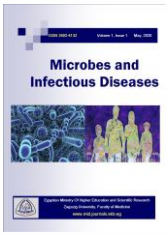
Microbes and Infectious Diseases 2025; Article-In-Press, **DOI:** 10.21608/MID.2025.370728.2649



Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Genome sequencing of endophytic bacterial species associated with *Datura stramonium* and *Sida acuta*

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ARTICLE INFO

Article history:
Received 23 March 2025
Received in revised form 9 May 2025
Accepted 28 May 2025

Keywords:
Endophytes
Datura stramonium
Sidaacuta
genome sequencing
antimicrobial products

ABSTRACT

Background: Endophytes are ubiquitous microbes that colonize plants' tissues without causing any harm to the host plants, but rather, they confer several adaptable characteristics on them. They could be of bacterial, fungal, algal, archaeal and actinomycetes origins. Endophytes also produce useful bioactive metabolites some of which are potent antimicrobials. In this study, bacterial endophytes were isolated from the leaves of *Datura stramonium* and *Sida acuta*, both of which are important medicinal plants. **Methods:** Freshly cut leaves were collected, rinsed and sequentially sterilized. The sterilized leaves were then cut into smaller pieces and incubated onto extract-seeded nutrient agar medium augmented with antifungal drug. Pure distinct bacterial colonies were morphologically and biochemically characterized. Molecular characterization of the strains was done using the 16S rRNA while phylogenetic evolutionary analysis was carried out using the maximum likelihood method and Tamura-Nei model with MEGA 11 software. **Results:** A total of 7 bacterial endophytes were isolated and identified, 3 from *D. stramonium* and 4 from *S. acuta*. 16S rDNA genome sequencing of the strains revealed that isolates from *D. stramonium* were *Pseudomonas aeruginosa*, *Atlantibacter hermannii* and *Enterobacter roggenkampii* while those isolated from *S. acuta* were identified as *Pseudomonas aeruginosa*, *Pseudomonas monteilii*, *Enterobacter pseudoroggenkampii* and *Aeromonas veronii*. **Conclusion:** The outcome of this research has indicated that numerous strains of endophytic bacteria inhabit leaves of plants. It is therefore essential to exploit these endophytic bacteria for their potentials to produce bioactive metabolites and other valuable antimicrobial products.

Introduction

Datura species are herbaceous, leafy, flowering annual plants, approximately two meters in height and belongs to the family Solanaceae [1]. Among about 25 species of *Datura* species that exist, *Datura stramonium* (jimson weed) is the most widespread [2]. *Sida acuta* (broom weed) on the other hand, is a flowering shrub that belongs to Malvaceae family [3]. Both *D. stramonium* and *S. acuta* are medicinal plants having various pharmaceutical uses in traditional medicine [1,3].

Endophytes are microbes that colonize both intercellular and intracellular tissues of plant species without causing any serious morphological

DOI: 10.21608/MID.2025.370728.2649
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alterations or diseases in the colonized plants. Endophytes could either be bacterial, fungal, archaeal, algal [4,5] and actinomycetes' endophytes [6] with bacterial and fungal endophytes the most reported [4]. Endophytes are ubiquitous in nature and found in all plant species where they colonize different parts of plants like leaves, petioles, stems and roots. Endophytic bacteria confer suitable characteristics and developmental features that enhance growth on plant host that they colonize. These characteristics range from adaptable features to environmental conditions, production of antioxidants, metabolites, antimicrobials, anti-cancer substances, antidiabetics and useful industrial enzymes [7].

Also, the biological compounds produced by endophytic bacteria play a pivotal role in the protection of medicinal and herbal plants against pests and pathogens of both human and plant origin. Living plants are a good source of bacterial endophytes which can be isolated for more efficient production of antimicrobial compounds with pharmacological importance. In turn, medicinal plants benefit from the endophytes in terms of growth promotion with less application of inputs such as fertilizers, fungicides, insecticides, or herbicides [8].

