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Isolation, Characterization and Identification of Putative Bacterial Endophytes from Some Plants in Hot Springs, South Dakota

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A B S T R A C T

Endophytic bacteria to promote plant growth by facilitating nutrient acquisition through the fixation of nitrogen, solubilizing phosphate, producing siderophores, producing plant growth hormones, or enzyme1- aminocyclopropane-1- carboxylate (ACC) deaminase and protecting plants from pathogens, via production of antibacterial or antifungal agents, or outcompeting pathogens for nutrients. Isolation and development of new selected plant growth promoting endophytic bacterial strains could be one of the many new approaches that are needed to aid the growth and health of agricultural crops, to eliminate or minimize the harmful effects of inorganic fertilizers, and to conserve organic and inorganic soil nutrients. The aim of this study was to isolate, characterize and identify endophytic bacteria from plants growing along the stream banks in Hot Springs, South Dakota. The bacterial endophytes were isolated, identified and screened in vitrofor morphological features (Gram stain, Gram morphology, and colony morphology). Further, isolates exhibiting difference in morphological features were selected for molecular identification through partial 16S-rRNA gene sequencing. Twenty-five endophytic bacteria strains were isolated from monocotyledons plants, viz. *Typha, Bromus tectorum* and *Festuca* and eight strains from a dicotyledonous plant, *Nasturtium officinale*. All the isolated endophytic bacteria were identified as different bacterial strains belonging to *Bacillus thuringensis, B. cereus, B. atrophaeus, Pseudomonas sp., Cedeceadavisae, Escherichia sp., Acinetobacter calcoaceticus, Lysinobacillus sp., Pantoea sp., and Citrobacter freundii*. Further investigation is needed to screen these isolated endophytic bacteria for different activities known to promote plant growth and protection from phytopathogen.

Keywords

Endophytic bacteria, Plant growth promotion, Isolation, Characterization, Identification.

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Introduction

Most plants in nature associate with varied species of endophytic bacteria. About 300,000 plant species exist on the earth, and evidence suggests that most of them host one or more endophytes. However, only a few of these plants have been researched in detail with respect to their endophytic biology. Hence, expanding the search to identify new, as well as interesting, endophytic bacteria is important (Strobel *et al.,* 2005). The term endophyte was first coinedby De Barry (endon: within; phyton: plant) (Bary, 1866). Endophytic bacteria can be referred to as bacteria that live all or part of their life cycle colonizing inter, or intra-cellular, healthy tissues of the host plant, without causing symptomatic effects to the plant (Wilson, 1995). Endophytic bacteria found in plant

hosts comprise several genera and species. Evidence suggests that mostly every plant is populated with a diversity of endophytes. The interactions between endophyte communities inside plants are not well understood; however, it has been anticipated that beneficial effects are the combined result of their activities. Distribution of endophytic bacteria within plants depends on their ability to colonize and obtain plant resources. Endophytes can enter the plant tissues through the root zone or aerial portions of plants (Kobayashi and Palumbo, 2000), while they also have the ability to colonize different compartments of the plant apoplast, including the intercellular spaces of the cell walls, and xylem vessels, as well as reproductive organs of plants, including flowers, fruits and seeds. These bacteria do not normally cause any morphological changes, or symptoms of disease in the plant. However, many endophytic bacteria can positively influence plant growth. Most studies show that the main source of these endophytic colonizers is the rhizosphere (Hallmann *et al.,* 2006), but can also include the phyllosphere, anthosphere, and seeds (Compant *et al.,* 2005). Endophytes contact and colonize the host plant through cracks formed at the emergence of lateral roots or at the zone of elongation and differentiation of the root, then can quickly spread to the intercellular spaces in the root (Chi *et al.,* 2005). For instance *Klebsiella* strain Kp342 forms aggregates at lateral-root junctions of wheat and alfalfa (Dong *et al.,* 2003).Cellulolytic and pectinolytic enzymes produced by these endophytes contribute to efficiency in contacting and colonizing the host (Hallmann *et al.,* 1997). For example, in *Klebsiella* strains, pectate lyase is involved in plant colonization (Kovtunovych *et al.,* 1999).

