Genetic potential of *Bacillus ginsengihumi* as a basis for a combined biofertilizer

Daria Itkina*, *Daria* Pudova, *Margarita* Drozdova, and *Margarita* Sharipova
Kazan (Volga region) Federal University, Kazan, Russia, 420008

Abstract. *Bacillus* species and their metabolites have potential alternative applications as biofertilizers that can limit the growth of potential phytopathogens and enhance crop yields. The aim of this study was to investigate the potential of *Bacillus ginsengihumi* M2.11 for plant growth promotion and control of micromycete phytopathogens. Identification of the genes of *B. ginsengihumi* M2.11 strain responsible for plant growth promotion and biofortification is an urgent research. The study evaluated the plant growth promotion potential of the strain by characterizing its traits. Genes responsible for the production of hydrolytic enzymes, indoleacetic acid, siderophores, motility, biofilm formation and phosphate solubilization are described.

1 Introduction

To maintain crop yields, chemicals are used against diseases, which unintentionally leads to environmental pollution and health hazards [1]. In this context, cultivation of resistant varieties and use of biological control agents will minimize the use of synthetic chemicals. This approach will protect the environment in addition to maintaining the ecological balance. The rhizosphere contains beneficial microorganisms that have the potential to be used as biopesticides in controlling plant diseases and inducing systemic resistance in the host. These rhizosphere bacteria such as *Bacillus* have been found to contain bioactive molecules with growth promoting and antagonistic effects against phytopathogens and trigger defense mechanisms in plants. Higher growth rate and tolerance to adverse environmental conditions due to endospore formation have made *Bacillus* a popular biological control agent [2]. In a recent study, we established the efficiency of the *B. ginsengihumi* M2.11 strain as a potential biocontrol agent and plant growth stimulator [3]. The strain was found to be capable of releasing ammonium and cyanide ions, cellulase and protease activity, nitrogen fixation, and mobilizing soil phosphates and phytates. The strain was found to be capable of synthesizing siderophores, the maximum production of which was detected after 48 hours of cultivation and amounted to 198 ± 8 μM in the *B. ginsengihumi* M2.11 strain. The bacteria produced the phytohormone indoleacetic acid (IAA), the yield of which after 24 hours of growth was 34 ± 3 μg/ml in *B. ginsengihumi* M2.11. It was found that the strain had fungicidal activity against phytopathogens of the genus *Fusarium*: the growth of representatives of different *Fusarium* species was

* Corresponding author: laia9301@mail.ru

suppressed by more than 90% in the presence of *B. ginsengihumi* M2.11 bacteria. It was concluded that, having multiple characteristics useful for plants, the *B. ginsengihumi* M2.11 strain can be used as an object for creating biofertilizer technologies and plant growth stimulants. The aim of this study was a comprehensive bioinformatics study of the strain with confirmation of the obtained characteristics.

2 Materials and methods

The object of the study is the bacterial strain *B. ginsengihumi* M2.11, isolated from the soil of the Republic of Tatarstan based on the maximum phytase activity on the differential PSM medium and identified by molecular genetic methods [4].

The genome of the soil isolate *B. ginsengihumi* M2.11 was sequenced and annotated. Genomic DNA was isolated using a set of reagents and the Thermo Fisher Scientific protocol. Genome sequencing was performed using the Illumina MiSeq platform; reads obtained using a 454 GS Junior sequencer were also used for assembly. The quality of raw reads was checked using FastQC version 0.11.8, filtered and trimmed using Trimmomatic version 0.39. Genome assembly was performed using the SPAdes version 3.15.5 program. Genome annotation was performed using Prokka version 1.14.6. To search for genes responsible for plant growth stimulation and bioenrichment, antiSMASH version 7.0 (https://antismash.secondarymetabolites.org/) and the BlastKOALA automatic annotation server, which is used to assign KEGG orthologs (https://www.kegg.jp/blastkoala/) were used. Gene sequence alignment was performed using BLASTn NCBI.

Motility and biofilm formation of soil plant growth-promoting rhizobacteria (PGPR) play a key role in their interaction with the root system of plants. To characterize the studied *B. ginsengihumi* M2.11 strain in more detail, its motility and biofilm formation were determined. Using the hanging drop and crushed drop methods with an immersion system with 0.1% methylene blue dye, the motility of bacteria was visualized [5]. The motility of microorganisms was determined on Peshkov's differential diagnostic semi-liquid nutrient medium [6]. Semi-liquid agar 0.3 and 0.6% was used, respectively, 5 µl of the overnight culture of *B. ginsengihumi* M2.11 (OD590 = 1) were added to the center of the dish. Then the radius of the colonies was analyzed.

