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MOLECULAR IDENTIFICATION AND ANTAGONISTIC ACTIVITY OF ENDOPHYTIC BACTERIA ISOLATED FROM *ARTEMISIA AUSTRIACA*

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Abstract

This study aims to molecularly identify and evaluate the antagonistic activity of endophytic bacteria isolated from *Artemisia austriaca*. Three bacterial strains, representing the families *Enterobacteriaceae*, *Bacillaceae*, and *Microbacteriaceae*, were isolated and their morphological and biochemical characteristics were determined. Molecular identification, performed via 16S rRNA gene sequencing, revealed that the isolates were *Pantoea agglomerans* (AG1), *Bacillus proteolyticus* (AY1), and *Microbacterium sp.* (AY2). Furthermore, the antagonistic activities of these strains were tested against several pathogenic bacteria, including *Staphylococcus aureus*, *Escherichia coli*, and *Proteus mirabilis*. Among the isolates, only *B. proteolyticus* (AY1) exhibited antimicrobial activity against all three indicator pathogens. In conclusion, this study represents the first report of endophytic bacteria isolated from *A. austriaca* and demonstrates their antagonistic effects against pathogenic bacteria.

Key words: *Artemisia austriaca*, endophytic bacteria, isolation, antagonistic activity

Introduction. The genus *Artemisia* L., belonging to the Asteraceae family, includes approximately 500 species and exhibits a wide distribution in the northern

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hemisphere [1]. One of the common species of this genus, *Artemisia austriaca* Jacq., is found in regions such as Central Europe, Siberia, as well as in certain areas of Türkiye and Iran [2]. *A. austriaca* is a medicinal plant popularly used for respiratory issues, insomnia, ulcers, and high body temperature. Additionally, its tinctures, infusions, and decoctions are used in the treatment of various ailments, including wound healing, appetite stimulation, as well as anticonvulsant, analgesic, antihelminthic, anti-edema, and dysmenorrhea conditions [3].

Bacteria living in the internal tissues of plants are called endophytic bacteria. These bacteria reside within the tissues of the host plant without causing harm. The symbiosis between endophytes and plants provides various benefits in agriculture, such as promoting plant growth, protecting plants against pests and pathogens, and increasing resistance to environmental stressors. Furthermore, the bacteria produce several secondary metabolites of medicinal importance, which may demonstrate antimicrobial, anticancer, and anti-inflammatory activities Understanding the functions of bacterial endophytes can help us utilize them more effectively to improve the yield and quality of medicinal plants [4].

The genus *Artemisia* is a potential source of many endophytic bacteria with various important properties and bioactive compounds. Numerous studies have focused on the isolation, characterization, and biological activities of endophytic bacteria associated with *Artemisia* species [5,6]. Research on *A. austriaca*, on the other hand, has predominantly concentrated on evaluating the antibacterial and antioxidant activities of plant-derived extracts and essential oil components [7–9]. Furthermore, the allelopathic potential of the essential oil derived from the aerial parts of *A. austriaca* has been documented, suggesting its broader ecological significance [2]. However, there are no studies in the literature on the endophytic bacteria inhabiting *A. austriaca* and their antagonistic activities.

This study aimed to isolate and identify the endophytic bacteria from *A. austriaca* growing in Artvin, Türkiye, and to investigate the antagonistic activity of these bacteria against some pathogenic bacteria.

Materials and methods. ***Plant material.*** The *A. austriaca* plant samples used in this study were collected during fieldwork in June 2022 in Artvin, Türkiye. While collecting the samples, the plant parts (stem and leaves) were visually examined, with particular attention given to selecting healthy plants. The specimens were then placed in clean plastic bags, with the top left open and the sides designed to allow airflow, ensuring that the samples were not compressed. Subsequently, the samples were transported to the laboratory and stored at $-20\,^{\circ}\mathrm{C}$ for further analysis. The location of the collected *A. austriaca* samples was recorded at an elevation of 592 m with geographic coordinates of $41^{\circ}08'54.2''$N latitude and $41^{\circ}55'29.6''$E longitude.

