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# Identification of Intracellular *Spiroplasma melliferum* Metabolites by the HPLC-MS Method

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**Abstract**—In contrast to the abundance of systems-oriented approaches describing changes on the transcriptome or proteome level, relatively few studies have employed the metabolome. The goal of the presented research was to identify as many intracellular metabolites as possible in a *Spiroplasma melliferum* extract by flow injection time-of-flight mass spectrometry. The Mollicutes class bacterium *S. melliferum* is a member of a unique category of bacteria that have in common the absence of a cell wall, a reduced genome, and simplified metabolic pathways. Metabolite identification was confirmed by fragmentation of previously detected ions by target mass spectrometry. The selected liquid chromatography approach, hydrophilic interaction chromatography with amino and silica columns, effectively separates highly polar cellular metabolites prior to their detection on a high accuracy mass spectrometer in positive and negative acquisition mode for each column. Here we present reliable measurement of 76 metabolites, including components of sugar, amino acid, and nucleotide metabolism. We have identified about a third of the possible intracellular *S. melliferum* metabolites predicted by genome annotation.

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*Key words*: *Spiroplasma melliferum*, metabolomics, mass spectrometry, LC-ESI-MS/MS, metabolite, hydrophilic interaction chromatography

The development of systems biology requires the integration of transcriptomic, proteomic, genomic, and metabolomic knowledge to obtain an integral model of the living organism. As opposed to the system-oriented approaches describing transcriptome and proteome changes, there are few studies describing metabolites. Metabolomics is an area of research that focuses on systematic chemical identification and quantification of all the metabolites of cells, tissues, or organisms [1]. Therefore, a metabolic profile of the organism represents a living imprint of cell activity.

The mRNA transcription data, decoding of DNA sequences, and results of proteomic analysis data do not reveal all the ongoing processes in cells. For example, there are many genes whose transcription is not directly regulated. Elucidation of metabolite composition can provide valuable insights into the processes of cell regulation and mechanisms of cell adaptation to different environments.

The overall objective of metabolomics is to perform a complete and possibly quantitative analysis of all known metabolites in a given biological sample [2]. At the present time, using a set of specially developed methods, almost all intracellular metabolites can be identified individually. However, there is no single method that can analyze the entire set of metabolites. Due to the variety of physical and chemical properties of metabolites, it is important to use customized methods of separation and subsequent detection for different types of compounds to obtain adequate results.

For the study of all cell metabolites the chosen analytical method must include two parts: the separation of

*Abbreviations*: FTICR, Fourier transform ion cyclotron resonance; HILIC, hydrophilic interaction liquid chromatography; Q, quadrupole detector; QQQ, triple quadrupole detector; TOF, time-of-flight.

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analytes according to their retention time and their subsequent detection. For the detection of metabolites, the following methods are most often used: detection of chemical shifts in the nuclear magnetic resonance spectrum (NMR); measurement of the analytes mass-to-charge ratio (mass spectrometry); detection of retention time during chromatographic separation; or a combination of several methods. NMR analysis techniques have several significant advantages over mass spectrometry: biological samples do not require additional preparation and the intensity of the signals depends linearly on the amount of the substance [3]. However, NMR has relatively low sensitivity, so it is only the most represented metabolites in the sample that are detected [4]. High sensitivity and specificity of the detection and a wide dynamic range of measurements could be achieved by a combination of high-precision mass spectrometry with effective sample preparation and chromatographic separation of substances [5-7]. Currently, there are available a large number of diverse chromatographic separation techniques and ionization and mass spectrometric detection of metabolites. Metabolite separation can be carried out by gas chromatography [6], capillary electrophoresis [8], or liquid chromatography [9], which includes HPLC (high performance liquid chromatography) and UHPLC (ultra-high performance liquid chromatography) [10-13]. In conjunction with liquid chromatography, the sample can be ionized by electrospray and chemical or photoionization at atmospheric pressure [14]. Mass spectrometric detection can be performed with a quadrupole mass analyzer (Q, QQQ), an ion trap (i-TRAP), a high precision time-of-flight mass spectrometer (TOF) [15], or it can be performed using a Fourier transform ion cyclotron resonance mass analyzer (FTICR) [16]. Different types of mass spectrometers can be combined in hybrid devices [17], for example, quadrupole and TOF mass spectrometers could be used together in the Q-TOF analyzer. In our study, we used a high-performance liquid chromatograph (Agilent 1200) coupled with a quadrupole TOF mass spectrometer of high resolution (Q-TOF Agilent 6520 series). The instrument provides a 10-fold increase in sensitivity of detection compared to similar MS systems and a wide dynamic range (about 3.5 orders of magnitude), which is combined with an excellent accuracy of mass detection where the working value is 5 ppm *m/z*.

The goal of the current study was to achieve the most complete identification of the intracellular metabolites of a Mollicutes class bacterium, *Spiroplasma melliferum* [18]. Due to its small size, small number of proteins, and the extreme simplicity of intrinsic organization, Mollicutes are a convenient model object for studying of the organizing principles of a maximally simple living system [19-21]. Owning to its parasitic life style (*S. melliferum* has two kinds of hosts, insects and plants in its natural habitat), it lacks many of metabolic pathways, which makes it easier to analyze its metabolome. We studied the metabolic profile of *S. melliferum* cells growing under optimal conditions in the current work. We detected 76 metabolites, which accounts for 30% of all the annotated metabolites of the bacterial genome of *S. melliferum* in accordance with results previously obtained in our lab [22].

## MATERIALS AND METHODS

***Spiroplasma melliferum* strain and growth conditions.** *Spiroplasma melliferum strain* KC3 was kindly provided by Prof. G. Vroblevsky of the University of Rennes, France. *Spiroplasma melliferum* was grown in SP4 media without any specific aeration and mixing; the time of growth and the pH of the solution was controlled [22]. The cells were harvested by centrifugation (15 min, 16,000*g*) in the log phase (20 h culture growth), which was determined by the values of pH (6.5-6.6). The cells were harvested from 50 ml of culture, centrifuged, and washed. The metabolic profiles were obtained from cultures grown from a single clone. Each of the experiments for the study of the *S. melliferum* metabolome was repeated at least five times.

**Reagents.** The following chemicals were used as standards: sodium pyruvate (100 mg/ml, disodium salt hydrate), D-fructose 6-phosphate, hydrate of sodium phospho(enol)pyruvate (97% purity, enzyme quality), dehydrated disodium salt of D-ribose 5-phosphate, diglyceraldehyde 3-phosphate (46.1 mg/ml). Purified (98%) amino acids, nucleotides, nucleosides (adenosine, deoxyadenosine, inosine, cytosine monophosphate, and thymidine) were also used as standards (Sigma-Aldrich, USA). The following samples were used for extraction and solution preparation: absolute methanol (HPLC grade) from Biosolve (The Netherlands), ammonium acetate (ultra clean grade) from Helicon (Russia), formic acid (98-100%) from Riedel-de Haen (Germany), ammonium hydroxide solution (29.73%) from Fisher Scientific (USA), water (HPLC-MS) and acetonitrile (HPLC-MS) from Panreac (Spain).

