

Functional characterization of endophytic bacteria isolated from feather grass (*Chloris virgata* Sw.)

Rusi Lata^{1,2}, Sandip Chowdhury^{1,2} and Surendra Kumar Gond^{1*}

¹ Department of Botany, MMV, Banaras Hindu University, Varanasi 221005, India

² Department of Botany, Institute of Science, Banaras Hindu University, Varanasi 221005, India

* Corresponding author, E-mail: surendragond@gmail.com

Abstract

The objective of this research was to isolate, identify, conduct biochemical characterization, and evaluate the plant growth-promoting (PGP) characteristics of endophytic bacteria obtained from various parts of feather grass collected from waterlogged rice fields. A total of 43 bacterial isolates from roots, rhizome and leaf of *Chloris virgata* were isolated. They were subjected to biochemical characterization (Catalase, Oxidase, Indole, Methyl red and Vogues Prausker, oxidative fermentation test). The isolates were tested qualitatively and quantitatively for PGP traits including indole acetic acid ($> 0.2\text{-}\mu\text{g mL}^{-1}$), gibberellic acid production ($> 21.03\text{ }\mu\text{g}\cdot\text{mL}^{-1}$), inorganic phosphate solubilization (PSI), siderophore production ($> 26.5\text{ PSU}$), ammonia production ($> 120.0\text{ }\mu\text{g}\cdot\text{mL}^{-1}$) and HCN production. The isolates were also subjected to salt (NaCl) and osmotic stress (PEG-6000). Endophytic bacteria were morphologically grouped and selected on the basis of their PGP traits for identification via 16s rRNA sequencing. The identified isolates belonged to two genera namely *Bacillus* and *Glutamicibacter* with the former being dominant in the plant. A total of 30 endophytic bacterial isolates identified by 16s rRNA gene sequencing were found to be of *Bacillus* spp. (25 isolates) and *Glutamicibacter* spp. (five isolates).

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Introduction

Plants exhibit diverse natural associations with microorganisms through various mechanisms. These associations with spatially and temporally complex land plants could be in the form of pathogens, PGPR (plant growth-promoting rhizobacteria) and endophytes^[1]. The symbiotic relationship between endophytic bacteria and plants plays a crucial role in enhancing seed germination, fostering plant growth, boosting crop yield, influencing secondary metabolite production, improving disease resistance, and bolstering tolerance to environmental stressors like drought and salinity^[2]. Given their widespread presence in plants and their ecological and economic significance, it is imperative to elucidate the intricate interactions among bacterial endophytes, their host plants, and agricultural practices. In recent decades, investigations into endophytic bacteria have predominantly concentrated on delineating their advantageous effects and ecological dynamics, aiming to enhance comprehension regarding the intricate interactions between bacterial endophytes and their respective host plants^[3,4].

Soil salinization exerts a substantial detrimental effect on agricultural productivity worldwide, affecting both irrigated and non-irrigated regions. It induces osmotic stress, water scarcity, stomatal closure, and diminished leaf growth. Additionally, salinity in the soil leads to the depletion of vital nutrients like potassium (K^+). Elevated Na^+ inside plants can decrease plant photosynthetic rates and biomass accumulation^[5-7]. Most crop and forage plants that feed the global population are sensitive to high salt concentration in soils^[8]. Investigating the community structure of endophytic bacteria in saline environments

could reveal the distribution characteristics of microbial populations and enable evaluations of homeostasis in the tissues of host plants. Advanced sequencing technologies have provided extensive data sources, which have facilitated the study of microbial species, community structure, community function, and genetic diversity. Over the past ten years, significant progress has been made in studies on environmental microbial diversity based on these technologies.

This work aimed to isolate bacterial endophytes from a halotolerant plant, *Chloris virgata*, a member of the grass family Poaceae. We examined potential *in vitro* beneficial physiological traits induced by the endophytes in plants under normal or salt-stressed conditions. In particular, the following traits of bacterial endophytes were analyzed in this study: siderophore production, phosphate solubilization, synthesis of auxin, capacity to promote plant growth, resistance to antibiotics, ACC deaminase synthesis, and the ability to increase plant tolerance to salinity stress.

Materials and methods

Collection of plant samples

Healthy plant samples of *C. virgata* were randomly collected during the month of April–May 2018 from five sites around the agricultural fields situated at the Institute of Agriculture Sciences, Banaras Hindu University, Varanasi, India.

Isolation of endophytic bacteria and biochemical characterization

The endophytic bacteria were isolated from the roots, rhizomes, and leaves of *C. virgata* plants following the surface

sterilization protocol^[9]. The plant parts were thoroughly washed with running tap water to remove soil particles and attached microbes. Root, rhizome and leaves were individually surface sterilized by serial washing in 70% ethanol (1 min), 2% sodium hypochlorite solution (available Cl⁻) for 3–4 min and 70% ethanol (60 s) followed by rinsing three times with sterile distilled water. Isolation was performed using the direct plating method on nutrient agar (NA) medium supplemented with 2% sodium chloride (NaCl). The sterilized leaves were cut into 0.5 cm × 0.5 cm sections while root and rhizome were cut into 2 mm sections. A minimum of five sections were placed on NA plates and incubated at 35 ± 2 °C for 24–72 h. The plates were checked every 24 h for bacterial colony emergence. The bacterial colonies were selected, sub-cultured, purified, and used for further studies. The bacterial isolates were characterized after 48 h for the following traits: colony color, form, elevation, margin, surface, opacity, and texture. Biochemical tests such as indole production, methyl red and Vogues-Proskauer tests, citrate utilization, catalase and oxidase production tests, nitrate reduction, and oxidative fermentation tests were performed for the endophytic isolates.

