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Proteome of the Bacterium *Mycoplasma gallisepticum*

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Abstract—Using modern proteomic assays, we have identified the products of gene expression and posttranslational modifications of proteins of the bacterium *Mycoplasma gallisepticum* S6. Combinations of different technologies of protein separation by electrophoresis and mass-spectrometric analysis gave us a total of 446 proteins, i.e. 61% of the annotated proteins of this microorganism. The Pro-Q Diamond and Pro-Q Emerald dye technology was used for fluorescent detection of ten phosphoproteins and two glycoproteins. The acylation of proteins was studied by electrophoresis after *in vivo* labeling with different ^{14}C-labeled fatty acids, followed by autoradiography. Sixteen acylated proteins were identified, with a quarter of them involved in plasma membrane construction and another quarter involved in cell energy metabolism.

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Key words: *Mycoplasma gallisepticum*, proteomics, 2-D electrophoresis, mass spectrometry, posttranslational modifications

Mycoplasmas are the conditionally pathogenic microorganisms most widespread in nature, representatives of the class Mollicutes. The characteristic features of this class as a whole are small genomes and absence of a rigid cell wall. These features are associated with the parasitic lifestyle of these microorganisms and bring them nearer (by characteristics) to the so-called "minimal cell", i.e. a theoretical organism with the minimal set of components necessary and sufficient for independent self-reproduction on artificial nutrient media [1].

Like other representatives of this class, *Mycoplasma gallisepticum* has a very simple cellular organization: a cytoplasmic membrane, a nucleoid, and ribosomes. Its membrane is about 10 nm thick and contains all lipids as well as up to 50% of total proteins. The lipid membrane composition substantially depends on growth medium composition. About 25% of lipids are sterols, and *M. gallisepticum* is absolutely dependent on their presence in the medium. It should be noted that a significant share of the proteins, as compared with membranes of other bacteria, are lipoproteins. The distinguishing features of *M. gallisepticum* are pear-shaped cells and tubular structures that probably function as a cytoskeleton and serve for the sliding of cells on a substrate [2].

Mycoplasmas are notable for the smallest genome size among prokaryotes. So, the genome of *M. gallisepticum* R_{low} is 996,422 bp and has the low GC pair content (31%); 91% of the genome is comprised by 726 protein-coding DNA sequences [3]. The genome of mycoplasmas is significantly reduced as a result of parasitism and lacks some important regulatory factors. Mycoplasmas have no complete tricarboxylic acid cycle, quinines, and cytochromes. Thus, their electron cascade is terminated on flavins, which results in their significant energetic insufficiency in the exponential growth phase [4]. Nevertheless, mycoplasmas are rather easily cultivated and adapted to variation of nutrient medium components. At the same time, one should expect noticeable changes in metabolism and, as a result, variations of cell proteome as regards the changes of relative quantities and possible modifications of different proteins. This work pursued the inventory of expressed gene products and determination of posttranslational modifications of *M. gallisepticum* S6 proteins.

MATERIALS AND METHODS

Reagents for electrophoretic separation of proteins and ^{14}C-labeled fatty acids were from Amersham Bioscience (UK), gels and equipment for electrophoresis

Abbreviations: COG, clusters of orthologous groups.
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were from Bio-Rad (USA), and fluorescent dyes were from Invitrogen/Molecular Probes (USA).

Cultivation of *M. gallisepticum*. *Mycoplasma gallisepticum* cell culture was grown in a liquid medium as described in [5]. Growth phase was determined by the change of light scattering of the culture measured at 600 nm.

Harvesting of *M. gallisepticum* cell precipitate. The culture of *M. gallisepticum* was centrifuged at 15,000*g*, 4°C, for 20 min for precipitation of cells. The precipitate was twice washed in medium containing 150 mM NaCl, 50 mM Tris, 2 mM $MgCl_2$, pH 7.4. The cell precipitate was frozen and stored at −20°C.

1D separation of *M. gallisepticum* proteins. *Mycoplasma gallisepticum* proteins were separated by 1D electrophoresis under reducing conditions in gradient 9-16% polyacrylamide gels (the concentrating gel contained 4% acrylamide), 20 cm in length, and Tris-glycine buffer, by Laemmli followed by staining with Coomassie R-250. Total protein (150 μg in 30 μl of the Laemmli buffer with 2% of sodium dodecyl sulfate (SDS) and 2.5% dithiothreitol (DTT)) was exposed to 96°C for 5 min. The sample was introduced into 10 mm wells. Electrophoresis was performed in the following conditions: 10 mA in the concentrating gel and 20 mA in the separating gel, under chamber cooling to 10°C.

