



Exploring antimycobacterial potential and profiling secondary metabolite gene clusters in the whole genome of *Bacillus* isolated from dogfruit (*Archidendron pauciflorum*)

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ARTICLE HISTORY

Received on: 05/06/2024

Accepted on: 14/09/2024

Available Online: XX

Key words:

Endophyte, *Bacillus*, Biofilm, Genome, *Mycobacterium smegmatis*, Secondary metabolite gene cluster

ABSTRACT

Bacillus spp. associated with tropical medicinal plants are potential sources of secondary metabolites possessing antimycobacterial properties. This study evaluated the antimycobacterial activity of endophytic *Bacillus* species against *Mycobacterium smegmatis* and analyzed putative secondary metabolite gene clusters (SMGCs) in the complete genome sequence of the selected isolate. Among the four isolates tested, colony and metabolite extract from *Bacillus* sp. strain DJ4 strongly inhibited the growth of *M. smegmatis*, a surrogate bacterium for *Mycobacterium tuberculosis* (*Mtb*). The extract was categorized as bactericidal, since the minimum inhibitory concentration (MIC) and MBC of the bacterial extract were 31.25 µg/ml and 125 µg/ml, respectively. The most effective inhibition of biofilm formation and eradication of *M. smegmatis* cells' biofilm was shown by the 2 × MIC extract treatment. Eighteen volatile compounds (VOCs) were identified by gas chromatography-mass spectrometry analysis. Some VOCs found in the extract have been reported to act as antibacterial agents. Whole-genome analysis revealed that *Bacillus* sp. strain DJ4 is similar to *Bacillus velezensis* strain KCTC 13012. Eight SMGCs were identified in the bacterial genomes. In conclusion, the present study indicates that endophytic *Bacillus* species, especially *Bacillus* sp. strain DJ4, are a new source of antimycobacterial compounds. This discovery may allow further exploration of secondary metabolites and genomic features of this endophytic bacterium to open up great prospects in the pharmaceutical industry to combat *Mtb* infection.

INTRODUCTION

Tuberculosis (TB) disease, caused by *Mycobacterium tuberculosis* (*Mtb*), is the most frequent single infectious illness leading to global mortality [1]. Nevertheless, anti-TB drugs are more limited compared to agents existing for other bacterial infections [2]. Despite TB being declared as a global health crisis by WHO in 1993, only three new anti-TB drugs were approved and introduced to the market from 2012 to 2019: namely

bedaquiline (approved by the Food and Drug Administration US in 2012), delamanid (approved by the European Medicines Agency Europe in 2014), and pretomanid (approved by the Food and Drug Administration US in 2019) [3,4]. However, the prevalence of TB drug resistance increases challenges in finding novel anti-TB drugs that are effective in shortening TB treatment [5]. Discovering new anti-TB agents involves time-intensive research due to the slow growth of *Mtb*. Another limitation in developing anti-TB agents is the necessity of a biosafety level 3 laboratory for conducting studies related to *Mtb*. Utilizing non-pathogenic and fast-growing *Mycobacterium smegmatis* for anti-TB investigation has exhibited several successes in past research. *Mycobacterium smegmatis* strains are commonly susceptible to TB medicines, such as isoniazid, rifampicin, and ethambutol [6]. It has also been well-studied that *M. smegmatis*

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is over 90% genetically identical to *Mtb*, shares common genes in stress response, and exhibits similarity in cell wall structure and metabolism [6,7]. Therefore, *M. smegmatis* serves as a suitable model for evaluating drug candidates against *Mtb*.

Endophytic microbes are promising sources of metabolites. These microbes live in plant tissues and play important roles in enhancing plant growth and protecting the host against pathogens [8]. Insights from ethnobotanical knowledge have proven valuable in identifying potential host plant reservoirs for endophytic microbes. Southeast Asia's native plant, dogfruit (*Archidendron pauciflorum*), is locally used as a vegetable and is exploited for its therapeutic functions to cure several diseases, such as diarrhea, headaches, fevers, colds, coughs, and stomach-aches [9]. Exploiting the endophytic bacteria from this plant may provide a new alternative source of antimycobacterial agents. *In vitro* study supported by a genome mining approach could be an effective way to screen and evaluate endophytic bacteria as sources of secondary metabolites. The development of next-generation sequencing and powerful computational tools in the big-data era has provided researchers with a useful genomic database to easily identify diverse and novel secondary metabolite gene clusters (SMGCs) in the entire bacterial genome, which is beneficial for drug discovery [10].

In our previous studies, four endophytic *Bacillus* spp. isolated from *A. pauciflorum* exhibited antibacterial activity against antibiotic-sensitive strains (*Staphylococcus aureus* ATCC6538, *Bacillus subtilis* ATCC19659, *Pseudomonas aeruginosa* ATCC15442, and *Escherichia coli* ATCC8739), along with antibiotic-resistant strains (*P. aeruginosa* M19, *B. subtilis* M18, *E. coli* M4, and *Klebsiella pneumoniae* M.9) [11,12]. However, the antimycobacterial activity of these isolates is yet to be investigated. Of note, numerous previous studies have shown that *Bacillus* spp. isolated from various environmental samples exhibit remarkable antimycobacterial activity. Some *Bacillus* strains isolated from the soil and water samples, as well as the endophyte *Salicornia brachiata* displayed antimycobacterial potency against *Mycobacterium* strains [13,14]. However, none of these studies have evaluated the effect of secondary metabolites derived from *Bacillus*

on the inhibition of biofilm formation and eradication of *Mycobacterium* cell biofilms of *Mycobacterium* strains. This is an important insight because *Mycobacterium* is a biofilm-forming bacterium, and the biofilm structure promotes the persistence of the bacterium in response to antimicrobial agents. In addition, genome profiling of *Bacillus* is still underexplored, although it is beneficial for identifying novel SMGCs and predicting possible biosynthetic pathways related to antimycobacterial compound production. Therefore, the present study aimed to evaluate the antimycobacterial properties of four endophytic *Bacillus* spp. from *A. pauciflorum* and their inhibitory effects on *M. smegmatis* biofilm formation and cell biofilm eradication. Additionally, whole-genome analysis was conducted to identify secondary metabolite gene clusters (SMGCs) within the genome of the most promising endophyte isolate. The composition of volatile compounds (VOCs) in the extract was determined through analysis using gas chromatography-mass spectrometry (GC-MS). From this study, we have successfully explained the potential of endophytic *Bacillus* as an anti-TB agent. The data were proven from the results of *in vitro* analysis through antibacterial assays supported with antibiofilm tests. At the same time, we also managed to report genomic aspects by providing the whole genome sequence of the most potential *Bacillus* and identifying its SMGCs. The most potential *Bacillus* extract has also been profiled using GC-MS. These results are important reports that could incorporate the analysis of the potential of endophyte bacterial active compounds as new anti-TB agents.

