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EXPERIMENTAL ARTICLES

Characterization of Propionic Acid Bacteria Using Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry

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Abstract—It was shown that matrix-assisted laser desorption/ionization (MALDI) mass spectrometry could be used for the diagnostic characterization of propionic acid bacteria (PABs). The spectra of proteins (whole PAB cells) with a molecular mass of 3000 to 11 000 were obtained and analyzed using three matrices: sinapinic (SA), 2,5-dihydroxibenzoic (DHB), and α-cyano-4-hydroxycinnamic acid (HCCA). The MALDI spectra of PAB revealed the protein peaks characteristic of (1) the genus *Propionibacterium* (3496, 5386, 5605, 10 470), (2) the groups of species sharing the common composition of their cell walls and fatty acids, and (3) a species (four species were investigated). Exemplified by the *P. shermanii* strains (the collection and mutant ones) producing and not producing vitamin B_{12}, the possibility of using MALDI profiles for strain differentiation was confirmed. The MALDI profiles of the propionic acid cocci of the genus *Luteococcus* differ substantially from the profiles of PAB strains of the genus *Propionibacterium*, which is an additional proof of the validity of whole-cell MALDI spectra for generic differentiation of bacteria. Our investigation shows that the bacterial groups determined using the MALDI profiles correlate with the phylogenetic 16S rRNA gene groups, thus demonstrating the high resolution of this method for the differentiation of intraspecific differences (subspecies, strains).

Keywords: propionic acid bacteria, cocci, MALDI spectra, MALDI–TOF matrices, proteins, biomarker molecules.

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Mass spectrometry (MS) of the fingerprint type was first used for identification of bacteria in 1975 [1]. MS is based on generating ions from the molecules of the studied substances and their separation in electric and/or magnetic field according to the ratio of the ion mass to its charge. Time-of-flight (TOF) mass analyzers and matrix-assisted laser desorption/ionization (MALDI–TOF) MS have become the most common techniques for the identification of biological molecules. The first report about the use of the MALDI spectra for analysis of whole microbial cells was published in 1996 [2–4].

The MALDI technique is regarded as supplementary to the biochemical and morphological methods of indication and identification of microorganisms [5]. It differs advantageously from other methods for microbial diagnostics in the simplicity of sample preparation, the possibility of using whole bacterial, fungal, or yeast cells, the high speed of analysis [3, 5, 6], and good reproducibility [7].

The MALDI-based characterization of whole microbial cells is based on obtaining the fingerprints of biomarker molecules, including proteins, peptides, and oligonucleotides, and comparing the results with the databases [8].

This method makes it possible to detect antibiotic resistance, posttranslational modifications and unmodified native versions of the same proteins, the presence of virulence factors, medically important biomarker molecules, etc. [5].

The most common and generally accepted methods for identification of bacteria are based on DNA and RNA analysis using the PCR for amplification of the nucleotide sequences of the marker genes. While the 16S rRNA gene sequence is widely used for species identification due to its conservative nature, it is not applicable to differentiation of strains and intraspecific phase variants, which is especially important for medicine and biotechnology [5]. In contrast to techniques based on analysis of nucleic acids, mass spectroscopy makes it possible to detect proteins produced by a certain strain or variant directly, for example, the proteins characteristic of a virulent variant.

It was shown [9] that MALDI–TOF permits the differentiation of the intraspecific differences. For example the similarity between 16S rRNA gene

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sequences of the members of *Arthrobacter globiformis* was 99–100%, whereas their MALDI profiles had only a 60–95% similarity [6]. Good MALDI-based differentiation was obtained for a number of *Bacillus* strains [10] and mycobacteria [11], as well as for the indication of environmental samples [12]. Importantly, the MALDI-based identification of microorganisms is presently limited by the possibilities provided by the libraries of available spectra and primarily deals with a small group of cultivated clinical bacterial species [9].

