Analysis of the probiotic activity of Bacillus velezensis RT-26 strain isolated from reindeer rumen by whole-genome sequencing

L.A. Ilina[1,2,*], *V.A.* Filippova[1,2], *E.S.* Ponomareva[1], *E.A.* Brazhnik[2], *T.P.* Dunyashev[1] and *K.A.* Laishev[3]

[1] OOO BIOTROF+, 19-1 Zagrebsky Blvd., St. Petersburg.
[2] St. Petersburg State Agrarian University, 2, Peterburgskoe sh, Saint-Petersburg, Russia
[3] St. Petersburg Federal Research Center of the Russian Academy of Sciences, 39, 1-ya liniya V.O., St. Petersburg, Russia

Abstract. The paper analyses the properties of Bacillus velezensis RT-26, a probiotic strain isolated from reindeer rumen, which has high activity towards fiber degradation, against bacterial and fungal pathogens. The analysis was performed using whole-genome sequencing of the strain using the Illumina platform. The study revealed that strain RT-26 possessed a complete set of metabolic pathways, including glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway. 411 genes were involved in carbohydrate metabolism in the strain genome, 229 genes were related to vitamin and coenzyme metabolism, 149 genes were involved in fatty acid metabolism. The synthesis pathways of various amino acids, most B vitamins (thiamine, riboflavin, nicotiamide, vitamin B5) were identified in the genome. A complete pathway for synthesis of the dipeptide antibiotic bacilisin was detected in the strain. In addition, the strain is capable of synthesizing class A beta-lactamase. No genes responsible for the degradation of mycotoxins and xenobiotics were detected in the genome of the strain studied. A number of glycosyl hydrolase families were detected in the strain genome: GH 1, 3, 4, 5, 6, 11, 13, 16, 18, 20, 23, 26, 28, 30, 32, 43, 46, 51, 53, 68, 68, 73, 101, 109, 126. Carbohydrate-binding proteins were of the SVM 50 family. Glycosyltransferases were of GT 1, 2, 4, 8, 26, 28, 30, 51, 83 families. In the genome of Bacillus velezensis strain RT-26, cellulases related to families GH 5, 6, 26, 51, chitinases related to families GH 18 and 23, and xylanases related to families GH 1, 3, 4, 16, 30, 43 were found. Thus, strain B. velezensis RT-26 has several phenotypically and genotypically proven properties that can characterize it as a good probiotic microorganism.

1 Introduction

Probiotics are live microorganisms that, when introduced in adequate amounts, benefit the health of the host [1]. Products based on them have many advantages, but the issues of their safety must not be forgotten [2]. For example, inaccurate labeling can be caused by

* Corresponding author: ilina@biotrof. ru

incorrect taxonomic identification of probiotic strains [3]. The product may be contaminated [4]. These problems can be caused by the limitations of classical microbiological identification and detection methods. In addition, the genome diversity of probiotic microorganisms is likely to affect the functionality of probiotics. So, quality assurance measures and stringent controls are needed to assess the stability of the genome of probiotic strains, especially mobile genetic elements [5]. It is also necessary to perform screening of common bacterial toxic metabolites harmful to human health, including hemolysins, D-lactic acid (D-lactate), biogenic amines involving key enzymes such as nitroreductase, amino acid decarboxylase enzyme and azoreductases [6-8].

Identification by sequencing methods at the strain level ensures that microbes stored in culture repository are correctly identified and handled [9]. It provides a genetic passport for monitoring both probiotic engraftment and probiotic-associated bacteremia, and provides a baseline sequence to assess whether the genetic composition of the strain changes over time.

Cumulatively, the whole-genome analysis is an expected method for accurate identification and safety assessment [10], which may address growing concerns about human health risks associated with probiotic products [11-13].

The aim of this study was to perform the whole-genome sequencing of the most promising probiotic strain RT-26 isolated from reindeer rumen and evaluate its biological activities related to the synthesis of biologically active, antimicrobial substances, vitamins, enzymes contributing to fiber degradation, mycotoxins, etc.