Different plant hosts have different susceptibilities to being colonized by the same bacterial endophytes. For example, two *Klebsiella* strains differ in their occupation in different plant hosts (*Medicago sativa, Arabidopsis thaliana, Triticumaestivum* and *Oryza sativa*). One of the bacteria (Kp342) was a better colonizer in all hosts, and it needed only a single cell to colonize the plants (Dong *et al.,* 2003). Endophytic bacteria can be located inside different parts of a plant, such as roots, stems, leaves, seeds, fruits, and also inside legume nodules (Hallmann *et al.,* 1997). As a rule, more endophytes are found in the roots of plants than other plant parts (Rosenblueth and Martínez-Romero, 2006). Endophytic bacteria have the ability to penetrate the plant cell wall and become systemically spread throughout the host plant, sometimes actively colonizing the apoplast, and conducting vessels (Hallmann *et al.,* 1997), and occasionally the intracellular spaces. Most researchers have found that intercellular spaces and xylem vessels are the most common locations for endophytic bacteria (Reinhold-Hurek and Hurek, 1998).

Some endophytic bacteria have positive effects on the host plant. These may include the promotion of plant growth by producing various compounds, providing the plant with nutrients, and antagonizing plant pathogens through biological control. Plants severely restrict the endophytes growth, while the endophytic bacteria employ a number of mechanisms to slowly conform to their surroundings. In order to maintain a stable symbiosis, endophytes secrete a number of compounds, which enhance plants' growth and assist the endophytes in adapting better to the surroundings (Uma Maheswari *et al.,* 2013).

Different mechanisms are employed by endophytic bacteria to promote plant growth. These include both direct and indirect mechanisms. Direct mechanisms include facilitating nutrient acquisition through the fixation of nitrogen, solubilization of

phosphate, production of siderophores, production of phytohormones (such as auxins, cytokinins, and gibberellins), or production of the enzyme1- aminocyclopropane-1-carboxylate (ACC) deaminase (Tsavkelova *et al.,* 2006). Indirect mechanisms entail prevention of infections by pathogens, via production of antibacterial or antifungal agents, or outcompeting pathogens for nutrients (Nair and Padmavathy, 2014).

There has been a great deal of interest in recent years among investigators concerning endophytic bacteria, which has been facilitated by newly available and applied molecular techniques for their isolation and identification (Hallmann *et al.,* 1997). Generally, the endophytic bacterial community aids in enhancing crop production and health.

The development of selected endophytic bacterial strains that can promote plant growth could be one of many new approaches that are needed to aid the growth and health of agricultural plants. Isolation and development of beneficial endophytes could lead researchers using them as commercial products to help eliminate or minimize commercial fertilizers, and allow practices to conserve organic and inorganic soil nutrients. Moreover, the ability of some endophytes to protect against plant pathogens could help minimize the use of commercial pesticides (Glick, 2012).

The objectives of this study were, to isolate endophytic bacteria from the roots of four different plants, viz. *Typha* (Cattail), *Bromus tectorum* (Downy brome or cheatgrass), *Festuca*. (Fescue), and *Nasturtium officinale* (Watercress), growing along the stream banks in Hot Springs, South Dakota; to study selected phenotypic characteristics of the recovered bacterial strains in vitro, and to identify these endophytic bacterial isolates through partial 16S-rRNA gene sequencing.

Materials and Methods

Collection of plant samples

Plant samples were collected from the banks of streams in Hot Springs, SD. As much as possible, whole plants were obtained including both root systems and aerial portions. Then, the plant samples were transported to the laboratory for processing. In the lab, the plants were kept in water at room temperature until processing.

Identification of plants

Identification of collected plants species was performed by Dr. Gary Larson (Biology/Microbiology Department, South Dakota State University, Brookings, SD). Four different plant species were identified *Typha*(Cattail), *Bromus tectorum* (Downy brome or cheatgrass), *Festuca* (Fescue), and *Nasturtium officinale* (Watercress).

Isolation of putative endophytic bacteria from plants

Thirty-three bacterial strains were recovered from the collected plant samples. Twenty-five of these strains were isolated from roots of monocotyledons plants, *Typha*, *Bromus tectorum*, and *Festuca*. The remaining eight strains were isolated from roots of dicotyledons plants, *Nasturtium officinale*. For the isolation of endophytes, plant samples were showing healthy appearing (no disease symptoms), and subsequently were washed with sterile tap water to remove soil. These were further treated with 70% ethanol for 10 seconds, then 1% chloramine-T for 10 minutes with vigorous shaking, and then washed with sterile distilled water several times to remove chloramine-T.

