Molecular and Cell Biotechnologies

ISSN 1993-6842 (on-line); ISSN 0233-7657 (print)
Biopolymers and Cell. 2023. Vol. 39. N 2. P 131–145
http://dx.doi.org/10.7124/bc.000A8D

UDC576+579.6

Komagataeibacter oboediens changes outer membrane vesicle-associated activities after exposure on the International Space Station

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Outer membrane vesicles (OMVs) carry various biomolecule cargo, including enzymes, and communicate with the surrounding via them. The role of the enzyme-associated OMVs from food-grade bacteria in human health and disease is not clear, in particular, after the impact of stressful factors (changed gravity and atmosphere, UV-radiation) in the context of astronauts' diet. **Aim.** We researched OMVs of *Komagataeibacter oboediens* isolated from kombucha microbial community samples exposed to the space/Mars-like stressors simulated on the International Space Station and cultivated within a five-year period to know more about their behaviour with biomolecules and mammalian cells. **Methods.** A series of *in vitro* experiments on the RNA cleavage, DNA and RNA transcription inhibition and cytotoxic assay with OMVs/Komagataeibacter were conducted. SignalP 6.0 was used for detecting signal peptides in predicted ribonucleases in *K. oboediens* genome, and ProtComp Version 9 served for identifying RNase sub-cellular location. **Results.** The behaviour of OMVs in bacteria before and after an impact of stressful conditions differed, relying on differences in associated nucleolytic activity, the inhibitory capabilities against the T7 bacteriophage RNA polymerase and *Taq* DNA polymerases and a cytotoxic effect, despite identical nucleotide sequences in homologous genes. The *in vitro* inhibition of RNA and DNA transcriptions was less pronounced in OMVs of exposed bacteria than in vesicles of the ground-based strain. It correlated with a lower RNase activity and a loss of cytotoxicity towards human malignant cells, even five years after the flight. Bacterial RNase I was predicted to be located in the OMV periplasm. **Conclusion.** Komagataeibacter's OMV-associated activities were modified after exposure to the International Space Station and inherited in a non-genetic manner.

K e y w o r d s: Outer membrane vesicles, stressful factors, RNase I.

Introduction

Bacteria produce a big variety of bioactive molecules and contribute them to human host physiology, in particular, to the immune system regulation and the gut–brain axis. A portion of them is enclosed in extracellular membrane vesicles. All living cells within three domains — Archaea, Bacteria and Eukaria — produce nano-scale membranous vesicles, which are described as: 'particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate' [1]. The extracellular vesicles from Gram-negative bacteria are referred to as outer membrane vesicles (OMVs). Their main function is to provide the communications between bacterial cells within a population or between different populations and domains of living organisms [2-4]. The interactions between microorganisms and host macroorganisms via OMVs are mediated largely by microbe-associated molecular patterns of the cell envelope, such as lipopolysaccharide and peptidoglycan; in addition, OMVs may carry nucleic acids, small bioactive metabolites, lipids and proteins involved in communications [5]. Recent research shows the interest raised in the enzymes associated with membrane vesicles in human pathophysiology, *e.g.,* OMVs-associated DNases are involved in the degradation of DNA backbones of extracellular traps used as the phagocytosis-independent defense system against pathogens [6], secretion of antibiotic degrading enzymes via OMVs enabling their survival [7] or triggering immunological responses in the respiratory epithelium [8]. However, less is known about the role of enzyme-associated OMVs from generally recognized as safe, food-grade bacteria in the human body [9]. Gram-negative bacteria of the *Komagataeibacter* genus are known to synthesize cellulose for a multi-species microecosystem — kombucha microbial community (KMC) composed of bacteria and yeasts [10, 11]. Being a dominant core KMC-member [12], these bacteria and their OMVs are present in fermented live kombucha drinks. Komagataeibacters are health-promoting agents, at least, as containing valuable antioxidants and unique lipids considered to improve cognitive function [13] and body's hydration [14]. The enzyme-bearing komagataeibacters' OMVs can work as the nanobioreactors when being consumed as a healthy kombucha drink and interact with exogenous nucleic acids, proteins, host cell nanovesicles, viruses, as well as gut microbiota in the human body microenvironments. The OMVs of beneficial health-promoting bacteria could be considered reasonable in the prophylaxis of diseases [15] and helpful for astronauts during long-duration space missions [16, 17]. OMVs assembled with diverse molecular cargo call for an interest in using them as therapeutics/vaccines and for diagnostic purposes [5, 18, 19]. The essential activity of bacterial OMVs is to transfer biomolecules to particular targets and serve as a drug-delivery tool [20].

In this study, we researched the activities associated with OMVs of *K. oboediens* isolated from KMC samples after five years since the exposure of the latter to the space/Mars-like stressors (UV, atmosphere, pressure) on the International Space Station (ISS) [21]. We showed that the OMVs exhibited differences in *in vitro* communications. OMVs of the ex-

posed bacteria did not exhibit toxicity toward human *in vitro* cells, showed a lower ribonucleolytic activity and decreased inhibition of transcriptase activities *in vitro*. These data will help to elucidate to what extent the stressors can impact changes in the OMVs and how changed vesicles may influence human health and microbiota in the distant future.

Materials and Methods

Bacterial strains

Strains of *K. oboediens* IMBG180 isolated from reference ground-based KMC specimens, *K. oboediens* IMBG185 — from UV-irradiated top-located KMC, *K. hansenii* IMBG183 — from UV-protected bottom-located KMC and *K. hansenii* IMBG184 — from a protected middle-located KMC on the ISS [22]. These strains were cultured in HS medium [23] at 28 °C for three days for OMVs isolation. After returning from the ISS, the re-isolated from KMC samples bacteria were maintained on HS agar medium (1 %) (Sigma-Aldrich, USA) being re-cultured monthly within a 5-year postflight period. *Escherichia coli* DH5α (Thermo Fisher Scientific, Lithuania), *E. coli* S-17 (kindly provided by Prof. A. Puehler, Germany) and *Serratia marcescens* IMBG291 (the collection of the Institute of Molecular Biology and Genetics, Kyiv, Ukraine) were cultured in LB medium (Sigma-Aldrich, USA) at 37 °C overnight.

Extracellular membrane vesicles isolation and visualization

The 3-day komagataeibacter cultures, derived from KMCs, and the overnight cultures of *E. coli* S-17 and *S. marcescens* were centrifuged at 17,000 rpm for 20 min at 4 ºC. The supernatants were collected and further ultracentrifuged at 100,000 g for 1 h at 4 ºC (Beckman Instruments Inc., L8M, rotor 55.2 Ti). The resulting pellets were resuspended in filtered (0.20 μ pore size filter; Minisart, Sartorius, Germany) phosphate-buffered saline (PBS), pH 7.4. The vesicle sizes were determined using Nanosight (NS300, Malvern Pananalytical, UK) and transmission electron microscope JEM-1400 (Jeol Inc., Japan). Zeta potential determination was performed using a Zetasizer Nano S (Malvern®Instruments, UK) at 25 °C. For this, the samples were irradiated with a helium-neon laser with $\lambda = 633$ nm, and the scattered light was recorded at an angle of 173°. Chromatography columns (qEVORIGINAL, IZON, France) were used to purify and concentrate OMV preparations. In this paper, the membrane vesicles from exposed on the ISS bacteria are designated as E-OMVs.

The electrophoretic mobility shift (EMS) assay

The interaction of the OMVs with the pTZ19R/*Eco*RI DNA (Fermentas, Lithuania) was indicated by slower electrophoretic mobility of their complexes in the agarose gel as described in [21].

Isolation of plasmid DNA and total RNA

Plasmid pTZ19R DNA was isolated from *E. coli* DH5α using the innuPREP Plasmid Mini Kit 2.0 (Analytik Jena AG, Germany). Total RNA extraction from *E. coli* DH5α was performed using TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA purity was assessed by measuring

the 260 nm/280 nm ratio, and a ratio between 1.8 and 2.1 was considered pure.

