



Section 1. Biology

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IDENTIFICATION OF NEW BACILLUS VELEZENSIS ENDOPHYTIC STRAINS – PROBIOTIC AGENTS FOR SOYBEAN SYMBIOTIC SYSTEMS DEVELOPMENT

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Abstract

The aim of the study was to identify soybean endophytic bacteria and determine the prospects for their use in soybean cultivation. It was found that the isolated strains belong to Gram-positive spore-forming bacteria that ferment a wide range of carbohydrates. Based on whole-genome sequencing and phylogenetic analysis, the endophytes were identified as *Bacillus velezensis* IMV B-8134 and IMV B-8135. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JBPUKF000000000 and JBRIKE000000000, BioProject PRJNA1294768. The application of the isolated endophytic strains together with nodule bacteria in soybean cultivation under biotic and abiotic stresses showed a positive effect on adaptation and productivity of plants. This provides grounds for further research on strains as promising for the creation of biological agents in soybean cultivation.

Keywords: *Bacillus velezensis*, *endophytes*, *Glycine max (L.) Merr.*, *whole-genome sequencing*, *stress-protective potential*

Introduction

Endophytic bacteria are described as those isolated from surface-sterilized plant tissues and inhabiting the intercellular space in various plant organs without harming the phytobiont (Santoyo et al., 2016). They promote the growth of the host plant and help it tolerate stressful conditions, having unique metabolic abilities: synthesis of phytohormones and siderophores, mobilization

of phosphates, nitrogen fixation, biocontrol of phytopathogens, protection against abiotic stresses (Burrage & Jeon, 2021). Due to these properties, endophytes have great probiotic potential for use in the cultivation of various agricultural crops (Afzal et al., 2019). The wide range of metabolic activity of endophytes determines the prospects for their use in plant growing, biotechnology, medicine, and remediation measures. Despite the fact

that the metabolic potential of endophytic bacteria has been studied sufficiently, there is limited data on their practical application as commercial biological products in agricultural practice. Therefore, the search for new effective strains of endophytic bacteria is currently relevant.

The aim of the study was to identify soybean endophytic bacteria and determine the prospects for their use in soybean cultivation.

Methods

The strains *Bacillus* sp. 4 and *Bacillus* sp. 5 were isolated from surface-sterilized nodules of cultivated soybean (*Glycine max* (L.) Merr.), grown in the Kyiv region (Ukraine). The morphology of the isolates was studied by light microscopy of Gram-stained 1-day-old cultures grown on agarised mannitol-yeast nutrient medium. The physiological and biochemical properties were studied using the Vitek 2 BioMérieux automated system. The ability of strains to utilize citrate and carbohydrates was tested according to Gordon et al. (1973), and gelatin hydrolysis was assessed according to Khatarkar et al., 2017).

DNA extraction was performed using the DNeasy® UltraClean® Microbial Kit. The quality of the extracts was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Whole-genome sequencing was performed on the Illumina NovaSeq platform (2×150 bp, GATC Biotech, Konstanz, Germany), yield-

ing approximately 6.2 million paired reads. Genome assembly was performed using BacFlux v1.1.8, including quality control, completeness and contamination assessment, and taxonomic verification. Primary assembly was performed using SPAdes v4.0.0; the average genome coverage was 420.53 ± 133.01 .

The similarity of the genomes of the studied isolates to the type strain *B. velezensis* FZB42 was checked using the Orthologous Average Nucleotide Identity using USEARCH (OrthoANIu) service, available at <http://www.ezbiocloud.net/tools/ani> (Yoon et al., 2017).

Results

In terms of morphological properties, both strains *Bacillus* sp. 4 and *Bacillus* sp. 5 are Gram-positive bacilli measuring $2,0 \times 0,7$ and $2,3 \times 0,5 \mu\text{m}$ respectively, located singly or in chains, with central endospores that do not change the shape of the cell. On meat-peptone and potato agar media, they form rounded beige matte colonies with a diameter of 5–7 mm and a paste-like consistency. When grown on meat-peptone broth, the strains form a film.

It was found that *Bacillus* sp. 4 and *Bacillus* sp. 5 are capable of liquefying gelatin, fermenting lactose, D-mannitol, L-arabinose, D-glucose, D-ribose, as well as hydrolyzing aesculin and growing at 6.5% NaCl, while they are not capable of fermenting D-tagatose and inulin (Table 1).

Table 1. Physiological and biochemical properties of new isolates *Bacillus* sp. 4 and *Bacillus* sp. 5 and type strain *B. velezensis* sp. nov. CR-502T

Reaction	<i>Bacillus</i> sp. 4	<i>Bacillus</i> sp. 5	<i>B. velezensis</i> sp. nov. CR-502T*
Citrate utilization	+	+	–
Gelatin hydrolysis	+	+	+
Lactose utilization	+	+	+
D-mannitol utilization	+	+	+
L-arabinose utilization	+	+	+
D-glucose utilization	+	+	+
Glycogen utilization	+	–	+
D-mannose utilization	–	+	+
D- melezitose utilization	–	+	–
N-acetyl-D-glucosamine utilization	+	–	–

Reaction	<i>Bacillus</i> sp. 4	<i>Bacillus</i> sp. 5	<i>B. velezensis</i> sp. nov. CR-502 ^T *
D-tagatose utilization	–	–	–
Inulin utilization	–	–	–
D-ribose utilization	+	+	+
Aesculin utilization	+	+	+
Growing in 6,5% NaCl concentrations	+	+	+

Note: “+” – test positive, “–” – test negative, * – according to data (Ruiz-Garcia et al., 2005).

