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EXPERIMENTAL ARTICLES

Adaptive Reactions of Mycoplasmas In Vitro: "Viable but Unculturable Forms" and Nanocells of *Acholeplasma laidlawii*

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Abstract—The adaptation of *Acholeplasma laidlawii* to conditions unfavorable for growth has been found to be accompanied by cell transformation into special morphological structures known as ultramicroforms (nanocells). The ratio of the cells of the two morphological types in the population depended on the growth conditions. Nanocells retained viability for a long time under conditions unfavorable for growth and showed resistance to stressors. Reduction in the cell size occurred due to unequal division, which involved the loss of cytoplasmic material. *A. laidlawii* ultramicroforms (nanocells) were able to restore proliferative activity and to revert to their initial vegetative form; they measured less than 0.2 μm and are the smallest cells known at present. Nanocells formed in vitro under exposure to abiogenic stressors may correspond to the *A. laidlawii* minibodies observed in infected plants upon exposure to biogenic stressors. The transformation of *A. laidlawii* cells into ultramicroforms was accompanied by condensation of the nucleoid, a change in the polypeptide spectrum, and a change in the availability of rRNA operons for in vitro amplification. All these changes are indicative of reorganization of the genetic and metabolic systems of mycoplasmas.

Key words: mycoplasmas, adaptation, nanoforms, viable but unculturable forms.

The mechanisms providing for the adaptation of the minute non-spore-forming prokaryotes, mycoplasmas (class *Mollicutes*), to biogenic and abiogenic stressors are as yet unknown [1, 2]. From the point of view of its adaptive capacities, *Acholeplasma laidlawii*, an ubiquitous mycoplasma found in soil, compost, sewage, cell cultures, and human, animal, and plant tissues, is a unique species. The fact that they have no cell wall, possess a miniature genome, and have limited metabolic capacities are not a formidable obstacle for these bacteria when overcoming various defense systems of higher organisms, surviving under unfavorable conditions, and withstanding competition against soil microflora and hydrobionts [3].

One of the contemporary conceptions of microorganism survival upon exposure to stressors implies the coordinated actions of a population, which are provided for by a three-component regulatory system [4]. Differential expression of gene cassettes determines the realization of different physiological programs in the bacterial population, including the transition of microorganisms to so-called unculturable but viable forms. A characteristic feature of these forms is their resistance to physical and chemical effects and a reduction in the cell size. This state is characterized by stability, and, for it to be abolished, the start of a new program is required [5].

Reduction in the cell size sometimes leads to the emergence of bacterial ultramicroforms, or nanocells. The phenomenon of nanotransformation has been revealed in a number of bacteria, sometimes as a response of microorganisms to stressful exposures in vitro [6]. However, in relation to mycoplasmas, no data from such investigations have been published.

The aim of our investigation was to study the biochemical, ultrastructural, and molecular–genetic features of *A. laidlawii* cells upon exposure to stressors that can cause in vitro nanotransformation of bacteria.

MATERIALS AND METHODS

Two strains of *Acholeplasma laidlawii*—reference strain PG8 (obtained from the Collection of the Gamaleya Research Institute of Epidemiology and Microbiology) and strain 118, isolated from wheat (*Triticum*) cells and kindly provided by I.G. Skripal' (Zabolotnyi Institute of Microbiology and Virology, National Academy of Sciences of Ukraine)—were used in this work.

The mycoplasma cells were grown for 48 h at 37°C in Edward's liquid medium. The full-value nutrient medium included 84% tryptic digest of calf heart mus-

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cle, 10% horse serum, 1% dry yeast extract (Difco, United States), 4% freshly prepared yeast extract, 0.4% glucose, and 1000 units/ml of penicillin [1]. The poor variant of the medium contained no glucose, yeast extract, or serum. Cell adaptation to survival under unfavorable conditions was carried out as follows: the cells were sampled in the logarithmic growth phase (14 h at 37°C), maintained at 8°C for 2 h, harvested by centrifugation (10000 *g* at 8°C for 20 min), washed in PBS buffer (50 mM sodium phosphate buffer (pH 7.2) with 100 mM NaCl), introduced into the poor medium, and incubated for 21 days at 30°C.