After the treatment with chloramine-T, the roots were cut into 0.5 to 1 cm sections with

sterile surgical blade under aseptic conditions. The samples of roots were placed on a plate of nutrient-agar medium (Difco), or J-agar medium (5 g tryptone, 15 g yeast extract, 3 g HP, 20 g-agar, 2 g-glucose, 1000 ml distilled water, pH 7.3 to 7.5), which is recommended for culturing Bacillus (Bacon and Dorothy, 2004). All plates were incubated at room temperature (25) for five days, and observed periodically for bacterial growth. Isolated colonies were re-streaked until judged to be pure cultures by uniform colony morphology.

Preliminary characterization of putative endophytic bacterial strains

All the thirty-three bacterial isolates mentioned above were evaluated for Gram stain, potassium hydroxide (KOH) "string test" (Sutton, 2006), and colony morphology.

Identification of the putative endophytic bacterial isolates by 16S rRNA partial sequencing

The selected bacterial isolates were cultured on medium for extraction of genomic DNA for 16S rRNA gene analysis to identify strains.

Extraction of genomic DNA for 16S rRNA sequence analysis

Twenty-seven bacterial isolates were amenable to extraction of their genomic DNA in our SDSU laboratory. Genomic DNA was obtained from bacterial colonies by growing them on NA medium for 24 h at 28°C using a commercial bacterial genomic DNA extraction (Zymo research miniprep kit, Zymo Research Corporation, Irvine, CA) following manufacturer instructions.

Colonies were suspended in 1 ml sterile distilled water in Eppendorf tubes and centrifuged at 10.000 xg for 5 min and the supernatant discarded. Briefly, pellets were suspended in 750 µL lysis solution and vortexed for 5 min, followed by centrifugation at 10 000 xg for 1 min. 400 µL of the upper aqueous phase was aliquoted into a new Eppendorf tube and centrifuged at 7000 xgfor 1 min. 1200 µL of buffer was added to the filtrate and 800 µL of the mixture was transferred to the new collection tube and centrifuged at 10.000 xg for 1 min. The filtered DNA was pre-washed by adding 200 µL DNA pre-wash buffer and centrifuged at 10.000 xg for 1 min. 500 µL of DNA wash buffer was added to the new collection tube and centrifuged at 10.000 xg for 1 min. Finally, 100 µL of DNA elution buffer was added to elute the DNA in a clean 1.5 ml micro-centrifuge tube. The concentration of DNA was visualized by agarose gel electrophoresis (0.8 % agarose gel was electrophoresis run at 80 Volt for 40 min).

Polymerase Chain Reaction (PCR) amplification

The 16S rRNA gene of each strain was amplified by PCR in a 30 µL reaction containing 1 µL of template genomic DNA, 0.125 µL Taq DNA polymerase, 3 µL Taq buffer, 0.6 µL dNTP, 2.4 µL Mg, and 0.6 µL gene-specific primers 27f (5′GAGTTTGATCCTGGCTCA-3′), and reverse primer 518r (5'-GTATTA CCG CGG CTG CTGG-3'), with the addition of sterile deionized O to obtain a final volume of 30 µL. PCR amplification was performed using a thermocycler (Eppendrof ®Mastercycler nexus®) with the following PCR conditions for 50 cycles, initial denaturation of 94°C for four min, followed 94°C for 45 seconds. Then 50°C for 55 seconds, and 72°C for one min with a final extension of 72°C for 10 min (Ngoma *et al.,* 2013).

The PCR product was visualized by agarose gel electrophoresis (1% agarose gel was

electrophoresis run at 80 volts for 40 min). The PCR products with the primers were sent in 96 well plate, for sequencing (single pass PCR sequencing) by Beckman Coulter Genomics Company, (36 Cherry Hill Drive, Danvers, MA; 01923 USA). Then, the sequence data were checked by BLAST analysis in the NCBI database for microbial identification. The phylogenetic analysis of the 16SrDNA sequences of the strains was conducted with MEGA 6 (Molecular Evolutionary Genetics Analysis, version 6) software, using the neighbor-joining method.

