

Original Research Paper

Phylogeny and Molecular Study of Some Entomopathogenic Rhizobacteria Isolated from Two Regions in Algeria

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Abstract: The use of bacteria in the control of insect pests is a form of biological control whose practice is still not widespread. It is in this context that the present work falls. It concerns the isolation, characterization and identification of local bacterial strains for the purpose of their use in the control of certain pests. Indeed, 20 bacteria were isolated from soil cultivated in the region of Boumerdes (center of Algeria) with a total of 21 bacterial strains isolated from Adrar region (Desert Algerian). After carrying out the efficacy tests against 2 insect pests; Migratory locust (*Locusta migratoria*) and wax moth (*Galleria mellonella*), 8 potentially interesting strains were identified based on their genetic traits. Molecular characterization of these strains was performed by isolation of DNA, PCR and sequencing of the 16S rRNA gene, followed by phylogenetic analysis. The rDNA16S sequences of the 8 strains named B1, B2, B3, B4, B5, B6, H1 and H2 were recorded in the EMBL/EBI database and their phylogenetic analysis revealed that they belong to the genera *Bacillus*, *Pseudomonas*, *Enterobacter* and *Delftia* with a very high percentage of similarity with *Bacillus thuringiensis* strains (NR_043403) (99%) for isolate B1, *Bacillus weihenstephanensis* (NR_024697) (99%) for isolate B2, *Pseudomonas fragi* (JCM5420) (99%) for isolates B3 and B4, *Bacillus thuringiensis* (CMBLBT-5) (99%) for isolate B4, *Enterobacter ludwigii* (EN-119) for B5, *Bacillus thuringiensis* (4916) (99%) for isolate H1 and *Delftia lacustris* (R-54734) (100%) for isolate H2.

Keywords: Isolation, Entomopathogenic Bacteria, Rhizosphere, Molecular Identification, Phylogeny, MEGA6.06

Introduction

Due to the use of a large amount of chemical products, continuous loss of soil fertility puts pressure on farmers toward more crop production in a sustainable manner. Therefore, it requires adapting an eco-friendly, sustainable and cost-effective approach for agricultural practices without arising environmental issues (Singh *et al.*, 2019).

Indeed, the soil is a reservoir of numerous microorganisms critical for the sustainable functioning of natural and managed ecosystems (Sharma *et al.*, 2019). Several natural rhizobacteria inhabiting the rhizospheric soil exist, their application reduces the utilization of chemical fertilizers, pesticides and other artificial growth regulators that cause severe health and environmental issues, soil infertility, water pollution and biodiversity losses (Singh *et al.*, 2019).

Multiple species of entomopathogenic bacteria may be isolated from this part of soil, they are the enemies of pest-insects, whose utility in agroecosystems has been studied since decades. These entomopathogens spend significant time period in soil, either as saprotrophs, resting spores or dormant endospores (Oulebsir-Mohandkaci *et al.*, 2016; Sharma *et al.*, 2019).

Among the key insect-pest in Algeria, *Locusta migratoria* (Orthoptera-Acrdidae) and *Galleria mellonella* (Lepidoptera-Pyralidae) exhibit different damage to agriculture and are distributed in all the country (Oulebsir-Mohandkaci *et al.*, 2016; Benzina *et al.*, 2017). With increased awareness towards the environment, biological methods to control these two pests such as biopesticides based on entomopathogenic have been receiving greater attention as alternatives to chemicals pesticides (Jaronski, 2010).

To facilitate the isolation of entomopathogenic strains from soil, insect baiting by *Galleria mellonella* or the ‘Galleria-bait method’ (Zimmermann 1986; Benzina *et al.*, 2017), is the most renowned methodology. The migratory locust was also used for this. The main advantage of the insect baiting method is that only entomopathogens are obtained selectively amongst other soil microbes (Vega *et al.* 2012; Sharma *et al.*, 2018).

Material and Methods

Sol Sampling

The soil samples were taken from Adrar region in the Algerian desert at the rhizosphere layer of the date palm (Tamietti and Pramotton, 1990). Other samples were taken from the region of Boumerdes located in the central part of northern Algeria, from rhizosphere layer of wheat (Fig. 1).

Isolation and Phenotypic Characterization of Bacterial Isolates

Ordinary nutrient agar (GN) medium, pH 7.2 was used for isolation bacterial strains. 0.1 mL of 10⁻¹ to 10⁻⁵ dilutions was seeded on the surface of isolation medium. The bacterial colonies obtained after incubation

at 32°C for 48 h underwent purification. The obtained strains were then stored at -20°C using glycerol (20%, v/v) as cryo-protector.

