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EXPERIMENTAL ARTICLES

Ribonucleolytic Activity of Mycoplasmas

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Abstract—Mycoplasmas are incapable of de novo synthesis of nucleotides and must therefore secrete nucleases in order to replenish the pool of nucleic acid precursors. The nucleolytic activity of mycoplasmas is an important factor in their pathogenicity. Bacterial ribonucleases (RNases) may produce a broad spectrum of biological effects, including antiviral and antitumor activity. Mycoplasma RNases are therefore of interest. In the present work, the capacity of *Acholeplasma laidlawii* and *Mycoplasma hominis* for RNase synthesis and secretion was studied. During the stationary growth phase, these organisms were found to synthesize Mg^{2+}-dependent RNases, with their highest activity detected outside the cells. Localization of *A. laidlawii* RNases was determined: almost 90% of the RNase activity was found to be associated with the membrane vesicles. Bioinformational analysis revealed homology between the nucleotide sequences of 14 *Bacillus subtilis* genes encoding the products with RNase activity and the genes of the mycoplasmas under study. Amino acid sequences of 4 *A. laidlawii p*roteins with ribonuclease activity and the Bsn RNase were also established.

Keywords: mycoplasmas, *Acholeplasma laidlawi, Mycoplasma hominis*, ribonuclease activity, localization, vesicles

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Interest to mycoplasmas (class *Mollicutes*) is caused by the unique biology of these smallest prokaryotes and a range of practical issues. Most mycoplasmas are parasites of humans, animals, and plants; some are the agents of socially important diseases, contaminants of cell cultures and vaccine preparations [1]. Control over mycoplasma infections and contaminations is thought to be possible upon investigation of the basic mechanisms of mycoplasma adaptation to environmental conditions, which are responsible for their abundance in nature and pathogenicity. Successful realization of a series of genomic projects related to a number of mycoplasmas proved the possibility of application of the post-genomic technologies to the subject. The well-known contaminants of cell cultures *Acholeplasma laidlawii* (causing agent of phytomycoplasmoses) and *Mycoplasma hominis* (causing agent of respiratory and urogenital disorders in humans) [2, 3] are unique in terms of their adaptive capabilities. Transcriptome–proteome analysis and nanoscopy studies of these microorganisms allowed for identification of their stress-reactive proteins [4, 5]; adaptation and virulence of the mycoplasmas were shown to be associated to a considerable degree with secretion of extracellular membrane vesicles [6, 7].

Nuclease activity is an important factor of mycoplasma pathogenicity. In contrast to other eubacteria, mycoplasmas are incapable of de novo synthesis of nucleic acid precursors. Nuclease activity provides for the possibility of obtaining the nucleic acid precursors essential for the cells [2, 3]. Ribonucleolytic (RNase) activity may determine, to a considerable extent, the genotoxic properties of these bacteria [8]. Earlier, nuclease activity of mycoplasmas was demonstrated to be associated mainly with the membrane [9]. Meanwhile, data of proteomic profiling evidence that extracellular membrane vesicles of a number of bacteria mediate the RNase traffic [10, 11]. In this connection, analysis of mycoplasma vesicles for the presence of RNase activity is of interest.

Small size of the mycoplasma genome is associated with its high information capacity [12, 13]. Despite the characteristic structural features of the mycoplasma genes, considerable homology between the DNA nucleotide sequences coding for proteins, in particular, RNases involved in metabolism of these microorganisms and those of the phylogenetically related bacteria (bacilli) were revealed [14, 15]. For example, the *rnhC* gene—one of the three *B. subtilis* genes coding for intracellular nonspecific endoribonucleases cleaving the 3'-O–P bond in RNA of the DNA/RNA duplex—is coding for RNase HIII (33.9 kDa) and is homologous to the genes of

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M. genitalium (GenBank accession no. MG199) and *M. pneumoniae* (GenBank accession no. C09_orf143b) [16]. As for the extracellular RNases, secreted RNase Bsn (241 amino acids, 27 kDa) of *B. subtilis* is coded by the *bsn* gene [17] and a homologous enzyme (binase-II, 292 amino acids, encoded by the *birB* gene) is also produced by *B. pumilus* [18]. These facts explain the feasibility of the search for the genes encoding secreted RNase homologous to those of bacilli, in the mycoplasma genomes.

