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Proteomic Characterization of *Mycoplasma gallisepticum* Nanoforming

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Abstract—The goal of this work was to create a model for the long persistence of *Mycoplasma gallisepticum* in depleted medium and under low growth temperature followed by proteomic study of the model. Nanoforms and revertants for *M. gallisepticum* were obtained. Proteomic maps were produced for different stages of the formation of nanoforms and revertants. It is shown that proteins responsible for essential cellular processes of glycolysis, translation elongation, and DnaK chaperone involved in the stabilization of newly synthesized proteins are crucial for the reversion of *M. gallisepticum* to a vegetative form. Based on the current data, it is assumed that changes in the metabolism of *M. gallisepticum* during nanoforming are not post-mortal, thus *M. gallisepticum* does not transform to uncultivable form, but remains in a reversible dormant state during prolonged unfavorable conditions.

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The phenomena of adaptation of bacteria to changing environment and their reaction to stress factors (temperature, antibiotics, pH change, etc.) motivate researchers to create experimental models for study of molecular mechanisms underlying specific and nonspecific bacterial resistance to stress conditions. The creation of experimental models and research on mechanisms controlling sporogenesis for Gram-positive [1] and forming unculturable Gram-negative microorganisms forms [2] have been conducted for a long time. The question of possible mechanism of survival in stress conditions for Mollicutes (mycoplasmas), which lack cell wall and do not form spores, is still open. All mycoplasmas have a reduced metabolic apparatus due to the restricted capacity of their genome, and they depend on either metabolites of a eukaryotic host cell or components of nutritional medium. When cultivated *in vitro* they need medium exceedingly rich in nutrients. Recently, synthetic cultivating medium for *Mycoplasma pneumoniae* consisting of 147 components was developed based on genomic metabolome analysis [3]. However, bacterial populations in natural environment almost never find themselves in such favorable conditions and commonly undergo various stresses, including nutrient limitations, pH and temperature changes, etc. The state of exponentional division in unfavorable conditions is more an exception than a rule. However, mycoplasmas have various mechanisms that allow them to stay in a dormant state or with limited metabolic activity and to survive in unfavorable conditions outside the host cell in which they spend most of their lifetime [4].

Resistance to antibacterial agents was proven for various mycoplasma species, as was the persistence of mycoplasmic infection in cell cultures without any visible signs of division with consequent multiplication (despite various decontamination procedures used). The phenomenon of nanoforming was described for *Acholeplasma laidlawii*, which is observed after limiting nutrients in the process of cultivation [5]. However, the mechanisms underlying this phenomenon remain poorly studied and experimental models have not been developed. There are studies underway to investigate the mechanisms of resistance of mycoplasma to various stress factors and to find

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possible regulatory mechanisms that become apparent after a significant reduction of the genome. For example, a transcription profiles shift of *Mycoplasma hyopneumoniae* was described under exposure to three stress factors—heat shock, hydrogen peroxide, and reduced iron content in the cultivation medium [5-7]. The overall reaction of *M. hyopneumoniae* to all of these three stress factors was the change in expression of two genes—*rplM* and mhp232. Ten genes changed expression under heat shock and hydrogen peroxide exposure (*ftsY*, *rluC*, *gatA*, mhp038, *glyS*, mhp232, *pfkA*, mhp325, mhp445, and *rplM*). Since they include genes of DNA translation and replication, such as *rluC* (mhp015) and *rplM* (mhp672) that expression lowers, the authors supposed that the high resistance of mycoplasmas is caused by a general reduction of DNA translation and replication in the adapting cell. Expression of four genes changed under iron deprivation and after peroxide exposure (*rpl4*, *spoU*, mhp325, and *rplM*).

The most significant increase in expression was noted for the *dnaK* gene under heat shock. This indicates the importance of protection of proteins from misfolding by chaperones not only during heat shock, but also under oxidative stress. These studies show that the reactions of *M. hyopneumoniae* to different stress factors are similar.