Isolation of endophytic bacteria. Bacterial isolation studies were carried out with utmost attention to sterility, using the stem and leaves of the plant for isolation. First, the plant samples were cleaned of dust, soil, and other particles on

their surfaces by serial washing with sterile, purified water. Next, the stem samples underwent a surface sterilization procedure, beginning with a 3-minute immersion in 70% ethanol, followed by a 3-minute exposure to 2% sodium hypochlorite, and concluding with a final 3-minute treatment in 70% ethanol. Similarly, the leaf samples were sterilized by first being immersed in 70% ethanol for 3 min, then exposed to 2% sodium hypochlorite for 2 min, and finishing with a second 2-minute exposure to 70% ethanol. These procedures were followed by five serial rinses with sterilized distilled water. After surface sterilization, the samples were gently dried using sterile filter paper, then sectioned into small fragments and homogenized in sterile nutrient broth. Serial dilutions were prepared from the homogenate and transferred to the medium, followed by incubation at 30 °C for 4–5 days. Endophytic bacteria were isolated from developing colonies, purified, and stored at −20 °C. The success of the sterilization process was verified by transferring the wash control water into the medium.

Characterization of endophytic bacteria. Some morphological characteristics (cell morphology, motility), biochemical tests (Gram reaction, catalase, oxidase) [10], as well as other enzymatic activities and phenotypic characteristics (with the application of the API 20E, API 20NE, and API 50CH test kits), were determined.

Molecular identification of endophytic bacteria. The bacterial colonies were cultured in nutrient broth at 30 °C for approximately 18 h. Subsequently, DNA was isolated from the bacterial samples using a commercially available DNA isolation kit (Norgen Biotek Corp., Canada) following the manufacturer's instructions.

The 16S rRNA gene region was PCR-amplified with universal primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (GGY TAC CTT GTT ACG ACT T). A 20 µl PCR reaction mixture was prepared following the PCR Master Mix (A.B.T.™) protocol. For each sample, the reaction mixture consisted of 10 µl of Hs-Taq master mix, 1 µl of forward primer, 1 µl of reverse primer, 4 µl of template DNA, and 4 µl of dH2O. The prepared samples were then placed into a PCR thermocycler (T100™ Thermal Cycler, Bio-Rad, Hercules, California, USA), programmed with the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min.

The PCR products were then examined through electrophoresis on a 1% agarose gel in 1x TAE buffer to confirm the presence of the desired amplicon. The sequencing of the amplified product was performed by a commercial sequencing service, with sequences obtained in both directions (Macrogen, Netherlands). The sequence results were used for identification through BLAST in NCBI GenBank [11]. The sequences were processed using BioEdit (version 7.09) and subsequently aligned using ClustalW [12]. Based on the results, a phylogenetic tree was generated using the MEGA 11 program [13] (Fig. 1).

