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Diversity and activity of AHL-lactonases in *Bacillus* spp. from various environments

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Abstract

Disrupting quorum sensing (QS) pathways in animal and plant pathogenic bacteria is an effective strategy to mitigate infections without promoting antibiotic and pesticide resistance. This approach inhibits the production of virulence factors, biofilm formation, and toxin production, reducing bacterial pathogenicity. In plant health protection, *Bacillus* spp. are extensively researched and utilized as biocontrol agents; however, the potential of their AHL-lactonase-producing ability, which plays a key role as a QS inhibitor of Gram-negative pathogens, remains largely unexplored. This study examined the activity and diversity of QQ enzymes from *Bacillus* spp. isolates obtained from various natural sources, confirming their presence in previously unreported environments associated with agricultural fields (straw and manure). Our findings show that AiiA lactonase is the most dominant and highly conserved AHL-lactonase among *Bacillus* isolates from bulk soil, manure, and straw. Despite its sequence conservation, we observed significant variation in AiiA lactonase activities toward the N-hexanoyl-DL-homoserine lactone (C6-HSL) substrate. Furthermore, *in silico* analysis suggested that the *Bacillus* sp. YtnP lactonase may have a lower affinity for C6-HSL compared to AiiA lactonase. Finally, this research presents a selection of *Bacillus* isolates with high AiiA lactonase activity for potential testing against plant pathogens.

Keywords: quorum quenching; *Bacillus*; Acyl-homoserine lactones; AiiA lactonase; YtnP lactonase

Introduction

Numerous plant and animal bacterial pathogens can optimize their infection strategies, evade host defenses, and establish successful infections through the coordinated expression of virulence factors in response to population density (Wu et al. 2020). The synthesis of these virulence factors is tightly regulated by quorum sensing (QS) mechanisms (Zhu et al. 2023) primarily mediated by Gram-negative bacteria through the secretion, diffusion, and recognition of signal molecules, including small, medium, and long-chained N-acyl homoserine lactones (AHLs) (Papenfort et al. 2016). QS systems regulate virulence factor production in human pathogens such as *Pseudomonas aeruginosa* and *Burkholderia cepacia*, impacting biofilm formation, antibiotic resistance, and other pathogenic traits (Slinger et al. 2019, Azimi et al. 2020). In plant pathogenic bacteria like *Pectobacterium*, *Ralstonia*, *Agrobacterium*, *Dickeya*, *Pseudomonas*, and *Xanthomonas* genera, QS regulates mechanisms such as toxin secretion, hydrolytic enzyme production, and biofilm formation (Bzdrenga et al. 2017, Zhu et al. 2023, Roca et al. 2024). These processes lead to severe diseases in plants, ranging from leaf spots to soft rot and necrosis of plant tissue, causing significant economic impact (Carezzano et al. 2023).

Interference with the molecular signals of Gram-negative animal and plant pathogens can be achieved through quorum-sensing inhibitors, which disrupt the signal molecules' synthesis pathways, or quorum quenching (QQ) enzymes, such as lactonases, amidases, and oxidoreductases, which enzymatically degrade and modify secreted signal molecules (Naga et al. 2023). The effects on both pathogen types are continuously researched due to the significant impact of these pathogens on agriculture and human/animal health (Wang et al. 2024).

For example, *Bacillus* spp. have been at the forefront of pioneering research on QQ enzymes, with *Bacillus* sp. 240B1 being the first strain in which the AHL-degrading enzyme AiiA lactonase was identified, demonstrating its efficacy in suppressing phytopathogens (Dong et al. 2000; Ayyappan et al. 2022). *Bacillus* spp. offer a plethora of biocontrol mechanisms against a wide range of plant pathogens, spanning from viruses to nematodes (Etesami et al. 2023). The most extensively studied mechanisms include antibiosis (via lipopeptides, bacteriocins, lytic enzymes, volatile organic compounds, and insecticidal proteins), competition for resources, and induced systemic resistance (Shafi et al. 2017, Hashem et al. 2019, Singh et al. 2024), forming the cornerstone of *Bacillus*-based biocontrol products. However, the interference with QS molecules of phytopathogens remains relatively underexplored (Zhang et al. 2023).

Since the discovery of AHL-lactonases in *Bacillus* spp., particularly the group of metallo-β-lactamases, these enzymes have been extensively studied (Fan et al. 2017, Ryu et al. 2020). AiiA lactonase, the most common metallo-β-lactamase in *Bacillus* spp., is encoded by a conserved gene with a highly conserved Zn^{2+} binding motif (^{104}HXHXDH^{109}) essential for its activity (Kim et al. 2005, Kachhadia et al. 2022). So far, AiiA lactonase remains the dominant QQ enzyme identified in *Bacillus*. AiiA exhibits a broad specificity for AHL molecules, acting on acyl chains ranging from C4 to C14, as well as modified AHLs with 3-oxo or 3-hydroxy substitutions (Chen et al. 2010). When not secreted, AiiA functions intracellularly to defend *Bacillus* cells from biotic stress caused

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by exogenous AHLs produced by plant pathogens, thereby aiding in survival and maintaining population stability—both of which are critical for its biocontrol activity (Zhang et al. 2023). However, the secretion of AHL lactonases into the environment can play an even more significant role, enabling an offensive strategy where these enzymes exhibit QQ activity to disrupt the virulence factors of phytopathogens. This activity is particularly notable in natural conditions, where no genetic engineering is applied to enhance secretion. It is also well established that AHL lactonases are frequently discovered in soil *Bacillus* spp. isolates, highlighting their strong potential for rhizospheric biocontrol. However, their occurrence and functionality in other environments related to agricultural spaces remain largely unexplored. To date, only one strictly novel AHL lactonase has been discovered in *Bacillus*: YtnP lactonase (Schneider et al. 2012). Despite being discovered more than a decade ago, YtnP lactonase is still relatively understudied. Comparative activity analyses with AiiA lactonase and detailed investigations into its substrate specificity are areas that remain largely unaddressed.

Utilizing AHL-lactonases from *Bacillus* spp. is considered a novel approach to attenuating phytopathogen virulence rather than attacking the phytopathogen directly, potentially slowing down the development of resistance (Zhu et al. 2023, Roca et al. 2024).

However, despite the aforementioned advantage, it is crucial to note that AHL-lactonases may inadvertently disrupt communication among beneficial bacteria in the plant microbiota (Molina et al. 2003, Hartmann et al. 2021, Sanchez-Mahecha et al. 2022). In light of these complexities, exploring *Bacillus* spp. as green agents of phytopathogen suppression on inert surfaces surrounding plants in commercial crops, such as growth substrate boxes, irrigation hoses, and other agricultural equipment, may be an alternative, especially for phytopathogens that form biofilms (Rana et al. 2020, Catara et al. 2020, Mishra et al. 2022).

We hypothesized that within our *Bacillus* spp. laboratory collection gathered from different natural environments and locations, there are isolates that produce metallo-β-lactamases, especially in unexplored environments such as straw and manure. Our objective was to observe the activity and diversity of secreted metallo-β-lactamases from *Bacillus* spp. isolates, as well as to compare the activity of YtnP and AiiA lactonase types through violacein inhibition test and substrate specificity *in silico*. This approach is particularly relevant for potential biocontrol applications, as it evaluates the functionality of *Bacillus* spp. strains in their native, applied settings, rather than relying solely on isolated enzyme activity. Additionally, we analyzed AiiA amino acid sequences to identify potential mutations affecting Zn^{2+} binding and catalytic motifs, with the end goal of suggesting the most promising isolates for potential research against phytopathogens on inert surfaces.

