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**Full Paper**

# Phylogenetic distribution of extracellular guanyl-preferring ribonucleases renews taxonomic status of two *Bacillus* strains†

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**The potential of microbial ribonucleases as promising antitumor and antiviral agents, determines today's directions of their study. One direction is connected with biodiversity of RNases. We have analyzed completed and drafted *Bacillus* genomes deposited in GenBank for the presence of coding regions similar to the gene of an extracellular guanyl-preferring RNase of *Bacillus amyloliquefaciens* (barnase). Orthologues of the barnase gene were detected in 9 species out of 83. All of these belong to "*B. subtilis*" group within the genus. *B. subtilis* itself, as well as some other species within this group, lack such types of RNases. RNases similar to barnase were also found in species of "*B. cereus*" group as a part of plasmid-encoded S-layer toxins. It was also found that taxonomic states of culture collection strains, which were initially described based on a limited set of phenotypic characteristics, can be misleading and need to be confirmed. Using several approaches such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), sequencing of genes for 16S ribosomal RNA and RNA polymerase subunit beta followed by reconstruction of phylogenetic trees, we have re-identified two RNase-secreting *Bacillus* strains: *B. thuringiensis* B-388 which should be assigned as *B. altitudinis* B388 and *B. intermedius* 7P which should be renamed as *B. pumilus* 7P. Therefore, small secreted guanyl-preferring RNases are the feature of "*B. subtilis*" group only, which is characterized by distinctive lifestyle and adaptation strategies to environment.**

**Key Words: *Bacillus altitudinis*; *Bacillus pumilus*; barnase; extracellular ribonuclease; phylogeny; *rpoB*; taxonomy revision; 16S rRNA gene**

## Introduction

Barnase, an extracellular alkaline guanyl-preferring ribonuclease first isolated from *Bacillus amyloliquefaciens* (Hartley and Rogerson, 1972), was shown to possess a wide range of biological activities and is of special interest for research and practical applications in biotechnology and medicine (Kempe et al., 2014; Spång et al., 2012; Sreenivasan et al., 2013; Ulyanova et al., 2011). Ribonucleases similar to barnase (UniProtKB/Swiss-Prot accession number P00648) were purified from *B. intermedius* 7P (P00649), *B. circulans* BCF-256 (P35078), *B. pumilus* KMM-62 (P48068), *B. coagulans* BCF-247 (P37203), and *B. thuringiensis var. subtoxicus* B-388 (Q9R5D7, GenBank acc No. AAB28984.1). *B. intermedius* RNase referred to as binase is as well studied as barnase. Its antitumor and antiviral properties are documented (Mironova et al., 2013; Mitkevich et al., 2014; Shah Mahmud and Ilinskaya, 2013; Ulyanova et al., 2011) while the strain itself is an undescribed taxon which is

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†The GenBank accession number for the 16S rRNA gene sequence of *Bacillus altitudinis* B-388 is JX129389, for the *rpoB* gene sequence of *Bacillus altitudinis* B-388 is JX129391, for the 16S rRNA gene sequence of *Bacillus pumilus* 7P is JX129390 and for the *rpoB* gene sequence of *Bacillus pumilus* 7P is JX129392 correspondingly.

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absent in the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (Parte, 2014). Primary structures of *B. intermedius* and *B. pumilus* RNases are identical and differ from that of *B. thuringiensis* by one amino acid only (Dement'ev et al., 1993; Shul'ga et al., 2000). Regulatory mechanisms of their biosynthesis are similar (Ul'yanova et al., 2007; Ulyanova et al., 2008, 2015). However, our searches for barnase-like gene in 22 complete and 30 draft *B. thuringiensis* genomes during comparative studies failed to find even somewhat similar sequences. It forced us to clarify the taxonomic states of RNase-secreting *Bacillus* strains 7P and B-388.

The strain of *B. thuringiensis var. subtoxicus* B-388 which is reported in the literature as RNase-secreting bacteria (Chepurnova et al., 1988; Dement'ev et al., 1993) was initially isolated by Steinhaus as *Bacillus finitimus var. subtoxicus* 58-1-1 in the second half of 20th century (Gordon et al., 1973) in USA from intestine of *Plodia interpunctella* (or Indian Meal Moth), one of the most common pests of stored food products. It was maintained in several culture collections under different names, such as *Bacillus entomocidus* subsp. subtoxicus, *Bacillus thuringiensis var. thuringiensis*, *Bacillus cereus var. thuringiensis*, *Bacillus thuringiensis var. subtoxicus* (NRRL HD-18, NRS 1124, CCEB 458, CIP 53.138, ATCC 19268, and VKM B-388). It differed from *B. thuringiensis* ATCC 10792 type strain by negative Voges-Proskauer test and lack of crystalline endotoxin (Gordon et al., 1973; Nakamura and Dulmage, 1988).