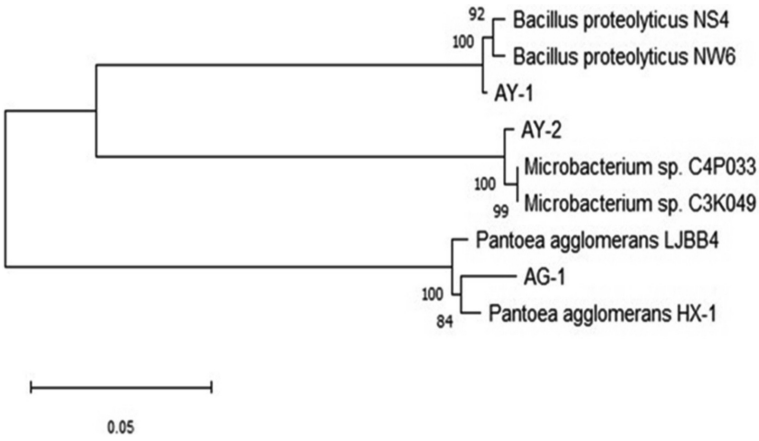

Fig. 1. Phylogenetic relationships of bacteria isolated from *A. austriaca*

Antagonistic activity assay. Antimicrobial activity of bacterial isolates was determined using the Mueller–Hinton Agar (MHA) by the agar diffusion method. In this method, one Gram-positive bacterium (*Staphylococcus aureus* ATCC 25923) and two Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Proteus mirabilis* ATCC 43071) were used as indicator pathogenic bacteria. First, the turbidity of 18–24 hour bacterial cultures was adjusted to the McFarland standard 0.5 scale (approximately 1×10^8 cfu/ml of bacterial cells). Then, 100 µl of each indicator pathogenic bacterial suspension was transferred to MHA and spread evenly over the entire surface with a sterile swab, followed by a 15-minute incubation. After this period, sterile 6 mm diameter empty disks (Bioanalsye) were placed on the Petri dish, and the disks were treated with 20 µl of the endophytic bacterial suspension. The agar plates were incubated at 37 °C for a period of 24 h, following incubation, the antimicrobial activity was subsequently quantified by measuring the diameters of the inhibition zones surrounding the discs. Sterile physiological saline was used as a negative control.

Results and discussion. ***Isolation and identification of endophytic bacteria.*** In this study, three distinct endophytic bacterial isolates were identified based on colony morphology from suspensions derived from the stem and leaf parts of *A. austriaca*. The AG1 isolate was obtained from the plant stem, while the AY1 and AY2 isolates were sourced from the leaf. On nutrient agar medium, the AG1 isolate formed smooth, yellow-coloured, translucent colonies; the AY1 isolate produced white-coloured, opaque, round colonies; and the AY2 isolate formed yellow-coloured, circular, convex colonies (Table 1). These isolates were characterized based on various morphological characteristics (such as cell morphology, colony colour, and motility) as well as biochemical properties (including Gram staining, oxidase, and catalase tests). Furthermore, other phenotypic and biochemical characteristics of the bacterial isolates were evaluated using API test strips (API 50CH, API 20E, and API 20NE).

Table 1

Isolation sources and colony characteristics of bacteria isolated from *A. austriaca*

Isolate Code	Colony characteristics	Plant parts
AG1	Yellow, smooth, translucent	Stem
AY1	White, round, opaque	Leaf
AY2	Yellow, circular, convex	Leaf

With the exception of the AG1 isolate, all other isolates were identified as Gram-positive. The AY1 and AG1 isolates exhibited rod-shaped morphology, whereas the AY2 isolate demonstrated a short rod morphology. The AG1 isolate was found to be motile, while the remaining isolates were non-motile. All isolates were catalase-positive. The oxidase test yielded positive results for the AY1 isolate and weakly positive results for the AY2 isolate.

The API 50CHB test revealed that the AY1 isolate was able to utilize glycerol, D-ribose, D-glucose, D-fructose, N-acetylglucosamine, arbutin, aesculin ferric citrate, cellobiose, maltose, D-sucrose, trehalose, starch, glycogen, and gentiobiose (Table 2). The results of the API 20E and 20NE tests showed that nitrate reduction was positive for both the AG1 and AY2 isolates, but negative for the AY1 isolate. The β-galactosidase (Onpg:Ortho-NitroPhenyl βDGalactopyranosidase) test was positive only for the AG1 isolate, while the arginine dihydrolase reaction yielded positive results exclusively for the AY1 isolate. Urease hydrolysis was observed only in the AY2 isolate. All three isolates demonstrated the ability to hydrolyze gelatin. In addition, the AG1 isolate has provided positive results for glucose, D-mannitol, L-rhamnose, D-sucrose, amygdalin, and L-arabinose. The detailed phenotypic and biochemical characteristics of the isolates are presented in Tables 2 and 3.

Based on the results of 16S rRNA sequence analysis, the bacteria were identified as *Pantoea agglomerans* (AG1), *Bacillus proteolyticus* (AY1), and *Microbacterium sp.* (AY2). GenBank accession numbers were obtained for each isolate: AG1 (PP905253), AY1 (PP905255), and AY2 (PP905256), and the sequences were submitted to GenBank. A phylogenetic tree was generated employing the Neighbour Joining method (Fig. 1).

Endophytic bacteria can serve as a source of natural products with various medicinal applications, including antimicrobials, antiviral agents, antioxidants, anticancer molecules, insecticides, antidiabetic compounds, and volatile substances [14]. As a result, interest in the ability of medicinal plant endophytes to produce bioactive compounds, including antimicrobial substances, is steadily increasing. Previous studies have reported that species from the genera *Bacillus*, *Microbacterium* and *Pantoea* were isolated from important medicinal plants such as *Artemisia annua* [15], *Artemisia nilagirica* [16] and *Artemisia absinthium* [17].