RNases are used in gene engineering, molecular biology, and biotechnology to remove RNA from biological material, in research on the structure–functional relationship of nucleic acids and their complexes with proteins, for development of vectors for positive selection of recombinants, and for production of sterile transgenic plants. A promising area of microbial RNase application is associated with their antiviral and antitumor potential [19, 20]. Study of mycoplasma RNases may widen our knowledge on the nature of their pathogenicity and reveal new aspects of RNase functions in physiological processes.

In this connection, the goal of the present work was to search for and characterize RNase activity in *Acholeplasma laidlawii* and *Mycoplasma hominis*, to reveal the optimal parameters of manifestation of such activity, and to establish the major sites of its localization in the cells.

MATERIALS AND METHODS

Microorganisms. Bacterial strains *Acholeplasma laidlawii* PG8 and *Mycoplasma hominis* PG37, obtained from the collection of the Gamaleya Research Institute of Epidemiology and Microbiology, were the subjects of the study.

Cultivation. *A. laidlawii* PG8 and *M. hominis* PG37 were cultured on the Edward's medium with insignificant modifications [21] at 37°C. Culture growth was followed visually by changes in the intensity of phenol red staining in the medium; the number of colony-forming units in a milliliter of the medium (CFU/mL) was determined simultaneously. Duration of cultivation for measurements of RNase activity of the cells and isolation of the membrane fraction was 30 h (stationary growth phase, 1.2×10^9 CFU/mL).

Isolation of cell membranes. Cells were precipitated by centrifugation (10000 *g*, 10 min), washed twice with the buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.01 M 2-mercaptoethanol), and lysed in deionized water (37°C, 30 min). Nonlysed cells were collected by centrifugation (10000 *g*, 10 min), and the supernatant was centrifuged at 37000 *g* for 40 min to precipitate the membrane fraction. The membranes were washed twice with the same buffer without 2-mercaptoethanol, resuspended in 1000 μL of the buffer, and stored at –20°C.

Isolation of membrane vesicles of *A. laidlawii* PG8. Membrane vesicles were isolated from 200 mL of the culture as described previously [6]. The cells were precipitated by centrifugation (15000 *g*, 40 min), and the supernatant was concentrated with a Vivacell 100 (Sartorius Stedim Biotech GmbH, Germany) apparatus. Concentrated supernatant was filtered through a sterile cellulose acetate filter (Sartorius Minisart, France) with pore diameter of 100 nm. The filtrate was then concentrated with an Amicon Ultra-15 100K (Millipore, United States) concentrator and washed with the buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl) six times. The suspension was stored at 8°C.

Isolation of peripheral proteins. To obtain various fractions of peripheral proteins, *A. laidlawii* PG8 and *M. hominis* PG27 were progressively resuspended in tenfold volumes of solutions with varying ionic strength and centrifuged for 20 min at 4400 *g* (4°C). Three solutions were used: no. 1 (50 mM Tris-HCl, 0.15 M NaCl, and 2 mM $MgCl_2$), no. 2 (50 mM Tris-HCl, 0.075 M NaCl, and 1 mM $MgCl_2$), and no. 3 (50 mM Tris-HCl, 0.0375 M NaCl, and 0.5 mm $MgCl_2$); in solution 1, the cells were resuspended twice. Thus, four fractions of peripheral proteins—Ia, Ib, II, and III—were obtained for both mycoplasmas.

Determination of RNase activity. Quantitative determination of ribonuclease activity was performed with a modified Anfinsen method by the acid-soluble products of RNA hydrolysis [22]. Activity of RNases was determined in the supernatant of the culture liquid, pure medium, membrane fraction, four fractions of peripheral proteins, fraction of cytoplasmic proteins after lysis (only for *A. laidlawii* PG8), and fraction of membrane vesicles (only for *A. laidlawii* PG8). Total protein values were determined according to Bradford [23]. The amount of enzyme causing an increase in the optical density of experimental samples by 1 unit against the control after 1 h incubation calculated for 1 mL enzyme solution was considered an activity unit. Specific activity was calculated per 1 mg protein.