The culture viability was assessed by determining the CFU number and by the end-point dilution method (EPDM). To determine the CFU number, 0.1 ml of the cell suspension was mixed with 4 ml of agarized semiliquid Edward's medium (0.4% agarose HS, Bio-Rad, United States) cooled to 40°C. After the medium had solidified at room temperature, incubation was carried out at 37°C for 10–14 days. In the case of the EPDM, tubes with serial tenfold dilutions made in the full-value nutrient medium in three replicates were incubated on a temperature-controlled rocking platform (37°C, 160 rpm) for up to 28 days or until characteristic growth appeared. In order to facilitate visual control of the growth, the dye Bromphenol Red (0.01%) was added to the tubes. The titer order confirmed in three replicate dilutions was considered to be reliable. The culture homogeneity was judged by the colony morphology in an Edward's solid medium containing 1.2% Bactoagar (Difco, United States). Strain typing was carried out by comparing the sets of DNA restriction fragments and PCR products after their electrophoretic separation in agarose gel [7]. The PCR primers used (5'-cccttatgacctgggctacaaacgtgatac-3' and 5'-caccttcttc-gaccgattttcccac-3', synthesized at NPO LITEKh, Moscow) were complementary to the sites of 16S rDNA [8] and 23S rDNA [9] adjacent to the intergenic spacer.

In the experiments aimed at determining the thermotolerance of mycoplasmas, the cells of the control cultures, occurring in the logarithmic growth stage, and cells of adapted (starved) cultures were harvested by centrifugation (10000 *g* at 25°C for 40 min), diluted or concentrated in a tryptic digest of calf heart muscle to a density of about 10^7 cells/ml, and dispensed in 1-ml portions into tubes. The cell suspensions were heated to 48°C, kept at this temperature for certain periods of time, and cooled to room temperature. In order to assess tolerance to hydrogen peroxide, the cells were placed in PBS buffer, hydrogen peroxide (0.2%) was introduced into the suspension, the cells were immediately transferred to a fresh buffer by means of two successive tenfold dilutions, and the number of surviving cells was determined as the CFU number. The cell survival rate in the presence of hydrogen peroxide and under heat shock was expressed as a percentage ratio of the viable cells after and before the exposure.

In order to compare the protein profiles of cells of differing size, the method of two-dimensional electrophoresis was used. The cells of the starved cultures (250–500 ml) were harvested by centrifugation at 10000 *g* (at 4°C for 40 min). The sediments were suspended in PBS buffer and loaded onto a 15 to 30% linear gradient of Percoll density (Sigma, United States) in 50-ml bucket-rotor tubes. After 1.5 h of centrifugation (2500 *g* at 4°C), the cells of the microorganisms concentrated in two zones (approximately, in the upper and lower quarters of the density gradient); a third, diffuse, layer occurred between these two zones. These three gradient fractions were separated, diluted with three volumes of fresh buffer, and recentrifuged at a small speed to avoid Percoll precipitation. The cellular precipitates obtained were again washed with the buffer and stored at –20°C. Preparation of the protein samples and electrophoresis were carried out as described in [10] at the Laboratory of Gene Engineering and Immunogenetics (headed by V.M. Govorun), Research Institute of Physicochemical Medicine, Ministry of Health of the Russian Federation).

Cell precipitates and colonies sampled with glass capillaries from the surface of the agarized medium provided the material for the preparation of ultrathin sections of mycoplasmas. A detailed description of the methods involved in electron microscopic studies of mycoplasmas was provided in [11]. The sections were examined and photographed using a JEM-100C electron microscope at an instrumental magnification of 10000 to 30000×. The cell diameter was measured in randomly selected micrographs, taking into account the image scale. More than 500 intact cells with a clearly pronounced membrane structure were measured in each variant.