Lactococcus lactis subsp. *lactis* was established to remain alive after a change in the nutrient concentration in the cultivation medium, but they turn into an unculturable state characterized by a decrease in metabolic activity [8].

The genomes of spore-forming clostridii as well as many other Gram-positive microorganisms have only 22 to 27% GC [4]. In spite of the fact that mycoplasma genomes also have lower GC content, they do not form spores because of the absence of a cell wall. However, in some cases after lowering the nutrient content without any additional stress factors the bacteria endure these conditions with lowered metabolic activity but without loss of cultivability [9]. Mycoplasma in the unculturable state is characterized by decrease in cell diameter, but there is no detailed description of processes taking place in them.

Therefore, the goal of this work was to create an experimental model of prolonged persistence in depleted medium under lowered temperature for one of the mycoplasma species, *Mycoplasma gallisepticum*, and to characterize it during this process.

MATERIALS AND METHODS

***Mycoplasma gallisepticum* cell culture and obtaining of nanoforms and revertants.** *Mycoplasma gallisepticum* S6 cells were cultured on liquid medium containing 2% tryptose, 0.5% glucose, 0.5% NaCl, 0.13% KCl, 0.3-0.5% Tris, 5% (v/v) yeast dialysate, 10% (v/v) horse serum, phenol red indicator, pH 7.2, at 37°C. To obtain nanoforms, *M. gallisepticum* cells were cultivated for 20 h in 25 ml of complete medium, then held for 2 h at 8°C and centrifuged at 15,000*g* at 8°C for 20 min. The cells were further resuspended in 100 ml of medium containing 2% tryptose, 0.5% Tris, 0.5% NaCl, and 0.13% KCl and incubated at 30°C for 6 weeks, 6 months, and one year.

For reversion, nanoforming cells were transferred into complete medium, resuspended, and cultivated at 37°C. Five passages were made. The cells were pelleted by centrifugation as described above and stored at −20°C. Samples from each passage were taken to create a 2D-map.

***Mycoplasma gallisepticum* cell pellet recovery.** *Mycoplasma gallisepticum* cell culture was centrifuged at 1500*g* for 10 min at 4°C to precipitate the suspension of horse serum proteins and lipids used for cultivation. Then the supernatant was centrifuged at 15,000*g* for 20 min at 4°C to obtain the cell pellet. The pellet was washed twice under the conditions described above in the following buffer: 150 mM NaCl, 50 mM Tris, 2 mM $MgCl_2$, pH 7.4, with addition of a protease inhibitor mixture (Amersham Bioscience, Sweden). The resulting pellet was resuspended in 1 ml of water for chromatography (Panreac, Spain) and centrifuged at 13,000*g* for 10 min at 4°C. The cell pellet was frozen and stored at −70°C.

Determination of culture viability during starvation. A differential method of live/damaged cell visualization with SYTO 9 and propidium iodide (Invitrogen, USA) was used to estimate the number of dead and live cells. Calculations were performed using data from scanning laser confocal microscopy with an Axiovert 200M LSM510 META microscope (Carl Zeiss, Germany) with Plan-Neofluar 10×/0.3, Plan-Neofluar 20×/0.5, and Plan-Neofluar 40×/1.3 lenses.

***Mycoplasma gallisepticum* vegetative form cell and nanoform size estimation.** Size was measured by dynamic light scattering using a 90Plus particle size analyzer (Brookhaven Instruments Corporation, USA) in liquid nutritional medium in a constant-temperature cuvette at 37°C for 10 min. The data were analyzed with ZetaPlus Particle Sizing Software v.4.02.

Two-dimensional separation of Chaps-soluble fraction of *M. gallisepticum* vegetative culture and nanoform proteins. Sample preparation and two-dimensional electrophoresis were carried out as described earlier [10]. After electrophoresis, gels were marked and stained with silver and thiosulfate [11].