In the present work, propionic acid bacteria (PAB), which have diverse practical applications [13], were selected as the objects of study of the specificity of their MALDI spectra. These organisms are used in cheese-making; the production of vitamin B_{12}, porphyrins, therapeutic and preventive preparations and probiotics for animals; in fodder ensilaging; etc. Skin PAB may cause certain diseases. Among PAB some hemolytic strains exist [13]. Mutated vitamin B_{12} overproducers of and mutants that do not synthesize corrinoids have been obtained. In connection with the variegated application of PAB in different fields of the national economy and medicine, it is important to have a reliable and time-saving technique for the identification of both productive strains and opportunistic pathogens. The MALDI–TOP MS meets these requirements.

The goal of the present work was to perform a comparative analysis of the protein spectra of propionic acid bacteria using whole-cell MALDI-TOF MS for their additional differentiation at the species and strain level.

MATERIALS AND METHODS

The objects of study. The following PAB strains were investigated: *Propionibacterium freudenreichii* KM 133, *P. jensenii* CCM 1864, *P. thoenii* CCM 1865, and *P. acidipropionici* CCM 1859 obtained from the Czechoslovak Collection of Microorganisms; *P. freudenreichii* subsp. *shermanii* VKM 2092; mutants: the vitamin B_{12} overproducer *P. shermanii* 1-63 and the zero mutant *P. shermanii* 0 not synthesizing the corrinoids obtained from the collection of microorganisms of the Bakh Institute of Biochemistry, Russian Academy of Sciences; and the strain *Luteococcus japonicus* subsp. *casei* we isolated from cheese and forming an exogenous reactivating factor (RF).

Cultivation. Bacteria were grown in the medium containing the following (%): glucose, 1; yeast extract, 1; peptone, 0.5; and tap water; pH 6.8–7.0. The bacteria were cultivated at 37° C for 48 h under static conditions. The solid medium of the same composition (with 1.5% agar) was inoculated with bacteria from submerged cultures; the petri dishes were incubated in anaerobic jars (Genbox 19124, Biomerjaux, France) for 8 days.

Acquisition of the mass spectra of whole cells. Sample preparation was carried out as follows [14]. A single colony was picked from a dish with a microbiological loop; the cells were resuspended in 500 μl of distilled water in a microcentrifuge test tube, mixed thoroughly for 1 min, and then centrifuged (5 min at 6000 *g*); and the pellet was washed twice with distilled water. The pellet was then resuspended in 150 μl of freshly prepared 50% aqueous acetonitrile solution containing 0.1% of trifluoroacetic acid; the suspension was mixed thoroughly.

In order to destroy the bacterial cell walls, the cell suspension in the acetonitrile solution was exposed to ultrasound for 20 min at 37°C on an UZV ultrasonic unit (PKF, Sapfir 187, Russia) at a working frequency of 35 kHz. In order to avoid cell sedimentation, the suspension was thoroughly mixed.

Sinapinic (SA), 2,5-dihydroxybenzoic (DHB), and α-cyano-4-hydroxycinnamic (HCCA) acids were used as matrices [14]. For analysis, the cell suspension in the matrix (1 : 1) was applied dropwise with an automatic dispenser onto a steel target plate and air-dried.

The protein spectra were recorded in the delayed ion extraction mode using an Autoflex II device (Bruker, Germany) equipped with a nitrogen laser with a working wavelength of 337 nm with a reflectron time-of-flight analyzer. The accelerating voltage was 20 kV. The range of recorded masses was 2–20 kDa in the positive ion mode. The resultant spectrum was obtained by summing up the spectra obtained at several sample points with 50 laser impacts.

The bacterial species were compared using the BioNumerics software package, version 6.5 (Applied Maths, United States). The matrix with the MALDI data was transposed, and the data were converted to binary form. The data were entered into the program as a text file, and each m/z value was entered as an individual performance experiment. The binary comparison was made using Jacquard's correlation, and the analysis results were depicted by the program as a dendrogram.

RESULTS AND DISCUSSION

The spectrum quality depends on the nature of a matrix, the composition of a solvent, the mode of sample preparation and application to the underlying material, and the growth phase of microorganisms. In the present work, we observed the standard conditions of the preparation and carrying out of the experiment described in [14–16].

