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EXPERIMENTAL ARTICLES

Adaptation of *Mycoplasma gallisepticum* to Unfavorable Growth Conditions: Changes in Morphological and Physiological Characteristics

V. M. Chernov[a, 1], O. A. Chernova[a], O. V. Gorshkov[a], A. A. Muzykantov[a], G. F. Shaimardanova[a], A. D. Pel'nikevich[b], A. B. Margulis[b], A. I. Kolpakov[b], and O. N. Il'inskaya[a]

[a] *Kazan Institute of Biochemistry and Biophysics, Kazan Scientific Center, Russian Academy of Sciences, ul. Lobachevskogo, 2/31,Kazan, Tatarstan, 420111 Russia*

[b] *Kazan State University*

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Abstract—Adaptation of *Mycoplasma gallisepticum* to unfavorable growth conditions results in altered morphological and physiological characteristics of the cells. *M. gallisepticum* populations in a complete nutrient medium contain pear-shaped vegetative cells ($d \sim 0.3$ μm; $l \sim 0.8$ μm) with pronounced polar and cytoskeleton-like structures. Such mycoplasma cells are able to induce damage in a bacterial genome, causing an SOS response of the test strain (*Escherichia coli* PQ37). In a starvation medium, *M. gallisepticum* produces nanoforms, small coccoid cells ($d \sim 0.15$–0.2 μm) without either polar or cytoskeleton-like structures. Unlike vegetative cells, nanoforms do not induce genome damage. Alleviation of unfavorable growth conditions results in a reversion of nanoforms to vegetative cells.

Key words: mycoplasmas, adaptation, cell ultrastructure, nanoforms, SOS response.

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Mycoplasma gallisepticum is a widespread agent causing poultry respiratory mycoplasmoses, which are transmitted both horizontally and vertically. Infection with this mycoplasma causes pathology and death of chick embryos, and contamination of the viral vaccines using them [1]. Control and suppression of infections caused by *M. gallisepticum* is a serious problem; to solve it, research into mycoplasma molecular and cell biology is required to understand their pathogenicity and adaptation to biotic and abiotic stressors.

We have demonstrated that the adaptation of an "ubiquitous" mycoplasma *Acholeplasma laidlawii*, to unfavorable growth conditions involves nanotransformation, i.e., transformation of the vegetative cells to ultramicroforms (nanoforms, NF) [2]. Vegetative forms (VF) and NF of *A. laidlawii* exhibit significant differences in morphology, ultrastructure, genome expression, and pathogenicity [3]. Changes in cell size, morphology, ultrastructure, and virulence in asporogenic bacteria caused by unfavorable growth conditions have been described in a number of publications [4]. These processes in *M. gallisepticum*, however, have not been studied.

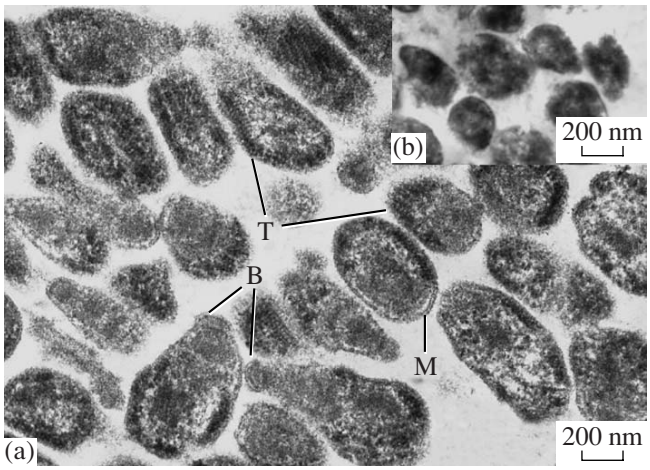
[1] Corresponding author; e-mail: chernov@mail.knc.ru

The goal of the present work was comparative analysis of morphology, ultrastructure, and capacity for induction of genome damage in *M. gallisepticum* cells grown in a complete nutrient medium and under unfavorable growth conditions (substrate limitation).

MATERIALS AND METHODS

The strain *Mycoplasma gallisepticum* S6 was obtained from the collection of the Gamaleya Research Institute of Epidemiology and Microbiology; the tester strain *Escherichia coli* PQ37, in which expression of the β-galactosidase gene is controlled by the SOS operon promoter, was obtained from the collection of Vavilov Institute of General Genetics, Russian Academy of Sciences.