The species belonging of *B. intermedius* is unclear as well. This name is absent in LPSN. The strain of *B. intermedius* 7P was initially isolated from soil of Tatarstan Republic of Russia in 1978 (Leschinskaya et al., 1978) and was described according to the Krasilnikov's Guide (Krasil'nikov, 1949). The strain is deposited in Russian National Collection of Industrial Microorganisms (VKPM) as *Bacillus* sp. 7P under accession number B3073.

The taxonomic status of RNase-producing strains must be clarified in order to use molecular approaches in comparative studies and modulate gene expression for enhancement of protein production in bacterial cells. Since both *B. thuringiensis var. subtoxicus* B-388 and *Bacillus intermedius* 7P were identified based on a limited set of phenotypic characteristics and that obviously does not reflect their actual taxonomic state, we have applied molecular methods to determine the relationships of these strains to other *Bacillus* species.

## Material and Methods

***Strains.*** *Bacillus intermedius* 7P was received from VPKM. *B. thuringiensis var. subtoxicus* B-388 which is deposited in VKM was kindly provided by Dr. A. A. Dement'ev, Centre for Bioengineering RAS, Moscow, Russia. *B. amyloliquefaciens* H2 was kindly provided by Dr. R. W. Hartley from the National Institutes of Health, Bethesda, Maryland, USA.

***RNase activity assay.*** RNases were purified from culture fluids of *B. amyloliquefaciens* H2, *B. intermedius* 7P and *B. thuringiensis* B-388 strains and ribonuclease activity was measured using methods described before (Dudkina et al., 2014, 2015). One unit of RNase activity corresponds to the amount of enzyme that increases the extinction of acid-soluble products of 1 mg/ml RNA hydrolysis at 260 nm per min at 37°C.

***Immunoprecipitation.*** Immunoprecipitation of RNases with anti-barnase polyclonal serum which was obtained as reported before (Ulyanova et al., 2015b), was performed on microscopic slides covered with 3.5 ml of 1% Difco agar in 0.9% NaCl solution. Wells 3 mm in diameter and 4 mm apart were filled with antigen (RNase sample) or serum. Slides were kept for 24 h at room temperature followed by 24 h storage at 4°C.

***MALDI-TOF.*** Identification of bacteria was performed using Microflex LT MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectrometer (Bruker Daltonik GmbH) according to the manufacturer's instructions. The method is based on a measurement of mass spectrum generated by cell proteins under laser ionization and its comparison to a reference database (Welker and Moore, 2011). Since MALDI-TOF-MS detects a large number of proteins, the technique allows discrimination between closely related species. For experimental procedures the strains were grown at 37°C in Luria-Bertani agar for 16–18 h. For each strain, two similar applications of colony material to a 384-well stainless steel target plate were made. Cell proteins were extracted on target plate with $\alpha$-cyano-4-hydroxycinnamic acid (5 mg/ml) in a 50:48:2 acetonitrile:water:trifluoroacetic acid matrix solution. The samples were allowed to air-dry at room temperature. Analysis was performed in MALDI-TOF mass spectrometer in automatic mode with default settings, and a minimum of 240 laser shots at different positions on the sample spot were collected for each isolate. Ionized proteins were separated based on their mass-to-charge ratio and mass spectra were detected and compared to reference database (Bruker Biotyper 3.0 software and library version 3.1.2.0). The higher score of the two reads was used. The bacterial test standard (Bruker Daltonik GmbH) was used as a positive control and the matrix alone as a negative control.

***PCR.*** Bacterial chromosomal DNA was prepared using Wizard Genomic DNA Purification Kit (Promega, USA) according to the instruction manual. The gene encoding 16S rRNA was amplified using two pairs of oligonucleotide primers 101F and 1110R and 933F and 1407 R (Domann et al., 2003). The gene encoding RNA polymerase beta subunit (*rpoB*) was amplified using primers 1206f (5′-atcgaaacgcctgaaggtccaaacat-3′) and 3202r (5′-acacccttgttaccgtgacgacc-3′) (Ki et al., 2009). Each PCR mixture contained 1× Phusion Flash PCR Master Mix (Thermo Fisher Scientific, USA), 5 $\mu$L of extracted DNA, 0.5 $\mu$M of each primer, and water to total volume of 50 $\mu$L. PCR was performed on a Pico Thermal Cycler (Thermo Fisher Scientific, USA) with the following cycling program: 98°C for 10 s, 35 cycles at 98°C for 1 s, 61°C for 5 s for 16S rRNA gene or 50°C for 5 s for *rpoB* gene, and 72°C for 30 s, with a final extension at 72°C for 1 min. Subsequently, 2 $\mu$L of each PCR product was subjected to electrophoresis on a 1% agarose gel in 1 X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3). The PCR products on the gel were visualized by UV