Three matrices most commonly employed in MALDI-TOF were used: 2,5-dihydroxybenzoic (DHB), sinapinic (SA), and α-cyano-4-hydroxycinnamic (HCCA) acids. When SA and HCCA were used as matrices, the differences in the sets of characteristic peaks of the propionic acid bacteria were small. They mainly concerned proteins with a high molecular mass and low relative intensity. Most peaks with identical

Table 1. The *m/z* and peak intensity values in the MALDI spectra of *P. shermanii* VKM 2092 with the use of sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid (HCCA) as matrices

Relative intensity, in % of the maximal ion			Intensity	
m/z	SA	HCCA	SA	HCCA
3176	8	7	128	89
3496	100	100	1627	1205
3532	–	16	–	188
3634	–	19	–	228
5238	6	24	99	285
5386	16	15	250	190
5601	10	12	156	152
7060	–	13	–	152
7270	64	73	1040	878
7303	–	6	–	75
7478	4	–	61	–
8407	–	7	–	89
9283	1	–	16	–
10470	10	13	257	156

Note: "–" stands for the absence of a protein peak.

m/z values were observed in the bacterial spectra with slight variations in the intensities, which is illustrated by the example of *P. shermanii* (Table 1). Figure 1 shows the spectra of *P. thoenii* and *P. acidipropionici* strains. Analysis of the spectra suggests that, with the use of HCCA, the spectral region of low-mass proteins was richer.

When bacterial cell proteins are analyzed, DHB is often used as a matrix, since it absorbs radiation well at 337 nm (the working wavelength of the nitrogen type of laser), has a low thermal desorption energy, and cocrystallizes well with intact proteins [5, 6]. In our experiments when we analyzed the MALDI spectra of *P. jensenii* cells, the use of DHB provided for higher resolution than the use of SA, which made it possible to record a comparatively larger number of biomolecules with low mass values (m/z). The dominant peaks with the use of DHB were the 2832, 3176, 4250, 6084, 6729, 7248, and 7267, and with the use of SA they were 3336, 3873, 4300, 6084, 6729, 7132, 7248, and 7266.

Table 2 shows the mass spectra of the most significant proteins in the spectra of different PAB revealed with the use of the SA-matrix. It was shown that all the strains contained proteins with molecular masses of 3496, 5386, 5605 (except *P. jensenii*), and 10 470 (except *P. freudenreichii* subsp. *shermanii* 1-63) as marker biomolecules. This set of peaks with the dominance of the protein with a molecular mass of 3496 seems to be characteristic of the genus *Propionibacterium*.

Based on the cell wall composition and the nature of fatty acids in the cytoplasmic membrane, the representatives of the genus *Propionibacterium* are divided into two groups [17]: (1) the group of *P. freudenreichii* that includes the closely related species *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* and (2) the group including the species *P. thoenii, P. jensenii*, and *P. acidipropionici*.

The species *P. freudenreichii* differs from the other species in its ability to ferment a limited number of carbohydrates, heat resistance, and high corrinoid content [18]. Apart from the genus-specific protein peaks, it is characterized by the peaks (on the SA matrix) 3176 and 4837 (except for strain 133). The similarity between their MALDI profiles reflects the close relationship between three strains of the species *P. freudenreichii* subsp. *shermanii*. The characteristic peaks for this subgroup (on the SA matrix) correspond to proteins with the molecular masses 4837 and 5883 Da. For the subspecies *P. freudenreichii* subsp. *freudenreichii*, the peaks of proteins with *m/z* 5112, 5356, 6729, and 7014, which were absent in the subspecies *P. shermanii*, differed. The traditional procedure for differentiation between these subspecies involves determination of their capacity for nitrate reduction (a positive reaction in *P. freudenreichii* and a negative reaction in *P. shermanii*) and lactose fermentation (a positive test for *P. shermanii* and a negative test for *P. freudenreichii*) [13].

For the second group of strains including *P. acidipropionici, P. thoenii*, and *P. jensenii*, proteins with *m/z* 7245 and 7267 absent in *P. shermanii* strains (group 1) are characteristic.

