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Communication

Whole-Genome Assembly and Antimicrobial Properties of *Bacillus atrophaeus* R7PjV2-12 from Spruce *Picea jezoensis*

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Abstract

The search for antagonistic microorganisms as alternatives to chemical pesticides is an urgent priority in sustainable agriculture. Previously, we isolated several bacterial isolates from spruce plants, and one of them, identified as *Bacillus atrophaeus* R7PjV2-12, showed strong antagonistic properties against plant pathogens such as *Magnaporthe oryzae*, *Fusarium avenaceum*, and *Erwinia billingiae*. Given its strong fungicidal properties, we decided to sequence the complete genome of this bacterium to determine how it can inhibit fungal growth. The whole genome size of *B. atrophaeus* R7PjV2-12 was 4,127,644 bp with 4032 open reading frames. *B. atrophaeus* R7PjV2-12 genome possessed clusters of secondary metabolites with a complete set of genes with 100% similarity representing clusters of biosynthesis of bacillin, bacillibactin, subtilosin A, and fungicin, which indicates the studied strain's ability to synthesize these substances. Thus, this paper has shown and discussed the potential importance of *B. atrophaeus* R7PjV2-12 for biocontrol of pathogenic microorganisms in agriculture.

Keywords: antimicrobial activity; genomic analysis; endophytes; *Magnaporthe oryzae*; *Fusarium avenaceum*; *Erwinia billingiae*

1. Introduction

In modern agriculture, the search for environmentally friendly and effective methods of protecting plants from pathogenic microorganisms is of particular importance [1]. Despite their high efficiency, traditional chemical agents have several negative effects on the environment, human health, and the sustainability of agricultural systems [2]. In this regard, the development of biological methods for plant protection is becoming increasingly important [3], one of which is the use of endophytes, microorganisms that are distributed asymptomatically in plant tissues throughout their life cycle, participate in their growth and development, and play an important role in shaping plant health [4].

Endophytes of coniferous trees are a promising research subject, as coniferous forests cover vast terrestrial areas and play critical roles in global carbon sequestration, biodiversity maintenance, and timber production, making them ecologically and economically significant. It has previously been shown that endophytes of spruce *Picea rubens* and *Picea mariana* spruces growing in the Acadian forest in North America produce substances with

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fungicidal properties [5,6]. Some spruce endophytes can stimulate plant growth and inhibit the development of pathogenic microorganisms. Bacterial strains that stimulate plant growth have been isolated from Scots pine trees in western North America. These bacterial strains significantly increased the length and biomass of their natural host (Scots pine), as well as their non-native host (hybrid white spruce) in a 540-day greenhouse experiment [7].

The spruce *Picea jezoensis* (Siebold et Zucc.) Carrière, which grows in the Russian Far East, is an interesting host of endophytic microorganisms, as it is a fairly successful widespread plant species growing in harsh climatic conditions. Moreover, the bark of *P. jezoensis* is a rich source of valuable secondary metabolites such as stilbenes (*trans*-isorapontin, [8,9]). Stilbenes have an antimicrobial effect, inhibiting the growth of certain pathogenic fungi [10,11], and it is known that endophytes can often synthesize some of the secondary metabolites of the host plant [12], which also increases interest in studying them as alternative sources of biologically active substances.

Previously, endophytic bacterial communities in healthy needles, branches, and wood of *P. jezoensis* from Primorsky Krai were analyzed using metagenomic analysis [13]. The most abundant bacterial classes were Alphaproteobacteria, Gammaproteobacteria, and Actinobacteria. In addition, many endophytes were isolated using microbiological cultures, and their effects on plant pathogens' growth were analyzed.

In Prymorsky territory, there are many phytopathogens that parasitize agricultural crops. For example, pathogenic fungus *M. oryzae* causes rice diseases such as rice blast fungus, rice rotten neck, rice seedling blight, blast of rice, or pitting disease [14]. Species of fungus genus *Fusarium* are of significant importance as pathogens that cause fusarium disease in the form of fusarium wilt in a wide range of plants [15]. Also, phytopathogenic bacteria of the genus *Erwinia* [16,17], which cause bacterial blight or canker in many fruit plants, are also widespread in the Russian Far East. One of the isolated endophytes from *P. jezoensis*—*B. atrophaeus* R7PjV2-12—showed strong antagonistic activity against phytopathogens like *M. oryzae*, *F. avenaceum*, and *E. billingiae*. Therefore, the goal of this study was to investigate this R7PjV2-12 isolate in detail.