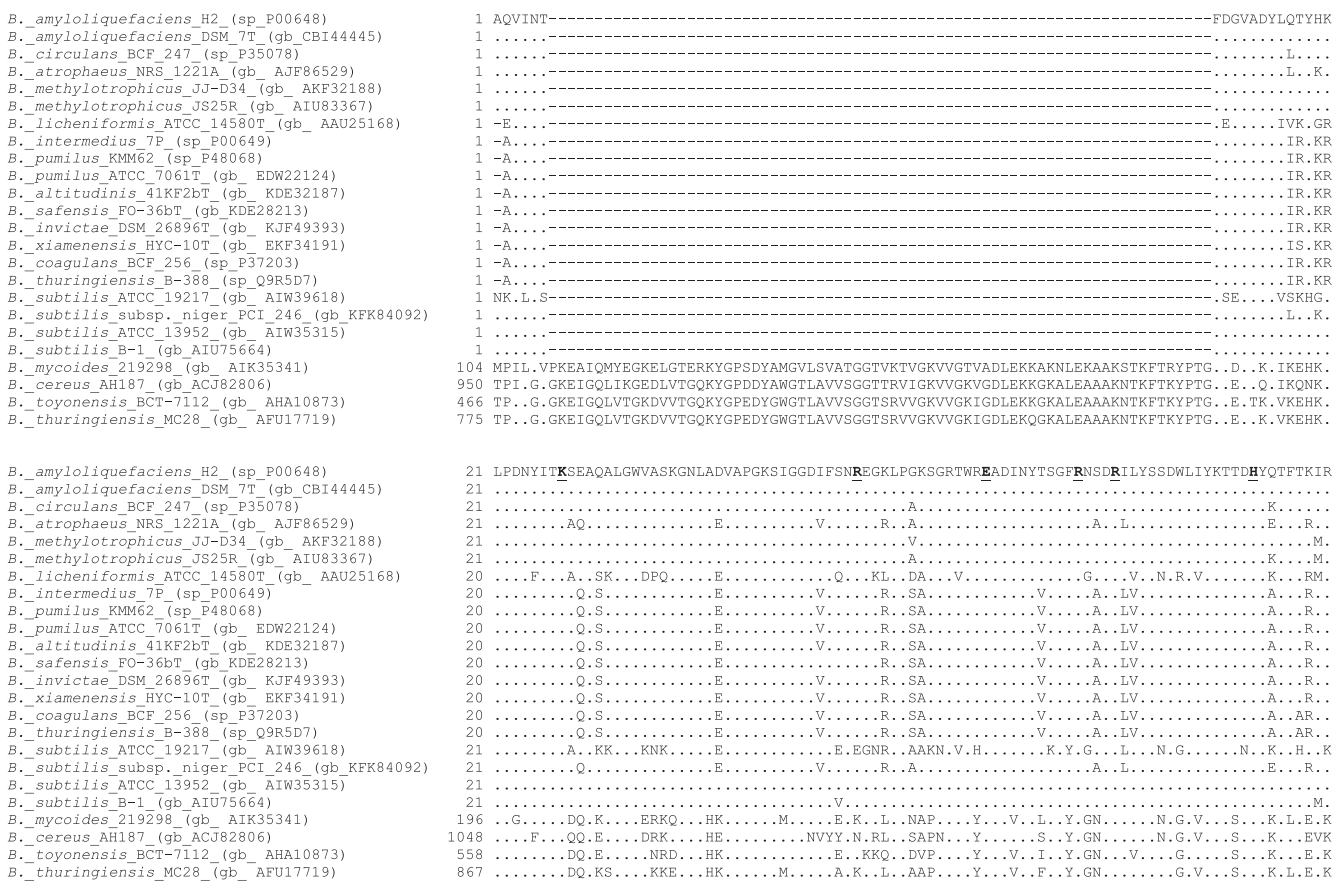


**Fig. 1.** Comparative sequence analysis of barnase homologues found in *Bacillus* species upon BLAST and UniProtKB/Swiss-Prot searches.

Only amino acid residues different from barnase are shown. Amino acid residues of barnase active site are bold and underlined. GenBank or Swiss-Prot accession numbers are indicated in parentheses.

illumination after staining with ethidium bromide. The obtained PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's instructions and submitted for Sanger sequencing to AGOWA (http://www.nucleics.com).