T a b l e 2

API 50CH test results of AY1 isolate

Tests	24 h	48 h
Glycerol	wp	+
Erythritol	−	−
D-Arabinose	−	−
L-Arabinose	−	−
D-Ribose	wp	+
D-Xylose	−	−
L-Xylose	−	−
D-Adonitol	−	−
Methyl-β-D-xylopyranoside	−	−
D-Galactose	−	−
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	−	−
L-Sorbose	−	−
L-Rhamnose	−	−
Dulcitol	−	−
Inositol	−	−
D-Mannitol	−	−
D-Sorbitol	−	−
Methyl-α-D-mannopyranoside	−	−
Methyl-α-D-glucopyranoside	−	−
N-Acetylglucosamine	+	+
Amygdalin	−	−
Arbutin	+	+
Esculin-ferric citrate	+	+
Salisin	−	−
D-Celiobiose	−	+
D-Maltose	+	+
D-Lactose (bovine origin)	−	−
D-Melibiose	−	−
D-Saccharose	+	+
D-Trehalose	+	+
Inulin	−	−
D-Melezitose	−	−
D-Raffinose	−	−
Starch	−	+
Glycogen	+	+
Xylitol	−	−
Gentiobiose	+	+
D-Turanose	−	−
D-Lyxose	−	−
D-Tagatose	−	−
D-Fucose	−	−
L-Fucose	−	−
D-Arabitol	−	−
L-Arabitol	−	−
Potassium gluconate	−	−
Potassium 2-ketogluconate	−	−
Potassium 5-ketogluconate	−	−

+: positive, wp: weak positive, −: negative.

Table 3

Morphological, biochemical, and phenotypic characteristics of the isolates

Tests	AY1	AG1	AY2
Gram reaction	+	−	+
Cell morphology	R	R	SR
Motility	−	+	−
Oxidase	+	−	wp
Catalase	+	+	+
$NO_3 \rightarrow NO_2$	−	+	+
β-galaktosidase (Onpg)	−	+	nd
Arginin dihydrolase	+	−	−
Lysine decarboxylase	−	−	nd
Ornithine decarboxylase	−	−	nd
Trisodium citrate	−	−	nd
H_2S (sodium thiosulphate)	−	−	nd
Urease	−	−	+
L-tryptophan	−	−	−
VP (sodium pyruvate)	+	+	nd
Gelatinase	+	+	nd
Fermentation			
Glucose	wp	+	−
D-mannitol	wp	+	nd
Inositol	−	−	nd
D-sorbitol	−	−	nd
L-rhamnose	−	+	nd
D-saccharose	−	+	nd
D-melibiose	−	−	nd
Amygdaline	−	+	nd
L- arabinose	−	+	nd
Esc	nd	nd	+
Gel (hydrolyse, protease)	nd	nd	+
Pnpg	nd	nd	−
Assimilation	nd	nd	
D-Glucose	nd	nd	−
L-Arabinose	nd	nd	−
D-Mannose	nd	nd	−
D-Mannitol	nd	nd	−
N-Acetyl-glucosamine	nd	nd	−
D-Maltose	nd	nd	−
Potasyum gluconate	nd	nd	−
Capric acid	nd	nd	−
Adipic acid	nd	nd	−
Malate	nd	nd	−
Citrate	nd	nd	−
Phenyl acetic acid	nd	nd	−

+: positive, wp: weak positive, −: negative, nd: not determined, R: Rod, SR: Short rod. Except for the first five tests, the other results were determined by API 20E and API 20NE tests.

Additionally, the diversity of endophytic bacteria in various *Artemisia* species has been reported to include species from the genera *Bacillus*, *Pseudomonas*, *Enterobacter*, and *Lysinibacillus* [18]. However, no endophytic bacteria have been isolated from *A. austriaca* in the literature.