The ability of the *B. ginsengihumi* M 2.11 strain to form biofilms was determined. For this, a method was used based on the ability of crystal violet to bind to cells and the matrix of biofilms. Flat-bottomed 24-well plates were used to cultivate biofilms. To obtain biofilms, 1% of the inoculum of the overnight culture of *B. ginsengihumi* M2.11 (OD590 = 1) was added to the LB nutrient medium in a volume of 3 ml. The sterile LB nutrient medium without addition was used as a control. The bacteria were cultivated at a temperature of 37 °C for a week. Then, the obtained samples were examined for the presence of biofilms.

After cultivation, the remaining planktonic cells were removed from the wells by washing with a sterile buffer Phosphate buffered saline (PBS g / l: NaCl - 8, KCl - 0.2, Na2HPO4 - 1.42, KH2PO4 - 0.24) 3 ml for 3 minutes. After the time has elapsed, the buffer was completely removed. 3 ml of filtered 0.1% gentian violet solution were added to the wells of the plates. Biofilms were incubated with the dye for 15 minutes at room temperature. After removing the dye, the plate was washed with water to remove unbound dye and dried at room temperature. 3 ml of 95% ethanol was added to each well and kept at room temperature for 15 min, during which the gentian violet bound to the biofilm structure dissociates into ethanol. The well contents were carefully mixed, 200 µl of the mixture were collected and transferred to the wells of a flat-bottomed 96-well plate for measurement on a SmartSpecPlus spectrophotometer (BioRad, USA) at a wavelength of 590 nm.

Biofilm detection was also tested using a method based on the binding of Congo Red dye to the main amyloid matrix protein [7]. For this purpose, 5 µl of the overnight culture of *B. ginsengihumi* M 2.11 were plated on solid medium E (Yeast Extract 0.5 g/l; Casaminic acids 10 g/l; Agar 20 g/l) containing Congo Red dye 25 µg/ml. Cultivated at 37°C for 2 days, then the formed stained colonies were scraped off and resuspended in sterile physiological solution 0.9% NaCl. Bacteria were sedimented by centrifugation for 10 min at 14,000 rpm. 1 ml of Congo Red solution at a concentration of 25 µg/m3 was added to the sedimented cells, incubated for 10, 30 and 60 min, respectively, then centrifuged and the density of the supernatants was measured at OD590. The intensity of binding of Congo Red dye by cells was assessed by the decrease in optical density relative to the initial 0.002% solution of Congo Red dye in 0.9% NaCl [8].

3 Results and discussion

Plant growth promoting bacteria (PGPB) are bacteria that when placed on or in the plant environment can act as a biofertilizer or biocontrol agent. This is an important association as it can lead to a reduction in the use of fertilizers and pesticides in agriculture while maintaining crop yields and safety [9, 10].

Bacillus strains are considered to be the best green alternative to chemical fungicides and fertilizers for crops. Many of them, isolated from the rhizosphere of plants, are reported to exhibit high antifungal activity against phytopathogens and promote plant growth [11].

Of interest is the study of the genome sequence of *B. ginsengihumi* strain M2.11 and the genes responsible for its potential ability to act as a PGPR, in particular as a biocontrol agent. As a result of this study, the genome was assembled into 42 scaffolds (>= 500 bp in length), with the total genome length being 3,752,461 bp with a GC content of 36.08%. The genome annotation was performed using Prokka version 1.14.6. A total of 3,560 CDS, 14 rRNA, and 89 tRNA were identified. Using the antiSMASH 7.0 server, 3 potential gene clusters encoding secondary metabolites with antimicrobial properties were identified in the strain genome. The bacillibactin biosynthesis gene cluster consisting of 9 genes was annotated (Figure 1A). The cluster is located in contig 5 of the genome at coordinates 234,870 – 286,076 bp. In addition, the analysis showed the presence of two gene clusters responsible for betalactone biosynthesis. One of these clusters has 40% similarity with the cluster of lipopeptide biosynthesis – fengycin (Figure 1B).