The following chromatographic analytical columns were used in the study: Luna NH2 (30 mm × 2 mm × 5 μm) from Phenomenex Torrance (USA) and Zorbax RX-SIL Narrow-Bore (150 mm × 2.1 mm × 5 μm) from Agilent Technologies (USA). To protect the separating phase of these highly effective analytical columns from chemical damage, the following safety cartridges were used: Zorbax NH2 4-Pack analytical guard column (4.6 mm × 12.5 mm × 5 μm) and Zorbax RX-SIL 4-Pack analytical guard column (4.6 mm × 12.5 mm × 5 μm) purchased from Agilent Technologies.

**Extraction of metabolites.** For metabolite analysis, 50 ml culture of *S. melliferum* grown in liquid medium under the conditions described above was used. The metabolism of the growing culture was rapidly quenched

by cold methanol extraction. There were five repetitions of the experiment. Aliquots of 50 ml of *S. melliferum* cultures grown from the same strain and with same sample preparation were used for each of the experiments in accordance with the standard protocol.

A cold methanol extraction method was developed on the basis of a previously reported cold methanol extraction protocol [23] as described below.

*Metabolite extraction protocol.* Cell culture aliquots of 50 ml were centrifuged for 15 min at 16,000*g* at 20°C, and then the supernatant was carefully removed. The precipitate was resuspended in 3 ml of 150 mM NaCl, and the resulting suspension was centrifuged for 10 min at 16,000*g* at 20°C. The supernatant was discarded. The precipitate was resuspended in 150 μl of 150 mM NaCl and centrifuged for 10 min at 16,000*g* at 4°C. The supernatant was discarded. The washing was repeated one more time. The precipitate was resuspended in 75 μl of 150 mM NaCl. The metabolites were extracted by addition of 1000 μl of methanol (–77°C) to the sample; the sample was thoroughly shaken (1 min) and then kept at –77°C for 15 min. The sample was warmed for 3 min and then thoroughly shaken again. The resulting sample was centrifuged for 30 min at 16,000*g* at 4°C. The supernatant was separated into aliquots and lyophilized. The dry extract was kept no longer the 4 days prior to the analysis. The dry extract was dissolved in mixture consisting of 20% acetonitrile and 80% water prior to analysis.

**Apparatus and HPLC/MS method.** Mass spectroscopic analysis was performed on a Q-TOF 6520 series time-of-flight mass spectrometer (Agilent Technologies).

The flow from the analytical column was introduced directly into the electrospray ion source of the mass spectrometer. Prior to the experiment, the mass spectrometer was calibrated so that its measure of accuracy was 2 ppm of the *m/z* value. The ionizing spray voltage was 3500 V in both positive and negative ionization modes. Nitrogen of various degrees of purity was used for both: to dry gas at a pressure of 20 psi and as the gas in the collision cell. The temperature of the quartz capillary was 325°C.

HPLC-MS and HPLC-MS/MS analysis was carried out using the series 1200 high performance liquid chromatograph (Agilent Technologies) coupled with the mass spectrometer. Chromatographic analysis was performed with the following parameters: auto-sampling temperature, 8°C; analytical column temperature, 18°C; injection volume, 2 μl; solvent flow rate, 50 μl/min. The following solvents were used as eluting solutions: eluent A was 20 mM ammonium acetate + 0.25 mM ammonium hydroxide in water/acetonitrile mixture of 95 : 5 ratio, pH 8.02; eluent B was pure acetonitrile. The gradient of the solvent transition was as follows: for positive ionization mode $t = 0$, 100% B; $t = 30$ min, 0% B; $t = 32$ min, 0% B; $t = 35$ min, 100% B; $t = 60$ min, 100% B. In negative ionization mode $t = 0$, 100% B; $t = 30$ min, 0% B; $t = 32$ min, 0% B; $t = 35$ min, 100% B; $t = 60$ min, 100% B.

Metabolite identification was confirmed by fragmentation spectra of the detected ions. The collision energy was fixed at 20 eV, and MS/MS spectra were recorded in the range of 30-1000 *m/z* with the minimal width of ion isolation window being 1.3 *m/z*.

**Identification of metabolites and data processing software.** Metabolite search and data processing were performed using the software Metabolomic Analysis and Visualization Engine (MAVEN) [24] and was performed using the Internet resource called the Trans-Proteomic Pipeline (TPP), where the data were converted into the mzXML format (MAVEN compatible).

*Spiroplasma melliferum* metabolites were identified using the list of all possible metabolites for these bacteria, the list being from all the protein annotation data previously reported in [22]. A list of all theoretically possible metabolites of *S. melliferum* was prepared in accordance with the KEGG database [25] and contained all the metabolites associated with the proteins that were annotated for this bacterium. The following parameters were used for the search: range of *m/z* values (extraction window), ±10 ppm *m/z*; minimum intensity of peak, 1000 a.u.; minimum value of baseline signal intensity ratio, 10 [26]. The mass spectra of the tested metabolites were aligned to increase the accuracy measure (up to 3 ppm of the values of *m/z*). The spectra were aligned using the mass values (*m/z*) of the isotopes of the tested metabolite and was carried out with the software package R [27] with additional libraries for analysis and annotation LC-MS data XCMS [28] and CAMERA [29].

## RESULTS

*Spiroplasma melliferum* bacterial cultures were grown in liquid media because this kind of cell culture growth allows easily determining the growth phase. Cold methanol extraction was used for instant quenching of the metabolism [30]. High effectiveness of this method together with low loss of compounds was shown previously [31]. HPLC analysis was used in combination with ionization in the quadrupole time-of-flight mass spectrometer electrospray (HPLC–MS Q-TOF) that was applied for analysis of *S. melliferum* metabolites. Metabolites were preliminarily identified using the Metabolomic Analysis and Visualization Engine (MAVEN) software [24]. Mass spectra of the detected compounds were aligned to increase the accuracy of measure (up to 3 ppm of the values of *m/z*). This was carried out with exact values of the isotopes and using software package R [27]. Identification of metabolites was confirmed from the fragmentation spectra of the detected ions using targeted mass spectrometry [32].

Parameters of the chromatographic method and of the mass spectrometric peak recording were chosen in accordance with the method developed by Rabinovitz [9].