***Bioinformatics.*** Amino acid sequences of *Bacillus* RNases which are similar to barnase were extracted from GenBank upon Basic Local Alignment Search Tool (BLAST) search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with experimentally described ribonucleases deposited in UniProtKB/Swiss-Prot database (http://www.uniprot.org/uniprot/?query=reviewed%3Ayes) using Muscle algorithm (http://www.ebi.ac.uk/Tools/msa/muscle/). Differences in primary structures were visualized by BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Editing and contig assembly of the sequenced DNA were performed using SeqMan (DNAStar Lasergene, USA). For additional verification, all the *rpoB* and 16S rRNA gene sequences were compared with nucleotide sequences in the GenBank database using the BLAST search algorithm. The data were analysed using MEGA5 software (Tamura et al., 2011). The near complete 16S rRNA (>1,400 nt) and *rpoB* (~3,500 nt) gene sequences of *Bacillus* strains were obtained from Genbank and aligned using built-in Muscle algorithm. Nucleotide sequences of *rpoB* genes were trimmed to the region limited by the primers used; codons were aligned and translated into amino acid sequences. The variable and ambiguously aligned sites were excluded. Multiple sequence alignments are available in Supplementary Materials (Tables S1 and S2). Unrooted neighbor-joining phylogenetic trees were generated using Kimura two-parameter (16S rRNA) or JTT (RpoB) models of substitution. The robustness for individual branches was estimated by bootstrapping with 1,000 replicates. Modeling of tertiary protein structures was performed on I-Tasser server (Yang et al., 2015). Models were visualized by Jmol: an open-source Java viewer for chemical structures in 3D (http://www.jmol.org/).

## Results and Discussion

Ribonucleases of bacterial origin are perspective tools for the development of new pharmaceutical and biotechnological agents which have a potential as antitumor (Mironova et al., 2013; Mitkevich et al., 2014) and antiviral (Shah Mahmud and Ilinskaya, 2013) drugs. In order to estimate the biodiversity of RNase-secreting strains, to conduct comparative studies and to find new RNase producers we have performed database searches for homologues of barnase. According to the List of Prokaryotic Names with Standing in Nomenclature (Parte, 2014) there are 299 validly published *Bacillus* species. According to Genome database of National Center for Biotechnology Information (NCBI) genomes of 24 *Bacillus* species are completely sequenced while 59 species are involved in draft projects (http://www.ncbi.nlm.nih.gov/genome/

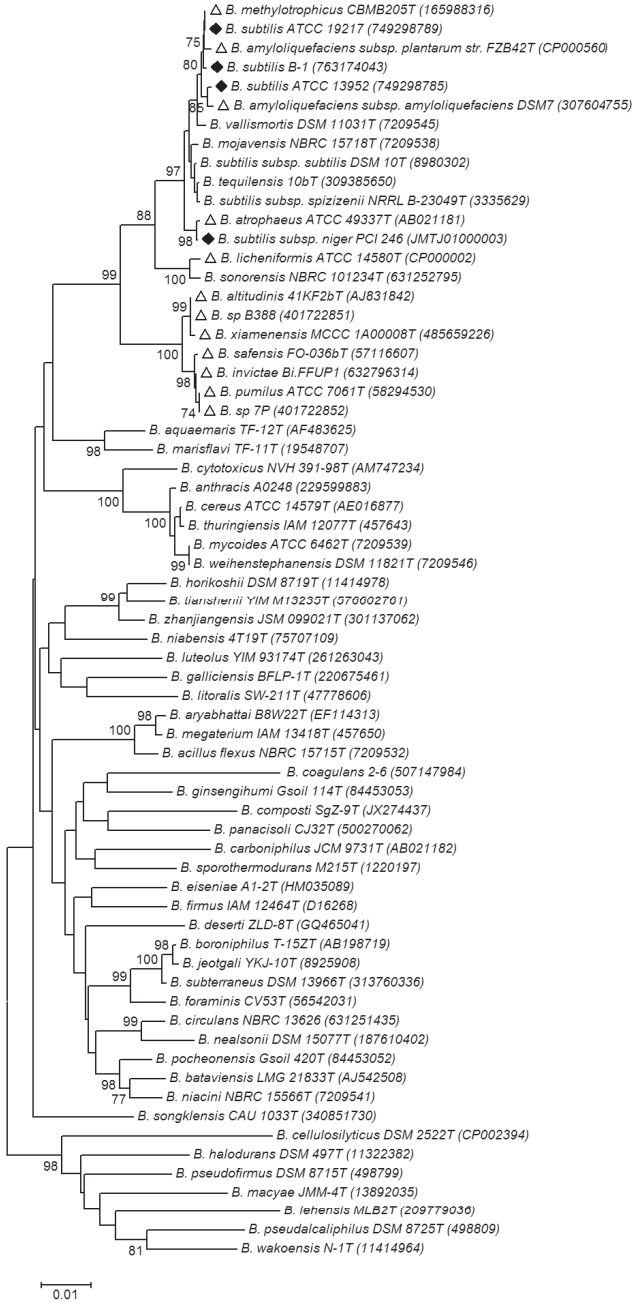


**Fig. 2.** Unrooted neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences and showing the relationships of *B. sp* B-388, *B. sp* 7P and RNase-harboring *B. subtilis* (diamonds) strains with other closely related *Bacillus* species.