Bioinformatics analysis. A search for the homologous genes coding for RNases of *A. laidlawii, M. hominis*, and *B. subtilis* was performed upon whole-genome comparison of these microorganisms using the CMR database (http://cmr.jcvi.org; Protein Scatter Plot subroutine). The sequences present in the genome of *B. subtilis* and the genome of at least one mycoplasma were selected.

Search for gene sequences of nucleolytic proteins of *A. laidlawii* PG8 homologous to extracellular RNases of bacilli—RNases of *B. pumilus* (binases) and Bsn RNase of *B. subtilis*—was conducted in the NCBI data base (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) basing on the phylogenetic proximity of *A. laidlawii* and gram-positive bacteria with low G+C content in the DNA (31%). The search for *A. laidlawii* PG-8A amino acid sequences similar to

Table 1. Homologous genes of *B. subtilis*, *A. laidlawii*, and *M. hominis*, the product of which exhibit RNase activity

Encoded protein*	Gene locus in the genome		
	B. subtilis	*A. laidlawii*	*M. hominis*
Ribonuclease III	BSU15930	ACL_0228	MHO_4690
Ribonuclease J1	BSU14530	ACL_0309	MHO_3380
Ribonuclease HII	BSU16060	ACL_0338	MHO_3300
ATP-dependent RNA helicase	BSU04580	ACL_0432	–
ATP-dependent RNA helicase	BSU04580	ACL_0481	–
Ribonuclease J1	BSU14530	ACL_0832	MHO_3690
Polynucleotide phosphorylase (PNPase)	BSU16690	ACL_0808	–
Ribonuclease M5	BSU00410	ACL_0015	MHO_2160
Ribonuclease III	BSU15930	ACL_0637	MHO_0830
Ribonuclease P	BSU41050	ACL_1432	MHO_0020
23S RNA maturation ribonuclease	BSU00950	ACL_0144	–
Putative exonuclease	BSU31470	ACL_0307	–
Ribonuclease R	BSU33610	ACL_0405	MHO_1900
Ribonuclease III	BSU28620	ACL_0815	MHO_3300

* Protein names correspond to those of the *B. subtilis* genome annotation and may be different in mycoplasmas.

the Bsn RNase was performed with the DELTA-Blast algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Mature form of ribonuclease of *B. subtilis* 168 (record number CAB15244.1) containing 262 amino acid residues was used as a reference sequence.

RESULTS AND DISCUSSION

Homology of RNase nucleotide sequences in *A. laidlawii, M. hominis*, and *B. subtilis*. In genomes of the three microbial species, 14 homologous genes encoding the proteins with RNase activity were revealed (Table 1). For 5 genes present in the genomes of *A. laidlawii* and *B. subtilis*, no homologue was found in *M. hominis*. In all three species, the RNase genes present either did not have a homologous gene in the other two genomes or the homologue was coded by the protein with another function (data not shown). Relatively recently, data on spatial arrangement of mycoplasma proteome and protein presentation in dynamic protein associates were obtained [13]. The possibility that a single protein may change its localization in the cell (membrane, cytoplasm, or extracellular space) depending on the proteins it interacts with was demonstrated. In addition to the classical pathways of secretion, when there is a signal peptide or a characteristic motif in the amino acid sequence, bacterial secretory pathways include another one, mediated by extracellular membrane vesicles, that has been actively studied in the past few years. No signaling sequences were detected in the proteins secreted via the vesicles [24]. The data of proteomic profiling demonstrate that the vesicles of a number of bacteria mediate RNase traffic [10, 11]. RNase activity may, to a considerable extent, cause the genotoxic properties of mycoplasmas and of the vesicles secreted by these bacteria [8]. In this connection, analysis of the distribution of RNase activity in mycoplasma cell fractions, including the vesicles, is of interest.