Materials and methods

Bacterial strains and growth conditions

In this study, a collection of 251 *Bacillus* spp. isolates from bulk soil (153), manure (64), and straw (34) isolated from different locations in Serbia were inoculated on Luria-Bertani (LB) broth supplemented with 10 µM of N-hexanoyl-DL-homoserine lactone [C6-HSL ≥97% (HPLC) Sigma-Aldrich].

Mutant strain *Chromobacterium violaceum* CV026 was used as a biosensor for AHL degradation. It was grown in an LB medium supplemented with 20 µg/ml of kanamycin.

All bacteria were incubated at 30°C, with 180 rpm agitation, for 24 h.

AHL-lactonase activity detection and activity assessment

Initial screening of *Bacillus* spp. isolates was done using the well-diffusion method (Reina et al. 2019). Briefly, 70 µl of *C. violaceum* CV026 overnight culture was mixed with 7 ml of LA-soft agar medium (0.7% agar) and overlayed on LA plates with well molds (5 mm diameter × 7 mm height). *Bacillus* spp. overnight cultures (16–24 h) were centrifuged at 10 000 rpm for 10 min, and 50 µl of supernatants were poured into each well. The highest levels of AHL lactonase secretion occur during the early stationary phase (Abdel-Aziz et al. 2017). Therefore, by using stationary-phase overnight cultures (Atanasković et al. 2020), we can ensure capturing the maximum amount of secreted enzymes. A 10 µM solution of C6-HSL in LB broth served as a negative control. Plates were incubated for 24 h at 30°C. The absence of violacein purple pigment around wells was counted as a confirmation of QQ activity by C6-HSL lactonase.

The AHL-lactonase activity of positive isolates from the well-diffusion test was assessed through a violacein inhibition assay (Packiavathy et al. 2021). Briefly, an overnight culture of *C. violaceum* CV026 was adjusted to optical density OD 0.1, supplemented with 10 µM C6-HSL and supernatants of AHL-lactonase producing *Bacillus* spp. isolates in concentrations of 5%, 10%, and 20% of the mixture, in triplicates. Negative control was prepared by mixing C6-HSL and LB medium without supernatants.

After incubation of *C. violaceum* CV026 cultures at 30°C, with agitation of 180 rpm for 24 h, followed by centrifugation (8000 rpm, 10 min), cell pellets were resuspended in equal volumes of dimethyl-sulfoxide. Suspensions were again centrifuged to collect supernatants. Changes in color intensity and, thus, violacein inhibition were measured spectrophotometrically at A_{585} nm. Inhibition of violacein was assessed using the following formula (Bali et al. 2019):

$$\textit{Violacein inhibition}\ (\%) = \frac{A_{585}\ (\textit{negative control}) - A_{585}\ (\textit{Bacillus supernatants addition})}{A_{585}\ (\textit{negative control})} * 100$$

Detection of AHL-lactonase autoinducer inactivation *aiiA* gene

Genomic DNA was isolated from AHL-lactonase-positive *Bacillus* spp. using the CTAB-chloroform method (Le Marrec et al. 2000), adapted for Gram-positive bacteria. Genomic DNA was added to PCR Master Mix prepared as follows: 25 µl DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific); 21 µl DNase/RNase Free water (Thermo Fisher Scientific); 1 µl of 10 µM forward primer *aiiA*-F (5′–ATGACAGTAAARAARCTTTATTTC–3′) and 1 µl of 10 µM reverse primer *aiiA*-R (5′–TCACTATATATAYTCMGGGAACTC–3′). Both primer sequences were designed based on the study by Pan et al. (2008). Amplification was done in MiniAmp™ Thermal Cycler (Thermo Fisher Scientific) and consisted of the following steps: initial denaturation at 94°C for 5 min; 30 cycles encompassing denaturation at 94°C for 1 min, hybridization at 55°C for 2 min, and elongation at 72°C for 2 min; final elongation was done at 72°C for 7 min. Amplification was confirmed by detecting a 793 bp band on 1% agarose gel.

Table 1. *Bacillus* spp. *aiiA* sequences used for comparative analysis.

Bacillus species	Accession Number	*Bacillus* species	Accession Number
B. amyloliquefaciens	WNN27081.1	*Bacillus* sp. SS-23.2	PP841971
B. albus	WP_336589945.1	*Bacillus* sp. SS-23.3.1	PP841972
B. anthracis	WP_326042145.1	*Bacillus* sp. SS-31.1	PP841973
B. arachidis	WP_286015682.1	*Bacillus* sp. SS-31.2	PP841974
B. atrophaeus	WP_328193263.1	*Bacillus* sp. SS-31.3	PP841975
B. cereus	WP_336458662.1	*Bacillus* sp. SS-35.1/2	PP841976
B. clarus	WP_042978938.1	*Bacillus* sp. SS-35.2	PP841977
B. dicomae	WP_140971148.1	*Bacillus* sp. SS-35.3	PP841978
B. gaemokensis	WP_033677072.1	*Bacillus* sp. SS-35.5	PP841979
B. fungorum	WP_099685082.1	*Bacillus* sp. SS-35.7	PP841980
B. luti	WP_151626741.1	*Bacillus* sp. SS-35.8	PP841981
B. megaterium	ACX55098.1	*Bacillus* sp. SS-36.1	PP841982
B. mobilis	WP_327798574.1	*Bacillus* sp. SS-36.2	PP841983
B. mycoides	WP_070146799.1	*Bacillus* sp. SS-36.4	PP841984
B. nitratireducens	WP_097809865.1	*Bacillus* sp. SS-36.6/1	PP841985
B. pacificus	WP_229136099.1	*Bacillus* sp. SS-36.6/2	PP841986
B. paramobilis	WP_336181747.1	*Bacillus* sp. SS-37.1	PP841987
B. paramycoides	WP_328064864.1	*Bacillus* sp. SS-37.1/2	PP841988
B. paranthracis	WP_284997747.1	*Bacillus* sp. SS-35.6	PP841989
B. proteolyticus	WP_071747546.1	*Bacillus* sp. SS-37.2/1	PP841990
B. subtilis	CUB50997.1	*Bacillus* sp. SS-37.2/2	PP841991
B. thuringiensis	WP_335466617.1	*Bacillus* sp. SS-37.3	PP841992
B. tropicus	WP_337679459.1	*Bacillus* sp. SS-37.4	PP841993
B. toyonensis	WP_335447769.1	*Bacillus* sp. SS-37.6	PP841994
B. wiedmannii	WP_327973071.1	*Bacillus* sp. SS-37.7	PP841995
Bacillus sp. SS-1.1	PP841946	*Bacillus* sp. SS-39.1	PP841996
Bacillus sp. SS-2.1.2	PP841947	*Bacillus* sp. SS-39.2	PP841997
Bacillus sp. SS-2.1.3	PP841948	*Bacillus* sp. SS-39.3	PP841998
Bacillus sp. SS-2.3/1	PP841949	*Bacillus* sp. SS-40.3	PP841999
Bacillus sp. SS-2.8	PP841950	*Bacillus* sp. SS-40.6/2	PP842000
Bacillus sp. SS-2.11	PP841951	*Bacillus* sp. SS-27.7	PP842001
Bacillus sp. SS-2.12.1	PP841952	*Bacillus* sp. SS-29.2	PP842002
Bacillus sp. SS-6.5	PP841953	*Bacillus* sp. SS-32.4	PP842003
Bacillus sp. SS-10.3	PP841954	*Bacillus* sp. SS-32.5	PP842004
Bacillus sp. SS-10.8.1	PP841955	*Bacillus* sp. SS-32.8	PP842005
Bacillus sp. SS-11.2.2	PP841956	*Bacillus* sp. SS-33.1	PP842006
Bacillus sp. SS-12.3	PP841957	*Bacillus* sp. SS-33.2	PP842007
Bacillus sp. SS-12.9.2	PP841958	*Bacillus* sp. SS-33.3	PP842008
Bacillus sp. SS-14.6	PP841959	*Bacillus* sp. SS-33.4	PP842009
Bacillus sp. SS-15.2	PP841960	*Bacillus* sp. SS-33.5	PP842010
Bacillus sp. SS-16.2	PP841961	*Bacillus* sp. SS-33.6	PP842011
Bacillus sp. SS-17.3	PP841962	*Bacillus* sp. SS-33.7	PP842012
Bacillus sp. SS-17.4	PP841963	*Bacillus* sp. SS-33.8	PP842013
Bacillus sp. SS-18.2	PP841964	*Bacillus* sp. SS-34.1	PP842014
Bacillus sp. SS-19.1	PP841965	*Bacillus* sp. SS-34.3	PP842015
Bacillus sp. SS-20.1	PP841966	*Bacillus* sp. SS-34.4	PP842016
Bacillus sp. SS-21.5/1	PP841967	*Bacillus* sp. SS-34.5	PP842017
Bacillus sp. SS-21.6	PP841968	*Bacillus* sp. SS-34.7	PP842018
Bacillus sp. SS-22.2	PP841969	*Bacillus* sp. SS-27.6	PP842019
Bacillus sp. SS-22.5/1	PP841970		