The mycoplasma cells were grown for 48 h in a liquid modified Edward medium. The complete medium contained the following (%): tryptose, 2; NaCl, 0.5; KCl, 0.13; Tris, 0.3; horse blood serum, 10; yeast extract, 5; glucose, 1; penicillin, 1000 U/ml; and phenol red, 0.3 ml of 1% solution [5]. Glucose, yeast extract, and serum were excluded from the starvation variant. In order to obtain an *M. gallisepticum* S6 culture adapted to unfavorable growth conditions, the procedure for induction of an nonculturable state in *Salmonella typh-*



Transmission electron micrographs of *M. gallisepticum* cells formed on complete Edward medium (a) and under substrate limitation (b): T, tubulin-like structures; B, terminal structures; M, cell membrane.

imurium (prolonged cultivation on a substrate-limited medium) [6] was modified for mycoplasma cultivation [5]. To adapt *M. gallisepticum* to unfavorable growth conditions, the cells were harvested at the logarithmic growth stage (20 h at 37°C), stored for 2 h at 8°C, collected by centrifugation (4500 *g*, 8°C, 20 min), resuspended in four volumes of a starvation medium, and stored at 30°C for 4.5 months. For comparative analysis of morphology, ultrastructure, and genotoxic properties, *M. gallisepticum* cells were grown for three days in a complete Edward medium (VF) and for 4.5 months under substrate limitation (NF).

Electron microscopy of in vitro grown mycoplasmas was carried out on a Hitach-110 electron microscope (Japan). Ultrathin sections were obtained on an LKB-III microtome (Sweden). The material was fixed for 12 h with glutaraldehyde (2.5%) in phosphate buffer (0.1 M, pH 7.2). After dehydration in acetone, the material was treated with 1% OsO_4 solution in the same buffer supplemented with sucrose (34 g/l).

Cell suspensions were centrifuged for 20 min at 7000 and 12000 *g* (VF and NF, respectively). The supernatant was collected, and the pellet was resuspended in 1 ml of the medium. Each sample was tested using the standard SOS chromotest [7].

Experiments for determination of the toxic and genotoxic effects of *M. gallisepticum* cells and culture liquid were carried out according to Quillardet [7] using methodical recommendations of [8]. The toxic effect of VF and NF samples, as well as of their culture liquid was determined by expression of the constitutive alkaline phosphatase; the SOS response was measured by activity of the inducible β-galactosidase considering the sample toxicity [8]. The dose-dependent increase of the calculated value of induction factor (IF, the ratio of β-galactosidase and alkaline phosphatase activities in the experimental and control variants) was used as an indicator of SOS response. The values of IF ≥ 2 indicate genotoxic effects [9]. The standard mutagen (ethylmethane sulfonate, 50 μg/ml) was used as a positive control.

The standard statistical programs from the Microsoft Excel software package were used for data treatment.

RESULTS

In a complete Edward medium, *M. gallisepticum* cells are pear-shaped, vegetative cells ($d \sim 0.3$ μm; $l \sim 0.8$ μm) with pronounced polar and cytoskeleton-like structures (Fig. a). Under unfavorable growth conditions with substrate limitation, *M. gallisepticum* forms NF. These have a small size ($d \sim 0.15$–0.2 μm), a coccoid shape, a condensed nucleoid, and an electron-dense membrane structure (figure, b). When unfavorable conditions are alleviated (in a complete Edward medium), NF undergo reversion and transform into *M. gallisepticum* VF.

Comparison of the toxic and genotoxic characteristics of VF and NF cells is presented in the table. Since no reliable changes were detected in the phosphatase activity of the *E. coli* tester strain treated with *M. gallisepticum* VF or their culture liquid (as compared to the mycoplasma-free control), toxic metabolites were not accumulated in the mycoplasma VF cells. Activity of β-galactosidase in *E. coli* cells treated with VF cells and their culture liquid were significantly higher than in the control; this result indicated expression of the SOS operon genes (table). For these samples, the values of induction factor of *E. coli* PQ37 SOS response were 19.54 and 7.8, significantly higher than the IF value of the standard supermutagen (3.07).

Both NF and their culture liquid did not affect the activity of alkaline phosphatase and β-galactosidase in the tester strain. The calculated IF values (0.9 and 0.71) were beyond the range of SOS response induction (table).

Our results demonstrate that the *M. gallisepticum* VF and NF cells differ significantly in morphology, ultrastructure, and genotoxic characteristics. VF and their culture liquid induce an SOS response in the tester strain *E. coli* PQ37. NF and their culture liquid do not exhibit these genotoxic effects.