RNA degradation assay

Extracellular vesicles (5 mg mL-1) were suspended in PBS (pH 7.4), filtered (0.20 μ), added to 300 ng of bacterial RNA, and a final mixture was incubated for 16 h at 37 °C in the DEPC-treated water. After incubation, the samples were separated by electrophoresis in 1.2 % agarose gel in Tris-acetate-EDTA buffer (pH 7.0). The stained gels were visualized under UV light. RNA band intensity was measured in an agarose gel and compared with the ImageJ software (https://imagej.net/).

Design and synthesis of primers

Oligonucleotides were designed as a pair of single-strand oligomers complementary to the promoter region of pTZ19R (pTZrepF1, 5'-TCTCTTACTGTCATGCCATCCG-3'; pTZrepR1, 5'-ATTTCCGTGTCGCCCTTAT-3') and purchased in LLC "TRADING HOUSE BIOLABTECH" (Kyiv, Ukraine). Single-strand DNAs were incubated with linear DNA (pTZ19R/*Eco*RI) in the presence of OMVs or E-OMVs or without pTZ19R at 37 ºC for 16 h.

In vitro T7 bacteriophage RNA polymerase activity assay

In vitro transcription assay with the T7 RNA polymerase was performed as described in [24]. In brief, the reaction mixture (20 μL) contained the *Eco*RI-linearized DNA template (0.5 μg of pTZ19R plasmid DNA with a 341 bp insert cloned in the Ecl136II site), ribonucleoside triphosphates (2 mM), a ribonuclease inhibitor (10 U), the T7 RNA polymerase (12 U) in the presence of Tris-HCl (40 mM, pH 7.9), $MgCl_2$ (6 mM), spermidine (2 mM), NaCl (10 mM) and dithiothreitol (10 mM). OMVs of different concentrations (125.0; 62.5; 31.25 μg/mL) were added to the reaction mixtures, and these mixtures were incubated at 37 ºC for 1 h. Then the reaction was stopped by cooling up to –20 ºC, and the products were separated by electrophoresis in 1.2 % agarose gel in Tris-acetate-EDTA buffer (pH 7.0) supplemented with ethidium bromide (0.5 μg mL^{-1}) and visualized using UV-transilluminator.

DNA polymerase activity assay

The PCR mix (final volume 20 μL) contained 10 pmol of each primer, 20 ng of template DNA, OMVs of different concentrations (125.0; 62.5; 31.25 μg/mL), DNA polymerase and the BioMix buffer (Neogen, Ukraine). Amplification was carried out with a Thermal Cycler T-CY (CreaCon Technologies, The Netherlands) equipped with a heated lid. The PCR conditions were as follows: denaturing at 95 °C for 5 min, denaturing at 94 °C for 45 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 60 s. The last three steps were repeated 30 times, and the final elongation was performed at 72 °C for 10 min. The amplicons were separated by electrophoresis in 1.2 % agarose gel in Tris-acetate-EDTA buffer (pH 7.0). The stained gels were visualized under UV light.

Cytotoxicity assay

Human *in vitro* cells K-562 (chronic myeloid leukemia, collection of IMBG NASU) were harvested by centrifugation and suspended at lg5 cells mL^{-1} in a complete medium. Fifty-μL aliquots of the cell suspension were transferred

in duplicate to the 96-well microtitre plate, containing diluted OMV samples (2 mg/mL) (total volume, 100 μL). As a positive control, amitozin (1 mg mL-1) [25)] kindly provided by Dr. A. Potopalsky (IMBG of NAS of Ukraine, Kyiv) was used, as a negative control, PBS (pH 7.4) served. Cytotoxicity test was performed using the MTT reduction assay [26]. The optical density of each well was measured with a microplate spectrophotometer (Multiscan Tirertek MMC 340, USA) equipped with a 540 nm filter.

Bioinformatic analysis of K. oboediens genomes on the presence of nucleolytic enzymes associated with OMVs and E-OMVs

Nuclease-like (ribonuclease) gene reads from complete bacterial genomes (the draft genomes are deposited in GenBank® (NIH) with accession numbers SAMN14942824 and SAMN14942470, respectively, for *K. oboediens* IMBG180 from the reference KMC and *K. oboediens* IMBG185 from the top-space-exposed KMC, were BLAST-searched against GenBank collection. SignalP 6.0 was used for detecting signal peptides (short amino acid sequences that control protein secretion and translocation) in predicted nucleases that provide spanning proteins from cytoplasm to periplasm, *i.e.,* the space between the inner cytoplasmic membrane and the bacterial outer membrane [27]. The ProtComp v. 9 (http://www.softberry.com/) predicted sub-cellular location of Komagataeibacter's ribonucleases.

Statistical tests

The results were expressed as mean ± standard deviation (SD) of independent experiments performed in triplicate. The difference between groups was compared by a two-tailed Student's t-test. The differences were considered to be significant, if $P \leq 0.05$.

Results and Discussion

The morphology and zeta potential were different in OMVs/IMBG180 and E-OMVs/IMBG185. OMVs from both strains exhibited different average mean sizes of ~ 80 nm and 100 nm, respectively (Fig. 1A, B), as showed nanoparticle tracking analysis and transmission electron microscopy. The size shift retained after a long period corresponds to the earlier observation in the postflight IMBG185 E-OMVs. The E-OMVs demonstrated the same shape deformation and tendency to aggregation after a 5-year postflight period (Fig. 1B) as it was determined after finishing the spaceflight, in contrast to the vesicles of *K. oboediens* IMBG180, which were stiffer (Fig. 1A). The zeta potential (the potential difference existing between the surface of a particle immersed in a conducting liquid and the bulk of the liquid) of OMVs/IMBG180 was — 5.5 mV, and E-OMVs/IMBG185 exhibited — 1.7 mV.

The ribonucleolytic activity has been exhibited in OMVs of K. oboediens IMBG180, and its change after the spaceflight was shown by electrophoresis of products after the incubation of bacterial RNA with OMVs (Fig. 2). The E-OMVs of top-located *K. oboediens* IMBG185 exhibited a lower RNase activity (Fig. 2, lane 5) compared to the homologous ground-based reference strain IMBG180 (Fig. 2, lane 4).

Interaction of OMVs with a double-stranded linear DNA (dsDNA), a linearized plasmid DNA (pTZ19R/*Eco*RI). The linear dsDNA