It should be noted that the species *P. thoenii* and *P. jensenii* have a high degree of DNA similarity (51–53%) [17] but differ in a number of phenotypic characteristics. Their MALDI spectra also differed in several differentiating proteins: in *P. jensenii*, those with *m/z* 3873, 4299, 6084, 6729, and 7132, which were not found in the spectra of the other strains of this group and, therefore, may be the markers for this species. In the *P. jensenii* strain, the peaks of proteins with *m/z* 3496 and 5605 characteristic of the genus were absent altogether or had a very low relative intensity, which could also be a feature of this species. As far as the set of proteins characteristic of the group (2) of PAB is concerned, *P. jensenii* was similar to both *P. thoenii* and *P. acidipropionici* (the peaks with *m/z* 7245 and 7267). *P. acidipropionici* also had a set of species-characteristic proteins with *m/z* 4299, 7376, and 7388.

The use of the HCCA matrix confirmed the presence of the proteins with a mass of 3491–3496, 7265–7270, and 10467–10470 in the MALDI spectra, which are characteristic of PAB at the generic level.

Thus, the bacterial MALDI spectra detected the proteins revealed at the generic level and characteristic of all the species of the genus *Propionibacterium,* a number of proteins characteristic of the groups of species (1) and (2), and of species and subspecies within