2 Materials and methods

The collection of bacterial strains was obtained by isolation from the rumen of reindeer (Rangifer tarandus L.). At the first stage, RT-26 strain with high activity against bacterial and fungal pathogens was selected from the collection. Whole-genome sequencing was performed for it to determine biological activities.

Total DNA was isolated from strains using the Genomic DNA Purification Kit (Thermo Fisher scientific, USA). The Nextera DNA Flex Library Prep kit (Illumina) was used to prepare samples for whole-genome sequencing using the Illumina Miseq platform. The MiSeq Reagent Kit v2-150 (Illumina) was used to sequence the obtained libraries.

The strain genome was combined using Illumina paired-end reads. The reads were analyzed using the Fastqc program. Trimming was performed using the trimmomatic program with HEADCROP, SLIDINGWINDOW, LEADING, and TRAILING filters. After filtering, the read quality was checked again with Fastqc, and when the results achieved the proper quality, assembly was started with SPAdes from paired-end Illumina reads varying the k-measure length. The output data were then loaded by scaffolds to the QUAST assembly evaluation tool. A mapping program was used to map the reads. The output file sam was converted into a binary sorted by coordinate bam, which was more convenient for work and storage. For ease of processing, bam files were indexed using the samtools software package. The bwa mapper was used. After executing the protocol for our sample, we obtained one sorted bam file and its corresponding index file, which were further used for analysis. Using the database of 16S rRNA bacteria and the blastn (as well as makeblastdb) program, we determined the location of the 16S rRNA gene in the assembly. The maximum match of our assembly according to 16S gene was with the bacterium *Bacillus velezensis*. This result was obtained using the NODE 12 scaffold with a length of 4767 bp, an alignment length of 1545 bp, and a similarity of 99.93% relative to the 16S reference gene. The strain was classified as *Bacillus velezensis*.

After a more accurate identification of the strain species, we re-evaluated the assembly in QUAST together with the *Bacillus velezensis* reference genome taken from the NSBI

database (https://www.ncbi.nlm.nih.gov/genome/?term=Bacillus%20velezensis[Organism]&cmd=DetailsSearch). The genome fractions score was 93.1%, which proved the high quality of our assembly. For further analysis and comparison with other databases, we used the Prokka program, a software tool for rapid annotation of genes and identification of coding sequences in prokaryotic genomes (conversion of nucleotide sequences to amino acids). For the analysis, we loaded the assembly into a functional annotation in the KEGG database using the KEGG Automatic Annotation Server program and the GHOSTX, bi-directional best hit (BBH) algorithm. It was also loaded in the SEED database (a platform to support genome comparison and annotation) and to the dbCAN2 meta-server (a web server for automated annotation of carbohydrate-active enzymes funded by the National Science Foundation). The CAZy database describes families of structurally related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds. CAZy data are available either by browsing sequence-based families or by browsing the contents of genomes in carbohydrate-active enzymes.

3 Results and discussion

The genome size of *Bacillus velezensis* strain RT-26 was 3,980,359 bp, with an average GC content of 46.3%. In the strain genome we identified all major groups of genes for proteins that jointly implement a particular biological process, more than 48 % of *B. velezensis* genes were involved in the functions of amino acid transport and metabolism; as well as transcription, translation, transport and metabolism of carbohydrate and proteins (Fig. 1). The strain had products for the functioning of a full set of metabolic pathways, including glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway. 411 genes were involved in carbohydrate metabolism in the strain genome, 229 genes were related to vitamin and coenzyme metabolism, 149 genes were involved in fatty acid metabolism. *Bacillus velezensis* is currently quite actively studied as a probiotic [14-17]. *Bacillus velezensis* is a plant growth-promoting bacterium that can also inhibit plant pathogens. However, there are several experiments with *B. velezensis* used as a substitute for feed antibiotics [15,16]. It has been suggested that these bacteria can inhibit the adhesion, replication, and virulence of intestinal pathogens [18]. In addition, it can play an important role in modulating the immune system [19]. Thus, *B. velezensis* can be used as a probiotic agent for animals. Now the probiotic characteristics of *B. velezensis* are the subjects of ongoing discussions on its potential probiotic utilization for fish and animal feed industry. Various strains of *B. velezensis* isolated from different sources have the ability to produce antimicrobial compounds and have a beneficial effect on the gut microbiota, which could become a probiotic in the feed industry [17, 20].

