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Morphological and Molecular characterization of *Flectobacillus roseus* isolated from rhizosphere of Pomegranate

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Abstract
Flectobacillus spp. was identified on morphological characters such as circular, smooth colony, rod shaped cell with orange pigmentation. It showed negative test for Gram's reaction, starch hydrolysis, acid production and gas production. The molecular phylogeny of sample was determined by analyzing 16S rRNA gene sequences. On the basis of the position of sequence of the bacterial samples in the phylogenetic tree, sample LP2 showed closest similarity to *Flectobacillus roseus.*

Keywords: Molecular characterization, *Flectobacillus roseus*, Pomegranate

1. Introduction
Microbial control of soil borne diseases is economically viable and environment friendly aimed in sustainable agriculture. Among the microorganisms, plant growth promoting rhizosphere microflora provide a great promise with the dual advantage of plant growth promotion and disease suppression. There is a need to isolate efficient rhizosphere microflora with a property of disease suppression, preferably from the same environment in which they are used. Such isolates will be more ecologically fit than the exotic strains. Hence, in the present study, an attempt was made to isolate, screen and select efficient promising rhizosphere microflora from pomegranate rhizosphere for their beneficial traits.

Material and methods
Identification of bacteria
Isolated rhizobacterial strains were identified upto generic level based on the morphological and biochemical tests as specified in Bergey's manual of Determinative Bacteriology. Molecular phylogeny of bacteria was determined by amplifying genomic 16S rRNA region. Two primers specific to 16S rRNA region used in this study were 519F and 1385R in order to amplify approximately 850bp sequence of bacterial 16srRNA gene.

DNA extraction and quantification:
DNA Extraction was carried out using HiPurA Bacterial Genomic DNA Purification Kit (Himedia, MB505). Loopful of culture was suspended in 200µl of lysozyme solution (2.115 x 10^6 unit/ml) and incubated at 37°C for 30 min. 20 µl of RNase A solution was added and incubated for 2min at room temperature. Then 20 µl of Proteinase K solution (20mg/ml) was added followed by 200µl of lysis solution C1. The mixture was vortexed and incubated at 55^0C for 10 min. DNA was precipitated by adding 200µl of ethanol to the lysate and mixed by vortexing. Lysate was then loaded on HiEluteMiniprep Spin column and centrifuge at 10,000 rpm for 3 min. Flow through liquid was discarded and the column was transferred to new 2 ml collection tube. 500 µl of Prewash Solution was then added to the HiEluteMiniprep Spin column and centrifuged at 10,000rpm for 3 minutes. Flow through liquid was discarded and 500 µl of Wash Solution was added to the column. It was centrifuged at 10,000rpm for 3 minutes. 200µl of elution buffer was then added to the column and incubated at room temperature for 5 minutes. Again it was centrifuged at 10,000 rpm for 3 minutes. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation A11454806498). The DNA was stored at -20°C for further use.

PCR amplification
The DNA isolated from bacteria was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48).

The PCR reaction mix contained 2.5µl of 10X buffer, 1µl of each primer, 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA and 8.5µl nuclease free water. The PCR amplification cycle consist of, a cycle of 5 min at 94°C; 35 cycles of 1min at 94°C, 1 min at 50°C, 2 min at 72°C; and additionally 1 cycle of 7 min at 72°C. The reagents used were procured from GeNei.

Gel electrophoresis

Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The size obtained was approx. 850bp for 16S rRNA region

DNA sequencing

The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). 100µl of PCR-A buffer was added to the 25µl of reaction. The sample was mixed and transferred to column placed in 2ml collection tube and centrifuge at 10,000 rpm for 1min. The filtrate was discarded.700µl of W2 buffer was added to the column and centrifuged at 10,000rpm for 2min. This step was repeated twice. The column was transferred to a new tube. 25µl of Eluent was added into the column and incubated at room temperature for 2min. Then it was centrifuged at 10,000rpm for 5min. It was further sequenced using Applied Biosystems3730xl DNAAnalyzer USA and chromatogram was obtained. For sequencing of PCR product 519F- 5'CAGCAGCCGCGGTAATAC3' sequencing primer was used.

Primers used for 16S rRNA region amplification

Primers	Primer Sequence (5'-3')
519F (Forward)	CAGCAGCCGCGGTAATAC
1385R (Reverse)	CGGTGTGTACAAGGCCC

Ute Hentschel *et al*. (2002) [7]

Bioinformatics analysis

The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of bacteria. Altogether twenty sequences, including sample were used to generate phylogenetic tree. The tree was constructed by using MEGA 5 software (Saitou N. and NeiM., 1987; Felsenstein J.1985 and Tamura K. *et al* 2011) [5, 2, 6].

Result and Discussion

Results presented in Table 1 revealed that LP2 isolate was identified as *Flectobacillus* spp. on the basis of morphological and biochemical characterization. The colony of *Flectobacillus* spp. was smooth and circular, cells were rod shaped and pigmentation was found in orange colour. It showed negative test for Gram's reaction, starch hydrolysis, acid production and Gas production.

