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Original Research

Comparative Genomics of Two Novel *Bacillus* Strains: Microbiomic Insights into the Sequences, Metabolomics, and Potential Safe Use in the Creation of Biopreparations

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Abstract

Background: *Bacillus* bacteria are often used in the production of biopreparations. Moreover, these bacteria can be used in agriculture as probiotics or starters for manufacturing fodder preserved by fermentation (silage). The ability of *Bacillus* bacteria to produce many biologically active molecules and metabolites with antimicrobial activity means that these bacteria can stimulate plant growth and restore the balance of the microbiome in the digestive system of certain animals. **Methods**: Using molecular biological analysis, bioinformatic annotation, and metabolic profiling of whole genome sequences, we analyzed two promising candidates for creating biopreparations, i.e., two *Bacillus* strains, namely *B. mucilaginosus* 159 and *B. subtilis* 111. We compared the genomes of these two strains and characterized both their microbiomic and metabolomic features. **Results**: We demonstrated that both strains lacked elements contributing to the formation of toxic and virulent properties; however, both exhibited potential in the biosynthesis of B vitamins and siderophores. Additionally, these strains could synthesize many antimicrobial substances of different natures and spectrums of action. *B. mucilaginosus* 159 could synthesize macrolactin H (an antibiotic from the polyketide group), mersacidin (a class II lanthipeptide), and bacilysin. Meanwhile, *B. subtilis* 111 could synthesize andalusicin (a class III lanthipeptide), bacilysin, macrolactin H, difficidin, bacillaene (a polyene antibiotic), fengycin (a lipopeptide with antifungal activity), and surfactin (another lipopeptide). Further, a unique pathway of intracellular synthesis of the osmoprotectant glycine betaine was identified in *B. subtilis* 111, with the participation of betaine aldehyde dehydrogenase (BetB); this is not widely represented in bacteria of the genus *Bacillus*. These compounds can increase osmotic stability, which may be key for manufacturing biological starters for silage preparation. **Conclusions**: These two *Bacillus* strains are safe for use as probiotic microorganisms or starters in producing preserved fodder. However, *B. subtilis* 111 may be preferable due to a wider spectrum of synthesized antimicrobial substances and vitamins. Our findings exemplify using genomic technologies to describe the microbiomic and metabolomic characteristics of significant bacterial groups such as *Bacillus* species.

Keywords: *Bacillus* spp.; biopreparations; probiotics; silage; whole genome sequencing; functional microbiomics; metabolomics; antimicrobial substances; vitamins; lipopeptides

1. Introduction

Efforts to transition to more environmentally friendly agriculture systems are now commonplace worldwide. The following are desirable aspects of environmentally friendly agriculture: (1) refusal to use particular chemicals in crop production; (2) refusal to use other certain chemicals for feed antibiotics in livestock farming; (3) increasing the efficiency of roughage assimilation; (4) improving the quality of plant feed preparation; (5) reducing the share of compound feed for ruminants and other animals [1–6]. Using microorganisms with various enzymatic and metabolic activities represents one of the main components of the new environmentally friendly approach to agriculture [7–13]. Enzyme, probiotic, prebiotic, and combined enzyme–

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probiotic preparations are increasingly used to improve efficiency and stimulate the growth and development of ruminants and other animals. This can enhance non-specific immunity and improve the quality of feed preparation [14–19]. The main purpose of using so-called "biopreparations" in feed production is to facilitate the creation of a microbial community that will lead to rapid acidification of the environment and prevent the development of pathogenic and opportunistic microorganisms [20–23], such as clostridia and enterobacteria [24–27]. Clostridia, which are found on crops and in the soil as spores, grow anaerobically. Clostridia also produce butyric acid and breaks down amino acids, making the silage tasteless and possessing reduced nutritional value. Enterobacteria are non-spore-forming facultative anaerobes that ferment sugars to acetic acid and other products and can break down amino acids [27–31].

The most commonly used microorganisms as probiotic preparations include bacilli, lactobacilli, and bifidobacteria [17,22,32,33]. Moreover, the functional microbiomics of probiotic bacteria and their influence on related and other gut microbes have received particular attention [34–40]. The field of "gut microbiomics" investigates the function of the intestinal microbiota by examining its overall "microbiome", including the genomes of its individual representatives [39,41]. Microbiomics provides molecular tools for analyzing microbial communities, facilitating their comprehensive characterization. Thus, using such microbiomic approaches can provide more information regarding the function of a certain bacterial group [12,42]. Strategies for developing food and feed preparations take advantage of incorporating genomic and microbiomic studies [38,43–45], which generally rely upon omics and multi-omics approaches [22,46–48]. The term "microbiomic" is currently widely used in the scientific literature [49–54] as an adjective to mean "*of, or relating to, the microbiome or to microbiomics*" [55]. Paleri [56] defines microbiomic research as "*Microbiomic studies cover the entire life systems as the collective genomes of the microbes that are composed of bacteria, bacteriophage, fungi, protozoa and viruses.*" Thus, the term "microbiomic" was used in the present study in relation to this accepted terminology.