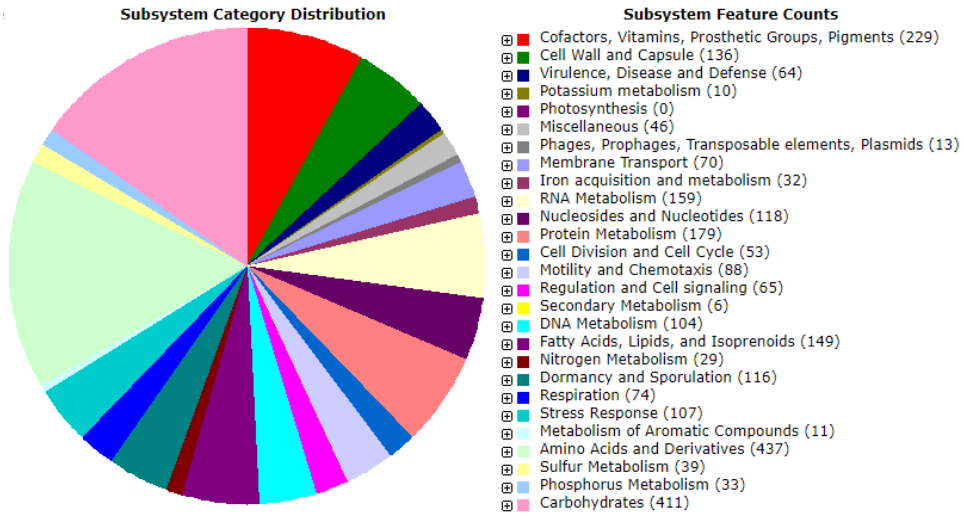

Fig. 1. Metabolic systems of Bacillus velezensis strain based on the results of functional annotation according to the RAST database (https://rast.nmpdr.org).

Pathways for the synthesis of various amino acids and most B vitamins were identified in the genome. Identified were genes of bacterial secretion systems (11 genes) annotated in the ko03070 pathway, mostly belonging to the Sec-SRP type. Bacterial secretion systems are protein complexes located in the cell membrane of bacteria that serve to secrete various proteins. Various types of ABC transporters have been identified, annotated in the ko02010 pathway (90 genes). Quorum sensing systems (54 genes) annotated in the ko02024 pathway, as well as various biofilm formation pathways have been identified.

The full synthesis pathway of the dipeptide antibiotic bacilisin (Fig. 2) and beta-lactamase of class A [EC:3.5.2.6] were detected in the strain. No genes responsible for the degradation of mycotoxins and xenobiotics were detected in the genome of the studied strain.

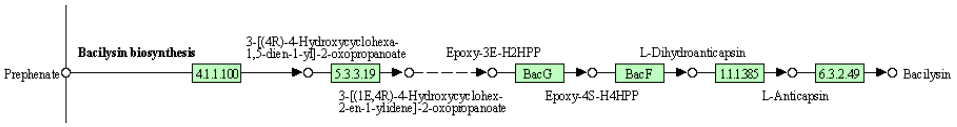

Fig. 2. Bacilisin biosynthesis

The bCAN carbohydrate-active enzyme annotation algorithm (CAZy) was used to analyze CAZy annotations to identify genes involved in lignocellulose degradation. Using the Cazy database, we identified a number of enzyme families responsible for carbohydrate degradation: glycosylhydrolases, glycosyltransferases, and carbohydrate-binding protein modules (CBMs). Glycosylhydrolases include enzymes responsible for the degradation of various polysaccharеads including cellulases, chitinases, xylanases, etc. Glycosyltransferases are enzymes that transfer monosaccharide residues from a donor carbohydrate to an acceptor molecule, most often alcohol. The products of the reaction can be monosaccharides, glycosides, oligosaccharides, polysaccharides, and glycoproteins. CBMs were previously known as cellulose-binding domains and are involved in the process of bacterial cell attachment to the polysaccharide substrate. CBMs are subdivided into numerous families based on amino acid sequence similarity.