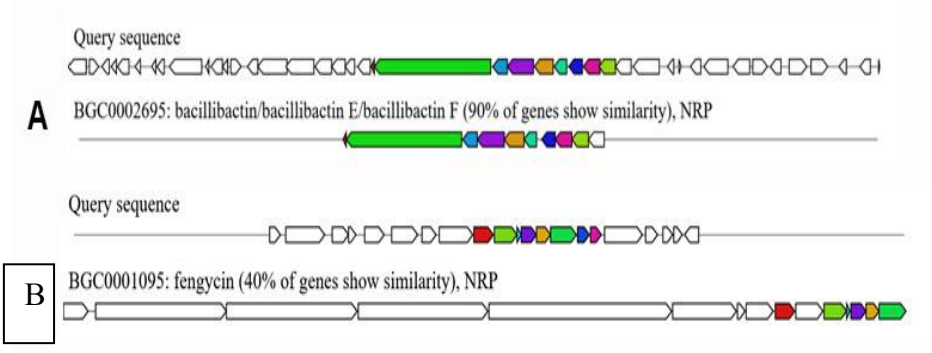


Fig. 1. Clusters of secondary metabolite genes identified in the *Bacillus ginsengihumi* M2.11 genome using antiSMASH 7.0.

To identify specific pathways for the development of plant growth-promoting traits, the functions of the annotated genes were analyzed and compared with the corresponding

pathways in the KEGG pathway database (https://www.genome.jp/kegg/pathway.html). A total of 57.5% of the genes were classified into functional categories (Figure 2).

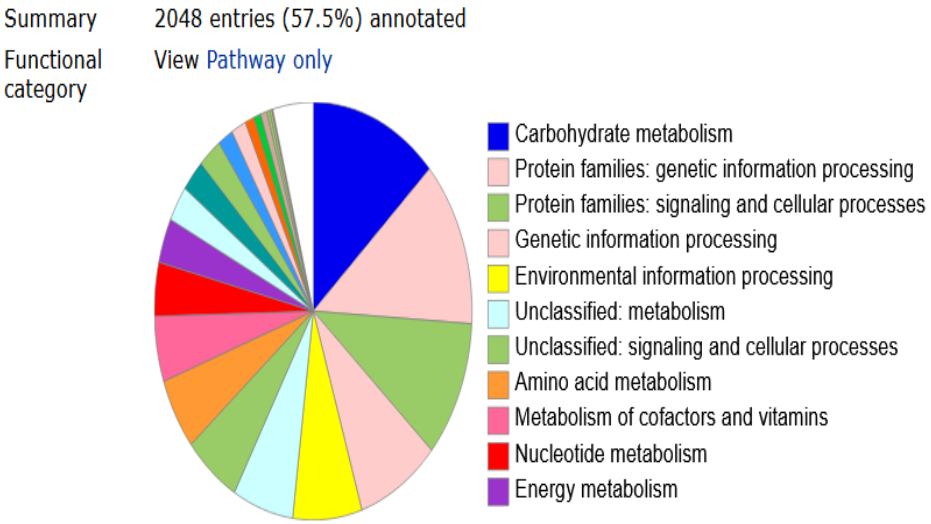


Fig. 2. The result of genome annotation using the BlastKOALA automatic annotation server.

Nitrogen metabolism.

Ammonia production, an important feature of PGPR bacteria, indirectly promotes plant growth and biomass accumulation. Our genomic study showed that B. ginsengihumi M2.11 includes nar genes that convert atmospheric nitrogen to nitrite, as well as nir genes that convert nitrite to nitrate, and nrt genes are responsible for nitrate transport (Figure 3).

Identified genes:

gudB - glutamate dehydrogenase [EC:1.4.1.2]
gltB - glutamate synthase (NADPH) large chain [EC:1.4.1.13]
gltD - glutamate synthase (NADPH) small chain [EC:1.4.1.13]
narG, narZ, nxrA - nitrate reductase / nitrite oxidoreductase, alpha subunit [EC:1.7.5.1 1.7.99.-]
narH, narY, nxrB - nitrate reductase / nitrite oxidoreductase, beta subunit [EC:1.7.5.1 1.7.99.-]
narI, narV - nitrate reductase gamma subunit [EC:1.7.5.1 1.7.99.-]
ncd2, npd - nitronate monooxygenase [EC:1.13.12.16]
formamidase [EC:3.5.1.49]
nitrilase [EC:3.5.5.1]
glnA - glutamine synthetase [EC:6.3.1.2]
NRT2, narK, nrtP, nasA - MFS transporter, NNP family, nitrate/nitrite transporter.

