ISSN 0026-8933, Molecular Biology, 2024, Vol. 58, No. 1, pp. 157–164. © The Author(s), 2023. This article is an open access publication.

PROTEOMICS

UDC 577.29579.22

The Proteome of Extracellular Membrane Vesicles from *Bacillus pumilus* 3-19

W. Kurdy[a], P. V. Zelenikhin[a], G. Yu. Yakovleva[a], M. N. Sinyagina[a], A. I. Kolpakov[a], and O. N. Ilinskaya[a, *]

[a] *Kazan (Volga-Region) Federal University, Kazan, 420008 Russia*

**e-mail: Ilinskaya_kfu@mail.ru*

Received August 1, 2023; revised September 6, 2023; accepted September 7, 2023

Abstract—Production of extracellular membrane vesicles plays an important role in communication in bacterial populations and in bacteria–host interactions. Vesicles as carriers of various regulatory and signaling molecules may be potentially used as disease biomarkers and promising therapeutic agents, including vaccine preparations. The composition of membrane vesicles has been deciphered for a limited number of Gram-negative and Gram-positive bacteria. In this work, for the first time, extracellular membrane vesicles of a streptomycin-resistant strain *Bacillus pumilus* 3-19, a producer of extracellular guanyl-preferring ribonuclease binase, are isolated, visualized, and characterized by their genome and proteome composition. It has been established that there is no genetic material in the vesicles and the spectrum of the proteins differs depending on the phosphate content in the culture medium of the strain. Vesicles from a phosphate-deficient medium carry 49 unique proteins in comparison with 101 from a medium with the high phosphate content. The two types of vesicles had 140 mutual proteins. Flagellar proteins, RNase J, which is the main enzyme of RNA degradosomes, phosphatases, peptidases, iron transporters, signal peptides, were identified in vesicles. Antibiotic resistance proteins and amyloid-like proteins whose genes are present in *B. pumilus* 3-19 cells are absent. Phosphate deficiency-induced binase was found only in vesicles from a phosphate-deficient medium.

Keywords: extracellular membrane vesicles, *Bacillus pumilus*, genome, proteome, RNase, binase, flagellar proteins, amyloid-like proteins

DOI: 10.1134/S0026893324010059

INTRODUCTION

Most bacteria have the ability to produce spherical membrane nanoparticles with a diameter of 20 to 400 nm, called membrane vesicles (MVs). MVs contain various molecules and perform specific functions, including transport of virulence factors, DNA transfer, interception of bacteriophages, antibiotics and eukaryotic host defense factors, cell detoxification and bacterial communication [1]. For the first time, MVs formed by swelling and bubbling (blebbing) of the outer membrane were discovered in Gram-negative bacteria. Recently, vesicles of Gram-negative bacteria have been described that are formed from both membranes (outer and inner) as a result of cell lysis triggered by endolysin [2]. Gram-positive bacteria also secrete vesicles that are formed from the cytoplasmic membrane, but their study began later, since a significant layer of murein in the cell wall of these bacteria causes low MV production [3].

The study of the composition of MVs is of significant interest, since it reveals the features of the interaction between bacterial cells, as well as the interaction of bacteria with the cells of eukaryotic organisms, including in the pathogen–host system. The composition of the vesicles includes not only the components of the outer membrane, lipopolysaccharides, phospholipids, proteins, but also periplasmic proteins, some cytoplasmic enzymes and nucleic acids. The variable composition of MVs indicates the still unexplored potential of their practical use, in particular, as new biomarkers of diseases, as well as for the creation of non-cellular vaccines.

The proteomic composition of MVs from Gram-negative bacteria *Bacteroides thetaiotaomicron* [4], *Fusobacterium nucleatum* [5], *Haemophilus parasuis* [6] and *Burkholderia multivorans* [7] has been deciphered. A strain was created using synthetic biology methods *Escherichia coli* BL21(DE3)Δ60, producing MVs with a minimized proteome, devoid of 59 endogenous proteins, capable of carrying up to 30% of recombinant proteins, as a base strain for the production of new vaccine preparations [8].

Abbreviations. MV, membrane vesicles; LPM, low phosphate medium; HPM is a high phosphate medium.

The proteome of vesicles of such Gram-positive bacteria as *Staphylococcus aureus* [9, 10], *Streptococcus pneumonia* [11], *Bacillus subtilis* [12], *Granulicatella adiacens* [13] was analyzed.

B. pumilus is a known producer of extracellular ribonuclease (RNase), which has antitumor [14–16] and antiviral properties [17–20]. The production of MVs and their composition in this bacterium are unknown.

This work confirmed the possibility of MV formation by a streptomycin-resistant strain *B. pumilus* 3-19; the composition of these MVs was analyzed. A special task is to compare the proteomes of MVs produced in media with high and low phosphorus content, since phosphate deficiency induces the biosynthesis of extracellular RNases by bacilli [21], which is of practical importance for therapeutic needs. Solving the issue of transfer of antibiotic resistance genes and proteins within MVs is also of fundamental importance.