2. Results and Discussion

2.1. Antipathogenic Activity of Bacteria Strain R7PjV2-12

At the beginning of our work, we isolated 104 bacterial isolates and studied their antagonistic properties against pathogenic fungi *M. oryzae* and *F. avenaceum*, as well as the bacterium *E. billingiae*. Using the double culture method, we found that among 104 isolates, only the R7PjV2-12 strain exhibited strong antifungal activity against *M. oryzae* and *F. avenaceum* (Figure 1). The average antagonistic activity index of R7PjV2-12 was 66.1% for *M. oryzae* and 56.8% for *F. avenaceum* in three independent experiments. Also using the agar plate method, it was shown that the strain R7PjV2-12 has strong antibacterial activity against *E. billingiae* (Figure 1). Therefore, we can conclude that the R7PjV2-12 strain possesses quite rare and strong antagonistic properties against phytopathogens. However, this is not the highest level of inhibition, as another strain, *Bacillus velezensis* AMR25, was shown to have a stronger inhibitory effect on the growth of *F. avenaceum* [18]. The inhibition zone achieved with *B. velezensis* AMR25 was 62.9%, which is 6.1% higher than that with R7PjV2-12; however, this difference falls within the measurement error.

2.2. Phylogenetic Identification

Based on phylogenetic analysis using 49 universal genes based on clusters of orthologous genes (COG), isolate R7PjV2-12 shows the closest genetic relationship to *Bacillus atrophaeus* (GCF_000742675.1) (Figure 2). These data are confirmed by an average nucleotide identity (ANI) of 98.5% (Figure 2) and digital DNA-DNA hybridization (dDDH) of 89.1%

(Tables S1 and S2) in comparison with seven closely related species. Based on phylogenetic analysis, and since the ANI and dDDH values obtained exceed the established species separation thresholds (>95% and >70%, respectively [19]), isolate R7PjV2-12 was assigned to the species *B. atrophaeus*.

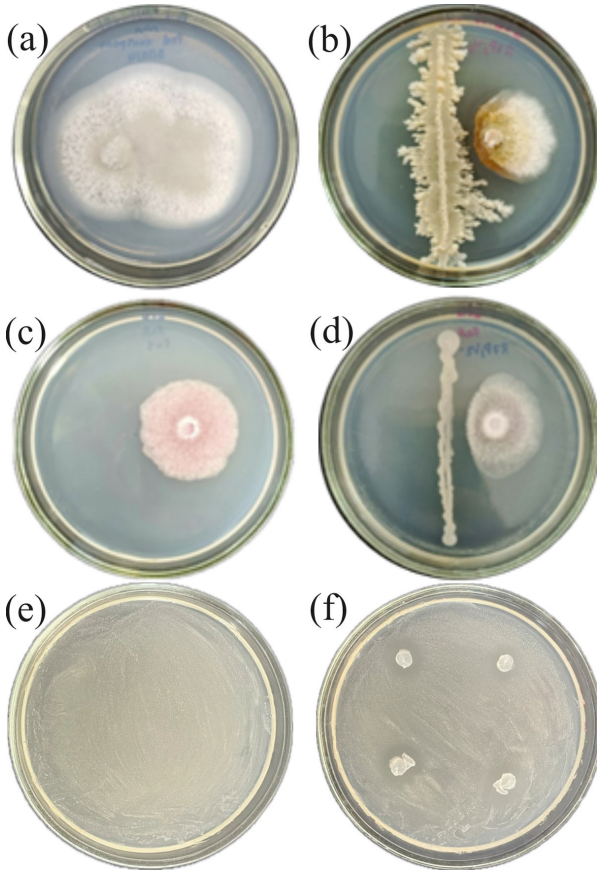

Figure 1. Antagonism of bacteria strain R7PjV2-12 against phytopathogenic fungi *Magnaporthe oryzae*, *Fusarium avenaceum*, and bacterium *Erwinia billingiae*. (**a**) Control (*M. oryzae* without R7PjV2-12); (**b**) cultivation of the *M. oryzae* with R7PjV2-12; (**c**) control (*F. avenaceum* without R7PjV2-12); (**d**) cultivation of the *F. avenaceum* with R7PjV2-12; (**e**) control (*E. billingia* without R7PjV2-12); (**f**) cultivation of the *E. billingia* with R7PjV2-12.

