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Comprehensive Morphological, Biochemical and Phylogenetic Analysis of *Flectobacillus* Strain Isolated from Soil

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***Abstract*:** This study aimed to isolate and identify *Flectobacillus* strain from a paddy field environment. The strain was characterized through detailed morphological, physiological and molecular analysis. Morphologically, the *Flectobacillus* spp. presented as circular, smooth colonies with rod-shaped cells and the O31 strain tested negative for Gram's reaction. Molecular identification was conducted using NCBI BLAST analysis of 16S rRNA gene sequences, which revealed a high sequence similarity of 98.41% to *Flectobacillus* sp. strain K37. Phylogenetic analysis using MEGA 12 software confirmed that the O31 strain belongs to the *Flectobacillus* genus and is closely related to strain K37. The findings suggest that this strain may play a significant role in the microbial ecology of paddy soils. Future research will focus on investigating the ecological functions, plant-growth promoting potential and stress tolerance mechanisms of this strain, alongside whole-genome sequencing for a deeper understanding of its genetic capabilities.

Index Terms: *Flectobacillus, Bacterial isolation, Molecular identification, 16S rRNA, Phylogenetics, Paddy field microbiology, Microbial ecology, Sustainable agriculture.*

I. INTRODUCTION

The phylum Bacteroidetes is a widespread and ecologically significant group of bacteria, with a pronounced presence in aquatic environments, where they play important roles in shaping ecosystem dynamics (Andra´s Ta´ncsics *et al.*, 2010). *Flectobacillus* is a genus of Gram-negative, elongated rod-shaped bacteria, initially described by Larkin *et al.* in 1977, predominantly isolated from aquatic habitats. Initially, two species were assigned to this genus: *Flectobacillus marinus* and *Flectobacillus glomeratus*. However, these species were later reclassified as *Cyclobacterium marinum* (Raj and Maloy, 1990) and *Polaribacter glomeratus* (Gosink *et al.*, 1998), respectively. The first recognized species of *Flectobacillus* was *F. major* (Larkin et al., 1977). Subsequent discoveries include *F. lacus* from a eutrophic pond (Hwang and Cho, 2006) and *F. roseus* in a freshwater environment (Harresh Adikesavalu et al., 2015).

Soil sample from paddy field in Kerala was collected and stored in a sterile bag. The sample was plated on Jensen agar, and after 3-day incubation at 28°C, white, convex colonies were observed. Bacterial strain was purified, selected for taxonomic analysis, and stored for further study. Genomic DNA was extracted from the bacterial isolate using the NucleoSpin® Tissue Kit (Macherey-Nagel). The 16S rRNA gene was amplified and sequenced using the primers 16S-RS-F (5'-CAGGCCTAACACATGCAAGTC-3') and 16S-RS-R (5'-GGGCGGWGTGTACAAGGC-3'). PCR amplification was carried out using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems; Wen-Ming Chen et al., 2017).

Phylogenetic analysis is a systematic approach used to reconstruct the evolutionary history and infer the relationships among a group of organisms. Through this method, phylogenetic trees are typically generated to visualize and elucidate the evolutionary connections and divergence patterns among different species (Tokumasa Horiike., 2016).

II. MATERIALS AND METHOD

i. Sample Collection and Isolation

Soil sample was collected from paddy field located in Thiruvalla, Kerala, and stored carefully in a sterile container to preserve its microbial content. For isolation, serial dilutions of the soil sample were spread onto Jensen agar plates. After incubation at 28°C for three days, colonies exhibiting a white, convex morphology were selected, purified through successive streaks and preserved for subsequent analysis. Preliminary identification of bacterial isolates was conducted based on their morphological and biochemical traits, following the guidelines outlined in Bergey's Manual of Determinative Bacteriology.

ii. Morphological and Biochemical Characterization of Bacterial Isolate

The bacterial isolate was subjected to detailed morphological and biochemical analyses to facilitate preliminary identification. Morphologically, the isolate was assessed for motility, cell shape, size, margin, coloration, appearance and Gram staining characteristics. The strain was cultured on trypticase soy agar and incubated at 28°C for 72 hours. Post-incubation, the colonies were examined microscopically to observe cellular features.