EXPERIMENTAL

Strain and cultivation conditions. Strain *B. pumilus* 3-19 (GenBank No. HQ650161.1; VKPM V-9862) were maintained on agar medium Luria-Bertani; to analyze vesicles, cells were seeded on glucose-peptone media (two options) of composition (g/L): peptone, 20.0; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.3; NaCl, 3.0; glucose, 10.0 (pH 8.4–8.5). The composition of a low phosphate medium (LPM) included peptone from Semipalatinsk (Russia), whereas high phosphate medium (HPM) contained peptone from the Pan-Eco company (Russia). The strain to obtain MVs was cultivated until it reached the logarithmic phase ($OD_{600} > 2.0$).

Determination of phosphorus concentration in the medium. The phosphorus content in the medium was determined colorimetrically by the formation of phosphorus-molybdenum blue after adding 0.8 mL of sodium molybdate solution (1.8% in 2.5 N sulfuric acid) and 0.2 mL of tin chloride (0.4% in concentrated hydrochloric acid) to 1 mL of medium [22]. Colorimetry was carried out on KFK-2 at a wavelength of 670 nm. The phosphorus content in the medium was determined using the formula: Phosphorus (μg/mL) = $C \times D$, Where C is the phosphate concentration found from the calibration curve, D is the dilution. Monopotassium phosphate containing 0.1 mg of phosphorus in 1 ml was used as a standard solution.

Isolation and purification of membrane vesicles (MVs). MVs were isolated according to a protocol developed for Gram-positive bacteria [9, 12]. Cells were pelleted by centrifugation at 6000 *g* for 20 min, after which the supernatant was filtered through a sterile Minisart cellulose acetate filter (Sartorius Stedim Biotech GmbH, Germany) with a pore size of 100 nm. The sterile filtrate was concentrated 20 times using a Vivacell 100 concentrator (Sartorius Stedim Biotech GmbH). Vesicles were pelleted by ultracentrifugation (100000 *g*, 2 h, 8°C) using an MLA-80 rotor (Beckman Coulter Optima™ MAX-E), the sediment was suspended in buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$). The resulting vesicle suspension was layered onto a stepwise gradient of 10–20–40% Optiprep (Sigma, United States) and separated by ultracentrifugation. Vesicle fractions were washed twice in buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM $MgCl_2$ pH 7.4) and pelleted again by centrifugation. The resulting sediment was resuspended in buffer with the addition of 1 mM PMSF (Fluka, Germany). The absence of cell impurities in the vesicle preparation was checked by microscopy and plating on LB agar medium.

DNA extraction and purification. Total DNA was isolated by phenol–chloroform extraction with pretreatment of samples with DNase I and proteinase K. The amount of isolated DNA was assessed using a Qubit 2.0 fluorometer (Invitrogen, United States).

DNA sequencing. DNA for sequencing on an Illumina Miseq instrument was fragmented using a Covaris S220 ultrasonic fragmenter (Covaris, United States), after which DNA libraries were prepared using the commercial NEBNext Ultra II DNA Library Prep kit for Illumina (NEB, United States). To quantify the libraries, a Qubit 2.0 fluorometer (Invitrogen) was used. The quality of the libraries was assessed using High Sensitivity, 2100 Bioanalyzer chips (Agilent Technologies, United States).

The resulting reads were mapped to the genome assembly of *B. pumilus* 3-19 (GenBank No. HQ650161.1) using Bowtie2 and counted using the featureCounts program [23].

Proteomic analysis of vesicles. Proteomic profiling of MVs was performed using LC/ESI-MS/MS. MB proteins were dissolved in buffer (25 mM Tris-HCl pH 6.8, 5% glycerol, 0.05% bromophenol blue, 1% SDS; 50 mM DTT), centrifuged (12000 *g*, 10 min) and the supernatant containing proteins was collected.

Proteins were separated by one-dimensional electrophoresis on a 12% polyacrylamide gel and stained with Coomassie G-250. Protein fractions were excised from the gel and washed in $ddiH_2O$ (15 min) and mixtures of acetonitrile: 200 mM NH_4HCO_3 (1 : 1) at 50°C for 30 min. A working solution of trypsin (Promega, United States) was added to the gel. Trypsinolysis was carried out at 37°C overnight. To extract peptides from the gel, 20 μl of a 0.5% TFA solution was added to each tube and incubated for 10 min in an ultrasonic bath. Trypsinolysis products were purified on columns (Pierce C18 Tips, Thermo Fisher) according to the manufacturer's instructions and dried in a centrifugal evaporator (Eppendorf, Germany).

MV proteins were identified using gas chromatography–mass spectrometry. Spectra were obtained on a Maxis Impact instrument (Bruker, Germany) equipped with a Dionex Ultimate 3000 Series HPLC

system (Thermo Fisher Scientific, United States). Samples were dissolved in a mixture of methanol (1%), formic acid (0.1%) and water (98.9%) (v/v), applied to an Acclaim PepMap RSLC column (Thermo Fisher Scientific) and eluted for 3 h, increasing the mixture content of acetonitrile (99.9%) and formic acid (0.1%) from 2 to 60%.