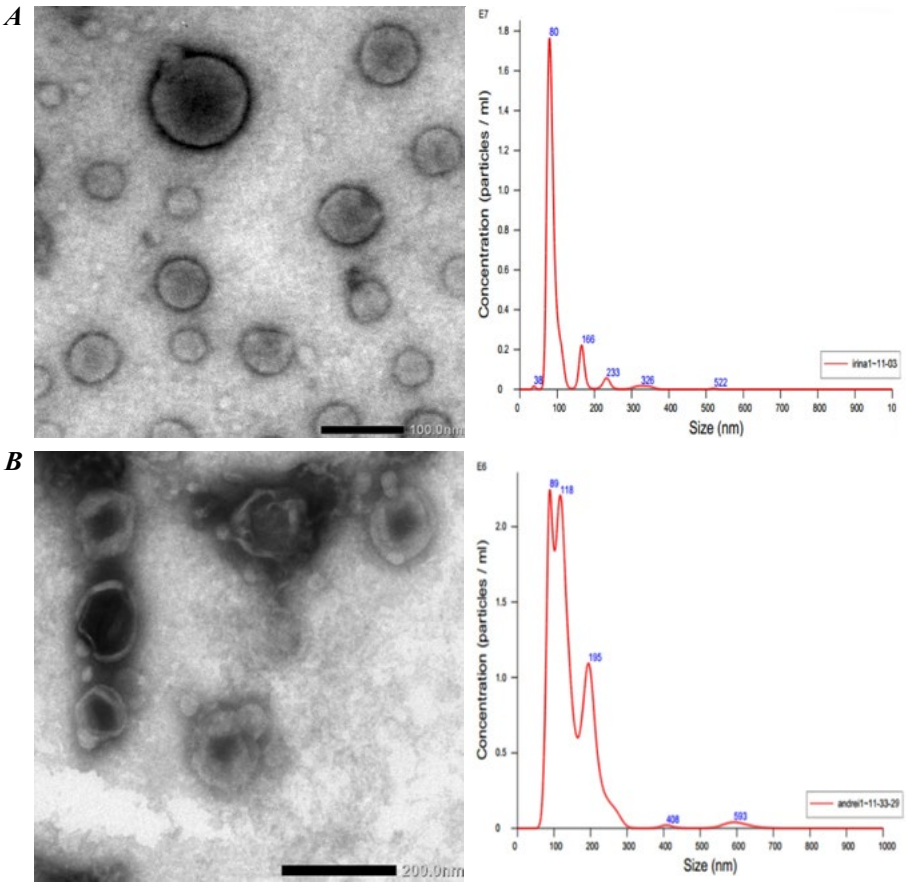

Fig. 1. Transmission electron microscopic images of the morphology of outer membrane vesicles from *Komagataeibacter oboediens* IMBG180 (ground-based reference) (*A*) and *K. oboediens* IMBG185 (exposed to space/Mars-like factors on the International Space Station) (*B*) (on the left) and distribution of sizes in vesicles' fractions determined with nanoparticle. Tracking analysis (on the right) after a 5-year post-flight period. The photo clearly shows the deformations of membrane vesicles of bacteria exposed on the International Space Station.

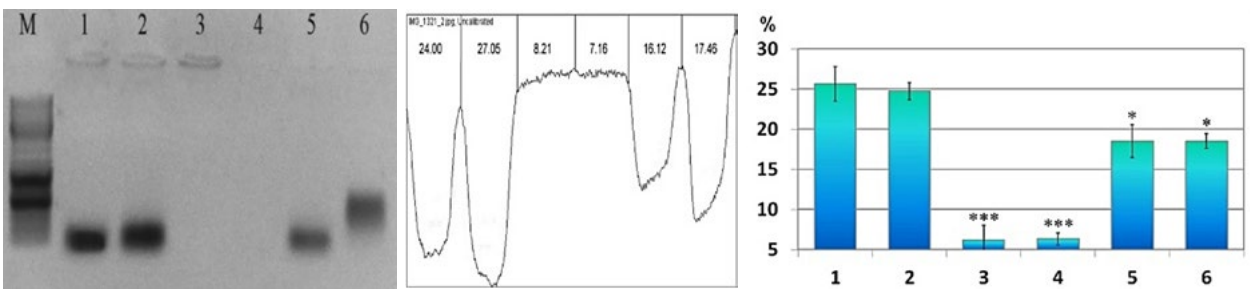

Fig. 2. Electrophoregram of RNA-derived products generated after RNA interaction with outer membrane vesicles (OMVs) of *Komagataeibacter oboediens.* On the left: a genomic *Escherichia coli* RNA after incubation with OMVs. M-DNA ladder, 1 — reference RNA (without incubation); 2 — reference RNA (incubation at 37 °C, 16 h); 3 — RNA + RNase A (Thermo Scientific) (incubation at 37^{0}C, 16 h); 4 — OMVs of *K. oboediens* IMBG180 isolated from ground-reference kombucha microbial community; 5 — E-OMVs of *K. oboediens* IMBG185 isolated from post-flight samples; 6 – OMVs of *Serratia marcescens* IMBG291 (incubation at 37 °C, 16 h); on the right: a densitogram and a histogram of the RNA degradation rate after incubation with bacterial OMVs. Data were shown as mean ± SD (n = 3). Statistical significance was assessed between control RNA without OMVs and RNA of experimental samples (with OMVs) ($P \leq 0.05$).

formed complexes with the *K. oboediens* IMBG180 vesicles and exhibited an electrophoretic mobility shift during the running in agarose gel (Fig. 3A, lanes 7–12). In contrast, the vesicles from the bacteria exposed on the ISS did not form supramolecular complexes with the linear dsDNA and did not exhibit EMS (Fig. 3A, lanes 1–6).

Influence of OMVs and E-OMVs from K. oboediens and K. hansenii on in vitro RNA transcription. It is known about the impact of ribonucleases from different sources on the activities of transcription enzymes, *e.g.,* reverse transcriptase inhibition [28, 29]. The components of the T7 bacteriophage RNA *in vitro* transcription system, more specifically, the *Eco*RI/pTZ19R, containing a T7-promoter sequence incorporated in the template, were used. The full-length RNA product was synthesized in the reference of the pTZ19R DNA-matrix without OMVs and in the presence of 31.25 μg/mL of E-OMVs from

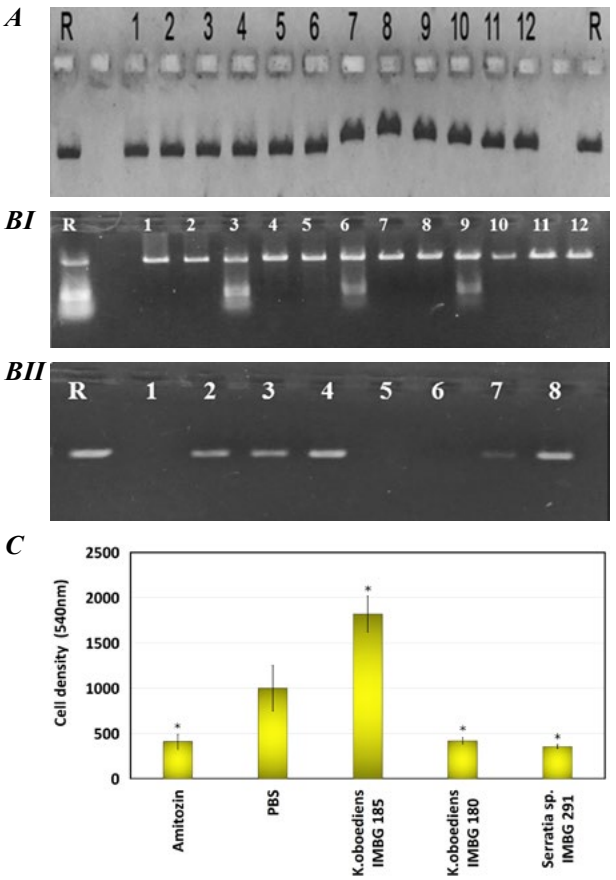

Fig. 3. Effects of extracellular outer-membrane vesicles (OMV) of *Komagataeibacter* spp. isolated from post-flight kombucha microbial community pellicle samples on the *in vitro* interactions with biomolecules. *A*, electropherogram of polynucleotide complexes generated after interaction OMVs with a target linearized (*Eco*RI) plasmid pTZ19R DNA (pDNA). *R* — the linearized pDNA; (1–6) — different concentrations of OMVs of *K. oboediens* IMBG185 from exposed in the International Space Station (ISS) samples (E-OMVs); (7–12) — OMVs of ground reference *K. oboediens* IMBG180. *B*, the T7 RNA-polymerase (I) and *Taq* DNA polymerase (II) activities in the presence of the linearized pDNA and OMVs. *I*, 1–3, E-OMVs of *K. oboediens* exposed to space/Mars-like factors at the UV-irradiated top level of the exposure platform on the ISS; 4–6, E-OMVs of *K. hansenii* from the UV-protected middle level of exposure; 7–9, E-OMVs of *K. hansenii* from the bottom level of exposure; 10–12, OMVs from reference strain *K. oboediens* (concentrations, from left to right: 125.0; 62.5; 31.25 μg/mL. R, a reference: RNA was synthesized on the pDNA matrix without OMVs. *II*, *Taq* DNA polymerase chain reaction products generated on the linearized pDNA in the presence of E-OMVs from *K. oboediens* IMBG185 (UV-irradiated KMC) (1–4) and ground reference *K. oboediens* IMBG180 (5–8) (concentrations, from left to right: 125.0; 62.5; 31.25; 6.25 μg/mL). R, a reference: PCR products were generated on pDNA without OMVs. *C*, a survival rate of human cells K-562 of chronic myeloid leukemia after co-cultivation with different concentrations of E-OMVs from *K. oboediens* IMBG185 or OMVs of K. oboediens IMBG180, in comparison with OMVs from *Serratia marcescens* IMBG291 and medicinal preparation amitozin.