Statistical data processing was carried out using the generally accepted significance criteria and Microsoft Excel software. The results of three experiments aimed at determining cell viability under heat shock and five experiments that employed hydrogen peroxide treatment were analyzed and consolidated using an analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Comparative analysis of the data obtained on the experimental and control *A. laidlawii* cultures by transmission electron microscopy, PCR, and inoculation of the nutrient media revealed that the cell population of mycoplasmas is represented by two classes of cells (Fig. 1) that differ in size: cells measuring 0.5–0.8 μm, which corresponds to the size of typical mycoplasma cells [11], and cells with a size of less than 0.2 μm, which is characteristic of bacterial nanoforms [6]. It was established that the quantitative ratio of the cells of these classes may vary depending on the growth conditions (Fig. 2).

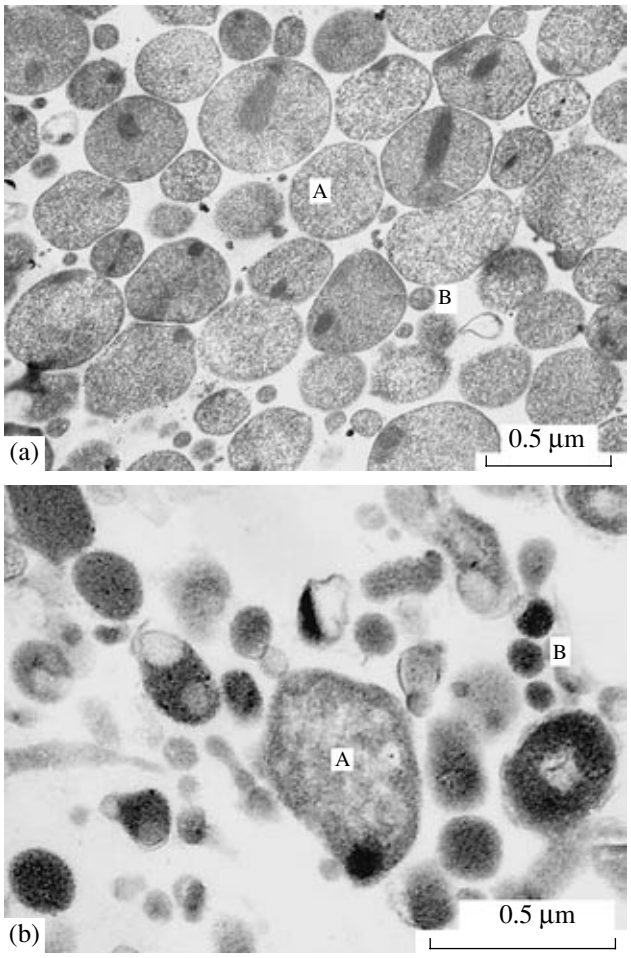

Fig. 1. Transmission electron micrographs of *A. laidlawii* cells (a) grown on poor Edward's medium and (b) at the periphery of a microcolony. "A" indicates typical cells and "B," nanoforms.

Under unfavorable growth conditions (poor medium), *A. laidlawii* nanoforms become the prevalent morphological type. The subpopulations of these cells retained their viability for a long time. While, in the full-value medium, the number of viable cells decreased considerably after three weeks and all the cells died after six weeks, the adapted cultures retained a high CFU titer for up to 120 days (Table 1). Upon prolonged starvation (for more than 70 days), some of the cells lost the capacity to form colonies but could restore their viability upon long-term cultivation in liquid media. The state of these cells may be classified as viable but unculturable [12]. The suspension cultures of these cells were characterized by a prolonged lag phase. The onset of growth in such cultures occurred after 14–20 days, whereas the control cultures entered the logarithmic growth phase not later than 4 days after the beginning of incubation. The existence of a viable, but unculturable, state of *A. laidlawii* cells was suggested by us earlier when we studied infected plants in model systems [13].