Mass spectra (MS1) were obtained with the following parameters: detection of molecular ions in the range of 300–2000 m/z and signal accumulation time 250 ms. To obtain MS2 spectra, ions with a signal-to-noise ratio of at least 400 and a charge from 2 to 5 were selected. Ion detection was carried out in the range of 200–2000 m/z, signal accumulation time was 50 ms for each parent ion. The measurement accuracy was 0.6 Da. The obtained MS/MS spectra were analyzed using the MASCOT program (Matrix Science, Inc.). Protein identification was considered reliable when at least two peptides with different amino-acid sequences with a PEPscore value ≥ 14 were detected.

Atomic force microscopy. Cell culture samples concentrated by centrifugation (5000 *g*, 15 min), applied to a glass slide and dried at room temperature. Bacteria were visualized in air at room temperature and constant humidity in semi-contact and contact modes on a Solver P47H atomic force microscope (manufactured by NT-MDT, Russia) [24]. We used standard silicon cantilevers, the radius of curvature of the tip of which did not exceed 10 nm. Scanning was performed with a resolution of 512 × 512 pixels.

Transmission electron microscopy. Samples were prepared using the standard method [25]. Vesicles were fixed for 16 hours in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2–7.4 at 4°C, centrifuged at 35000 *g* (8°C, 70 min), saturated with 1% OsO_4 vapor, for dehydration, the sediment was washed with alcohol and acetone. Propylene oxide was added to the sediment, incubated for 30 min, and then the sediment was impregnated with epon resin. The blocks obtained after polymerization were cut and transmission electron microscopy was performed (JEM-100 CX microscope, Japan).

Statistics and bioinformatics. To predict the subcellular localization of proteins from MV bacteria grown on LPM and HPM, we used their amino-acid sequences and the PSORTb v3.0.3 software, available online at https://www.psort.org/psortb/ [26]. Sequences were also used to assign K (KO) numbers to proteins and for their functional annotations using BlastKOALA software on the KEGG (Kyoto Encyclopedia of Genes and Genomes) website (https://www.kegg.jp/blastkoala/) [27]. Microsoft Excel 2019 from the microsoft office package was used to calculate the level and statistical significance of changes between the MV proteins of bacteria grown on LPM and HPM, for the purpose of their subsequent graphical representation in accordance with the VolcaNoseR software (https://huygens.science.uva.nl/VolcaNoseR/) [28].

RESULTS AND DISCUSSION

Formation and Characteristics of MVs

Under conditions of phosphate starvation, the synthesis of extracellular guanyl-preferring RNases is activated in bacteria, which confirms the participation of these enzymes in the extraction of nutrients from hard-to-reach substrates [20]. To identify the possible secretion of RNases into MVs, we chose two variants of bacterial cultivation media: HPM, in which the phosphorus content was 82 ± 8 μg/mL, and LPM, containing 20 ± 2 μg/mL phosphorus, taking into account that there is practically no secretion of RNases on the HPM medium. The formation of MVs, which are spherical formations 80–120 nm in size, was detected during bacterial growth on both media (Fig. 1).

The maximum number of genes identified in *B. pumilus* is 3679 (Gen Bank No. CP054310.1). We sequenced DNA fragments of bacteria grown in LPM medium and identified 3507 genes, of which 686 encode hypothetical proteins (Table S1, see Supplementary Information). Some differences in the number of identified genes are associated with bacterial DNA methylation patterns, technical features of the sequencing process, and imperfect genome assembly, which varies depending on cultivation conditions [29]. A preliminary genome study was carried out on *B. pumilus*, aimed at studying the possible existence of any genes into MVs. Previously, it was believed that MVs of Gram-positive bacteria are characterized by the absence of genetic material [30]. Although recently the presence of DNA in vesicles has been recorded *Clostridium perfringens* [31], *S. mutans* [32], in MVs of *B. subtilis* no DNA was detected [12]. In vesicle fractions of *B. pumilus* 3-19 we were also unable to detect DNA. Thus, MVs of *B. pumilus* 3-19 are lipoprotein complexes that are not involved in DNA transfer.

Functional Analysis of the MV Proteome

Proteomic analysis showed 189 proteins in vesicles of *B. pumilus* grown on LPM medium. The proteome of bacterial vesicles grown on HPM revealed 241 proteins. As can be seen from the Venn diagram "(Fig. 2), both types of vesicles contain 140 mutual proteins (listed in Table S2, see Supplementary Information).