Numerous endophytes, including bacterial endophytes, have been isolated and reported by several authors, some of which were highlighted in Table 1. However, due to the difficulty in culturing most of these endophytes, a large number of them remains unidentifiable [9]. There is therefore the need to device more and more suitable methods that will enhance the cultivability and thus identification of more endophytes. In this research work, endophytic bacterial species from 2 prominent medicinal plants, *D. stramonium* and *S. acuta*, were isolated, using a newly modified method that was not only friendly, but also enable the isolates to be viable. The isolated strains were thereafter characterized biochemically and then identified using 16S rDNA genome sequencing.

Materials and methods

Collection and identification of plant materials

Plant sample was collected within Ilorin (8.4627º N, 4.4888º E) Kwara State, Nigeria. The plant specimens were identified at the herbarium of the Department of Plant Biology, University of Ilorin. Voucher numbers UILH/001/766/2024 and UILH/002/1256/2024 were deposited for *Sida acuta* and *Datura stramonium* respectively.

Preparation of plant materials

Healthy fresh leaves from the plant species were aseptically cut into ziplock bags and immediately transferred to the laboratory for further analysis.

Isolation of endophytic bacteria

Isolation of endophytic bacteria was done using standard methods [4,7,13,15] with slight modifications. The leaves collected from the plant species were gently washed under running tap water to remove any residual particles. The surface area of the leaves was then sterilized with 70% ethanol for about 1 minute followed by 3% sodium hypochlorite (NaClO) for about 2 minutes and then again with 70% ethanol for 30 seconds. This was then followed by a 5-fold rinsing in sterilized distilled water in order to neutralize the excess sterilants from the leaves. The leaves were then left to dry on sterilized aluminium foil. The sterilized leaves were then cut into smaller pieces of about 1cm in length using sterilized scalpel. The cut leaves were then seeded with nutrient agar medium augmented with antifungal drug (Fluconazole) in order to prevent fungal growth. Already dried powdered samples and extracts from the leaves were also added to the nutrient agar before sterilization. Aliquots of last tissue washing were inoculated onto nutrient agar to serve as control and also to confirm the effectiveness of the sterilization procedure. All plates were incubated at 37ºC for 48 hours in duplicates. After incubation, emergence of endophytic bacteria was observed. Distinct colonies were subcultured several times unto fresh nutrient agar medium to get pure colonies. Pure colonies were kept at 4ºC for further characterizations and uses. All isolates were kept on nutrient agar slants that have been seeded with powdered leaves/ extracts.

Morphological and Biochemical tests

Bacterial strains were subjected to Gram's reaction test and biochemical tests like motility, catalase, urease, methyl red, Voges-Proskauer, indole, citrate tests amongst others [16, 17].

DNA extraction and molecular identification of isolated endophytic bacteria

DNA extraction was done using the Quick-DNA bacterial Miniprep kit (Zymo Research) following the manufacturer's instructions. The 16S rDNA target region was amplified in OneTaq

Quickload 2X MasterMix (NEB) using the universal eubacterial primers 27f (5'-AGAGTTTGATCATGGCTCAG-3') and 1492r (5'-TACGGTTACCTTGTTACGACTT-3').

Amplifications were carried out in Thermal Cycler (CS Cleaver, Scientific Ltd., TC 32/80). The cycling conditions were as follows: One denaturing cycle at 94ºC for 4 min, followed by 40 denaturing cycles at 94ºC for 30 s, annealing at 45ºC for 30 s, and polymerization at 72ºC for 45 s. The amplification was terminated with an extension cycle at 72ºC for 7 min [18-20]. The PCR products were run on a gel electrophoresis and cleaned up enzymatically [21]. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, Brilliant Dye Terminator Cycle Sequence Kit V3.1, BRD3-100/1000) and purified using (Zymo Research ZR-96 bDNA sequencing clean-up kit). The purified fragments were analysed on the ABI 3500xl genetic analyser (Applied Biosystems). BioEdit sequence alignment editor version 7.2.5 was used to analyze the .abl files generated by the ABI 3500XL genetic analyzer. The homology of the 16S rDNA sequences of the isolates were using the Basic Local Alignment Search Tool (BLAST) from GenBank database (https://www.ncbi.nlm.gov/BLAST/). Alignment of the sequences was performed using the ClustalX (1.81) [22,23]. The seven endophytic bacteria sequences were submitted to GenBank and accession numbers were assigned.