Plant growth promotion traits

Pure cultures of the bacterial isolates were inoculated onto solid diagnostic media supplemented with respective substrates to test enzyme activity. Protease, cellulase, lipase and amylase activity were assessed on Gelatin Yeast extract Peptone agar (GYP agar), Carboxymethyl cellulose agar (CMC agar), Tween 20 agar, and starch agar, respectively. The agar plates for enzyme activity testing were spot inoculated with the bacterial isolates and incubated at 30 ± 2 °C for 24–36 h. Proteolytic activity was detected by flooding the plates with saturated ammonium sulfate solution. The formation of a clear zone around bacterial colonies indicated substrate breakdown by proteases. Cellulase activity was evaluated by adding 0.2% (w/v) Congo red solution to the plates followed by washing with 5 M NaCl solution. The appearance of clear zones around colonies against a red background signified cellulase activity. Amylolytic activity was assessed by flooding the plates with 2% iodine solution and observing for clear zones, indicating substrate hydrolysis. Lipase activity was denoted by crystallization around bacterial colonies^[10–12].

Indoleacetic and Gibberellic Acid production

Nutrient broth (NB) media supplemented with 0.01% w/v tryptophan was used for IAA production and analyzed with Salkowski reagent (35% HClO₄ 50 mL, 0.5 M FeCl₃ 1 mL). Uninoculated growth medium was used as the negative control. The IAA concentration in the culture was estimated based on the IAA standard curve^[13]. Gibberellic acid was estimated following the modified protocol of Graham & Thomas^[14].

Phosphate solubilization

Isolates were spot inoculated onto the National Botanical Research Institute's phosphate growth medium (NBRIP) containing 2% w/v tricalcium phosphate [Ca₃(PO₄)₂] and incubated at 30 °C for 7–10 d. Formation of clear zones around growth indicated the phosphate solubilizing activity of bacterial isolates^[15].

ACC deaminase activity

Endophyte bacterial strains were cultured in nutrient broth to test the activity of ACC deaminase. Bacterial cells were harvested (centrifuged at 5,000 rpm for 15 min), washed with

sterilized 0.1 M Tris-HCl (pH 7.5) and were inoculated onto a medium of minimal saline agar. A medium with the addition of 0.2% NH₄(SO₄)₂ was used for positive control. DF (Dworkin and Foster) medium was used as a negative control. The cultures were incubated for 36 h at 28 ± 2 °C. The appearance of endophytic bacterial colonies in DF minimal saline medium was evaluated as a positive control^[16].

Siderophore, ammonia, and HCN production

The Chrome azurol assay (CAS) was followed for siderophore estimation^[17]. All bacterial isolates underwent spot inoculation onto CAS agar plates and were then incubated at 30 °C for 24 h. The observation of a yellow to light orange halo formation around the colonies served as an indicator for siderophore production. Siderophore production was also assessed by using a modified microtitre plate method^[18]. The bacterial cultures were cultivated in broth, and subsequently, 100 µL of the supernatant from each bacterial culture was introduced into individual wells of a microplate, followed by the addition of 100 µL of CAS reagent. Following the designated incubation period, absorbance readings were measured at 630 nm using a microplate reader (Agilent BioTek Synergy HTX Multimode Reader).

PSU (% siderophore unit) =

$$\left[\frac{\text{Absorbance (reference)} - \text{Absorbance (sample)}}{\text{Absorbance (reference)}} \right] \times 100\%$$

To assess ammonia production, 1 mL of Nessler's reagent was introduced to bacterial cultures cultivated in peptone water. The emergence of a light yellow to brown coloration indicated the presence of ammonia^[19]. For the examination of hydrogen cyanide (HCN) production, the bacterial cultures were inoculated onto NB medium supplemented with 0.4% glycine. Additionally, Whatman No.1 filter paper strips soaked in 0.5% picric acid in 2% sodium carbonate were placed within the Petri dish lid, which was then sealed with Parafilm. Incubation at 28 °C for duration of four days was ensued. A change in the color of the strips from yellow to brown signified a positive result for HCN production^[20].

Nitrogen free growth

Bacteria cultured in Jensen's medium (Sucrose 20 g; K₂HPO₄ 1.0 g; MgSO₄·7H₂O 0.5 g; NaCl 0.5 g; FeSO₄·7H₂O 0.1 g; Na₂MoO₄ 0.005 g; CaCO₃ 2.0 g; Agar 15.0 g) were incubated for 7 d at 28 ± 2 °C. The emergence of endophytic bacterial colonies in Petri dishes during the incubation period indicates that the bacterial cultures possess the ability to grow in N-free medium^[21].

In vitro evaluation of stress response to osmotic and salinity

To characterize salt tolerance, the isolates were cultivated at 35 ± 2 °C for 72 h in NA medium with varying NaCl concentrations (5%, 10%, 15%, and 20%). The assessment of osmotic stress tolerance involved the isolates' growth observation in nutrient agar medium supplemented with polyethylene glycol (PEG-6000) at 35 ± 2 °C for 24 h. The osmotic potentials in the media ranged from -0.15 MPa to -1.03 MPa^[22].

Molecular identification of endophytic isolates

A total of nine out of 13 isolates were selected for identification based on their PGP activities. Genomic DNA was extracted according to Wilson^[23]. The isolated genomic strains were analyzed on 0.8% agarose gel (Himedia) and amplification of the 16S rRNA (~1,500 bp) gene was carried in PCR using

universal primers [forward primer (27F) AGAGTTTGATCCTG GCTCAG, Reverse primer (1492R), CGGTTACCTGTTACGACTT (Eurofin Genomics, India)]. PCR was performed with 25 μ L reaction mixture composed of PCR buffer 2.5 μ L; $MgCl_2$ 1.5 μ L; 0.2 mm dNTPs 2 μ L; Primers each 1.25 μ L; Taq DNA polymerase 0.25 μ L (Thermo Fischer Scientific); template DNA 2 μ L; PCR water 14.75 μ L. The PCR conditions were as follows: initial denaturation 94 $^{\circ}C$ for 4 min, followed by 30 cycles of 30 s denaturation at 94 $^{\circ}C$, 1 min annealing at 63 $^{\circ}C$, 1 min extension at 72 $^{\circ}C$ and 7 min final extension at 72 $^{\circ}C$. The PCR products were purified before they were sent for sequencing.