Table 1: Morphological and biochemical characteristics of *Flectobacillus* spp.

Isolate code	Colony morphology	Cell shape	Pigmentation	Gram Staining	Starch hydrolysis	Acid production	Gas Production
LP2	Smooth, Circular	Rod	Orange	Negative	Negative	Negative	Negative

Molecular phylogeny of bacteria was determined by amplifying genomic 16S rRNA region. Two primers specific to 16S rRNA region used in this study were 519F and 1385R in order to amplify approx. 850bp sequence of bacterial 16srRNA gene. DNA sequencing was done as per the procedure described in Material and Methods, whereas, bioinformatics analysis was done using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of bacteria. Altogether twenty sequences, including sample were used to generate phylogenetic tree (figure 1). The tree was constructed by using MEGA 5 software (Saitou N. and Nei M., 1987; Felsenstein J. 1985 and Tamura K. *et al*. 2011) [5, 2, 6]. The result revealed that the isolate code LP2showed 98 per cent homology for *Flectobacillus roseus*.

Identification of isolates by 16S rRNA sequencing.

Sr. No.	Isolate code	Isolate Identity	Percentage homology
1	LP2	*Flectobacillus roseus*	98%

Sample no.: LP2

Partial 16S rRNA gene Sequence (869bp)

GGGGATTGTGGACAGAAGCAGTTCGGATTATTGGGT
TTAAGGGTGCGTAGCGGTTTATTAAGTCAGTGGTGA
AAGACGGTCGCTCAACGATTGCAGTGCCATTGATAC
TGGTAGACTTGAGTTCGTATGAGGTAGCTGGAATGG
ATAGTGTAGCGGTGAAATGCATAGATATTATCCAAA
ACTCCAATTGCGTAGGCAGGTTACTAATACGATACT
GACGCTGATGCACGAAAGTGTGGGGATCAAACAGG
ATTAAATACCCTGGTAGTCCACACTGTAAACGATGA
TGACTAACTGTTTGCCCTTTGGGGTGAGTGGTACAG
AGAAATCGTTAAGTCATCCACCTGGGGAGTACGCCG
GCAACGGTGAAACTCAAAGGAATTGACGGGGGTCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCTATGAT
ACGCGAGGAACCTTACCTAGGCTAGAATGTGAAGGA
ATAATTTAGAGATAAATTAGTCTGCAAAGACCTGAA
ACAAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGT
GAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
CTGTTGTTAGTTGCCATCAAGTTATGTTGGGCACTCT
AGCAAGACTGCCTACGCAAGTAGAGAGGAAGGAGG
GGACGACGTCAAGTCATCATGGCCCTTACGCCTACG
GCGACACACGTGCTACAATGGGCGGTACAGAGGGTA
GCTACCTGGCAACAGGATGCCAATCTCGAAAGCCGC
TCTCATTTCGGATTGGGGTCTGCAACTCGACCCTATG
AAGCTGGAATCGCTAGTAATCGGGTATCACCATGAC
CCGGTGAATACTTTCCCGGGCCTTGTTCACACCGGA
GGGGAA

Sequence length: 869bp

Table 2: Phylogenetic neighbors of LP2 based on partial 16S rRNA gene sequence through NCBI.

Description	Max score	Query cover	E value	Ident	Accession
Flectobacillus roseus strain GFA-11 16S ribosomal RNA gene, partial sequence	1441	96%	0.0	98%	NR_116312.1
Flectobacillus major strain ATCC 29496 16S ribosomal RNA gene, complete sequence	1315	96%	0.0	95%	NR_044736.2
Flectobacillus lacus strain CL-GP79 16S ribosomal RNA gene, partial sequence	1262	96%	0.0	94%	NR_043554.1
Arcicella aquatica strain NO-502 16S ribosomal RNA gene, partial sequence	1206	96%	0.0	93%	NR_029000.1
Arcicellarigui strain NSW-5 16S ribosomal RNA gene, partial sequence	1205	96%	0.0	93%	NR_108997.1
Arcicella rosea strain TW5 16S ribosomal RNA gene, partial sequence	1201	96%	0.0	93%	NR_042707.1
Arcicella aurantiaca strain TNR-18 16S ribosomal RNA gene, partial sequence	1195	96%	0.0	92%	NR_116735.1
Pseudarcicella hirudinis strain E92 16S ribosomal RNA gene, partial sequence	1114	96%	0.0	91%	NR_108865.1
Siphonobacte raquaeclarae strain P2 16S ribosomal RNA gene, partial sequence	968	96%	0.0	88%	NR_116562.1
Mongoliita lealutea strain MIM18 16S ribosomal RNA gene, partial sequence	950	96%	0.0	87%	NR_122086.1
Belliella pelovolcani strain CC-SAL-25 16S ribosomal RNA gene, partial sequence	950	96%	0.0	87%	NR_116416.1
*Belliella balticas*train DSM 15883 16S ribosomal RNA gene, complete sequence	939	96%	0.0	87%	NR_102864.1
Belliella baltica strain BA134 16S ribosomal RNA gene, partial sequence	939	96%	0.0	87%	NR_025599.1
Algoriphagus alkaliphilus strain AC-74 16S ribosomal RNA gene, complete sequence	933	96%	0.0	87%	NR_042278.1
Algoriphagus faecimaris strain LYX05 16S ribosomal RNA gene, partial sequence	926	96%	0.0	87%	NR_108679.1
Algoriphagus boritolerans strain NBRC 101277 16S ribosomal RNA gene, partial sequence	922	96%	0.0	87%	NR_113997.1
Algoriphagus hitonicola strain 7-UAH 16S ribosomal RNA gene, partial sequence	922	96%	0.0	87%	NR_044249.1
Algoriphagus boritolerans strain T-22 16S ribosomal RNA gene, partial sequence	920	96%	0.0	87%	NR_041271.1
Nitritaleahal alkaliphila strain LW7 16S ribosomal RNA gene, complete sequence	917	96%	0.0	86%	NR_116930.1
Algoriphagus boseongensis strain BS-R1 16S ribosomal RNA gene, partial sequence	915	96%	0.0	86%	NR_118640.1