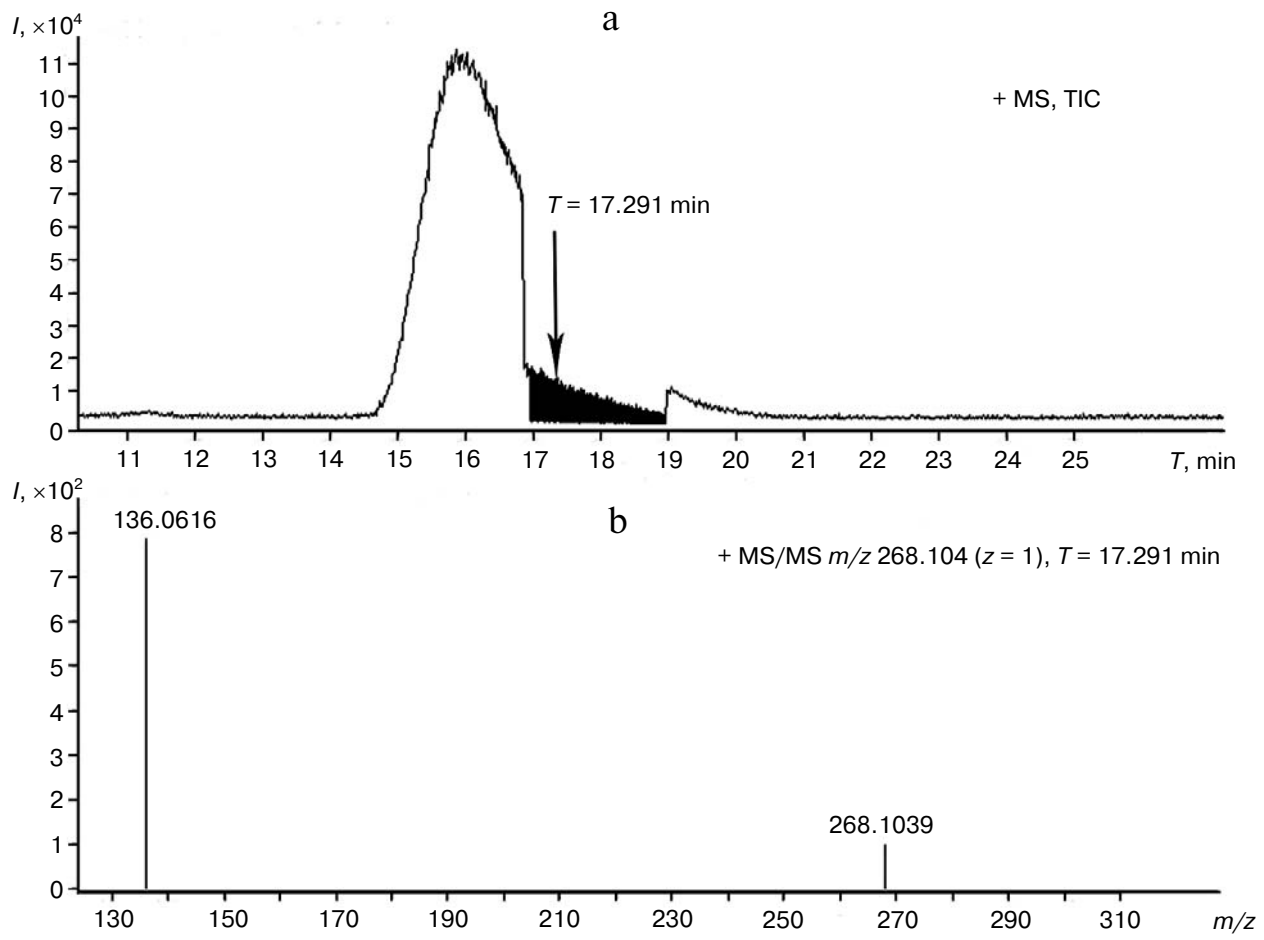


**Fig. 1.** a) Chromatographic peak (data extracted from ion current) of adenosine retention (268.104). Injection was 2 µl of standard sample of 1 ng/ml in positive ionization mode; b) adenosine fragmentation spectrum (fragment *m/z* 136.0620) at 20 V.

However, the equipment used in the current study (HPLC–MS Q-TOF) was slightly different from the equipment that was used by the authors of [9] (HPLC–MS QQQ). So, to obtain the most reliable data it was necessary to adjust the parameters of the detection system (time of analysis, flow rate of elution systems, solvents gradients, values of ionizing spray voltage, etc.). The analysis conditions were optimized (see section "Apparatus and HPLC/MS method" above) in the following preliminary experiments. The separation standards (fructose 6-phosphate, ribose 5-phosphate, fructose, adenosine, deoxyadenosine, inosine, thymidine, and the twenty amino acids) were used to optimize the separation parameters. The detection limit was determined for the testing conditions, which was 10 pg/ml with injection volume of 2 µl (taking into account that the identification peaks should be of at least 1000 units). Also, fragmentation spectra of some standards were obtained at different values of collision energies (CE), which were in the range of (10 to 60 V). The results allowed selection of a fixed value of CE = 20 V, which provided the highest intensity of the peaks corresponding to parent ions and to its fragments for substances with *m/z* = 30-1000 in the fragmentation spectra (see Fig. 1).

There were five independent repeats for analytical detection of *S. melliferum* bacterial metabolites. The average number of metabolites, which was identified during each analysis, was about 120 compounds. However, to increase the reliability or the reading an additional selection step was introduced. Only metabolites that were observed at least three times in any of the ionization modes (positive or negative) and that were registered with intensity of the corresponding peak of at least 1000 units were selected. Among the detected metabolites were compounds that participate in the main metabolic pathways such as glycolysis, amino acid, sugar, and amino sugar metabolism, synthesis of terpenoids, purine and pyrimidine bases, etc.

There were 76 metabolites of *S. melliferum* that were reliably detected (see Table 1). This accounts for 30% of the total number of metabolites that were listed using the annotated genome of the bacterium [22].