MATERIALS AND METHODS

Source of endophyte isolates

Four endophytic bacteria were isolated from *A. pauciflorum*, namely *Bacillus* sp. strain DJ4, *B. subtilis* strain DJ7, *Bacillus velezensis* strain DJ9, and *Bacillus megaterium* strain AJ5 [12]. These isolates were stored at the Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Indonesia. The 16S rRNA sequences of these isolates could be accessed under the NCBI

Table 1. Colony and cell characteristics of the four endophytic *Bacillus* spp. isolated from *A. pauciflorum*.

Characteristics	Bacterial strains			
	<i>Bacillus</i> sp. strain DJ4	<i>B. subtilis</i> strain DJ7	<i>B. velezensis</i> strain DJ9	<i>B. megaterium</i> strain AJ5
Colony morphology				
Shape	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire
Elevation	Flat	Flat	Convex	Convex
Optic	Opaque	Opaque	Opaque	Opaque
Pigmentation	Pearl white	Light ivory	Pale beige	Dark ivory
Cell morphology				
Shape	Bacilli	Bacilli	Bacilli	Bacilli
Arrangement	Monobacilli	Monobacilli	Diplobacilli	Diplobacilli
Gram	Positive	Positive	Positive	Positive

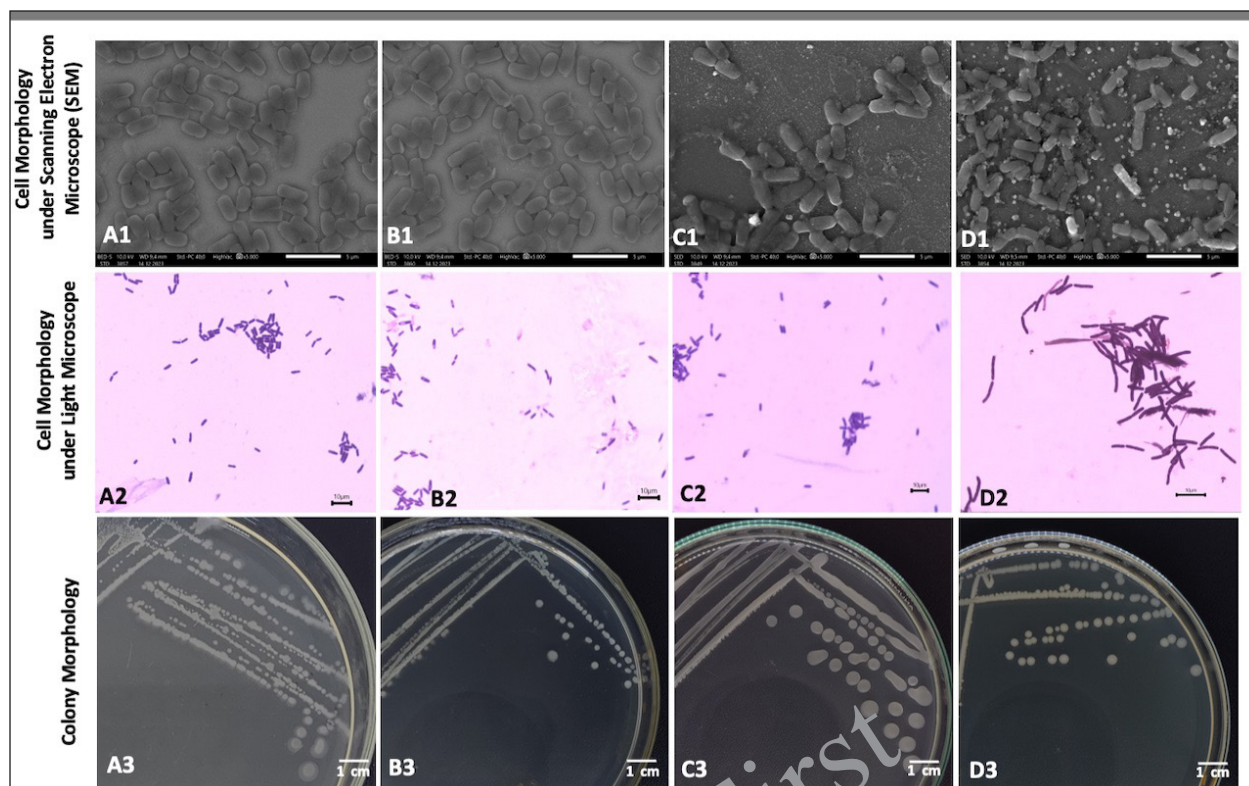


Figure 1. Colony and cell morphology of the four endophytic *Bacillus* spp. isolated from *V. pauciflorum*. Cell morphology under SEM: (A1) *B. megaterium* strain AJ5, (B1) *Bacillus* sp. strain DJ4, (C1) *B. subtilis* strain DJ7, (D1) *B. velezensis* strain DJ9. Cell morphology under light microscope after stained using Gram technique: (A2) *B. megaterium* strain AJ5, (B2) *Bacillus* sp. strain DJ4, (C2) *B. subtilis* strain DJ7, (D2) *B. velezensis* strain DJ9. Colony morphology: (A3) *B. megaterium* strain AJ5, (B3) *Bacillus* sp. strain DJ4, (C3) *B. subtilis* strain DJ7, (D3) *B. velezensis* strain DJ9.

Table 2. Antimycobacterial activity of endophytic *Bacillus* spp. against *M. smegmatis*.

No.	Isolates	Diameter of inhibition zone (mm)	
		Endophytic bacteria colony	Endophytic bacteria extract
1	<i>Bacillus megaterium</i> strain AJ5	9 ± 0.81 ^a	8.6 ± 0.47 ^b
2	<i>Bacillus</i> sp. Strain DJ4	29.33 ± 0.47 ^d	17.7 ± 0.47 ^e
3	<i>Bacillus subtilis</i> strain DJ7	10 ± 0 ^b	8.3 ± 0.47 ^b
4	<i>Bacillus velezensis</i> strain DJ9	28 ± 0.81 ^c	16 ± 0 ^d
5	Tetracycline	Not tested	15 ± 0.81 ^c
6	DMSO	Not tested	0 ± 0 ^a

Note: Distinct superscript letters within the identical column denote statistically significant variances (*p*-value of each treatment group is 0.000).

accession numbers: PP178167.1, OR511995.1, OP164675.1, and OP164672.1, respectively.

Colony and cell morphology characterization

Four endophytic isolates were cultured in nutrient agar (NA) medium (Oxoid) and incubated for 24 hours. Colony characteristics, including shape, margin, elevation, optic, and pigmentation, were observed. Approximately 18-hour-old cultures were stained using the Gram staining technique to characterize their cell characteristics, such as shape, arrangement, and Gram reaction, and observed under a light microscope (Olympus CX31) with 1,000 × resolution. Additionally, the cell morphology of each isolate was also observed using a scanning

electron microscope (SEM). The pellets from bacterial cultures aged 24 hours were rinsed using 100 µl of sterile distilled water. Afterward, 10 µl of the suspension was applied onto the surface of a single-polished Silicon Wafer (Sigma) and left to incubate for 18 hours. Subsequently, the SEM specimen was coated with sputter-gold particles (Hitachi®, Tokyo, Japan) and observed using an SEM-JEOL JSM-IT200 (JEOL, South Korea) at a resolution of 5,000 ×, with a working distance of 5 µm and an accelerating voltage of 10 kV.