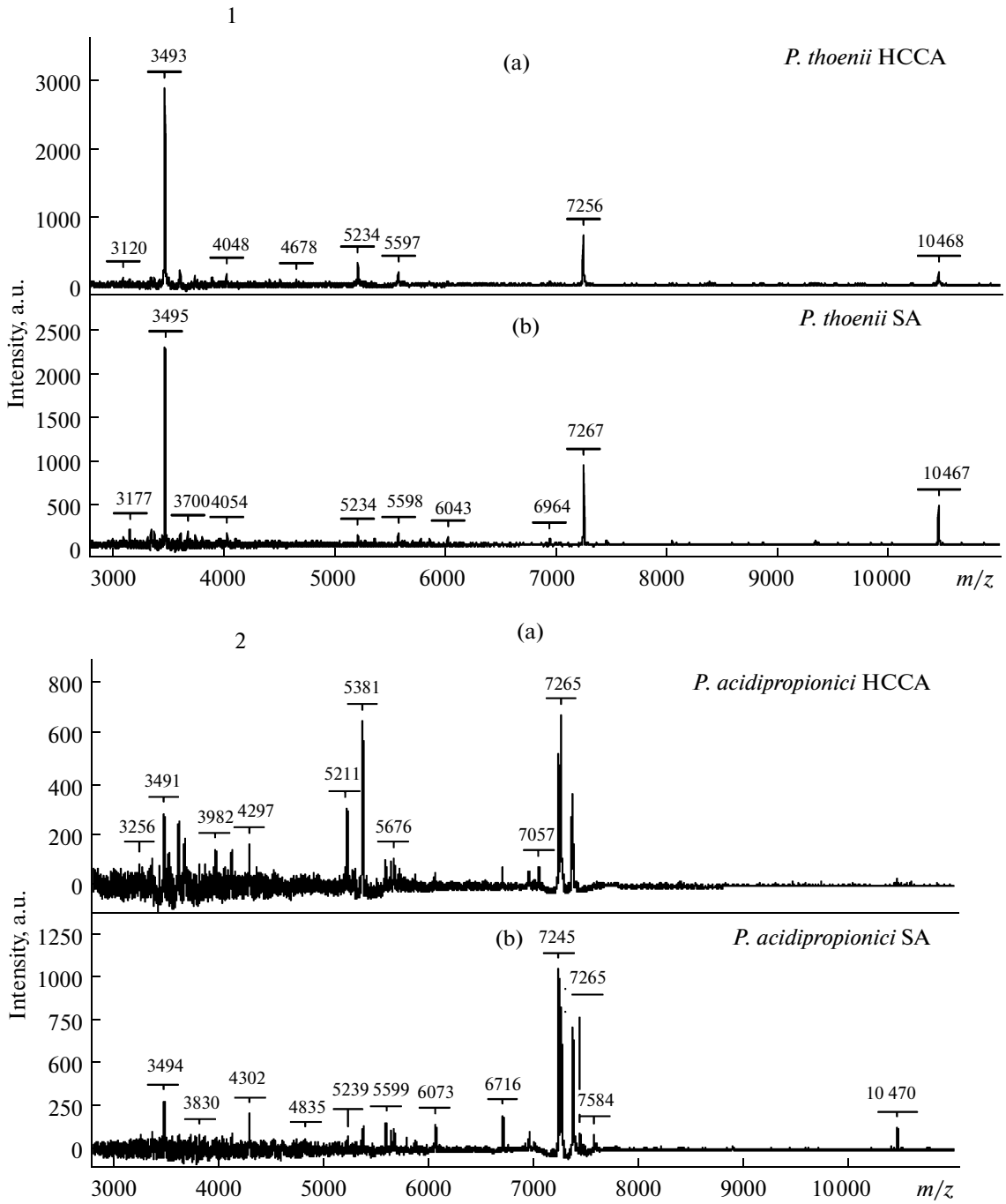

Fig. 1. Comparison between the MALDI–TOF spectra of *P. thoenii* (*1*) and *P. acidipropionici* (*2*) cells with the use of α-cyano-4-hydroxycinnamic acid (HCCA) (a) and sinapinic acid (SA) (b) as matrices.

these groups. Under the standard experimental conditions, the method was reproducible in relation to the set of proteins but not their amount (expressed by the intensity of the signals). In our investigations, the strains of the genus *Propionibacterium* differed in relation to the dominant proteins. The proteins with molecular masses of 3496, 5386, 5356, 4248, 6729, and 3496 Da exhibited 100% relative intensity in *P. shermanii* VKM and *P. shermanii* 0, *P. shermanii* 1-63, *P. freudenreichii*, *P. acidipropionici*, *P. jensenii,* and *P. thoenii*, respectively. Variations in the peak intensities are related to the fact that, first, even under controlled conditions, bacteria respond to the uncontrolled fluctuations in the growth conditions by changing the protein content [5] and, second, the standard conditions may vary in the degree of optimality for bacteria of different species. In addition, the presence of more than one protein, as well as overlapping with

Table 2. Mass spectra of the biomarkers of the representatives of the genus *Propionibacterium* with the use of sinapinic acid as a matrix

Relative intensity, in % of the maximal ion							
*m/z**	*P.* VKM	*P. shermanii*, "0"	*P. shermanii*, 1-63	*P. fråudenreichii* 133	*P. acidipropionici* 1859	*P. thoenii* 1865	*P. jensenii* 1864
3176	8	14	55	7	–	–	–
3496	**100**	**100**	68	8	21	**100**	–
3873	–	–	24	–	–	–	12
4299	–	–	–	–	16	–	16
4837	28	15	11	–	–	–	–
5112	–	–	–	43	–	–	–
5356	–	–	–	**100**	–	–	–
5386	15	14	**100**	10	11	2	1
5605	10	6	61	14	12	6	–
5883	12	–	32	–	–	2	–
6084	–	–	–	–	–	–	25
6729	–	–	–	9	–	–	**100**
7014	–	–	–	29	–	–	–
7132	–	–	–	–	–	–	10
7245	–	–	–	33	**100**	1	59
7267	–	–	–	11	79	37	8
7376	–	–	–	6	69	–	–
7388	–	–	–	19	33	–	–
10470	16	6	–	8	12	19	10

* The dominant peaks are bold-faced.

salt clusters or modificants formed when a protein loses small molecules (for example, H_2O), may be reflected in the peak areas. It is, therefore, assumed [5] that a characteristic set of proteins, rather than their amount (the intensity value), is of differentiating importance; thus, cell identification must be based on a group of characteristic proteins. We were guided by this principle when assessing the results obtained.