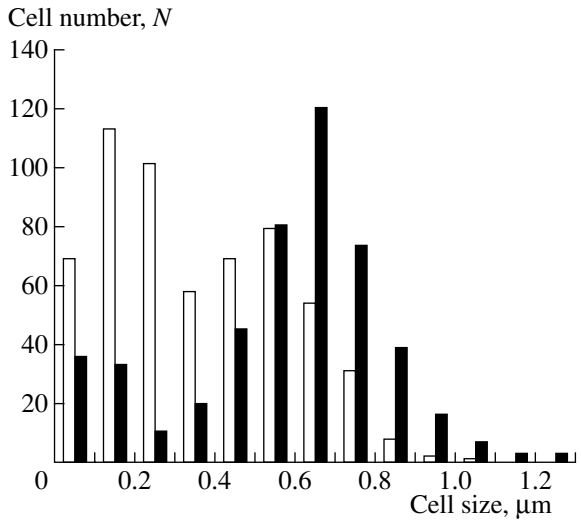

Fig. 2. Histogram of the size distribution of *A. laidlawii* cells grown in full-value nutrient medium (dark columns) and under starvation conditions (light columns).

The adapted cultures were more tolerant to the effect of high concentrations of hydrogen peroxide than the control cultures (Fig. 3a). A comparison of tolerance to increased temperatures revealed that the starved cultures showed lower death rates at extreme temperature values and during long-term exposure to increased temperatures. The data presented in Fig. 3b provide evidence of a monophasic process of death in relation to the cells grown under optimal conditions and of a biphasic process of death with respect to the cells grown under stressful conditions. These results confirm the presence of two subpopulations, one of which is thermotolerant, in the stressed culture. The colony morphology of this subpopulation on solid media corresponded to the colony morphology of the mycoplasma nanocells.

On agarized media, the nanoforms formed microcolonies of less than 0.1 mm in diameter; they lacked the nonopalescent peripheral zone that usually determines the characteristic appearance of mycoplasma colonies ("fried eggs"). Similar microcolonies have been revealed on solid media during the isolation of the mycoplasma *A. laidlawii* from soil samples [14]. Transmission electron microscopy of ultrathin sections of the peripheral microcolony zone revealed active division of ultramicroforms and the emergence of typical mycoplasma cells measuring 0.5–0.8 μm (Fig. 1b). The data obtained allow us to conclude that the minimal sizes of bacteria capable of proliferation and reversion may be smaller than those reported for *Nanobacterium sanguineum* isolated from human blood [15].

Comparative analysis of the polypeptides of the cells of the two morphological types by two-dimensional electrophoresis suggested the existence of differences in gene expression in the two mycoplasma subpopulations (Fig. 4). A considerable number of the

Viability of *A. laidlawii* cells in full-value and poor nutrient media

Culture age, days	Experiment no.	Culture in the full-value nutrient medium		Adapted culture in the poor medium	
		CFU number	EPDM titer	CFU number	EPDM titer
2	1	2.4×10^9	10^9	2.4×10^7	10^8
	2	1.5×10^9	10^9	3.0×10^8	–
	3	3.3×10^9	10^9	7.8×10^7	–
21	1	1.4×10^4	10^4	1.1×10^8	10^8
	2	3.1×10^4	10^4	7.9×10^7	–
	3	9.1×10^3	10^3	2.8×10^6	–
42	1	0	0	2.9×10^7	10^7
	2	0	0	7.1×10^6	10^5
	3	0	0	6.5×10^5	–
70	1	0	0	2.5×10^6	10^6
	2	0	0	1.2×10^5	10^6
	3	0	0	3.3×10^4	10^5
120	1	–	–	1.6×10^5	10^6
	2	–	–	6.8×10^3	10^5
	3	–	–	0	10^1
170	1	–	–	6.1×10^4	10^5
	2	–	–	2.5×10^4	10^5
	3	–	–	0	10^1

Note: "–" means "not determined".

polypeptides revealed in the typical *A. laidlawii* cells were absent from the nanoforms; at the same time, new polypeptides appeared.