Differential staining. Before the 2D-separation, the samples of solubilized proteins of *M. gallisepticum* vegetative culture and nanoforms were re-precipitated in a methanol–chloroform system to remove DNA and lipids and solubilized in 100 µl of buffer with the following content: water for chromatography (Panreac), 8 M urea, 2 M thiourea, 10 mM Tris-HCl, 167 µl 30% Chaps, and 10% NP 40 solution, pH 8.0. Then the samples were centrifuged at 13,000*g* for 15 min.

Protein concentration in the samples was measured with Bradford reagent (BioRad, USA). Proteins were covalently tagged with CyDye-DIGE Cy3 and CyDye-DIGE Cy5 (Amersham Bioscience) in accordance with the producer's instructions (400 pmol for 50 μg protein). Samples were incubated for 30 min in the dark at 4°C, and then the reaction was stopped with 10 mM lysine solution. One-dimensional electrophoresis was conducted in 7 × 8 cm 12% polyacrylamide gel to test the efficiency of covalent protein tagging with fluorescent tags. Tagged protein samples were mixed in 1 : 1 proportion by total fluorescence intensity before isoelectric focusing; DTT was added to 80 mM and Ampholine 3-10 was added to 0.2%. Isoelectric focusing and the second direction electrophoresis were conducted as described above.

The resulting gels were scanned on a Typhoon Trio instrument (Amersham) at the wavelength of 532 nm (Cy3), 633 nm (Cy5) and laser intensity of 600 pmt. Afterwards the gels were stained with silver and thiosulfate by the method described in [10]. The data was analyzed with PDQest 8.0 software (BioRad).

Protein hydrolysis and mass-spectrometry of *M. gallisepticum*. Proteins were hydrolyzed and extracted from the gel according to the previously described protocol [12].

MALDI-TOF mass-spectrometry. Mass-spectrometric analysis was conducted under previously described conditions [13]. Proteins were identified after two-dimensional separation using a MALDI-TOF/TOF Ultraflex II (Bruker Daltonics, Germany) in positive ions mode in the range 500-4000 Da. The mass spectrum was calibrated using the known masses of internal standards. Proteins were identified by the masses of proteolytic fragments with Mascot Peptide Fingerprint software (Matrix Science, USA). MH^+ ion mass identification accuracy was 0.007%; possible modifications of cysteine residues by acrylamide and methionine oxidation were taken into consideration. Proteins were identified from peptide fingerprints, the search being carried out with Mascot software (Matrix Science). The US National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov) database was used for the search; it contains a full genome of *M. gallisepticum* strain R [14]. The cutoff was 54 ($p < 0.01$).

***dnaK* mRNA content measurement by semiquantitative real-time polymerase chain reaction (PCR).** Total cell RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol. The RNA sample (1 μg) was treated with 4 units of deoxyribonuclease (Fermentas, Lithuania) for 2 h in the presence of 40 activity units of RiboLock ribonuclease inhibitors (Fermentas). The reverse transcription reaction was carried out with a First Strand cDNA synthesis kit (Fermentas) according to the provided protocol. Amplification with real-time detection was carried out on IQ5 amplifier (BioRad) in 50 μl of mixture containing 200 μM of each dNTP in 1× PCR buffer (Lytech, Russia), 2 units of Taq polymerase, 20 ng of cDNA in the presence of 1× SYBR Green I (Invitrogen) with oligonucleotide primers to *dnaK* gene fragments flanking a gene section of 300 nucleotide pairs (n.p.). Amplification was conducted in the following mode: 95°C for 30 sec, 45 cycles of 95°C for 10 sec, 60°C for 10 sec, 72°C for 30 sec. Fluorescence data was gathered after each cycle. Melting from 60 to 95°C was conducted after the end of the reaction to determine the reaction specificity and draw the melting curve.