Figure 2 shows the dendrogram based on the analysis of the MALDI-TOF spectra of the experimental strains (Fig. 2a) and the phylogenetic tree constructed on the basis of the 16S rRNA gene sequences of the strains of the classical species of propionic acid bacteria (Fig. 2b). The main phylogenetic groups determined from the branching order are a large cluster including *P. acidipropionici, P. jensenii*, and *P. thoenii* with a 99% significance of branching and by a small cluster consisting of *P. freudenreichii* and *P. cyclohexanicum* with a 100% significance of branching, which is distinctly separated from the first cluster. Two similar groups were present in the MALDI dendrogram: *P. acidipropionici, P. jensenii* and *P. freudenreichii, P. shermanii.* In contrast to the phylogenetic tree, in which the group of *P. shermanii* is not designated, the MALDI dendrogram shows that the *P. freudenreichii* and *P. shermanii* strains represent closely related, but discernible, groups. The high-resolving capacities of the MALDI spectra are also confirmed by the special localization of the mutant strain *P. shermanii* 1-63 within the group of similar species. The positions of the *P. thoenii* strain in the dendrogram and the phylogram are different. In the dendrogram *P. thoenii* does belong to the first cluster and occupies a special position with the same level of similarity to both clusters. The latter finding requires the investigation of additional strains of this species.

It was established that the protein ions recorded by MALDI belonged mostly to the main medium-hydrophilic proteins, such as the ribosomal proteins of many bacteria [20]. Thus, the ribosomal proteins isolated from different *Escherichia coli* strains exhibited a significant taxonomic specificity. Many ribosomal proteins had the same molecular mass as those detected by the characteristic peaks in the whole-cell spectra in MALDI analysis. Moreover, it was shown that the well-detectable ribosomal proteins mask most of the other proteins in the spectrum with similar *m/z* values.

It is essential that, in many cases of bacterial identification [5], the MALDI profile-based groups correlate with the phylogenetic 16S rRNA groups, probably

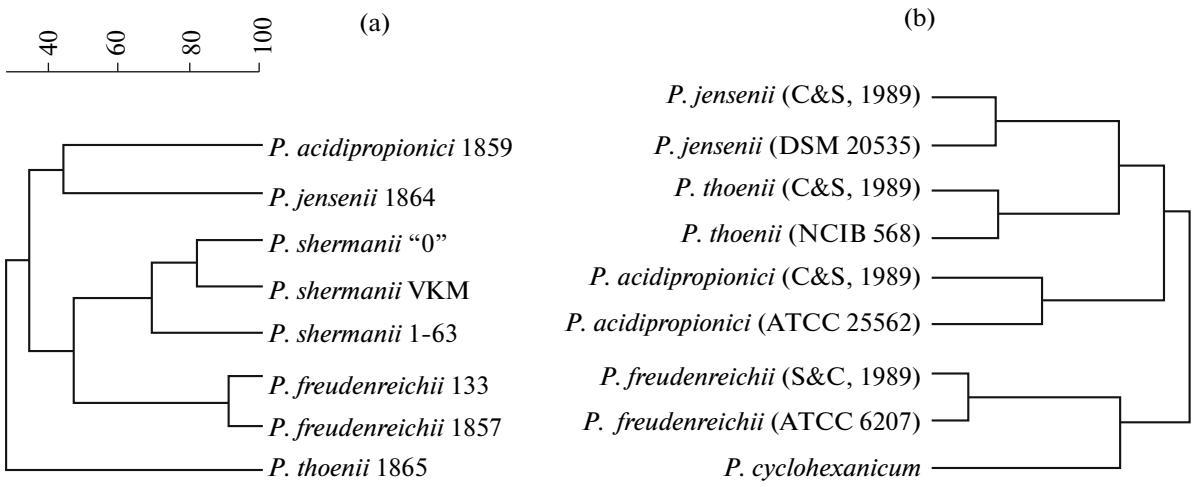

Fig. 2. Comparison between the MALDI–TOF spectrum-based dendrogram (a) and the phylogenetic tree obtained by sequencing of the 16S rRNA genes of the propionic acid bacterial strains (b). The significance of branching was established with bootstrap analysis of 1000 alternative trees [19].