2D separation of CHAPS-soluble fraction of *M. gallisepticum*. Before 2D electrophoresis, the cell precipitate of *M. gallisepticum* was treated with a mixture of nucleases. Then proteins were re-precipitated with methanol–chloroform and dissolved in buffer containing 8 M urea, 2 M thiourea, 2% ampholines (pH 3-10), 100 mM DTT, 16.7% solution (30% CHAPS + 10% NP-40) for complete dissolution of proteins in the samples. The samples were centrifuged at 15,000*g* for 15 min. Protein concentration in the samples was measured by the Bradford method using Quick Start Bradford dye (Bio-Rad). Isoelectrofocusing was performed on 18 cm strips, pH 7-10, under conditions recommended by the manufacturer or in 18 cm glass tubes in 4% polyacrylamide gel (8 M urea, 4% acrylamide/methylene bis-acrylamide, 2% ampholines (pH 3-10), 4% ampholines (pH 5-8), 6% solution containing 30% CHAPS and 10% NP-40, 0.1% TEMED, 0.02% ammonium persulfate). The total protein of *M. gallisepticum* was applied to 500 μg to the strips and to 250 μg to the tubes. Isoelectrofocusing was performed in the following mode: 100, 200, 300, 400, 500, and 600 V for 45 min; 700 V for 10 h; 900 V for 1 h. On completion of isoelectrofocusing, the tubes were equilibrated in buffer containing 6 M urea, 30% glycerol, 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20 mM DTT, and bromophenol blue, for 30 min. Then the tubes were transferred to the surface of gradient polyacrylamide gel (9-16%) and fixed with 0.9% agarose with bromophenol blue. Electrophoresis was performed in Tris-glycine buffer under cooling in the following mode: 20 mA on glass for 20 min; 40 mA on glass for 2 h; 35 mA on glass for 2.5 h under chamber cooling to 10°C. On completion of electrophoresis, the gels were stained with silver with thiosulfate as described in [6]. The results were analyzed by PDQuest 8.0 software (BioRad).

Fluorescent staining of phosphorylated and glycosylated proteins. Phospho- and glycoproteins were stained by specific dyes Pro-Q Diamond and Pro-Q Emerald 488, respectively. Staining and scanning were performed following the manufacturer's protocols. Then the gels were stained by Sypro Ruby for total protein detection and scanned in a Typhoon Trio scanner (Amersham Biosciences). The resulting pictures were analyzed by PDQuest software. All specifically stained spots were identified by MALDI mass spectrometry.

Detection of acylated proteins. Acylated proteins were identified by addition of ^{14}C-labeled palmitic, linoleic, and oleic fatty acids to the incubation medium on cultivation of *M. gallisepticum* as described in [11]. Specific activity was 7.4 MBq/ml for ^{14}C-labeled palmitate, 3.7 MBq/ml for ^{14}C-labeled linoleic acid, and 3.7 MBq/ml for ^{14}C-labeled oleic acid. Toluene used for storage of fatty acids was eliminated by evaporation under vacuum, the precipitate was transferred into 96% alcohol, and the resulting emulsion was added to the incubation medium on the basis of 1 : 100 for ^{14}C-labeled palmitate and 1 : 50 for ^{14}C-labeled linoleic and oleic acids. The culture was grown as described above. Then the standard procedure of 2D separation of proteins was performed and the silver-stained gels were dried for further accumulation of radioactivity on a Storage phosphor screen. After one month, the screen was scanned using the Typhoon Trio scanner. The stained spots containing radioactive inclusions were cut out, exposed to hydrolysis, and identified.

Hydrolysis and mass spectrometry of *M. gallisepticum* proteins. Protein was hydrolyzed and extracted from the gel using the protocol described in [7].

LC-ESI-MS analysis. Not only stained spots, but also the spaces between them were cut out from the 1D gel. After 1D separation, proteins were identified by mass spectrometry. The products of trypsin hydrolysis of *M. gallisepticum* proteins were separated in a nano-ESI/MSD Trap Agilent 1100 Series functioning in the nanoflow mode (0.3 μl/min) and equipped with a Zorbax 300-SB C18 column in 60-min gradient of aqueous acetonitrile (5-80%) with the addition of 0.1% formic acid. The Agilent 1100 MSD Trap SL with a device for electrospray ionization (Nano-ESI) was used as a mass-spectrometric detector. Mono- and polyvalent molecular ions were detected in the range of 200-2200 *m/z* at optimization of 800 and capillary voltage of 1800 V. The maximal storage time was 30 msec. Ion fragmentation spectra were taken automatically when the value of total ionic current exceeded the background level. The accuracy of measurement was 0.5 Da. Proteins were identified using Mascot

software (Matrix Science, USA). The pruning limit was 40 with two identified peptides.

MALDI analysis. Mass-spectrometric analysis was performed under conditions described in [8]. The search was performed using the database of the US National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov) containing the full genome of *M. gallisepticum* strain R [9]. The pruning limit was 44 ($p < 0.05$).

RESULTS

Annotation of *M. gallisepticum* proteins. The main objectives of this work were identification of the products of expressed genes, the study of variation of their expression in different phases of mixed culture growth, and determination of posttranslational modifications of *M. gallisepticum* S6 proteins. Proteins were searched and annotated by the sequenced genome of closely related strain *M. gallisepticum* R [3] coding 726 proteins.