Several glycosyl hydrolase families were identified in the strain genome: GH 1, 3, 4, 5, 6, 11, 13, 16, 18, 20, 23, 26, 28, 30, 32, 43, 46, 51, 53, 68, 68, 73, 101, 109, 126. Carbohydrate-binding proteins of the SVM family 50 were identified. Glycosyltransferases of GT 1, 2, 4, 8, 26, 28, 30, 51, 83 families were identified.

4 Conclusion

The study revealed that strain RT-26 possessed a complete set of metabolic pathways, including glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway. 411 genes were involved in carbohydrate metabolism in the strain genome, 229 genes were related to vitamin and coenzyme metabolism, 149 genes were involved in fatty acid metabolism.

In the annotations of KEGG *B. velezensis* RT-26 metabolism, carbohydrate and amino acid metabolism, which are considered its main functions, contain a large number of genes. It was also shown that the strain has a broad capacity for fatty acid biosynthesis. We identified all the major enzymes responsible for fatty acid formation (C3-C18): FabD, FabF, FabG, FabZ, FabI (FabK, FabL), etc. The synthesis pathways of various amino acids, and most B vitamins (thiamine, riboflavin, nicotiamide, vitamin B5) were identified in the genome.

The strain was found to have a complete synthesis pathway of the dipeptide antibiotic bacilisin. Despite its simple structure, it is active against a wide range of bacteria and fungi, including *Candida albicans*. In addition, the strain is capable of synthesizing class A beta-lactamase. No genes responsible for the degradation of mycotoxins and xenobiotics were detected in the genome of the strain studied.

The bCAN carbohydrate-active enzyme annotation algorithm (CAZy) was used to analyze CAZy annotations to identify genes involved in lignocellulose degradation. Several glycosyl hydrolase families were identified in the strain genome: GH 1, 3, 4, 5, 6, 11, 13, 16, 18, 20, 23, 26, 28, 30, 32, 43, 46, 51, 53, 68, 68, 73, 101, 109, 126. Identified were carbohydrate-binding proteins of SVM family 50, and glycosyltransferases of GT 1, 2, 4, 8, 26, 28, 30, 51, 83 families. In the genome of *Bacillus velezensis* strain RT-26, cellulases belonging to families GH 5, 6, 26, 51, chitinases belonging to families GH 18 and 23, and xylanases belonging to families GH 1, 3, 4, 16, 30, 43 were found.

Thus, strain *B. velezensis* RT-26 has several phenotypically and genotypically proven properties that can characterize it as a good probiotic microorganism.

The study was supported by grant RFF № 17-76-20026 "Microbiocenosis of the rumen of Rangifer tarandus of the Arctic regions of Russia as a fundamental basis for promising biotechnologies for farm animals".

References

1. FAO/WHO. Guidelines for the evaluation of probiotics in food, report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. London Ontario: World Health Organization (2002).
2. J.S. Weese, H. Martin, Assessment of commercial probiotic bacterial contents and label accuracy, Can Vet J,52(1), 43-6 (2011).
3. J.N. Patro, P. Ramachandran, T. Barnaba, M.K. Mammel, J.L. Lewis, C.A. Elkins, Culture-Independent Metagenomic Surveillance of Commercially Available Probiotics with High-Throughput Next-Generation Sequencing. mSphere, 1(2), e00057-16 (2016).