Biochemical tests were conducted to determine metabolic and enzymatic properties, including oxidase, catalase, indole, citrate utilization, urease activity and starch hydrolysis. These tests were performed following standard protocols outlined in Bergey's Manual of Determinative Bacteriology, allowing for a reliable preliminary identification of the bacterial strain.

iii. DNA Barcoding and Sequencing

The bacterial samples were subjected to DNA barcoding at the Rajiv Gandhi Centre for Biotechnology. The 16S rRNA gene was amplified using specific primers: forward primer (16S-RS-F: 5'-CAGGCCTAACACATGCAAGTC-3') and reverse primer (16S-RS-R: 5'-GGGCGGWGTGTACAAGGC-3') (Kim et al., 2014). A 1µL aliquot of DNA template was used in each PCR reaction. The PCR products were purified and sequenced commercially. The sequence data were initially evaluated for quality using Sequence Scanner Software. Subsequently, the sequences were refined, edited and alignments were performed using Geneious Pro software.

iv. Sequence Identification and Phylogenetic Analysis

The high-quality sequences were uploaded to NCBI BLASTn for taxonomic identification by comparing them with existing sequences in the NCBI nucleotide database. To analyse the evolutionary relationships among the isolates, multiple sequence alignments were conducted with related reference sequences retrieved from GenBank, using ClustalW in MEGA 12 (Kumar et al., 2018). Phylogenetic tree was constructed via the Maximum Likelihood method in MEGA 12, with 1000 bootstrap replicates to assess the statistical support of the inferred phylogenies. This analysis facilitated the determination of genetic proximity and taxonomic positioning of the bacterial isolate.

III. RESULTS

i. Morphological Characterization

The isolated bacterial strain from the soil was characterized as Gram-negative, non-motile and straight rod-shaped bacteria. The colonies appeared as cream white, convex and smooth with entire margins. These morphological features are depicted in **Figure 1.**

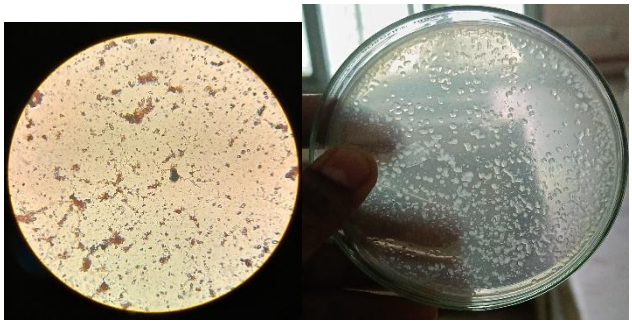


Figure 1. Morphological features of the isolate:
(A) Gram-stained bacterial cells observed under the microscope
(B) Colonies grown on agar plates.

Table 1. Morphological features of the isolated strain

Gram nature	Shape Colony	Colony Surface	Colony Margin	Colony Colour	Colony Texture
Gram-negative	Rod shape	Smooth Convex	Entire margins	Cream white	Mucoid

ii. Biochemical Characterization:

The biochemical analysis of the strain revealed positive activities for catalase and oxidase, indicating its aerobic nature and ability to neutralize reactive oxygen species, which is characteristic of many environmental bacteria. The negative results for the Methyl Red and Voges-Proskauer tests suggest that the organism does not utilize mixed acid or butylene glycol pathways for glucose fermentation, reflecting its specific metabolic pathways. Additionally, the absence of indole production and urease activity points to the lack of tryptophanase enzyme and urease enzyme, respectively, further defining its metabolic profile. These biochemical characteristics collectively enhance our understanding of the organism's physiological properties, supporting its classification and providing insights into its potential ecological roles.