After separation of the CHAPS-soluble protein fraction by 2D electrophoresis within the pH range of 3-10, all protein spots were cut out and hydrolyzed by trypsin in the gel. Electrophoregrams with the above range demonstrated about 300 protein spots on average (Fig. 1) corresponding to about 150 individual proteins. More than half of *M. gallisepticum* proteins have calculated p*I* over 8, and their separation by isoelectrofocusing is extremely difficult. An IPGstrip with pH 7-10 made it possible to slight-

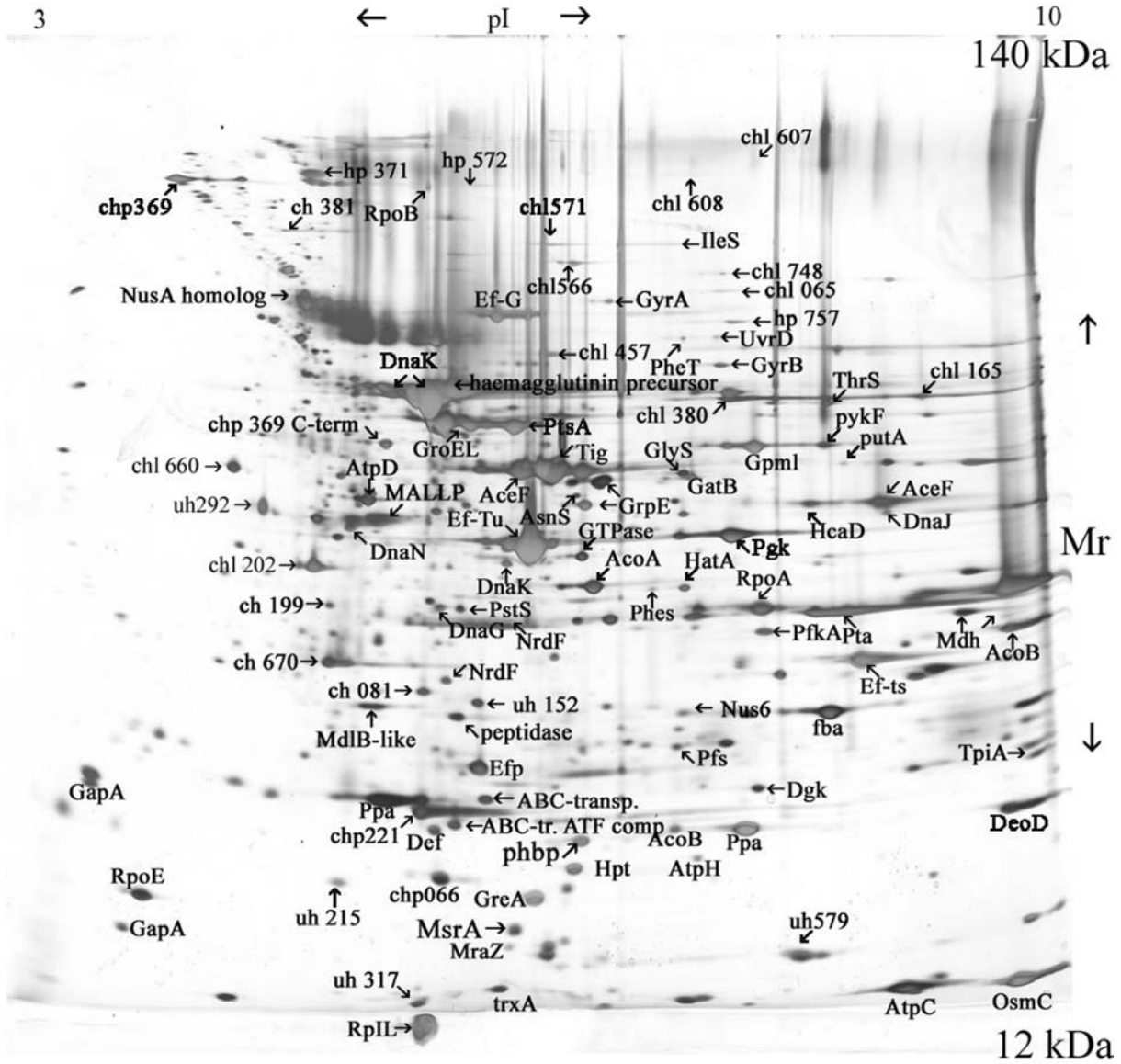


Fig. 1. Proteomic map of *Mycoplasma gallisepticum*. CHAPS-soluble protein fraction is divided horizontally by isoelectrofocusing in the pH range of 3-10 and vertically by electrophoresis in gradient 9-16% polyacrylamide gel followed by gel staining with silver.

ly improve the situation and to identify 20 additional proteins. Besides, resolution of 2D electrophoresis was enhanced by preliminary preparative liquid fractionation of total protein mixture under denaturing conditions in a Rotofor device. Each of the 10 obtained fractions was analyzed separately. As a result, resolution of the neutral range substantially improved, allowing us to identify 11 more proteins.

