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EXPERIMENTAL ARTICLES

Application of MALDI-TOF Mass Spectrometry for Differentiation of Closely Related Species of the *"Arthrobacter crystallopoietes"* Phylogenetic Group

N. V. Prisyazhnaya[a, 1], E. G. Plotnikova[b], O. V. Bueva[a], E. S. Korsakova[b], L. V. Dorofeeva[a], E. N. Il'ina[c], A. T. Lebedev[d], and L. I. Evtushenko[a]

[a] *All-Russian Collection of Microorganisms, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia*

[b] *Institute of Ecology and Genetics of Microorganisms, Ural Branch, Russian Academy of Sciences, Perm', Russia*

[c] *Scientific Research Institute of Physical-Chemical Medicine, Moscow, Russia*

[d] *Department of Chemistry, Moscow State University, Moscow, Russia*

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Abstract—MALDI mass spectra were generated for the type strain of *Arthrobacter crystallopoietes* VKM Ac-1107^T and for closely related (99.6–100% 16S rRNA gene similarity) halotolerant *Arthrobacter* strains, as well as for some other *Arthrobacter* species. Results of the cluster analysis of the spectra were in agreement with the genotypic characteristics of bacteria (DNA–DNA hybridization and BOX-PCR). The data obtained in this study indicate that the halotolerant strains belong to two new *Arthrobacter* species. Specific peaks which can serve as chemotaxonomic markers of the species composing the phylogenetic group "*Arthrobacter crystallopoietes*" were revealed.

Keywords: Arthrobacter crystallopoietes, MALDI mass spectrometry, DNA–DNA hybridization, BOX-PCR

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Various genotyping techniques with taxonomic resolution at the "strain–species" level are presently applied for species identification of bacteria [1–3]. The key characteristics used to unambiguously assign closely related bacteria (16S rRNA gene similarity above 97%) to the same species are the DNA–DNA hybridization level (~70% and higher), high similarity of phenotypic properties, and distinct differences from other species [2, 4, 5]. At the same time, the difficulties in differentiating the organisms of closely related genomic groups (genomospecies) at the phenotypic level have been emphasized repeatedly [1, 6]. Search for reliable phenotypic properties differentiating microbial species and development of comprehensive databases for phenotypic characteristics are among the essential issues of bacterial taxonomy [2, 7].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) of bacterial cells is a relatively new technique which is increasingly used for identification and classification of microorganisms at the species and subspecies levels [8–10]. Peaks registered in the mass range of 2000–20000 m/z mainly correspond to peptides and proteins, a significant proportion of which are ribosomal ones [11–15].

Clusters of closely related bacteria revealed by MALDI mass-spectra usually correlate well with grouping based on the 16S rRNA gene sequence analysis and other genotypic characteristics allowing higher taxonomic resolution [16–18]. Further, MALDI mass spectral analysis enables detection and identification of the marker ions (peptides and proteins) that are indicative of species and the intraspecies groups (subspecies) [11, 14, 16]. The advantages of MALDI mass spectrometry also encompass rapidity of analysis and a small amount of material required (one colony is enough). MALDI mass spectrometry is therefore often preferable for identification of bacteria at the species (subspecies) level as compared to the laborious genotypic and classic microbiological methods, especially in the case of a large number of isolates to be tested. However, at present there are no clear criteria for the species assignment of strains on the basis of mass-spectral analysis (e.g., the similarity threshold of mass spectral fingerprints) [10]. The wide application of MALDI mass-spectrometry for identification of environmental isolates is also limited by the absence of mass-spectral data on the majority of type strains of known species and, consequently, incomplete relevant MALDI mass-spectral data-bases [10].

Bacteria of the genus *Arthrobacter* (the phylum *Actinobacteria*) are ubiquitous in soils and other habitats,

[1] Corresponding author; e-mail: labyrinth@land.ru

including the extreme ones, and are known for their ability to degrade various natural compounds and xenobiotics, including the toxic ones [19, 20]. Development of methods for fast and accurate species identification of the newly isolated *Arthrobacter* strains, especially the closely related ones (>99% of the 16S rRNA gene similarity), is undoubtedly important to enhance the efficiency of studies in several fields. At the time of writing, only several groups of isolates of the genus *Arthrobacter* were subjected to a study by MALDI mass spectrometry [21, 22].

