Acholeplasma laidlawii is a unique mycoplasma species (class Mollicutes) due to its adaptive capacity. This mycoplasma is widespread in nature, it is found in soil and sewage as well as in tissues of higher eukaryotes. This is one of the five major contaminants of cell cultures and a causative agent of phytomycoplasmoses [1, 2]. The control of mycoplasma infections is a serious problem, the solution of which is associated with the elucidation of mechanisms of adaptation of mycoplasmas to environmental conditions, formation of the “parasite–host” system, and realization of virulence [2, 3]. The sequencing of the genome of A. laidlawii [4] made it possible to use post-genomic technologies to determine the molecular-genetic basis of the survival of mycoplasmas in different environmental conditions [2]. The use of transcriptomic and proteomic analysis and nanoscopy made it possible to identify stress-reactive proteins and genes of A. laidlawii PG8 and show that the adaptation of mycoplasmas to unfavorable factors is associated with the secretion of extracellular membrane vesicles (ECMVs) [2]. It has been found that A. laidlawii ECMVs, in addition to the membrane components, comprise the nucleotide sequences of several genes as well as soluble proteins involved in manifestation of bacterial virulence and exhibit a high mutagenicity with respect to the cells of higher eukaryotes [5]. ECMVs mediate secretion in bacteria, are involved in signaling, cell-cell interactions and pathogenesis [6]. It is assumed that ECMVs can significantly contribute to the phytopathogenicity of bacteria [7–9], including mycoplasma [2]. For example, the phytopathogenicity of stress-adapted A. laidlawii, whose cells, unlike unadapted bacteria, cannot penetrate through the root system of Oryza sativa L., can be mediated by the ECMVs of mycoplasma. However, information on the studies of phytopathogenicity of bacterial ECMVs is missing in the literature. The purpose of this study was to elucidate the characteristics of interaction of extracellular membrane vesicles of A. laidlawii PG8 with plants (O. sativa L.).

In this study, we used the A. laidlawii strain PG8, which was obtained from the collection of microorganisms of the Gamaley Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences. A. laidlawii PG8 cells were grown at 37°C in Edward’s liquid medium with some modifications [3]. Extracellular membrane vesicles were isolated from A. laidlawii PG8 culture (stationary growth phase, 45 h) according to [5]. Rice (O. sativa L.) plants were grown in a medium containing A. laidlawii PG8 membrane vesicles as described in [2].

Transmission electron microscopy was performed according to [10].

DNA from mycoplasmas and plant tissues were isolated as described in [11]. DNA from ECMVs was isolated using the DNA Express commercial kit (Lytech, Moscow). Prior to extraction of nucleic acids, samples of mycoplasma membrane vesicles were treated with DNase I (37°C, 30 min). Total RNA from O. sativa L. leaf tissues was isolated using the SV Total RNA Isolation System commercial kit (Promega, Germany) according to manufacturer’s instructions.

The nucleotide sequences of genes tuF (105 bp) and ftsZ (1133 bp) of A. laidlawii PG8 were amplified using the following primers (tuF: AqF3, 5’-cagcctcgcgatcaaggt-3’, AqR3, 5’-cagctgctgttggcatttg-3’; ftsZ: AlaF1, 5’-gggtggatggtgaaatcga-3’, AlaR1, 5’-ggtggagccttccgctattt-3’), which were constructed on the basis of data on the nucleotide sequences of A. laidlawii PG8-A genes (GenBank, NC_010163) at the Lytech Science and Production Association (Moscow).
The reverse transcription was performed with oligo(dT)$_{18}$ according to the recommendations of the manufacturer of RevertAidTM M-MuLV reverse transcriptase (Fermentas, Lithuania). Residual amounts of DNA in the RNA preparation were monitored by amplifying the RNA samples using the specific primers: OsqF1, 5'-gccagcagcagcagcagcagc-3'; OsqR1, 5'-ccttgattccaccgcagc-3'; OsqF2, 5'-gcggcatcaccatcaacacc-3'; OsqR2, 5'-tgtgctctttggtctgcggc-3'; OsqF3, 5'-aggtgacatcgcgcggtaacc-3'; OsqR3, 5'-ctctgtgtctctgtctggt-3'; OsqF4, 5'-accggaggccccgcaagac-3'; and OsqR4, 5'-gggaggccgcaagatggt-3'.