During mass-spectrometric identification of proteins, the pruning limit was 44 ($p < 0.05$). An additional factor that enhanced identification reliability was positioning of a spot on the 2D map in accordance with its calculated isoelectric point and molecular weight values. In the case of significant discrepancy between the calculated and actual parameters of a protein, the latter was exposed to MS/MS analysis. Protein GapA proved to have two N-terminal fragments with identical isoelectric point and slightly different weights, which were reproducibly presented on a 2D map in the strong-acid region of separation.

As a result, 171 individual proteins were identified using the above approach. The major proteins on the 2D map of *M. gallisepticum* are chaperone DnaK, a surface protein (hemagglutinin precursor), elongation factor TufB, and pyruvate dehydrogenase AceF. Thus, from among the most actively expressed proteins it is possible to identify representatives of three major functional cell systems: protein stabilization and folding, protein synthesis, and energy metabolism. Seventeen of the identified proteins are encoded by two and more genes but represented by a single spot on the electrophoregram, evidencing the prevalent expression of one of the genes in the *M. gallisepticum* population. On the other hand, some proteins encoded by a single gene are present in several protein spots. For example, inorganic pyrophosphatase PpA and elongations factors TufB and FusA are observed as a set of isoforms of unknown nature. In some cases, the presence of several protein spots of the same protein may be evidence of strictly determined proteolysis during the cell lifecycle. So, the hypothetical protein with gi|31541369 (accession number in the NCBI database) is reproducibly presented as a series of spots that differ from each other both in weight and in p*I*. More complete inventory of *M. gallisepticum* proteins was performed by 1D electrophoresis followed by chromato-mass spectrometry. A gel track was divided into approximately 120 sections, with the cutting of both heavily and weakly stained zones. After trypsin hydrolysis of complex protein

Table 2. Distribution of identified proteins of *M. gallisepticum* by COG functional categories

COG functional categories	Annotated proteins according to NCBI	Identified proteins
[J] Translation, ribosome structure, biogenesis	107	82
[K] Transcription	19	18
[L] DNA replication, recombination, and repair	54	31
[D] Cell cycle control, mitosis, and meiosis	8	6
[O] Posttranslational modifications, protein turnover, chaperones	41	31
[M] Cell membrane, outer membrane	12	9
[N] Cell motility and secretion	1	1
[P] Transport and metabolism of inorganic ions	26	20
[T] Mechanisms of signal transduction	7	6
[C] Energy production and processing	27	21
[G] Transport and metabolism of carbohydrates	40	26
[E] Transport and metabolism of amino acids	28	24
[F] Transport and metabolism of nucleotides	29	23
[H] Metabolism of coenzymes	14	7
[I] Metabolism of lipids	15	5
[Q] Biosynthesis, transport, and catabolism of secondary metabolites	2	0
[R] General prediction of functions	54	33
[V] Protective mechanisms	23	13
[U] Intracellular transport and secretion	8	4
[S] Unknown function	22	8
(–) Absent in COGs	230	98

mixtures present in each fraction, peptide extracts were separated by reverse-phase HPLC, and fragmentation mass spectra were obtained for all peptides automatically. The data were combined and analyzed by the Mascot software. The proteins detected by at least two peptides with the total score of 40 and more were taken into account. A total of 427 proteins were identified, including 256 proteins not present on the two-dimensional map. Such method of separation and identification resulted in detection of 100% of proteins with molecular weight of more than 81 kDa, about 40% of 31- to 81-kDa proteins, and 20% of 9- to 31-kDa proteins. Proteins with the weight below 9 kDa were not identified.

All in all, we detected 446 individual proteins, i.e. about 60% of the total number of annotated proteins of this microorganism (Table 1; available as Supplement to the article at the site of *Biochemistry* (Moscow) (http://www.protein.bio.msu.ru/biokhimiya)). Functional affiliation of the proteins was determined by the category of COG (clusters of orthologous groups). Proteins were classified by 20 categories of functional activity (http://www.ncbi.nlm.nih.gov/COG/) (Table 2). We did not identify the proteins from the group of biosynthesis of secondary metabolites. The most widely represented proteins are involved in translation and construction of ribosomes. The finding of 94% of proteins of the transcription group proves them to be a pool of very common cell proteins. At present, 230 of the studied proteins have no COG index in the NCBI database.