aiiA gene sequencing and sequence analysis

*aii*A gene was purified from PCR mix using GeneJET PCR Purification Kit (Thermo Fisher Scientific). Five microliter of purified *aii*A gene sequence and 5 µl of 5 µM forward *aii*A-F primer were mixed and sequenced via Sanger sequencing method Mix 2Seq/Light Run by Eurofins Genomics GmbH (Ebersby, Germany). The quality of the sequences was observed using FinchTV software (Geospiza, Inc.).

Nucleotide *aii*A sequences were translated using the EMBOSS TransSeq online tool, with the following parameters selected: reading frame one and the Bacteria codon table.

To explore the phylogenetic relationship and diversity between amino acid sequences of isolates, a condensed Neighbor-joining tree (cut-off value 70%) with bootstrap consensus for 1000 replicates, based on the Jones-Taylor-Thompson model and γ-distribution, was computed using the MEGA11 software (Tamura et al. 2021). Isolates were compared to chosen AiiA protein sequences of 25 different *Bacillus* species avilable in the NCBI database (Table 1).

To determine the potential effects of amino acid sequence diversity and their influence on AiiA lactonase activity, we conducted an AiiA amino acid sequence analysis. To calculate the percentage of identical, similar, and variable amino acids us-

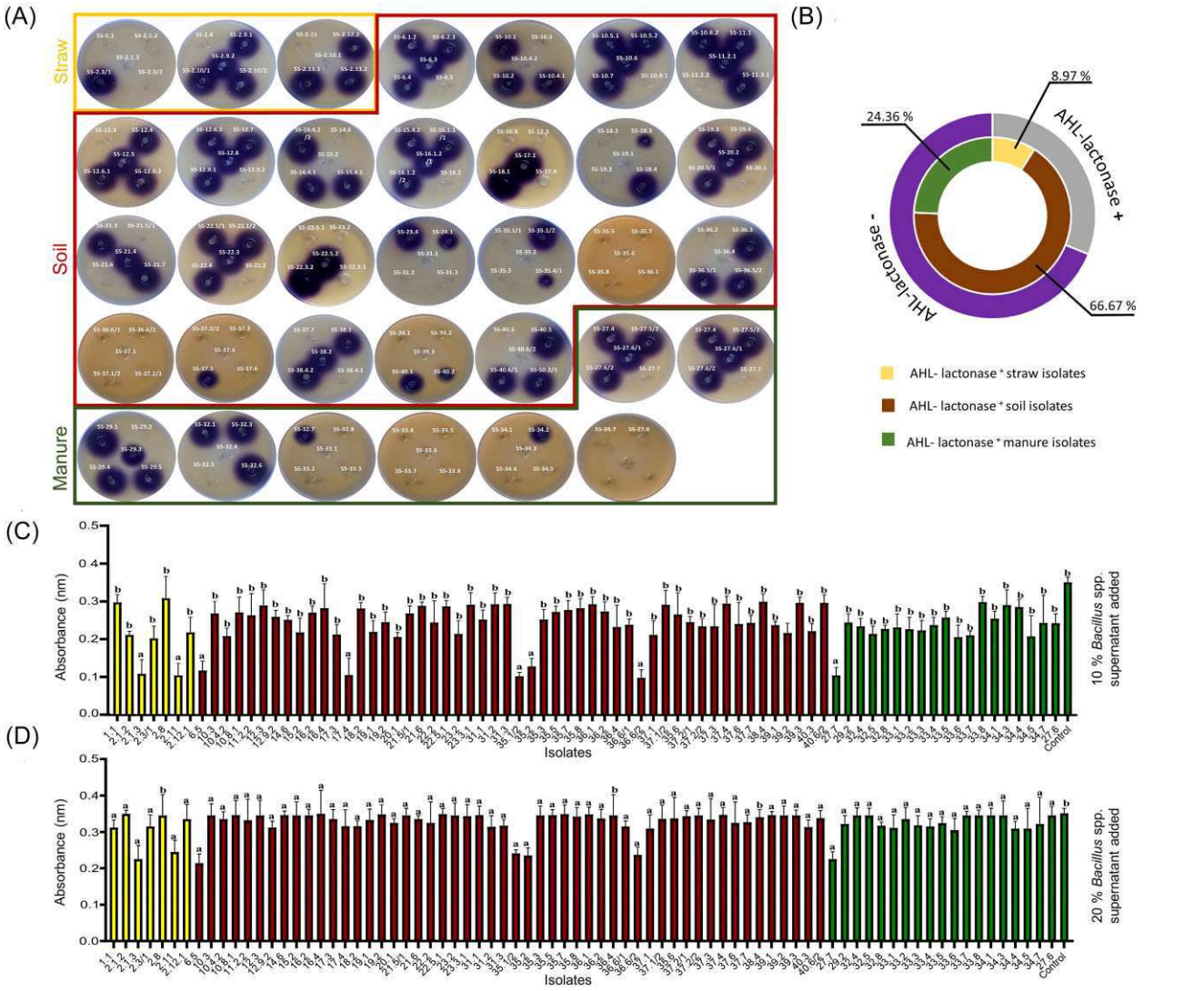

Figure 1. AHL-lactonase activity and distribution in Bacillus spp. isolates: AHL-lactonase activity of *Bacillus* spp. isolates observed through the absence of violet ring around wells containing supernatants (A). The arrangement of positive strains on Petri dishes follows the order of the bars in the graphs c and d. Percentage of AHL-lactonase positive and negative isolates and distribution of AHL-positive isolates across environmental samples (B). Only eight isolates showed a significant decrease ($P \leq .05$) in absorbance (marked with the letter "a") compared to the control when supernatants were added at a 10% concentration (C). At a 20% concentration, 76 isolates exhibited a significant decrease ($P \leq .05$) in absorbance compared to the control (D). Isolates are color-coded as follows: yellow for straw, red for soil, and green for manure.

ing multiple sequence alignment using the Fasta Fourier transform(MAFFT) tool. Further, using NCBI Conserved Domain Search (Wang et al. 2023), we searched for differences in the active site of the AiiA enzyme, along with Zn^{2+} binding motifs, to detect potential differences in signature amino acids that could affect the activity and diversity of AiiA enzymes. The SwissModel online tool (Waterhouse et al. 2018) was used for Ytnp and AiiA lactonases model building, while the CB-Dock2 online tool was used for molecular docking analysis, with the following parameters selected: five cavities for docking and option "Auto Blind docking." Docking results were analyzed based on structure-based blind docking, where no prior knowledge of the binding site is used.