Screening of antimycobacterial activity

Preliminary screening for antimycobacterial activity was performed using the streak plate method against

M. smegmatis ATCC700084 (collection of the Research Center for Pharmaceutical Ingredients and Traditional Medicine, National Research, and Innovation Agency (BRIN), South Tangerang, Indonesia). Approximately 1 ml of *M. smegmatis* inoculum (1×10^8 CFU/ml) was added into 100 ml of Mueller Hinton Agar medium (Himedia) and poured into a petri dish. After the medium had solidified, the overnight endophyte colony was spot inoculated onto the surface of the medium and cultivated for 24 hours at $\pm 37^\circ\text{C}$. The diameter of the inhibition zone was expressed in millimeters [12].

Metabolites extraction

Each endophytic *Bacillus* strain was cultured overnight in a nutrient broth (NB) medium (Oxoid). The culture (1% v/v) was seeded to 1 l of NB medium and incubated for 3 days at $\pm 28^\circ\text{C}$ with agitation at 120 rpm to optimize extracellular secondary metabolite production. The cultures were extracted by mixing an equal volume of ethyl acetate and shaking for 20 minutes. The upper layer was then dried at 50°C using an evaporator. The extract was re-dissolved in 1% DMSO (Merck) for antimycobacterial and antibiofilm analyses [12].

Antimycobacterial test of endophytic extracts

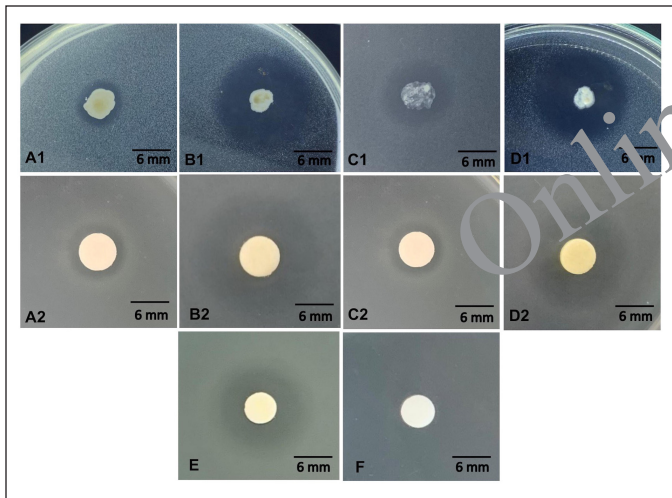


Figure 2. Antimycobacterial activity of endophytic bacterial colony (A1) *B. megaterium* strain AJ5, (B1) *Bacillus* sp. strain DJ4, (C1) *B. subtilis* strain DJ7, (D1) *B. velezensis* strain DJ9, and endophytic bacterial extracts (A2) *B. megaterium* strain AJ5, (B2) *Bacillus* sp. strain DJ4, (C2) *B. subtilis* strain DJ7, (D2) *B. velezensis* strain DJ9, compared to (E) tetracycline, and (F) 1% DMSO.

The antimycobacterial activity of each endophytic extract (1 mg/ml) was analyzed using the disk diffusion method as described previously. Approximately 200 $\mu\text{g/ml}$ tetracycline (Sigma-Aldrich) and 1 % DMSO (Merck) were used as positive and negative controls, respectively [15].

Evaluation of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

The extract from each endophytic isolate underwent further assays to evaluate the MIC using a microdilution test [15]. Briefly, microdilution was performed using Mueller Hinton (MH) Broth (Himedia) containing extract (100 μl) ranging from 1,000 $\mu\text{g/ml}$ to 1.95 $\mu\text{g/ml}$ in 96 sterile well plates (Biologix). Subsequently, the *M. smegmatis* suspension (100 μl) at a density of 0.5 McFarland (1×10^8 CFU/ml, Thermo Fisher Scientific) was added into the wells (final bacterial concentration of 5×10^7 CFU/ml) and incubated overnight at 37°C . The MIC was defined as the lowest extract concentration that suppressed the visible growth of *M. smegmatis*. The MBC was determined by plating 100 μl suspension from the well with no growth of *M. smegmatis*. The lowest concentration of endophytic extract demonstrating complete killing of the target bacteria was considered the MBC. One percent of DMSO served as the negative control, while tetracycline (Sigma-Aldrich) was employed as the positive control. The MBC/MIC ratio was analyzed to classify the antimycobacterial properties of the endophytic extracts as either bacteriostatic or bactericidal. If the ratio was ≤ 4 , the extract was categorized as bactericidal, but if the ratio exceeded 4, the extract was defined as bacteriostatic.

Antibiofilm analysis

The four endophytic extracts underwent antibiofilm analysis using a microtiter plate test [15]. In brief, 100 μl of *M. smegmatis* cells at a density of 1×10^8 CFU/ml were inoculated into brain heart infusion (BHI) medium (Merck) in the 96-well plates containing various extract concentrations ($2 \times \text{MIC}$, $1 \times \text{MIC}$, $\frac{1}{2} \times \text{MIC}$, $\frac{1}{4} \times \text{MIC}$) and incubated overnight. The medium was then removed from each well, and the wells were washed twice with phosphate buffer saline (PBS, Sigma-Aldrich). The formed biofilm was stained with 200 μl of 0.1% crystal violet (Merck) for 30 minutes at 37°C . The excess staining solution was removed by washing once with PBS. Subsequently, the stained biofilm was eluted in 200 μl of DMSO (99%). The optical density of the suspension was measured at 595 nm using an ELISA reader (Thermo Scientific Varioskan Flash-Thermo Fischer). Control plates without any

Table 3. The MIC, MBC, and MBC/MIC ratio of the endophytic *Bacillus* species against *Mycobacterium smegmatis*.