K. oboediens (Fig. 3B-I, lanes 3, 6, 9); higher concentrations of OMVs inhibited RNA synthesis. Notably, E-OMVs of both these space-exposed strains exhibited the same inhibiting activities. In contrast, the total absence of transcript formation by OMVs in *K. oboediens* has been observed at a lower concentration (Fig. 3B-I, lane 12).

Influence of the OMVs on* in vitro *DNA transcription. As in the case of the T7 RNA polymerase assay, E-OMVs of stress-exposed bacteria inhibited DNA transcription at a higher concentration (125 μg/mL) (Fig. 3B-II, lanes 1–4) than OMVs of the reference strain (62.5 μg/mL) (Fig. 3B-II, lanes 5–8). We can predict that difference in the rates of a DNA amplicon pool generation could be explained by different conformational changes of the linear DNA template in PCRs under the influence of vesicles.

The vesicles of K. oboediens IMBG180 showed a concentration-dependent cytotoxic effect (IC_{50}) towards human cells K-562 of chronic myeloid leukemia at the same level as therapeutic preparation amitozin and OMVs from opportunistic bacterium *S. marcescens* IMBG291 (Fig. 3C). This is in line with findings that bacterial RNases show cytotoxicity towards *in vitro* cells [30]. Interestingly, the E-OMVs of *K. oboediens* IMBG185 (UV-irradiated KMC), which showed a low RNase activity, had a clear cytostimulating effect on K-562 cells.

In silico analysis of K. oboediens genome reveals the presence of a gene encoding subcellular location of Ribonuclease I. To determine the genetic basis of extracellular DNase/RNase activities of *K. oboediens*, we sequenced and assembled genomes of both reference and spaceflight-related isolates [22]. The genomes were found with minor differences, despite the exposure to stressful factors. The set of 15 genes was revealed by BLAST-searching against completed bacterial genomes for nuclease-like ribonuclease gene reads. Using SignalP 6.0, we detected a signal peptide (1–22) in predicted Ribonuclease I (ABPAJMHB_02574) (Table 1). The ProtComp v. 9 predicted the sub-cellular periplasmic location of RNase I with a score of 7.2 and membrane score of 2.8 (it is neither cytoplasmic nor secreted enzyme). RNase I is an endoribonuclease that preferentially hydrolyzes single-stranded RNA and also possesses a Ca^{2+}-dependent strong dsRNase activity [31]. The RNase I belongs to the RNase T2/S-RNase group of endoribonucleases, transferase-type RNases [32].

In this study, we experimentally detected ribonycleolytic activity of OMVs obtained in *K. oboediens.* A proteogenomic approach predicted membrane translocation by the Sec translocon and a periplasm location for RNase I in *K. oboediens,* as in *E. coli* [33]. The rate of RNase activity differed in OMVs of wild type and post-flight bacteria, in spite of identical nucleotide sequences encoding komagataeibacter's RNase [22]. Since we have observed differences in the RNase activities persisting in five years, we hypothesize that in these bacteria after long-term exposure in the harsh hybrid environment, non-genetic inheritance of acquired changes occurs via a range of mechanisms, including DNA methylation [34] or protein aggregates [35]. In this case, transgenerational inheritance of amyloid proteins could be most probable (will be published).

E-OMVs poorly interacted with DNA polynucleotides and probably did not form or

formed unstable complexes with them. The rate of *in vitro* DNA and RNA transcription inhibition was less pronounced in E-OMVs than in vesicles of the reference strain, and it correlated with their both lower RNase activity and a loss of cytotoxicity toward *in vitro* human cells. Different eukaryotic and microbial RNases are selectively cytotoxic for tumor cells and have been investigated as anticancer drugs [36].

To our knowledge, RNase I associated with OMVs was detected in *K. oboediens* for the first time. In bacteria, extracellular nucleases perform different roles to fit bacterial lifestyles: from accommodating bacteria in hosts [7] to degrading extracellular nucleic acids for nutrient sources [32]. In *K. oboediens*, RNase I has periplasmic location in cell membrane, however, it considered to play roles in degradation of RNA species, resulting in scavenging of ribonucleic acids in microenvironments, modulating host metabolism response to stressors (*e.g.,* by formation of biofilm) or roles independent of the ability to hydrolyse RNA [37, 38]. In *E. coli* and in *Salmonella enterica* ser. Typhimurium, RNase I cleaves short RNAs in 2′,3′-cyclic nucleotide monophosphates (2′,3′-cNMPs), second messengers in signalling pathways, responding to environmental changes [37-39]. The 2′,3′-cNMPs are positional isomers of the 3′,5′-cNMPs, signalling molecules, playing key roles in both prokaryotes and eukaryotes [37, 40]. RNase I-dependent elevation of 2′,3′-cNMP levels in *E. coli* correlated with reduced biofilm production and activation in response to aminoglycoside antibiotics [38, 41]. Recently, it was shown that 2′,3-cNMP levels could modulate bacterial physiology by binding to bacterial ribosomes and rapidly affecting *in vitro* translation in response to environmental factors [40]. Bacterial RNase I, which can degrade viral RNA [41, 42], is promising to reduce virus particles and also to combat highly transmissible COVID-19 variants [43]. As ribonucleases contribute to destroying RNA viruses, we may assume a role of the OMV-carrying nucleases in the defense of non-pathogenic komagataeibacters against alien nucleic acids as these bacteria exist in a semi-closed multimicrobial microecosystem. The presence of lytic enzyme in the OMVs might mean that antiviral activities could be focused more on the OMV-associated defense in the proximity of cells than on, *e.g.,* the CRISPR/Cas system, which shows some signs of degeneration in *K. oboediens* (none CRISPR arrays available in CAS-VI-B and CAS-III-D subtypes [22]). It is known that the nuclease activity over the RNA can lead to the formation of a set of oligonucleotides and nucleotide monophosphates, some of which have immunomodulatory properties [31], and this is in line with the fact that KMC (where komagataeibacters occupy leading positions) possesses immunostimulatory capacity in mammals [44].

Taken together, the above observations identified the RNase-I-bearing OMVs and E-OMVs to be promising in enzyme-delivery platforms, also loaded with other biologicals. This is the first step towards understanding the role of enzymatic properties of the OMVs from *Komagataeibacter* spp. in innate defense against mobile genetic elements and exogenous nucleic acids in the host gut environment, *e.g.,* after consumption of kombucha products under normal and spaceflight stressful conditions.

Conclusions

The difference in behaviour of OMVs in komagataeibacters five years after an impact of stressful conditions retains (despite unchanged appropriate genes), taking into account differences in associated enzymatic activity and *in vitro* inhibition of both DNA and RNA polymerases. OMVs from exposed bacteria exhibited a loss of cytotoxicity which correlated with a lower level of the RNase I activity and an *in vitro* inhibition of DNA and RNA transcriptions, comparing the OMVs of ground-based strain. We have predicted membrane translocation by the Sec translocon for RNase I in *K. oboediens* and its sub-cellular location. This can be engineered into a sub-surface display system for expression in OMVs for different purposes, *e.g.,* as an edible kombucha prophylaxis vaccine.