The identified proteins were classified into functional groups using BLAST analysis. It was found that in vesicles from cells grown on LPM medium, the number of proteins whose functions are associated with the biosynthesis and metabolism of lipids, amino acids, glycans, as well as with the processing of genetic and environmental information increased. The pro-

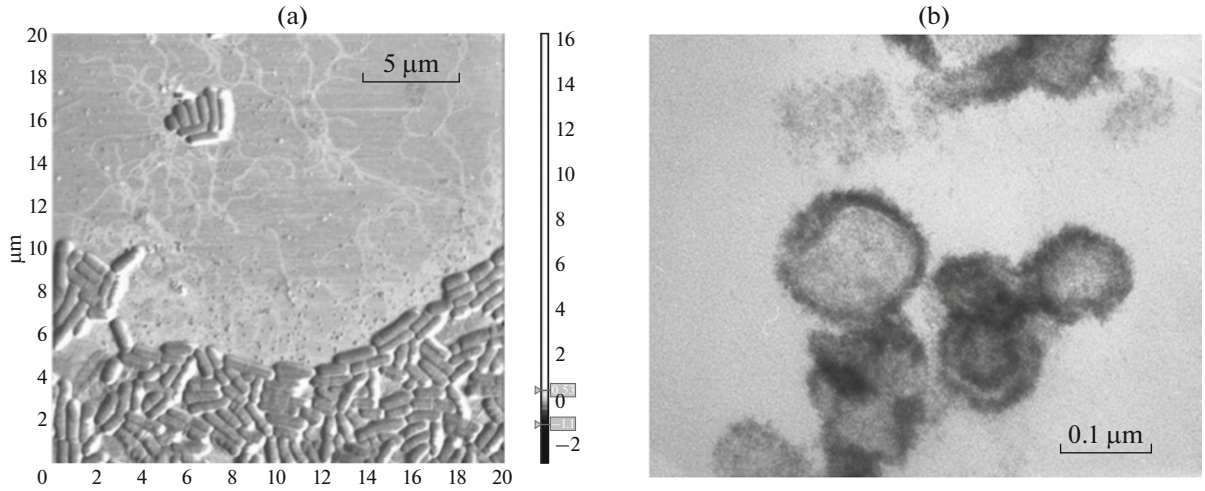

Fig. 1. Visualization of the extracellular vesicles of *Bacillus pumilus* 3-19 using atomic force (a) and transmission electronic (b) microscopy.

portion of proteins involved in energy and carbohydrate metabolism, on the contrary, decreased (Fig. 3).

Protein localization analysis showed that vesicles from bacteria grown on LPM medium included more proteins annotated as extracellular and associated with the cytoplasmic membrane compared to vesicles from HPM, which contained more cytoplasmic proteins (Fig. 4).

RNase was detected in vesicles of bacteria cultured on LPM medium (no. 34, Table S2, see Supplementary Information). As shown by BLAST analysis, the amino-acid sequence of this protein is completely identical to the sequence of extracellular RNase binase (Gen Bank No CAA37735.1). This protein is absent in the vesicles of bacteria grown on HPM medium. Thus, we have shown for the first time that on a low phosphorus medium *B. pumilus* 3-19 secretes binase as part of MVs (Fig. 5a).

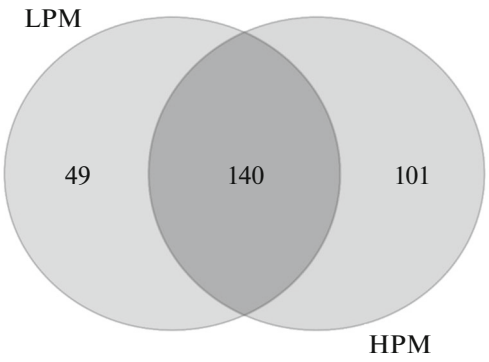

Fig. 2. A Venn diagram showing the number of proteins in vesicles obtained by culturing *B. pumilus* 3-19 on LPM and HPM.

RNase J, the main enzyme of the RNA degradosome, plays an essential role in global mRNA decay. This enzyme was identified in the composition of MVs produced by cells growing in both LPM and HPM media (Fig. 5a).

For limited movement (gliding) on moist agar surfaces, many bacilli use filamentous outgrowths called fimbriae (pili). Individual cells in populations move at speeds from 76 to 116 μm/s [33]. The transition from motility to biofilm formation involves inhibition of fimbriae gene transcription; in the absence of fimbriae de novo synthesis they disappear, which is accompanied by stabilization of the biofilm [34]. We grew bacteria in a liquid medium where biofilm did not form and were able to visualize the fimbriae using atomic force microscopy (Fig. 1a).

We found fimbriae proteins in MVs of *B. pumilus*. Since these proteins are present in motile flagellated bacteria, they are classified as flagellar. In the genome of *B. pumilus* 3-19 there are 33 genes encoding flagellar proteins. The level of the FlgG (Flagellar basal body rod protein) protein is increased in the MVs of bacteria grown on LPM medium, while the level of the flagellar motor protein MotA is increased in the vesicles of bacteria grown on HPM. FlgK and flagellin proteins are present in equal amounts in MVs of both types (Fig. 5b).