Phylogenetic evaluation

Phylogenetic evolutionary analysis of the 16S rDNA base nucleotide gene sequence was carried out by using the maximum likelihood method and Tamura-Nei model [24, 25]. The evolutionary analysis was conducted using MEGA 11 software. The percentage of tress whereby the associated taxa clustered together were shown next to the branches while the initial tree for the search was automatically derived by using Neighbour-Join and BioNJ algorithms to a matrix of pairwise estimated using the Tamura-Nei model. The topology with superior log likelihood was then selected for all the sequences involved. Also, 1000 bootstrap value was used.

Results

Isolation of colonies on leaf samples

Colonies of endophytes (Plate 1) showing various forms of growth characteristics of isolates.

Biochemical characteristics of isolates

Biochemical characteristics of the endophytes isolated from the leaves of *D. stramonium* and *S. acuta* were represented in Table 2. All the isolated bacteria were Gram negative motile rods having a diverse of other characteristics as represented in the Table.

Agarose gel electrophoresis

Photographic image of the agarose gel was represented in Figure 1. It showed lane 1 (DNA ladder), the distribution of the 7 bacterial DNA bands from lanes 2 to 8 (ASA 1 to ASA 7) and the last lane(control) which has no DNA (NTC).

BLAST results of the isolated endophytes

The BLAST results of the endophytes that were isolated from the leaves of *D. stramonium* and *S. acuta* were represented in Table 3.

Phylogenetic evolutionary relationship

Pseudomonas aeruginosa and *Pseudomonas monteilii* were placed in different groups due to the fact that they belong to the same species level but different strains level. Also, all the other bacterial species were placed in their respective groups based on their similarity levels. The phylogenetic tree is shown in Figure 2.

Table 1. Some endophytic bacteria isolated from plants.

Host plant	**Isolated bacteria**	**References**
Tinospora cordifolia	*Bacillus* species *Aneurinibacillus* sp. *Pseudomonas* sp.	[10]
Platycodon grandiforum	*Pseudomonas aeruginosa*	[11]
Cacao sp.	*Bacillus subtilis*	[12]
Curcuma aeruginosa	*Bacillus* sp. *B. amyloliquefaciens* *B. cereus*	[13]
Curcuma zedoaria	*Bacillus* sp.	[13]
Crinum macowanii	*Bacillus megaterium* *Bacillus cereus*	[14]
Gloriosa superba	*Bacillus* sp. *Escherichia* sp. *Providencia* sp.	[15]
Datura stramonium	*Staphylococcus* sp. *Bacillus* sp. *Rhodococcus* sp.	[9]
Swertia chirata	*Cupriavidus*sp. *Bacillus* sp. *Rhodococcus* sp. *Cupriavidus metallidurans* *Staphylococcus* sp.	[9]

Table 2. Biochemical characteristics of isolated endophytes.

ISO	**GS**	**CS**	**CL**	**MT**	**CIT**	**CAT**	**OX**	**MR**	**VP**	**IND**	**UR**	**CO**
1	-	rod	G	+	+	+	+	-	-	-	-	-
2	-	rod	Y	+	-	+	-	+	-	+	-	-
3	-	rod	W	+	-	+	-	+	-	-	-	-
4	-	rod	G	+	+	+	+	-	-	-	-	-
5	-	rod	W	+	+	+	+	-	-	-	+	-
6	-	rod	W	+	-	+	-	+	-	-	+	-
7	-	rod	WY	+	+	+	+	+	-	+	+	-

ISO = Isolate; GS = Gram staining; CS = Cell shape; CL = Colour; MT = Motility; CIT= Citrate; CAT = Catalase; OX = Oxidase; MR = Methyl red; VP = Voges Proskauer's; IND = Indole; G= Greenish; W= Whitish; C= Creamy; Y= Yellowish; WY= Whitish yellow.

Table 3. BLAST results of isolated endophytic bacteria.