For PCR we used 850 ng cDNA of samples of both a vegetative form and nanoform and also specific primers computed from the known nucleotide sequence of the genes. PCR conditions were the following: 40 cycles on cDNA and DNA templates with a mixture of 5 units/μl Taq-polymerase (Lytech) at hybridization temperature of 66°C. PCR was conducted on a 700 sequence detection system (Applied Biosystems, USA). Amplification product accumulation in consecutive cycles was detected by the increase in SYBR Green I fluorescence. The presence of specific cDNA was confirmed on the basis of PCR product melting curve analysis. Genomic DNA of *M. gallisepticum* vegetative form (65 ng for a reaction) was used as a positive control. We used a DNA-free sample (reaction mixture and specific primers) as a negative control. DNA amplificates were analyzed by electrophoresis in agarose gels with ethidium bromide staining. Specific gene product size was determined by the positions of 100-3000 n.p. markers (Fermentas).

RESULTS AND DISCUSSION

Parameters of mycoplasma restriction on substrate composition and cultivation temperature were previously determined. The conditions induce a unique response of these microorganisms – a nanotransformation [5]. Because *M. gallisepticum* is mobile, normally growing cells move in the medium while being in suspension. When incubating cells in "poor" medium, we noted that the cells sediment, whereas the supernatant fluid remains clear. This is caused first by transition of the cells to round shape, a decrease in their volume, and an increase in relative density. As a result, most of the cells lose mobility and sink to the bottom of the vessel.

Analysis of cell sizes in vegetative forms and nanoforms showed that nanoforms remaining in the suspended state have one peak of light scattering corresponding to 450 ± 25 nm. *Mycoplasma gallisepticum* vegetative form cells do not have one distinct peak, but rather a distribution from 525 to 1050 nm with a maximum at 746 nm. This indicates not only cell size decrease during prolonged starvation, but also their transition from pear-shaped to round.

To study the protein composition shift of starving *M. gallisepticum* cells during prolonged incubation under the

conditions described above, we determined two-dimensional maps of protein extracts after 3, 7, and 10 days and then after 2, 3, 4, and 5 weeks. It was found that isoforms of some proteins which differ in p*I* value but not in molecular mass become visible on the two-dimensional map by the third day after transfer of *M. gallisepticum* to the poor medium. These changes first affected elongation factors such as FusA, glycolysis system proteins (glyceraldehyde-triphosphate-dehydrogenase, pyruvate kinase, α-subunit of pyruvate dehydrogenase), and also DnaK chaperone. As time passed, more proteins underwent such changes and all emergent protein stains were shifting to the acidic area relative to the prior protein position. Cells attained coccal form on the sixth week and decreased in size, cytoskeleton-like elements were undetectable by electron microscopy, and the cytoplasm became more electron-transparent [15].

Reversion (conversion to cultivatable state) of nanoforms was achieved by their transfer to complete nutritional medium and incubation at 37°C. We used 6-week-, 6-month-, and 1-year-old nanoforms for this experiment. Six-week-old nanoforms grew normally, which was determined by the color change of phenol red indicator introduced into the cultivation medium. Their reversion was found. For 6-month- and 1-year-old nanoforms, reversion to vegetative culture was not found after 1 month of cultivation in complete medium.

Cell samples from each of the five passages were chosen to characterize the changes accompanying reversion of nanoforms to vegetative culture; the two-dimensional protein distribution map was demonstrated to undergo gradual reverse changes resulting in decrease in the number of isoforms. The proteomic maps of revertants and the initial culture were identical by the fifth passage. Therefore, the protein spectrum changes under unfavorable conditions are not postmortal for a fraction of the cells, and *M. gallisepticum* does not turn into an uncultivatable form but stay in a state of reversible dormancy. However, this is correct only for a limited period of cell exposure to stress factors. We did not succeed in obtaining revertants after longer (6 months and 1 year) periods of bacterial cultivation under restricted substrate conditions and under lowered temperature.