The intensity of bands after electrophoretic separation of the amplification products was quantified using the Phoretix 1D software (v. 2003.02) (Nonlinear Dynamics Ltd., Newcastle upon Tyne, Great Britain). Normalization was performed by the level of expression of the genes encoding TufA and RuBisCO. The expression of the gene was expressed as the ratio of the signal intensity of the amplicon derived from the cDNA sequence of the studied gene (Oi) to the signal intensity of the amplicon derived from the cDNA sequence of the control gene (Ki), which was converted into a base 2 logarithm: log$_2$(Oi/Ki).

DNA sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, United States) and 3130 Genetic Analyser (Applied Biosystems, United States). The nucleotide sequences were analyzed in the Sequencing Analysis 5.3.1 program (Applied Biosystems, United States) as well as using the NCBI database (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The ability of _A. laidlawii_ to induce chronic oxidative stress in plants largely determines the toxigenicity of mycoplasma [12]. Note that in the plants that are

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**Fig. 1.** Ultrastructural organization of cells of _O. sativa_ L. plants grown in media (a) containing and (b) not containing _A. laidlawii_ PG8 ECMVs. Designations: Vc, vacuole; Gr, granules; CW, cell wall; Mt, mitochondria; Pr, peroxisome; Chl, chloroplast; N, nucleus.
not specific indicators of phycomycoplasmoses, *A. laidlawii* can cause inapparent infections when clear signs of morphological abnormalities (dwarfism, the development of side shoots, stunting, and yellowing of leaves) are not expressed but reorganization of the proteome and destructive changes in the ultrastructure of tissues are recorded [2].

Our studies showed that, in the plants grown in the medium containing ECMVs of *A. laidlawii* PG8, pronounced morphoses were not detected. Apical necrosis and tillering were observed only in single plants. However, the analysis of ultrathin sections of leaves of plants grown in the ECMV-containing medium revealed significant disturbances in the ultrastructure of tissues (Fig. 1): chloroplasts in the leaf parenchymal cells were located near the wall, had an electron-dense stroma, and contained no starch grains. Vacuoles of vessel lining and parenchymal cells were filled with a loose content, mitochondria had a cleared loose matrix and contained single cristae. The observed disturbances in the ultrastructural organization of parenchymal cells of *O. sativa* L. leaves are characteristic of plants experiencing oxidative stress [13] and plants infected with *A. laidlawii* PG8 cells [2].

A similar pattern of plant response to ECMVs and mycoplasma cells was also observed during the analysis of expression of the genes encoding the *O. sativa* L. proteins, the modulation of the content of which was detected by us in the case of infection with *A. laidlawii* PG8 cells [2]. The obtained raw data for the amplification products of the genes encoding the stress-reactive proteins TufA (translation elongation factor Tu), MAT (methionine adenosyl transferase, an enzyme involved in the secondary metabolism of plants), RASIPs (precursor of activase of ribulose biphosphate carboxylase, which is involved in photosynthesis), and

| Protein name                                           | NCBI*              | Change in protein expression* | Change in gene expression |
|--------------------------------------------------------|--------------------|-------------------------------|---------------------------|
| Unnamed protein (UPP)                                  | gi|215686337         | **1.7**                      | **1.37**                  | **1.45**                  |
| Translation elongation factor Tu (TufA)                | gi|17225494          | **1.6**                      | **1.51**                  | 0.07                      |
| Ribulose biphosphate carboxylase (RASIPs))            | gi|62733297          | **3**                        | **1.91**                  | **0.46**                  |
| Methionine adenosyl transferase (MAT)                  | gi|3024122           | **1.23**                     | **0.98**                  | **1.79**                  |

Note: * Identification number of protein in the NCBI database.
     * Numerals (bold font) indicate an increase in expression.
     C—plants infected with the control *A. laidlawii* PG8 culture cells.
     M—plants grown in the medium containing *A. laidlawii* PG8 ECMVs.