Homology of amino acid sequences of *A. laidlawii* PG8 and bacillary RNases. To search for mycoplasmal secreted RNases, two bacillary RNases were chosen as homologues. Upon comparison of amino acid sequences of *A. laidlawii* with that of binase, we did not find any statistically significant match. In the case of the Bsn RNase, ten matches were found, but four of them were statistically significant (nos. 1–4), since the probability of their matching by chance (E-value) did not exceed 10^{-3} (Table 2). Homology of the revealed nucleases to Bsn was 33–41%, or 44–55%, taking into account conserved substitutions. Alignment of the four revealed sequences and Bsn, containing a conserved endonuclease 1 superfamily domain, demonstrated that similar fragments belonged to the same conserved domain (Fig. 1). These data evidenced that the identified sequences belonged to the extracellular endonucleases, which provided ground for further experimental determination of RNase activity in the culture liquid and cell fractions of the mycoplasmas.

Table 2. Amino acid sequences of *A. laidlawii* PG-8A similar to the Bsn RNase

No.	NCBI database ID number	Description	Estimated probability of random matching (E-value)	Overlap	Identity	Similarity	Gaps
1	YP_001620119.1	Putative extracellular endonuclease anchored at cell surface	4×10^{-50}	84%	76/228 (33%)	107/228 (46%)	16/228 (7%)
2	YP_001620162.1	Extracellular endonuclease comprising a domain of endonuclease I	3×10^{-47}	85%	88/245 (36%)	120/245 (48%)	39/245 (15%)
3	YP_001620156.1	Putative extracellular endonuclease anchored at cell surface	1×10^{-38}	57%	55/151 (369%)	78/151 (51%)	26/151 (17%)
4	YP_001620157.1	Hypothetical protein containing a domain of extracellular endonuclease	3×10^{-37}	83%	69/220 (31%)	95/220 (43%)	42/220 (19%)

Activity of RNases in extracellular medium and cell fractions of the mycoplasmas. We obtained four fractions of peripheral proteins progressively extracted by solutions of different ionic strengths, as well as fractions of the membranes, cytosol, and vesicles of *A. laidlawii* PG8. It should be noted that due to the specific conditions of mycoplasma cultivation, the cultivation medium and, consequently, the culture fluid, contain considerable amounts of proteins (Fig. 2), among which there are those possessing RNase activity (Table 3). The homology of the *A. laidlawii* gene sequence with Bsn RNase, which nonspecifically hydrolyzes RNA to oligonucleotides with 5'-terminal phosphate and is a Mg^{2+}-dependent enzyme [17], motivated verification of the possibility of activation of the revealed ribonucleolytic activity by magnesium ions. It turned out that RNases of the cultivation media exhibited the highest activity at pH 5 in the absence of Mg^{2+} ions, in contrast to RNases of the culture liquid and cell fractions exhibiting the highest activity at weakly alkaline pH in the presence of magnesium. RNases of the cultivation media, which were

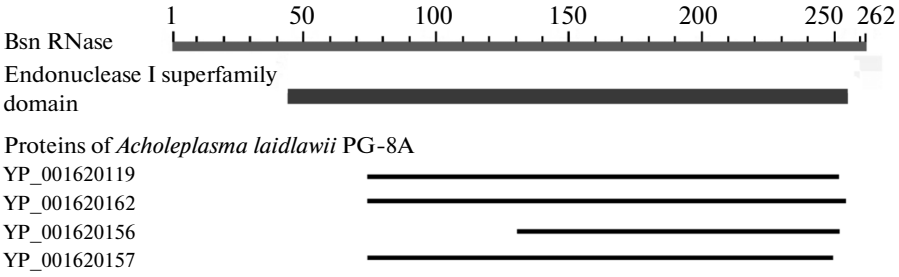

Fig. 1. Regions of *Acholeplasma laidlawii* PG-8A amino acid sequences overlapping with the Bsn RNase sequence, including the conserved domain of the endonuclease I superfamily.