RNase-producing species are marked with triangles. The tree was generated using 1427 bp nucleotide sequences of 60 *Bacillus* species. Genbank accession numbers are in parentheses. Numbers at the nodes indicate bootstrap values based on 1000 replicates. Numbers less than 70% are not shown. Bar, 0.01 changes per nucleotide position.

browse/). Using BLAST algorithm we have found that genomes of 10 *Bacillus* species, namely *B. altitudinis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. invictae*, *B. licheniformis*, *B. methylotrophicus*, *B. pumilus*, *B. safensis*, *B. subtilis*, *B. xiamenensis*, encode guanyl-preferring ribonuclease similar to barnase (Fig. 1). All of them belong to "*B. subtilis*" group within *Bacillus* genus. However, the obtained data were in a contradiction with the fact that

**Table 1.** Proteins found in species of "*Bacillus cereus*" group upon delta-BLAST search using barnase as a query.

| Species | Strain | Plasmid | GenBank accession number | Protein name | Protein length (aa) | N-terminal domain(s), position (aa) | Pre-Toxin domain, position (aa) | RNase domain, position (aa) | RNase active site (aa) |
|---|---|---|---|---|---|---|---|---|---|
| *B. toyonensis* | BCT-7112 | pBCT77 | AHA10873.1 | hypothetical | 647 | — | 455–535 (80) | 544–647 (103) | 593, 610, 624, 639 |
| *B. thuringiensis* | MC28 | pMC319 | AFU17719.1 | S-layer homology domain ribonuclease | 956 | glucan-binding domains, 31–411 | 764–844 (80) | 853–956 (103) | 902, 919, 933, 948 |
| *B. mycoides* | 219298 | Unnamed_4 | AIK35341 | RNase domain protein | 285 | — | 101–173 (72) | 182–285 (103) | 231, 248, 262, 277 |
| *B. cereus* | A1 Hakam | pBTZ_2 | AJI14540.1 | ribonuclease domain protein | 285 | — | 101–173 (72) | 182–285 (103) | 231, 248, 262, 277 |
| *B. cereus* | AH820 | pAH820_272 | ACK92711.1 | S-layer domain-containing ribonuclease | 1131 | SLH domains, 36–194 | 939–1019 (80) | 1028–1131 (103) | 1077, 1094, 1108, 1123 |
| *B. cereus* | AH187 | pAH187_270 | ACJ82806.1 | S-layer domain ribonuclease | 1131 | SLH domains, 36–194 | 939–1019 (80) | 1028–1131 (103) | 1077, 1094, 1108, 1123 |

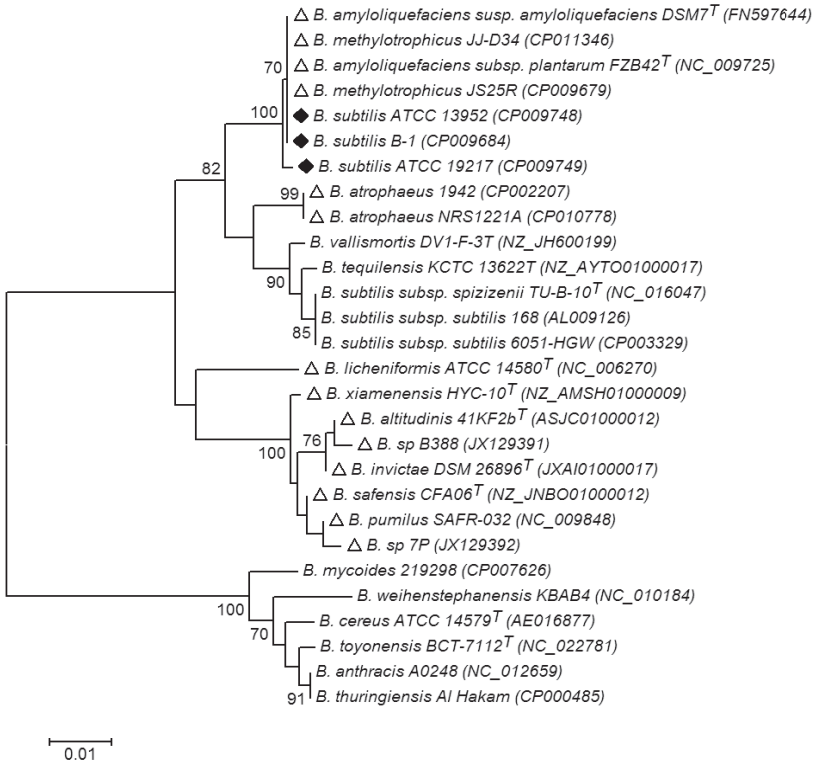


**Fig. 3.** Unrooted neighbor-joining phylogenetic tree highlighting the position of *B. sp* B-388, *B. sp* 7P and RNase-harboring *B. subtilis* (diamonds) strains among related *Bacillus* species based on multiple alignment of deduced amino acid sequences of 1337 bp *rpoB* fragments.