4. W. Sybesma, D. Molenaar, I.J. Wv, K. Venema, R. Kort, Genome instability in Lactobacillus rhamnosus GG. Appl Environ Microbiol, 79(7), 2233-9 (2013) 10.1128/AEM.03566-12.
5. C. Llor, L. Bjerrum, Antimicrobial resistance: risks associated with antibiotic overuse and initiatives to reduce the problem, Ther Adv Drug Saf, 5(6), 229-41 (2014). 10.1177/2042098614554919.
6. S. Fu, Q. Yang, F. He, R. Lan, J. Hao, P. Ni, Y. Liu, R. Li, National safety survey of animal-use commercial probiotics and its spillover effects from farm to human: an emerging threat to public health, Clin Infect Dis, 70(11), 2386-95 (2019).
7. D. Pradhan, R.H., Mallappa, S. Grover, Comprehensive approaches for assessing the safety of probiotic bacteria, Food Control, 108, 106872 (2020).
8. L.G. Pell, R.G. Horne, S. Huntley et al, Antimicrobial susceptibilities and comparative whole genome analysis of two isolates of the probiotic bacterium Lactiplantibacillus plantarum, strain ATCC 202195. Sci Rep 11 15893. 10.1038/s41598-021-94997-6 (2021)
9. A. Rouanet, S. Bolca, A. Bru, I. Claes, H. Cvejic, H. Girgis, A. Harper, S.N. Lavergne. S. Mathys, Live biotherapeutic products, A Road Map for Safety Assessment,Front Med.,7, 237 (2020).
10. R. Soni, S. Nanjani, H. Keharia, Genome analysis reveals probiotic propensities of Paenibacillus polymyxa HK4, Genomics, 113(1), 861-73 (2020).
11. E. Salvetti, L. Orrù, V. Capozzi, A. Martina, A. Lamontanara, D. Keller, H. Cash, G. Felis, L. Cattivelli, S. Torriani, G. Spano, Integrate genome-based assessment of safety for probiotic strains: Bacillus coagulans GBI-30, 6086 as a case study. Appl Microbiol Biotechnol., 100(10),4595-605 (2016). 10.1007/s00253-016-7416-9.
12. A. Lerner, Y. Shoenfeld, T. Matthias, Probiotics: If It Does Not Help It Does Not Do Any Harm. Really?, Microorganisms, 7(4), 104 (2019).
13. J.M. Castro-González, P. Castro, H. Sandoval, D. Castro-Sandoval, Probiotic lactobacilli precautions, Front Microbiol (2019), 10:375. 10.3389/fmicb.2019.00375.
14. J. Li, Z.B. Wu, Z. Zhang, J.W. Zha, S.Y. Qu, X.Z. Qi, G.X. Wang, F. Ling, Effects of potential probiotic Bacillus velezensis K2 on growth, immunity and resistance to Vibrio harveyi infection of hybrid grouper (Epinephelus lanceolatus♂ × E. fuscoguttatus♀),Fish Shellfish Immunol., 93, 1047-1055 (2019). 10.1016/j.fsi.2019.08.047.
15. Y. Yi, Z. Zhang, F. Zhao, H. Liu, L. Yu, J. Zha, G. Wang, Probiotic potential of Bacillus velezensis JW: Antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on Carassius auratus, Fish Shellfish Immunol., 78, 322-330 (2018). 10.1016/j.fsi.2018.04.055.
16. M. Ye, C. Wei, A. Khalid, Q. Hu, R. Yang, B. Dai, H. Cheng, Z. Wang, Effect of Bacillus velezensis to substitute in-feed antibiotics on the production, blood biochemistry and egg quality indices of laying hens, BMC Vet Res., 16(1), 400 (2020). 10.1186/s12917-020-02570-6.
17. F. Khalid, A. Khalid, Y. Fu, Q. Hu, Y. Zheng, S. Khan, Z. Wang, Potential of Bacillus velezensis as a probiotic in animal feed: a review, J Microbiol., 59(7), 627-633 (2021). 10.1007/s12275-021-1161-1.
18. L.J. Fooks, G.R. Gibson, In vitro investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens, FEMS Microbiol Ecol., 39(1), 67-75(2002). 10.1111/j.1574-6941.2002.tb00907.x.

19. F.M. Elshaghabee, N. Rokana, R.D. Gulhane, et al., Bacillus as potential probiotics: status, concerns, and future perspectives, Front Microbiol., 81490 (2017). 10.3389/fmicb.2017.01490.
20. M. Ye, X. Tang, R. Yang et al, Characteristics and application of a novel species of Bacillus: Bacillus velezensis, ACS Chem Biol., 13(3), 500-5 (2018). 10.1021/acschembio.7b00874.