**Table 1.** Identified metabolites of *S. melliferum*

| No. | Peak intensity | Compound | Compound ID | *m/z*, positive | Δppm | *m/z*, negative | Δppm |
|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | 1.62E + 04 | serine | C00065 | | | 104.0352 | 2 |
| 2 | 1.69E + 04 | threonine | C00188 | | | 120.0660 | 3 |
| 3 | 4.13E + 05 | valine | C00183 | 118.0863 | 1 | 116.0715 | 1 |
| 4 | 2.26E + 03 | asparagine | C00152 | | | 131.0457 | 3 |
| 5 | 2.25E + 03 | glutamine | C00064 | 147.0760 | 2 | | |
| 6 | 2.63E + 05 | lysine | C00047 | 147.1133 | 3 | 145.0980 | 1 |
| 7 | 4.44E + 05 | leucine | C00123 | 132.1016 | 1 | 130.0870 | 1 |
| 8 | 4.44E + 05 | isoleucine | C00407 | 132.1016 | 1 | 130.0870 | 1 |
| 9 | 4.26E + 03 | histidine | C00135 | 156.0769 | 1 | 154.0625 | 1 |
| 10 | 6.89E + 03 | methionine | C00073 | 150.0580 | 2 | 148.0432 | 3 |
| 11 | 1.06E + 04 | proline | C00148 | 116.0708 | 2 | 114.0561 | 1 |
| 12 | 2.33E + 04 | tryptophan | C00078 | 205.0965 | 2 | 203.0820 | 2 |
| 13 | 1.69E + 05 | phenylalanine | C00079 | 166.0855 | 3 | 164.0720 | 1 |
| 14 | 5.65E + 04 | tyrosine | C00082 | 182.0808 | 2 | 180.0660 | 3 |
| 15 | 7.12E + 03 | glutamate | C00025 | 148.0600 | 2 | 146.0452 | 3 |
| 16 | 3.20E + 03 | cysteine | C00097 | 122.0271 | 1 | | |
| 17 | 3.59E + 03 | aspartate | C00049 | | | 132.0297 | 2 |
| 18 | 1.33E + 04 | 5-methylthioadenosine | C00170 | 298.0962 | 1 | | |
| 19 | 3.65E + 04 | 5-methylthio-D-ribose | C03089 | 181.0500 | 2 | 179.0384 | 2 |
| 20 | 2.50E + 03 | phenyl pyruvate | C00166 | 165.0550 | 2 | | |
| 21 | 2.19E + 03 | N-carboyl putrescine | C00436 | 132.1130 | 1 | | |
| 22 | 4.07E + 02 | 2-oxoglutarate | C00026 | 147.0290 | 1 | 145.0148 | 2 |
| 23 | 1.36E + 05 | ornithine | C00077 | 133.0970 | 1 | 131.0824 | 1 |
| 24 | 9.06E + 04 | creatine | C00300 | 132.0766 | 1 | | |
| 25 | 1.25E + 05 | citrulline | C00327 | 176.1026 | 1 | 174.0879 | 2 |
| 26 | 4.13E + 05 | betaine | C00719 | 118.0863 | 1 | 116.0715 | 2 |
| 27 | 3.25E + 04 | creatinine | C00791 | 114.0663 | 1 | | |
| 28 | 1.14E + 04 | 2-keto-isovalerate | C00141 | 117.0550 | 3 | 115.0409 | 2 |
| 29 | 1.84E + 03 | mercaptopyruvate | C00957 | 120.9940 | 2 | 118.9805 | 3 |
| 30 | 3.83E + 02 | D-4-hydroxy-2-oxoglutarate | C05946 | 163.0225 | 2 | 161.0100 | 3 |
| 31 | 3.06E + 03 | xanthine | C00385 | | | 151.0262 | 2 |

**Table 1** (Contd.)

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 32 | 2.62E + 04 | uracil | C00106 | | | 111.0210 | 3 |
| 33 | 1.28E + 05 | adenine | C00147 | 136.0616 | 1 | 134.0467 | 2 |
| 34 | 7.04E + 03 | guanine | C00242 | 152.0562 | 2 | 150.0414 | 4 |
| 35 | 1.71E + 03 | hypoxanthine | C00262 | 137.0463 | 3 | 135.0315 | 1 |
| 36 | 3.21E + 03 | adenosine | C00212 | 268.104 | 1 | 266.0874 | 1 |
| 37 | 2.59E + 03 | cytosine | C00380 | 112.0508 | 3 | | |
| 38 | 2.72E + 03 | thymidine | C00214 | | | 241.0822 | 3 |
| 39 | 1.11E + 03 | xanthosine | C01762 | | | 283.0676 | 1 |
| 40 | 3.21E + 03 | deoxyguanosine | C00330 | 268.1042 | 1 | | |
| 41 | 1.13E + 03 | deoxyadenosine | C00559 | 252.1091 | 1 | | |
| 42 | 3.86E + 03 | AMP | C00020 | 348.0690 | 2 | 346.0547 | 3 |
| 43 | 1.49E + 04 | ADP | C00008 | 428.0350 | 3 | 426.0225 | 1 |
| 44 | 1.06E + 03 | CMP | C00055 | 324.059 | 2 | 322.0450 | 1 |
| 45 | 2.62E + 03 | UMP | C00105 | 325.0415 | 3 | 323.0274 | 2 |
| 46 | 2.16E + 03 | GMP | C00144 | 364.066 | 1 | 362.0501 | 2 |
| 47 | 7.01E + 02 | dAMP | C00360 | 332.0740 | 3 | 330.0599 | 3 |
| 48 | 5.09E + 02 | ITP | C00081 | 508.9880 | 2 | 506.975 | 2 |
| 49 | 1.03E + 04 | IDP | C00104 | 429.0213 | 1 | 427.0100 | 3 |
| 50 | 9.71E + 02 | UDP | C00015 | 405.0099 | 1 | 402.9938 | 2 |
| 51 | 7.24E + 02 | dCMP | C00239 | 308.0635 | 2 | 306.0485 | 3 |
| 52 | 1.72E + 03 | uridine | C00299 | 245.0760 | 3 | 243.0620 | 3 |
| 53 | 2.90E + 04 | lactose/α-α-trehalose | C00243/C01083 | | | 341.1079 | 2 |
| 54 | 1.5E + 03 | lactate | C00186 | | | 89.0245 | 1 |
| 55 | 3.38E + 04 | fructose 1-phosphate | C05345 | 261.0360 | 2 | 259.0215 | 3 |
| 56 | 2.66E + 03 | fructose 1,6-bisphosphate | C05378 | 341.0040 | 1 | 338.9901 | 1 |
| 57 | 1.23E + 03 | mannitol 1-phosphate/sorbitol 6-phosphate/galactinol 6-phosphate | C00644/C02888 | 263.0514 | 3 | | |
| 58 | 1.07E + 03 | glycerol | C00116 | 93.0549 | 1 | | |
| 59 | 7.09E + 03 | gycerol-3-phosphate | C00093 | | | 171.0060 | 2 |
| 60 | 8.48E + 02 | sn-glycero-3-phosphocholine | C00670 | 258.1091 | 2 | 256.0960 | 2 |
| 61 | 6.22E + 03 | D-glyceraldehyde 3-phosphate/ dihydroxyacetone phosphate | C00118/C00111 | 171.0050 | 1 | 168.9899 | 3 |
| 62 | 9.47E + 02 | D-galactosamine 6-phosphate/ D-glucosamine 6-phosphate | C06377 | 260.0530 | 1 | | |

**Table 1** (Contd.)

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 63 | 3.73E + 03 | bisphosphate | C00013 | 178.9510 | 1 | 176.9381 | 2 |
| 64 | 1.30E + 03 | 5-phospho-α-D-ribose 1-bisphosphate | C00119 | 390.9592 | 1 | 388.943 | 3 |
| 65 | 6.99E + 04 | glucosamine/galactosamine | C00329 | 180.0872 | 1 | | |
| 66 | 4.00E + 03 | 6-deoxygalactose | C01019 | 165.0758 | 1 | 163.0610 | 1 |
| 67 | 1.40E + 03 | $NAD^+$ | C00003 | 664.1145 | 2 | 663.0835 | 3 |
| 68 | 1.50E + 03 | FAD | C00016 | 786.1635 | 1 | 744.1477 | 2 |
| 69 | 2.76E + 03 | deamino-$NAD^+$ | C00857 | 666.1120 | 2 | 664.0968 | 3 |
| 70 | 8.55E + 04 | nicotinamide | C00153 | 123.0549 | 3 | | |
| 71 | 2.60E + 03 | (R)-4-phosphopantotheinocysteine | C04352 | 403.0933 | 1 | | |
| 72 | 1.83E + 04 | 1-hydroxy-2-methyl-2-butenyl 4-bisphosphate | C11811 | 263.0071 | 1 | 260.9942 | 2 |
| 73 | 3.76E + 04 | L-erythro-4-hydroxyglutamate | C05947 | 164.0550 | 3 | 162.0401 | 3 |
| 74 | 1.69E + 03 | 2-metylthioerythritol 4-phosphate | C11434 | 217.0475 | 1 | | |
| 75 | 8.31E + 02 | D-4-phosphopantothenate | C03492 | 300.0845 | 1 | 298.0710 | 3 |
| 76 | 1.71E + 04 | pantothein | C00831 | 279.1370 | 1 | | |