No.	Endophytic extracts	MIC and MBC ($\mu\text{g/ml}$)			Category
		MIC	MBC	MBC/MIC	
1	<i>Bacillus megaterium</i> strain AJ5	125	500	4	Bactericidal
2	<i>Bacillus</i> sp. Strain DJ4	31.25	125	4	Bactericidal
3	<i>Bacillus subtilis</i> strain DJ7	125	1,000	8	Bacteriostatic
4	<i>Bacillus velezensis</i> strain DJ9	31.25	250	8	Bacteriostatic
5	Tetracycline	7.81	15.62	2	Bactericidal

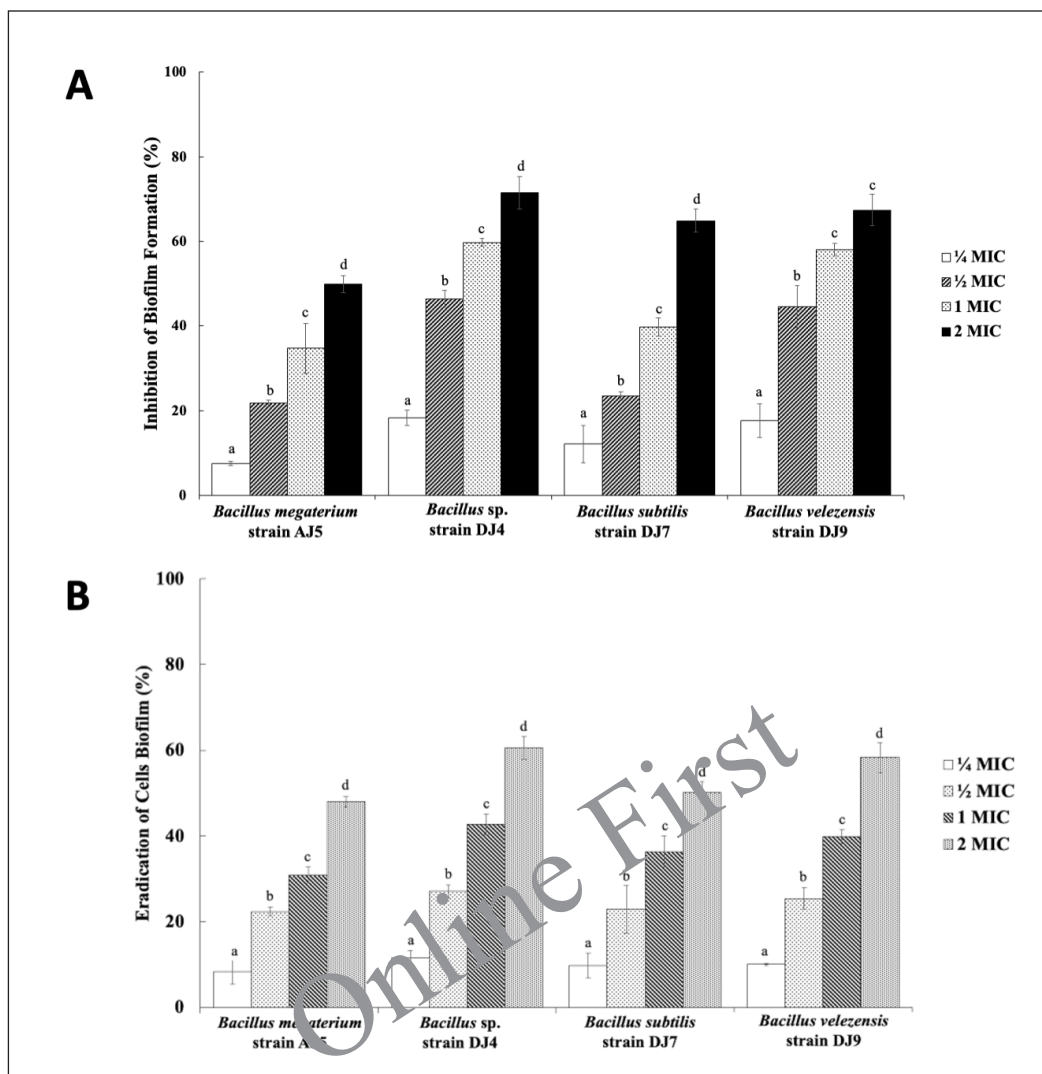


Figure 3. The effect of endophytic *Bacillus* spp. extracts on (A) the biofilm-forming capacity of *M. smegmatis*, and (B) in eradicating *M. smegmatis* cell biofilm. Dissimilar letters following the bars in each treatment mean statistically significant difference (p -value of each treatment group is 0.000).

treatment were used as reference. The percentage of inhibition of biofilm formation was determined in comparison to that of the control. The experimental data were expressed as average \pm standard deviation from triplicates. The biofilm structure of each treatment was observed using an SEM. The biofilm matrix was collected and applied onto the surface of a single-polished Silicon Wafer (Sigma), and then incubated for 18 hours. Subsequently, the SEM specimens underwent coating with sputter-gold particles (Hitachi®, Tokyo, Japan), and examination was conducted using a SEM-JEOL JSM-IT200 (JEOL, South Korea) at a resolution of 5,000 \times , with a working distance of 5 μ m and an accelerating voltage of 10 kV.

Biofilm cells eradication assay

To examine the biofilm cells eradication effect of endophytic extracts on preformed cell biofilm (a five-day biofilm), a methyl tetrazolium test was conducted following the procedure outlined by Wintachai *et al.* [16]. In the experiment, 200 μ l of *M. smegmatis* culture (1×10^8 CFU/ml) was introduced

into 96-well plates containing BHI medium (Merck) and left for 5 days at 37°C to allow for mature biofilm formation. The medium was replaced daily (every 24 hours) with fresh BHI medium supplemented with 0.25% glucose. Additionally, the endophytic extracts were added at different concentrations ($2 \times$ MIC, $1 \times$ MIC, $\frac{1}{2} \times$ MIC, $\frac{1}{4} \times$ MIC) and incubated for 24 hours at 37°C. The medium was removed from the well, and the cell biofilm was stained with 10 μ l of 5 mg/ml methyl tetrazolium solution (Merck), and then incubated for 4 hours at 37°C. Subsequently, the formazan crystals were dissolved with 200 μ l of 99% DMSO. Cell biofilms of *M. smegmatis* that did not undergo any treatment were utilized as controls. Optical density was measured at 595 nm using an ELISA reader (Varioskan Flash-Thermo Fischer, Thermo Scientific). The experimental data were presented as average \pm standard deviation from three replications.

Analysis of VOCs composition

The composition of VOCs in the endophyte extract was analyzed using a GC-MS system (Agilent Technologies

6890N Inert C, USA) according to the manufacturer's instructions. Mass spectra and chromatograms were processed using the MSD ChemStation Data Analysis software (G1701EA E.02.02.1431).

Whole-genome sequencing and detection of secondary metabolic gene clusters

The bacterial cells from the 24-hour-old culture underwent centrifugation at 15,000 rpm for 1 minute. Genomic DNA isolation was conducted using the Presto™ Mini gDNA Bacteria Kit (Geneaid) following the kit's protocol. The bacterial genome sequence was acquired utilizing Oxford Nanopore Technology. *De novo* assembly was executed employing the Flye (v.2.8.3). Subsequently, mapping and genome polishing were iteratively performed four times polishing using Minimap2 (v.2.24-r1122) and Racon (v.1.5.0). The assembled sequence and genome completeness of closely related species were assessed using dfast-qc (v.0.4.2) and BUSCO (v.5.4.4). The quality assessment of the assembled sequence was carried out with Quast software (v5.0.2). Visualization of the bacterial genome was accomplished using Circos (v.0.69–8). AntiSMASH bacterial version v5.1.2 was employed to annotate SMGCs within the bacterial genome (<https://antismash.secondarymetabolites.org>) [17].