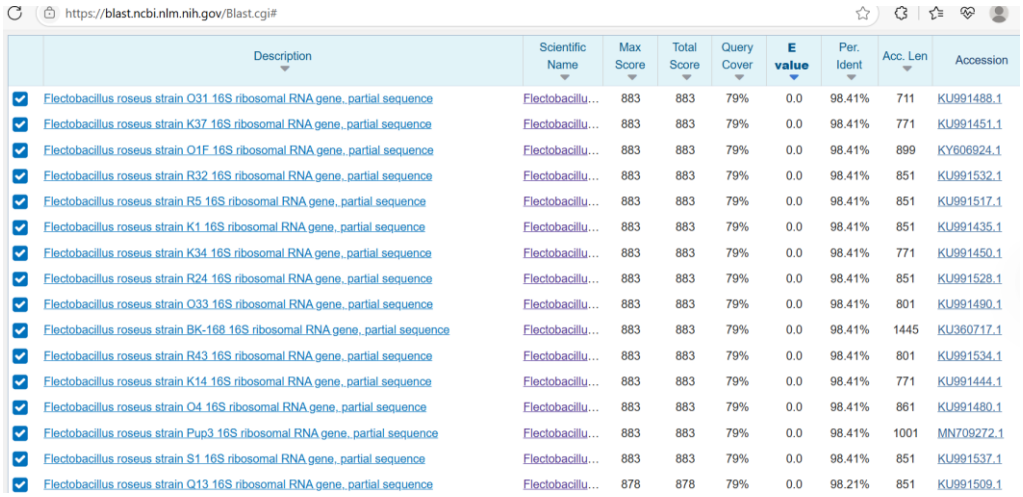
Table 2. Biochemical properties of the isolate strain.

S.NO	BIOCHEMICAL TEST	RESULT
1.	INDOLE TEST	**NEGATIVE**
2.	UREASE TEST	**NEGATIVE**
3.	CITRATE UTILIZATION TEST	**NEGATIVE**
4.	OXIDASE TEST	**POSITIVE**
5.	CATALASE TEST	**POSITIVE**
6.	METHYL RED TEST	**NEGATIVE**
7.	VOGERS- PROSKAUER TEST	**NEGATIVE**

iii. Molecular Identification and Phylogenetics

The identification and taxonomic placement of the bacterial strains were supported by both NCBI BLAST analysis and phylogenetic tree construction. The BLAST results revealed that the studied strain share high sequence similarity with known *Flectobacillus* species, notably *F. roseus* and related uncultured bacteria, confirming their placement within the genus. The similarity scores and close genetic relationships observed in the BLAST analysis underscore the conserved nature of these bacteria within the genus *Flectobacillus*.

Further, phylogenetic analysis was conducted using the MEGA 12 software, employing the maximum likelihood method with 1000 bootstrap replicates to ensure robust support for the inferred relationships. The resulting phylogenetic tree clearly shows that all of the studied strains cluster within the *Flectobacillus* genus, forming distinct clades with high bootstrap support (mostly 100), indicating confidence in their evolutionary relationships. These strains exhibit close clustering with *F. roseus* and uncultured clones, emphasizing their conserved evolutionary lineage. The high bootstrap values across most nodes reinforce the reliability of the phylogenetic groupings.



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Flectobacillus roseus strain O31 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	711	KU991488.1
Flectobacillus roseus strain K37 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	771	KU991451.1
Flectobacillus roseus strain O1F 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	899	KY606924.1
Flectobacillus roseus strain R32 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	851	KU991532.1
Flectobacillus roseus strain R5 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	851	KU991517.1
Flectobacillus roseus strain K1 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	851	KU991435.1
Flectobacillus roseus strain K34 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	771	KU991450.1
Flectobacillus roseus strain R24 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	851	KU991528.1
Flectobacillus roseus strain O33 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	801	KU991490.1
Flectobacillus roseus strain BK-168 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	1445	KU360717.1
Flectobacillus roseus strain R43 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	801	KU991534.1
Flectobacillus roseus strain K14 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	771	KU991444.1
Flectobacillus roseus strain O4 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	861	KU991480.1
Flectobacillus roseus strain Pup3 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	1001	MN709272.1
Flectobacillus roseus strain S1 16S ribosomal RNA gene, partial sequence	Flectobacillu...	883	883	79%	0.0	98.41%	851	KU991537.1
Flectobacillus roseus strain Q13 16S ribosomal RNA gene, partial sequence	Flectobacillu...	878	878	79%	0.0	98.21%	851	KU991509.1

Figure 2: The BLASTn alignment results of a 16S rRNA gene sequence query against the NCBI nucleotide database. The matched sequences belong to various strains of *Flectobacillus roseus*.