Fragmentation spectra were obtained for most of the metabolites by means of target mass spectroscopy. The fragmentation spectra of the detected metabolites were compared with standard spectra of the fragmentation of such compounds, which were obtained under similar ionization conditions and were provided by the Metlin database [30] (polarity of ionization and collision energy values were taken into account). Figure 2 shows an example of a confirmation of the identification of a metabolite (guanosine monophosphate, GMP, as an example). Interestingly, all the fragmentation spectra confirmed the primary identification of the compounds made by means of the *m/z* value alone. There were no compounds whose fragmentation spectra contradicted the identification made by *m/z* values alone provided that *m/z* values did not deviate from the theoretical value by more than 2 ppm. Thus, we have shown high precision mass determination with the TOF mass spectrometer, i.e. Q-TOF provides qualitative identification of metabolites even without obtaining their MS/MS spectra (see Table 2).

In accordance with analysis of the metabolome map, the metabolic pathways were reconstructed (using data from the KEGG database [25]) with all the identified metabolites. Identified metabolites and annotated proteins of *S. melliferum* are shown in the maps. Purine and pyrimidine metabolism maps were the most uniformly and densely filled with identified tagged metabolites (Figs. 3 and 4, respectively). Proteins were annotated that are functionally associated with the synthesis and metabolism of purines and pyrimidines expressed in *S. melliferum* according to our previous study [22]. Detection of the nitrogenous bases adenine, guanine, xanthine, hypoxanthine, uracil, and their mono-, di- and deoxyribonucleotides is in agreement with the proteomic data.

For amino acids, metabolites of the biosynthesis of arginine and proline were detected. Also found were metabolites of aspartate, alanine, and glutamate, as well as the metabolism of fructose.

There are many nutrient carriers in the *S. melliferum* proteome, so the description of membrane fluxes and the discovery of exogenous metabolites via membrane transport pathways is an important question in metabolome research. Sugars are the most important energy source for bacteria. Sugar importing pathways always involve a sugar phosphorylation step. Carbohydrates and their derivatives were detected among *S. melliferum* metabolites, including ascorbic acid, fructose 1,6-bisphosphate, sorbitol-6-phosphate, and fucose 6-phosphate. Detection of these metabolites is in agreement with protein-genomic annotation for *S. melliferum* [22], where the sugar transporter protein group was present. As expected, free forms of fructose and glucose were not found in the metabolome of *S. melliferum*.

As a result of our investigation, we detected 17 of the 20 amino acids in *S. melliferum* cells. Their presence in the metabolome can be attributed to their exogenous

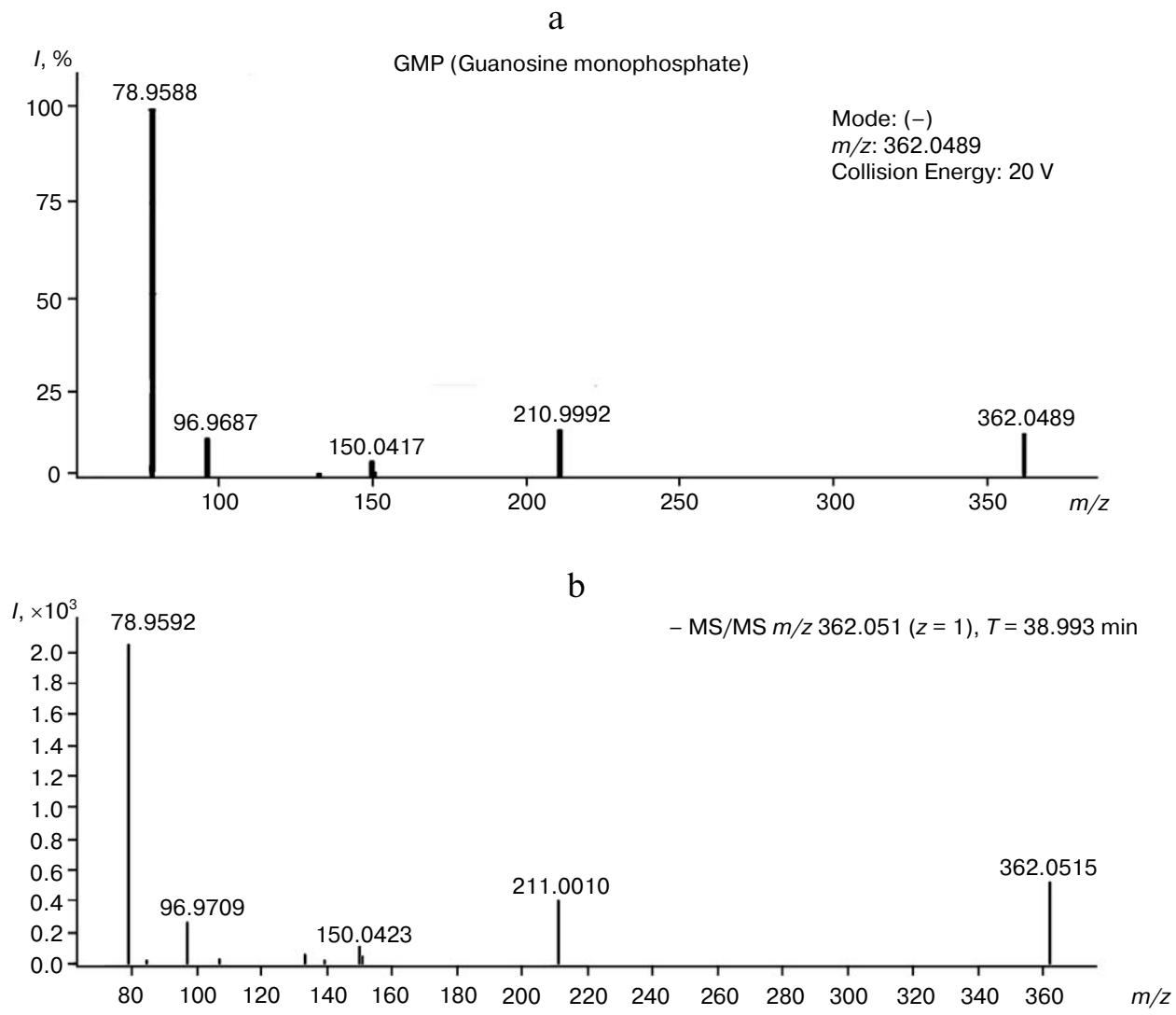


**Fig. 2.** a) Standard GMP fragmentation spectrum in negative ionization mode, collision energy 20 eV from Metlin Metabolites database [30]; b) GMP fragmentation spectrum obtained for the *S. melliferum* sample: $m/z$ = 362.05069, Δ = 2.099 ppm.

nature and active import into the cells through specific transporters (permeases), since no amino acid synthesis enzymes there have been annotated in this organism [22]. The peaks in the MS spectra that corresponded to amino acids were characterized by the highest in intensity (intensity values up to $10^5$ units). Glycine, alanine, and arginine were not detected. However, in similar experiments for the metabolome of the bacterial species *Acholeplasma laidlawii,* we were able to detect all 20 amino acids (data not shown). This suggests that the concentration of the three amino acids, which were not found in *S. melliferum*, was significantly lower than concentration of the seventeen detected amino acids.