Posttranslational modifications of *M. gallisepticum* proteins. *Phosphorylation*. We have constructed a phosphoproteome of the CHAPS-soluble protein fraction of *M. gallisepticum*. For this purpose, we used 2D separation of proteins followed by staining with fluorescent dye Pro-Q Emerald 488 (Fig. 2) for detection of phosphoproteins and with Sypro Ruby for general staining. The resulting gels were scanned in the Typhoon Trio scanner. Computer overlay of the images from two channels using ImageQuant software (Amersham Bioscience) revealed proteins carrying phosphoric acid residues. Two independent experiments were performed. The MALDI mass spectrometry was used for identification of 15 phosphorylated proteins in each experiment, which was 2% of the total number of *M. gallisepticum* proteins (Table 3). However, only 10 out of 15 proteins were identified twice. Protein DnaK was selected for characterization of specific binding site of phosphoric acid residues, because phosphorylation of this protein was described in the literature for many bacterial species. Peptide 401QIFSTAQDNQSESVDVSIYQGERPMAR429 (*m/z* 3032.9) was exposed to MS/MS analysis (Fig. 3). It was shown that the phosphoric acid residue was bound with serine in position 421.

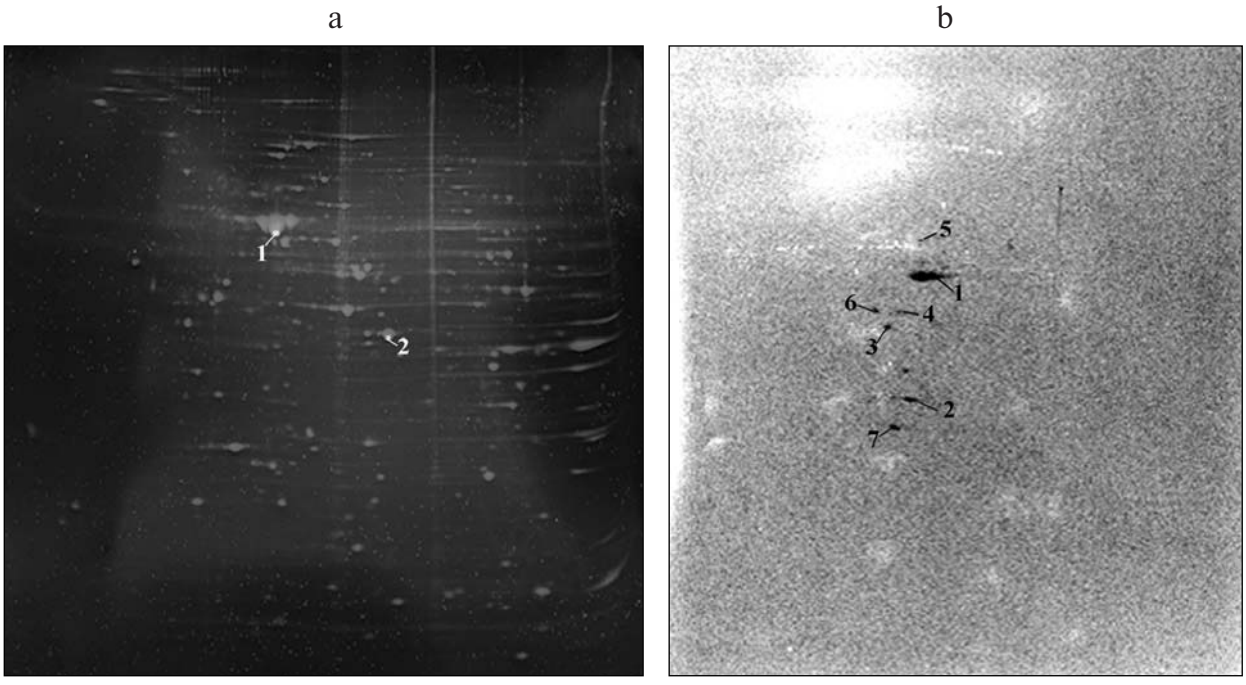


Fig. 2. Mapping of posttranslational modifications of *M. gallisepticum* proteins. a) Glycoproteome; the gel was stained with ProQ Emerald 488 for detection of glycosylated proteins and with Sypro Ruby for visualization of total protein fraction. 1) Chaperone DnaK; 2) α-subunit of E1-dehydrogenase AcoA. b) Detection of acylated proteins; autoradiography (four weeks of exposure) of the 2D map of separation of *M. gallisepticum* proteins after labeling with ^{14}C-palmitic acid. 1) Hemagglutinin precursor; 2) conservative hypothetical protein gi|31541080; 3) conservative hypothetical lipoprotein gi|31541199; 4) β-chain of ATP synthase AtpD; 5) conservative hypothetical lipoprotein gi|31541571; 6) protein similar to macrophage-activating lipoprotein; 7) unique hypothetical protein gi|31541215.