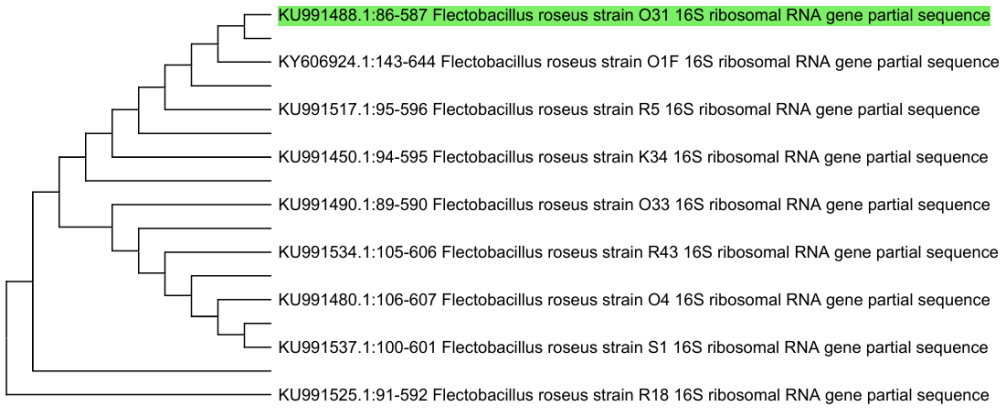


Figure 2: Shows a **phylogenetic tree** constructed using **MEGA 12 software**, based on the partial 16S ribosomal RNA gene sequences of *Flectobacillus roseus* strains. The evolutionary relationships among the strains were inferred using aligned nucleotide sequences. The green highlight marks the reference strain KU991488.1.

IV. DISCUSSION

The profile of this bacterial isolate supports its classification as a member of the genus *Flectobacillus*. Morphologically, the Gram-negative, rod-shaped cells and colonial features align with previously reported characteristics for *Flectobacillus* species. The mucoid, smooth colonies suggest the production of extracellular polysaccharides, which may play roles in environmental adaptation or biofilm formation in soil ecosystems.

Biochemically, the positive catalase and oxidase activities suggest an aerobic lifestyle, consistent with many soil bacteria that encounter oxidative stress. The absence of fermentative pathways such as methyl red and Voges-Proskauer reactions indicates a metabolic specialization leaning toward respiration rather than fermentation, which aligns with its environmental niche. Urease activity, often associated with nitrogen metabolism, was absent, implying that this strain might not play a significant role in nitrogen fixation or urease-mediated nitrogen cycling.

The high bootstrap support in the phylogenetic tree strongly confirms the strain's placement within *Flectobacillus*, especially its close relationship to *F. roseus*. This phylogenetic proximity not only corroborates morphological and biochemical observations but also emphasizes the conserved evolutionary lineage within this genus. The genetic similarity suggests functional and ecological similarities, possibly contributing to organic matter decomposition or nutrient cycling in soil environments.

Overall, this integrated characterization enriches our understanding of *Flectobacillus* diversity and ecological roles. Future studies will aim to explore the ecological roles, ability to enhance plant growth, and stress resilience mechanisms of this strain, in addition to conducting whole-genome sequencing to gain comprehensive insights into its genetic potential.

V. CONCLUSION

The isolated soil bacterium exhibits typical morphological, biochemical and genetic features of *Flectobacillus*, particularly close to *F. roseus*. Its physiological traits suggest adaptation to aerobic soil habitats, with potential roles in organic matter degradation and nutrient cycling. Further investigations into its functional capabilities could elucidate its ecological significance and possible applications.

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