Data analysis

A one-way analysis of variance, with Fisher's Least Significant Difference *post-hoc* test without correction for multiple comparisons and statistical significance set at a threshold of *P* values below .05, was used to detect significant differences in AHL-lactonase activity based on absorbance values in violacein inhibition assay. Data analysis was performed in GraphPad Prism v.8.4.3.

Results and discussion

Natural *Bacillus* spp. isolates tested displayed diverse AHL-lactonase activity

Following screening, 78 isolates lacked purple violacein color, indicating C6-HSL lactonase activity (Fig. 1A). The majority of AHL-lactonase-positive isolates were from soil (52), with seven from straw and 19 from manure (Fig. S1; Fig. 1B). Soil isolates not only outnumbered others but also exhibited higher AHL-lactonase activity, establishing soil as a key source of biocontrol *Bacillus* spp. (Vincze et al. 2024). This underscores that bulk soil, beyond the rhizosphere, is a significant source of AHL-lactonase-producing *Bacillus* spp., as evidenced by a number of studies using *Bacillus* spp. isolates from soil (Huma et al. 2011, Easwaran et al. 2015, Lee et al. 2022). Significantly, straw and manure emerged as po-

tential novel sources of these *Bacillus* spp., a revelation not previously reported in existing studies. Therefore, the presence of AHL-lactonase-producing *Bacillus* spp. in soil, straw, and manure—integral components of crop cultivation processes—could potentially exert a significant impact on phytopathogen suppression as a natural plant defense (Prazdnova et al. 2022).

To further assess the diversity in AHL-lactonase activity, we assessed violacein inhibition of AHL-lactonase-positive *Bacillus* spp. isolates. The absorbance of samples containing 5% supernatants closely matched that of the negative control (A_{585} 0.351) (Table S1). Since none of these absorbance values differed significantly from the negative control, no further analysis was conducted at this concentration. However, at a 10% supernatant concentration, eight isolates exhibited significantly lower absorbance than the control. These included two straw isolates (SS-2.1.3 and SS-2.11), five soil isolates (SS-6.5, SS-17.4, SS-35.1/2, SS-35.2, and SS-36.2/2), and one manure isolate (SS-27.7) (Fig. 1C; Table S2). At a 20% concentration, the number of isolates whose supernatants affected violacein synthesis increased substantially, with only two isolates not showing significantly lower absorbance compared to the negative control (Fig. 1D; Table S3). The absorbance values obtained with 20% supernatants were used to calculate AHL-lactonase activity based on the percentage of violacein inhibition. The highest violacein inhibition (71.7%) was observed with the supernatant of isolate SS-36.6/2, while the lowest (14.52%) was detected with the SS-38–4 supernatant (Fig. S2). The soil isolates exhibited the highest and lowest C6-HSL lactonase activity assessed through violacein inhibition. While none of the isolates' supernatants containing AHL-lactonases exhibited notably high inhibition levels at similar concentrations to those observed in other studies (80%–90% inhibition) (Musthafa et al. 2011, Devi et al. 2018), it is worth noting that factors such as pH, temperature, and the potential presence of interfering substances in the media can influence AHL-lactonase activity (Chen et al. 2010; Garge et al. 2016). Despite that, assessing AHL lactonase activity through violacein inhibition offers a rapid, cost-effective, and straightforward method that serves as a valuable guide for selecting AHL-lactonases for further testing (Reina et al. 2019).

Four AHL-lactonase positive isolates have different AHL-lactonase genes

A 759 bp amplicon corresponding to the AiiA lactonase gene was detected in 74 of the 78 isolates that showed AHL-lactonase activity (Fig. S3). In straw and manure isolates, only the typical AiiA lactonase specific to *Bacillus* spp. was found. However, four soil isolates—SS-10.4.2, SS-16.4, SS-19.2, and SS-38.4—did not display the characteristic band after gel electrophoresis, and the *aiiA* gene was not identified despite observed AHL-lactonase activity.

One notable isolate, *B. velezensis* SS-38.4, which exhibited the lowest AHL-lactonase activity, was found to harbor the *ytnP* lactonase gene after whole genome sequencing (GCF_035600155.1). Initially reported as an unidentified gene in *B. subtilis* (Schneider et al. 2012), studies on this lactonase remain limited, with no comparison yet made regarding its substrate specificity, catalytic efficiency, and stability with the AiiA lactonase. The sole research comparing the activity of these two metallo-β-lactamases was conducted with the enzymes isolated from *Burkholderia* sp. (Malešević et al. 2020), revealing variable substrate specificity and efficiency in AHL degradation between YtnP and AiiA lactonase.

It is important to highlight that one of the objectives of this study was to compare different isolates based on *aiiA* sequencing. Notably, the taxonomy of *Bacillus* is highly complex, and precise identification and differentiation of strains often require whole genome sequencing. However, in most studies, 16S rRNA sequencing and genotyping are sufficient to differentiate between isolates when precise identification is not necessary. In previous studies on this collection, it was confirmed that the isolates are distinct. This was established using 16S rRNA and *tuf* gene sequencing, as well as genetic profiling techniques such as RAPD (random amplified polymorphic DNA) and rep (repetitive element)-PCR and PFGE (pulsed-field gel electrophoresis) (Berić et al. 2009, Dimkić et al. 2017, Berić et al. 2018, Draganić et al. 2018, Rosić et al. 2023).

Bacillus spp. isolates from different natural origins have very similar AiiA lactonase amino acid sequence

The 74 translated *aii*A gene sequences underwent phylogeny and diversity analysis. Despite originating from isolates across various natural habitats and locations, the isolates were generally closely related to each other. Over half of the isolates (26 from soil and 16 from manure) formed a distinct cluster separate from NCBI reference strains (Fig. 2). One straw isolate (SS-2.8) and two soil isolates (SS-12.3 and SS-22.2) were classified as separate branches on the phylogenetic tree. Six isolates (one from straw, three from soil, and two from manure) exhibited high relatedness to *B. anthracis*, clustering together on the tree. Additionally, 12 isolates (three from straw, eight from soil, and one from manure) showed high relatedness to *B. toyonensis*, evidenced by their clustering. Notably, AiiA amino acid sequences from nine isolates (two from straw and seven from soil) displayed significant similarity to those of *B. toyonensis* (Fig. 2). Overall, most isolates demonstrated greater relatedness to *Bacillus* species commonly found in the three analyzed natural habitats, such as species of *B. subtilis* (Rooney et al. 2009, Caulier et al. 2019) and the *B. cereus* species complex (Rasko et al. 2005, Ehling-Schulz et al. 2019). While the AiiA sequence is conserved among *Bacillus* spp. and can assist in determining the similarity and relatedness of isolates (Molina et al. 2003, Noor et al. 2022), the *Bacillus* genus comprises closely related species that are phenotypically and genotypically very similar and to distinguish between them, analysis of more conserved genes is required (Fan et al. 2017, Hernández-Flores et al. 2020). This could explain the unexpected classification of some isolates with *B. anthracis*.

Sequence alignment analysis was conducted to investigate potential differences in conserved amino acids of the AiiA lactonase sequence that could contribute to observed variations in AHL-lactonase activities. AiiA sequences displayed high similarity among all isolates, with 79.63% of identical amino acid residues and 7.24% of similar residues (Fig. S4, black and gray marked). However, when the isolates' AiiA sequences were compared to those of chosen reference strains, the number of variable sequences was notably higher, with 81.9% differing amino acids. Identical amino acids between isolates and reference strains predominantly comprised residues forming the active site of the lactonase. Notably, all sequences exhibited an identical active site to the consensus sequence of *B. thuringiensis* cd07729 MBL-fold lactonase (Fig. S4, yellow marked). No differences in amino acids forming the active site and Zn^{2+} binding domain were observed across all sequences.