Identification of bacterial genera was based primarily on the study of several macroscopic characters (the colonies type) and microscopic examinations (the form of bacterial cells and their mobility). The characterization of isolates needed physiological traits (highlighting the respiratory type, presence and position of spores and type of Gram) and biochemical (catalase, oxidase, nitrate reductase, levane sucrase, arginine di hydrolase, Voges Proskauer reaction (VP), degradation of certain substrates, gelatin, starch, casein, urea, indole, sorbitol, mannitol, citrate, tartrate and the production of lipase) (Bergey *et al.*, 1974; Dong and Cai, 2001; Guiraud, 2003).

Molecular Identification

The molecular study concerns the 8 best performing strains, those which gave the best results on the migratory locusts for the strains isolated from Adrar and on the wax moth for the strains isolated Boumerdes.

The 16S rRNA gene was amplified in three overlapping fractions with the following universal bacterial primers:

Primer	Sequence	Fragment	Size
16S1FOR_0008MF	AGAGTTTGATCATGGCTCAG	16S1	832 bp
16S1REV_0800M R	ACCAGGGTATCTAATC		
16S2FOR_0514MF	GTGCCA GCAGCCGCGGTA	16S2	599 bp
16S2REV_1073M R	CACGAGCTGA CGA CA		
16S3FOR_0775MF	GAGCRAACA GGATTAG	16S3	805 bp
16S3REV_1540M R	AAGGAGGTGATCCAACCGCA		



Fig. 1: Geographical situation of the sampling site

The Forward primers (FOR) were tailed by the M13-20FOR (CTGTAAAACGACGGCCAGT) primer whereas the Reverse ones (REV) by the M13-26REV (AGGAAACAGCTATGACCATG). Two colony PCR trials were performed per sample and per fragment and the obtained amplicons were run on agarose gel, cleaned-up and Sanger sequenced. Sanger sequencing was performed using the BigDye v3.1 chemistry on 3730XL machines (Applied Biosystems; www.appliedbiosystems.com). All obtained amplicons were sequenced from the forward and reverse directions using the universal primer-tailing of the 16S amplification primers. The sequences were base called status with the software program Sequencing Analysis v.5.2 (Applied Biosystems; www.appliedbiosystems.com). All generated chromatograms were assembled per sample using the TraceEditPro software (Ridom Bioinformatics; <http://www.ridom.de/traceedit/>). The consensus sequences were analyzed by human expert for base calling errors and IUPAC code was applied for any mixed position (originating from the presence of several copies of the 16S rRNA gene for one species). The primer sequences chromatograms were manually trimmed and the resulting consensus sequences were exported in FASTA format. The obtained FASTA consensus sequences were analyzed by similarity searches with BlastN/Megablast against the non-redundant nucleotide database of GenBank (NCBI GenBank; <http://blast.ncbi.nlm.nih.gov>). Further on, the degree of statistical support for the branches was determined by 1000 bootstrap replicates. The maximum likelihood trees were estimated by using the rapid bootstrap option (100 replicates) (Tamura *et al.*, 2011).

Results

Phenotypic Characterization of Isolates

20 bacterial strains were isolated from soil in the region of Boumerdes (coastal region located in the center of Algeria) with a total of 21 bacterial strains isolated from Adrar region (Sahara Desert, Algeria). Appearance of the colonies obtained on agar was very variable. The isolates were aerobic, mobile and with a positive catalase. Microscopic observation permitted their division into two groups, the first consisted of long rods, Gram positive and form a propriated sporal shape and the second contained short Gram negative rods and which non sporulating.

The isolates carrying the B1 and B2 codes provide well-isolated colonies, broad, smooth, flat, circulars, cream color with irregular edges. Observation under the light microscope, has shown that the two strains are Gram positive and are long stick shaped with square end.

Malachite green staining revealed that both isolates have a spore form. The spore has an oval shape nondeforming center position. Moreover, the two isolates B1 and B2 have shown positive results for the test of catalase, nitrate reductase and mannitol-mobility. The results of the pyruvic acid derivatives appeared positive for the Voges Proskauer reaction and the two isolates show a positive response for the hydrolysis of starch, gelatin and casein.

This isolates have all the cultural characteristics of the genus *Bacillus* already described by Brossard and Terry (1984) and Euzéby (2007).