Endophytic bacteria were isolated following complete surface sterilization. *Pantoea agglomerans*, a Gram-negative bacterium, was the only isolate obtained from the stem. This result can be interpreted in two ways: either *Pantoea agglomerans* is the only dominant bacterium on the stem, or the superficial isolation process may have killed other, more sensitive endophytes. Additionally, there may be bacteria present that cannot be cultured under normal laboratory conditions. However, the fact that only Gram-positive bacteria were obtained from the leaf isolation suggests that Gram-positive endophytes found a more suitable habitat in this part of the plant.

Antagonistic activity of endophytic isolates. In this study, the antimicrobial activities of endophytic bacterial isolates were tested using the disk diffusion method against some pathogenic Gram-positive and Gram-negative bacteria. The antimicrobial activity of the isolates was evaluated by measuring the growth inhibition zones surrounding the discs. The results of the agar diffusion test indicated that some pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, and *Proteus mirabilis*) exhibited resistance to the endophytic isolates of *Pantoea agglomerans* and *Microbacterium sp.* However, another endophytic isolate, *Bacillus proteolyticus*, showed inhibitory activity against all of the tested pathogenic microorganisms, showing inhibition zones between 19.3 and 25.3 mm. This isolate demonstrated the highest antagonistic activity against *S. aureus* (with an inhibition zone of 25.3 mm) and the lowest against *P. mirabilis* (with an inhibition zone of 19.3 mm). The *Pantoea agglomerans* and *Microbacterium sp.* strains showed no antagonistic activity against the test cultures.

Most members of the *Bacillus* species exhibit antagonistic effects against various pathogenic bacteria [19]. Akpor et al. [20] assessed the antibacterial activity of metabolites synthesized by *Bacillus* species against both Gram-negative and Gram-positive pathogens. Their studies also indicated that *Bacillus* metabolites have pesticidal potential against sugar ants. In another study, scanning electron microscope images demonstrated that the protease synthesized by *B. proteolyticus* CFR3001 exerts an antagonistic effect against various pathogens by causing the lysis of pathogenic bacterial cells [21]. *B. proteolyticus* (AY1), a strain from the *Bacillaceae* family, showed strong antimicrobial activity against all pathogens tested. This strain is recommended for use in certain biotechnological applications.

In this study, *Pantoea agglomerans* (AG1) and *Microbacterium sp.* (AY2) isolates exhibited no antagonistic activity against the tested pathogens. Damavandi et al. [17] reported that most of the ten endophytic bacteria isolated from *Artemisia absinthium* belonged to the genera *Pantoea* and *Bacillus*, with *P. aerug-*

inosa SD01 showing significant antibacterial and antitumour activity. However, not all endophytic bacteria from the same plant are expected to exhibit antagonistic activity. A similar observation was made in a study of twelve plant-associated bacteria isolated from *Artemisia nilagirica*. Among the bacterial isolates from the genera *Arthrobacter*, *Pseudomonas*, *Microbacterium*, *Psychrobacter*, *Enterobacter*, *Bacillus*, *Kosakonia*, *Chromobacterium*, *Serratia*, and *Burkholderia*, only two strains demonstrated antibacterial properties targeting clinical pathogens [16].

On the other hand, endophytes such as *Pantoea* sp., isolated from medicinal plants, can be used as inoculants to promote plant growth, enhance soil fertility, and sustain crop production systems [22]. Although isolate AG1 does not exhibit antagonistic activity, it may still be evaluated for these beneficial properties. Similarly, *Microbacterium sp.* is a bacterium commonly associated with plants, though it may not always produce antimicrobial metabolites. The absence of antagonistic activity could be attributed to the environmental conditions in which the plant grows. Strains of the genus *Microbacterium* have been reported to possess hydrocarbon biodegradation potential [23], and certain species exhibit plant growth-promoting (PGP) properties [24]. Therefore, isolate AY2 will be further evaluated in subsequent studies, taking these characteristics into account.

Conclusion. This study is the first to report the isolation of three endophytic bacteria from stem and leaf samples of *A. austriaca*, with the isolates belonging to the families *Enterobacteriaceae*, *Bacillaceae*, and *Microbacteriaceae*. Furthermore, the *Bacillus proteolyticus* AY1 isolate demonstrated significant efficacy in inhibiting the growth of both Gram-positive and Gram-negative pathogens tested. These findings suggest that the AY1 isolate may possess valuable antibacterial compounds, highlighting its potential for biotechnological applications.

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