RNase-producing species are marked with triangles. Genbank accession numbers are in parentheses. Numbers at the nodes indicate bootstrap values based on 1000 replicates. Numbers less than 70% are not shown. Bar, 0.01 changes per nucleotide position.

type strain of *B. subtilis* lacks barnase-like RNase (Condon and Putzer, 2002). Moreover, other representatives of "*B. subtilis*" group such as *B. mojavensis*, *B. sonorensis*, *B. tequilensis*, *B. vallismortis* do not have barnase gene as well. It encouraged us to examine phylogenetic relations of RNase-encoding *B. subtilis* strains ATCC 19217, ATCC 13952, B-1 and PCI 246 to *B. subtilis* DSM10$^T$. Sequence analysis of 16S rRNA and housekeeping RNA polymerase sub-unit beta (*rpoB*) genes showed that they are closely related to the species like *B. amyloliquefaciens* or *B. methylotrophicus* (Fig. 2 and 3) that supports the absence of barnase-like RNases in *B. subtilis* strains. Therefore, sequence data of non-type strains that were isolated long ago and described without involvement of molecular genetics approaches should be treated very carefully even if the strains are deposited in the international culture collections.

Using Delta-BLAST algorithm which is specialized on a sensitive protein-protein search barnase-like RNases were also identified in *B. cereus* A1 Hakam, *B. cereus* AH820, *B. cereus* AH187, *B. thuringiensis* MC28, *B. toyonensis* BCT-7112, *B. mycoides* 219298. However, these RNases represented C-terminal domains of large S-layer toxins which are encoded in operons together with the gene for RNase inhibitor on plasmid (Table 1). The level of similarity of amino acid sequences between barnase and RNase domains of S-layer proteins was 78–82% (identity 61–66%) with overall coverage of 94–97% (Fig. 1). Amino acid residues of barnase active site (K27, R59, E73, R83, R87, H102) are conserved in S-layer proteins (except for R59 in some cases). Tertiary structures of S-layer toxins which were generated using I-Tasser server have less in common with three-dimensional structure of barnase (PDB: 1BNR) until the RNase domain is detached (Fig. 4). RNase domain within S-layer proteins has a nearly identical to barnase three-dimensional structure and is oriented so that its assumed active site is faced outside the whole molecule. This observation together with conservation of amino acid residues of RNase active site suggests that S-layer toxins have among others an RNase function. Composition and arrangement of RNase-containing toxins suggest the distinct physiological role for them. Chromosome-encoded RNases are likely to be involved in stress response of bacterial cells whereas plasmid-encoded RNases serve as aggression factor (Van Melderen, 2010; Yamaguchi et al., 2011). Being the part of S-layer the RNase-containing toxins should be considered as contributing to virulence or defense strategy of the organism (Mignot et al., 2001; Sleytr and Beveridge, 1999). Nevertheless, it is difficult to find correlation between the presence of S-layer, the occurrence of RNase-containing toxin, the pathogenic lifestyle or phylogenic relations. The plasmid-encoded gene for RNase-containing toxin can easily be distributed between strains and endows them competitive advantages in certain conditions.