According to many studies, the genus *Bacillus* has several useful properties, making these bacteria effective biopreparations [57–61]. These properties include (1) high preservation, (2) stability during storage, (3) antagonistic activity to a wide range of pathogenic and opportunistic microorganisms, and (4) high enzymatic activity. Bacteria in the genus *Bacillus* can form endospores, which allow them to remain viable under extreme conditions, i.e., at high or low temperatures, radiation, suboptimal pH, pressure, and in the presence of toxic chemicals that damage vegetative cells [62–64]. The ability to synthesize antibiotics, bacteriocins, cyclic lipopeptides, and lytic enzymes with antimicrobial activity is characteristic of the probiotic activity of bacteria in the genus *Bacillus* [22,23,26,65–68]. The genomes of microorganisms from this taxonomic group have many loci that determine the biosynthesis of antimicrobial compounds [19]. This provides one of their most important functional properties that are considered when choosing suitable microorganisms for drug production [22]. Herewith, the metabolomic data [69,70] suggest that *Bacillus* spp. can perform metabolism via the pentose phosphate pathway, making them effective producers of vitamins, among which the most significant are cobalamin, riboflavin, folic acid, and biotin [71–73]. Whole genome sequencing (WGS) allows us to assess fully the biotechnological capabilities of promising strains [61,74,75]. As a result, we can identify the genetic determinants that are associated with the possibility of biosynthesis of various biologically active substances important for creating biopreparations for agriculture. At the same time, we can confirm the absence of different virulence factors that designate the biological safety of using microorganism strains [22,46].

Among the issues associated with creating probiotic preparations, it is important to reveal the presence of unknown properties in strains, both positive (synthesis of antimicrobial substances and various enzymatic activities) and negative (synthesis of undesirable substances and the presence of pathogenicity factors). The development of new technologies in molecular genetics makes it possible to rapidly determine the complete genome of microorganisms [61,74,75]. The use of next-generation sequencing (NGS) technologies, which include WGS, for the analysis of strains of probiotic microorganisms allows for a complete characterization of a strain under study and predicts its capabilities [61], thus facilitating the creation of an effective and safe probiotic drug [22].

Among studies on the genomes of bacilli strains, Kapse *et al*. [60] performed WGS to examine the *B. coagulans* HS243 strain. This genome study revealed the presence of several marker genes that conform to probiotic features. In particular, genome analysis of HS243 revealed the presence of multi-subunit ATPases, hydrolases, and adhesion proteins necessary for survival and colonization of the intestine. The HS243 genome also contains genes for the biosynthesis of vitamins and essential amino acids, which suggests that HS243 can be used as a food additive. The strain was found to have genes responsible for producing bacteriocins; thus, it is apparent that the *B. coagulans* HS243 strain can potentially prevent diseases. Sulthana *et al*. [61] confirmed the genetic safety of the *B. subtilis* UBBS-14 strain using WGS. It was shown that this strain lacks antibiotic-resistance genes (within its mobile genetic elements) and pathogenicity factors. Therefore, it can be considered biologically safe.

The present study aimed to generate and compare the genomes of two promising strains to create biopreparations: *B. mucilaginosus* 159 and *B. subtilis* 111. A comprehensive characterization and assessment of *B. mucilaginosus* 159 and *B. subtilis* 111 were achieved using molecular biolog-

ical analysis and bioinformatic annotations regarding their biological properties and potential use in antimicrobial feed additives.

2. Materials and Methods

2.1 Strains, DNA Extraction, and Whole Genome Sequencing

The *B. subtilis* 111 and *B. mucilaginosus* 159 strains were obtained from the BIOTROF LLC collection (Pushkin, St. Petersburg, Russia). Total DNA was isolated from the strains using the Genomic DNA Purification kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Furthermore, we employed the Nextera DNA Flex Library Prep kit (Illumina, Inc., San Diego, CA, USA) to prepare the DNA library samples for WGS using the Illumina MiSeq platform. The MiSeq Reagent kit v3 (300 cycles; Illumina) was utilized to sequence the resultant libraries [76–78]. The above purification, preparation, and reagent commercial kits were used following the in-house protocols [38,78–80] based on the manufacturers' instructions.

2.2 Bioinformatics Analysis

Sequence quality assessment was performed using the specialized FastQC software [81]. Unreliable sequences and adapters were removed using the Trimmomatic-0.38 program [82]. Paired-end nucleotide sequences, filtered by a length of at least 50 base pairs (bp), were assembled *de novo* using the genome assemblers SPAdes-3.11.1 (for *B. subtilis* 111) and SPAdes v. 3.12.0 (for *B. mucilaginosus* 159) [83]. The genome coverage for *B. subtilis* 111 and *B. mucilaginosus* 159 was 100× and 160×, respectively. The resultant genome assemblies in the form of contigs were validated and deposited using the NCBI BioSample and BioProject databases [84], with the following assigned respective BioSample and BioProject identifiers (accession numbers): SUB14629276 and PRJNA1141124 for *B. subtilis* 111, and SAMN33397894 and PRJNA937299 for *B. mucilaginosus* 159. The contigs assembled and obtained this way were used for subsequent analysis, including annotation for genes (protein-coding genes) using NCBI RefSeq version 6.7 [84]. Additionally, for the independent comparison, a well-documented and annotated genome of the related model microorganism *B. subtilis* subsp. *subtilis* str. 168 [85], with the BioSample and BioProject identifiers SAMEA3138188 and PRJNA76, respectively, was considered.