All the physiological and biochemical properties of *Bacillus* sp. 4 and *Bacillus* sp. 5 strains corresponded to those of spore-forming bacteria of the *Bacillus subtilis* group. In most tests, the results coincided with the properties of the typical strain *B. velezensis* sp. nov. CR-502^T (Ruiz-Garcia et al., 2005).

To determine the species boundaries of the studied bacteria, the results of molecular

genetic studies were used, in particular, the average nucleotide identity (ANI) index (Table 2). ANI calculation involves fragmenting genome sequences, searching for nucleotide sequences, aligning and determining the identity of nucleotide sequences of the genomes of two prokaryotic strains during the classification and identification of bacteria.

Table 2. Identification of nucleotide genome sequences of new isolates *Bacillus* sp. 4 and *Bacillus* sp. 5 and strain *B.velezensis* FZB42

Compared genomes	<i>B.velezensis</i> FZB42 / <i>Bacillus</i> sp. 4	<i>B.velezensis</i> FZB42 / <i>Bacillus</i> sp. 5
OrthoANIu value (%)	98.86	98.87
Genome length (bp) of <i>B.velezensis</i> FZB42	3 917 820	
Genome length (bp) of endophytic strain	3 967 800	4 038 180
Average aligned length (bp)	2 646 004	2 777 125
Genome coverage (%) of <i>B.velezensis</i> FZB42	67.54	70.88
Genome coverage (%) of endophytic strain	66.69	68.77

Comparative genomic analysis using OrthoANIu showed a high level of similarity between both endophytic isolates and the strain *B.velezensis* FZB42. OrthoANIu values were 98.86% for *Bacillus* sp. 4 and 98.87% for *Bacillus* sp. 5 with 66.69 and 68.77% reciprocal coverage, indicating that the studied strains and *B. velezensis* FZB42 belong to the same species.

Thus, according to whole-genome sequencing and phylogenetic analysis, the isolated endophytes were identified as *Bacillus velezensis* IMV B-8134 and IMV B-8135 and deposited in the D. K. Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine Depository.

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under

the accession JBPURK000000000; BioSample SAMN50125348 and JBRIKE000000000; BioSample SAMN52024099 respectively; BioProject PRJNA1294768. The versions described in this paper are versions JBPURK000000000 and JBRIKE000000000.

The results of *in vitro* and *in vivo* experiments showed that the studied endophytic strains exhibited a wide range of antagonistic activity against bacterial and fungal pathogens of soybean diseases (Shevchuk et al., 2025 b). As shown by studies on soybean cultivation under drought conditions in the Kyiv region (Ukraine), the use of complex seed inoculation with nodule bacteria together with the studied endophytic bacteria contributed to an increase in adaptive potential, better

formation of a symbiotic system and an increase in yield of soybean variety Sculptor by 36.2% (Shevchuk et al., 2025a).

Discussion

Representatives of the *Bacillus velezensis* species have agricultural, biotechnological, and ecological applications due to their probiotic effects: stimulating plant growth, improving nutrition, suppressing pathogens, etc. Correct identification of *B. velezensis* strains is important due to their potential applications. More often, *B. velezensis* strains are isolated from the rhizosphere soil of various plants, including soybeans. In contrast to these data, our research concerns strains isolated directly from surface-sterilized soybean nodules. Data on the use of *B. velezensis* in soybean cultivation are very limited. In particular, it has been reported that *B. velezensis* S141, isolated from a soybean rhizosphere in Thailand, enhanced soybean growth by stimulating nodule formation with the *Bradyrhizobium diazoefficiens* USDA110 strain (Kondo et al., 2023). The co-inoculation showed the highest efficiency in nitrogen fixation, number of nodules, dry root weight, and total dry plant weight (Sibponkrung et al., 2020). It has been established that *B. velezensis* enhances the symbiosis of *B. japonicum* with soybean by secreting flavonoids, in particular rutin, which modulates the expression of nodule genes (Cheng et al., 2025).

Comparing these data with ours, it should be noted that our studies were conducted in a different climatic zone. We also used other strains of nodule bacteria, *B. japonicum*. However, we obtained similar results regarding the positive effect of co-inoculation on soybean plants. This indicates a general pattern of complementary synergistic action of nodule and endophytic bacteria. In addition, our data add to the knowledge on how co-inoculation of soybean seeds with endophytic *B. velezensis* together with nodule bacteria increases stresses tolerance and yield of plants under adverse conditions.

Conclusion

Non-rhizobial endophytic bacterial strains were isolated from soybean nodules. Based on morphological, cultural, physiological, and biochemical characteristics, as well as whole-genome sequencing and phylogenetic analysis, the isolates were identified as *B. velezensis* IMV B-8134 and *B. velezensis* IMV B-8135. Their positive effect on the symbiosis of soybean and rhizobia was revealed, which is manifested in the ability to maintain the physiological homeostasis of the phytobiont under adverse conditions and increase plant productivity. New *Bacillus velezensis* endophytic strains are prospective probiotic agents for the development of stress-protective biotechnologies.

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