To determine the causes of this phenomenon, comparative proteomic analysis of nanoforms was conducted by two-dimensional electrophoresis with differential staining; the nanoforms were kept in poor medium for 6 weeks, 6 months, and 1 year. Mass-spectroscopic identification allowed the preparation of a table of expression distinctions for proteins of glycolysis and elongation present on comparative electrophoregrams of nanoforms and vegetative culture (Fig. 1; see color insert) and also on the electrophoregrams of 6-month- and 1-year-old nanoforms (Table 1).

It was shown that not only the present isoforms of many proteins shift to the acidic area with the passage of time, but the whole proteomic map becomes significantly depleted (Fig. 2; see color insert). However, the iso-

Table 1. Expression change of glycolysis system proteins and elongation factors of *M. gallisepticum*

Protein name	6 weeks	6 months	1 year
Glucose-specific IIC component of PTS system (PtsA)	+	+	–
Glucose-6-phosphate-isomerase (Pgi)	0	0	0
6-Phosphofructokinase (PfkA)	+	+	–
Fructose-bisphosphate aldolase (Fba)	+	+	–
Triosephosphate isomerase (TpiA)	+	–	–
Glyceraldehyde-triphosphate dehydrogenase	+	–	–
Phosphoglyceromutase (Pgk)	+	+	–
Phosphoglyceromutase (GpmI)	+	+	+
Phosphopyruvate hydratase (Eno)	0	0	0
Pyruvate kinase (PykF)	+	+	–
Lactate dehydrogenase (Mdh)	+	+	–
α-Subunit of E1-component of pyruvate dehydrogenase (AcoA)	+	+	–
β-Subunit of E1-component of pyruvate dehydrogenase (AcoB)	+	+	–
Elongation factor G (FusA)	+	–	–
Elongation factor Ts (Tsf)	+	+	–
Elongation factor Tu (TufB)	+	+	+

Note: +, present on proteomic map; –, absent on proteomic map; 0, do not enter differentiation zone because of physicochemical properties.

forms do not disappear – they differ only in their isoelectric points, as before. We conducted mass-spectrometric studies of individual isoforms to search for possible posttranslational modifications. Proteins generally change their mass during posttranslational modifications because of addition of various functional groups (acetyl-, methyl-, and phosphate groups), attachment of acyl and carbohydrate groups, amino acid substitutions, structure changes, etc.

Similar phenomena have been described for other mycoplasmas. For example, two-dimensional maps of *Mycoplasma penetrans* [16], *M. suis* [17], *M. pulmonis* [18], etc. also have a number of notable protein stains corresponding to isoforms of a single protein. But a certain explanation to the nature of this phenomenon has still not been found. It is supposed that this may be a result of some protein modifications in the course of solubilized protein storage and extraction. As samples of vegetative culture and nanoforms were processed simultaneously in the course of our experiments, we can state that the described changes have another cause. It is supposed that protein glycosylation may result in this, but this process is characterized very poorly for bacteria and is even doubted to exist. Several reports for the existence of glycosylated protein for membranes of *M. pneumoniae* [19] and *M. gallisepticum* [20] have been published, but direct proof is absent. Taking into consideration the fact that, for example, *M. gallisepticum* is cultivated *in vitro* in medium containing a high content of horse serum, its components, which bind to membranes, may be identified in the course of study as mycoplasma proteins. A report of glycosylated protein in three stains of *M. gallisepticum* was published in 1990 [21]. Two *O*-glycosylated proteins of *Mycobacterium tuberculosis* have been described [22].

It should be noted that despite the decrease in protein diversity in the process of nanoforming, the main protein chaperones, proteins of glycolysis, and elongation factors were represented in full. Prolonged (at least for 1 year) preservation of a relatively large amount of chaperone DnaK is important in correct conformation and consequent stabilization of other protein molecules.