Results and Discussion

Isolation of putative endophytic bacteria

Nine strains of the bacteria were isolated from *Typha*, five strains from *Bromus tectorum*, ten strains from *Festuca*, and eight strains from Nasturtium officinal (Table 1).

From the surface sterilization procedure for the isolation of putative endophytic bacteria, an adequate number of colonies were obtained in the culture using nutrient agar and J-agar media plates. Based on the distinct colony characteristics, the bacterial isolates obtained from 10 plates of nutrient agar (NA) and 2 plates of J-Agar (J) were grouped into different groups named as M1RNA, M2RNA, M3RNA, M4RNA, M2RJA, M3RJA, and D1RNA. Each distinct colony type was characterized as a putative bacterial endophyte.

Characterization of putative endophytic bacterial strains

For morphological characterization, the putative endophytic bacterial isolates were grown on NA to look for differences between colonies, in shape, color, elevation, margin, and texture (Willgohs and Bleakley, 1999). In addition, Gram stains were performed to evaluate Gram reaction, cell shape, and arrangement. Twenty-two isolates were Gram positive and negative for the KOH string test.

The remaining eleven isolates were Gram negative and positive for the KOH string test. This diversity of morphological characteristics of putative endophytic bacterial isolates indicated that they were different bacterial species (Table 2).

Identification of putative endophytic bacterial isolates by 16S rRNA partial sequencing

The 16S rRNA gene sequencing of the twenty-seven bacterial isolates were amplified and obtained from Beckman Coulter Genomics Company. The data BLAST analysis of 16S rRNA gene sequences for selected bacterial isolates showed alignments of these sequences with reported 16S rRNA gene sequences in the NCBI database. The highest similarities found with different bacterial genera for the bacterial isolates are summarized (Table 3).

The sequence analysis of 16S rDNA sequences of isolated bacteria showed the maximum identity (97%-100%) to different bacterial species belonging to the genera of *Bacillus, Pseudomonas, Escherichia, Lysinibacillus, Acinetobacter, Pantoea,* and *Citrobacter*. The bacterial isolates, (M2RNA 1-1, M2RNA 1-2, M3RNA4-6, M4RNA 3-4, M1RNA 10-2, M1RNA 12-1, M1RNA 12-2, M1RNA 12-3, D1RNA 7-2, D1RNA 7-3, and D1RNA8-1), Gram positive, rod shaped morphology, negative for potassium hydroxide, belonged to *Bacillus thuringensis* with 97% to 100% similarity. In addition, isolates (M2RNA 2-1,M2RJA 6-1,M4RNA 3-2,M1RNA 11-1,D1RNA 7-1, andD1RNA 13-5), Gram negative short rod shaped morphology, positive for potassium hydroxide, with 99% similarity, belonged to *Pseudomonas* sp. Isolates (M3RNA 4-3, M3RNA 4-8, and D1RNA 13-3),Gram

positive, rod shaped morphology, negative to potassium hydroxid, belonged to *Lysinibacillus* sp. Isolates (M3RNA 4-2, and M1RNA 10-1) were closely related with 99% to *Bacillus cereus*. In addition, Gram negative, short rod shaped morphology, positive to potassium hydroxide, (M3RNA 4-5, and D1RNA 8-2) isolates, denominated with 98% to 99% to be members of *Citrobacter fruendii*. Similarly, isolate (M2RJA 6-2) had a sequence similarity of 99%, to the type strain of *Pantoea* sp. The two isolates (M3RJA 5-1 and M3RJA 5-2) were classified as Cedeceadavisae/ *Escherichia hermannii* and *Escherichia* sp. (M1RNA 11-4) isolate belonged to *Bacillus atrophaeus*.

Fig.1 Phylogenetic analysis of 16S rRNA sequences of the putative endopytic bacterial isolates

Table.1 Isolation of putative endophytic bacteria from monocotyledon and dicotyledon plants

Type of plant	Number of bacteria isolated	Scientific name of the plant	Common name for the plant
Monocot 1	9	*Typha*	Cattail.
Monocot 2	5	*Bromus tectorum*	Downy brome, Cheatgrass.
Monocot 3	9	*Festuca.*	Fescue
Monocot 4	2	*Festuca.*	Fescue.
Dicot 1	8	*Nasturtium officinale*	Watercress.