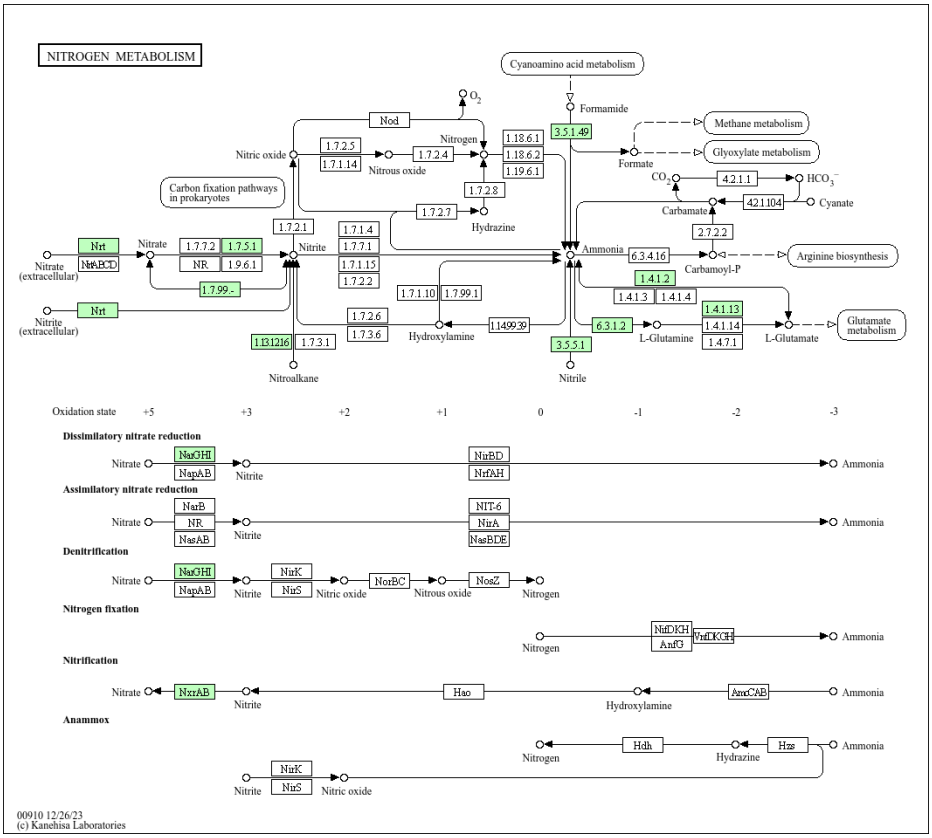


Fig. 3. Nitrogen metabolism. Identified genes.
*The genes that were found in the genome are highlighted in green in the diagram.

Biosynthesis of nonribosomal peptides of the siderophore group. Siderophores

It has been shown that high-affinity iron chelates are produced mainly by plant-associated bacterial strains and can assist them by collecting iron from the environment [12]. *B. ginsengihumi* M2.11 has the ability to synthesize the enterobactin siderophore, and its ent ABCEF genes have been annotated. This siderophore is responsible for iron recovery by complex formation after its export from the cell. *B. ginsengihumi* M2.11 also has multiple receptors for siderophores, an efflux pump protein that helps in transport and binding (Figure 4).

Identified genes:

entA - 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase [EC:1.3.1.28]
entB - bifunctional isochorismate lyase / aryl carrier protein [EC:3.3.2.1 6.3.2.14]
entC - isochorismate synthase [EC:5.4.4.2]
entE - 2,3-dihydroxybenzoate---[aryl-carrier protein] ligase [EC:6.3.2.14 6.2.1.71]
menF - menaquinone-specific isochorismate synthase [EC:5.4.4.2]
dhbF - glyine---[glycyl-carrier protein] ligase [EC:6.2.1.66]