Molecular docking confirms lower affinity of YtnP lactonase toward C6-HSL substrate than AiiA

For comparison of AiiA's affinity toward C6-HSL, we constructed a model of the AiiA lactonase from the isolate SS-36.6/2, which displayed the most effective violacein inhibition. This model was

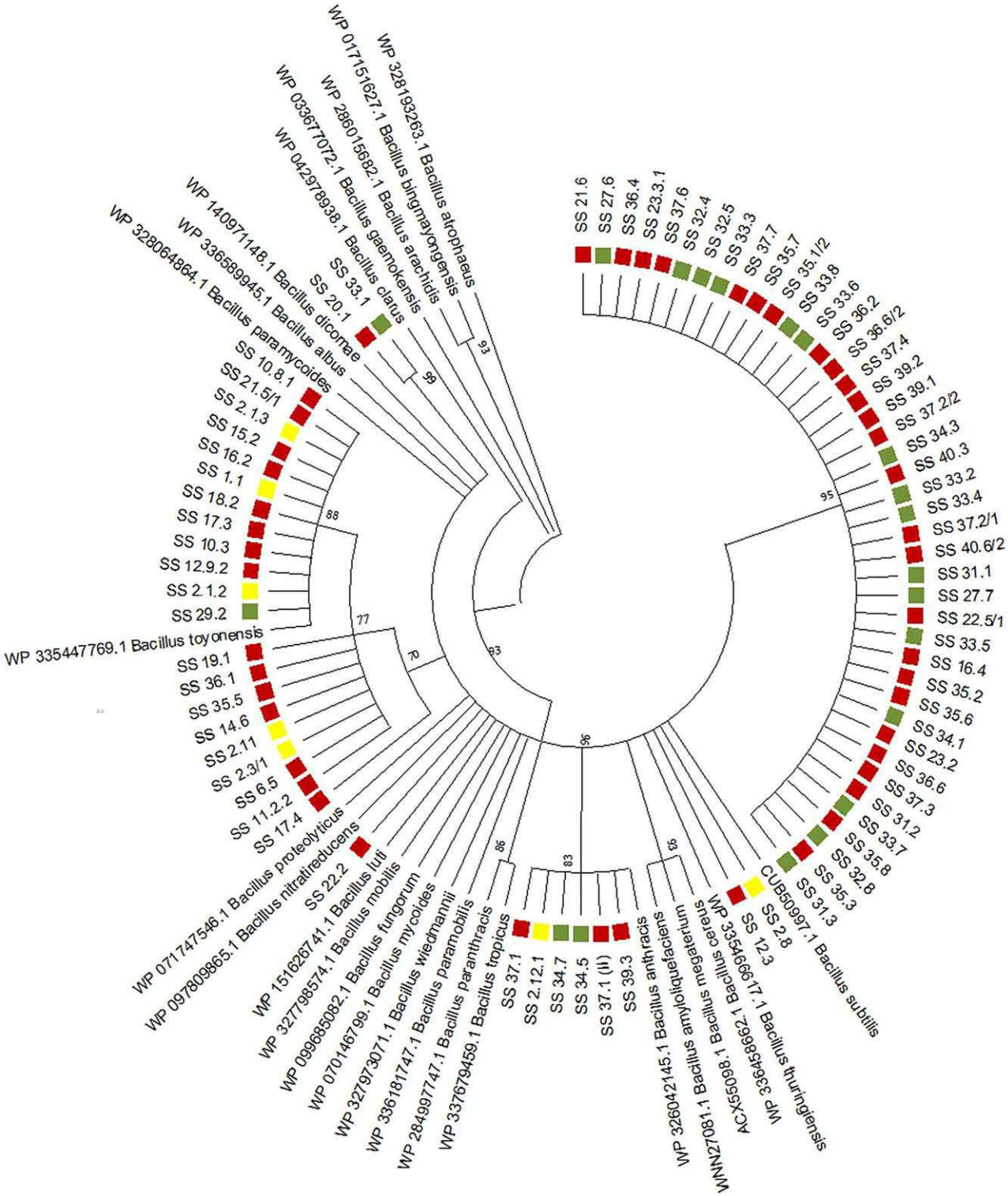

Figure 2. Diversity of *Bacillus* spp. AHL-lactonases: phylogenetic relationships between *Bacillus* spp. isolates (red squares mark soil isolates, yellow straw isolates, and green manure isolates) and strains from the NCBI database.

based on A0A2C1D257.1.A QQ N-acyl-homoserine lactonase to build a model: AlphaFold DB model of A0A2C1D257_BACCE (gene: A0A2C1D257_BACCE, organism: *B. cereus*) of 93.21% and Global Model Quality Estimate (GMQE) of 0.98 (Fig. 3A). To model the 3D structure of YtnP lactonase from *B. velezensis* SS-38.4, we utilized a template based on the A0A6I7TWQ4.1.A metallo-beta-lactamase family protein to build a model: AlphaFold DB model of A0A6I7TWQ4_9BACI (gene: A0A6I7TWQ4_9BACI, organism: *B. paralicheniformis*), exhibiting a sequence identity of 76.51% and a GMQE of 0.98 (Fig. 3B). Both models are homologous 3D models of proteins, based on template structures available in the Protein Data Bank and deposited in the SWISS-MODEL Repository under