Table 3. Posttranslational modifications of *M. gallisepticum* proteins (phosphorylation, glycosylation, acylation)

	NCBI No.	Name	Molecular weight (Da)	Score	Isoelectric point (p*I*)	Function
1	2	3	4	5	6	7
		Phosphorylated proteins				
	gi|1905870	hemagglutinin precursor	70206	195	5.91	cell membrane, other
	gi|31541275	elongation factor TufB	43606	129	5.76	protein synthesis, translation factor
	gi|31541369	conservative hypothetical protein	98877	209	4.37	not defined
	gi|31541381	conservative hypothetical protein	85818	256	4.66	not defined
	gi|31541519	permease component of saccharo-spermidine-putrescine ABC-transport system MalK/PotA	58056	60	5.16	transport and binding of proteins, amino acids, peptides, and amines
	gi|31541527	chaperone GroEL/Hsp60	56875	175	5.38	purines, pyrimidines, nucleosides, and nucleotides; biosynthesis of pyrimidine ribonucleotides
	gi|31541553	conservative hypothetical protein	224370	126	4.27	not defined
	gi|31541561	translation factor Efp	21240	64	5.35	protein synthesis, translation factor
	gi|31541589	chaperone DnaK	64647	76	5.25	protein degradation, folding, and stabilization
	gi|31541601	conservative hypothetical protein	228550	119	4.23	not defined
		Glycosylated proteins				
	gi|31541535	α-subunit of E1-dehydrogenase AcoA	40553	136	5.81	energy metabolism, glycolysis/ gluconeogenesis
	gi|31541589	chaperone DnaK	64647	227	5.25	protein degradation, folding, and stabilization
		Acylated proteins				
lin	gi|31541188	50S ribosomal protein L2 RplB	18025	148	5.58	protein synthesis, ribosomal proteins: synthesis and modifications
lin	gi|31541240	transcription elongation factor GreA	17933	157	5.56	transcription, transcription factor
lin	gi|31541485	predicted phospholipid-binding protein	24046	86	6.44	unknown
lin	gi|31541670	conservative hypothetical protein	32900	126	5	central intermediary mechanism, other
lin	gi|31541690	guanylate kinase Gmk	22459	83	6.93	purines, pyrimidines, nucleosides, and nucleotides
ol	gi|31541066	conservative hypothetical protein	19893	107	5.28	transport and binding of proteins, transfer of cations and iron
ol	gi|31541100	ribosomal protein L4 RplD	23177	106	9.9	protein synthesis, ribosomal proteins: synthesis and modifications

Table 3. (Contd.)

1	2	3	4	5	6	7
ol	gi|31541381	conservative hypothetical protein	85818	55	4.66	not defined
ol	gi|31541586	conservative hypothetical protein	66091	131	8.52	cell membrane, other
pal	gi|1905870	hemagglutinin precursor	70206	214	5.91	cell membrane, other
pal	gi|31541080	conservative hypothetical protein	82535	42	8.92	cell membrane, other
pal	gi|31541199	conservative hypothetical lipoprotein	45216	172	5.5	prokaryotic lipoprotein signal
pal	gi|31541215	unique hypothetical protein	29760	75	6.1	cell membrane, other
pal	gi|31541355	β-chain of ATP-synthase AtpD	51513	249	5.12	energy metabolism
pal	gi|31541571	conservative hypothetical lipoprotein	120325	324	5.83	energy metabolism, prokaryotic lipoprotein signal
pal	gi|31541648	protein similar to macrophage-activating lipoprotein	62181	80	6.37	not defined

It is accepted that the main sites of phosphorylation of bacterial proteins are histidine and aspartate [9]. However, it has been shown previously that serine, threonine, and tyrosine residues can be phosphorylated not only in eukaryotes, but also in prokaryotes [10].

Glycosylation. For the search for glycosylated proteins of *M. gallisepticum*, we used the method of 2D gel staining with fluorescent dye Pro-Q Emerald specific to sugar residues followed by total protein staining with Sypro Ruby. Pro-Q Emerald is a low-molecular-weight fluorophore and does not prevent subsequent mass-spectrometric identification of proteins. Computer overlay in the ImageQuant software of the images obtained from two different channels using the Typhoon Trio scanner made it possible to detect specific staining in two points identified by MALDI-MS as DnaK (the chaperone involved in protein stabilization and folding) and AcoA (α-subunit of E1-dehydrogenase from pyruvate/2-oxoglutarate dehydrogenase complex).

Acylation. Prior to introduction of fatty acids in 96% alcohol into the incubation medium, an experiment was performed on cultivation of *M. gallisepticum* in the presence of respective volumes of alcohol without fatty acids. It was shown that the alcohol in the used amounts had no effect on culture growth parameters.

The autoradiography after 2D separation of the CHAPS-soluble protein fraction was used for MALDI-mass spectrometric identification of 17 acylated proteins; 7, 6, and 4 of them contained the chains of palmitic, linoleic, and oleic acids, respectively (Table 3). Nearly a quarter of the identified proteins participate in plasma membrane construction and another quarter is involved in cell energy metabolism.