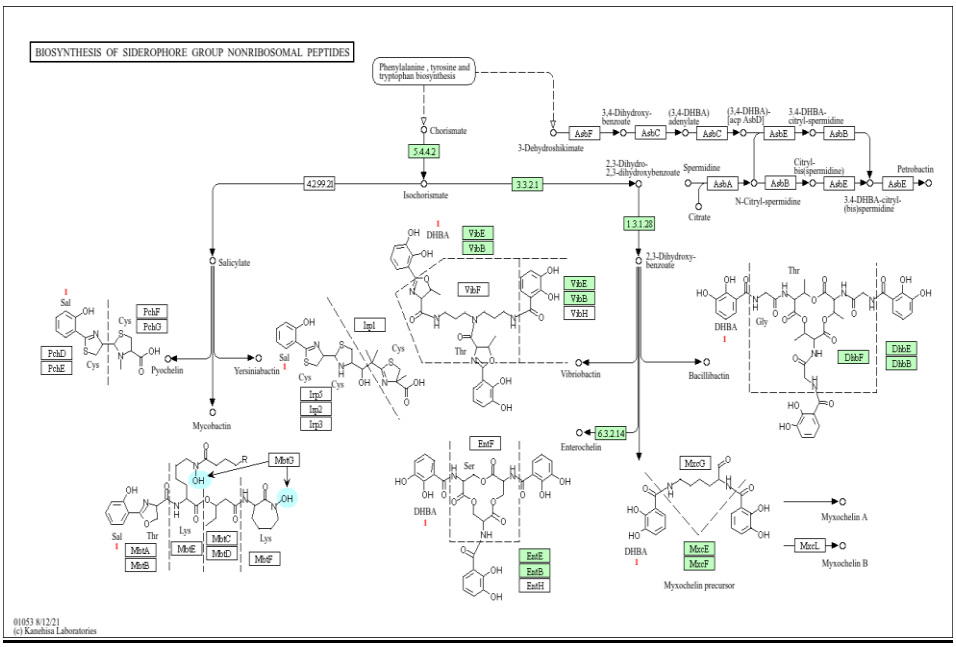


Fig. 4. Siderophore biosynthesis. Identified genes.
*****on the diagram, genes that were found in the genome are marked in green.

Tryptophan metabolism. Indole-3-acetic acid

The studied strain produces indole-3-acetic acid (IAA), which is necessary for plant development processes and plays an important role in plant-microbe interactions.

Identified genes:

ALDH - aldehyde dehydrogenase (NAD+) [EC:1.2.1.3]
DLD - dihydrolipoyl dehydrogenase [EC:1.8.1.4]
ACAT - acetyl-CoA C-acetyltransferase [EC:2.3.1.9]
sucB - 2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase) [EC:2.3.1.61]
amiE - amidase [EC:3.5.1.4]
nitrilase [EC:3.5.5.1]
katE - catalase [EC:1.11.1.6]
dmpC - aminomuconate-semialdehyde/2-hydroxymuconate-6-semialdehyde dehydrogenase [EC:1.2.1.32 1.2.1.85]
FAD reductase [NAD(P)H] [EC:1.5.1.45]

Inositol phosphate metabolism. Phosphate and organic acids.

It is known that after application as a fertilizer, a significant portion of inorganic phosphates is immobilized, making phosphate unavailable to plants [13]. As a result, it is extremely important for some bacterial species to produce acid phosphatases and organic acids, especially gluconic acid (GA), and to solubilize insoluble or poorly soluble mineral phosphates (Figure 5).

Identified genes:

iolG - myo-inositol 2-dehydrogenase / D-chiro-inositol 1-dehydrogenase [EC:1.1.1.18 1.1.1.369]
iolA - malonate-semialdehyde dehydrogenase (acetylating) / methylmalonate-semialdehyde dehydrogenase [EC:1.2.1.18 1.2.1.27]

suhB - myo-inositol-1(or 4)-monophosphatase [EC:3.1.3.25]
tpiA - triosephosphate isomerase (TIM) [EC:5.3.1.1]
iolE - inosose dehydratase [EC:4.2.1.44]
iolD - 3D-(3,5/4)-trihydroxycyclohexane-1,2-dione acylhydrolase (decyclizing) [EC:3.7.1.22]
iolB - 5-deoxy-glucuronate isomerase [EC:5.3.1.30]
iolC - 5-dehydro-2-deoxygluconokinase [EC:2.7.1.92]
iolJ - 6-phospho-5-dehydro-2-deoxy-D-gluconate aldolase [EC:4.1.2.29]
iolI - 2-keto-myo-inositol isomerase [EC:5.3.99.11]
iolW - scyllo-inositol 2-dehydrogenase (NADP+) [EC:1.1.1.371]