Furthermore, the data characterize ATP transformation in the cell. Adenosine monophosphate, adenosine diphosphate, and pyrophosphate were detected in free form, while adenosine triphosphate (ATP) was not detected among the metabolites of *S. melliferum*. This indicates that ATP could actively bind to ABC-transport proteins, which are represented in the proteome of the bacteria [6] and which cause a rapid decay of ATP into mono- and diphosphates of adenosine and free pyrophosphate during energy consumption by cellular processes.

Glycolysis is the central metabolic pathway in Mollicutes. Metabolites that are formed during glycolysis serve as precursors for many other metabolic pathways. Detection of glycolysis intermediates is a difficult task because glycolytic intermediates are quickly drawn into metabolic reactions due to the fact that the genes of glycolytic enzymes have a high level of expression. We were able to detect some metabolites of glycolysis – fructose 1,6-bisphosphate, fucose 6-phosphate, glyceraldehyde-3-

**Table 2.** Fragmentation spectrum results for detected metabolites of *S. melliferum* and fragmentation spectra the analyzed ion standards from the Metlin database [30] for the experimental conditions

| Compound | Ionization mode | Parent mass (*m/z*) | Product mass (*m/z*) from Meltin database | Found product mass (*m/z*) | Collision energy, eV |
|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 |
| Adenine | + | 136.0575 | 136.062; 119.035; 94.040 | 136.0575; 119.030; 94.034 | 20 |
| Hypoxanthine | – | 135.0296 | 92.025; 65.015; 66.0097 | 92.0305; 65.0187; 66.015 | 20 |
| Uracil | – | 111.0209 | 41.998 | 41.999 | 20 |
| Guanine | – | 152.0560 | 152.056; 135.030; 110.035; 82.041 | 152.056; 135.030; 110.0361; 82.038 | 20 |
| GMP | – | 362.0509 | 78.959; 96.968; 150.042; 210.999 | 78.959; 96.970; 150.042; 211.001 | 20 |
| AMP | – | 346.0558 | 134.047; 96.969; 78.959 | 134.0476; 96.9698; 78.9598 | 20 |
| ADP | – | 426.0220 | 328.045; 276.952; 158.924; 134.046; 78.959 | 328.0505; 276.952; 158.9235; 134.0475; 78.9549 | 20 |
| UDP | – | 403.0000 | 323.028; 111.020; 96.969; 78.959 | 323.028; 111.020; 96.969; 78.959 | 20 |
| CMP | – | 322.0447 | 110.033; 96.969; 78.958 | 110.025; 96.960; 78.950 | 20 |
| Glutamate | + | 147.074 | 84.045; 56.0502; 41.039 | 84.0445; 56.0488 | 20 |
| Tryptophan | – | 203.0824 | | 142.071; 116.055; 74.0307 | 20 |
| Asparagine | – | 131.0460 | 41.999; 72.009; 70.0304; 58.030 | 42.003; 70.0322; 58.0368 | 20 |
| Leucine | + | 132.1019 | 86.097; 44.050; 43.055; 30.034 | 86.09499; 44.050; 43.055; 30.034 | 20 |
| Isoleucine | + | 132.1019 | 86.097; 57.058; 44.050; 43.055; 30.034 | 86.0965; 57.059; 44.051; 43.056; 30.0345 | 20 |
| Cysteine | + | 122.0256 | 58.996 | 58.996 | 20 |
| Threonine | – | 118.0507 | 74.059; 57.035; 56.050 | 74.0601; 56.0498 | 20 |
| Proline | + | 116.0710 | 70.066 | 70.064 | 20 |
| Phenylalanine | + | 166.0859 | 120.081; 103.054 | 120.0801; 103.0539 | 20 |
| Histidine | + | 156.0761 | 110.071; 95.061; 93.045; 83.061 | 110.0668; 95.0575; 81.046 | 20 |
| Lysine | + | 147.1121 | 84.081 | 84.0766 | 20 |
| Serine | – | 104.0357 | 74.025 | 74.0272 | 20 |
| Valine | + | 118.0853 | 72.0815; 57.0576; 56.0503; 55.0547 | 72.0805; 57.0531; 55.0549 | 20 |
| Lactate | – | 89.0251 | 43.019; 41.0017 | 43.0151 | 20 |
| D-Glyceraldehyde 3-phosphate/ dihydroxyacetone phosphate | – | 168.9902 | 96.969; 78.959 | 96.974; 78.964 | 20 |

**Table 2** (Contd.)

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| Lactose | – | 341.1094 | 89.0239; 59.0144; 71.0138; 101.024; 113.024; 119.034 | 89.0239; 59.0144; 71.0138; 101.024; 113.024; 119.034 | 20 |
| Trehalose | – | 341.1094 | 89.0239; 59.0144; 71.0138; 101.024; 113.024; 119.034 | 89.0239; 59.0144; 71.0138; 101.024; 113.024; 119.034 | 20 |
| Aspartate | – | 132.0301 | 115.003; 88.0401; 72.008; 71.014; 59.0133; 42.0328 | 115.011; 88.0454; 72.032; 71.014; 59.0187; 42.0384 | 20 |
| $NAD^+$ | + | 665.1149 | 542.062; 524.060; 428.035; 323.084; 136.061 | 542.061; 524.059; 428.0389; 232.084; 136.061 | 20 |
| Tyrosine | – | 180.06653 | 72.0093; 93.0343; 119.0500; 163.038; 74.0253 | 72.010; 119.0516; 163.0389 | 20 |
| UMP | – | 323.0290 | 211.001; 150.980; 138.980; 111.0200; 96.969; 78.959; 192.990 | 211.0014; 193.000; 150.913; 138.980; 111.027; 96.972; 78.959 | 20 |

phosphate, and lactate. Lactate is an indication of a transition point from glycolytic metabolism to propionate metabolism.

The intermediates of terpenoid biosynthesis form an important group of metabolites that are characteristic for *S. melliferum*. α-Hydroxy-2-methyl-2-butenyl-4-diphosphate and methyl-erythritol-4-phosphate, which are involved in the biosynthesis of terpenoids in the synthesis of pyrophosphate iso-pentenyl, were detected. Also detected was nicotinamide, a product of the synthesis of NADH among the metabolites of *S. melliferum*.