In bacteria, filamentous structures are part of the extracellular matrix, which surrounds the cell and thereby provides protection from unfavorable environmental conditions. Similar structures, which have been well studied in Enterobacteriaceae [35], consist of so-called functional amyloids or, more correctly, amyloid-like proteins, which have a constructive function in contrast to amyloid fibers associated with diseases. Amyloid-like proteins are involved in the formation of biofilms, fimbriae, and cell adhesion. These

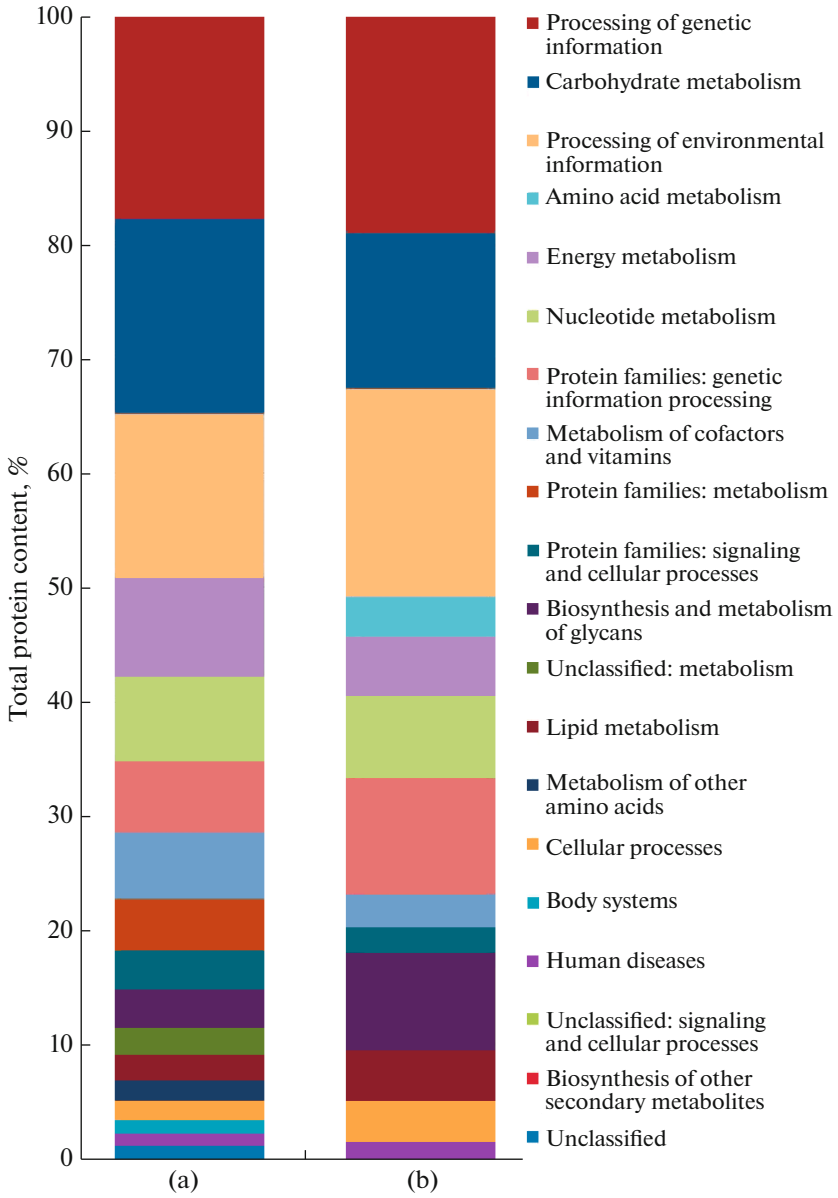

Fig. 3. Groups of proteins with known functions in membrane vesicles of *B. pumilus* 3-19 obtained by cultivating the strain on HPM media (a) and LPM (b). (BlastKOALA Software).

proteins have also been described in Gram-positive bacteria *Streptomyces coelicolor* and *B. subtilis* [36].

The amyloid-like protein TasA is the main matrix protein in biofilms *B. subtilis* [37, 38]. The *TasA* gene nucleotide sequences have been identified from *B. subtilis, B. velezensis,* and *S. pneumoniae,* as well as *B. pumilus* (https://www.ncbi.nlm.nih.gov/nuccore/KC576815.1). Genome Analysis of *B. pumilus* 3-19 identified the gene for the amyloid-like protein TapA (No. 2767, amyloid fiber anchoring/assembly protein, table S1, see Supplementary Information), which serves as a primer for TasA oligomerization. However, this protein is not part of the vesicles.

Thus, in this work, the formation of extracellular MVs was detected for the first time in *B. pumilus* 3-19 and their composition was determined. It has been established that MVs do not contain genetic material and differ in the spectrum of proteins, which depends on the phosphate content in the strain cultivation medium. MVs carry 140 mutual proteins; 49 unique proteins were identified in vesicles of cells grown in LPM medium, and 101 in HPM medium. Flagellar proteins were found in the vesicles, but antibiotic resistance proteins and amyloid-like proteins, whose genes are present in the cells, were not found in MV of *B. pumilus* 3-19. Both types of vesicles contain RNase J, the main enzyme of the RNA degradosome.