DISCUSSION

We found that, similar to *Acholeplasma laidlawii, M. gallisepticum* cells are capable of nanotransformation, i.e., under unfavorable growth conditions they form NF, differing from VF in their morphology and ultrastructure (with smaller size, condensed nucleoid and membranes, and deprived of the polar and cytoskeleton-like structures).

Such changes of cell morphology and ultrastructure are known to involve significant changes in the genome

Induction of an SOS response of *E. coli PQ*37 tester strain treated by VF and NF of the mycoplasma *M. gallisepticum* and their culture liquid

Variant		Alkaline phosphatase activity, U	β-galactosidase activity, U	IF, U
VF	Medium for VF*	0.36 ± 0.02	0.003 ± 0.0005	1
	M. gallisepticum cells	0.35 ± 0.03	0.06 ± 0.001	19.54
	Culture liquid	0.43 ± 0.05	0.03 ± 0.001	7.8
NF	Medium for NF	0.34 ± 0.04	0.13 ± 0.01	1
	M. gallisepticum cells	0.38 ± 0.05	0.13 ± 0.02	0.9
	Culture liquid	0.44 ± 0.05	0.12 ± 0.02	0.71

* Sterile medium for mycoplasma cultivation was used as control.

expression [4]. Our results on comparative proteome analysis of NF and VF indeed indicate significant differences in the expression of *M. gallisepticum* genome in the course of nanotransformation, including dissociation of the cell population into sublines carrying isoforms of the proteins controlling the key reactions of bacterial cells (signaling, energy production, translation, transcription, replication, and adhesion) [10].

Significant changes in the expression of the bacterial genome may result in significant changes of their metabolism and virulence [11, 12]. In asporogenic bacteria, reorganization of the genome expression under unfavorable growth conditions was shown to result in transition to a dormant (hypometabolic) state with attenuated virulence [13, 14]. Our work included, apart from analysis of morphology and ultrastructure, comparative investigation of the genotoxic characteristics of *M. gallisepticum* VF and NF formed under favorable (in a complete Edward medium) and unfavorable conditions (substrate limitation). The standard SOS chromotest permits detection of multiple genomic damage resulting in formation of single-strand DNA and therefore in the switching on of the SOS operon genes [7]. Our results indicate the absence of toxic effects of the investigated samples against the tester bacteria. A significant increase of the values of β-galactosidase activity in *E. coli* PQ37 cells treated with VF and their culture liquid (but not NF and their cultural liquid) compared to control confirms the mutagenic potential in VF and its absence in NF. Since the genotoxic effects against *E. coli* PQ37 were observed both for VF and for their culture liquid, genotoxic metabolites are possibly secreted into the medium; changes of some components of the medium caused by the metabolites secreted by *M. gallisepticum* vegetative cells is another possible explanation. The genotoxic metabolites of VF probably interact closely with the mycoplasma surface structures and gradually penetrate into the medium. This may be the explanation of a more pronounced genotoxic effect of VF proper (IF = 19.54) compared to their culture liquid (IF = 7.8). The nature of these metabolites is highly interesting from the point of view of the pathogenic potential of this widespread mycoplasma against infected host cells and microorganisms in microbiocenoses.

The absence of an SOS response in *E. coli* PQ37 tester cells treated with NF and their culture liquid is evidence of the absence of genotoxicity in NF samples. Thus *M. gallisepticum*, like some other asporogenic bacteria, probably loses its virulence in the course of adaptation to unfavorable growth conditions. The molecular basis of this phenomenon is to be investigated. It is evident, however, that infection of an organism with *M. gallisepticum* NF with attenuated genotoxicity may present a serious danger due to NF capacity for reversion (transition to the VF with a pronounced mutagenic effect).

Thus, we established that adaptation of *M. gallisepticum* to unfavorable growth conditions is accompanied by significant changes in morphology, ultrastructure, and genotoxicity of the mycoplasma cells. The presence of a mechanism for switching between the programs responsible for the adaptation to different conditions requires a new approach to investigations of the molecular base in the formation and evolution of the "parasite–host" system in order to develop technologies for efficient control of mycoplasma infections and for the safety of vaccine preparations.