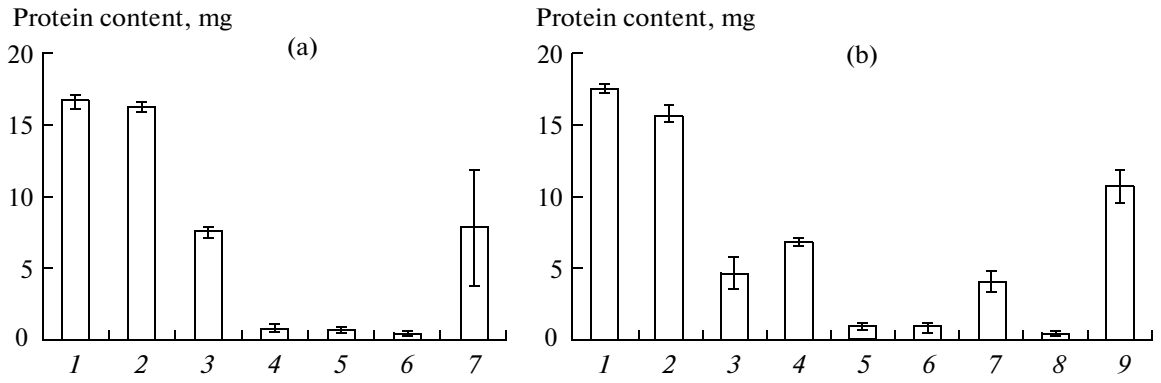

Fig. 2. Protein content in the cultivation medium, culture liquid, and cell fractions of *M. hominis* PG37 (a) and *A. laidlawii* PG8 (b): culture liquid (*1*); cultivation medium (*2*); peripheral proteins Ia (*3*); peripheral proteins Ib (*4*); peripheral proteins II (*5*); peripheral proteins III (*6*); membranes (*7*); cytosol (*8*); and vesicles (*9*).

introduced together with horse blood serum and yeast extract were probably essentially different from the RNases of mycoplasmas. The only exception was the activity of membrane RNases of *A. laidlawii* PG8 activated by Mg^{2+} at pH 5 (Table 3). A high level of ribonucleolytic activity was detected in culture liquids of both mycoplasmas, with activity of *A. laidlawii* PG8 being almost twice as high as the activity in *M. hominis* PG37. We also obtained the cytosolic and vesicular fractions of *A. laidlawii* PG8 cells and found that the RNases of secreted vesicles possessed the maximum activity (Table 3).

Distribution of RNase activity of mycoplasmas in cell fractions. Distribution of RNase activity over cell fractions showed that at pH 7.4 ribonucleolytic activity of both mycoplasmas was mainly detected outside the cells: the level of activity of secreted RNases was 95% of the total activity of these enzymes in the cells and culture liquid for *A. laidlawii* PG8 and 86%, for *M. hominis* PG37 (Table 4). RNase content in the membrane fraction and in the fraction of total peripheral proteins was not high. In *M. hominis* PG37, it was approximately 3 and 19% respectively, which exceeded the values for *A. laidlawii* PG8. This was probably due to the fact that we did not isolate the vesicles of *M. hominis* PG37 and the RNases they comprised were distributed over the culture liquid and peripheral proteins fractions. Distribution of RNase activity of *M. hominis* PG37 at alkaline pH of 8.5 remained the same as at pH 7.4. In *A. laidlawii* PG8, where we succeeded in isolation of a fraction of membrane vesicles, the major share of ribonucleolytic activity (89%) at alkaline pH was observed in the vesicles (Table 4).

Data on localization of nucleolytic activity of mycoplasmas in the cell membranes were obtained in the 1990s [9]. During the past twenty years, studies on bacterial membrane vesicles—subcellular organelles capable of various functions, including transport of nucleic acids and virulence determinants, cell protection from antimicrobial factors, and elimination of cell toxins—have experienced great progress [25]. Vesicles have been detected and characterized in gram-negative bacteria *Escherichia coli* [26] and *Pseudomonas aeruginosa* [27], gram-positive bacteria *Staphylococcus aureus* [28], etc. The latest works revealed the presence of such vesicles in the mycoplasma *A. laidlawii* PG8 [6, 7].