## DISCUSSION

The technique used for detection of metabolites in this work was hydrophilic interaction liquid chromatography (HILIC). This is a method of chromatographic separation of polar compounds where a polar phase used as a separating phase of the analytical column and a gradient from nonpolar to polar liquids is used for elution (where water or buffer solutions serve as a polar component). Hydrophilic chromatography is a very productive method for the separation of polar water-soluble substances [9]. Two types of analytical columns were used with silica and amino stationary phase where ions were subsequent detected in positive and negative ionization modes (for each column).

It was possible to detect 40 metabolites when amino propyl silica gel was used as the separating phase, and 24 metabolites were detected in positive ionization mode and more than 26 metabolites were detected in negative ionization mode. Eleven metabolites were detected in both positive and negative ionization mode, eight of which were amino acids having bipolar properties; two of these were adenine and guanine, which are prone to form negatively and positively charged ions in both ionization modes, and deoxygalactose was also detected in negative and positive ionization modes (Table 2).

Hydroxyl or carboxyl group-containing metabolites can release a proton and tend to form negative ions, so they are tested in the negatively charged form. The main of this kind of metabolites includes: 1) carbohydrates; 2) some amino acids (threonine, serine, tyrosine, aspartate, and glutamate) which are characterized by the presence of an additional oxygen atom (excluding the characteristic group $NH_2$-(CHR)-COOH) within carboxyl groups; 3) nucleosides and nucleotides; 4) phosphate-containing compounds.

The following metabolites were detected in the negative ionization mode: nitrogenous bases uracil and xanthine, nucleotides thymidine and xanthosine, which have several oxygen atoms in the ring; four amino acids (threonine, aspartate, serine and asparagine), and also some carbohydrates. Some of the carbohydrates are isomers and have similar *m/z* values for the precursor ions and for its fragments, which makes it impossible to distinguish between them (lactose/trehalose).

Seventeen compounds of *S. melliferum* were detected in positive ionization mode (see Table 1). This group of metabolites includes compounds that contain two or more amino groups. There are several metabolites that are prone to the formation of negative ions, which were, however, detected in the positive ionization mode due to their protonation (glycerol, mannitol phosphate/sorbitol phosphate/galactinol phosphate (isomers, which cannot be distinguished by MS or MS/MS analysis), methyl-erythritol phosphate, and phenyl pyruvate).

Among the 40 amino acids detected using the metabolite amino column, there were 20 amino acids that

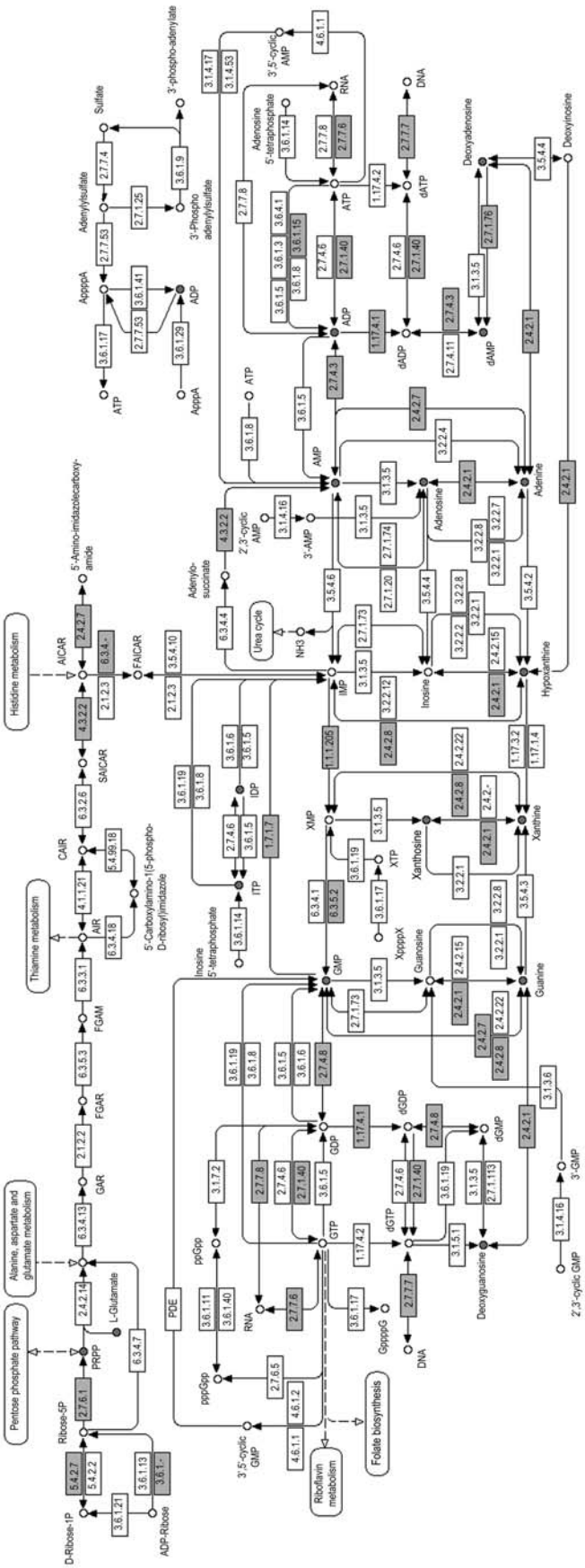


**Fig. 3.** Reconstruction of metabolism map of purines based on the KEGG scheme [25]. The identified metabolites and the annotated proteins of *S. melliferum* [22] are shown in gray (circles and rectangles, respectively).

