms of oxygen and increasing of catalase activity in rhizobia could be useful to improve the nitrogen-fixing efficiency of nodules by the reduction of H\(_2\)O\(_2\) content [34]. Hence, all of tested isolates were evaluated for their catalase activity by adding two drops of H\(_2\)O\(_2\) to isolates growing on YEM plates and checked for formation of oxygen gas bubbles as indicator of catalase activity. Results in Table 2 and Figure 1 showed that all of \( rlv \) isolates were positive in their production of Catalase enzyme except RLZ isolate. It is not understood why RLZ isolate was negative in catalase production. One of explanations is that there are different kinds of catalase and may be one kind was impaired.

**B. IAA production test:** Since Indole-3-acetic acid (IAA) plays a significant role in regulating cellular elongation, differentiation, cellardivision, apoptosis, and nodule formation in plants [35,36]. Rhizobia are known to produce significant levels of IAA both in free
living conditions and also symbiotically in nodules [37]. Hence, the *Rlv* isolates were screened for their ability to produce plant growth regulator, IAA. All isolates showed different colors after treatment with Salkowski reagent indicating variation in their abilities to produce IAA. The range of IAA production varied from 2.04 (RLK of Kaha) to 4.56 µg/ml (RLZ of Zefta). These results are in accord with previous studies showing that Production of IAA by microbial isolates varies greatly among different species and strains and depends on the availability of substrate(s) (Figure 2) [38].

**Molecular characterization of RLV isolates**

### A. Plasmid profiles: RLV isolates were analyzed for their plasmid content and profiles. In general, all the isolates harbored one plasmid with size of 10 kb, while two isolates (RLB of Benisuef and RLG of Giza) contained an additional plasmid with size of 2 kb (Figure 3A). These results are in agreement with previous studies have shown that most of the Rhizobial species harbour plasmids that vary in number (1 to 10) and in size [39, 40]. Two restriction endonucleases, MspI and ECORI were used to digest the plasmids and studying their restriction profiles. The isolates of RLQ, RLA, RLZ, RLM and RLK have the same profile after digestion by ECORI and shared two bands of 7 and 3 kb (Figure 3). On the other hand, the isolates RLB and RLG gave three different bands. It must be mentioned that digestion with MspI showed more bands than ECORI. Moreover, the isolates RLA, RLM and RLK have the same profile as indicated with ECORI, while isolates RLQ, RLS and RLZ shared same profile and produced three bands of 5, 3 and 2 kb (Figure 3C). In addition, the isolates RLB, RLI and RLG produced the highest number of bands with 4, 4 and 5 bands respectively. These results show variation between isolates based on plasmid restriction profiles and are homologous with these obtained

| RLV  | RLV  | RLV  | RLV  | RLV  | RLV  | RLV  | RLV  | RLV  |
|------|------|------|------|------|------|------|------|------|
| RLQ  | 1.00 |      |      |      |      |      |      |      |
| RLS  | 0.54 | 1.00 |      |      |      |      |      |      |
| RLZ  | 0.54 | 0.54 | 1.00 |      |      |      |      |      |
| RLB  | 0.63 | 0.90 | 0.72 | 1.00 |      |      |      |      |
| RLT  | 0.54 | 1.00 | 0.54 | 0.81 | 0.90 | 1.00 |      |      |
| RLA  | 1.00 | 0.54 | 0.54 | 0.83 | 0.54 | 0.54 | 1.00 | 1.00 |
| RLM  | 1.00 | 0.54 | 0.54 | 0.83 | 0.54 | 0.54 | 1.00 | 1.00 |
| RLK  | 1.00 | 0.54 | 0.54 | 0.83 | 0.54 | 0.54 | 1.00 | 1.00 |
| RLG  | 0.90 | 0.63 | 0.63 | 0.72 | 0.63 | 0.90 | 0.90 | 0.90 |
| RLI  | 1.00 | 0.54 | 0.54 | 0.63 | 0.54 | 0.54 | 1.00 | 1.00 |

**Table 3: Similarity matrix among RLV isolates as revealed by protein banding patterns based on Jacard’s coefficient.**

![Figure 4: SDS-PAGE protein banding patterns of RLV isolates grown in Broth YEM medium. M: Molecular weight standard (PageRuler™ protein ladder, Fermentas).](image)

![Figure 5: The relationship between RLV isolates based on Jacard’s coefficient as revealed by protein banding patterns.](image)
by [16] who used different restriction endonucleases to digest the amplified DNA.

B. Protein banding patterns analysis: The protein banding patterns of the Rlv isolates were detected using SDS-PAGE analysis and were used to classify these isolates as shown in Figure 4. The size of detected bands ranged from 14 to 70 KD. The similarity matrix among the isolates was calculated based on Jacard’s coefficient as revealed by protein banding profiles (Table 3). The highest values of similarity were between isolates RLS and RLT; RLA and RLQ; RLM and RLQ; RLA and RLM; RLA and RLK; also between RLI and RLM, RLA and RLT with 100% similarity (Table 3). While, the lowest values of similarity were recorded between the RLQ and RLS, RIZ and RLT; RLS and RLM; RLA and RLT; RLZ and RLM; also between RLI and RLTZ. In general, the values of similarity among isolates based protein profiles are high, it is possible that these isolates have originated from the same genotypes and the human activities like soil and plant transfer limited the genetic diversity between these isolates. These results are in agreement with previous studies showing that different isolates of Rhizobia may have the same origin [41-43]. Moreover, it was indicated that rhizobia population in China probably originated from those of Japan and North America [44].

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