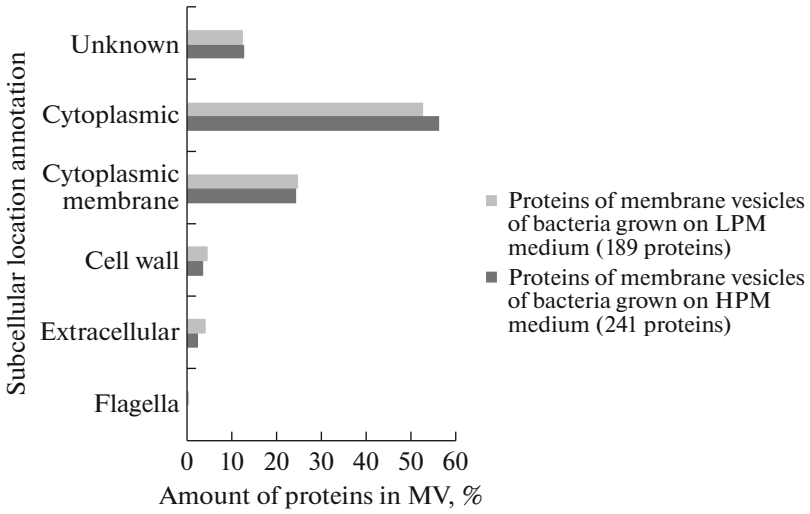

Fig. 4. Localization of MV proteins *B. pumilus* 3-19 grown on LPM and HPM (PSORTb v3.0.3 software). The number of proteins in the vesicles of each fraction is taken as 100%.

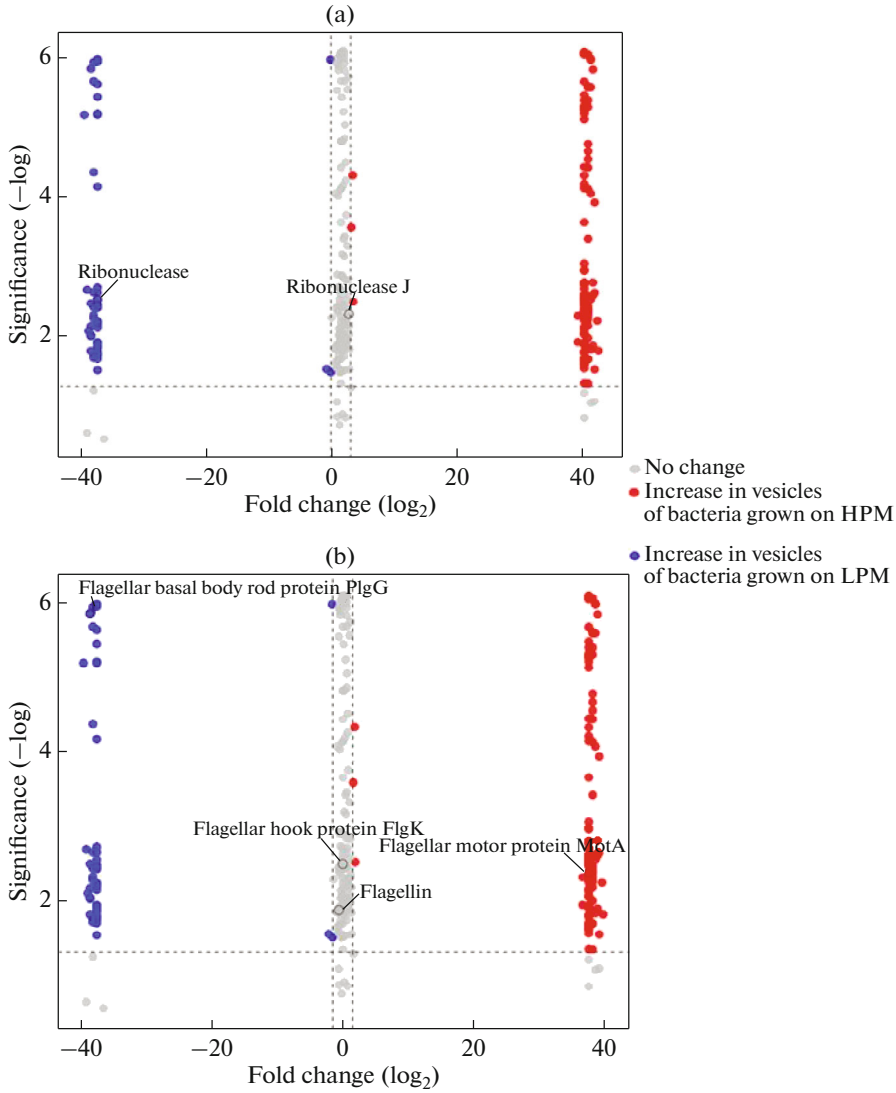

Fig. 5. Comparison of the protein content ((a) RNases, (b) flagellar) in MV of *B. pumilus* 3-19, cultured on LPM and HPM (VolcaNoseR software).

Secreted RNase binase whose synthesis is induced by phosphate deficiency, is part of MV cells cultured in LPM, but not HPM, media.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S0026893324010059

ACKNOWLEDGMENTS

The authors thank the director of the Interdisciplinary Center for Proteomic Research of KFU T.V. Grigorieva for assistance in the work.