Taking into account the cytotoxic potential of many bacterial RNases [20], one may assume that the consequences of cell infection with mycoplasma are different at least with respect to the level of secretion and activity of RNases in these bacteria. For example, *M. fermentas* and *M. penetrans* efficiently support growth of (IL)-3-dependent lymphoblasts of murine bone marrow; on the contrary, *M. hominis* and *M. salivarium* induce apoptosis of these cells, and *M. genitalium* does not possess any apoptosis-modulating effects [29]. In this context, the detected high RNase activity in *M. hominis* PG37 and its extracellular localization (10.35 U/mg protein per hour; 86% to the total activity of the cells and culture liquid) confirm the possibility of apoptogenic effects of the enzymes. Here, we report for the first time that in the stationary growth phase, the major share of magnesium-dependent RNase activity in mycoplasmas is detected at physiological pH values and is characterized by extracellular localization. RNases of *A. laidlawii* PG8, active at alkaline pH values, make up to 89% of the total cellular and extracellular RNases of this microorganism and are localized in membrane vesicles. Further detailed characteristics of the vesicle functional proteins certainly will contribute to understanding of the mechanisms of the interaction of these infectogens with different cells.

Table 3. Activity of RNases in the extracellular medium and cell fractions of mycoplasmas

Fraction	pH	*A. laidlawii* PG8		*M. hominis* PG37	
		no $MgCl_2$	2 μM $MgCl_2$	no $MgCl_2$	2 μM $MgCl_2$
Medium	5.0	10.51	2.27	1.87	0.00
	7.4	0.59	4.73	0.40	0.00
	8.5	0.85	2.44	0.00	2.30
Culture liquid	5.0	1.80	1.19	3.17	5.57
	7.4	0.00	22.00	2.48	10.35
	8.5	3.01	4.74	4.53	4.30
Peripheral proteins Ia	5.0	0.18	0.17	0.26	0.83
	7.4	0.11	0.11	0.23	1.24
	8.5	0.22	0.13	0.23	0.90
Peripheral proteins Ib	5.0	0.26	0.22	0.04	0.03
	7.4	0.71	0.21	0.00	0.03
	8.5	0.32	0.23	0.02	0.04
Peripheral proteins II	5.0	0.02	0.04	0.03	0.02
	7.4	0.07	0.11	0.01	0.03
	8.5	0.04	0.08	0.04	0.02
Peripheral proteins III	5.0	0.05	0.07	0.02	0.02
	7.4	0.07	0.06	0.02	0.03
	8.5	0.05	0.09	0.02	0.05
Membranes	5.0	0.32	1.52	0.15	0.03
	7.4	0.10	0.07	0.22	0.35
	8.5	0.03	0.02	0.67	0.00
Cytosol	5.0	0.13	0.12	Not determined	
	7.4	0.03	0.21		
	8.5	0.04	0.06		
Vesicles	5.0	0.05	0.44	Not determined	
	7.4	0.12	0.33		
	8.5	2.62	43.28		

Data of three independent experiments are presented. Standard deviation did not exceed 12%.

Table 4. Percentage of ribonucleolytic activity (activated by Mg^{2+}) in the culture liquid and cell fractions of the mycoplasmas. Total activity of the culture liquid (minus the activity of the medium) and all cellular fractions at a certain pH value was accepted as 100%

Fraction	pH	*A. laidlawii* PG8	*M. hominis* PG37
Culture liquid	7.4	95.19	86.03
	8.5	9.74	81.12
Total peripheral proteins	7.4	2.16	10.02
	8.5	1.08	18.88
Membranes	7.4	0.30	2.91
	8.5	0.04	0.00
Cytosol	7.4	0.92	1.04
	8.5	0.15	0.00
Vesicles	7.4	1.43	Not determined
	8.5	88.99	Not determined

Data of three independent experiments are presented. Standard deviation did not exceed 12%.

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