S/N	**Source**	**Percentage ID**	**GenBank Accession**	**Organisms**	**Strain Names**
1	*D. stramonium*	99.53 %	PQ661161.1	*Pseudomonas aeruginosa*	AKA_ASA_NBRDA_UIL1
2	*D. stramonium*	98.48 %	PQ661162.1	*Atlantibacter hermannii*	AKA_ASA_NBRDA_UIL2
3	*D. stramonium*	99.85 %	PQ661163.1	*Enterobacter roggenkampii*	AKA_ASA_NBRDA_UIL3
4	*S. acuta*	99.67 %	PQ661164.1	*Pseudomonas aeruginosa*	AKA_ASA_NBRDA_UIL4
5	*S. acuta*	99.87 %	PQ661165.1	*Pseudomonas monteilii*	AKA_ASA_NBRDA_UIL5
6	*S. acuta*	93.36 %	PQ661166.1	*Enterobacter pseudoroggenkampii*	AKA_ASA_NBRDA_UIL6
7	*S. acuta*	99.87 %	PQ661167.1	*Aeromonas veronii*	AKA_ASA_NBRDA_UIL7

Figure 1. A photographic image of an agarose gel electrophoresis indicating the amplification of the 16S rDNA target region

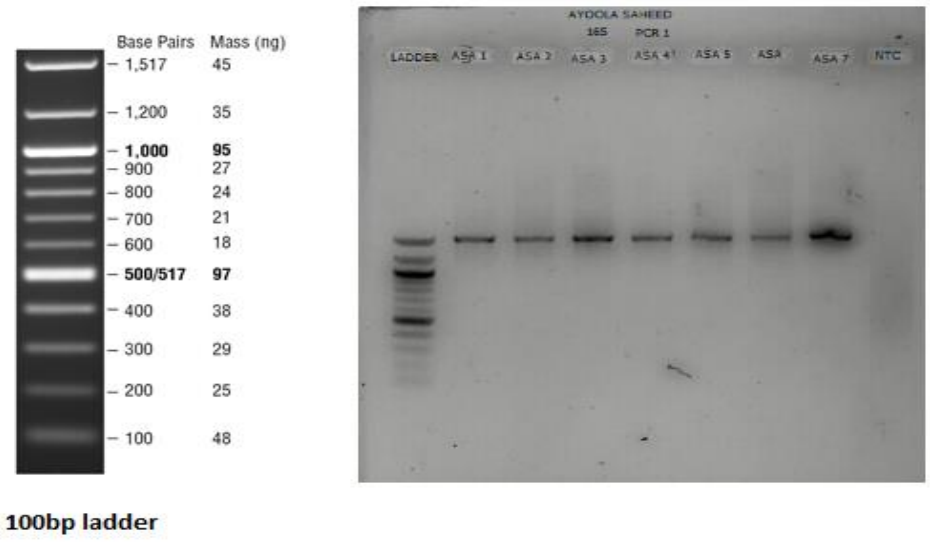


Figure 2. Phylogenetic tree of the 16S rDNA of the sequenced endophytic bacteria

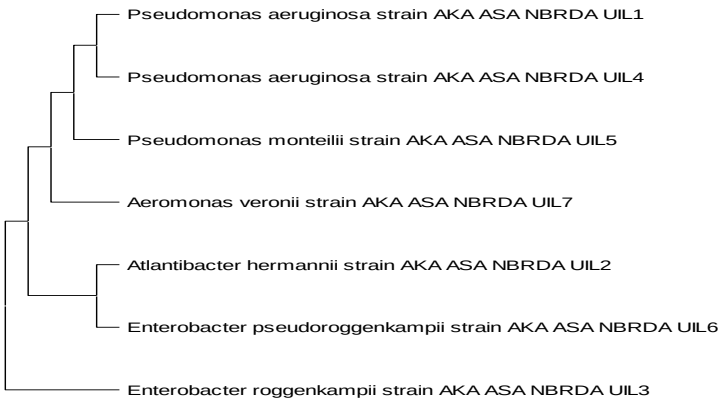
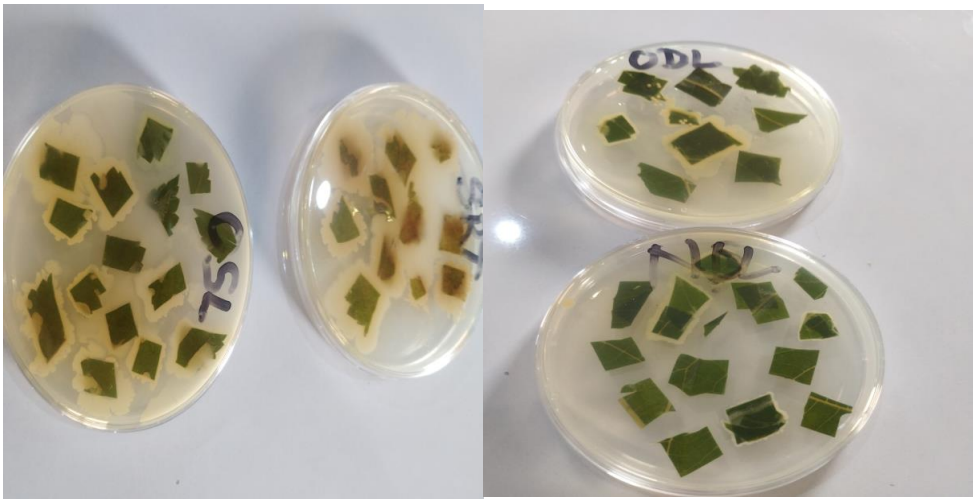