Statistical analysis

The experimental data obtained from the antibacterial assay, determination of MIC and MBC, antibiofilm analysis, and biofilm eradication assays were conducted in triplicate. Subsequently, the data underwent one-way analysis of variance,

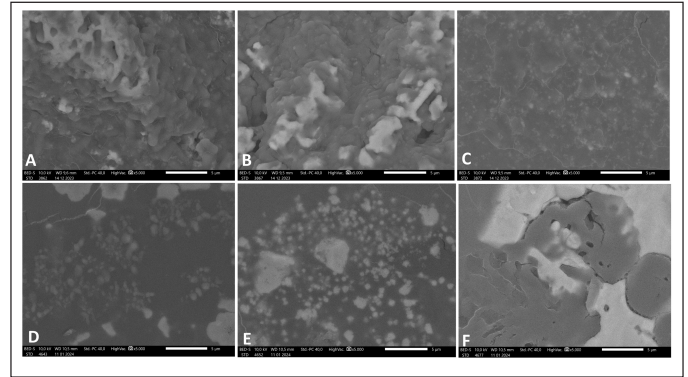


Figure 4. Biofilm structure of *M. smegmatis* after treated with (A) DMSO, (B) medium only, and extract derived from (C) *Bacillus* sp. strain DJ4, (D) *B. megaterium* strain A15, (E) *B. subtilis* strain DJ7, (F) *B. velezensis* strain DJ9, at the concentration $0.2 \times$ MIC.

Table 4. VOCs profile of the metabolite extract from *Bacillus* sp. strain DJ4.

No	Compounds	Retention times (mins)	Quantity (%)	Similarity (%)	Bioactivity	Other sources	References
1	2,4-di-tert-butylphenol	14.749	4.05	97	Antibacterial, cytotoxic, Antifungal, antioxidant	Endophytic <i>Streptomyces</i> sp. KCA1; <i>Lactococcus</i> sp.;	[18,19]
2	1-Octadecene	18.039	0.44	97	–	–	–
3	Cyclo(L-prolyl-L-valine)	18.436	3.40	96	–	–	–
4	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	19.261	0.41	91	Alpha amylase inhibitor	Cestrum octurnum	[20]
5	Dibutyl phthalate	19.740	2.60	78	Antibacterial	soil <i>Streptomyces albidoflavus</i> 321.2	[21]
6	Cycloeicosane	20.055	0.96	99	–	–	–
7	Palmitic acid	20.522	0.32	99	–	–	–
8	9,12-Octadecadienoic acid (Z,Z)- methyl ester	21.026	0.29	92	–	–	–
9	Methyl stearate	21.316	0.25	99	–	–	–
10	Tributyl acetylcitrate	22.374	1.73	90	–	–	–
11	Phthalic acid	23.345	0.46	93	–	–	–
12	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)	23.761	1.84	98	Antibacterial, antioxidant, antifungal	<i>Bacillus tequilensis</i> MS145; <i>Streptomyces</i> sp. VITPK9	[22,23]
13	Oxacyclopentadecan-2-one	24.328	0.36	90	–	–	–
14	Lauric acid	25.021	6.66	93	Antimicrobial	coconut oil	[24]
15	1-Hexacosene	25.223	0.71	99	–	–	–
16	Squalene	26.836	0.73	98	Antimicrobial and cytotoxic	<i>Stichopus hermanni</i>	[25]
17	Dodecanamide	27.075	0.34	96	–	–	–
18	DL-alpha-tocopherol	29.533	0.92	98	antioxidant	palm	[26]

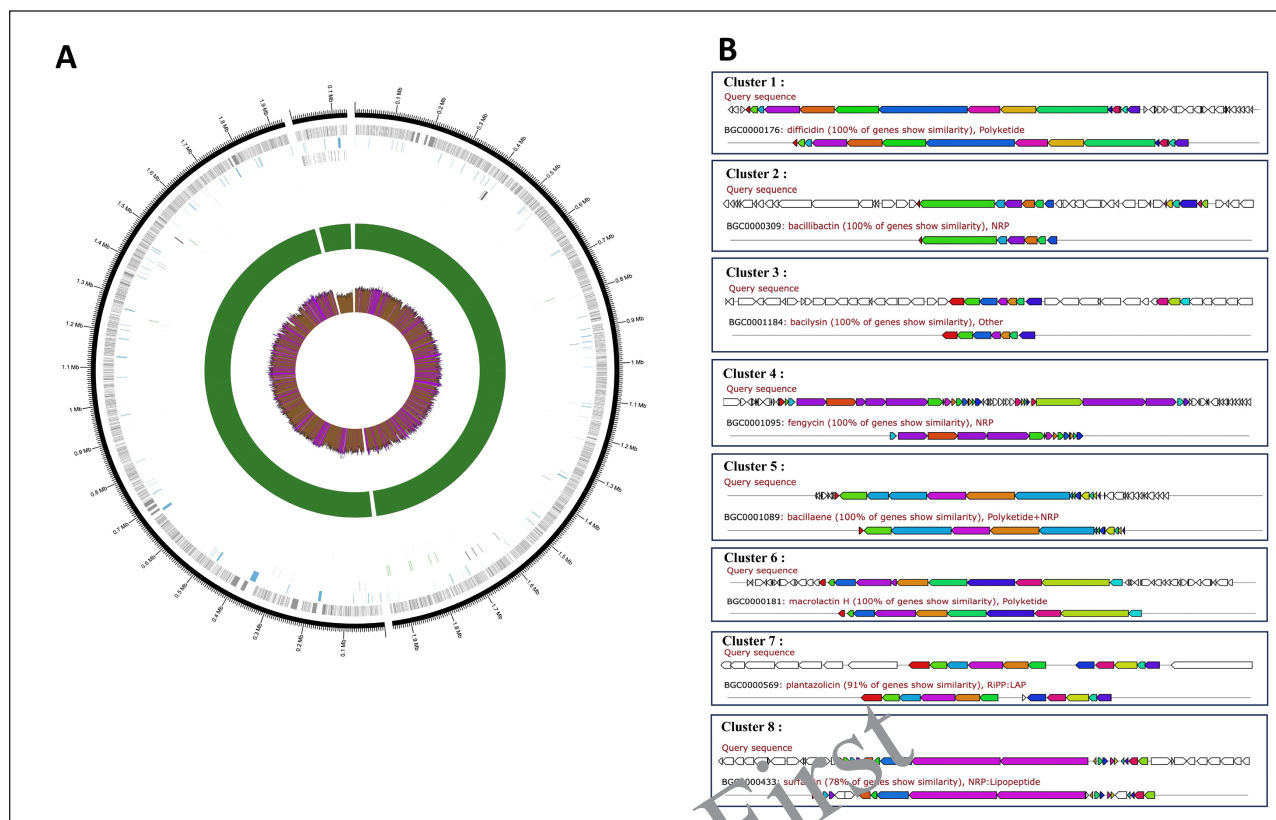


Figure 5. (A) Annotated genome of *Bacillus* sp. strain DJ4. Figure Legend (from outer): contig (blue), genes (grey), pseudogenes (blue), coding sequences (CDS; black), rRNA (green), tRNA (purple), depth (depth >50 = green; depth <50 = red), gc content (gc content >50% = purple; gc content <50% = brown); (B) Putative SMGCs from the *Bacillus* sp. strain DJ4 genome with the antiSMASH database.