Table.2 The morphological characteristics of putative endophytic bacteria on Nutrient Agar (NA)

Plate Code	Plate number	Culture number	KOH test	Gram Result	Gram Morphology	Cell Morphology	Cultural Morphology
M2RNA	1.00	1.00	Negative	Positive	Singles and clumps	Rods	circular, Entire, flat, large, rough, dull, non-pigmented, opaque
		2.00	Negative	Positive	Chains	Rods	circular, curled, flat, large, rough, dull, non-pigmented, opaque
M2RNA	2.00	1.00	Positive	Negative	Clumps	Short rods	circular, Entire, raised, small, smooth, shiny, non-pigmented, opaque
M4RNA	3.00	2.00	Positive	Negative	Singles and clumps	Short rods	circular, entire, convex, small, smooth, shiny, pigmented, opaque
		4.00	Negative	Positive	Single and chain	Rods	circular, curled, raised, large, rough, dull, non-pigmented, opaque
M3RNA	4.00	1.00	Negative	Positive	Chains	Rods	circular, curled, raised, large, rough, dull, non-pigmented, opaque
		2.00	Negative	Positive	Chains and clumps	Rods	circular, entire, flat, moderate, rough, dull, non-pigmented, opaque
		3.00	Negative	Positive	Clumps	Rods	circular, entire, raised, small, smooth, shiny, non-pigmented, opaque
		5.00	Positive	Negative	Clustered	Short rods	circular, entire, flat, small, smooth, shiny, non-pigmented, opaque
		6.00	Negative	Positive	Chains	Rods	circular, curled, flat, moderate, rough, dull, non-pigmented, opaque
		7.00	Negative	Positive	Single and clumps	Rods	circular, curled, raised, large, rough, dull, non-pigmented, opaque
		8.00	Negative	Positive	Chains and clumps	Rods	circular, entire, convex, punctiform, smooth, shiny, non-pigmented, opaque
M3RJA	5.00	1.00	Positive	Negative	Clustered	Coccobacilli	circular, entire, raised, small, smooth, shiny, non-pigmented, opaque
		2.00	Positive	Negative	Clustered	Coccobacilli	circular, entire, flat, small, smooth, shiny, non-pigmented, opaque
M2RJA	6.00	1.00	Positive	Negative	Clumps	Short rods	circular, entire, raised, small, smooth, shiny, non-pigmented, opaque
		2.00	Negative	Positive	Clumps and single	Coccobacilli	circular, entire, raised, small, smooth, shiny, non-pigmented, opaque
D1RNA	7.00	1.00	Positive	Negative	Single and clumps	Short rods	circular, rhizoid, flat, small, smooth, shiny, non-pigmented, opaque
		2.00	Negative	Positive	Chains	Rods	circular, entire, raised, large, rough, dull, non-pigmented, opaque.

Plate Code	Plate number	Culture number	KOH test	Gram Result	Gram Morphology	Cell Morphology	Cultural Morphology
		3.00	Negative	Positive	Chains and clumps	Rods	circular, entire, flat, moderate, rough, dull, non-pigmented, opaque
D1RNA	8.00	1.00	Positive	Negative	Chains and clumps	Rods	circular, entire, raised, small, smooth, shiny, non-pigmented, opaque
		2.00	Positive	Negative	Singles and chump	Short rods	circular, entire, raised, small, rough, dull, non-pigmented, opaque
M1RNA	10.00	1.00	Negative	Positive	Chains and clumps	Rods	circular, entire, raised, large, rough, dull, non-pigmented, opaque
		2.00	Negative	Positive	Chains and clumps	Rods	circular, entire, raised, moderate, rough, dull, non-pigmented, opaque
M1RNA	11.00	1.00	Positive	Negative	Clumps	Short rods	circular, Entire, raised, small, smooth, shiny, non-pigmented, opaque
		2.00	Positive	Negative	Singles and clumps	Short rods	circular, entire, raised, small, smooth, shiny, non-pigmented, opaque
		3.00	Negative	Positive	Chains and single	Rods	circular, entire, raised, moderate, rough, dull, non-pigmented, opaque
		4.00	Negative	Positive	Singles and clumps	Rods	Irregular, entire, Raised, moderate, smooth, shiny, non-pigmented, translucent
M1RNA	12.00	2.00	Negative	Positive	Singles and clumps	Rods	circular, entire, raised, large, rough, dull, non-pigmented, opaque
		3.00	Negative	Positive	Chains	Rods	circular, entire, raised, moderate, rough, dull, non-pigmented, opaque
		1.00	Negative	Positive	Chains and clumps	Rods	circular, entire, raised, moderate, rough, dull, non-pigmented, opaque
D1RNA	13.00	3.00	Negative	Positive	Chains and clumps	Rods	circular, entire, raised, small, smooth, shiny, non-pigmented, opaque
		4.00	Negative	Positive	Clustered	Coccobacilli	circular, Entire, flat, small, smooth, shiny, non-pigmented, translucent
		5.00	Positive	Positive	Clumps	Short rods	circular, filamentous, flat, small, smooth, shiny, non-pigmented, opaque