Functional annotation of genomic sequences was performed using the RAST 2.0 web server (RAST, Rapid Annotations Using Subsystems Technology; [86]). Herewith, the annotation scheme followed Classic RAST, while the FIGfams protein collection version 70 [87] was used to process the genome. An additional annotation was performed using Prokka version 1.13.3 [88] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database [89,90]. In particular, the resultant amino acid sequences were uploaded to the KEGG Automated Annotation Server (KAAS). The sequences were processed using the BBH (bi-directional best hit) method and the BLAST search program [89,90].

To search for gene clusters involved in the biosynthesis of secondary metabolites [46], the antiSMASH version 7.1.0 web service [91] was used in the relaxed detection strictness mode, followed by refinements in the NCBI Protein database [92]. The Norine database [93] was utilized to identify non-ribosomal peptides (NRPs). The search for prophage sequences was performed using the PHASTER web server [94]. The virulence of genomes was analyzed using the VirulenceFinder-2.0 (version 2.0.5), with the parameters set to the %ID threshold of 90% and the minimum sequence length of 60% program, and with the support of the Center for Genomic Epidemiology web service (National Food Institute, Technical University of Denmark, Lyngby, Denmark; [95]). The antibiotic resistance determinants were searched and assessed using the ResFinder program (version 4.6.0), with all parameters specified by default [96].

3. Results and Discussion

3.1 Major Characteristics of the Bacillus spp. Strain Genomes

As illustrated in Table 1, the genome sizes of *B. subtilis* 111 and *B. mucilaginosus* 159 were established as similar: 3,949,468 and 3,970,760 bp, respectively. The guanine-cytosine (GC) composition was similar in percentage terms and amounted to 46.6% for *B. subtilis* 111 and 46.3% for *B. mucilaginosus* 159. According to the ResFinder assessment, no antibiotic resistance determinants were found in the genomes of *B. subtilis* 111 and *B. mucilaginosus* 159. A search for genes encoding putative virulence factors by the VirulenceFinder program also showed that these were absent in *B. subtilis* 111 and *B. mucilaginosus* 159.

We identified all the main groups of genes for proteins that characterize the metabolomic profiles in the genomes of the *B. subtilis* 111 and *B. mucilaginosus* 159 strains. These proteins jointly mediate and modulate certain biological processes involved in the functions of amino acid transport and metabolism, as well as transcription, translation, and carbohydrate/protein transport and metabolism. The strains had products that resembled a full set of functional metabolic pathways, including glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway.

As presented in Table 2 (data produced using RAST 2.0 [86]) and Fig. 1 (data produced using RAST 2.0 [86] and antiSMASH 7.1.0 [91]), analysis of the genomes of *B. subtilis* 111 and *B. mucilaginosus* 159 in comparison to one another showed that the genomes of the studied bacteria contained a large number of genetic determinants. These define the metabolomic profiles of the strains, including the metabolism of carbohydrates, proteins, and amino acids.

Table 1. The main characteristics of the two probiotic *Bacillus* strain genomes compared to the model microorganism.

Assembly characteristics	*B. subtilis* 111	*B. mucilaginosus* 159	Model microorganism *B. subtilis* subsp. *subtilis* str. 168
Genome assembly size, bp	3,941,553	3,970,760	4,214,814
Contig N50	2,109,194	500,910	4,215,606
Guanine-cytosine (GC) composition, %	46.6	46.3	43.5
Number of open reading frames (coding sequences)	3991	4002	4114
Genes	3964	3983	4536
Protein-coding genes	3807	3817	4237
Number of subsystems	444	438	515
Number of RNAs	87	73	181

Table 2. Comparative analysis of the cellular metabolic systems of the two *Bacillus* bacteria as performed using RAST 2.0 (https://rast.nmpdr.org/).

Metabolic systems	Distribution of the number of genes by functions of bacterial metabolic systems					
	B. mucilaginosus 159		*B. subtilis* 111		Model organism *B. subtilis* subsp. subtilis str. 168	
	Total	%	Total	%	Total	%
Protein metabolism	175	6.8	190	7.3	242	7.1
Carbohydrate metabolism	372	14.5	373	14.4	535	15.8
Metabolism of amino acids and their derivatives	410	16.0	401	15.4	450	13.3
Metabolism of nucleosides and nucleotides	110	4.3	105	4.0	145	4.3
Metabolism of fatty acids, lipids and isoprenoids	136	5.3	134	5.2	102	3.0
Metabolism of aromatic compounds	11	0.4	11	0.4	10	0.3
RNA metabolism	156	6.1	155	6.0	198	5.8
DNA metabolism	79	3.1	84	3.2	136	4.0
Sulfur metabolism	39	1.5	39	1.5	44	1.3
Phosphorus metabolism	33	1.3	28	1.1	25	0.7
Nitrogen metabolism	29	1.1	31	1.2	30	0.9
Systems determining potassium balance	8	0.3	8	0.3	26	0.8
Iron entry into the cell	19	0.7	28	1.1	32	0.9
Synthesis of cofactors, vitamins, prosthetic groups, and pigments	202	7.9	230	8.9	311	9.2
Cell division and cell cycle	52	2.0	52	2.0	48	1.4
Motility and chemotaxis	84	3.3	83	3.2	69	2.0
Regulation and signaling systems	63	2.5	61	2.3	50	1.5
Secondary metabolism	6	0.2	5	0.2	6	0.2
Dormancy and sporulation	114	4.4	114	4.4	141	4.2
Respiration process	59	2.3	59	2.3	91	2.7
Response to stress factors	102	4.0	100	3.9	111	3.3
Cell membrane transport systems	60	2.3	58	2.2	84	2.5
Cell wall and capsule synthesis	128	5.0	125	4.8	142	4.2
Cellular protection	64	2.5	63	2.4	77	2.3
Others	56	2.2	60	2.3	290	8.5
Total	2567	100	2597	100	3395	100