Thus, species belonging to "*B. cereus*" group do not

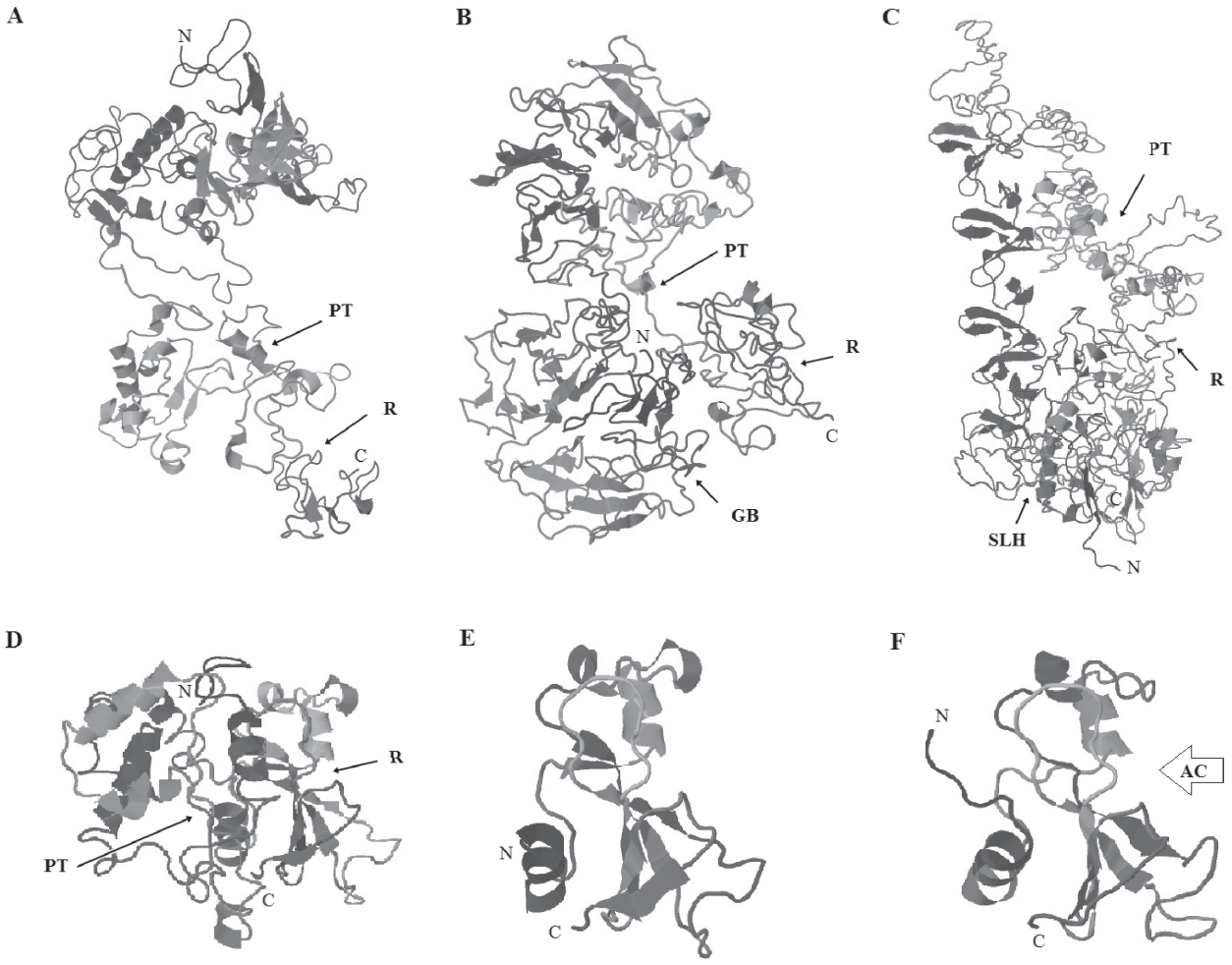


**Fig. 4.** Three-dimensional structures of RNase-containing S-layer toxins (A–E) and barnase (F, PDB: 1BNR).

Models were generated using amino acid sequences of *B. toyonensis* BCT-7112 (A), *B. thuringiensis* MC28 (B), *B. cereus* AH820 (C), *B. mycoides* 219298 (D) S-layer toxins and RNase domain of *B. thuringiensis* MC28 S-layer toxin (E). PT - pre-toxin domain, R - RNase domain, GB - glucan-binding domain, SLH - surface layer homology domain, AC - active center of barnase. N- and C-termini of the proteins are labeled.

possess standalone extracellular RNase which can be secreted into the environment. Previously, extracellular RNases differing from each other by one amino acid only and identical to RNase of *B. pumilus* were purified from supernatants of *B. thuringiensis var. subtoxicus* B-388 (Chepurnova et al., 1988; Dement'ev et al., 1993) and *B. intermedius* 7P (Aphanasenko et al., 1979). It forced us to clarify their taxonomic states.

Initially, we have tested *B. intermedius* 7P and *B. thuringiensis* B-388 strains for extracellular RNase. Strain of *B. amyloliquefaciens* H2 was used as a reference. In each case the level of RNase activity reached maximal values in the beginning of stationary phase of bacterial growth and amounted to 8,000 ± 1,200 units for *B. amyloliquefaciens*; 9,000 ± 1,300 units for *B. intermedius* and 18,000 ± 2,500 units for *B. thuringiensis* B-388. Using immunochemical assay of precipitation in agar gel it was shown that RNases from *B. intermedius* (binase) and *B. thuringiensis* are antigenically identical to each other and both share partial antigenic identity with *B. amyloliquefaciens* ribonuclease (Fig. 5). The results are in agreement with previous data regarding 85% identity of primary structures of barnase and binase as well as near identical amino acid sequences of binase and RNase from *B. thuringiensis* B-388 (Aphanasenko et al., 1979; Dement'ev et al., 1993; Ulyanova et al., 2015a).

There are several approaches that are currently applied in bacterial identification. Compared with phenotypic and molecular tests, MALDI-TOF MS is a rapid, accurate, uncomplicated method which allows discriminating between closely related species and can be used to classify organisms even at the subspecies level (Murray, 2010; Welker and Moore, 2011). In this study, spectra generated by MALDI-TOF MS during analysis of two *Bacillus* strains B-388 and 7P and compared by Biotyper software to the reference database resulted in matching of the first strain to *B. altitudinis* and the second one to *B. pumilus* (Biotyper database contains type strains for both species). The value scores for B-388 strain were 2.024 and 2.011, for 7P strain were 2.064 and 2.005. As specified by the manufacturer, scores between 2.000 and 2.299 indicate secure genus and probable species identification. The probes were classified into category A that implies species consistency. Since MALDI-TOF MS is a rather new