Phylogenetic characterization

The PCR products of the endophytes were sequenced by BOKART INDIA Pvt. Ltd (Bengaluru, India) by Sanger's dideoxy nucleotide sequencing method. All obtained sequences were compared with those in the GenBank database by using the BLASTN search program. Similar sequences were further aligned by CLUSTALW. Phylogenetic analysis was conducted by the neighbor-joining method. Bootstrap analysis was performed with 1,000 replications to determine the support for each clad with grouping by the neighbor-joining method. A phylogenetic tree was constructed based on evolutionary distance data by using MEGA 11^[24].

Nucleotide accession number

The 16S rRNA nucleotide sequences acquired have been submitted to the National Center for Biotechnology Information (NCBI) GenBank to secure accession numbers. The unique accession numbers assigned to the bacterial isolates are MW680956 through MW680964.

Statistical analyses

All samples underwent triplicate analysis, and the data were reported as the mean value accompanied by the standard deviation for each data point. Data processing and visualization software options include GraphPad Prism (version 10.1.0), Molecular Evolutionary Genetics Analysis (MEGA version 11.0.13), and InteractiVenn for Venn diagram analysis^[25].

Results

Isolation of bacterial endophytes and biochemical characterization

A total of 43 endophytic bacterial isolates were recovered from different parts of *C. virgata*. The number of bacterial

isolates obtained from leaf, rhizome, and root were 9, 12, and 22 respectively. However they were grouped as per their morphological similarity (appearance of colonies) for further study into 13 strains (Table 1). All the isolates were oxidase negative but catalase positive and most of the isolates exhibited positive result for Voges-Proskauer test, oxidative fermentation, citrate utilization and nitrate reduction test (Table 2; Fig 1). The leaf isolate was indole negative, whereas all the root and rhizome isolates were indole positive.

Plant growth promoting traits

Amylase activity was present in 84.6% of the isolates. All root isolates except CHVRTB3 and CHVRTB7 (84.6%) isolates were positive for cellulase activity, except isolates CHVRTB4 and CHVRTB7 all isolates had shown cellulase activity. Protease activity was evident for (69.2%) isolates CHVLB1, CHRB2–CHVRB5; CHVRTB1, 2, 3 and 7. All isolates except CHVLB1 and CHVRTB3 were positive for lipase activity (Table 3, Fig 2).

IAA production of all isolates ranged between 0.033 to 0.054 μ g·mL⁻¹ (without tryptophan supplement) and between 11.53 to 29.53 μ g·mL⁻¹ (with tryptophan supplement). The isolated strains demonstrated the ability to biosynthesize indole-3-acetic acid (IAA) analogs in a minimal liquid medium containing 100 μ g·mL⁻¹ of tryptophan (Table 4). All strains exhibited the ability to produce > 10 μ g·mL⁻¹ of IAA. Specifically, 38.46% of the isolates produced over 20 μ g·mL⁻¹ of IAA, while 61.53% of the isolates yielded > 20 μ g·mL⁻¹ of IAA analogs. Notably, the root endophyte CHVRTB4 (identified as *Bacillus* sp.) displayed the highest average IAA production at 29.53 μ g·mL⁻¹, whereas CHVRTB3 (again *Bacillus* sp.) exhibited the lowest average production at 11.53 μ g·mL⁻¹.

Similar to IAA production all isolates tested produced GA3. Gibberellic acid production of all isolates ranged between 22.2–149.03 μ g·mL⁻¹ with 92.30% of the isolates producing > 50.0 μ g·mL⁻¹, highest mean being 149.03 μ g·mL⁻¹ (CHVRTB1 identified as *Glutamicibacter* sp.), CHVRB1 (146.72 μ g·mL⁻¹) as the second highest and lowest mean being 22.22 μ g·mL⁻¹ by CHVLB1. All isolates had shown positive result for qualitative ammonia production test (Fig. 3). The quantitative assessment revealed ammonia production levels ranging from 35.45–276.13 μ g·mL⁻¹. Specifically, 53.84% of the isolates exhibited ammonia production within the range of 70–90 μ g·mL⁻¹, while 38.46% of the isolates demonstrated production levels between 90–270 μ g·mL⁻¹. Notably, isolates CHVRTB1 and CHVLB1 displayed the highest (276.13 μ g·mL⁻¹) and the lowest

Table 1. Morphological characteristic of endophytic bacterial isolates from *Chloris virgata*.

Isolates	Colour	Surface	Shape	Edge/margin	Elevation	Opacity
CHVLB1	Off-white	Shiny	Circular	Smooth	Raised	Opaque
CHVRB1	Off-white	Irregular	–	Lobate	Flat	Opaque
CHVRB2	Off-white to pink	Irregular	–	Undulate	Raised	Opaque
CHVRB3	Off-white to pink	Shiny	Circular	Lobate	Raised	Opaque
CHVRB4	Off-white to pink	Mucoidal, glistening	–	Entire	Raised	Opaque
CHVRB5	Off-white	–	–	Lobate	Flat	Opaque
CHVRTB1	Off-white	Dull	Circular	Entire	Raised	Opaque
CHVRTB2	Yellow	–	Irregular	Undulate	Raised	Opaque
CHVRTB3	Off-white	–	Irregular	Undulate	Raised	Opaque
CHVRTB4	Off-white	–	–	–	Flat	Opaque
CHVRTB5	Off-white	Irregular	–	Lobate	Flat	Opaque
CHVRTB6	Off-white	Irregular	–	Lobate	Raised	Opaque
CHVRTB7	Off-white	Slimy, glistening	Circular	Entire	Raised	Opaque

* CHVLB: *Chloris virgata* Leaf bacteria; CHVRB: *Chloris virgata* Rhizome bacteria; CHVRTB: *Chloris virgata* Root bacteria.