The reduction in the bacterial cell size during nanotransformation occurs as a result of unequal cell division, which is preceded by pronounced condensation of the nucleoid. The DNA of the *A. laidlawii* cells that had experienced long-term (90-day) starvation and that of the cells of the starved culture that had resumed growth exhibited a restricted availability of rRNA operons for in vitro amplification: analysis of the products of PCR with specific primers showed predominant amplification of the 832-bp spacer region of one of the 16S–23S rRNA operons. The presence of a second operon copy with a spacer elongated by 207 bp in the genome is characteristic of all acholeplasmas and was confirmed in our PCR experiments with morphologically typical cells of the studied strains by the presence of the 1039-bp amplicon. The changed availability of the rRNA operons that we revealed in the nanoforms may be a consequence of the occurrence of a genomic reorganization due to nucleoid condensation during the nanotransformation and associated masking of the PCR template. Suppression of the formation of PCR products has been reported for the P1 gene of an avirulent strain of *Mycoplasma pneumoniae* that has a high tolerance to hydrogen peroxide and in which the P1 gene expression was repressed [16]. The transition to the virulent state was accompanied by improvement of the template availability for amplification. The authors of [16] believe that the phenomenon they observed may be determined by the action of a previously revealed DNA-binding mycoplasma protein that performs the function of a transcription repressor. This protein is involved in the

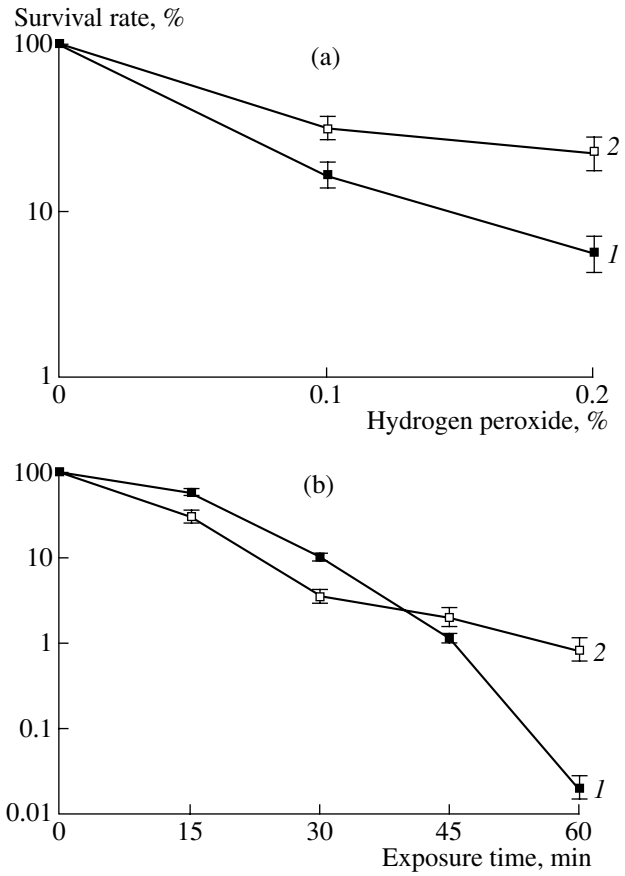

Fig. 3. Tolerance of the *A. laidlawii* cells of the (*1*) control and (*2*) experimental populations to exposure to stressors: (a) relationship between survival and hydrogen peroxide concentration and (b) relationship between survival and heat shock (48°C) duration.

regulation of topology (condensation) of DNA and provides for its local defense against oxidizing agents.

It is known that the transformation into dwarf forms during oxidative stress may be a consequence of the activation of the expression of protein Rec-A, which is necessary for triggering the mechanism of stable replication [5]. The resistance, revealed in our investigations, of the *A. laidlawii* nanoforms to high concentrations of oxidizing agents may also be related to the activity of similar proteins involved in nanotransformation of mycoplasmas.

The direct relationships existing between virulence, culturability, and resistance to unfavorable factors have previously been shown for several phytopathogenic gram-positive bacteria [17]. In our investigations [13], it was established that infecting plants with *A. laidlawii* induces the activation of nonspecific defense mechanisms, as a result of which conditions of chronic oxidative stress are established and the plant cells are colonized by *A. laidlawii* transformants, which are minibodies whose size (less than 0.2 μm) and structure correspond to bacterial nanoforms [6].