DISCUSSION

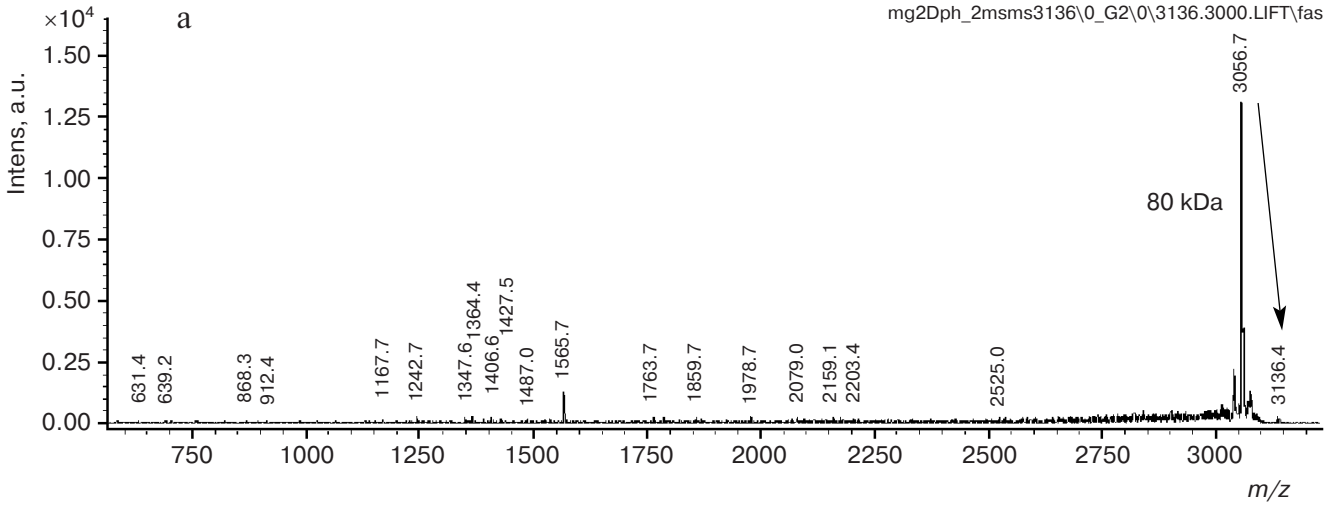
In this work, we have successfully identified 446 individual proteins accounting for 61% of total annotated proteins of this microorganism in conformity with the closely related strain *M. gallisepticum* R.

First of all, we used a combination of 2D electrophoretic separation in polyacrylamide gel with the MALDI-mass spectrometric analysis of peptide charts of individual proteins. The 2D electrophoresis with isoelectric focusing in the first direction ensures qualitative distribution of proteins over the isoelectric point that can be calculated by primary structure. These data are used for additional verification of the identified proteins and for quantitative calculations of relative numbers of proteins in different experiments. The 171 individual proteins were identified using additional methods of resolution improvement.

A more complete inventory of the *M. gallisepticum* proteins was performed by 1D electrophoresis followed by chromato-mass spectrometry. A total of 427 proteins were detected, including 256 proteins that were absent on the 2D map. Nineteen proteins present on the 2D map were not identified by this method. Thus, a total of 446 individual proteins were identified.

The functions of more than one third of *M. gallisepticum* proteins are still unknown. Identification and tracing of quantitative changes of most proteins of the microorganism, which underlie present-day metabolic reconstructions, suggest the existence of previously unknown functions of some proteins.

Covalent protein modifications such as phosphorylation, acylation, and glycosylation are the main mecha-



b

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  1 MTMSNNNGLI IGIDLGTTNS CVSVMEGAQK VVIENPEGKR TTPSVVSYKN
 51 GEIIVGDAAK RQMLTNPNTI VSIKRLMGTS KKVKINDKGV EKELTPEEVS
101 ASILSYLKDY AEKKTGQKIS RAVITVPAYF NDAERQATKT AGKIAGLTVE
151 RIINEPTAAA LAYGIDKGHR EMKVLVYDLG GGTFDVSLLD IADGTFEVMA
201 TAGDNRLGGD DWDNKIIEWI IAEIKKDHPS LDLKSDKMAM QRLKEAAERA
251 KIELSAQLET LISLPFIAVT PEGPVNAELT LSRAKFEELT KDLLERTRNP
301 IADVLKEAKV DPSQVDEILL VGGSTRMPAV QKLVESMIPN KAPNRTINPD
351 EVVAIGAAVQ GGVLRGDVKD ILLLDVTPLT LAIETLGGVA TPIIKRNTTI
401 PVSKSQIFST AQDNQESVDV SIYQGERPMA RENKSLGTFS LGGIQPAPKG
451 KPQIEITFNI DANGILNVKA KDLTTGKENS ITISNSSELD ENEIQRMIRD
501 AEANKERDAI VKQRIEMRYE GEGIVNTINE ILGSKEAEAL PAQEKASLTK
551 IVDGINGALK AEKWDELKEQ IDGFKKWRDD MSKKYGGGEA PAEPK
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Fig. 3. Detection of DnaK phosphorylated peptide by MALDI MS/MS. a) Mass-spectrum of phosphorylated peptide of DnaK protein; b) DnaK amino acid sequence; studied peptide is shown in bold underlined.

nisms of control and regulation of many paramount intracellular processes. Identification of proteins involved in these processes is necessary for establishment of regulation pathways.