Table 2. Biochemical characterization of endophytic bacteria.

Isolates	Indole production	Catalase	Oxidase	Methyl Red test	Voges-Proskauer test	Citrate utilisation	Oxidative fermentation		NO ₃ ⁻
							With oil	Without oil	
CHVLB1	-	+	-	-	+	+	+	+	+
CHVRB1	+	+	-	-	+	+	+	+	-
CHVRB2	+	+	-	-	+	+	+	+	+
CHVRB3	+	+	-	-	+	+	-	+	+
CHVRB4	+	+	-	-	+	+	+	+	+
CHVRB5	+	+	-	-	+	+	+	+	+
CHVRTB1	+	+	-	-	+	+	+	+	+
CHVRTB2	+	+	-	-	+	+	+	+	-
CHVRTB3	+	+	-	-	+	+	+	+	+
CHVRTB4	+	+	-	+	+	+	+	+	+
CHVRTB5	+	-	-	-	+	+	+	+	-
CHVRTB6	+	-	-	+	-	-	+	+	+
CHVRTB7	+	+	-	-	+	+	+	+	+

(+) = presence of activity; (-) = absence of activity.

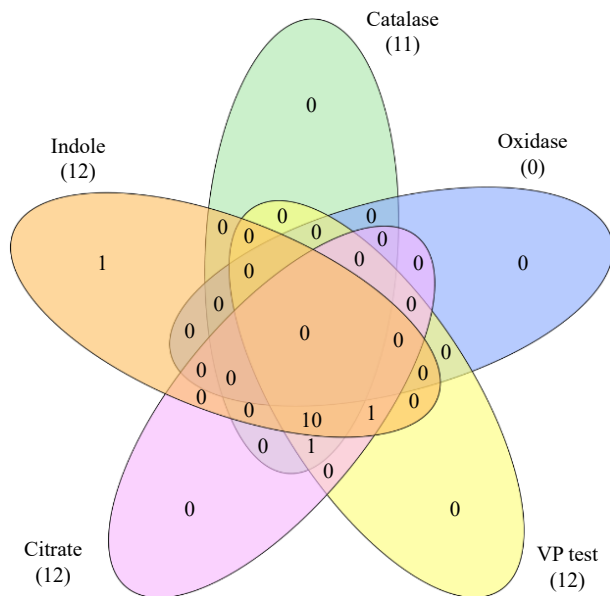


Fig. 1 Venn diagram showing biochemical characterization of bacterial endophytes.

(35.45 µg·mL⁻¹) concentrations of ammonia production, respectively.

Conversely, a lower percentage of the examined bacterial endophytic isolates, specifically 76.9% and 61.5%, exhibited positive outcomes for siderophore production and phosphate solubilization respectively. Phosphate solubilization was performed using both tricalcium (TCP) and dicalcium (DCP) phosphates as substrate. A total of 76.9% isolates showed DCP solubilization while 53.8% solubilized TCP. Zinc solubilization was altogether absent in all the isolates. Siderophores function by chelating iron and other metals, facilitating their absorption by plants. Additionally, they play a role in disease management by outcompeting phytopathogens for essential trace metals. Percent siderophore unit (PSU) was calculated for the isolates which was maximum for isolate CHVRTB4 (69.77%) and least for CHVRB5 (8.16%), unexpectedly leaf isolate CHVLB1 showed 32.71% siderophore units (Fig. 4). No bacterial isolates tested exhibited hydrogen cyanide (HCN) production. The ACC deaminase activity, known for aiding plants in resilience against both biotic and abiotic stress, was identified in 10 out of the 13 bacterial isolates. The majority of the isolated endophytic bacteria possessed more than three PGP traits out of the eight

Table 3. Qualitative observation of PGP traits of the endophytic isolates.

Isolates	Enzyme activity				Phosphate utilisation		Siderophore production	Ammonia production	HCN production	Acc deaminase	Nitrogen free growth
	Amylase	Cellulase	Protease	Lipase	TCP	DCP					
CHVLB1	-	+++	+++	-	-	+	+++	++	-	+	+++
CHVRB1	+++	++	-	+++	-	+	+	+++	-	+	+
CHVRB2	+++	+++	++	+++	+	+	++	+++	-	+	+
CHVRB3	+++	+++	+++	+++	+	+	+	+++	-	+	+
CHVRB4	+++	+++	+++	+++	+	+	+++	+++	-	+	+
CHVRB5	+++	+++	++	+++	-	+	-	+++	-	+	+
CHVRTB1	+++	+++	+++	+++	-	+	+++	++	-	+	+++
CHVRTB2	+++	+++	+++	+++	+	+	+++	++	-	+	+++
CHVRTB3	-	+++	+++	-	+	+	+++	++	-	+	+++
CHVRTB4	+++	-	-	+++	+	-	+++	++	-	-	-
CHVRTB5	+++	+	-	+++	-	+	-	+++	-	-	+
CHVRTB6	+++	+	-	+++	-	-	+	+++	-	-	+
CHVRTB7	+	-	+++	+	+	-	-	+++	-	+	+

* HCN: Hydrogen cyanide; TCP: Tricalcium phosphate, DCP: Dicalcium phosphate; Number of (+) sign indicates strength of activity, (-) indicates absence of activity.