Here, the strain *B. subtilis* 111 was found to have a higher proportion of genes involved in protein metabolism, i.e., 190 vs. 175 genes in *B. mucilaginosus* 159. A higher proportion of genes were also involved in the biosynthesis of cofactors, vitamins, prosthetic groups and pigments (230 vs. 202 genes) and more genes were involved in iron entry into the cell (28 vs. 19 genes). In contrast, the strain *B. mucilaginosus* 159 had more genes involved in amino acid metabolism (410 vs. 401 genes).

Annotation of genes in the KEGG Pathway database [89,90] demonstrated that strain 111 is capable of biosynthesizing B vitamins (thiamine, riboflavin, pantothenic acid, B_{12}), biotin, tetrahydrofolate, lipoic acid, and siderophores (bacillibactin). The siderophore bacillibactin

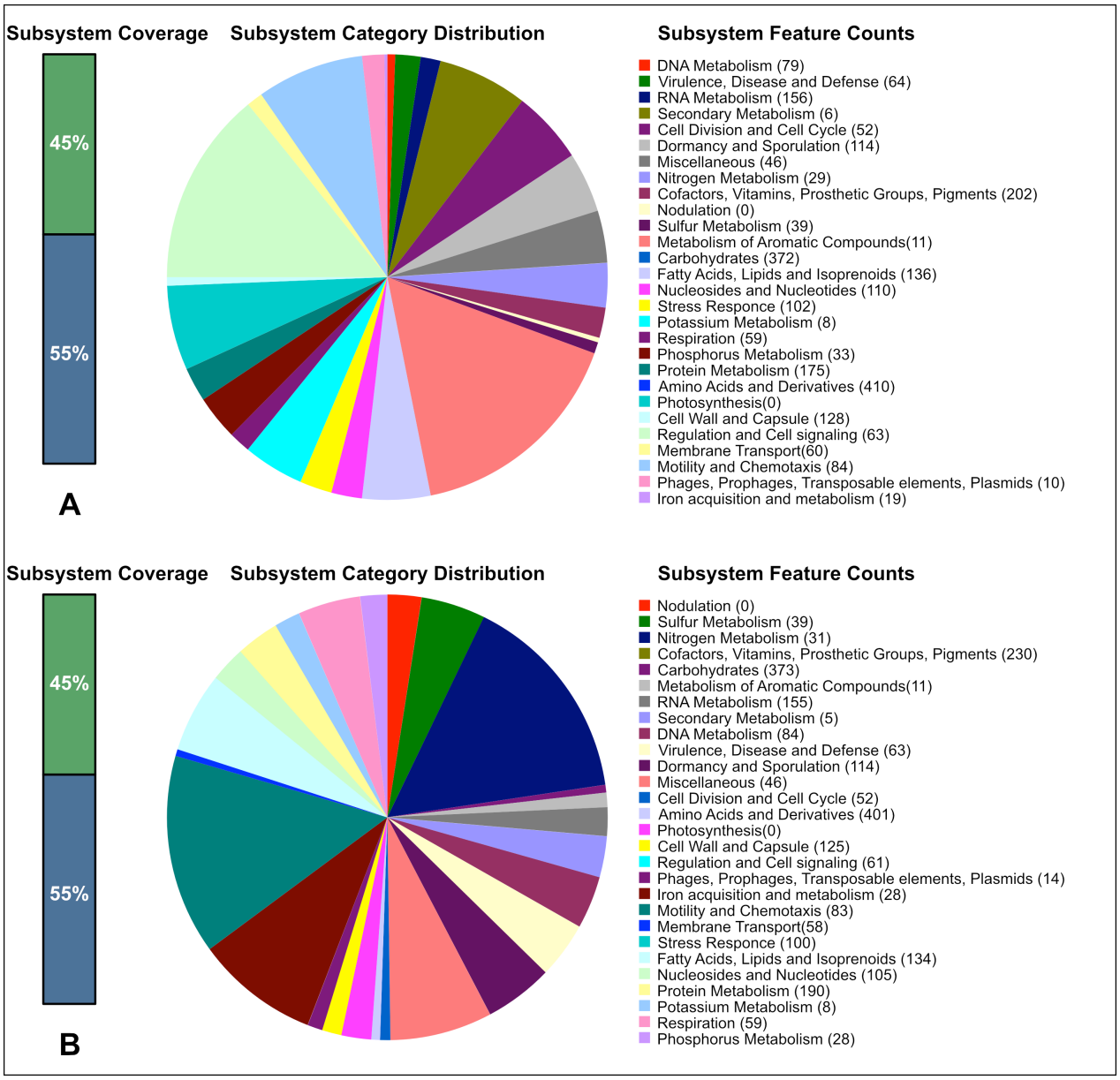


Fig. 1. Analysis of the cellular metabolic systems of the two *Bacillus* bacteria as performed using RAST 2.0 (https://rast.nmpdr.org/) and antiSMASH 7.1.0 web service (https://github.com/antismash/antismash/releases). (A) *B. mucilaginosus* 159. (B) *B. subtilis* 111. To visualize the analysis results, the data were pre-displayed on a computer screen and saved as images using macOS Sequoia version 15 and the screenshot function. The screenshots were then edited using Adobe Photoshop (Adobe Inc., San Jose, CA, USA) version 26.1 to achieve the required quality.

is a catecholic trilactone secreted by various bacilli species for iron absorption during periods of iron limitation. Strain 159 could biosynthesize riboflavin, thiamine, and the siderophore bacillibactin.