Cultural characteristics on GN of the strains B3, B4 and B6 showed generally bacterial colonies whose contour was with a regular staining often beige cream. The elevation was convex for all strains with a glossy smooth appearance and varying diameters 1 to 2 mm after 24 h of culture. The bacterial strain B5 had smooth medium-sized colonies with an irregular edge. Moreover, the strains B5 and B6 showed positive results for some tests like nitrate reductase and mannitol. The strain B3 showed a positive result for the tests of mannitol and citrate. However, almost all strains B3, B4, B5 and B6 expressed negative results towards hydrolysis test of indole.

Molecular Characterization

In the present study, three bacterial genera *Bacillus*, *Pseudomonas* and *Enterobacter* were selected where the main members of the entomopathogenic bacteria belong to these genera (Greathead *et al.*, 1994).

The selected strains B1, B2, B3, B4, B5 and B6, were isolated from Adrar (Algerian desert) and on the other side strains H1 and H2 were isolated from Boumerdes (Algerian coast).

The 16S rDNA sequences of the three strains B1, B2 and B5 were obtained with 1504, 760 and 1504 nucleotides and stored in the database EMBL/EBI under reference accession numbers HE799656, HE805963 and HF911367, respectively. Sequence analysis of two strains B1 and B2 has shown they are affiliated to the genus *Bacillus* (the family of *Bacillaceae*) showing very high sequence identity (99%) with the strains *Bacillus thuringiensis* (NR_043403), *Bacillus cereus* AM7 (JQ435684), *Bacillus weihenstephanensis* (NR_024697.1) and *Bacillus mycoides* (NR_036880). Otherwise, the strain B5 showed a high sequence identity (99%) with the closely related type strains of *Bacillus* species, *B. anthracis* strain Ames, *B. cereus* strain HN and *B. thuringiensis* strain CMBLBT-5), which indicated that this strain can belong to one of these species. Moreover, the strains H1 isolated from the region of Boumerdes is identified as *Bacillus* strain H1 (LT547812) with a percentage of similarity (99%) with *Bacillus thuringiensis* strain Se13 (KX959986) (Table 1).

The rDNA 16S sequences of the named strains B3, B4 and B6 were recorded in the EMBL/EBI data bank under the accession numbers HF911369, HF911366 and HF911368, respectively. The phylogenetic analysis showed that the strains B3 and B4 had a sequence identity higher than 98% with those of the genus *Pseudomonas*. A significant similarity (based on partial 16RNAr sequence) for possible species relatedness (99%) was found with the validly described species *P. fragi* strain JCM5420, *P. syringae* isolate Lz4W and *P. psychrophila* strain HA-4. The distance matrix indicated that strains B3 and B4 might belong to one of these

species (Table 2). Further, the strain B6 was similarly closed to the species *Enterobacter ludwigii* strain EN-119 and *E. aerogenes* strain MDAZTVIIR105a with 99% of sequence identity. The strain H2 from the northern Algeria is identified as *Delftia* strain H2 (LT547813), with 100% similarity to *Delftia lacustris* strain: 6bw_2 (LC191530) (Table 2).

Finally, the four strains are in the same cluster as that of *Bacillus thuringiensis* (NR_043403) and *Bacillus cereus* AM7 (JQ435684), the most widely used entomopathogenic bacteria in the world. The other strains are distributed in different clusters (Fig. 2).