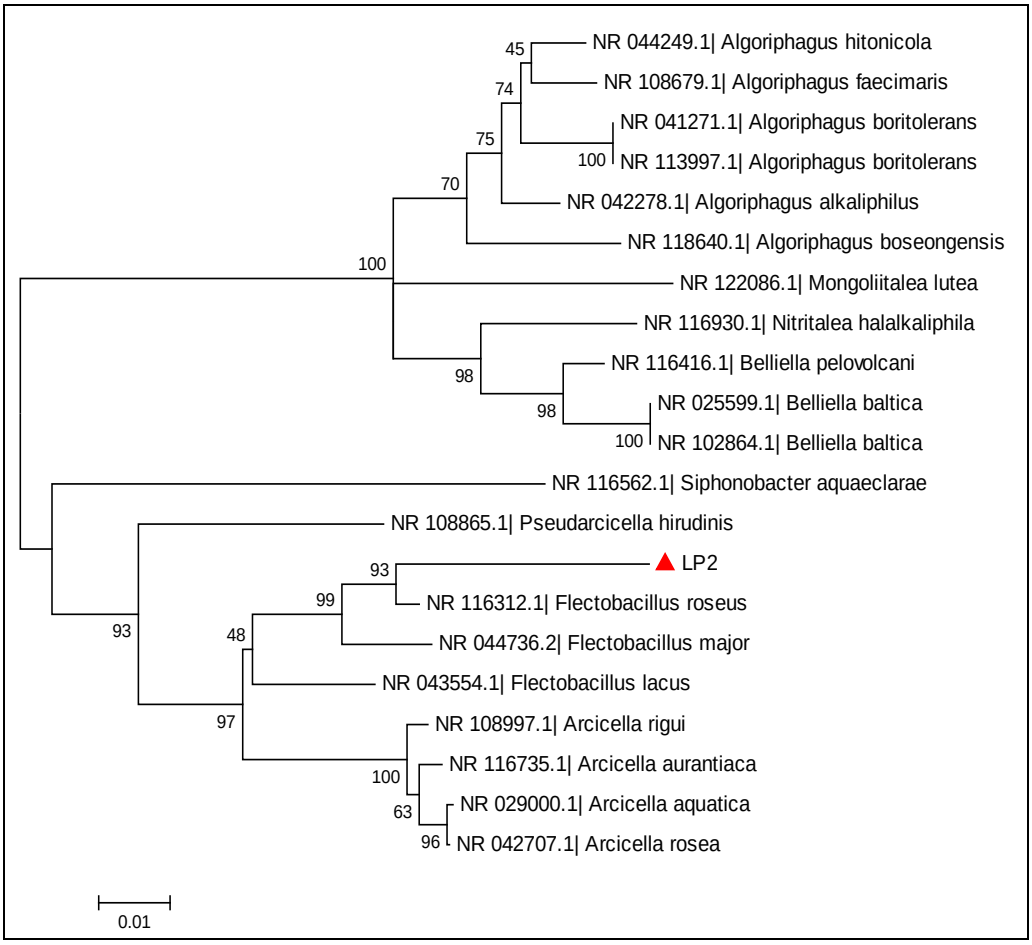

Fig 1: Phylogenetic tree for LP2 using partial 16S rRNA gene sequence through NCBI.

Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide

sequences. All positions containing gaps and missing data were eliminated. There were a total of 851 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. The molecular phylogeny of sample was determined by analyzing 16S rRNA gene sequences. On the basis of the position of sequence of the given bacterial samples in the phylogenetic tree, sample LP2showed closest similarity to *Flectobacillus roseus.*

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