FUNDING

The work was carried out within the framework of the Priority 2030 program and supported by a grant from the Russian Science Foundation (No. 22-24-00036).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

OPEN ACCESS

REFERENCES

1. Toyofuku M., Nomura N., Eberl L. 2019. Types and origins of bacterial membrane vesicles. *Nat. Rev. Microbiol*. **17**, 13–24.
2. Vitse J., Devreese B. 2020. The contribution of membrane vesicles to bacterial pathogenicity in cystic fibrosis infections and healthcare associated pneumonia. *Front. Microbiol*. **11**, 630.
3. Cao Y., Lin H. 2021. Characterization and function of membrane vesicles in Gram-positive bacteria. *Appl. Microbiol. Biotechnol*. **105** (5), 1795–1801.
4. Stentz R., Jones E., Juodeikis R., Wegmann U., Guirro M., Goldson A.J., Brion A., Booth C., Sudhakar P., Brown I.R., Korcsmáros T., Carding S.R. 2022. The proteome of extracellular vesicles produced by the human gut bacteria *Bacteroides thetaiotaomicron* in vivo is influenced by environmental and host-derived factors. *Appl. Environ. Microbiol*. **88** (16), e0053322.
5. Liu J., Hsieh C.L., Gelincik O., Devolder B., Sei S., Zhang S., Lipkin S.M., Chang YF. 2019. Proteomic characterization of outer membrane vesicles from gut mucosa-derived *Fusobacterium nucleatum*. *J. Proteomics*. **195**, 125–137.
6. Zhang K., Chu P., Song S., Yang D., Bian Z., Li Y., Gou H., Jiang Z., Cai R., Li C. 2021. Proteome analysis of outer membrane vesicles from a highly virulent strain of *Haemophilus parasuis*. *Front. Vet. Sci*. **8**, 756764.
7. Terán L.C., Distefano M., Bellich B., Petrosino S., Bertoncin P., Cescutti P., Sblattero D. 2020. Proteomic studies of the biofilm matrix including outer membrane vesicles of *Burkholderia multivorans* C1576, a strain of clinical importance for cystic fibrosis. *Microorganisms*. **8** (11), 1826.
8. Zanella I., König E. Tomasi M., Gagliardi A, Frattini L., Fantappiè L., Irene C., Zerbini F., Caproni E., Isaac S.J., Grigolato M., Corbellari R., Valensin S., Ferlenghi I., Giusti F., Bini L., Ashhab Y., Grandi A., Grandi G. 2021. Proteome-minimized outer membrane vesicles from *Escherichia coli* as a generalized vaccine platform. *J. Extracell Vesicles*. **10** (4), e12066.
9. Lee E.Y, Choi D.Y., Kim D.K. 2009. Gram-positive bacteria produce membrane vesicles: Proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles. *Proteomics*. **9** (24), 5425–5436.
10. Bitto N.J., Cheng L., Johnston E.L., Pathirana R., Phan T.K., Poon I.K.H., O'Brien-Simpson N.M., Hill A.F., Stinear T.P., Kaparakis-Liaskos M. 2021. *Staphylococcus aureus* membrane vesicles contain immunostimulatory DNA, RNA and peptidoglycan that activate innate immune receptors and induce autophagy. *J. Extracell. Vesicles*. **10** (6), e12080.
11. Olaya-Abril A., Prados-Rosales R., McConnell M.J., Martín-Peña R., González-Reyes J.A., Jiménez-Munguía I., Gómez-Gascón L., Fernández J., Luque-García J.L., García-Lidón C., Estévez H., Pachón J., Obando I., Casadevall A., Pirofski L.A., Rodríguez-Ortega M.J. 2014. Characterization of protective extracellular membrane-derived vesicles produced by *Streptococcus pneumoniae*. *J. Proteomics*. **106**, 46–60.
12. Kim Y., Edwards N., Fenselau C. 2016. Extracellular vesicle proteomes reflect developmental phases of *Bacillus subtilis*. *Clin. Proteomics*. **13**, 6.
13. Alkandari S.A., Bhardwaj R.G., Ellepola A., Karched M. 2020. Proteomics of extracellular vesicles produced by *Granulicatella adiacens*, which causes infective endocarditis. *PLoS One*. **15** (11), e0227657.
14. Ilinskaya O.N., Mahmud R.S. 2014. Ribonucleases as antiviral agents. *Mol. Biol*. **48** (5), 615–623.

15. Shah Mahmud R., Efimova M.A., Ulyanova V., Ravilov R.K., Shuralev E.A., Kolpakov A., Ilinskaya O. 2020. *Bacillus pumilus* ribonuclease rescues mice infected by double-stranded RNA-containing reovirus serotype 1. *Virus Res*. **286**, 198086.

16. Ulyanova V., Shah Mahmud R., Laikov A., Dudkina E., Markelova M., Mostafa A., Pleschka S., Ilinskaya O. 2020. Anti-influenza activity of the ribonuclease binase: Cellular targets detected by quantitative proteomics. *Int. J. Mol. Sci*. **21** (21), 8294.

17. Garipov A.R., Nesmelov A.A., Cabrera-Fuentes H.A., Ilinskaya O.N. 2014. *Bacillus intermedius* ribonuclease (BINASE) induces apoptosis in human ovarian cancer cells. *Toxicon*. **92**, 54–59.