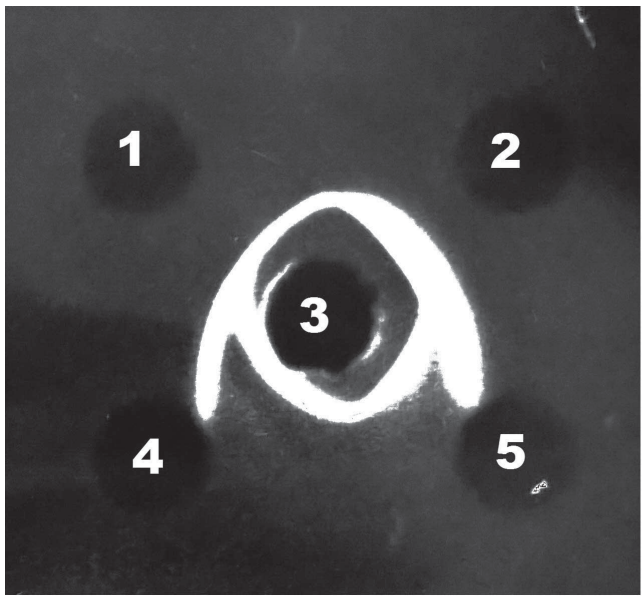


**Fig. 5.** Reaction of two-dimensional immunodiffusion of *Bacillus* RNases and anti-barnase serum.

1 and 2 - ribonuclease of *B. amyloliquefaciens* (barnase), 3 - polyclonal anti-barnase serum, 4 - ribonuclease of *B. intermedius* 7P, 5 - ribonuclease of *B. thuringiensis* B-388.

technique we compared the obtained results to data generated by standard method for species identification, namely 16S rRNA gene sequencing.

The sequences of the 16S rRNA genes of B-388 and 7P strains were determined and aligned against sequences downloaded from GenBank database for *Bacillus* type strains. A phylogenetic tree based on a neighbor-joining analysis of 1427 bp sequences common to all the strains showed that 7P strain formed a cluster with *B. pumilus*, while B-388 grouped together with *B. altitudinis* and *B. xiamenensis* (Fig. 2). The 16S rRNA gene sequence of strain 7P and *B. pumilus* ATCC 7061$^T$ as well as B-388 strain and *B. altitudinis* 41KF2bT were identical and there is only one nucleotide difference between 16S rRNA genes of B-388 and *B. xiamenensis* HYC-10$^T$. Both strains under study turned to be closely related. 1419 out of 1425 bp of their 16S rRNA genes were identical (99.6%).

Differentiating among species that have little variation in their 16S rRNA gene sequences can be attained by examination of genes less highly conserved than that of 16S rRNA. Therefore, we have included in the analysis housekeeping *rpoB* gene, that has demonstrated its usefulness for molecular identification (Ki et al., 2009). Phylogenetic tree reconstructed on the basis of amino acid sequences deduced from 1337 bp fragments of *rpoB* genes proved the grouping of B-388 and 7P strains (Fig. 3).

Phylogenetic trees derived from the 16S rRNA and *rpoB* genes confirmed the MALDI Biotyper identification of both *Bacillus* strains under study. The strains are closely related which reflects the high level of similarity of ribonucleases secreted by them. Therefore, the strain *B. thuringiensis* B-388 should be regarded as *B. altitudinis* B-388 and *B. intermedius* 7P should be renamed as *B. pumilus* 7P.

The results obtained also argue for the observation that extracellular guanyl-preferring ribonucleases similar to barnase are the exclusive feature of "*B. subtilis*" group within the *Bacillus* genus. This group is also known as a "soil" one. It differs from "pathogenic" *B. cereus* group by the lifestyle and adaptation strategies to the environment (Alcaraz et al., 2010). The representatives of "*B. subtilis*" group are often found in rhizosphere and are able to enhance growth of plants through direct and indirect mechanisms, in particular by the production of extracellular RNases (Kolpakov and Kupriianova-Ashina, 1992). Ribonucleolytic activity facilitates the breakdown of polymeric molecules and supplies other enzymes with the substrates. The complex of bacterial extracellular enzymes provides the pool of low-molecular weight nutrients which can be absorbed by plants. Being a cytotoxic, RNases can contribute to anti-pathogen defense (Deshpande and Shankar, 2002; Filipenko et al., 2013). Finally, 2′-3′-cGMP which is formed during RNA hydrolysis by guanyl-preferring RNases can act as signal molecule which has an influence on proliferation and programmed cell death (Kessler and Steinberg, 1973; Sokurenko et al., 2015; Van Damme et al., 2014). At the same time, not all members of "*B. subtilis*" group possess barnase-like extracellular RNases that could be due to the loss of the gene copy under ecological pressure. The soil is a very heterogeneous environment with complex spatio-temporal interactions between the species (Torsvik and Øvreås, 2002). Further deep genomic and metagenomic studies will help to shed light into ecology and evolution of RNase-secreting strains. On the whole, our study emphasized the need of accurate taxonomic information in comparative studies for understanding of the biodiversity and relationships among living organisms from different ecological niches.

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**Supplementary Materials**

Supplementary tables are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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