Table 5. Genomic features of *Bacillus* sp. strain DJ4.

Genomic features	
Size of the genome assembly (bp)	4,016,867
GC content (%)	46.39
Number of contigs	3
Largest contig (bp)	1,945,594
Mean read length (bp)	9,605.3
Read length N50 (bp)	10,774
Coding sequences (CDSs)	3,909
Genome completeness (%)	99.1

followed by further statistical analysis through Tukey's test using Statistical Product and Service Solutions software version 29.0. The level of statistical significance was established at $p < 0.05$ to determine significance.

RESULTS

Colony and cell characteristics

The four isolates of endophytic *Bacillus* spp. used in this study had different colony characteristics, especially in terms of elevation and pigmentation, but the four isolates had the same shape, margins, and optics, which were circular,

entire, and opaque, respectively (Table 1). These isolates also have bacilli in shape, and are stained purple using Gram staining procedure. The cell arrangement of *Bacillus* sp. strain DJ4 and *B. subtilis* strain DJ7 were monobacilli, whereas the cell arrangement of *B. velezensis* strain DJ9 and *B. megaterium* strain AJ5 were diplobacilli (Fig. 1).

Antimycobacterial activity of *Bacillus* species isolated from *A. pauciflorum*

The four endophytic *Bacillus* spp. exhibited varied antimycobacterial activities against *M. smegmatis*. Inhibition zone formation was determined using endophytic bacterial colonies and their metabolite extracts (Table 2). Among the four endophytic isolates, *Bacillus* sp. strain DJ4 consistently displayed the strongest antimycobacterial activity, showcased by its colony and extract with inhibition zone diameters of 29.33 ± 0.47 mm and 17.7 ± 0.47 mm, respectively. Overall, the endophytic bacterial colonies demonstrated larger inhibition zone diameters against *M. smegmatis* compared to their metabolite extracts. The inhibition zone was also observed with tetracycline as the positive control (diameter: 15 ± 0.81 mm) and absent in 1% DMSO as the negative control (Fig. 2).

The MIC and MBC of the four endophytic extracts were determined using a microdilution assay. The MIC ranged from 31.25 μ g/ml to 125 μ g/ml, while the MBC ranged from 125 μ g/ml to 1,000 μ g/ml (Table 3). However, the MIC

Table 6. Putative SMGCs present in the genome of *Bacillus* sp. strain DJ4.

Cluster	Length (bp)	Type	Most Similar Known Gene Cluster	Similarity (%)	MIBiG ID	References
Cluster 1	93,789	Polyketide	Difficidin from <i>Bacillus velezensis</i> FZB42	100	BGC0000176	[27]
Cluster 2	51,794	Nonribosomal peptide	Bacillibactin from <i>Bacillus subtilis</i> 168	100	BGC0000309	[28]
Cluster 3	7,298	Other*	Bacilysin from <i>Bacillus velezensis</i> FZB42	100	BGC0001184	[29]
Cluster 4	135,850	Nonribosomal peptide	Fengycin from <i>Bacillus velezensis</i> FZB42	100	BGC0001095	[30]
Cluster 5	96,451	Hybrid polyketide + Nonribosomal peptide	Bacillaene from <i>Bacillus velezensis</i> FZB42	100	BGC0001089	[31]
Cluster 6	88,218	Polyketide	Macrolactin H from <i>Bacillus velezensis</i> FZB42	100	BGC0000181	[32]
Cluster 7	22,117	Linear azole (in)-containing peptides (LAP)	Plantazolicin from <i>Bacillus velezensis</i> FZB42	91	BGC0000569	[33]
Cluster 8	41,884	Nonribosomal peptide + lipopeptide	Surfactin from <i>Bacillus velezensis</i> FZB42	78	BGC0000433	[34]

*A cluster harboring a protein related to secondary metabolites that does not align with any other classification.

and MBC of endophytic extracts are still higher than that of tetracycline, which had MIC and MBC of 7.81 µg/ml and 15.62 µg/ml, respectively. Furthermore, based on their MBC/MIC ratios, two bacterial extracts derived from *B. megaterium* strain AJ5 and *Bacillus* sp. strain DJ4 were categorized as bactericidal, whereas extracts from *B. subtilis* strain DJ7 and *B. velezensis* strain DJ9 were categorized as bacteriostatic. The remarkable antimycobacterial activity of the endophytic extract was demonstrated by *Bacillus* sp. strain DJ4 with the lowest MIC and MBC of 31.25 µg/ml and 125 µg/ml, respectively.

***Mycobacterium smegmatis* biofilm inhibition and biofilm cell eradication activities**

The endophytic extracts reduced the biofilm-forming capability of *M. smegmatis*. The biofilm-forming capacity of *M. smegmatis* decreased in a dose-dependent manner with increasing extract concentration (Fig. 3A). The greatest inhibition of biofilm formation was observed for all extracts at 2 × MIC. At this extract concentration, biofilm formation was inhibited by 49.87%–71.50%. Extracts from *Bacillus* sp. strain DJ4 demonstrated remarkable antibiofilm activity, with the highest inhibition percentage of biofilm formation reaching up to 71.50%. The four endophytic extracts also exhibited biofilm cell eradication effects on the preformed *M. smegmatis* biofilms. Similar to the prevention of biofilm formation, the biofilm cell eradication effect of endophytic extracts was dose-dependent (Fig. 3B). The higher the endophytic extract applied, the greater the percentage of biofilm cell eradication effect was achieved. Consequently, 2 × MIC of endophytic extracts showed the highest cell eradication percentage, ranging from 47.99% to 60.53%. The most significant cell eradication effect was also demonstrated by *Bacillus* sp. strain DJ4-derived extract, with a biofilm cell eradication percentage of 60.53%.