Acknowledgments

Authors thank Ms. M. Horbunova for technical assistance.

Finances

This project was supported by the National Academy of Sciences of Ukraine (grant 2.2.4.19, 2022).

REFERENCES

1. *Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles.* 2018; **7**(1):1535750.
2. *Gill S, Catchpole R, Forterre P.* Extracellular membrane vesicles in the three domains of life and beyond. *FEMS Microbiol Rev.* 2019; **43**(3):273–303.
3. *McMillan HM, Rogers N, Wadle A, Hsu-Kim H, Wiesner MR, Kuehn MJ, Hendren CO.* Microbial vesicle-mediated communication: convergence to understand interactions within and between domains of life. *Environ Sci Process Impacts.* 2021; **23**(5):664–77.
4. *Sim S, Lee DH, Kim KS, Park HJ, Kim YK, Choi Y, Park HS. Micrococcus luteus*-derived extracellular vesicles attenuate neutrophilic asthma by regulating miRNAs in airway epithelial cells. *Exp Mol Med.* 2023; **55**(1):196–204.
5. *Jalalifar S, Morovati Khamsi H, Hosseini-Fard SR, Karampoor S, Bajelan B, Irajian G, Mirzaei R.* Emerging role of microbiota derived outer membrane vesicles to preventive, therapeutic and diagnostic proposes. *Infect Agent Cancer.* 2023; **18**(1):3.
6. *Jhelum H, Sori H, Sehgal D.* A novel extracellular vesicle-associated endodeoxyribonuclease helps *Streptococcus pneumoniae* evade neutrophil extracellular traps and is required for full virulence. *Sci Rep.* 2018; **8**(1):7985.
7. *Dhital S, Deo P, Bharathwaj M, Horan K, Nickson J, Azad M, Stuart I, Chow SH, Gunasinghe SD, Bamert R, Li J, Lithgow T, Howden BP, Naderer T. Neisseria gonorrhoeae*-derived outer membrane vesicles package β-lactamases to promote antibiotic resistance. *Microlife.* 2022; **3**:uqac013.
10. *Yamada Y, Yukphan P, Lan Vu HT, Muramatsu Y, Ochaikul D, Tanasupawat S, Nakagawa Y.* Description of *Komagataeibacter* gen. *nov.*, with proposals of new combinations (Acetobacteraceae). *J Gen Appl Microbiol.* 2012; **58**(5):397–404.
11. *Lin SP, Huang YH, Hsu KD, Lai YJ, Chen YK, Cheng KC.* Isolation and identification of cellulose-producing strain *Komagataeibacter intermedius* from fermented fruit juice. *Carbohydr Polym.* 2016; **151**:827–33.
12. *Reva ON, Zaets IE, Ovcharenko LP, Kukharenko OE, Shpylova SP, Podolich OV, de Vera JP, Kozyrovska NO.* Metabarcoding of the kombucha microbial community grown in different microenvironments. *AMB Express.* 2015; **5**(1):124.

13. *Fukami H, Tachimoto H, Kishi M, Kage T, Tanaka Y, Koga Y, Shirasawa T.* Continuous Ingestion of Acetic Acid Bacteria: Effect on Cognitive Function in Healthy Middle-Aged and Elderly Persons. *Anti-aging Med.* 2009; **6**:60–5.
14. *Tsuchiya Y, Ban M, Kishi M, Ono T, Masaki H.* Safety and Efficacy of Oral Intake of Ceramide-Containing Acetic Acid Bacteria for Improving the Stratum Corneum Hydration: A Randomized, Double-Blind, Placebo-Controlled Study over 12 Weeks. *J Oleo Sci.* 2020; **69**(11):1497–508.
15. *Krzyżek P, Marinacci B, Vitale I, Grande R.* Extracellular Vesicles of Probiotics: Shedding Light on the Biological Activity and Future Applications. *Pharmaceutics.* 2023; **15**(2):522.
16. *Kozyrovska N, Reva O, Podolich O, Kukharenko O, Orlovska I, Terzova V, Zubova G, Trovatti Uetanabaro AP, Góes-Neto A, Azevedo V, Barh D, Verseux C, Billi D, Kołodziejczyk AM, Foing B, Demets R, Vera J-P.* To Other Planets With Upgraded Millennial Kombucha in Rhythms of Sustainability and Health Support. *Front Astron Space Sci.* **8**:701158.
17. *Orlovska I, Podolich O, Kukharenko O, Zubova G, Reva O, di Cesare A, Góes-Neto A, Azevedo V, Barh D, de Vera J.-P, Kozyrovska N.* The Conceptual Approach to The Use of Postbiotics Based on Bacterial Membrane Nanovesicles for Prophylaxis of Astronauts' Health Disorders. *Space Sci Technol.* 2022; **28**(6):34–51.
18. *Jiang L, Driedonks TAP, Jong WSP, Dhakal S, Bart van den Berg van Saparoea H, Sitaras I, Zhou R, Caputo C, Littlefield K, Lowman M, Chen M, Lima G, Gololobova O, Smith B, Mahairaki V, Riley Richardson M, Mulka KR, Lane AP, Klein SL, Pekosz A, Brayton C, Mankowski JL, Luirink J, Villano JS, Witwer KW.* A bacterial extracellular vesicle-based intranasal vaccine against SARS-CoV-2 protects against disease and elicits neutralizing antibodies to wild-type and Delta variants. *J Extracell Vesicles.* 2022; **11**(3):e12192.
19. *Suri K, D'Souza A, Huang D, Bhavsar A, Amiji M.* Bacterial extracellular vesicle applications in cancer immunotherapy. *Bioact Mater.* 2023; **22**:551–66.
20. *Zhang Y, Lu Y, Xu Y, Zhou Z, Li Y, Ling W, Song W.* Bio-Inspired Drug Delivery Systems: From Synthetic Polypeptide Vesicles to Outer Membrane Vesicles. *Pharmaceutics.* 2023; **15**(2):368.
21. *Podolich O, Kukharenko O, Haidak A, Zaets I, Zaika L, Storozhuk O, Palchikovska L, Orlovska I, Reva O, Borisova T, Khirunenko L, Sosnin M, Rabbow E, Kravchenko V, Skoryk M, Kremenskoy M, Demets R, Olsson-Francis K, Kozyrovska N, de Vera JP.* Multimicrobial Kombucha Culture Tolerates Mars-Like Conditions Simulated on Low-Earth Orbit. *Astrobiology.* 2019; **19**(2):183–96.
22. *Santana de Carvalho D, Trovatti Uetanabaro AP, Kato RB, Aburjaile FF, Jaiswal AK, Profeta R, De Oliveira Carvalho RD, Tiwar S, Cybelle Pinto Gomide A, Almeida Costa E, Kukharenko O, Orlovska I, Podolich O, Reva O, Ramos PIP, De Carvalho Azevedo VA, Brenig B, Andrade BS, de Vera JP, Kozyrovska NO, Barh D, Góes-Neto A.* The Space-Exposed Kombucha Microbial Community Member *Komagataeibacter oboediens* Showed Only Minor Changes in Its Genome After Reactivation on Earth. *Front Microbiol.* 2022; **13**:782175.
23. *Hestrin S, Schramm M.* Synthesis of cellulose by *Acetobacter xylinum*. *II.* Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. *Biochem J.* 1954; **58**(2):345–52.
24. *Novikov VV, Varzatskii OA, Negrutska VV, Bubnov YN, Palchykovska LG, Dubey IY, Voloshin YZ.* Size matters, so does shape: Inhibition of transcription of T7 RNA polymerase by iron(II) clathrochelates. *J Inorg Biochem.* 2013; **124**:42–5.
25. *Hermant B, Gudrun A, Potopalsky AI, Chroboczek J, Tcherniuk SO.* Amitozyn impairs chromosome segregation and induces apoptosis via mitotic checkpoint activation. *PLoS One.* 2013; **8**(3):e57461.
26. *Mosmann T.* Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; **65**(1–2):55–63.
27. *Teufel F, Armenteros JJA, Johansen AR, Gíslason MH, Pihl SI, Tsirigos KD, Winther O, Brunak S, von Heijne G, Nielsen H.* SignalP 6.0 achieves signal peptide prediction across all