The issue of synthesis of biologically active substances by bacteria of the genus *Bacillus* is relevant. During the annotation of the genome, genes responsible for the biosynthesis of antibiotics of the vancomycin group were identified:
rfbB, rmlB, rffG – dTDP-glucose 4,6-dehydratase [EC:4.2.1.46]
Гены, ответственные за биосинтез антибиотиков группы стрептомицины:
iolG - myo-inositol 2-dehydrogenase / D-chiro-inositol 1-dehydrogenase [EC:1.1.1.18 1.1.1.369]
rfbD - dTDP-4-dehydrorhamnose reductase [EC:1.1.1.133]
rfbA - glucose-1-phosphate thymidylyltransferase [EC:2.7.7.24]
suhB - myo-inositol-1(or 4)-monophosphatase [EC:3.1.3.25]
rfbB - dTDP-glucose 4,6-dehydratase [EC:4.2.1.46]
rfbC - dTDP-4-dehydrorhamnose 3,5-epimerase [EC:5.1.3.13]
pgm - phosphoglucomutase [EC:5.4.2.2]
glk - glucokinase [EC:2.7.1.2]
Биосинтез различных вторичных метаболитов:
aroE - shikimate dehydrogenase [EC:1.1.1.25]
metK - S-adenosylmethionine synthetase [EC:2.5.1.6]

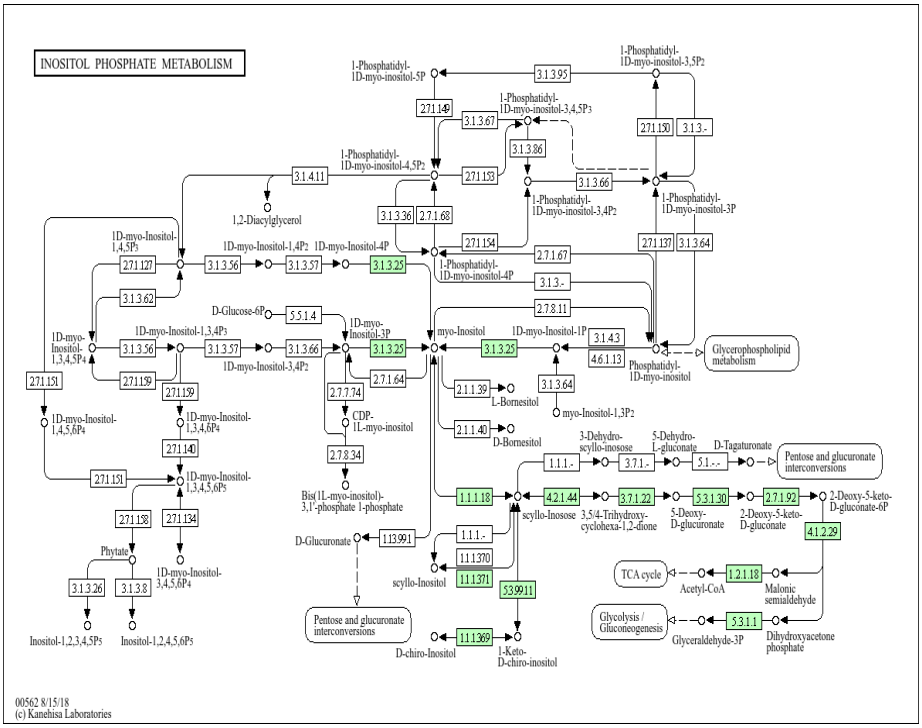


Fig. 5. Inositol phosphate metabolism.
*****The genes in the diagram that were found in the genome are highlighted in green.

Quorum sensing. Biofilm formation.

This is a process in which microbial cells come together to form collectives embedded in a self-produced extracellular matrix. The genus *Bacillus* is a gram-positive bacterium that has been used to analyze the mechanisms that control matrix production and the subsequent transition from a motile planktonic state to a sessile biofilm [14]. The collective nature of life in biofilm allows for the expression of new properties, and bacterial biofilms have been linked to new industrial applications, as well as probiotic and plant biocontrol processes (Figure 6).

Identified genes: LuxS, Hfq, phnA,blcC, rpfB, Clp, toxE, opp, dpp, lacD, ccfA, comK, Sec, DegU, Spo0F/0B/0A.