2.3. Genomic Features and Comparison of Genetic Characteristics

The genome of *Bacillus atrophaeus* R7PjV2-12 associated with *P. jezoensis* has the following characteristics: size, 4,127,644 bp; number of contigs, 51; GC content, 43.2%; and number of protein-coding sequences (CDSs) equal to 4032 (Table 1). For comparative genomics, we used the genomes of *B. atrophaeus*, which act as biological control agents, namely the endophytic strain SW isolated from wheat roots [20] and the rhizosphere strain HAB-5 [21]. Data on the characteristics of these genomes are presented in Table 1.

Comparative genomic analysis of three strains related to *B. amyloliquefaciens* using OrthoVenn revealed differences in the number of genetic clusters: *B. atrophaeus* R7PjV2-12 had 3867 clusters, SW had 3951, and Hab-5 had 3811 (Figure 3). The analysis revealed a common conservative core of 3356 orthologous clusters present in all three strains. At the same time, *B. atrophaeus* strains (R7PjV2-12 and SW) showed the greatest similarity to each other, having 3547 common clusters; notably, this strain was also isolated from the internal tissue of the plant. In addition, R7PjV2-12 has 262 unique gene clusters, absent in other isolates studied, distributed evenly throughout the genome as single genes and in

groups (Figure 3a,c). However, most of the 181 unique sequence data belong to hypothetical proteins (Figure 3b).

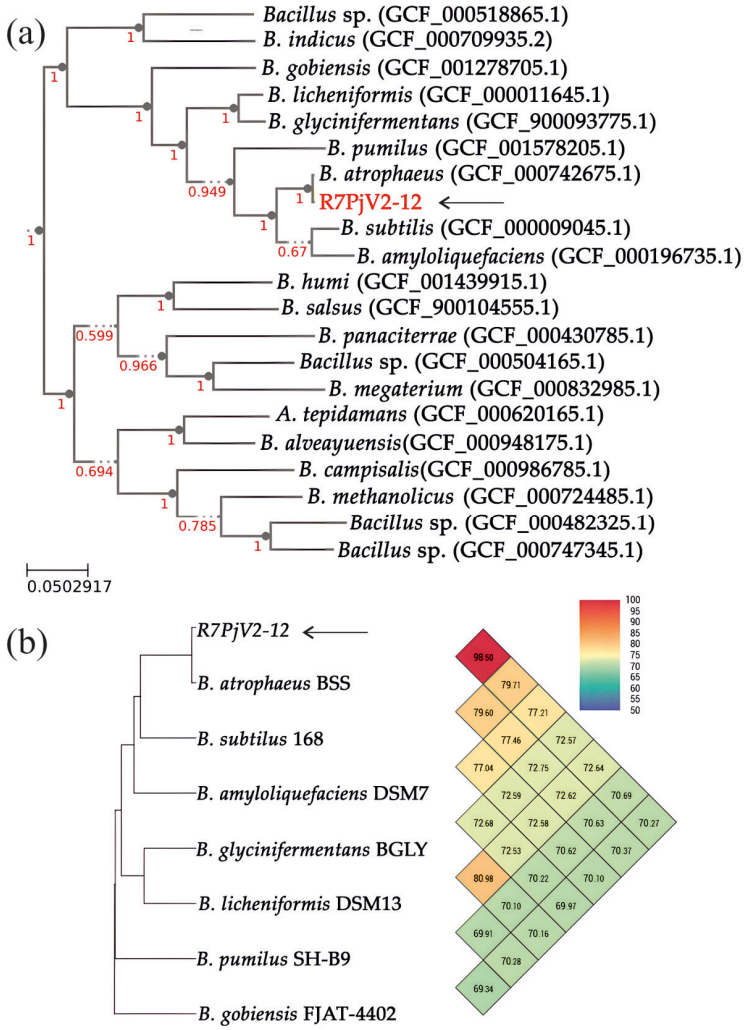

Figure 2. The phylogenetic analysis of *Bacillus atrophaeus* R7PjV2-12 (highlighted with an arrow and red font): (**a**) phylogenetic tree based on 49 genetic markers using the Species Tree-v2.2.0. NCBI RefSeq assembly numbers are listed next to the species name and duplicated in Table S4. (**b**) Orthologous average nucleotide identity analysis was determined using the Orthologous Average Nucleotide Identity Tool.