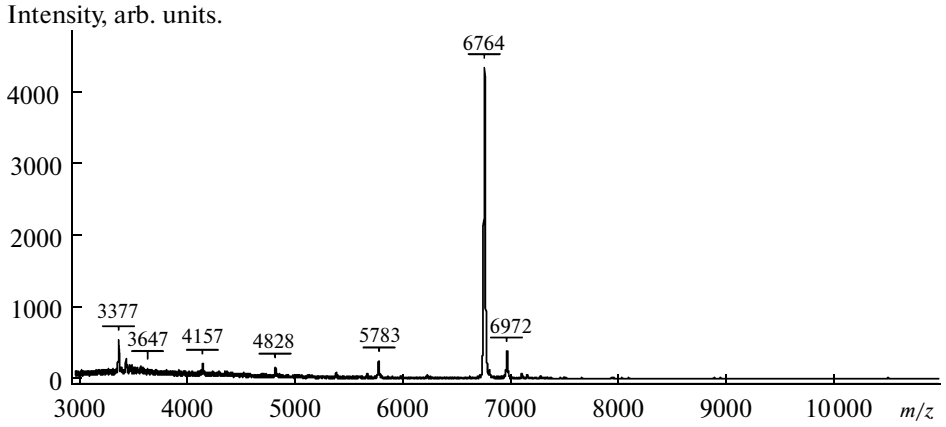

Fig. 3. The MALDI–TOF spectrum of *L. casei* cells with the use of sinapinic acid as a matrix.

as a result of the coevolution of the ribosomal proteins and ribosomal nucleic acids [5].

On the whole the results of the analysis of the MALDI spectra of propionic acid bacteria show a correlation with the data on the biochemical and genetic analysis of PAB.

The possibility of strain identification with the MALDI spectra is important for medical and biotechnological purposes. For example, successful discrimination between methicilline-sensitive and methicilline-resistant *Staphylococcus aureus* strains was described [21].

We had the following mutant strains at our disposal: the vitamin B_{12} overproducer *P. shermanii* 1-63 and the zero strain *P. shermanii* 0, which does not synthesize corrinoids at all. A comparative picture of significant and dominant peaks in the mutant strains and the nonmutant strain *P. shermanii* VKM presented in Fig. 2 shows that the overproducer was characterized by the presence of protein with m/z 3873, which was not found in the initial strain, and the absence of a protein with a molecular mass of 10 470 (when the SA and DHB matrices were used). It is presently difficult to say whether the differences between the MALDI spectra of the mutant and the wild strain are linked to the synthesis of corrinoids or other rearrangements as a result of mutations. Additional studies of the vitamin B_{12} producer are required. However, these specific features may serve as the identification characteristics for this mutant. The MALDI spectrum of strain *P. shermanii* 0 did not differ from that of the initial strain, except in the fact that it lacked the protein with a molecular mass of 5883. Thus, the MALDI–TOF spectra make it possible to differentiate between bacteria at the generic, species, and strain level.

The family *Propionibacterium* includes the propionic acid cocci we isolated by from cheese [22]. These high G + C cocci contained LL-DAP in the cell wall,

which makes them different from the coccoid described earlier [23]. Since these features, together with other properties, suggested high similarity of the propionic acid cocci to PAB, they were originally identified as *Propionibacterium coccoides* [22]. However, molecular biological investigations, ribotyping, the restriction analysis of the 16S and 23S rRNA fragments, and 16S rRNA gene sequencing showed that the propionic cocci should be assigned to a different genus, *Luteococcus*, under the name of *L. japonicus* subsp. *casei* [19]. *L. casei* is in the same cluster with the type strain of *L. japonicus* at a level of 80% and is related to the cluster of the *Propionibacterium* species at a level of 72%. The MALDI spectrum of *L. casei* is shown in Fig. 3 and is markedly dissimilar to the spectra of propionic bacteria. The peaks with m/z 3377, 5783, 6764, and 6972 with the dominant peak 6764 were well-marked. None of the peaks characteristic of the genus *Propionibacterium* was revealed in *L. casei*. Hence, the peak identity in the MALDI spectra of the propionic acid bacteria is confined to the level characteristic of the genus, but not the family *Propionibacteriaceae*.

The results of our studies show that bacterial groups determined using the MALDI profiles correlate with the phylogenetic 16S rRNA gene groups, thus demonstrating the high resolving capacity of this method.

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