types using protein language models. bioRxiv. 2021.06.09.447770.
28. *Zhang R, Zhao L, Wang H, Ng TB.* A novel ribonuclease with antiproliferative activity toward leukemia and lymphoma cells and HIV-1 reverse transcriptase inhibitory activity from the mushroom, *Hohenbuehelia serotina*. *Int J Mol Med.* 2014; **33**(1):209–14.
29. *Xu L, Geng X, Chang M, Meng J, Wang H, Ng TB.* A Novel Ribonuclease with HIV-1 Reverse Transcriptase Inhibitory Activity Purified from the Field Blewit Mushroom Lepista personata (Agaricomycetes). *Int J Med Mushrooms.* 2020; **22**(10):991–1000.
30. *Kumar R, Katwal S, Sharma B, Sharma A, Puri S, Kamboj N, Kanwar SS.* Purification, characterization and cytotoxic properties of a bacterial RNase. *Int J Biol Macromol.* 2021; **166**:665–76.
31. *Grünberg S, Coxam B, Chen TH, Dai N, Saleh L, Corrêa IR, Nichols NM, Yigit E. E. coli* RNase I exhibits a strong Ca2+-dependent inherent double-stranded RNase activity. *Nucleic Acids Res.* 2021; *49*(9):5265–77.
32. *Luhtala N, Parker R.* T2 Family ribonucleases: ancient enzymes with diverse roles. *Trends Biochem Sci.* 2010; **35**(5):253–9.
33. *Neu HC, Heppel LA.* The release of ribonuclease into the medium when *E. coli* cells are converted to spheroplasts. *Biochem Biophys Res Commun.* 1964; **14**:109–12.
34. *Sánchez-Romero MA, Casadesús J.* The bacterial epigenome. *Nat Rev Microbiol.* 2020; **18**(1): 7–20.
35. *Jackson EK.* Discovery and Roles of 2',3'-cAMP in Biological Systems. *Handb Exp Pharmacol.* 2017; **238**:229–52.
36. *Duggal Y, Fontaine BM, Dailey DM, Ning G, Weinert EE.* RNase I Modulates *Escherichia coli* Motility, Metabolism, and Resistance. *ACS Chem Biol.* 2020; **15**(7):1996–2004.
37. *Duggal Y, Kurasz JE, Fontaine BM, Marotta NJ, Chauhan SS, Karls AC, Weinert EE.* Cellular Effects of 2',3'-Cyclic Nucleotide Monophosphates in Gram-Negative Bacteria. *J Bacteriol.* 2022; **204**(1): e0020821.
38. *Kalia D, Merey G, Nakayama S, Zheng Y, Zhou J, Luo Y, Guo M, Roembke BT, Sintim HO.* Nucleotide, c-di-GMP, c-di-AMP, cGMP, cAMP, (p)ppGpp signaling in bacteria and implications in pathogenesis. *Chem Soc Rev.* 2013; **42**(1):305–41.
39. *Fontaine BM, Martin KS, Garcia-Rodriguez JM, Jung C, Briggs L, Southwell JE, Jia X, Weinert EE.* RNase I regulates *Escherichia coli* 2',3'-cyclic nucleotide monophosphate levels and biofilm formation. *Biochem J.* 2018; **475**(8):1491–506.
40. *Chauhan SS, Marotta NJ, Karls AC, Weinert EE.* Binding of 2',3'-Cyclic Nucleotide Monophosphates to Bacterial Ribosomes Inhibits Translation. *ACS Cent Sci.* 2022; **8**(11):1518–26.
41. *Ilinskaya ON, Mahmud RS.* Ribonucleases as antiviral agents. *Mol Biol.* 2014; **48**(5):615–23.
42. *Andreeva IS, Mazurkova NA, Zakabunin AI, Puchkova LI, Filippova EI, Safatov AS.* Evaluation of the Effectiveness of Metabolites of Bacterial Strains *Bacillus thuringiensis* against Human Influenza Virus A/Aichi/2/68 (H3N2) *In Vitro* and *In Vivo*. *Bull Exp Biol Med.* 2020; **169**(5):653–6.
43. *Xu SY, Fomenkov A, Chen TH, Yigit E.* Expression of Human ACE2 N-terminal Domain, Part of the Receptor for SARS-CoV-2, in Fusion With Maltose-Binding Protein, *E. coli* Ribonuclease I and Human RNase A. *Front Microbiol.* 2021; **12**:660149.
44. *Chakravorty S, Bhattacharya S, Bhattacharya D, Sarkar S, Gachhui R.* Kombucha: A Promising Functional Beverage Prepared From Tea. *In Non-Alcoholic Beverages, 6: The Science of Beverages (eds Grumezescu, A.*M. & Holban, A.M.), 285–327 (Woodhead Publishing, 2019).

Komagataeibacter oboediens **змінює активність, асоційовану із зовнішньо-мембранними везикулами, після експозиції на Міжнародній космічній станції**

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Зовнішньо-мембранні везикули (ЗМВ) несуть різноманітний біомолекулярний вантаж, включаючи фер-

менти, через які комунікують з навколишнім середовищем. Роль фермент-асоційованих ЗМВ харчових бактерій у здоров'ї та хворобах людини не досліджена, зокрема, після впливу стресових факторів (зміни гравітації та атмосфери, УФ-випромінювання) у контексті харчування астронавтів. **Мета.** Ми досліджували ЗМВ *Komagataeibacter oboediens*, виділених із зразків мікробного угруповання чайного гриба, які піддалися впливу космічних/марсоподібних стресорів, змодельованих на Міжнародній космічній станції, і культивованих протягом п'яти років, з ціллю дізнатися більше про їх поведінку з біомолекулами та клітинами ссавців. **Методи.** Було проведено серію експериментів *in vitro* з розщеплення РНК, інгібування транскрипції ДНК і РНК і аналіз цитотоксичності ЗМВ/*Komagataeibacter*. SignalP 6.0 використовувалася для виявлення сигнальних пептидів у передбачених рибонуклеазах у геномі *K. oboediens*, а ProtComp версії 9 слугувала для визначення субклітинного розташування РНКази. **Результати.** Поведінка ЗМВ бактерій до та після впливу стресових умов відрізнялася, спираючись на відмінності у пов'язаній нуклеолітичній активності, інгібуючій здатності проти РНК-полімерази бактеріофага T7 та *Taq* ДНК-полімерази та цитотоксичного ефекту, незважаючи на ідентичні послідовності нуклеотидів у гомологічних генах. *In vitro* інгібування транскрипції РНК і ДНК було менш вираженим у ЗМВ експонованих бактерій, ніж у везикулах наземного штаму. Це корелювало з нижчою активністю РНКази та втратою цитотоксичності щодо злоякісних клітин людини навіть через п'ять років після польоту. Було виведено, що бактеріальна РНКаза I розташована в периплазмі ЗМВ. **Висновок.** Діяльність *Komagataeibacter*, пов'язана з ЗМВ, була змінена після експозиції на Міжнародній космічній станції та успадкована негенетично.