Table 1. Genomic features of the *Bacillus atrophaeus* R7PjV2-12 and related members of the *Bacillus* species.

	***B. atrophaeus* R7PjV2-12**	***B. atrophaeus* SW**	***B. amyloliquefaciens* Hab-5**
GeneBank	PRJNA1401447	GCF_039519175.1	GCF_045278795.1
Sequence size, bp	4,127,644	4,292,910	4,083,597
Number of contigs	51	2	1
GC content (%)	43.2	43.2	43.5
Shortest contig size	562	17,976	4,083,597
Median sequence size	38,496	4,274,934	4,083,597
Mean sequence size	80,980.8	2,146,455	4,083,597
Longest contig size	436,320	4,274,934	4,083,597
N50 value	211,794	4,274,934	4,083,597
L50 value	7	1	1
Protein-coding genes (CDSs)	4032	4021	3833

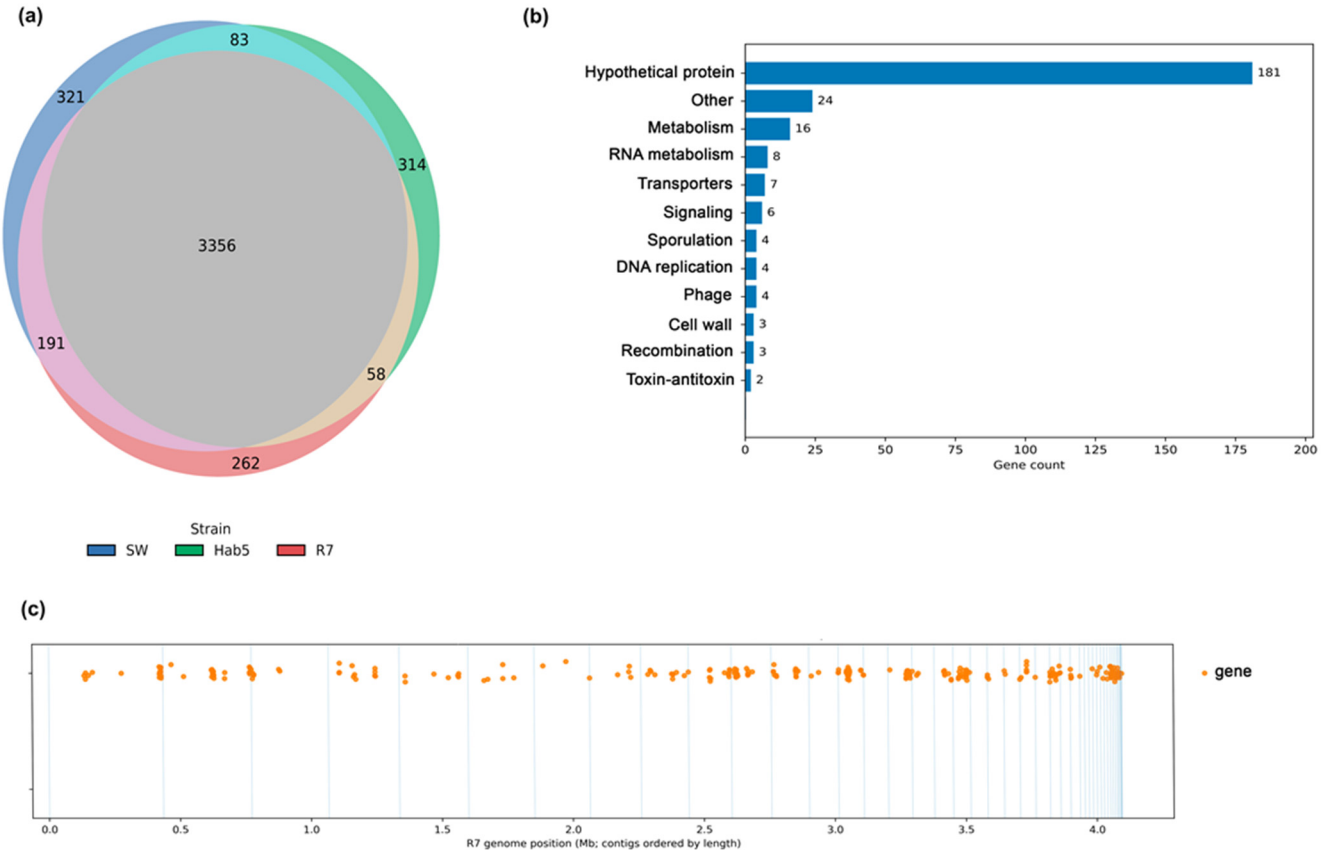

Figure 3. Comparative genomic analysis of three strains related to *Bacillus amyloliquefaciens*: (**a**) Venn diagram of orthologous clusters R7PjV2-12, SW, and Hab-5. Deep blue indicates unique clusters for the SW strain, green for Hab-5, red for R7. Pale blue common only for SW and Hab-5, yellow for Hab-5 and R7, pink for R7 and SW. Common conservative core of 3356 orthologous clusters for all three strains highlighted in gray; (**b**) graph of functional distribution of unique genetic sequences in R7PjV2-12; (**c**) distribution of unique sequence in the R7PjV2-12 genome.

The most interesting unique genetic clusters in the framework of this study were the genes related to the *sbo-ald* operon. These encode the genes responsible for the biosynthesis of subtilosin [22] and the genes of the *yeeF*/*yezG* toxin/antitoxin system involved in the formation of biofilms [23], which promotes the survival of bacteria in the rhizosphere [24] and facilitates plant colonization [25]. These provides R7PjV2-12 an advantage as a potential biological control agent relative to other strains analyzed (Table S2).

2.4. Search for Genes for Biosynthesis of the Antagonize Fungi and Bacteria Compounds