Many proteins and secreted peptides undergo various structural changes as a result of co-translational and posttranslational modifications. At present, more than 100 various posttranslational protein modifications have been described. The role of most of these modifications is still unclear; some of them are random and currently there is no data on their functional significance, while others are necessary for cell life and thoroughly controlled by specific enzymes.

Phosphorylation modifies proteins through addition of negatively charged groups to serine, threonine, and tyrosine residues. These neutral hydroxy-amino acid residues are typically exposed on the surface and often occur between the regulatory subunits of a protein. In this work, the MALDI mass spectrometry was used to identify 10 phosphorylated proteins, i.e. 1.5% of the total number proteins of *M. gallisepticum* (Table 3).

Previously it has been shown that *M. pneumoniae* has 3% of proteins identified as phosphorylated [12], *E. coli* has 79 phosphorylated proteins (2% of those annotated) [13], and *Bacillus subtilis* has 1.9% of such proteins [14]. However, these values are not final because many proteins that can be phosphorylated are not expressed in amounts sufficient for identification by the available methods; besides, 2D electrophoresis as a method of detection has significant limitations by p*I* and protein solubility, therefore most membrane proteins are not included in the region of separation.

As phosphorylation is not related to specific amino acid sequence and may actually occur at any available serine, threonine, or tyrosine residue, prediction of this modification is a complicated bioinformatics problem. For solving this problem, one must have reliable information about three-dimensional structure of a protein and/or its homologs in other microorganisms, where phosphorylation has already been demonstrated. Proteins DnaK, GroEL, and TufB have been previously annotated as phosphorylated in *E. coli* [15], *M. penetrans* [16], *M. genitalium*, and *M. pneumoniae* [12]. All proteins defined as phosphorylated have calculated p*I* below 6. The identified proteins are related to the systems of protein synthesis, pathways of energy metabolism, and protein transport. The functions of four proteins have not yet been determined. The number of regulatory proteins in *M. gallisepticum* is low, and many metabolic pathways are regulated by reversible phosphorylation.

Glycosylation of proteins in bacterial cells has been described rather poorly. Previously such modification has been considered impossible in bacteria due to the absence of intracellular organelles corresponding to those of eukaryotes. However, recent studies suggest the presence of similar (but not identical to eukaryotic) systems of protein glycosylation. In the 1970s, a few publications reported the presence of glycosylated proteins in the membranes of *Mycoplasma pneumoniae* [17] and *M. gallisepticum* [18], but no direct evidence was obtained. (For example, *M. gallisepticum* is grown *in vitro* in a medium with a high content of horse serum, but its components are tightly bound with the membrane and could be detected in the course of studies as mycoplasma proteins.)

In 1990, Thomas and Sharp reported the presence of glycosylated proteins in three strains of *M. gallisepticum* [19]. N-Glycosylation of asparagine residues and O-glycosylation of serine and threonine residues have been distinguished. O-Glycosylation is a highly conservative posttranslational modification in all eukaryotes, from yeast to man. At present, two O-glycosylated proteins have been described in *Mycobacterium tuberculosis* [20]. It is notable that O-glycosylation is associated with Sec-translocation of proteins in *Mycobacterium* sp. This mechanism is analogous to the eukaryotic translocation system containing Sec61 and an oligoglycosyltransferase complex responsible for both O- and N-glycosylation. The biological role of O-glycosylation is still unclear.

N-Glycosylation plays a key role in the folding, oligomerization, sorting, and transport of secretory and membrane proteins [21]. This modification, independent of translocation mechanisms, was first discovered in 2002 by Young et al. in the proteins of *Campylobacter jejuni* [22].

One of the major distinctive features of mycoplasmas is an ample quantity of membrane proteins with covalently bound fatty acid chains. At the same time, they are incapable of independent biosynthesis of fatty acids and therefore extremely dependent on cultivation conditions. Mycoplasmas are characterized by an extremely high quantity of membrane lipoproteins; however, far from all of them are acylated. Lipoproteins play an important role in adhesion, pathogenicity, and antigenic variability of mycoplasmas. Acylated proteins were revealed in the membrane of *M. gallisepticum* for the first time in 1992 by Forsyth et al. [23].

We have identified 16 acylated proteins. Three quarters of these are involved in plasma membrane construction, others participating cell energy metabolism, while the function of three proteins is unknown. Nine of the above proteins are hypothetical and specific for the given microorganism. Modification by palmitic acid residue is the most frequent, which is in agreement with earlier data on occurrence of fatty acids in various mycoplasma species [24].

This work reports the first proteomic analysis of *M. gallisepticum*. The number of sequenced genomes has been swiftly increasing in recent years. In this context, the functional analysis of gene expression products becomes relevant. Identification of changes under different states of a cell is particularly interesting, as they are helpful for establishing the functions of hypothetical and unique proteins as well as for determination of the factors of resistance, virulence, etc. Establishing and tracing of posttranslational modifications of numerous proteins will make up a complete picture of regulatory events occurring under the influence of various environmental conditions.

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