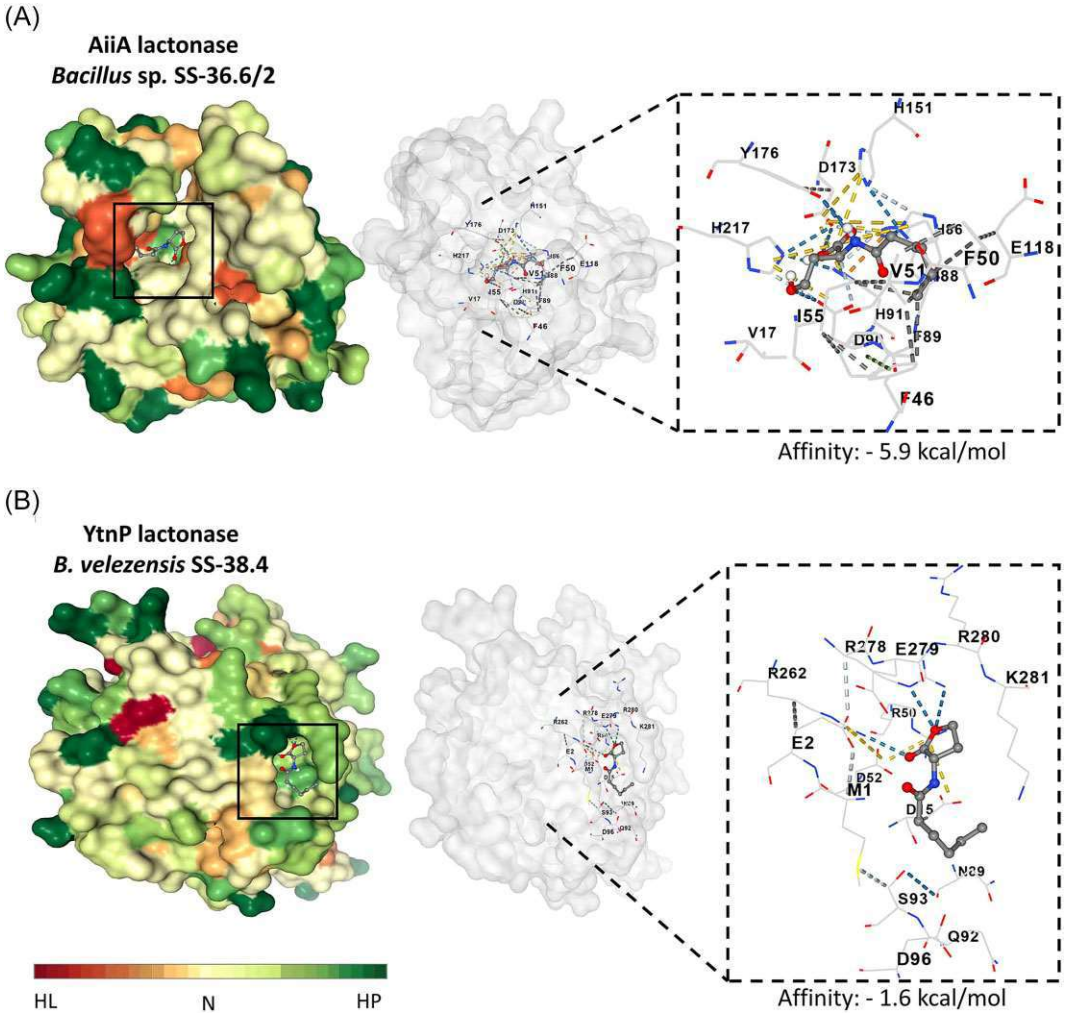

Figure 3. Three-dimensional models of YtnP and AiiA lactonases: Hydrophobicity color scheme for amino acids [legend: HL, hydrophilic, N, neutral; HP, hydrophobic], show the position of C6-HSL in the binding site of each lactonase. In the binding pocket (cavity volume 2487 Å3) of the AiiA lactonase, the amino acids interacting with the ligand are: SER1, SER2, VAL3, ASN4, ASN11, LEU12, LEU13, ASN14, LEU15, PRO16, VAL17, MET35, PHE46, PHE50, VAL51, GLN54, ILE55, LEU56, PRO57, LYS58, HIS86, HIS88, PHE89, ASP90, HIS91, GLU118, HIS151, ASP173, TYR176, ALA188, and HIS217 (A). In the binding pocket (cavity volume 190 Å3) of the YtnP lactonase, the amino acids interacting with the ligand are: THR67, GLY70, ARG71, GLY72, LYS73, SER88, ASN89, LEU90, LYS91, GLN92, ARG95, CYS101, CYS118, GLY119, THR121, GLU122, TYR123, VAL131, and PHE132 (B).

the specified names. To investigate whether the low activity of SS-38.4 YtnP lactonase in violacein inhibition was due to reduced affinity toward the C6-HSL substrate, we conducted molecular docking analysis. Our results revealed that SS-38.4 YtnP lactonase has nearly five times lower affinity toward C6-HSL (−1.6 kcal/mol) compared to SS-36.6/2 AiiA lactonase (−5.9 kcal/mol), as indicated by the greater negative values of free energy, signifying higher affinity (Mohamed et al. 2023). Models of ligand interaction with the cavity, where the interactions had the highest pose ranking based on the lowest binding affinity score, are shown in Fig. 3. A cut-off criterion of H-bond distance ≤3.5 Å was used to determine whether a hydrogen bond between a ligand and a receptor was considered valid. In both models, the binding pocket is characterized by a mix of hydrophobic (green) and hydrophilic (red) regions, matching the amphiphilic nature of C6-HSL (Liu et al. 2017). The hexanoyl chain primarily forms hydrophobic interactions, while the lactone ring engages in hydrophilic interactions. However, the AiiA binding pocket shows better pocket complementarity (Fig. 3A), as well as a greater number of interactions and tighter bonds, including hydrogen (blue dashes), hydrophobic (gray dashes), ionic (yellow dashes), and π-π stacking (green dashes) interactions. The higher binding affinity, the greater number of interactions, the tight fit of the ligand, and the more complex interaction network support the findings from the violacein inhibition test, where the AiiA lactonase of SS-36.6/2 demonstrated the highest inhibition, while the YtnP lactonase of SS-38.4 exhibited the lowest percentage of inhibition.

Some researchers (Djokic et al. 2022) have suggested that YtnP lactonase may exhibit higher affinity toward long-chained AHLs (C12-HSL and C14-HSL), while others have reported the opposite (Schneider et al. 2012). This could potentially be a genus-specific characteristic, as long-chain specificity was observed in YtnP from *B. paralicheniformis*, whereas short-chained HSL specificity was noted in YtnP from *Burkholderia cepacia*.

In conclusion, our study highlights that besides soil, manure and straw are also significant sources of *Bacillus* spp. isolates exhibiting high AHL-lactonase activity. Additionally, we reaffirm that the AiiA lactonase is the predominant and highly conserved enzyme, even among *Bacillus* spp. isolates from diverse environmental sources and regions. However, despite the conservation of the AiiA amino acid sequence, there are notable differences in their activities toward C6-HSL. Ultimately, this research presents a cu-

rated selection of isolates with AHL-lactonase activities that warrant further investigation for their potential in inhibiting QS in various phytopathogens and their application in biocontrol.

Supplementary data

Supplementary data are available at *FEMSLE Journal* online.

Conflict of interest: None declared.

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Data availability

Bacillus spp. aiiA sequences are available in NCBI database under accession numbers provided in the manuscript. Additional data will be made available by authors upon a request.

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03 – 17 Poster

IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF PLANT-GROWTH PROMOTING BACTERIA ISOLATED FROM SOILS AND ROOTS OF RED RASPBERRY (*RUBUS IDEAUS* L.)

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Red raspberry (*Rubus ideaus* L.) is a perennial plant with significant nutritional value and commercially important for Serbia. Plants recruit beneficial microorganisms in the rhizosphere and roots, forming a complex system called holobiont. These beneficial microorganisms contribute to plant-growth promotion (PGP) through various mechanisms, thereby enhancing plant productivity. This study aims to identify and elucidate phylogenetic relationships between PGP bacteria isolated from the roots and rhizosphere of organic and pesticides-treated red raspberries. Recently, 5 highly efficient PGPB strains from organic and 9 from pesticide-treated raspberries have been isolated and characterized. The partial sequence of the *16s rRNA* gene was amplified, while sequences were edited using the FinchTV program and identified via the BLASTn algorithm. A phylogenetic tree was constructed using neighbor-joining method in MEGA XI software. Initial identification revealed strains of *Pseudomonas*, *Variovorax paradoxus*, *Paraburkholderia gardini*, *Bacillus*, *Streptomyces*, *Brachybacterium*, *Cryocola* sp. and *Leifsonia lichenia*. Phylogenetic analysis revealed two main branches. One branch comprised a group with *Pseudomonas* species and a cluster containing *Variovorax paradoxus* and *Paraburkholderia gardini*. The other primary branch included a group of *Bacillus* species and a sub-branch containing three distinct groups: *Streptomyces* sp., *Brachybacterium* sp. and a cluster with *Cryocola* sp. and *Leifsonia lichenia*. The results indicate that in the rhizosphere of chemically-treated red raspberry, *Bacillus* species are predominant, while the organic rhizosphere exhibits a greater diversity of bacterial communities. Additionally, PGPB from both organic and pesticide-treated roots belong to the same phylogenetic group, suggesting a close phylogenetic relationship between communities regardless of treatment type.

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PHYLOGENETIC ANALYSIS, PLANT-GROWTH PROMOTION, RED RASPBERRY, SUSTAINABLE AGRICULTURE

03 – 12 Poster

PHYLOGENETIC ANALYSIS AND DETECTION OF A HIGHLY VIRULENT HAPLOTYPE AMONG *PSEUDOMONAS SYRINGAE* ISOLATES FROM THE DANUBE-TISA-DANUBE CANAL NETWORK

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Pseudomonas syringae (Psy) is a widespread complex of plant pathogenic bacterial species categorized into 23 clades across 13 phylogroups. Among the various genetic lineages, the dominant haplotype DD.1, identified based on a partial sequence of the citrate synthase (*cts*) housekeeping gene, is particularly common in disease outbreaks and aquatic environments and includes potentially highly virulent strains.