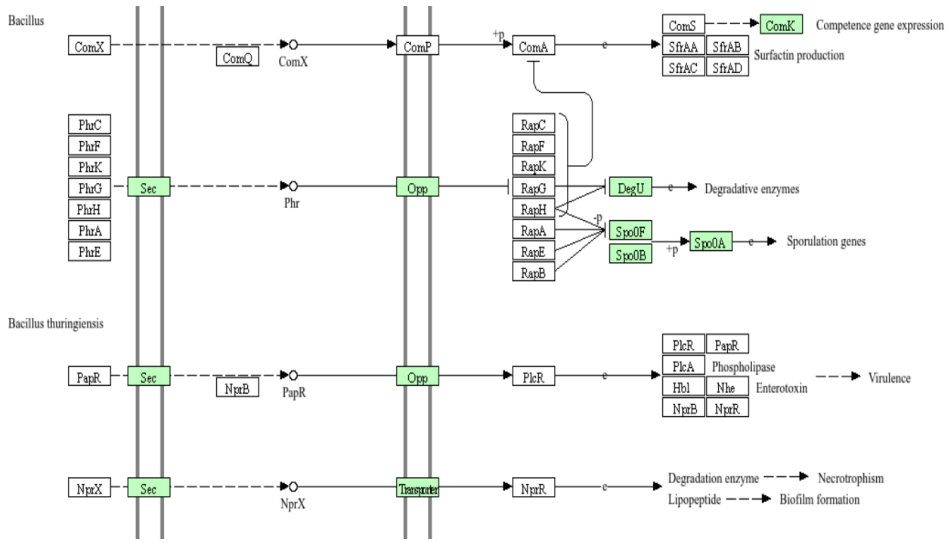
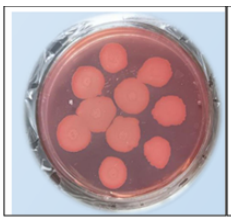
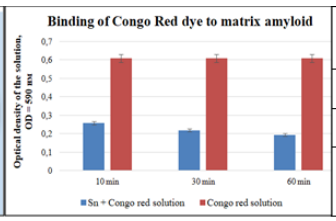


Fig. 6. Biofilm formation.
***** the genes found in the genome are marked in green in the diagram.

The ability of the *B. ginsengihumi* M2.11 strain to form biofilms was also studied. For this purpose, the method of determining the biofilm density through crystal violet staining was used. This method is based on the ability of crystal violet to bind to cells and the matrix of biofilms [15, 16]. This method allows one to obtain relative indicators of the density of the entire biofilm on the surface of the substrate. The results obtained were interpreted according to the optical density values of the stained solvent. The assessment of the efficiency of biofilm formation by gentian violet staining was assessed according to the criteria of biofilm production. The ability to produce a dense biofilm (OD = 2.438-2.937) was established (Figure 7).





Optical density	Biofilm production	Optical density of biofilm of strain *B. ginsengihumi*
ODc ≤ ~ ≤ 2*ODc	No/weak	
2*ODc < ~ ≤ 4*ODc	Moderate	
> 4*ODc	Dense	ODc*4 = 0,229±0,03*4 = 0,916 < 2,677±0,2

Fig. 7. Indirect identification of amyloid fibers with Congo Red dye and staining of biofilms with methylene blue.

We also established the ability to synthesize amyloid-like proteins in the biofilm of the *B. ginsengihumi* M2.11 strain. On medium E with Congo Red dye, it was found that on the 2nd day of cultivation, the colonies on the agar medium acquired a red color, the intensity of staining increased, and the production of amyloids by bacteria increased, which indicated the presence of amyloid proteins in the *B. ginsengihumi* M2.11 biofilm.

The binding of Congo Red dye by extracellular amyloids of biofilms of the *B. ginsengihumi* M2.11 strain was studied. It was recorded that with increasing incubation time, the density of the solution decreased, which may indicate the presence of amyloid protein capable of binding to the dye. Since the longer the incubation time, the greater the amount of Congo Red dye will bind to the amyloid protein.

Chemotaxis, motility and attachment of genes

The entire genome of *B. ginsengihumi* M2.11 contains genes such as the fli gene cluster, sec gene, che gene, mot gene: responsible for endophytic behavior, such as chemotactic movement and attachment to the host (Figure 8).

Identified genes:

cheA - two-component system, chemotaxis family, sensor kinase CheA [EC:2.7.13.3]
cheB - two-component system, chemotaxis family, protein-glutamate methylesterase/glutaminase
cheC - chemotaxis protein CheC
cheD chemotaxis protein CheD
cheW- purine-binding chemotaxis protein CheW
cheV- two-component system, chemotaxis family, chemotaxis protein CheV
cheR- chemotaxis protein methyltransferase CheR [EC:2.1.1.80]
mcp- methyl-accepting chemotaxis protein
fliG - flagellar motor switch protein FliG
fliM - flagellar motor switch protein FliM
fliN - flagellar motor switch protein FliN
motA- chemotaxis protein MotA
motB - chemotaxis protein MotB
rbsB- ribose transport system substrate-binding protein