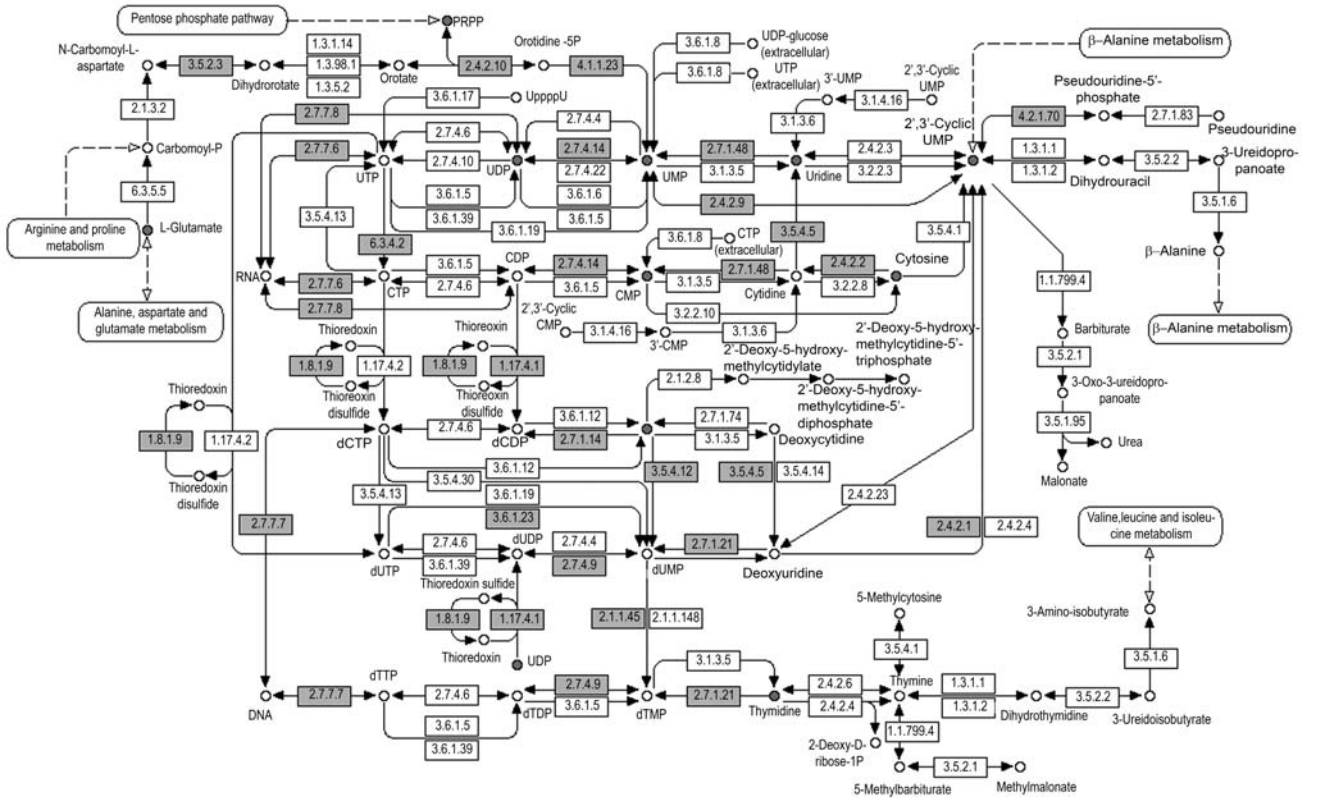


**Fig. 4.** Reconstruction of metabolism map of pyrimidines based on the KEGG scheme [25]. The identified metabolites and the annotated proteins of *S. melliferum* [22] are shown in gray (circles and rectangles, respectively).

have been previously described by the group of Rabinovitz [9], where the metabolites of *E. coli* were analyzed by a method similar to that we used in our work. We also found a few metabolites (they were detected in our work with high intensity peaks using amino column – xanthine, phenyl pyruvate, and glycerol-3-phosphate) that were not detected by the authors of [9]. This is probably due to the fact that we used high-precision quadrupole TOF mass spectrometer of high resolution (Q-TOF Agilent 6520 series) for MS spectral recording, which has higher sensitivity compared to the triple quadrupole mass spectrometer QQQ used by Rabinovitz's group.

The analytical column based on silica gel with deactivated surface was used in this study, allowing detection of 60 metabolites, where 40 of these metabolites were not detected when the amino column was used. Obviously, it is the application of both columns that reveals the most complete set of data when it is used for identification of the metabolites of *S. melliferum*.

There were some metabolites that were not detected in our experiment but were present in the list of metabolites based on the bacterial proteome [22]. However, it was possible to detect most of those metabolites using this method, as we have shown in metabolome studies of other bacteria (data not shown). Therefore, we assume that these metabolites were not detected in *S. melliferum* cells because their content was not sufficient for detection by our method. This could also be due to the rapid utilization of these metabolites because of their active involvement in metabolic processes. There are data suggesting the possible loss of intracellular metabolites during sample preparation, i.e. during the cold methanol extraction procedure. It was shown in the cold methanol extraction of metabolites of *S. cerevisiae* that metabolites like organic acids, *cis*-aconitate and phosphoenolpyruvate were lost, while pyruvate, sugar phosphates, and nucleotides were fully extracted [31]. There were 17 metabolites that we were not able to detect in any of our experiments. Among these metabolites some are unstable [31] (erythrosine phosphate, phosphoglycerate, tetrahydrofolate and formyltetrahydrofolate, inosine monophosphate). Due to the presence of acetate ions in the elution buffer A, it was not possible to detect acetate from the cell sample. Acetyl-coenzyme A is a major intermediate of many metabolic pathways, which explains its low contents in the bacterial cells. There were also metabolites that were not detected although they are precursors for synthesis of the most important compounds of the cell. These are nicotinate ribonucleotide, ribosyl nicotinamide, deoxyribose phosphate, deoxyuridine base, and cytidine, inosine

monophosphate, and the amino acid alanine. Possibly, guanosine, cytidine, and deoxyadenosine diphosphates could not be analyzed by our approach because they are lost during the sample preparation.

Most of the metabolites were detected in both positive and negative ionization in conjugation with each of the chromatography columns (aminopropyl silica gel and silica gel) (see Table 1). This could be explained by the fact that most of the metabolites form bipolar ions (amino acids, amino sugars, nucleosides and its derivatives), or it could be due to the affinity of some ions to the separation phase, which is caused by ionic interactions or by hydrogen bond formation or induced interactions. The use of water as a main component of one of the elution systems leads to its irreversible adsorption on the surface of silica gel, which creates favorable conditions for analysis of polar compounds, such as acidic and basic compounds, which includes a large number of metabolites (nucleotides and some amino acid derivatives, etc.).

Two different analytic columns were used due to the fact that the amino column allows separation and identification of such metabolites as low molecular weight amino acids (glycine, lysine, and serine), carbohydrates (glucosamine, fructose, and its derivatives), and these are more difficult to analyze using silica gel as the separating phase. On the other hand, analytical columns with silica gel with deactivated surface of the separating phase, small specific area, and decreased metal content in the matrix reveal polar compounds with an improved shape of the corresponding peaks. It also allows detection of some metabolites that were not detected by the amino column (for example, threonine and proline).

The negative ionization mode allowed us to detect phosphate-containing compounds (for example, nucleotides, derivates of phosphorylated sugars, etc.) that are prone to formation of negatively charged ions, while the positive ionization mode is a favorable method for detection of amino group-containing metabolites (amino acids, amino sugars, nitrogenous bases, etc.). Application of both analytical columns and both ionization modes make the identification of metabolites reliable and simple.

Thus, the combination of HPLC with a quadrupole TOF mass detector comprised of chromatographic separation using deactivated silica gel or aminopropyl silica gel and recording of MS spectra in positive or negative ionization modes makes it possible to detect amino acids, nucleotides, nucleosides, and nitrogenous bases, as well as sugar derivatives, amino sugars, organic acids, and some other classes of compounds.

The identified metabolites are highly conserved and widely represented among the intracellular metabolites of *S. melliferum*. The identification of these metabolites characterizes the major metabolic pathways of *S. melliferum* and opens great perspectives for further studies of intracellular regulation of the Mollicutes class of bacteria under the influence of various factors. Metabolic correlations provide a valuable insight into fine organization of intracellular activity and indicate changes in gene expression levels and mRNA transcription.

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