Plate 1: Plates showing bacterial colonies (endophytes) growing from the leaves of(A) *Sida acuta*; (B) *Datura stramonium*.



(A) (B)

Discussion

Three bacterial species were isolated from *D. stramonium* leaves while four bacterial species were isolated from *S. acuta* leaves. *Pseudomonas aeruginosa*, *Atlantibacter hermannii* and *Enterobacter roggenkampii* were isolated from *D. stramonium* while *Pseudomonas aeruginosa*, *Pseudomonas monteilii*, *Enterobacter pseudoroggenkampii* and *Aeromonas veronii*were isolated from *S. acuta.*

Endophytic bacterial species have been isolated from different plants by several researchers. *Stenotrophomonas maltophilia* and *Bacillus mojavensis* were isolated from *D. stramonium* [18] while *Staphylococcus* species, *Bacillus* species, and *Rhodococcus* species were isolated from *D. stramonium* and *Cupriavidus metallidurans* from *Swertia chirata* [9]. Species of *Bacillus* and *E. coli* were also isolated from *Gloriosa superba* [15]; *Pseudomonas* species, *Enterobacter* species and *Azotobacter* species [26].

Several endophytes have been isolated from numerous other plant species, some of which are medicinal plants. Interestingly, some of the bacterial strains that were isolated in this research work (Table 3) have not been reported for the plants under study. Also, to our knowledge and findings, endophytic bacterial species have not been reported for *Sida acuta* which is a very useful medicinal plant. This research has also successfully employed the use of general-purpose agar medium seeded with plants' powder/ extracts to isolate endophytic bacterial species from plants.

Conclusions

In this study, bacterial species of diverse strains, some of which have not been previously reported, were isolated from the leaves of *D. stramonium* and *S. acuta*, both of which are useful medicinal plants. The isolation, as demonstrated in this study, was carried out using a general-purpose medium (nutrient agar) which has been seeded with powdered leaves/extracts from these respective plants. The bacterial species were morphologically and biochemically screened; thereafter characterized using 16S rDNA genomic sequencing. A total of 7 endophytes consisting of 3 strains of *Pseudomonas* species, 2 strains of *Enterobacter* species, and 1 strain each of *Atlantibacter hermannii* and *Aeromonas veronii* were isolated and genomically sequenced. Due to the difficulties associated with culturing endophytic bacteria, identification of several strains of endophytes, including bacterial endophytes, has not been an easy task. There is therefore the need for development of methodologies that will make it easier for more endophytes to be culturable, identifiable and thus analyzed further for production of enzymes, bioactive antimicrobial metabolites and other useful products.

Authors' contributions

Conception and design of the study: SA Ayoola, AK Ajijolakewu. Field work and acquisition of data: SA Ayoola, AK Ajijolakewu, AE Ajadi. Analysis and interpretation of data: SA Ayoola, AK Ajijolakewu, AE Ajadi, RF Zakariyah, MO Kazeem, JO Oyedele. Drafting the article and revising critically for intellectual content: SA Ayoola, AK Ajijolakewu, AE Ajadi, RF Zakariyah, MO Kazeem, JOOyedele. Final approval of version to be submitted: All authors have approved the submission of the final article.

Disclosure of potential conflicts of interest

The authors report no conflicts of interest.

Funding

Non declared

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