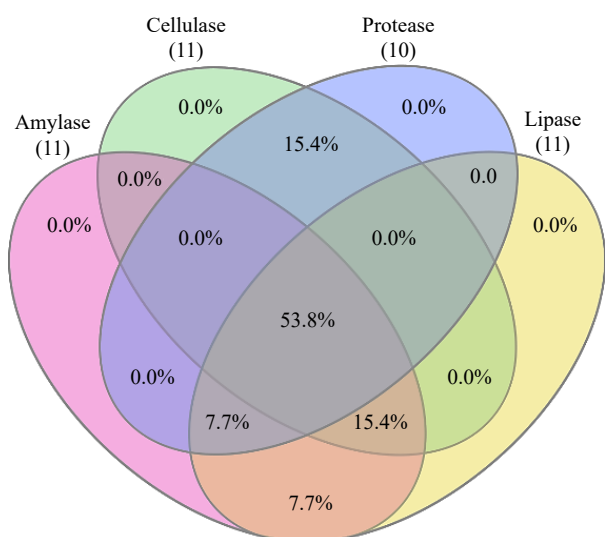


Fig. 2 Venn diagram showing extracellular enzyme assay exhibited by bacterial endophytes.

tested traits. The isolate CHVRTB4 obtained from the root exhibited majority of the PGP traits. When compared to the isolates from roots and leaves, the rhizome isolates showed positive outcome for the majority of the plant growth-promoting traits tested.

In vitro evaluation of bacterial response against salinity and osmotic stress

All 13 isolates exhibited the capacity to tolerate and thrive in NA medium supplemented with 5% NaCl. However, among these isolates, CHVRB1, CHVRTB4, and CHVRTB6 demonstrated limited growth in NA medium supplemented with NaCl, only up to 10%. No observable growth occurred at 15% and 20% salt concentrations. Regarding osmotic stress, when utilizing PEG 6000, 12 out of the 13 isolates demonstrated growth up to a water potential of -1.02 MPa (Table 5). In the context of bacterial osmotic stress tolerance, research has elucidated the biological mechanisms underlying the adaptation and survival of microorganisms in extreme environments. Under osmotic stress conditions, bacterial cells accumulate small compatible solutes, known as osmolytes. These include amino acids such as

glutamate, glutamine, proline, alanine, quaternary amines like glycine betaine, and sugars like sucrose, trehalose, and polyglucosyl granules. These osmolytes play a crucial role in improving cell growth under adverse osmotic conditions, serving as effective osmoprotectants.

Phylogenetic analysis of *Chloris virgata* bacterial endophytes based on 16s rRNA sequencing

All isolates exhibited 96%–100% similarity to sequences in the National Center for Biotechnology Information (NCBI) database. The phylogenetic analysis was divided into two groups according to genus: *Bacillus* and *Glutamibacter*.

Eight bacteria belonged to the *Bacillus* group (isolates CHVRB1, CHVRB2, CHVRB3, CHVRB4, CHVLB1, CHVRTB2, CHVRTB3, and CHVRTB4) and one belonged to the *Glutamibacter* group (isolate CHVRTB1). The analysis is represented by order Bacillales and Micrococcales. The 16s rDNA sequences were deposited in the GenBank with accession numbers MW680956–MW680964 (Table 6). The resulting phylogenetic tree of bacterial isolates revealed that isolates and reference sequences were clustered according to established taxonomic orders, with high bootstrap support (Fig. 5). The majority of the isolates were categorized under Bacillales order.

Discussion

In this study isolation of endophytes from *C. virgata* following surface sterilization resulted in 43 bacterial isolates where highest (37.2%) from root, rhizome (27.9%) and least from leaf (20.9%). However when grouped on morphological and biochemical characteristics and identified on molecular basis they fell only into two categorical orders (Bacillales and Micrococcales). Functional characterization of culturable endophytic bacteria is integral to enhancing our understanding of microbial communities existing in association with plants, such as endophytic bacteria. A significant proportion of the isolates exhibited notable enzymatic activities, including cellulase, amylase, lipase, and protease. These enzymes, encompassing cutinase, pectinase, cellulase, hemicellulase, protease, and lignin-peroxidases are predominantly linked to their involvement in penetrating plant tissues^[26]. Phosphate-solubilizing bacteria, when utilized as inoculants, augment phosphorus absorption by plants, consequently enhancing crop yield. The

Table 4. Quantitative observation of PGP traits of the endophytic isolates.

Isolates	Indole acetic acid production ($\mu\text{g}\cdot\text{mL}^{-1}$)		Gibberellic acid production ($\mu\text{g}\cdot\text{mL}^{-1}$) (540 nm)	Siderophore production (CAS ASSAY) % PSU	Ammonia production ($\mu\text{g}\cdot\text{mL}^{-1}$)
	Without trp	With trp			
CHVLB1	0.033 \pm 0.006	13.50 \pm 0.004	22.22 \pm 0.003	32.71 \pm 0.008	35.45 \pm 0.004
CHVRB1	0.036 \pm 0.006	16.91 \pm 0.008	146.72 \pm 0.004	41.05 \pm 0.012	71.28 \pm 0.031
CHVRB2	0.039 \pm 0.003	17.62 \pm 0.006	70.64 \pm 0.009	49.40 \pm 0.003	95.27 \pm 0.009
CHVRB3	0.038 \pm 0.005	20.76 \pm 0.003	74.92 \pm 0.007	49.30 \pm 0.003	98.33 \pm 0.006
CHVRB4	0.044 \pm 0.004	17.84 \pm 0.004	73.93 \pm 0.009	48.96 \pm 0.006	94.86 \pm 0.005
CHVRB5	0.028 \pm 0.009	23.95 \pm 0.005	97.64 \pm 0.008	8.16 \pm 0.003	73.64 \pm 0.032
CHVRTB1	0.038 \pm 0.004	12.19 \pm 0.007	^c 149.03 \pm 0.012	49.08 \pm 0.002	69.05 \pm 0.003
CHVRTB2	0.035 \pm 0.014	18.14 \pm 0.004	136.84 \pm 0.006	27.50 \pm 0.025	97.29 \pm 0.031
CHVRTB3	0.037 \pm 0.01	11.53 \pm 0.002	103.57 \pm 0.005	48.83 \pm 0.002	74.89 \pm 0.015
CHVRTB4	0.032 \pm 0.007	^b 29.53 \pm 0.008	96.33 \pm 0.005	^d 69.77 \pm 0.063	^e 276.13 \pm 0.13
CHVRTB5	^a 0.054 \pm 0.012	16.42 \pm 0.008	92.05 \pm 0.003	36.55 \pm 0.10	79.97 \pm 0.01
CHVRTB6	0.033 \pm 0.016	22.96 \pm 0.004	102.26 \pm 0.005	32.04 \pm 0.004	70.09 \pm 0.007
CHVRTB7	0.037 \pm 0.02	24.25 \pm 0.005	57.79 \pm 0.003	11.01 \pm 0.002	66.54 \pm 0.003