Ключові слова: зовнішньо-мембранні везикули, стресові фактори, негенетична модифікація, РНКаза I

Supplementary Material

Supplementary Table S1. **The BLAST-searched set of genes against completed *Komagataeibacter oboediens* IMBG180 bacterial genome for nuclease-like gene reads**

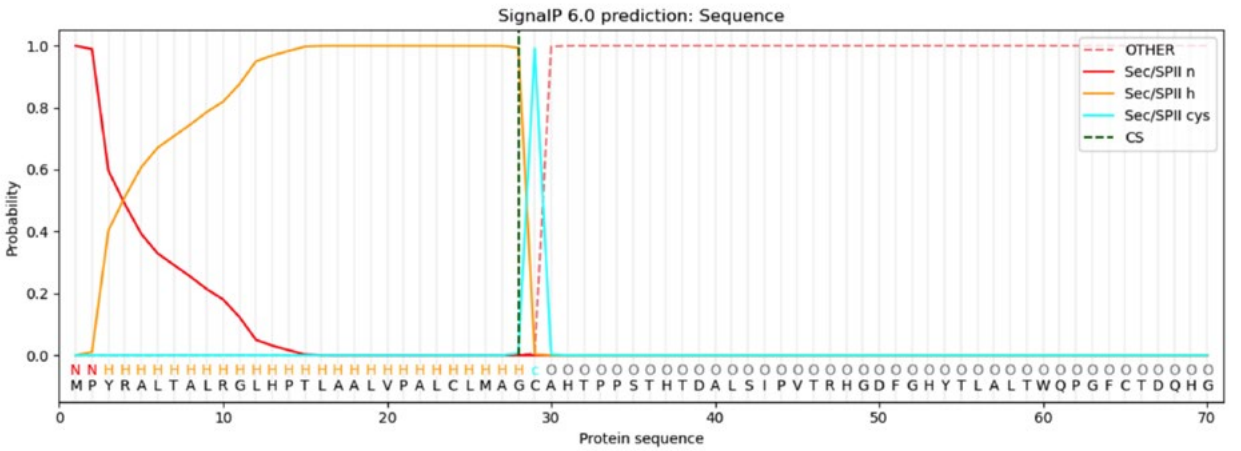
#ABPAJMHB_01047 Endoribonuclease_YbeY	MPVLPAGLDGPDITVADRRWNRAVRHPARLVARAVDAALRATGRHGPVTVVLADDRTVAQMNWRHRGRNK PTNVLTFEYAPAHAGGGLWGGDIIIAFETVVHEARAAGRTVAAHLAHLVIHGVLHLAGYDHHHPGEAREMEM LEASTLSTLGIANPWKQGRMATARGMRS
#ABPAJMHB_01047_ Endoribonuclease_YbeY	MPVLPAGLDGPDITVADRRWNRAVRHPARLVARAVDAALRATGRHGPVTVVLADDRTVAQMNWRHRGRNK PTNVLTFEYAPAHAGGGLWGGDIIIAFETVVHEARAAGRTVAAHLAHLVIHGVLHLAGYDHHHPGEAREMEM LEASTLSTLGIANPWKQGRMATARGMRS
#ABPAJMHB_00850_ Ribonuclease_PH	MRPSGRAIDALRPVFIQPGFARHAEGSALIRMGGTEVLCAATVETRVPSFLRGKGQGWVTAEYGMLPRATH SRGQREAAKGKQTGRTQEIQRLIARALRAVVDLKALGEISVTVDCDVLNADGGTRCAAITGAWVALRLALE KLVRAGTLPALPIKGQVAAISCGLTDDGPVLDLDYREDSAASADANFVLTADGGIVEIQGTAEKDPFTEAQFNTLM RLARTGTAELFRIQTAIVEEALKA
#ABPAJMHB_00069_ Ribonuclease_E	MSKRMLIDTTHAEETRVVVMDGNHLEDYDVEAASKKQLKGNIYLAKVIRVEPSLQAAFVDYGGNRHGFLAFSE IHPDYYQIPVADREKLLALQEEERRAEEERRARDDEEDDAPRAEDEADAPVDQDGDEPETVGGENDTGDEAQAQ RRIARFLRNYKIQEVIRRRQILLVQVVKEERGNKGAALTTYVSLAGRYCVLMPNSLRGGGVSRKITSVADRRRLRD IITELQIPRGMAMIVRTAGAQRPRAEIMRDCEYLLRLWDDIRQHTLESVAPALIYEEASLIKRAIRDIYTREVSEILIDG EPGWKSAREFMRMLMPQNAQKVKLWQDHEQTLFSHYKVEGLLDSMLSPSVQLKSGGYLVINQTEALVAIDVNSG RATRERNIEETALRTNLEAAEEVARQLRLRDLAGLIVIDFIDMESRKHNGMVERKLKDALRTDRARIQVGQISHFGL LEMSRQRLRPSIAESAFMPCPHCQGTGIVRGIESSALHVLRAIEEEGMQRKAAEISVGVAPEIAFYILNHKRNWLSEI ETRHKMQVSFAPDAALQPQECKIERVRQQTARFDAPVIEHAPAESSHVREIQIQAEPPAADATPDAVATTDEDEDIAS GTDHRRRRRRRRRRGGAAAQAGQTQAAEDTATEQAPAPVAPTPAPEPAPREVYRGPTPADPYGDAIIDIFDVIEQTT APSEVVVEADPVVVEVEEAATPPVAEEETTKPRRTRRRTRRRTTPVENGAAPVTQDAEATAAPVAEPAVPAVEAEA AEPEAAPAPAEPAAEEAPKRRRRASTTTTRRTTSRRKAAEPVTEEPAEETKAKPVDEPAAEEAAPARPARKRAASR STGTTATTRRRTTRTAAKTADKAEEPVVETPATTEAAAPATEEAPKRRRVTRRKKAEPVADAAADATPVEAEAKTA DAKAEKAEKPAPKKRTTTRKTATTTTATRRRRKKADEAAEATDTPVDTAITPVDVDATPTTHRRTGWWKR
#ABPAJMHB_02574_ Ribonuclease_I	MPYRALTALRGLHPTLAALVPALCLMAGCAHTPPSTHTDALSIPVTRHGDFGHYTLALTWQPGFCTDQHGPSCQP DQPHAPLIGLHGLWASRPSDLMREGLPVTQWWIKGCDIYQHDDTPPQLSPALSKRLSGTVAHTRSSLVTHEYTK HVQCFGMNAERFFTVASTLRDRFAAGPVGRELDHDAGRDISKADVIGLFERTYGTLPERGLQFRCNTDAQGQPIL AQLWFTLDPRGLGRFPAASGFLPSPDVQDNCPARFHVPTWPVTAQAG
#ABPAJMHB_01498_ Ribonuclease_J	MNDMTGVSFLPLGGTGEIGMNLNLYRQGETWLAVDCGIGFSGNDTPEAEIILPDPVFIAERRDRLAGLVVTHAHED HLGAIAHLWPQLGCPVYVTPFAAAVLRRKLGEAGLLQQVPIHVVAPGSAFTVGPFDLRFVSVTHSVPESQSLVLRT PQGIIVHTGDWKIDPDPQVGPPTDLETFAELGREGVLAMVCDSTNVRTEGPSASEADVRREMTRLIASLEGRIAVT CFASNVARVETLAMAAKAAGRRVAVVGRSLRNLEVAARECGYLSDVPHFLSEQDANSIPDNQILLIVTGSQGEPRS ALSRIAADTHPNIALGEGDTVIYSSRMIPGNEQAVIQVQDSLTRRGVHVITDKDHLVHVSGHATGGDVRRLYDL VRPRFLIPVHGEWRHLTANAAIAQELNIEPVLLEDGDILDIRADGVEIGDTAPTGRLVLDGGRILPMNGGVLSARRR MLFNGMVMGSFAVDDEGYLIGDPKVSAPGLLDADDPETQRVTEEFGNALDEIPDELRENDATFREAAKTALRRAL GRKLQKRPLVDVHLLRV
#ABPAJMHB_02461_ Endonuclease_III	MTTARSPKKPARRAMTLAEVRSFITQLAAANPNAESELDFVDDYTLLVAVVLSAQATDASVNRATKALFRDAPTP RAMVELGEEKVGAHIRTIGLWRTKARNVVALSQQLLERFDGKVPYDRAALESLPGVGRKTANVVMNVAFGDSTM AVDTHIFRIGNRTGLAPGATPRAVEDQLVRRIPADMLRPAHHWLILHGRYVCKARRPECWRCPAFDPCQYRMKDD LRAVGAPAASSKE
#ABPAJMHB_01570_ Ribonuclease_HI	MNEDMAPAENAATATDLVEIWTDGGCKPNPGPGGWGALLCYRGQERELSGGEAETTNNRMELTAAAEALEALK RPCRIVLHTDSEYVRNGITRWSTGWVRRKWRNASGDPVANMDLWRRLLDVSAKHDIEWKWVRGHSGDVNNER VDQLATAARDAMGIPYPKRGK
#ABPAJMHB_01662_ Ribonuclease_HII	MRIGPERFDSHTIRKEWMPDYALENAHGGRVAGVDEVGRGPLAGPVVAAAVMFLSGVPGVLADRLDDSKKLKPA VRQQLYDVLHATPGVLIGVGAASVSEIERYNILRASWVAMQRAVGRLPQSPELVLVDGNAAPDFGCPARCVVGGD AISLSISAASVVAKVIRDRLMTRLAQRWPSYGWDRNAGYGTPIHRAALMADGISPHHRAAFGTVRRIVQASVSPY SPQSAEQPSC
#ABPAJMHB_02405_ Ribonuclease_E	MRICASSSPGEVRIAVTTDGIMQDFALWRPDIADGVGDIYRARVSAHVPALGGTFVTLPGLEQAGFLPDSEGLGPLT QGQTVLVTITRSAQGGKGVRLGARNLPPDLPGGAEPQLLRRGPSPLERLARYYPHAPIIIDDAAIAARLPAVFHPRL ARVSSAFDSDLRAQADALEDPVVSLPGGMSASITPTPALVAIDMDGGATSMDRRPKQTAQFASNRDALPELLHQL RLRNLSGAIIVDVAGLAIRKRRALSETVETLLKNDPLRPRFLGFTALGLAEIVRPRVHPPLHELFQSTEGRLLRALRG QMQAHRGMPPDGRPVTMGCGHGIMTLLQDHPDWMEDFVRLTGRALQPVMDQDLPANGWRFDHG
#ABPAJMHB_00237_ Ribonuclease_P_protein_ component	MSLHKGRLKVADKSTRLKKRAEFLRVAAKGRKAPVPGLVLQALERGDTQAARYGFTVTKKVGNSVVRNRARRR LREVVRLLDREQALTGVDIVVIGRSGTCGRRFDALMGDYRKALRKAGVKEES
#ABPAJMHB_03207_ Ribonuclease_toxin_YhaV	MITINGWTILAHPLFLDQLEKLTDAVEVLKAKKPEKYQREANTKLLAALSKLVFQTIPADPTATVYRQGSTLGVAH RHWFRAKFGNGRFRLFFRYDSTAKIIIFAWVNDETTLRTYGAKTDAYRVFKGMLEAGDPPDDWIALREAASDQAA IDRLEKTSPSGP