18. Mitkevich V.A., Kretova O.V., Petrushanko I.Y., Burnysheva K.M., Sosin D.V., Simonenko O.V., Ilinskaya O.N., Tchurikov N.A., Makarov A.A. 2013. Ribonuclease binase apoptotic signature in leukemic Kasumi-1 cells. *Biochimie*. **95** (6), 1344–1349.

19. Ilinskaya O.N., Singh I., Dudkina E., Ulyanova V., Kayumov A., Barreto G. 2016. Direct inhibition of oncogenic KRAS by *Bacillus pumilus* ribonuclease (binase). *Biochim. Biophys. Acta*. 1863 (7 Pt A), 1559–1567.

20. Faizullin D., Valiullina Y., Salnikov V., Zelenikhin P., Zuev Y., Ilinskaya O. 2023. Fibrin-rhamnogalacturonan I composite gel for therapeutic enzyme delivery to intestinal tumors. *Int. J. Mol. Sci*. **24** (2), 926.

21. Kharitonova M.A., Kolpakov A.I., Kupriyanova-Ashina F.G. 2018. Intensification of secreted Bsn ribonuclease *Bacillus subtilis* production under salt stress. *Vestn. Biotekhnol. Fiz.-Khim. Biol. im. Ovchinnikova*, **14** (2), 42–47.

22. Arinushkina E.V. 1970. *Rukovodstvo po khimicheskomu analizu pochv* (Guide to the Chemical Analysis of Soils). Moscow: Mosk. Gos. Univ.

23. Liao Y., Smyth G.K., Shi W. 2014. featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. **30** (7), 923–930.

24. Konovalova O.A., Yakovleva G.Yu., Steryakov O.V., Trushin M.V. 2013. Scanning probe microscopy in the study of morphometric changes and physical parameters of *Escherichia coli* bacteria under the action of 2,4,6-trinitrotoluene. *W. Appl. Sci. J*. **23** (4), 507–509.

25. Chernov V.M., Chernova O.A., Mouzykantov A.A., Efimova I.R., Shaymardanova G.F., Medvedeva E.S., Trushin M.V. 2011. Extracellular vesicles derived from *Acholeplasma laidlawii* PG8. *Sci. World J*. **11**, 1120–1130.

26. Yu N.Y., Wagner J.R., Laird M.R., Melli G., Rey S., Lo R., Dao P., Sahinalp S.C., Ester M., Foster L.J., Brinkman F.S.L. 2010. PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics*. **26** (13), 1608–1615.

27. Kanehisa M., Sato Y., Morishima K. 2016). BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol*. **428**, 726–731.

28. Goedhart J., Luijsterburg M.S. 2020. VolcaNoseR is a web app for creating, exploring, labeling and sharing volcano plots. *Sci. Rep*. **10**, 20560.

29. Adhikari S., Curtis P.D. 2016. DNA methyltransferases and epigenetic regulation in bacteria. *FEMS Microbiol. Rev*. **40** (5), 575–591.

30. Dorward D.W., Garon C.F. 1990. DNA is packaged within membrane-derived vesicles of Gram-negative but not Gram-positive bacteria. *Appl. Environ. Microbiol*. **56** (6), 1960–1962.

31. Jiang Y., Kong Q., Roland K.L., Curtiss R. 2014. Membrane vesicles of *Clostridium perfringens* type A strains induce innate and adaptive immunity. *Int. J. Med. Microbiol*. **304** (3–4), 431–443.

32. Liao S., Klein M.I., Heim K.P., Fan Y, Bitoun J.P., Ahn S.J., Burne R.A., Koo H, Brady L.J., Wen Z.T. 2014. *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. *J. Bacteriol*. **196** (13), 2355–2366.

33. Mendelson N.H., Bourque A., Wilkening K., Anderson K.R., Watkins J.C. 1999. Organized cell swimming motions in *Bacillus subtilis* colonies: Patterns of short-lived whirls and jets. *J. Bacteriol*. **181** (2), 600–609.

34. Guttenplan S.B., Kearns D.B. 2013. Regulation of flagellar motility during biofilm formation. *FEMS Microbiol. Rev*. **37** (6), 849–871.

35. Barnhart M.M., Chapman M.R. 2006. Curli biogenesis and function. *Annu. Rev. Microbiol*. **60**, 131–147.

36. Dragoš A., Kovács Á.T., Claessen D. 2017. The role of functional amyloids in multicellular growth and development of Gram-positive bacteria. *Biomolecules*. **7** (3), 60.

37. Malishev R., Abbasi R., Jelinek R., Chai L. 2018. Bacterial model membranes reshape fibrillation of a functional amyloid protein. *Biochemistry*. **57** (35), 5230–5238.

38. Böhning J., Ghrayeb M., Pedebos C., Abbas D.K., Khalid S., Chai L., Bharat T.A.M. 2022. Donor-strand exchange drives assembly of the TasA scaffold in *Bacillus subtilis* biofilms. *Nat. Commun*. **13** (1), 7082.