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REFERENCES

1. McGarrity, G.J, Kotani, H, and Butler, H, Mycoplasmsa and Tissue Culture Cells, in *Mycoplasmas: Molecular Biology and Pathogenesis*, Maniloff, J., McElhaney, R.N., Finch, L., and Baseman, J.B., Eds., Washington, 1992, pp. 445–456.
2. Chernov, V.M., Mukhametshina, N.E., Gogolev, Yu.V., Nesterova, T.N., and Chernova, O.A., Mycoplasma Adaptation to Biogenic and Abiogenic Stressful Factors; *Acholeplasma laidlawii* Nannotransformation and Minibodies, *Dokl. Akad. Nauk*, 2004, vol. 396, no. 3, pp. 417–420 [*Doklady Biol. Sci.* (Engl. Transl.), vol. 396, pp. 251–254].
3. Chernov, V.M., Mukhametshina, N.E., Gogolev, Yu.V., Abdrakhimov, F.A., and Chernova, O.A., Adaptive Reactions of Mycoplasmas in vitro: "Viable but Unculturable Forms" and Nanocells of *Acholeplasma laidlawii*, *Mikrobiologiya*, 2005, vol. 75, no. 4, pp. 498–504 [*Microbiology* (Engl. Transl.), vol. 75, no. 4, pp. 428–433].
4. Golovlev, E.L., An Alternative State of Asporogeneous Bacteria, *Mikrobiologiya*, 1998, vol. 67, no. 6, pp. 725–735 [*Microbiology* (Engl. Transl.), vol. 67, no. 6, pp. 601–609].
5. Borkhsenius, S.N., Chernova, O.A., Chernov, V.M., and Vonskii, M.S., *Mikoplazmy* (Mycoplasmas), St. Petersburg: Nauka, 2002.
6. Romanova, Yu.M., Terekhov, A.A., and Gintsburg, A.L., Isolation and Characterization of the *Salmonella typhimurium* Mutants with Impaired Formation of Nonculturable Forms, *Genetika*, 1995, no. 6, pp. 34–37.
7. Quillardet, P., Huisman, O., Ari, R.D., and Hofnung, M., SOS-Chromotest, a Direct Assay of a SOS-Function in *Escherichia coli* K12 to Measure Genotoxity, *Proc. Natl. Acad. Sci. USA*, 1982, vol. 79, pp. 5971–5975.
8. Il'inskaya, O.N. and Margulis, A.B., *Kratkosrochnye test-sistemy dlya opredeleniya genotoksichnosti. Metodicheskoe rukovodstvo* (Genotoxicity Short-Term Test Systems. Methodical Guide), Kazan: Kaz. Gos. Univ., 2005.
9. Mersch-Sundermann, V., Kern, S., and Wintermann, F., Genotoxicity of Nitrated Polycyclic Aromatic Hydrocarbons and Related Structures on *Escherichia coli* PQ37 (SOS-Chromotest), *Environ. Mol. Mutagen.*, 1991, vol. 18, pp. 41–50.
10. Chernov, V.M., Chernova, O.A., Govorun, V.M., Demina, I.A., Gorshkov, O.V., Muzykantov, A.A., Shaimardanova, G.F., and Trushin, M.V., Adaptation of Mycoplasma to Unfavorable Growth Conditions: Ultrastructure and Genome Expression in *Mycoplasma gallisepticum* cells, *Dokl. Akad. Nauk*, 2008, vol. 421, no. 5 [*Dokl.* (Engl. Transl.), vol. 421, no. 5].
11. Chernov, V.M., Mukhametshina, N.E., Gogolev, Yu.V., Nesterova, T.N., and Chernova, O.A., Mycoplasma Adaptation to Adverse Growth Conditions: Nanotransformation and Phytopathogenicity of *Acholeplasma laidlawii* PG8, *Dokl. Akad. Nauk*, 2007, vol. 413, no. 2, pp. 271–275 [*Dokl. Biochem. Biophys.* (Engl. Transl.), vol. 413, pp. 57–60].
12. Khmel', I.A., Regulation of Expression of Bacterial Genes in the Absence of Active Cell Growth, *Genetika*, 2005, vol. 41, no. 9, pp. 1183–1202 [*Rus. J. Genet.,* vol. 41, no. 9, pp. 968–984].
13. Hentzer, M., Wu, H., Andersen, J.B., Riedel, K., Rasmussen, T.B., Bagge, N., Kumar, N., Schembri, M.A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J.W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Hoiby, N., and Givskov, M., Attenuation of *Pseudomonas aeruginosa* Virulence by Quorum Sensing Inhibitors, *The EMBO Journal*, 2003, vol. 22, pp. 3803–3815.
14. Oliver, J.D., The Viable But Nonculturable State in Bacteria, *J. Microbiol.,* 2005, vol. 43, pp. 93–100.