^aa = highest IAA produced without trp supplement; ^bb = highest IAA produced with trp supplement; ^cc = highest GA3 concentration produced; ^dd = highest PSU released; ^ee = highest concentration of ammonia released.

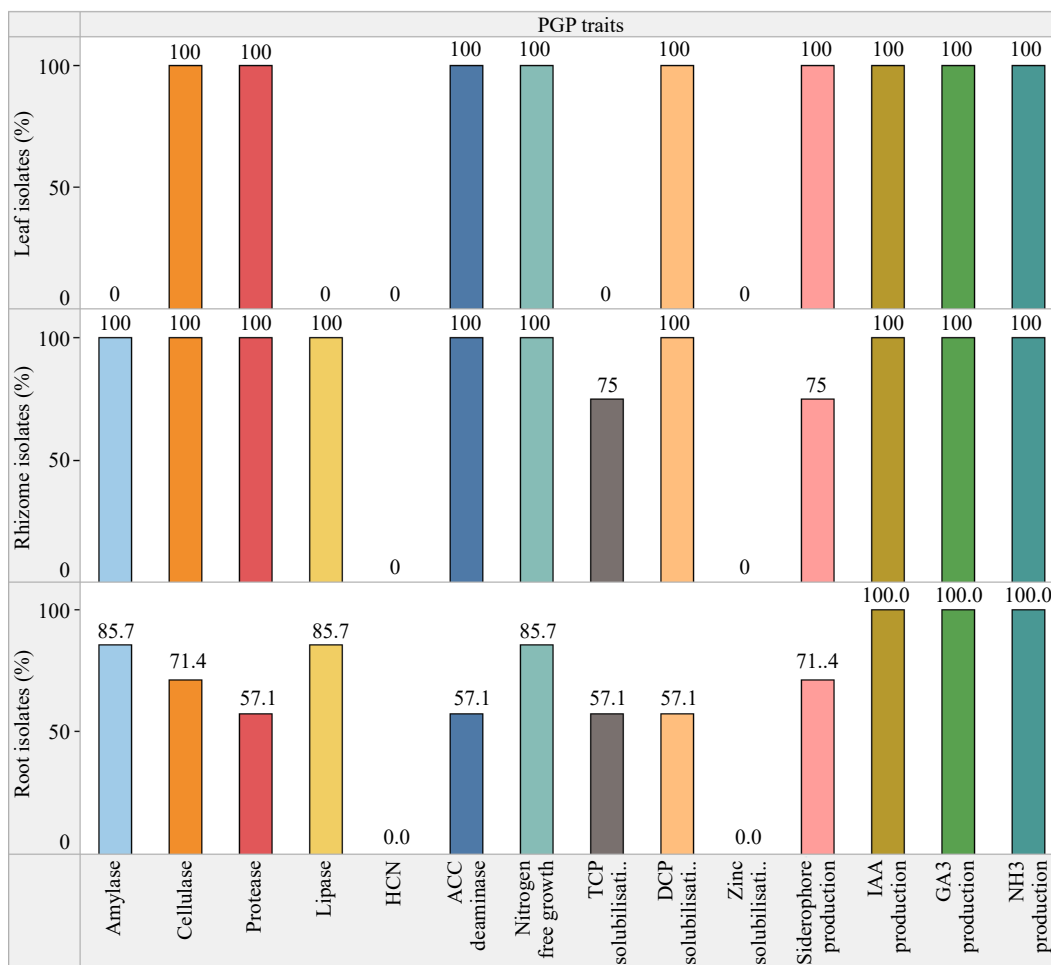


Fig. 3 % Bacterial endophytes of *C. virgata* with PGP activity.

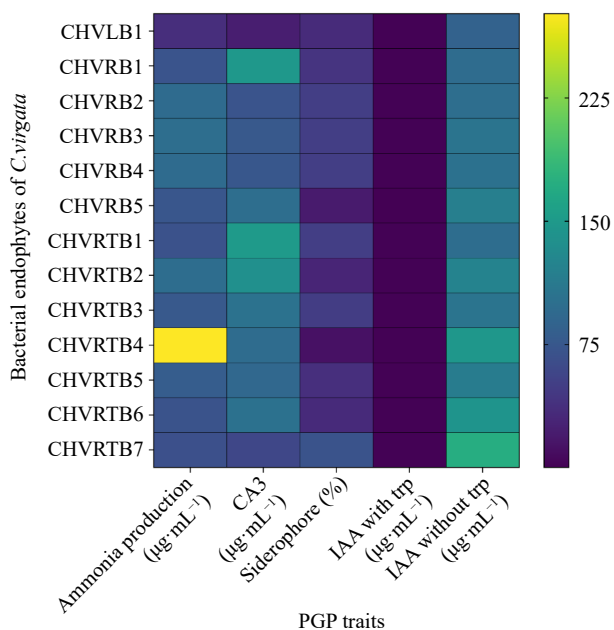


Fig. 4 Heatmap for plant growth promoting traits of the bacterial endophytes was prepared in terms of color intensity by normalisation of mean values of the results.