Furthermore, SEM images revealed a pronounced accumulation of the exopolysaccharide matrix in both the DMSO (Fig. 4A), and medium-only treatment (Fig. 4B). Conversely, a notable decrease in the exopolysaccharide matrix was observed in the treatments with endophytic extracts derived from *Bacillus* sp. strain DJ4 (Fig. 4C), *B. megaterium*

strain AJ5 (Fig. 4D), *B. subtilis* strain DJ7 (Fig. 4E), and *B. velezensis* strain DJ9 (Fig. 4F). Following endophytic extract treatments, the exopolysaccharide layer of *M. smegmatis* was thinner, less compact and separated into small pieces, indicating that the endophytic extract treatments inhibited the production of exopolysaccharide and the formation of the biofilm matrix. Notably, the sample treated with extract derived from *Bacillus* sp. strain DJ4 exhibited the thinnest biofilm structure compared to untreated control, DMSO, and other endophytic extract treatments.

VOCs composition of extract from *Bacillus* sp. strain DJ4

GC-MS analysis identified 58 VOCs peaks in the extract from *Bacillus* sp. strain DJ4. Eighteen compounds were found in the bacterial extract by matching with the spectra of the recognized compounds (Table 4). These compounds include 2,4-di-tert-butylphenol (4.05%), 1-octadecene (0.44%), cyclo (L-prolyl-L-valine) (3.40%), 9-di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione (0.41%), dibutyl phthalate (2.60%), cycloeicosane (0.96%), palmitic acid (0.32%), 9,12-octadecadienoic acid (Z,Z)-methyl ester (0.29%), methyl stearate (0.25%), tributyl acetyl citrate (1.73%), phthalic acid (0.46%), pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) (1.84%), oxacyclopentadecan-2-one (0.36%), lauric acid (6.66%), 1-hexacosene (0.71%), squalene (0.73%), dodecanamide (0.34%), dl-alpha-tocopherol (0.92%), and other unknown compounds.

Whole genome sequence and SMGCs of the most potential isolate

Based on the complete genome sequence, *Bacillus* sp. strain DJ4 exhibited a close relation (97.55%) to *B. velezensis* strain KCTC 13012 (accession no: GCA_001267695.1). The genome assembly size of this bacterium was 4,016,867 bp, having a 46.3% GC content (Fig. 5A). The genome assembly reached a completion rate of 99.1%, with the largest contig length at 1,945,594 bp and a read length N50 of 10,774 bp. Genome annotation via NCBI PGAP identified 3,909 coding sequences within the bacterial genome (Table 5). The complete

genome sequence of this bacterium is accessible in the NCBI GenBank database under accession number CP144358.1.

The presence of SMGCs within the genome of *Bacillus* sp. strain DJ4 was predicted using antiSMASH. Annotation results revealed eight SMGCs, encompassing gene clusters involved in the production of non-ribosomal peptides, polyketides, linear azole (in)e-containing peptides (LAP), hybrid non-ribosomal peptides and polyketides, hybrid non-ribosomal peptides and lipopeptides, as well as an unknown compound. Some genes shared their similarity with biosynthesis clusters of known compounds, such as difficidin, bacillibactin, bacilysin, fengycin, bacillaene, macrolactin H, plantazolicin, and surfactin, with similarities ranging from 78% to 100% (Fig. 5B). Interestingly, all these gene clusters were identified across the *Bacillus* group, encompassing *B. velezensis* and *B. subtilis* (Table 6).

DISCUSSION

Bacillus species isolated from the internal tissues of medicinal plants are considered promising sources of compounds with valuable pharmacological functions, particularly as antimicrobial agents [35]. Various chemical substances produced by endophytic bacteria may contribute to their antimicrobial activity. In this study, antimycobacterial properties of four endophytic *Bacillus* from *A. pauciflorum*, namely *Bacillus* sp. strain DJ4, *B. subtilis* strain DJ7, *B. megaterium* strain AJ5, and *B. velezensis* strain DJ9, have been evaluated against *M. smegmatis*, a surrogate bacterium commonly used in preliminary study of antituberculosis candidates.

The four isolates had different colony and cell characteristics, particularly different in colony pigmentation, and elevation, as well as their cell arrangement. All isolates also belonged to Bacilli Gram-positive bacteria according to Gram staining. Interestingly, all endophytic colonies showed antagonistic activity against *M. smegmatis*, as indicated by the formation of a clear zone around the endophytic colony. Endophytic bacteria produce antibacterial compounds that are excreted extracellularly near the colony. Consequently, *M. smegmatis* growth in this area was inhibited. Secondary metabolites from the four endophytic bacteria were extracted and assessed using a disc diffusion test. The results showed that their metabolites also showed antimycobacterial activities with different degrees of significance. Extract from *Bacillus* sp. strain DJ4 displayed the strongest antimycobacterial activity with the largest inhibition zone of 17.7 ± 0.47 mm, followed by *B. velezensis* strain DJ9, *B. megaterium* strain AJ5, and *B. subtilis* strain DJ7. The diverse antimycobacterial effects of the endophytic isolates indicate the diverse chemical constituents produced by these endophytic bacteria.

The MIC of the four endophyte extracts was investigated to define the lowest extract concentration capable of inhibiting the visible growth of *M. smegmatis*, along with the MBC, as the lowest concentration that could kill *M. smegmatis* cells completely. The MIC of the extracts varied from 31.25 $\mu\text{g/ml}$ to 125 $\mu\text{g/ml}$, and MBC from 125 $\mu\text{g/ml}$ to 1,000 $\mu\text{g/ml}$, indicating that *M. smegmatis* has different sensitivity to each endophytic extract tested. However, the extracts with MIC less

than 1,000 $\mu\text{g/ml}$ are considered to have noteworthy antibacterial properties [36]. In this case, extract from *Bacillus* sp. strain DJ4 demonstrated the strongest antimycobacterial activity because the extract had the lowest MIC and MBC (MIC: 31.25 $\mu\text{g/ml}$; MBC: 125 $\mu\text{g/ml}$) compared to that of other extracts. Extracts from *Bacillus* sp. strain DJ4 and *B. megaterium* strain AJ5 were categorized as bactericidal (MBC/MIC ratio: 4), whereas extracts from *B. subtilis* strain DJ7 and *B. velezensis* strain DJ9 were categorized as bacteriostatic (MBC/MIC ratio: 8). These results indicate that endophytic *Bacillus* spp. isolated from *A. pauciflorum* have potential as antimycobacterial agents. Several studies have investigated the antimycobacterial mechanisms of natural compounds against *Mycobacterium*. Well-known mechanisms include destructing bacterial cell membranes [37], blocking specific metabolic pathways [38], inhibiting cell wall synthesis [39], inhibiting ribosome function [40], inhibiting efflux pumps [41], and inhibiting DNA replication [42].