*M= Monocot plant. *D= Dicot plant. *R= Isolated from Root. *NA= Nutrient Agar. *JA= J- Agar.

Table.3 Identity of putative endophytic bacterial isolates by alignment of 16S rRNA gene sequences

Isolate code	Sequence length (bp)	Closest related in database	Accession number in NCBI	Similarity (%)	E-value	Plant name
M2RNA 1-1	484	*Bacillus thuringiensis*	HQ432809.1	100%	0	*Bromus tectorum*
M2RNA 2-1	517	*Pseudomonas sp.*	AB247229.1	99%	0	*Bromus tectorum*
M2RNA 1-2	1517	*Bacillus thuringiensis*	KF475852.1	99%	0	*Bromus tectorum*
M2RJA 6-1	517	*Pseudomonas sp.*	AB247229.1	99%	0	*Bromus tectorum*
M2RJA 6-2	2097	*Pantoea sp.*	FJ445213.1	99%	0	*Bromus tectorum*
M3RNA 4-1	537	*Bacillus thuringiensis*	C1504156520	97%	0	*Festuca.*
M3RNA 4-2	537	*Bacillus cereus*	C1504156521	99%	0	*Festuca.*
M3RNA 4-3	1506	*Lysinibacillus fusiformis*	KM817206.1	99%	0	*Festuca.*
M3RNA 4-5	784	*Citrobacterfreundii*	JQ267509.1	99%	0	*Festuca.*
M3RNA 4-6	1517	*Bacillus thuringiensis*	KP284269.2	99%	0	*Festuca.*
M3RNA 4-7	537	*Bacillus thuringiensis*	C1504156522	97%	0	*Festuca.*
M3RNA 4-8	1506	*Lysinibacillus fusiformis*	KM817206.1	99%	0	*Festuca.*
M3RJA 5-1	1385	*Cedeceadavisae/ Escherichia hermannii*	KC951923.1 HF585334.1	99%	0	*Festuca.*
M3RJA 5-2	1505	*Escherichia sp.*	KJ855238.1	99%	0	*Festuca.*
M4RNA 3-2	1531	*Pseudomonas mosselii*	KF784932.1	99%	0	*Festuca.*
M4RNA 3-4	1518	*Bacillus thuringiensis*	KJ767310.1	99%	0	*Festuca.*
M1RNA10-1	1066	*Bacillus cereus*	JQ912681.1	99%	0	*Typha*
M1RNA10-2	1517	*Bacillus thuringiensis*	KF475852.1	99%	0	*Typha*
M1RNA11-1	1484	*Pseudomonas sp*	FR823441.1	99%	0	*Typha*
M1RNA11-2	522	*Pseudomonas entomophila P. monteillii/P.putida*	C1504156523	100%	0	*Typha*
M1RNA11-3	537	*Bacillus thuringiensis*	C1504156524	97%	0	*Typha*
M1RNA11-4	1559	*Bacillus atrophaeus*	NR_075016.1	99%	0	*Typha*
M1RNA12-1	512	*Bacillus thuringiensis*	FJ755917.1	99%	0	*Typha*
M1RNA12-2	1517	*Bacillus thuringiensis*	KP284269.2	99%	0	*Typha*
M1RNA12-3	515	*Bacillus thuringiensis*	FJ755919.1	99%	0	*Typha*
D1RNA7-1	1502	*Pseudomonas sp.*	AJ785569.1	99%	0	*Nasturtium officinale*
D1RNA 7-2	1517	*Bacillus thuringiensis*	KF475852.1	99%	0	*Nasturtium officinale*
D1RNA 7-3	1517	*Bacillus thuringiensis*	KP284269.2	99%	0	*Nasturtium officinale*
D1RNA 7-3	1517	*Bacillus thuringiensis*	KP284269.2	99%	0	*Nasturtium officinale*
D1RNA 8-1	484	*Bacillus thuringiensis*	HQ432809.1	100%	0	*Nasturtium officinale*
D1RNA 8-2	1504	*Citrobacterfreundii*	KF145194.1	98%	0	*Nasturtium officinale*
D1RNA13-3	674	*Lysinibacillus sp.*	KC867319.1	99%	0	*Nasturtium officinale*
D1RNA13-4	1485	*Acinetobacter calcoaceticus*	KC900897.1	99%	0	*Nasturtium officinale*
D1RNA13-5	1484	*Pseudomonas sp.*	FR823441.1	99%	0	*Nasturtium officinale*