Table 5. Screening for salinity and osmotic stress tolerance on minimal DF (Dworkin and Foster) media.

Isolates	5% NaCl	10% NaCl	15% NaCl	20% NaCl	0.15 MPA	0.49 MPA	1.03 MPA
CHLB1	+	+	-	-	+	+	+
CHRB1	+	+	-	-	+	+	+
CHRB2	+	+	-	-	+	+	+
CHRB3	+	+	-	-	+	+	+
CHRB4	+	+	-	-	+	+	+
CHRB5	+	-	-	-	+	+	+++
CHRTB1	+	+	-	-	+	+	+
CHRTB2	+	+	-	-	+	+	+++
CHRTB3	+	+	-	-	+	+	++
CHRTB4	+	+	-	-	+	+	+++
CHRTB5	+	+	-	-	+	+	++
CHRTB6	+	-	-	-	+	+	++
CHRTB7	+	-	-	-	+	+	+++

Number of (+) sign indicates the increased appearance of colonies on media, (-) indicates absence of activity.

capability to solubilize inorganic phosphate is frequently observed in rhizospheric bacteria. DCP solubilization was exhibited by 2/3 isolates, while 1/3 isolates could solubilize TCP. Siderophores sequester iron (Fe³⁺, Fe²⁺) from the environment, making it accessible for use by both microbial and plant cells. Moreover, siderophores possess the ability to chelate undesirable metal ions (like Zn, Cd, Cr, Al, and Pb) in the rhizosphere,

Table 6. Taxonomic identification and sequence homology of isolated bacterial endophytes with genus or species affiliation and corresponding identity percentage in the NCBI database.

Endophytic isolates	Accession number	Bacterial genus with highest similarity	Accession number of closest match	Identity %
CHVLB1	MW680956	<i>Bacillus</i> sp.	NR_116240.1	99.45
CHVRB1	MW680957	<i>Bacillus</i> sp.	NR_148786.1	98.97
CHVRB2	MW680958	<i>Bacillus</i> sp.	NR_112686.1	98.64
CHVRB3	MW680959	<i>Bacillus</i> sp.	NR_112686.1	99.75
CHVRB4	MW680960	<i>Bacillus</i> sp.	NR_112686.1	99.84
CHVRTB1	MW680961	<i>Glutamicibacter</i> sp.	NR_026190.1	99.21
CHVRTB2	MW680962	<i>Bacillus</i> sp.	NR_113265.1	99.84
CHVRTB3	MW680963	<i>Bacillus</i> sp.	NR_112686.1	99.92
CHVRTB4	MW680964	<i>Bacillus</i> sp.	NR_157609.1	99.25

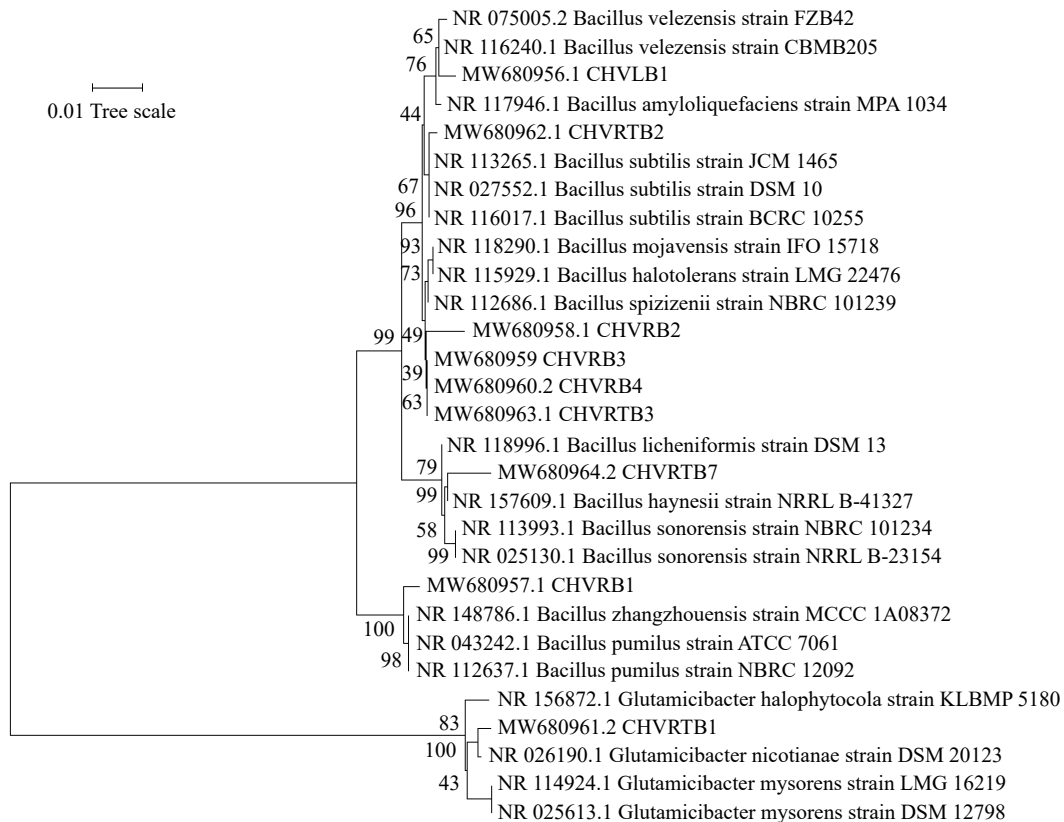


Fig. 5 Neighbor-joining phylogenetic tree based on the partial sequence of the 16S rRNA gene of bacterial isolates from *C. virgata* and their related type strains. The evolutionary distances were computed using the p-distance. Bootstrap values are given at branch nodes and are based on 1,000 replicates.

thus alleviating potential toxicity for plants. Furthermore, siderophores play a pivotal role in plant defense by actively acquiring nutrients and outcompeting phytopathogens in a competitive manner^[27]. Among the 13 bacterial endophytic isolates, 10 exhibited the ability to produce siderophores. The volatile secondary metabolite HCN is naturally synthesized by various soil microbes and endophytic bacteria^[28]. Moreover, HCN possesses broad-spectrum toxicity against fungi, nematodes, insects, and plants due to the sensitivity of heme groups in the targeted eukaryotic cells^[29]. However, none of the isolates displayed any detectable HCN production activity.