In addition, the strains exhibited broad capabilities for the biosynthesis of fatty acids. We identified all the main enzymes responsible for forming fatty acids (C3—C18): FabD, FabF, FabG, FabZ, FabI (FabK, FabL), etc.

The genome of *B. subtilis* 111 contains genes for the biosynthesis of vitamin B_6: thiamine monophosphate kinase [EC:2.7.4.16], thiamine phosphate phosphatase [EC:3.1.3.100], and 18 genes involved in this process. Both strains contain genes for enzymes involved in the biosynthesis of riboflavin, as well as its coenzyme derivatives, i.e., flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD): riboflavin synthase [EC:2.5.1.9], riboflavin kinase [EC:2.7.1.26], FAD synthetase [EC:2.7.7.2], FMN reductase [EC:1.5.1.38] and FMN reductase [NAD(P)H] [EC:1.5.1.39], as well as other enzymes. Strain 111 contains a gene for purine nucleoside phosphorylase [EC:2.4.2.1] involved in the biosynthesis of nicotinamide and nicotinic acid. The biological role of pan-

tothenic acid is due to its participation in the biosynthesis of coenzyme A (CoA). The genome of strain 111 contains the enzyme pantoate-beta-alanine ligase [EC:6.3.2.1] (catalyzes the biosynthesis of B_5 from pantoic acid and beta-alanine) and pantothenate kinase [EC:2.7.1.33] (the first enzyme in the pathway of CoA biosynthesis). Pantothenate kinase phosphorylates pantothenate (vitamin B_5) to form 4′-phosphopantothenate using an adenosine triphosphate (ATP) molecule. Other genes were also identified in the biosynthesis of pantothenic acid from pyruvate, catalyzing its further conversion to CoA. The studied strain also possessed the biotin biosynthesis pathway and the key enzyme biotin synthase [EC:2.8.1.6].

3.2 Synthesis of Antimicrobial Compounds

Fig. 1 shows the metabolic capacities of the two strains to synthesize antimicrobial substances. According to the complete genomic sequence analysis, the *B. mucilaginosus* 159 strain can simultaneously synthesize several antimicrobial compounds of different natures and spectrums of action (Fig. 2A; data produced using antiSMASH 7.1.0 [91] and KAAS [89,90]). This analysis allowed us to identify sequences encoding biosynthesis of macrolactin H (an antibiotic from the polyketide group), mersacidin (a class II lanthipeptide), and bacilysin. The *B. subtilis* 111 strain genome (Fig. 2B) can synthesize andalusicin (class III lanthipeptide), bacilysin, the polyketide antibiotic macrolactin H, difficidin, and bacillaene (a polyene antibiotic).

Both strains could synthesize the antibiotic bacilysin (Fig. 3; data produced using KEGG Pathway database and KAAS [89,90]). The strains possessed all the genes controlling this process (gene cluster *bacABCDE*). Bacilysin is a non-ribosome-synthesized dipeptide antibiotic with an l-alanine residue at the N-terminus and a non-proteinogenic amino acid, l-anticapsin, at the C-terminus. Despite its simple structure, bacilysin is active against a wide range of bacteria and fungi, including *Candida albicans*. The antibacterial action of bacilysin depends mainly on its transport into host cells, its hydrolysis by intracellular peptidases to anticapsin, and inhibition of glucosamine-6-phosphate (GlcN6P) synthase by anticapsin.

3.3 Lipopeptide Synthesis

As follows from Fig. 1B, the *B. subtilis* strain 111 can synthesize fengycin (a lipopeptide with antifungal activity) and surfactin (another lipopeptide).

It was previously established that *Bacillus* spp. lipopeptides are small metabolites with a cyclic structure formed by 7–10 amino acids (including 2–4 D-amino acids) and a beta-hydroxybutyric acid with 13–19 C atoms [97]. These lipopeptides have a variety of biological activities, including biofilm interactions and antifungal, anti-inflammatory, antitumor, antiviral, and antiplatelet properties. The multiple activities of lipopeptides have stimulated significant interest in the exploitation of these lipopeptides for use as antibiotics, feed additives, antitumor agents, acute thrombolytic therapeutics, and drug delivery systems. Hence, gaining knowledge of the inherent roles played by these structurally varied lipopeptides in *Bacillus* species is essential for effectively producing more potent products and understanding microbial regulatory systems. To increase the efficiency of their biosynthesis for industrial applications, ongoing efforts are required as there is currently a lack of knowledge regarding the direct target of these lipopeptides [97,98].

Surfactin is one of the powerful surfactants of biological origin and has a wide range of potential applications in industry and medicine. The main factor limiting its active usage is the high production cost. Due to the complexity of the structure, the chemical synthesis of surfactin is unprofitable, meaning microbiological synthesis is the main approach to its production [75]. Fengycin is an antifungal lipopeptide first discovered in the *B. subtilis* F-29-3 strain. Fengycin inhibits mycelial fungi but is ineffective against yeast and bacteria [99,100]. Thus, we may conclude that the *B. subtilis* strain 111 has a broader spectrum of activity, including fungicidal properties.