The identification of bacteria was further confirmed at phylogenetic level. The phylogenetic analysis of 16S rRNA sequence of the isolates along with the sequences retrieved from the NCBI database was carried out with MEGA 6 software using the neighbor-joining method. These results showed distinct clustering of the isolates (Figure 1).

The putative endophytic bacterial isolates were found associated with tissues of different plants, *Typha* (Cattail), *Bromus tectorum* (Downy brome or cheatgrass), *Festuca* (Fescue), and *Nasturtium officinale* (Watercress), growing along stream banks in Hot Springs, SD. These plants are relatively unstudied and being considered as potential source for natural products to be used in researches or agriculture fields.

In this study, a total of 33 bacterial strains were isolated from roots of different plants. The population of endophytes was found to be more in the roots than stems and leaves (Uma Maheswari *et al.,* 2013). The surface sterilization of roots tissue after rinsing with sterilized distilled water, and by sequential immersion in 70% ethanol and 1% chloramine-T ensured the removal of surface microbial flora. These chemical disinfectants have been employed for surface sterilization of excised roots tissue to remove epiphytes microbes; however, immersion of the tissues in ethanol and chloramine-T has shown significant success in different studies (Bacon and Dorothy, 2004). The processed tissues after dividing in to small pieces (0.5 cm to 1 cm sections), with sterile surgical blade under aseptic conditions were shifted to the isolation media. The putative endophytic bacterial colonies were purified by repeated sub culturing on NA or J agar, similar results were reported by Zinniel *et al.,* (2002). Subjection of the selected endophytic bacterial isolates to combinatory of morphological characterization, and 16S rRNA gene sequencing provided a specific identification of the bacterial isolates.

In morphological characterization, the putative endophytic bacterial isolates showed the diverse colony shapes, colors, margins and texture including round to irregular colonies, opaque to translucent with entire, curled, filamentous margins, for different endophytic bacterial isolates.

In addition, 22 among 33 putative endophytic bacteria isolates exhibited positive results for Gram staining while negative results for potassium hydroxide. In addition, 11of the putative endophytic bacteria isolates showed negative results for Gram staining while positive results for potassium hydroxide. These results indicated that potassium hydroxide was used to ensure the Gram staining results.

Furthermore, the putative endophytic bacterial isolates were determined by 16S rRNA gene sequencing. The BLAST analysis of 16S rRNA gene sequence data of the putative endophytic bacterial isolates showed alignments of these sequences with the reported 16S rRNA gene sequences in NCBI. The highest similarities found with different bacterial genera and NCBI accession number for the 33 bacterial strains were summarized in table 3.

The results indicated that the putative endophytic bacterial were isolated from Hot Springs, were found to be belonging to genera of *Bacilli, Pseudomonas, Citrobacter, Acinetobacter*, *Pantoea*, and *Enterobacter* after identified by 16S rRNA analysis.

Using different innovative tools of biotechnology will assist in fortifying the understanding of the interactions of plants and endophyte - such as their growth in plants,

and their secretion of new bioactive compounds, endophytes may be enhanced for biological control activity, and for decreasing the debris and other wastes that are otherwise harmful to the ecosystem. Putting all of this into consideration, endophytic bacteria have beneficial effects on the environment, industries, and agriculture. The utilization of their molecular activities that enhance our ability to better understanding and management of the endophytes could lead to new products with improved effectiveness.