End of Supplementary Table S1

#ABPAJMHB_02453_ Ribonuclease_R	MKRQRPATSEMPDTLPTGMPQARAGGLPDREQLRAFIENATGRIGKREISREFGLGPEHKQALRQMLREMALDGM LAPAGARRFRVSDRLPESMVVQVTGTDSDGDPIARPVQWDGDGSAPVIFMHPELRGRAALAPGERVVARLRRV GPGRYEGRTLRRLTDAPMEVVGLFRTLDETDPMASAPARYRPAGTLTPADRRAKAQWVIPVGEDADSPDNEIVIAT PLPQSGPGLHPARIIERLGPQGDARTISMIAVAMLGIPHVFPTEALAQARAARGVTAQGRTDLRDMPLVTIDGADA RDFDDAVYAEPDGEGFRITVAIADVAWYVRPGSALDREAHLRGNSVYFPDRVIPMLPEDLSNGWCSLRPGEDRG CIFVNMDVAADGAISNARFGRGIMRSAARLTYEQVQAARDGAPDATVAALPDGLLDSLFGAWRCLSTARAQRG TLDLDLPERQVRLNDHGQIAAIQPRIRLLDSHRLVEEFMIAANVAAARCLEGRHLPCLYRIHAPPTPERLESLRRTLD TMGLKLPPVGSLRAADLDHILQQVRDTDQAGLVNELILRAQNQAEYSPEPVGHFGLSLAAYAHFTSPIRRYADLLT HRALLVATGLEPGPAPSHDELEEAGLAITRTERRAAQAERETLERYAATWLQARVGTVMDAHISSLSRFGIFTVLDA TGTSALLPMSALPEDQWHHDEKTQTLHARDTTMVFRPGQALRVRIEEACAIRGTVLLALPDTPPSHRRRRT
#ABPAJMHB_00255_ Ribonuclease_D	MAASPAPDAIHFHRGDLPAGVTFPGAVAVDTETMGLNPHRDRLCLVQLSAGDGHAHLVQMPRGATAADCPNLVR VLTDPQRPKLMHFARFDVAILQHTLGITVAPNICTKIASKLVRTYTDRHGLAHLCRELLGVELSKQQQTSDWGAPD LTPEQLAYAASDVLYLHALWEKLEALLHRENRRELAQACYDFLPTRARLDILGYEEPDIFAHRAA
#ABPAJMHB_00221_ Ribonuclease_3	MDAERVPAAEIARLEACLDYHFTRADLLLRALTHRSAAHERNGGRRTRQSTAKRGAGSNERLEFIGDRVLGLLM AEWLLERFPDEQEGALGPRHAHLVSRTVLAQVAHAMGLQSALDVAEHEARAGVRQMANVLADAVEAILGAMY LDGGLDPARAFVRRMWNDSIVAQARPPKDPKTALQEWVLGRGLPLPQYRVVSSDGPSHAPRFVIAVDAQGRTGQ GIAGSKRAAESDAASDLLRQLGADGQAASSTDRKGQ
#ABPAJMHB_00409_ Ribonuclease_D	MARVSRPEFPAPVLVTTTAELEAVTARLRHEPFVTIDTEFVRERTYWPELCLVQLAGEKDVVVIDTTAPGIDLTSLG ALLDDASVVKVFHAARQDLEIFLHLFDHLPAALFDTQVAAMVAGYGDQVGYDNLVSSLLGVQIDKSHRFSDWAA RPLSPAQIGYAAADVTYLRLVYEKLLVQLEREGRLDWVAAELDILNNPTTFRPDPLTLWEKMRPRTNNRRMLGIL RAVAAWREGEAQRVNVPRQRLLKDESLMEIAATAPATIDALARVRGVSRGFAEGRSGQALLETVAQAVEEPEAAL PRLPKGRAKDAQRPSPALIALLKVLLASCCETHRVAPRLVASSEDLDRFALDDAADIPAFHGWRNEVFGKLARELK AGHLTMGVAGGKVRLIHD

Supplementary Table S2. **Prediction of lipoprotein signal peptide (Sec/SPII) in Ribonuclease I (#ABPAJMHB_02574) with Program Signal P 6.0.**

Protein type	Other	Signal Peptide (Sec/SPI)	Lipoprotein signal peptide (Sec/SPII)	TAT signal peptide (Tat/ SPI)	TAT Lipoprotein signal peptide (Tat/SPII)	Pilin-like signal peptide (Sec/SPIII)
Likelihood	0	0.0001	0.0001	0	0	0



Supplementary Fig. S1. The cleavage site, removing signal peptide, is predicted between positions 28 and 29 in RNase I as determined with the program SignalP 6.0. The probability is 0.993463. SignalP 6.0 relies on a start codon being correctly identified before application.

Received 24.04.2023