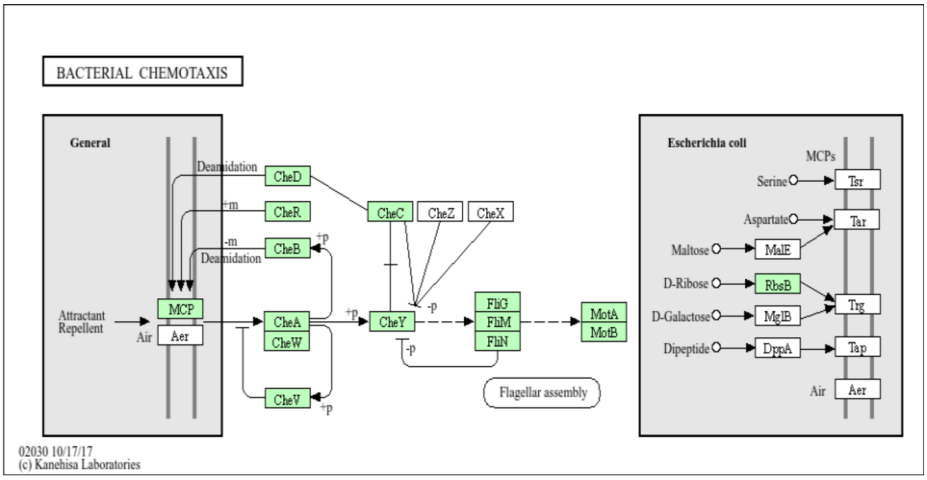


Fig. 8. Chemotaxis, motility and attachment of genes
*on the diagram, genes that were found in the genome are marked in green.

On a medium containing 0.6% agar, the colony radius increased by 2.5 cm in 48 hours, and on a medium with 0.3% agar, the culture covered the entire surface of the medium in 24 hours, which may indicate the ability of the studied strain to different types of movement (swimming and swarming). Optimization of their use in agriculture requires an in-depth study of environmental and genetic aspects. The introduction of PGPR-based biopreparations can become the basis for sustainable agriculture, minimizing the anthropogenic load on ecosystems (Figure 9).

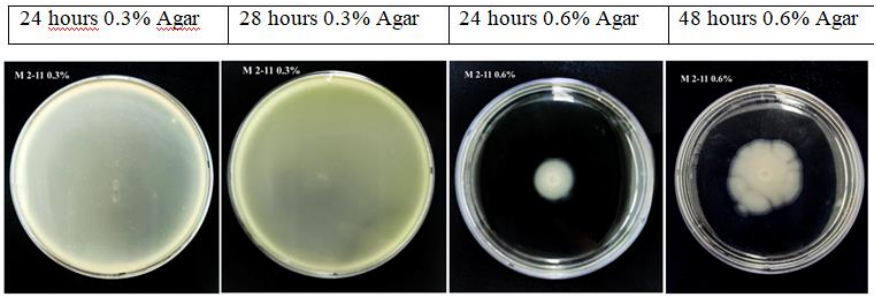


Fig. 9. Determination of the motility of the B. ginsengihumi strain M2.11 in semi-liquid agar (0.3% and 0.6%)

Based on the above facts, our study aimed to unravel the complexity of the *B. ginsengihumi* M2.11 genome to identify the genetic factors underlying its plant growth promotion and biocontrol properties. Combined with detailed bioinformatics analysis, the studies of this strain provide insights into its use as an effective biofertilizer.

Plant-associated endophytic bacteria have evolved unique biosynthetic pathways to help them interact with their plant host. These various endophytic metabolites promote plant growth; therefore, their genomic studies have attracted considerable interest recently [17].

The genomic analysis of *B. ginsengihumi* M2.11 revealed the presence of several genes involved in PGP such as phosphate solubilization and nitrate reduction, as well as in the production of siderophores and IAA. Moreover, it has genes related to chemotaxis, motility, adhesion, which are necessary for efficient colonization. It lacks the complete cluster of pathogenic genes. This study provides insights into the ability of *B. ginsengihumi* M2.11 to promote plant growth and highlights its potential as a biofertilizer. Thus, this strain is considered safe and can be further studied as a bioinoculant and used as an effective biofertilizer.

The widespread use of chemical fertilizers has led to negative consequences for both human health and natural ecosystems. Biofertilizer is a cost-effective and environmentally friendly way to increase plant growth and produce healthy food in a sustainable manner [18].

Acknowledgements

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