Comparative analysis of expression levels of genes and respective proteins in the cells of *O. sativa* L. plants infected with *A. laidlawii* PG8 cells and ECMVs

Fig. 2. Electrophoretograms of amplification products of the nucleotide sequences of genes *ftsZ* (lanes 1, 3, 5, 7) and *tufB* (lanes 2, 4, 6, 8) of *A. laidlawii* PG8, obtained by PCR with total DNA used as a template. Total DNA was isolated from (1, 2) cells and (3, 4) ECMVs of *A. laidlawii* PG8 as well as from the leaves of plants grown in the medium with (5, 6) and without (7, 8) ECMVs. The arrow denotes the localization of the corresponding amplification signal. M—fragment length marker.
UPP (unnamed protein, presumably a carbonic anhydrase involved in photosynthesis, respiration, pH homeostasis, and ion transport) indicate that the characteristics of expression of the genes encoding corresponding *O. sativa* L. proteins in the plants grown in media with mycoplasma cells and ECMVs largely coincide (table).

The ability of *A. laidlawii* PG8 ECMVs to induce pathological processes in the plants growing in the media containing mycoplasma cells and ECMVs largely coincide (table).

![Fig. 3. Results of alignment of (a) nucleotide and (b) amino acid sequences of the fragment of the *tufB* gene from (1) cells and (2) ECMVs of *A. laidlawii* PG8 and (3) amplicon obtained by PCR using the primers for the detection of the nucleotide sequence of the *tufB* gene of *A. laidlawii* PG8-A. DNA of the plants grown in the medium containing *A. laidlawii* PG8 ECMVs was used as a template in PCR. The sequences of the forward and reverse primers are shown in italic. The nucleotide and amino acid substitutions are enclosed in the rectangle. The asterisk indicates the nucleotide substitutions that lead to the substitution of valine with cysteine.](image-url)

The results of PCR experiments in which DNA of *A. laidlawii* PG8 cells, ECMVs, and plants grown in the medium with and without ECMVs was used as a template are shown in Fig. 2. In the case of samples from the plants grown in the medium containing *A. laidlawii* PG8 membrane vesicles, the PCR signal for the *ftsZ* gene was not detected on the electrophoregrams of PCR products, whereas the amplicon corresponding in size to the amplicon of the mycoplasma *tufB* gene was detected. The PCR signal of the infectious agent was first detected in *O. sativa* L. leaf tissues 2 h after the beginning of incubation of plants with ECMVs and then was detected throughout the observation period (9 days). The analysis of the nucleotide and amino acid sequences of the resulting amplicon (Fig. 3) made it possible to assign them to the *tufB* gene of *A. laidlawii* (the identity of the amino acid sequences of the fragments of the *tufB* gene from ECMVs and from the plants grown in the ECMV-containing medium was 100% (e-value 3e-25)).

The data obtained suggest that *A. laidlawii* PG8 ECMVs are able to penetrate through the root system into the tissues of the aboveground parts of plants and, therefore, exhibit virulent properties defined as infectivity and invasiveness. Note that ECMVs, similarly to *A. laidlawii* PG8 cells, can exhibit toxigenicity with respect to *O. sativa* L. and induce changes in gene expression and ultrastructural organization of plants. The similar pattern of plant responses to ECMVs and *A. laidlawii* PG8 cell may indicate that the phytopathogenicity of mycoplasma is largely associated with the vesicles secreted by bacterial cells, characteristics
of their composition, and localization of elicitors on the membranes of infectious agents [12, 14].

Thus, as a result of the study of the interaction of O. sativa L. and membrane vesicles secreted by A. laidlawii PG8 cells, we demonstrated for the first time that extracellular vesicles of mycoplasmas exhibit the properties of an infectious agent, taking into account the criteria of virulence (invasiveness, toxigenicity, and infectivity), and can make a significant contribution to the phytopathogenicity of bacteria. In this context, the determination of the complete composition of A. laidlawii PG8 vesicles (proteins, lipids, and nucleic acids) is highly relevant from the standpoint of basic research of the biology of the smallest prokaryotes and for practical development of tools for the control of mycoplasmal infections and contaminations of cell culture and vaccines.

ACKNOWLEDGMENTS

This study was supported by the Russian Foundation for Basic Research (project nos. 11-04-01406-a and 12-04-01052-a) and the program of the Russian Federation “Leading Scientific Schools” (project no. NSh-825.2012.4).

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Translated by M. Batrukova