References

Bacon, C.W.H. and Dorothy M. 2004. Techniques for Manipulating the Bacterial Endophyte *Bacillus* mojavensis. Methods in Biotechnology: Enviromental Microbiology: Methods and Protocols. A. L. R. S. John F. T. Spencer New Jersey, Humana Press Inc: 365.

Bary, A. 1866. Morphologie und physiologie der pilze, flechten und myxomyceten, W. Engelmann.

Chi, F., S.H. Shen, H.P. Cheng, Y.X. Jing, Y.G. Yanni and Dazzo, F.B. 2005. Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Appl. Environ. Microbiol.,* 71(11): 7271-7278.

Compant, S., B. Duffy, J. Nowak, C. Clément and Barka, E.A. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.,* 71(9): 4951-4959.

Dong, Y., A.L. Iniguez and Triplett, E.W. 2003. Quantitative assessments of the host range and strain specificity of endophytic colonization by *Klebsiella* pneumoniae 342. *Plant and Soil,* 257(1): 49-59.

Glick, B.R., D.M. Penrose and J. Li, J. 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J. Theoretical Biol.,* 190(1): 63-68.

Hallmann, J., A. Quadt-Hallmann, W. Mahaffee and Kloepper, J. 1997. Bacterial endophytes in agricultural crops. *Canadian J. Microbiol.,* 43(10): 895-914.

Hallmann, J., G. Berg and Schulz, B. 2006. Isolation procedures for endophytic microorganisms. Microbial root endophytes, *Springer,* 299-319.

Kobayashi, D. and Palumbo, J. 2000. Bacterial endophytes and their effects on plants and uses in agriculture. *Microbial Endophytes,* 199-233.

Kovtunovych, G., O. Lar, S. Kamalova, V. Kordyum, D. Kleiner and Kozyrovska, N. 1999. Correlation between pectate lyase activity and ability of diazotrophic *Klebsiella* oxytoca VN 13 to penetrate into plant tissues. *Plant and Soil,* 215(1): 1-6.

Nair, D.N. and Padmavathy, S. 2014. Impact of endophytic microorganisms on plants, environment and humans. *The Scientific World J.,* Article ID 250693, 11pp.

Ngoma, L., B. Esau and Babalola, O.O. 2013. Isolation and characterization of beneficial indigenous endophytic bacteria for plant growth promoting activity in Molelwane Farm, Mafikeng, South Africa. *African J. Biotechnol.,* 12(26): 4105-4114.

Reinhold-Hurek, B. and Hurek, T. 1998. Life in grasses: diazotrophic endophytes. *Trends in Microbiol.,* 6(4): 139-144.

Rosenblueth, M. and Martínez-Romero, E. 2006. Bacterial Endophytes and Their Interactions with Hosts. *Mol. Plant-Microbe Interactions*, 19(8): 827-837.

Strobel, G., B. Daisy and Castillo, U. 2005. The biological promise of microbial

endophytes and their natural products. *Plant Pathol. J.,* 4(2): 161-176.

Sutton, S. 2006. The Gram Stain, the Microbiology Network.

Tsavkelova, E., S.Y. Klimova, T. Cherdyntseva and Netrusov, A. 2006. Microbial producers of plant growth stimulators and their practical use: a review. *Appl. Biochem. Microbiol.,* 42(2): 117-126.

UmaMaheswari, T., K. Anbukkarasi, T. Hemalatha and Chendrayan, K. 2013. Studies on phytohormone producing ability of indigenous endophytic bacteria isolated from tropical legume crops. *Int. J. Curr. Microbiol. App. Sci.,* 2(6): 127-136.

Willgohs, J. and Bleakley, B. 1999. Laboratory Manual for General Microbiology, Pearson Custom Publishing.

Wilson, D. 1995. Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos,* 274-276.

Zinniel, D.K., P. Lambrecht, N.B. Harris, Z. Feng, D. Kuczmarski, P. Higley, C.A. Ishimaru, A. Arunakumari, R.G. Barletta and A.K. Vidaver, A.K. 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl. Environ. Microbiol.,* 68(5): 2198-2208.

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