Plants benefit from ACC deaminase-producing PGPEs as they aid in alleviating various stress-related symptoms, stimulating growth, and supporting adaptation and survival. These beneficial microbes degrade ACC into ammonia and α -ketobutyrate,

providing plants with nitrogen and the necessary energy for optimal growth under stressful conditions^[30]. In this study bacterial endophytes were grown on DF minimal media to identify isolates able to synthesize ACC deaminase and grow in the medium. Positively all the isolates grew on DF medium. Jensen's N-free medium is usually used for isolation of diazotrophic microbes^[31]. However here it was used to check the growth of the isolates in N-free medium thus attempting to establish their role as a free living diazotroph.

Phytohormone production raises tolerance of the plant to abiotic stresses and promotes plant growth^[32]. Microbial IAA acts as a signaling molecule in plant-microorganism interactions. IAA production is known to be one of the most common traits among the endophytic bacteria. IAA has been associated with developmental processes of vascular tissue, adventitious

root initiation, tropistic responses, apical dominance, and the development of flowers and fruits. It also influences functions like cellular differentiation, expansion, and division^[33]. In this study the majority of the isolates were able to produce IAA, GA3, and ammonia. IAA production was exhibited by all the isolates. Gibberellic acid (GA) plays a pivotal role in plants for seed germination, elongation growth, flowering time regulation, and floral development. GA3 stands out as one of the key bioactive gibberellins, among others^[34]. *Bacillus* spp. (*B. siamensis*, *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, etc.) have been emerging as the largest and most promising group of naturally occurring (PGPR and PGBEs) that enhance plant growth via GA^[35]. GA3 production was comparatively higher in the root isolates.

A significant portion of plants can utilize only inorganic forms of nitrogen, such as ammonium and nitrate, and cannot directly utilize atmospheric nitrogen^[36]. In this study the root and rhizome isolates of *C. virgata* were the primary ammonia producers.

Salinity stress is one of the major abiotic stresses directly responsible for drastic effects on the physical and chemical properties of soil but also suppresses the growth and assortment of soil microbiota, nematodes and crop plants, resulting in stunted plant growth that eventually lead to reduced crop production^[37]. Remarkably, almost all endophytic bacteria (isolated and characterized in the present study) grew on nutrient agar medium supplemented with NaCl (5%–20%). All isolates tolerated upto 10% NaCl in the medium. The primary focus of the study centered on evaluating the plant growth-promoting (PGP) traits, as well as the tolerance of selected endophytes associated with *C. virgata* to salinity and osmotic stress. Out of the 13 isolates, nine were identified, with the majority (8) belonging to the Bacillales order, mainly *Bacillus* sp., and the remaining one to the Micrococcales order, identified as *Glutamicibacter* sp. A previous study on diversity of endophytic bacteria of *C. virgata* growing on oil contaminated sites showed *Bacillus* as dominant genera. The study in itself was a first on the community structure of endophytic bacteria in *C. virgata*. *Bacillus* sp. is the most common colonizing genus in the rhizosphere and roots of gramineous plants, which may be the cause of its predominance among hydrocarbon-degrading bacteria^[38].

Traditionally, cultivation-dependent techniques were employed to analyze the community structure of endophytic microbes. Culture dependent methods are commonly cost-effective. However, a notable drawback of cultivation-dependent approaches in elucidating endophyte diversity lies in the bias towards fast-growing, ubiquitous species. This bias may result in the oversight of rare species with lower competitive strength and more specialized requirements, limiting the scope of functional analysis and the potential agricultural applications of these overlooked species^[39,40]. Conversely, when only a small amount of nucleic acid of suboptimal quality is obtainable from the source, the application of culture-independent molecular methods becomes challenging. The majority of bacteria are not amenable to cultivation, leading to a restricted understanding of the mechanisms governing the interactions between plants and beneficial endophytic bacteria. This limitation impedes the practical utilization of endophytes. Therefore, in the future employing a sophisticated and comprehensive omics approach to investigate both the structural and

functional aspects of microbial communities can serve as a means to overcome this obstacle.

Conclusions

The findings reported here suggest that the endophytic bacteria isolated from *C. virgata* could have potential applications in plant disease management by promoting plant growth, mitigating stress, and enhancing disease resistance in host plants. It will also help in conducting detailed studies to understand the mechanisms behind the functional characteristics exhibited by the endophytic bacteria, such as enzyme activities, phosphate solubilization, siderophore production, and ACC deaminase activity. Investigating the interactions between the isolated endophytes and *C. virgata* to understand how these microbes contribute to plant growth, stress tolerance, and disease resistance. Performing field trials to assess the effectiveness of these endophytic bacteria will further enhance the potential application in sustainable agriculture.

Author contributions

The authors confirm contribution to the paper as follows: Lata R has conducted all the experiments, data collection, draft manuscript preparation under guidance of Gond SK; Chowdhury S has analysed and participated in the interpretation of results. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information (Supplemental File S1).

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Conflict of interest

The authors declare that they have no conflict of interest.

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