3.4 Osmotic Tolerance

The osmotic stability of strains may be crucial for developing biological starters for silage production. If the dry matter content in the grass is 30% or more, the increased osmotic pressure in plant cells consequently leads to inhibiting the development of lactic acid bacteria and causing a slowdown in the acidification of the grass mass, especially in the first, most crucial stage of its ensiling [101]. However, using osmotolerant bacteria in silage preparations helps overcome this problem [102–105]. Osmoprotectants, also known as compatible solutes, are tiny organic compounds that function as osmolytes to assist organisms in resisting excessive osmotic stress. Osmoprotectants have a neutral charge and are not hazardous at high concentrations. Overall, osmoprotectants can be divided into three chemical classes: (1) betaines and related molecules, (2) sugars and polyols, and (3) amino acids. These molecules accumulate in cells and balance the osmotic difference between the cell environment and the cytoplasm. There are two pathways for the accumulation of osmoprotectants in the cell: transport from the external environment and the biosynthesis of osmoprotectants inside the cell [106].

The uptake of osmotic stress protectants occurs via the Opu family of transporters, a system of central importance for managing osmotic stress in bacteria. The osmolarity of the environment controls opuD-mediated glycine betaine transport activity in *Bacillus* spp. High osmolarity maximizes glycine betaine absorption activity by activating pre-existing OpuD proteins and stimulating *de novo* OpuD synthesis. Strains have different mechanisms of tolerance to high osmotic pressure [107].