Many bacteria of the genus *Bacillus* are widely known as biological control agents with high potential in combating phytopathogenic bacteria and fungi [26]. This genus uses a variety of mechanisms to implement biological control, one of the main ones being the synthesis of secondary metabolites [27].

Using antiSMASH 8.0.2, 17 biosynthetic gene clusters located on 14 scaffolds were discovered in the genome of *B. atrophaeus* R7PjV2-12 (Table 2). Among them were annotated bacillaene, bacillibactin, surfactin, sporulation killing factor, subtilosin A, mycosubtilin, fengycin, 1-carbapen-2-em-3-carboxylic acid, plipastatin, and six biosynthetic clusters related to terpenes, azole-containing-RiPP, lanthipeptide-class-v, and nonribosomal peptide synthetases (NRPSs), for which no closest known clusters were found (Table 2). However, in *B. atrophaeus* R7PjV2-12, genetic clusters of secondary metabolites with a complete set of genes with 100% similarity represent clusters by antiSMASH of biosynthesis of bacillin,

bacillibactin, subtilosin A, and fungicin, which indicates the ability to synthesize these substances by the studied strain (Figure 4, Tables 2 and S3).

Table 2. Secondary metabolism gene clusters identified using antiSMASH 8.0.2 in *Bacillus atrophaeus* R7PjV2-12. PKS—polyketide synthase; NRPS—nonribosomal peptide synthetase.

Scaffold	Type of the Cluster	Size (nt)	Most Similar Known Cluster	Similarity, %
3	PKS-like	115,205	Bacillaene	100
5	NRPS	51,867	Bacillibactin	100
10	Sactipeptide	21,490	Subtilosin A	100
17	NRPS	27,857	Fengycin	100
8	NRPS	43,255	Sporulation killing factor	85
16	NRPS	76,011	Mycosubtilin	83
31	NRPS	15,535	Surfactin	77
34	NRPS	12,983	Plipastatin	74
8	NRPS	28,267	Surfactin	73
32	NRPS	14,085	Plipastatin	73
38	NRPS	8909	-	73
10	Lanthipeptide-class-v	41,873	-	56
21	T3PKS	41,149	1-carbapen-2-em-3-carboxylic acid	54
5	Azole-containing-RiPP	30,104	-	53
2	Terpene	20,843		50
7	Terpene	20,891	-	49
14	Terpene	21,890	-	36