In Serbia, the diversity of Psy strains beyond agricultural contexts remains underexplored. This study aimed to elucidate the phylogenetic diversity of Psy isolates from the Danube-Tisa-Danube (DTD) canal network, an important source for crop irrigation, and to ascertain the presence of the DD.1 haplotype.

A partial sequence of the *cts* gene of 42 isolates was amplified and sequenced. Phylogenetic relationships were analyzed by constructing a phylogenetic tree using the neighbor-joining method in Mega 11 software.

The phylogenetic analysis revealed seven clades within five phylogroups, indicating significant strain diversity within the DTD canal network. The isolates predominantly belonged to phylogroup 2 (36%), followed by phylogroup 1 (31%), 12 (26%), 7 (5%), and 13 (2%). Comparison with the cts sequence of DD.1 confirmed that three isolates from our collection matched this highly virulent haplotype. The first detection of the DD.1 haplotype in the DTD canal network suggests a potential threat to irrigated crops. Investigating the Psy population in irrigation sources is an important step in crop risk management.

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PSEUDOMONAS SYRINGAE, PHYLOGENY, DIVERSITY, CITRATE-SYNTHASE

03 – 05 Oral

GENOME ANALYSIS OF *B. VELEZENSIS* SS- 38.4 REVEALED THE GENETIC BASIS OF ITS BIOCONTROL ACTIVITY

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Bacillus spp. are regarded as one of the most successful bacterial biocontrol agents. In this study, we investigated the phylogenetic status of *Bacillus* sp. strain SS-38.4 in comparison to other proven biocontrol and non-biocontrol *Bacillus* strains. Our aim was to understand its biocontrol mechanisms, focusing on its repertoire of genetic traits related to plant growth promotion, biocontrol, and environmental adaptation.

Genomic DNA from SS-38.4 was extracted and sequenced using the PacBio Sequel II system. De novo assembly and error correction were performed with Canu and Racon. Genome annotation was carried out using PGAP and RAST. The phylogenetic analysis involved 50 representative Bacillus spp. genomes, and comparative genomics was conducted using the EDGAR pipeline.

The SS-38.4 genome, comprising two contigs with a total size of 4,007,389 bp and 56.7% GC content, included 4,219 genes. Phylogenetic analysis confirmed SS-38.4 as *B. velezensis*, closely related to biocontrol strain *B. velezensis* FZB42. Comparative genomics identified 78.1% of the core genome and 97 unique genes, indicating high similarity within the species. The genome revealed a wide spectrum of genes responsible for siderophore production, biofilm formation, motility, and antimicrobial compounds, alongside plant-growth-promoting traits. These genes provide SS-38.4 with competitive advantages in nutrient acquisition and plant colonization, indirectly affecting phytopathogens and directly killing them through antimicrobial compounds, highlighting its strong biocontrol potential.

The genome analysis of SS-38.4 elucidates its biocontrol potential through plant growth promotion, environmental adaptation, and antimicrobial activity. This genetic insight supports its use in sustainable agriculture for managing plant pathogens and enhancing crop growth.

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BACILLUS SP, BIOCONTROL, PLANT GROWTH PROMOTION, COLONISATION

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Optimization of Iron Nanoparticle Biosynthesis Using Bacterial Isolates from Natural Environments

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Iron nanoparticles (FeNPs) have a wide range of applications in various industries due to their unique properties, such as high surface area, superparamagnetism, and favorable surface-to-volume ratio [1]. Key applications include the electromagnetic industry (battery development), bioremediation, wastewater treatment, pharmaceuticals (as vesicles for targeted drug delivery), and nanomedicine for diagnostics like magnetic resonance imaging [2]. However, conventional physical and chemical synthesis methods are expensive, especially for biological applications requiring special biocompatible coatings [3]. These methods also consume significant energy and use toxic reagents, posing environmental risks [4]. Therefore, biological synthesis using bacteria that reduce iron extracellularly or intracellularly presents a sustainable alternative. This method aligns with global sustainability goals, as it relies on bacterial metabolic reactions. It is also simple and economical, given the widespread availability of bacteria in various environments, the ease of laboratory cultivation, and their short generation time. The FeNPs have an inherent biocompatible coating and reduced toxicity [5]. However, two major challenges hindering the industrial success of this approach are inconsistent size uniformity and low yield compared to conventional methods [6].

The main objective of our ongoing study is to optimize iron bionanoparticles production using bacteria from natural environments by comparing different conditions for FeNP synthesis. We aim to isolate and identify iron-reducing and magnetotactic bacteria, test various synthesis conditions (effects of strain selection, iron salt type and concentration, pH, and temperature), and isolate, purify, and characterize FeNPs using physical methods (UV-Vis, FTIR, SEM). The goal is to develop a protocol with yields and uniformity comparable to physicochemical methods at an industrial scale.

Here, we present results from the first step of optimization, focusing on the isolation and selection of bacteria that demonstrated intracellular and extracellular synthesis of FeNPs. Bacteria were isolated from samples collected from seven locations in Serbia, including river waters and mud, rusty surfaces, rocks, soil, and mining sites. Pure strains were identified via 16S rRNA sequencing and tested for FeNP synthesis.

A total of 62 isolates were obtained. Four isolates (two *Stenotrophomonas* sp. and two *Bacillus megaterium*) exhibited magnetosomes and synthesized intracellular magnetite nanoparticles, confirmed by movement towards a magnetic field under a microscope. Extracellular synthesis was observed in 36 isolates (22 from $FeCl_3 \times 6\ H_2O$ and 14 from $FeCl_2 \times 4\ H_2O$) from 11 genera, based on yellow-orange precipitates indicating iron reduction. Notably, two *B. megaterium* isolates exhibited both intracellular and extracellular synthesis, while eight isolates synthesized iron from both salts. The most common genera in FeNP synthesis were *Bacillus* and *Pseudomonas*.

This part of the study confirmed extracellular FeNP synthesis by known nanoparticle-producing genera, such as *Enterobacter* sp., *Achromobacter* sp., and *Stenotrophomonas* sp. It also demonstrated FeNP synthesis by strains from genera previously known for silver and gold nanoparticle synthesis, including *Lysinibacillus* sp., *Geobacillus* sp., *Serratia* sp., and *Arthrobacter* sp. Additionally, FeNP synthesis was observed in *Buttiauxella* sp. and *Pseudoarthrobacter* sp., for which nanoparticle synthesis had not been previously studied, opening new research opportunities.

In conclusion, this study presents a first step toward optimizing bacterial FeNP synthesis by selecting strains for both intracellular and extracellular production. The potential for FeNP synthesis in these bacteria is promising, especially for sustainable nanotechnology development.

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Plant-growth-promoting potential of bacteria from organic and chemically-treated red raspberry roots and rhizosphere

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Raspberries are commercially important fruit crops in Serbia and all temperate regions. The excessive use of synthetic fertilizers in agriculture poses a serious threat to the environment and health of all living organisms. Application of beneficial plant-growth-promoting (PGP) bacteria is an eco-friendly alternative that will ensure crop productivity in an efficient and safe manner.

The objective of this research was to determine the plant-growth-promoting potential of bacteria from organic and chemically-treated red raspberry (*Rubus idaeus* L.) roots and rhizosphere.

Isolation of bacteria was performed by a high-throughput microtiter plate dilution method. Bacterial isolates were screened for five PGP traits – phosphate solubilization, production of siderophores, indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, and ammonia.