The transformation of *A. laidlawii* cells into minibodies is accompanied by substantial reorganization of their genomes. The persistence of these microorganisms determines morphophysiological, biochemical, molecular–genetic, and ultrastructural changes in plants due to the modulation of their signaling networks [18].

The *A. laidlawii* nanoforms formed in vitro as a result of the transformation of *A. laidlawii* cells upon exposure to abiogenic stressors that induce reorganization of the genetic and metabolic systems of microorganisms most probably correspond to *A. laidlawii* mini-

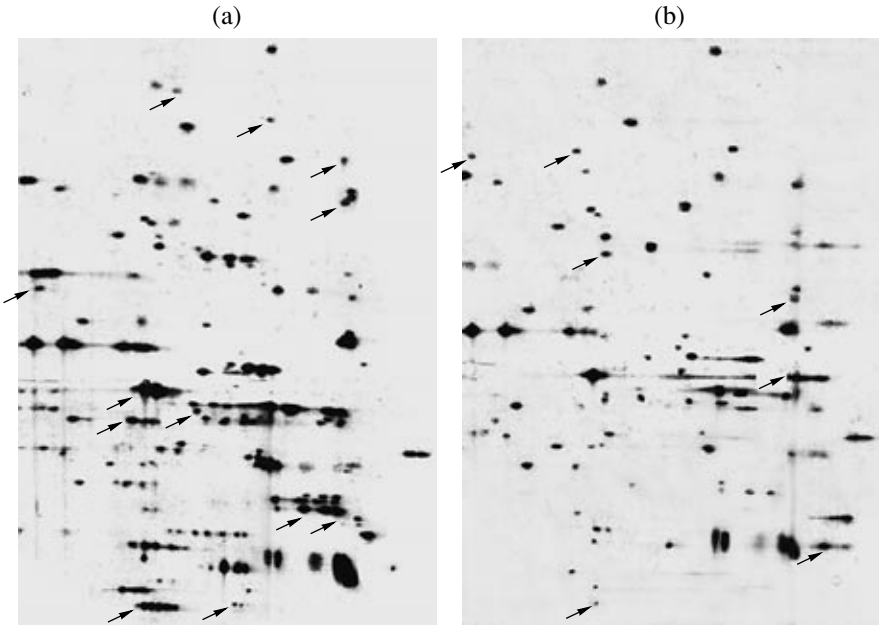

Fig. 4. Electrophoresis of (a) polypeptides of typical *A. laidlawii* cells and (b) nanoforms. The polypeptides distinguishing subpopulations are indicated by the arrows.

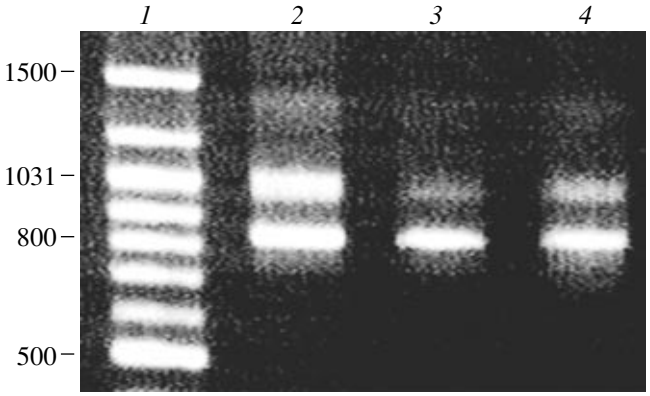

Fig. 5. Electrophoresis in the presence of (*1*) a molecular weight marker (500–1500 bp) of the products of amplification with primers complementary to the flanking regions of the ribosomal operon spacer. The amplification templates were DNAs from the *A. laidlawii* cells (*2*) grown in the full-value medium, (*3*) experiencing long-term starvation, and (*4*) that had resumed growth after starvation.

bodies formed in vivo in plant cells upon exposure to biogenic stressors. In this context, investigation of the molecular–genetic features of nanoforms is of special interest from the point of view of the molecular basis of the immunocompromise that determines the persistence of mycoplasmas in cells of higher eukaryotes.

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