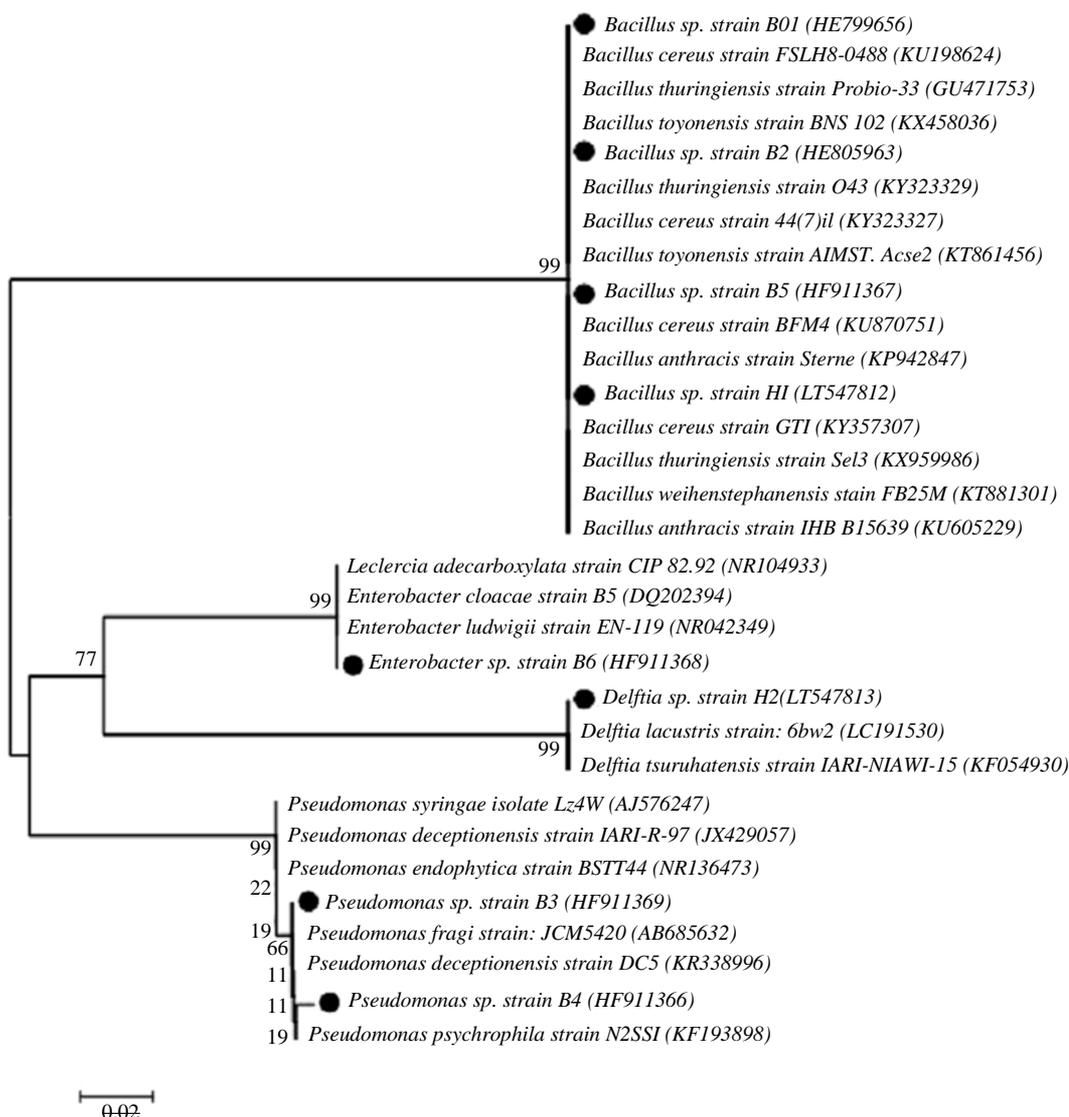


Fig. 2: Dendrogram indicating the position of the strains B1, B2, B3, B4, B5, B6, H1 and H2 (black circles) based on their 16S rDNA sequences. The tree was constructed using the neighbour-joining method. The sequence data used for the following strains were obtained from the sequences collected from Genbank database. The values at the tree nodes indicate percentages of recur- ring branches (1000 bootstraps for resampling)

Table 1: Percentage identity to sequence of 16S rDNA of the strains B1, B2, B5 and H1 with some species

Isolated strain	Similar strains	Identity (%)
Strain B1 (1504 pb)	<i>Bacillus cereus</i> strain FSL H8-0488 (KU198624)	99
	<i>Bacillus thuringiensis</i> strain Probio-33 (GU471753)	99
	<i>Bacillus toyonensis</i> strain BNS 102 (KX458036)	99
Strain B2 (760 pb)	<i>Bacillus thuringiensis</i> strain O43 (KY323329)	100
	<i>Bacillus cereus</i> strain 44(7)il (KY323327)	100
	<i>Bacillus toyonensis</i> strain AIMST. Acse2 (KT861456)	100
Strain B5 (1504 pb)	<i>Bacillus cereus</i> strain BFM4 (KU870751)	99
	<i>Bacillus anthracis</i> strain Sterne (KP942847)	99
Strain H1 (806 pb)	<i>Bacillus cereus</i> strain GT1 (KY357307)	99
	<i>Bacillus thuringiensis</i> strain Se13 (KX959986)	99
	<i>Bacillus weihenstephanensis</i> strain FB25M (KT881301)	99
	<i>Bacillus anthracis</i> strain IHB B 15639 (KU605229)	99