The main source of energy in *M. gallisepticum* cells is glycolysis, the enzymes (and correspondingly, genes) of which are fully present in vegetative cells. The complete set of glycolysis enzymes identified in the vegetative state was preserved in nanoforms for 6 weeks. However, 3 of 12 observed enzymes were not present on the map at 6 months, and the amount of glycolytic enzymes become so little after 1 year that it cannot be identified by the methods available to us. Similar dynamics were observed for elongation factors. All three factors are present in 6-week-old nanoforms, but after a year only TufB remains visible. Therefore, for *M. gallisepticum* to come out of stress state, the availability of all components of two main systems–glycolysis and all elongation factors–is necessary. Removal or critical decrease in amount of at least one of the component proteins leads to the impossibility of reversion. It should be noted that reversion does not require elongation factors to be present in the same amount as in vegetative form. For example, NusA-protein homolog (gi|31541161), which is detected in vegetative state, becomes undetectable during nanoforming in 6 weeks, but the cells are still able to revert.

Systematic analysis of *M. pneumonia* cells revealed a comparatively large number of non-translated transcripts, mainly from the antisense sequence class [23]. It is possible that when *M. gallisepticum* cells come into unfavorable cultivation conditions, similar transcripts regulate general transcription, and repressor regulates a gene whose regulatory functions are extremely limited [24].

Real-time PCR proved the existence in 6-week-old nanoforms of cDNA and, therefore, mRNA of the *dnaK* gene coding a chaperone that participates in protein folding and stabilization and also belongs to the heat-shock proteins. Therefore, we suppose that protein synthesis in nanoforms does not stop, and the cells that lack specific heat-shock proteins respond to environmental temperature decrease by an increase in expression of general heat-shock proteins.

One of the most interesting questions that arise while studying nanoforming is the functional state of cells undergoing proteomic analysis. Is it allowed to consider them all alive, or do we have to deal with a mixture of dead and live cells, like when we analyze a vegetative culture? In this case a series of stains may be considered as a set of different protein states in the cells of different phases of the life cycle. It is also unknown which cells exactly are able to revert. Is selection pressure present, or is any cell retaining a minimal set of necessary proteins able to return to vegetative state?

Table 2. Time of reversion of *M. gallisepticum* nanoform colonies

Time of reversion	Numbers of colonies
9.00	5, 7, 18
10.00	4, 19, 24
11.00	1, 9, 12, 16, 20
12.00	3, 11, 15, 29
13.00	25, 27
14.00	10, 22
15.00	6
16.00	2, 28
17.00	26
18.00	14, 23
19.00	13

To evaluate viability, a differential method of visualization of live and damaged cells with SYTO 9 and propidium iodide was used. SYTO 9 reveals nucleic acids and weakly stains cytoplasm of living cells, and propidium iodide marks damaged cells by staining nucleic acids. The calculation was performed with scanning laser confocal microscopy. It was shown that vegetative culture of *M. gallisepticum* in the middle of logarithmic growth phase has 80% of cells alive, while nanoform culture has only 30%.

Experiments were conducted to clone and subsequently nanotransform and revert *M. gallisepticum*. In all, 29 clones of vegetative form were obtained, and all of them were transferred to poor medium and held in it for 6 weeks; then the reversion was conducted. All 29 clones returned from nanoform state virtually simultaneously with a maximum delay of 10 h (Table 2). These results show that selection pressure is minimal and, most probably, undamaged *M. gallisepticum* cell is capable of effective reversion.

Hence, we have developed and characterized an experimental model of prolonged persistence of mycoplasma in poor cultivation medium at lowered temperature. In conclusion it should be noted that this article is the first attempt to describe mycoplasma cell state in which they have lowered activity but retain growth potential for 6 weeks of cultivation under stress conditions. Further systematic analysis of this state is considered topical, and is planned to be conducted in the next part of our study.

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