Total of 482 bacterial isolates were obtained from chemically-treated and 351 from organic root and rhizosphere samples. Among the isolates from chemically-treated samples, 35.5% were phosphate solubilizers, while this trait was detected in 29.6% of isolates from organic samples. Out of these, 115 isolates from chemically-treated and 60 from organic samples were able to produce siderophores. IAA production was detected in 55 isolates, ACC deaminase was produced by 47 isolates, and 14 were able to produce ammonia. Nine isolates from chemically-treated and five from organic samples were positive for all five tested PGP traits.

Both chemically-treated and organic red raspberry roots and rhizosphere present a valuable source of PGP bacteria. Fourteen bacterial isolates with multifaceted PGP activities were selected as most promising candidates for potential applications to improve the growth and yield of raspberry.

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Watermelon growth in Mars regolith simulant following seed treatment with plant growth-promoting bacteria

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Mars is currently the most extensively studied planet and the primary target for future human exploration and long-term settlement. The Martian surface is covered by regolith, a layer of loose, dusty material that lacks organic matter and microbiota, with physico-chemical properties unfriendly to plant growth. Nevertheless, every scenario for the long-term settlement of humans on extraterrestrial bodies includes plants as key components of bioregenerative life support systems (BLSS). To reduce reliance on Earth-based inputs, native Martian regolith could be utilized for plant cultivation. The environmental stressors may be mitigated using plant growth-promoting bacteria (PGPB). In this way, the combination of the *in situ* resource utilization (ISRU) approach and BLSS may enable sustainable food production in space. Therefore, the aim of this study was to investigate the potential for growing watermelon plants in a Mars regolith simulant and to evaluate the possible beneficial effects of PGPB.

Watermelon seeds were treated with suspensions of PGPB previously confirmed to be metal-tolerant. The bacterial strains used were *Pseudomonas chlororaphis* (strain B0), *Bacillus safensis* (D0), and *Bacillus thuringiensis* (F4). The treatments included: F4 alone, a combination of D0 and F4, and a mixture of B0, D0, and F4. Sterile distilled water was used as a negative control. Following treatment and drying, the seeds were sown in soil and regolith simulant substrates, with 14 seeds per substrate type. Plants were grown in a growth box under a 14/10 h light/dark photoperiod at room temperature. After four weeks, the following growth parameters were measured: plant height, root length, number of leaves, total leaf area, fresh weight, total chlorophyll, and chlorophyll a and b content. The results were analyzed using a PERMANOVA test (Permutational multivariate analysis of variance) and pairwise permutation as a post-hoc test.

Plant growth was observed in both substrates, but growth in soil was generally more successful than in regolith. Statistically significant differences ($p < 0.05$) were found between plants grown in soil and regolith for all measured growth parameters, except for chlorophyll content, which remained similar regardless of the substrate. Regarding the effect of PGPB treatment on plants in regolith, the combination of strains B0+D0+F4 enabled germination of all seeds, compared to 78% germination in the untreated control. Based on mean values, treatment F4 had the most positive effect on chlorophyll content in regolith, while the combination D0+F4 showed the highest fresh weight. Although overall plant growth was influenced by substrate, treatment, and their interaction, no statistically significant differences were observed between the most effective treatments in regolith and the negative control.

This study demonstrated that watermelon can grow in a substrate simulating Martian regolith and that PGPB may enhance germination and improve certain growth parameters. Future research should incorporate additional stressors such as low temperatures and radiation to more accurately mimic Martian conditions.

Abstract EANA2025-43

Isolation and Experimental Evolution of Thermophilic Bacteria from Serbia's Extreme Environment Toward Higher Temperature Growth

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With the establishment of the Laboratory for Experimental Astrobiology (LEA) at the Institute of Physics in Belgrade just a year ago, Serbia marked its first official venture into the field of astrobiology. With that, the first experiments were initiated - an appropriate beginning being the exploration of microbiological life and its potential in extreme environments across Serbia. These habitats remain largely unexplored, with the most prominent being thermal and acidic springs, as well as saline soils and lakes.

Here, we present our initial exploration of Serbia's hottest known habitat - the hyperthermophilic spring of Vranjska Banja, where water temperatures reach up to 96 °C. We investigated its bacterial diversity and the thermal growth limits of the isolated microorganisms. Water was collected from the spring and transported in thermal bags to the laboratory, where it was analyzed within a 12-hour window. One liter of water was filtered using a vacuum filtration system. Filters were then placed on three different solid media (M9, Nutrient Agar (NA), and R2A), each containing 30 g/l of agar, and incubated at 60 °C for 48 hours.

Most bacterial growth was observed on NA medium, from which colonies were picked. The isolates were grown in pure culture and subjected to 16S rRNA gene sequencing. All 22 isolates were identified as members of the *Geobacillus* genus, though no species could be precisely determined. We further aimed to investigate whether there were differences in the optimal growth temperatures among the isolates. Twelve morphologically distinct *Geobacillus* strains were selected, and their growth was measured over five hours by monitoring optical density (OD600) at 60, 65, 70, and 75 °C. A growth constant was calculated for each isolate at each temperature. It was found that 60 °C was the optimal temperature for all isolates, with one strain, VB_14, showing the highest growth constant (k = 1.35) and weak growth at 65 °C. This prompted us to experimentally test whether VB_14 could be adapted to grow at a temperature at least 10 °C higher than its original optimum.

To pursue this, we set up an experimental evolution experiment. VB_14 was first grown overnight in Nutrient Broth (NB) at 60 °C with shaking (180 rpm). The resulting culture was divided into three evolutionary lines by inoculating fresh NB medium in a 1:100 ratio, incubated at 62 °C, also with shaking. Transfers into fresh medium were performed daily, and growth was monitored spectrophotometrically at OD600. Once the OD curve matched that of the optimal growth at 60 °C, the temperature was increased

again by 2 °C. So far, VB_14 has adapted to grow optimally at 66 °C, evolving over the course of approximately 1000 generations.

This research marks the beginning of the exploration of thermophilic bacteria from Serbia's hot springs, with the broader astrobiological goal of testing their thermal growth limits. The adaptation of *Geobacillus* spp. to increasing temperatures may serve as a model for exploring the potential for life in high-temperature environments on other planetary bodies, such as Venus clouds.

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Selection and Experimental Evolution of a Bacterial Isolate under Acid Stress

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Acidophilic microorganisms serve as valuable models in astrobiology due to their ability to thrive under extreme pH conditions analogous to those found on planetary bodies such as Venus. This study aimed to select and experimentally evolve bacterial isolates capable of tolerating and adapting to increasingly acidic environments, with the long-term goal of obtaining extremophiles suitable for application in astrobiology.

Initially, from the pool of 24 environmental bacterial isolates, we identified growth capabilities across a pH range through OD600 measurements after 24 hours. Ten isolates that demonstrated robust growth at approximately pH 5.0 were subjected to dynamic optical density monitoring (every 30 minutes over 5 hours) in 96-well microtiter plates at four different pH conditions (from 7.0 to 5.0), following 1:50 dilutions. Based on the calculated growth rate constants (k), one superior isolate exhibiting a high growth rate constant at pH 5.0 was selected for a long-term evolution experiment.

This isolate underwent serial daily passages (1:100 dilution) in liquid medium at pH 5.0 over one week, followed by high-resolution growth monitoring, which served as a baseline for guiding subsequent adaptation experiments. Isolates were then transferred to pH 4.8, with only those showing improved growth compared to the baseline were retained. These bacterial isolates were cryopreserved at –80 °C for further experimentation.

Further adaptation was performed at a pH of 4.6, with 8 out of 10 isolates successfully growing by the fourth week.

These eight acid-adapted strains will be sequentially transferred into media with pH decreased by 0.2 units each cycle, aiming to select isolates capable of growth at pH below 2.0. Such acidophilic strains may serve as proxies for microbial life in acidic extraterrestrial environments, contributing to the search for biosignatures and habitability assessments on planetary bodies like Venus.