Table 2: Percentage identity to sequence of 16S rDNA of the strains B3, B4, B6 and H2 with some species required parameters are missing or incorrect

Isolated strain	Similar strains	Identity (%)
Strain B3 (1204 pb)	<i>Pseudomonas fragi</i> strain: JCM 5420 (AB685632)	100
	<i>Pseudomonas eceptionensis</i> strain DC5 (KR338996)	100
	<i>Pseudomonas syringae</i> isolate Lz4W (AJ576247)	100
	<i>Pseudomonas deceptionensis</i> strain IARI-R-97 (JX429057)	100
Strain B4 (538 pb)	<i>Pseudomonas deceptionensis</i> strain DC5 (KR338996)	99
	<i>Pseudomonas psychrophila</i> strain N2SS1 (KF193898)	99
	<i>Pseudomonas endophytica</i> strain BSTT44 (NR_136473)	99
Strain B6 (1492 pb)	<i>Enterobacter ludwigii</i> strain EN-119 (NR_042349)	99
	<i>Leclercia adecarboxylata</i> strain CIP 82.92 (NR_104933)	99
	<i>Enterobacter cloacae</i> strain B5 (DQ202394)	99
Strain H2 (817 pb)	<i>Delftia lacustris</i> strain: 6bw_2 (LC191530)	100
	<i>Delftia tsuruhatensis</i> strain IARI-NIAW1-15(KF054930)	100

Discussion

Bacteria represent the most commercially successful entomopathogenic microbial group, with most commercialized insecticides containing Gram-positive bacteria in the Bacillaceae family (De Bortoli and Jurat-Fuentes, 2019).

In fact our study allowed us to isolate, 20 bacteria from soil cultivated in the region of Boumerdes and with a total of 21 bacterial strains from the Adrar region. After carrying out the efficacy tests against 2 insect pests; *Locusta migratoria* and *Galleria mellonella*, 8 potentially interesting strains were identified based on their genetic traits. Partial sequencing of ARN16S. allowed to affiliate 4 strains to the genus *Bacillus*, 2 strains to the genus *Pseudomonas*, 1 strain to the genus *Enterobacter* and one strain to the genus *Delftia*. The first 3 genera are known by their entomopathogenic effect (Greathead *et al.*, 1994) but for the last one no study has demonstrated its insecticidal efficacy.

Entomopathogenic spore forming bacteria, most notably *Bacillus thuringiensis* (Bt), are the most widely used Microbial Pest Control Agents (MPCA). The broad spectrum of susceptible hosts, production on artificial media and ease of application using conventional equipment have resulted in widespread use against several insect pests in crops, forest and aquatic habitats (Lacey and Siegel, 2000).

Indeed, the majority of bacteria of the genus *Bacillus* live in the soil or they persist with their spores. These are bacteria from land who derive their nutritional needs from an organic matter, nitrogen and minerals present in the soil (Brossard and Terry, 1984; Ashnaei *et al.*, 2009).

Presence of *Pseudomonas* bacteria in the rhizosphere can be explained by their rapid growth, ubiquitous nature and ability to adapt to various environmental conditions, which was reported in many studies (Bossis *et al.*, 2000). The facility of in vitro culture and the ability of some species of this genus of bacteria to produce antibiotics and siderophores in case

of iron deficiency confer special characters in the biological control (Mamoun and Olivier, 1989).

Finally, the complete 16S rDNA sequence analysis and DNA: DNA hybridizations are suggested for an extensive study for the identification of the obtained isolated strains.

Conclusion

As part of our study, sustainable use of rhizobacteria has been suggested to be an eco-friendly and cost-effective approach which increases crop yields and directly or indirectly protects plant from pathogens and the most redoubtable pests including the two pests having been the subject of our study, *L. migratoria* and *G. mellonella*.

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Author’s Contributions

Oulebsir-Mohandkaci Hakima: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Benzina Farida: Participated in the experimental study, re-wrote and edited major sections of the manuscript.

Khemili-Talbi Souad: Participated in the phylogenetic study, wrote specific sections and edited the final version of the submitted manuscript.

Mohammedi Arezki and Halouane Fatma: Designed and optimized the research plan and organized the study.

Hadjouti Ryma: Analyzed the data.

Ethics

The present paper is an original work with no conflict of interest.

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