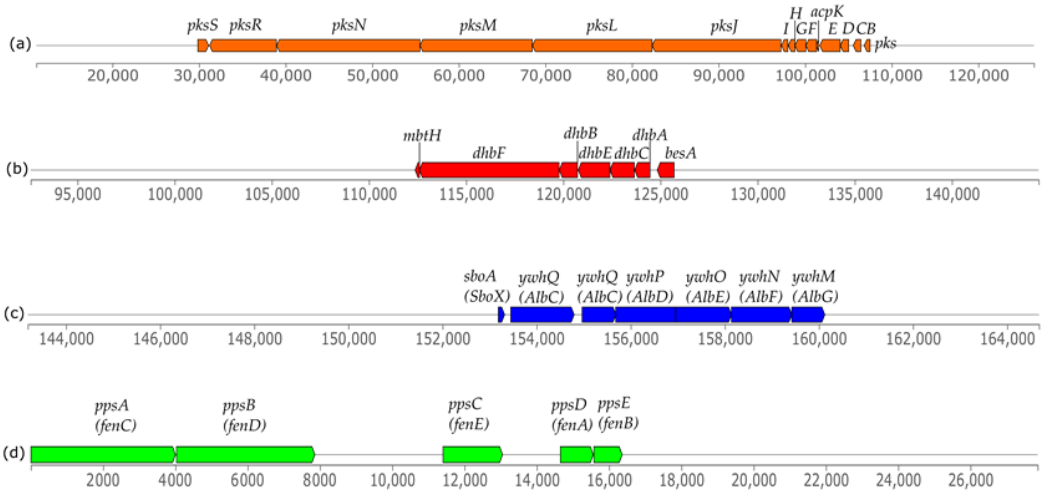

Figure 4. Organization of putative genetic clusters of secondary metabolism with 100% similarity represent clusters by antiSMASH in *Bacillus atrophaeus* R7PjV2-12 genome: (**a**) bacillaene cluster with 14 genes: *acpK*, *pksB*, *pksC*, *pksD*, *pksE*, *pksF*, *pksG*, *pksH*, *pksI*, *pksJ*, *pksL*, *pksM*, *pksN*, *pksR*, *pksS*; (**b**) bacillibactin cluster with 7 genes: *besA*, *dhbA*, *dhbC*, *dhbE*, *dhbB*, *dhbF*, *mbtH*; (**c**) subtilosin A clusrew with 7 genes: *ywhM* (*AlbG*), *ywhN* (*AlbF*), *ywhO* (*AlbE*), *ywhP* (*AlbD*), *ywhQ* (*AlbC*), *ywiA* (*AlbA*), *sboA* (*SboX*); (**d**) fengycin cluster with 5 genes: *ppsA* (*fenC*), *ppsB* (*fenD*), *ppsC* (*fenE*), *ppsD* (*fenA*), *ppsE* (*fenB*).

A comparative analysis of biosynthesis, using antiSMASH 8.0.2 with strict search settings, clusters R7PjV2-12 with other representatives of B. atrophaeus SW and HAB-5. Only clusters showing a high level of similarity were used in the analysis. The search results for SW and HAB-5 are presented (Figure 5). Comparative analysis showed that R7PjV2-12 does not have a large number of secondary metabolism clusters, but compared to SW and HAB-5, it has a subtilosin A biosynthesis cluster (Figure 5). However, the presence of the

subtilosin A biosynthesis cluster is not unique, since the ability to synthesize this substance has been demonstrated in other strains of *B. atrophaeus* [28].

Figure 5. Comparison of secondary metabolism clusters in genomes of *Bacillus atrophaeus* R7PjV2-12, SW, and HAB-5 using antiSMASH 8.0.2.

Synteny analysis of the matching biosynthesis clusters of secondary metabolites showed that the bacillaene biosynthesis clusters in R7PjV2-12, SW, and HAB-5 are completely identical; in turn, the bacillibactin biosynthesis cluster is identical in R7PjV2-12 and HAB-5 (Figure S1a,b). However, in the fengycin biosynthesis cluster, it was shown that R7PjV2-12 has a low level of identity of the *ppsA* (*fenC*) and *ppsD* (*fenA*) genes, but the order of these genes is preserved (Figure S1c). The observed divergence of the key genes of the NRPS module suggests possible functional changes in this cluster and suggests that the R7PjV2-12 strain either synthesizes a structurally modified variant of fengycin or has a limited ability to biosynthesize it.