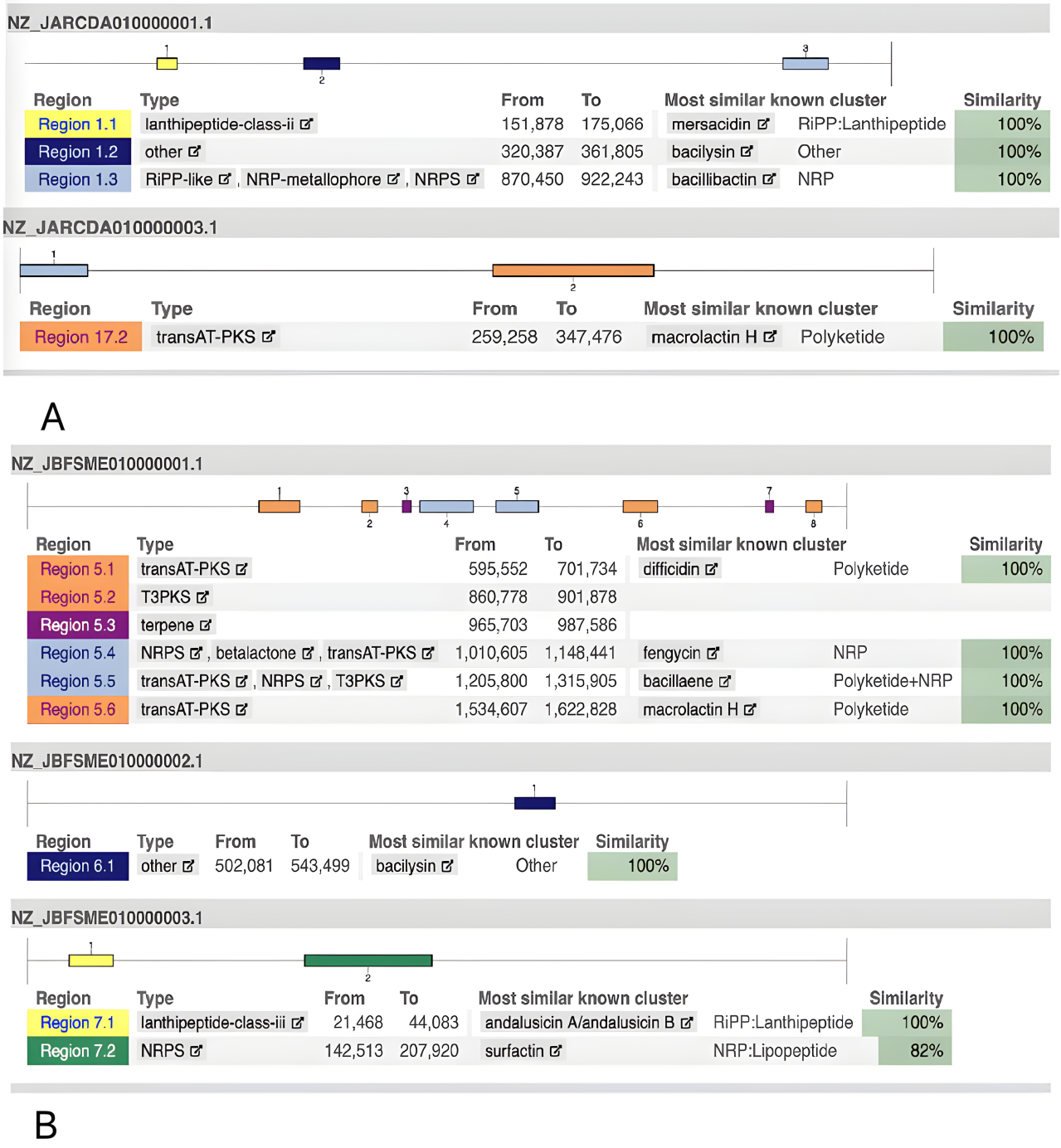


Fig. 2. Gene clusters involved in the biosynthesis of secondary metabolites that were determined using the antiSMASH 7.1.0 web service (https://github.com/antismash/antismash/releases) and KAAS (KEGG Automated Annotation Server; https://www.genome.jp/kegg/kaas/). (A) *B. mucilaginosus* 159. (B) *B. subtilis* 111. To visualize the analysis results, the data were pre-displayed on a computer screen and saved as images using macOS Sequoia version 15 and the screenshot function. The images were then edited using Adobe Photoshop version 26.1 to achieve the required quality.

Previously, bacilli were believed to be mainly capable of synthesizing only one osmoprotective substance within themselves, i.e., proline. However, one of the adaptation mechanisms that was identified in both studied *Bacillus* spp. strains (as shown in Fig. 4; data produced using KAAS [89,90]) turned out to be the biosynthesis mechanism of glycine betaine, which is a very effective osmoprotectant that accumulates in high concentrations in response to increased osmotic pressure from choline under the action of the choline dehydrogenase (BetA(GbsB)) and betaine alde-

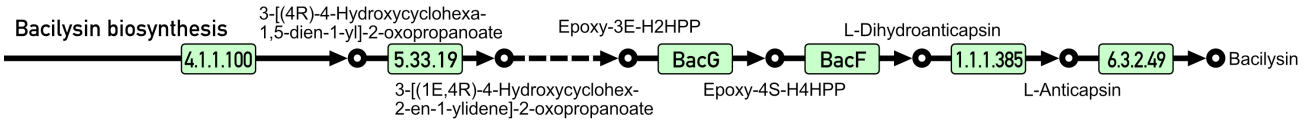


Fig. 3. Biosynthesis of the antibiotic bacilysin by the strains *B. subtilis* 111 and *B. mucilaginosus* 159 (according to the KEGG Pathway database (https://www.genome.jp/kegg/pathway.html) and using KAAS (KEGG Automated Annotation Server; https://www.genome.jp/kegg/kaas/)). To visualize the analysis results, the data were pre-displayed on a computer screen and saved as images using macOS Sequoia version 15 and the screenshot function. The images were then edited using Adobe Photoshop version 26.1 to achieve the required quality.