Biofilm formation stands as the primary virulence factor in *Mycobacteria*. TB treatment spans an extended period (around 6–9 months) due to *Mtb* cell's ability to survive within human lungs, forming biofilms for shielding against the host's immune response and various antibiotics [43]. Consequently, the infection persists, showing high persistence and tolerance to available anti-TB drugs. Hence, targeting the inhibition and destruction of biofilms holds promise for enhancing TB treatment success. Encouragingly, the four endophytic extracts exhibited inhibitory effects on *M. smegmatis* biofilm formation *in vitro*. Notably, metabolites extracted from *Bacillus* sp. strain DJ4 displayed a significant effect. At a concentration of $2 \times \text{MIC}$, the extract inhibited biofilm formation by up to 71.50% after 24 hours of treatment. The effectiveness increased with higher extract concentrations, suggesting a need for a higher concentration than that required to suppress the bacterium's growth. However, the same bacterial extract showed lower effectiveness in eradicating *M. smegmatis* cells within the biofilm, evidenced by a lower percentage (60.53%) of cell eradication. This indicates increased resistance of the target bacterium to the endophytic extract after biofilm formation. Hence, a combination of antibacterial agents might be necessary to eliminate biofilms and bolster TB chemotherapy efficacy.

VOCs produced by the most potent bacteria (*Bacillus* sp. strain DJ4) were identified using GC-MS analysis. Surprisingly, some substances with a high similarity to the spectra database ($\geq 90\%$) found in the extract are known to possess pharmaceutical properties (e.g., antimicrobial, antioxidant, alpha-amylase inhibitor, and cytotoxic) (Table 5). Other compounds not listed in Table 5 showed low similarity ($< 90\%$), indicating that VOCs might be unidentified or new compounds. The VOCs identified in the extract are also produced by other microbial sources, such as endophytic *Streptomyces* sp. KCA1, *Lactococcus* sp., soil *Streptomyces albidoflavus* 321.2, *Bacillus tequilensis* MS145, and *Streptomyces* sp. VITPK9, by plants (such as *Cestrum octurnum*, and coconut oil), and by animals (such as *Stichopus hermanni*) (Table 4). VOCs derived from *Bacillus* and other natural sources exert antimicrobial action, possibly by destroying the bacterial cell membrane, damaging DNA, interfering with protein structure and function, and downregulating the expression of genes involved in pathogen

virulence [44,45]. Additionally, particular VOCs, such as eugenol inhibit biofilm formation by interfering with quorum-sensing signals and controlling transmembrane transport [46]. VOCs also have an eradicating effect on established biofilms due to their ability to diffuse and destabilize the polysaccharide matrix in mature biofilms [47]. Therefore, VOCs found in the extract may contribute to the antimycobacterial and antibiofilm activities of the *Bacillus* sp. DJ4 derived extract.

Bacillus sp. strain DJ4 was deemed the most potent isolate based on its antibacterial and antibiofilm activities. In a prior study, the bacterium showed a close relation (similarity 100%) to *Bacillus amyloliquefaciens* strain B29 through the 16S rRNA sequence [12]. However, the 16S rRNA-based identification has limitations as the sequence is not sufficient for species-level bacterial identification and intra-strain differentiation due to its short size (~1,300 bp) compared to the general 4–6 million bp size of complete bacterial genomes [48]. Therefore, a more comprehensive bacterial identification requires sequencing the complete bacterial genome. In this study, the complete genome of this bacterium was sequenced. According to the average nucleotide identity from *dfast_qc*, the bacterium was closely related (97.55% similarity) to *B. velezensis* strain KCTC 13012. *Bacillus velezensis* strain KCTC 13012 was suggested as a relative of *B. amyloliquefaciens* strain KCTC 13012, a well-known producer of broad-spectrum antibiotics [49].

Secondary metabolic gene clusters within the genome of *Bacillus* sp. strain DJ4 were annotated by AntiSMASH. Polyketide, non-ribosomal peptide, LAP, and lipopeptide coding genes were found in the bacterial genome. The clusters shared high similarity (similarity: 78%–100%) with known products, such as difficidin (100%), bacillibactin (100%), bacilysin (100%), fengycin (100%), acillanone (100%), macrolactin H (100%), plantazolicin (91%), and surfactin (78%), which were synthesized by *B. velezensis* and *B. subtilis* (Table 6). Interestingly, bacillibactin produced by endophytic *B. subtilis* NPROOT3 has previously been reported as having antimycobacterial activity against *M. smegmatis* MTCC6 [14]. Additionally, all similar gene products showed antibacterial properties against a broad spectrum of bacteria (Table 5). Moreover, the bacterium may also carry genes contributed in the biosynthesis of VOCs because the products of these genes have been detected in bacterial extracts. However, these genes could not be identified through this methodology because of the limited genome database of VOCs-related genes in bacteria. *Bacillus* VOCs are mostly derived from amino acid degradation, sulfur reduction, terpene synthesis, glucose oxidation, and lactate fermentation [50]. Further studies are necessary to investigate whether the secondary metabolic gene clusters found in the bacterium are expressed under controlled laboratory conditions.

CONCLUSION

The current study showcases the potential of the four endophytic *Bacillus* species, especially *Bacillus* sp. strain DJ4 isolated from *A. pauciflorum*, as promising sources of novel antimycobacterial substances. Extract from this bacterium showed significant inhibition of growth, biofilm formation, and

eradication of *M. smegmatis* cell biofilm. The identification of eight secondary metabolic gene clusters within the bacterial genome suggests their potential contribution to the production of antimycobacterial compounds. These findings offer compelling evidence for the antimycobacterial capacity of endophytic *Bacillus* and highlight its genomic features, which hold promise for drug discovery. This opens avenues for uncovering new antimycobacterial agents from endophytic bacteria, fostering opportunities within the pharmaceutical industry. Future investigations should prioritize isolating active constituents and optimizing metabolite production using diverse biological, chemical, and molecular techniques. Additionally, in-depth exploration of the functionality of the identified secondary metabolic gene clusters in *Bacillus* sp. strain DJ4 through mutagenesis or heterologous overexpression, coupled with understanding the biosynthesis pathways of antimycobacterial compounds, will enhance the exploration and harnessing of novel compounds from this endophytic bacterium.

ACKNOWLEDGMENTS

All authors acknowledge Dr. Tjandrawati Mozef (BRIN, Indonesia) for providing *Mycobacterium smegmatis* ATCC700084 used in this study.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

FINANCIAL SUPPORT

Funding for this research was provided by the Directorate of Research and Innovation at IPB University, through the Young Lecturer Research Program (Penelitian Dosen Muda) 2023 (grant number: 11425/IT3/PT.01.03/P/B/2023) awarded to Jepri Agung Priyanto.

CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This is an observational study did not involve human or animal participants. Ethics approval was not required for this study.

DATA AVAILABILITY

All experimental data collected in this research are provided in this paper.

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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How to cite this article:

Priyanto JA, Prastya ME, Hening ENW, Astuti RI. Exploring antimycobacterial potential and profiling secondary metabolite gene clusters in the whole genome of *Bacillus* isolated from dogfruit (*Archidendron pauciflorum*). *J Appl Pharm Sci*. 2024. <http://doi.org/10.7324/JAPS.2025.204561>