A polyene antibiotic, Bacillaene, and cyclical peptide, subtilosin A, are known to have antibacterial activity against a wide range of bacterial plant pathogens, and bacillaene has been shown to have antibacterial activity against *Erwinia amylovora*, which causes bacterial burns in plants [29,30]. Subtilosin A has activity against *Acidovorax citrulli*, which causes bacterial spotting of pumpkin crops [31].

Fengycin is an antifungal lipopeptide that demonstrates antivenom against a wide range of phytopathogenic fungi such as *Colletotrichum gloeosporioid*, which causes anthractosis of many crops [32,33]; *Magnaporthe grisea*, causing rice explosion [34,35]; and *Fusarium graminearum*, causing fusarium of common wheat [36]. In addition, fengycin has antibacterial activity against potato brown rot caused by *Ralstonia solanacearum* and black bacterial blotch caused by *Xanthomonas euvesicatoria* [37,38].

Bacillibactin is a bacterial siderophore produced by bacteria of the genus *Bacillus*, which has antibacterial activity against a wide range of plant pathogens such as *Pseudomonas syringae*, *Xanthomonas oryzae*, and *Erwinia amylovora* [39,40]. In addition, the production of siderophores is also associated with antifungal activity [41].

The presence of complete sets of genes for the biosynthesis of the above substances in the genome of *B. atrophaeus* R7PjV2-12 is consistent with the data showing that this strain has antifungal and antibacterial properties, as demonstrated in the antagonism experiments described above (Figure 1).

3. Conclusions

The complete genomic sequence (4.1 Mb) of *B. atrophaeus* R7PjV2-12 was determined through sequencing, assembly, and subsequent annotation. Phylogenetic analysis con-

firmed the classification of strain R7PjV2-12 within the *B. atrophaeus* species. *B. atrophaeus* R7PjV2-12 endophytic bacterial isolate possesses four genetic clusters of secondary metabolites with a complete set of genes with 100% similarity, representing clusters of biosynthesis of bacillin, bacillibactin, subtilosin A, and fungicin, which indicates the studied strain's ability to synthesize these antimicrobial substances.

Thus, the *B. atrophaeus* R7PjV2-12 endophytic bacterium, obtained *from P. jezoensis*, holds promise for use in creating biological control agents against plant diseases caused by species such as *M. oryzae*, *F. avenaceum*, and *E. billingiae*. The precise mechanisms by which *B. atrophaeus* R7PjV2-12 inhibits these pathogens are currently unclear. But it is likely that bacillin, bacillibactin, subtilosin A, and fungicin are actively involved in the process of inhibiting the growth of plant pathogens; however, further research is needed using metabolomics and transcriptomics to understand this mechanism. So, our experimental data has shown the potential importance of *B. atrophaeus* R7PjV2-12 for biocontrol of pathogenic microorganisms in agriculture.

4. Materials and Methods

4.1. B. atrophaeus R7PjV2-12 Isolation

The R7PjV2-12 isolate was obtained from the tissues of a visually healthy *P. jezoensis* spruce branch using the previously described method [42]. In short, the branches of *P. jezoensis* were briefly washed under running water, soaked in 70% ethanol for one minute and 10% H_2O_2 for two minutes, and then washed five times in sterile distilled water. Next, a 150 mg fragment of sterile tissue was homogenized in a laboratory mortar with diameter of 10 cm and diluted with 200 µL of sterile water. Then, 70 µL of the resulting solution was plated on Reasoner's agar 2A (R2A) medium and incubated at 25 °C for 24 h. The resulting isolate was placed in a laboratory collection (Laboratory of Biotechnology, Federal Scientific Center of the East Asia Terrestrial Biodiversity, FEB RAS, Vladivostok, Russia).

4.2. Analysis of Antimicrobial Activity of R7PjV2-12

Antibacterial activity was evaluated using the phytopathogenic bacterium *E. billingiae* by the agar plate method [43]. Overnight-cultured R7PjV2-12 with concentration of 2×10^8 CFU/mL was placed in Petri dishes with R2A medium and incubated at 25 °C for 12 h. Next, using a cork drill, agar blocks with a diameter of 0.6 cm were cut out of the R7PjV2-12 cup and placed on Petri dishes with R2A medium and previously seeded *E. billingiae*. The dishes were incubated for 12 h at 25 °C. The antagonistic activity against bacteria was assessed using the following criteria: a growth delay zone diameter of more than 16 mm indicates very strong inhibition, 11–15.9 mm indicates strong inhibition, 6–10.9 mm indicates moderate inhibition, 1–5.9 mm indicates weak inhibition, and 0 mm indicates no inhibition [43].