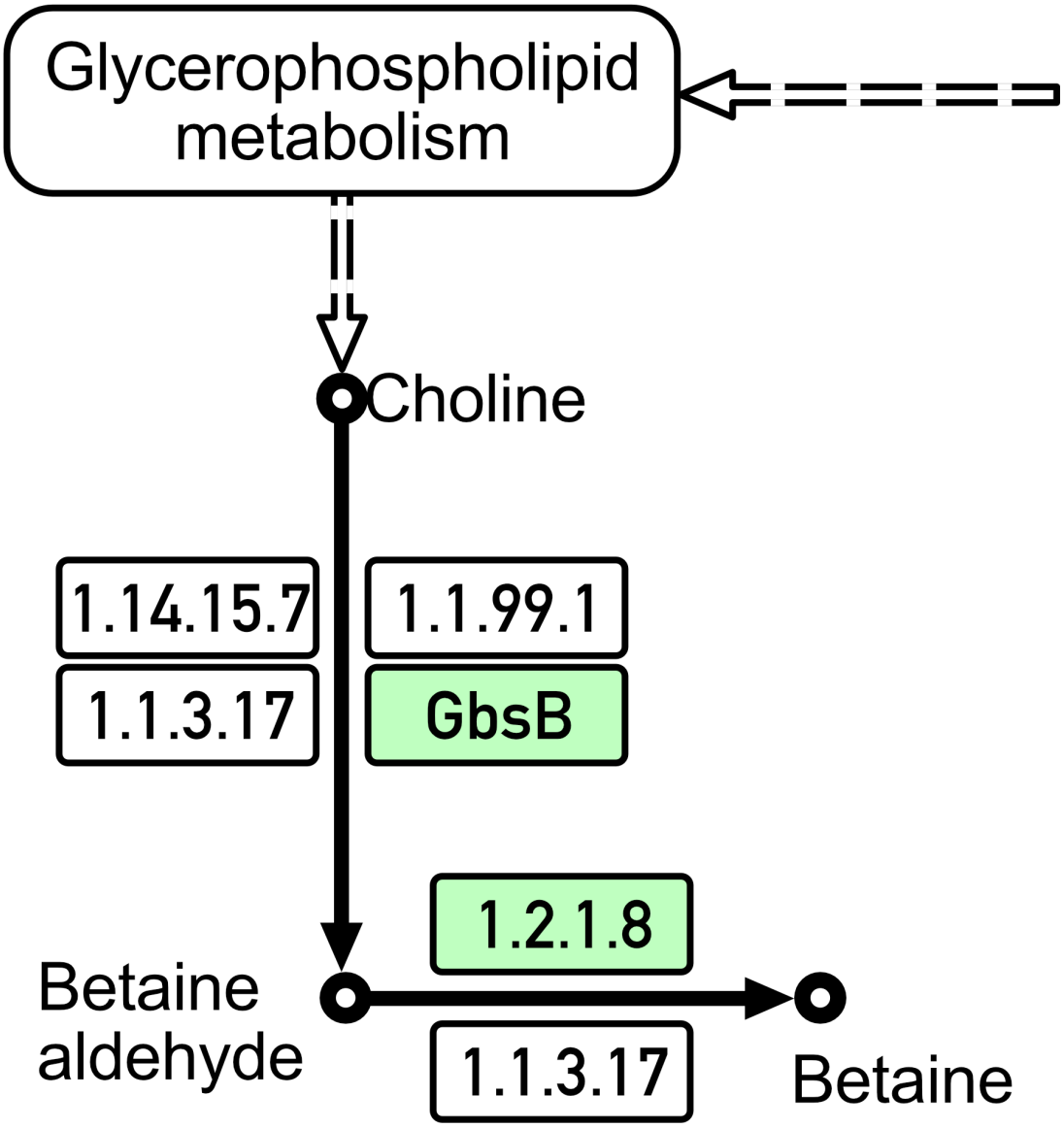


Fig. 4. Biosynthesis of glycine betaine by *B. subtilis* 111 strain and *B. mucilaginosus* 159 strain (according to the KEGG Pathway database; https://www.genome.jp/kegg/pathway.html). To visualize the analysis results, the data were pre-displayed on a computer screen and saved as images using macOS Sequoia version 15 and the screenshot function. The image was then edited using Adobe Photoshop version 26.1 to achieve the required quality.

hyde dehydrogenase (BetB) enzymes. Glycine betaine is synthesized in cells from choline in a two-step pathway under the action of these two enzymes [108,109]. Among the compatible solutions used, glycine betaine plays a partic-

ularly important role for bacilli since it can be both synthesized and imported through three high-affinity and osmotically induced transport systems [107]. Glycine betaine importation is mediated by the betaine–choline–carnitine transporter (BCCT)-type transporter OpuD and the ABC transporters OpuA and OpuC [110].

Thus, the strains we studied here are characterized not only by their ability to pump osmoprotectants from the environment but also by their ability to synthesize proline and glycine betaine independently (Fig. 4).

Our findings provide comprehensive information about the metabolomic profiles of the two strains and the functional properties of the important proteins they are capable of synthesizing. Among them, we also highlight the genetic determinants for such a crucial metabolic system as response to stress factors (Table 2) [111–113]. The present study was performed in accordance with state-of-the-art investigations aimed at describing and defining a healthy gut based on important characteristics of microbial functionality that may be derived from microbiomics and its research methodologies [13,38,114]; these, in turn, benefit from omics and multi-omics approaches [19,22,46,47,115].

4. Conclusions

Analysis of the genomic sequences of *B. subtilis* 111 and *B. mucilaginosus* 159 demonstrated that these bacteria were similar in terms of their metabolomic profiles based on the sets of proteins they produce. Moreover, these strains did not contain antibiotic resistance determinants or virulence factors. Antimicrobial activity against Gram-positive and Gram-negative bacteria is among the important requirements for promising strains used as silage starters. Both strains can synthesize many antimicrobial substances of various natures and spectrums of action. The *B. mucilaginosus* 159 strain can synthesize macrolactin H (an antibiotic from the polyketide group), mersacidin (a class II lanthipeptide), and bacilysin. The *B. subtilis* 111 strain can synthesize andalusicin (class III lanthipeptide), bacilysin, polyketide antibiotic macrolactin H, difficidin, bacillaene (a polyene antibiotic), as well as fengycin (a lipopeptide with antifungal activity) and surfactin (another lipopeptide). Thus, the spectrum of biosynthesized antimicrobial substances is wider in the *B. subtilis* 111 strain. The osmotic stability of strains can also be a determining property for generating biological starters for silage preparation. The studied strains are characterized by the ability to pump osmoprotectants from the environment and synthesize them independently, both proline and glycine betaine. In general, it can be stated that these strains are safe for use as probiotic microorganisms or as starters for obtaining preserved feed. The *B. subtilis* 111 strain may be preferable due to a wider spectrum of synthesized antimicrobial substances and vitamins. Our study demonstrates the applicability of modern genomic technologies to characterize functional microbiomic and metabolomic features of such important bacterial groups as *Bacillus* spp. and their subsequent use as antimicrobial feed additives.

Availability of Data and Materials

This article contains a presentation of all the experiments and findings from the study. Further details can be obtained upon request from the corresponding author.

Author Contributions

Conceptualization, GYL, LAI, VAF and EAY; methodology, LAI and VAF; software, ESP and EAB; validation, VAF, NIN and DKG; formal analysis, ESP; investigation, IAK, ASD, KAS and VAZ; resources, DGT and NIN; data curation, DGT and MNR; writing—original draft preparation, EAY and MNR; writing—review and editing, EAY, MNR and DKG; visualization, AVD, EAY and MNR; supervision, GYL and DKG; project administration, GYL and NIN; funding acquisition, LAI. All authors have read and agreed to the published version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflicts of interest. Authors Valentina A. Filippova, Georgi Yu. Laptev, Larisa A. Ilina, Elena A. Yildirim, Evgeni A. Brazhnik, Kseniya A. Sokolova, and Irina A. Klyuchnikova are affiliated with BIOTROF Ltd., which sponsored this study. The study design, data analysis, interpretation, and manuscript preparation were carried out independently and were not influenced by the sponsor.

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