Antagonistic activity against phytopathogenic fungi was carried out using *M. oryzae* and *F. avenaceum* by the method of counter cultures. *B. velezensis* R7PjV2-12 was placed on a Petri dish at a distance of 2 cm from the pathogen. The inhibition area was measured on the 5th day of cultivation at a temperature of 26 °C. The inhibition index was calculated using the following formula:

$$(R1 - R2)/R1 \times 100,$$

where R1 is the radius of colony growth in the opposite direction of the candidate microorganism, and R2 is the radius of colony growth in the direction of the candidate microorganism [44]. Pathogenic strains *E. billingiae* and *F. avenaceum* were isolated earlier, as described [42], from the wild grapevine *Vitis amurensis*. *M. oryzae* strain VPL14 was passed on by colleagues from the Federal Scientific Center of Agricultural Biotechnology of the Far East named after A.K. Chaiki (Timiryazevsky settlement, Russia). All plant pathogens were

added to the collection of microorganisms of the laboratory of Biotechnology at the Federal Scientific Center of Biodiversity FEB RAS (storage number for *F. avenaceum* is R9V3№2F1; R7MakVsik for *E. billingiae*; VPL14 for *M. oryzae*). The experiments were conducted at least three times with three replicates in each experiment, and the results are presented as the average value ± standard error.

4.3. Genome Sequencing, Assembly, and Annotation

The total DNA of the R7PjV2-12 isolate was isolated using cetrimonium bromide (CTAB) as described previously [45,46]. The complete genome has been sequenced by MiSeq Illumina method (Illumina, San Diego, CA, USA) in the Department of Scientific and Technical Services of Xi'an Haorui Gene Technology Co., Ltd. (Xi'an, China) and published in GenBank (acc. no PRJNA1401447). The full-genome sequence was assembled using SPAdes v4.2.0 [47], and the assembly quality was assessed using BUSCO [48].

The genome annotation was performed using the Prokka 1.14.6 program [49] and the RAST 2.0 server (http://rast.nmpdr.org/, accessed on 20 December 2025). The antiSMASH 8.0.2 program was used to identify a gene cluster associated with secondary metabolites in the genome [50].

4.4. Phylogenetic Analysis and Comparative Genomics

The phylogenetic analysis was performed using the Kbase server (https://www.kbase.us, accessed on 20 December 2025) data on the set of genes presented in Table S4. The phylogenetic tree was constructed based on 49 genetic markers employing the maximum likelihood method using the Species Tree function v2.2.0 [51].

Orthologous average nucleotide identity was determined using the Orthologous Average Nucleotide Identity Tool (OAT) program [52], and digital DNA-DNA hybridization (dDDH) was calculated using GGDC 3.0 [53]. The UpSet diagram was constructed using the OrthoVenn3 application [54] based on the genomes of R7PjV2-12, SW (GCF_039519175.1), and Hab-5 (GCF_045278795.1). The search for homologous proteins and sequence clustering was carried out using MMseqs2 [55], comparative analysis of gene synthesis, and visualization of homologous clusters by clinker v0.0.32 [56].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres17020039/s1. Table S1: Comparison of *Bacillus atrophaeus* R7PjV2-12 with *B. atrophaeus* BSS using digital DNA-DNA hybridization (dDDH). Table S2: Some characteristics of bacterial genomes used for upset construction. Table S3: Genes for the biosynthesis of antimicrobial substances. Table S4: Genomes of the strains included in the set of genomes. Figure S1: Analysis of the synteny of secondary metabolism gene clusters: (a) bacillaene; (b) bacillibactin; (c) fengycin.

Author Contributions: A.S.D. and K.V.K. were responsible for contributing to the research design, interpretation, and paper preparation. O.A.A. and A.A.D. grew fungus and bacteria for experiments and conducted the agar slab method and data analysis. A.A.A., N.N.N. and H.X. conducted genome sequencing analysis. All authors have read and agreed to the published version of the manuscript.

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