The goal of the present work was to perform a MALDI mass-spectral analysis of halotolerant *Arthrobacter* strains phylogenetically related to *A. crystallopoietes* and able to degrade polycyclic aromatic hydrocarbons to determine their species affiliation, and to evaluate the efficiency of MALDI mass spectrometry for discrimination of closely related species of the group studied.

MATERIALS AND METHODS

Subject of investigation. Strains of the genus *Arthrobacter* (VKM Ac-2548, VKM Ac-2549, VKM Ac-2550, VKM Ac-2551, VKM Ac-2552, VKM Ac-2063, and VKM Ac-2064) isolated from the bottom silt of Zyryanka river and from a salt-mining area technogenic soils, contaminated with the waste of chemical and salt-production industry (Berezniki, Perm' krai) [20], as well as the type strains of *A. crystallopoietes* and some other *Arthrobacter* species were used in the work.

Strains were grown for five days on trypticase soy agar (Difco) at 28°C. The strains were analyzed in triplicates using the 5-day cultures grown in intervals of 7 days.

MALDI mass spectrometry. The surface cultures (~5–10 μg) were transferred with a thin sterile spatula into a plastic tube containing 50 μL of the freshly prepared 50% aqueous acetonitrile solution (Sigma–Aldrich) supplemented with 2.5% trifluoroacetic acid and mixed thoroughly. The suspension was sonicated (35 kHz, 30 min, 37°C) in an UZV-1.3 (PKF Sapfir, Russia) ultrasound bath. To avoid precipitation, the mixture was stirred periodically. The suspension (0.8 μL) was then applied to a polished-steel MALDI sample target, mixed with an equal volume of the matrix solution (α-cyano-4-hydroxycinnamic acid (HCCA) in 50% aqueous solution of acetonitrile containing 0.1% trifluoroacetic acid), and air-dried at room temperature.

Mass spectra were registered in linear mode with delayed ion extraction on an Autoflex II MALDI-TOF mass-spectrometer (Bruker Daltonics) equipped with a nitrogen laser (337 nm), time-of-flight analyzer, and a reflectron. Spectra were recorded in positive ion mode using delay time of 350 ns, and an accelerating voltage of 20 kV. The mass range was 2–20 kDa. Protein 1 Calibration Standard (Bruker Daltonics) mixture was used for external calibration; spectra resolution was ±2 Da (200 ppm). The resulting spectra of each strain sample were obtained by summation of the spectra registered at 10–15 points of the analyte upon 50 laser shots. Mass spectra were processed using the Flex analysis 2.2 and Biotyper 2.0 software packages (Bruker Daltonics).

Amplification of the *boxA* chromosomal DNA sequences (BOX-PCR) was performed according to the procedure described previously [23]. The reaction products were separated by electrophoresis in 1.5% agarose gel in 1× TBS buffer solution [24].

DNA–DNA hybridization using membrane filters was performed as described previously [25].

16S rRNA sequence similarities of the bacteria from the technogenic soils of the salt-mining area (determined previously [20]) and the type strains of the known species were obtained using the EzTaxon server (http://www.eztaxon.org; [26]). The phylogenetic tree was built using the neighbor-joining algorithm of the TREECONW version 1.3B software package [27]. The evolutionary distance expressed as a number of substitutions per 100 nucleotides was calculated according to [28]. Evaluation of the statistical significance of the branching was determined by bootstrap analysis using the relevant function of the TREECONW software package with 1000 iterations.

RESULTS AND DISCUSSION

For each strain, a total spectrum of approximately 50 peaks was compiled from MALDI mass spectra of three independent cultivations of bacterial cells. All peaks of the total spectra within the range of 2000–20000 m/z and relative intensity above 1% of the maximal value were included in cluster analysis using the BioTyper 2.0 software package (Bruker Daltonics). It was revealed that the type strain *A. crystallopoietes* VKM Ac-1107^T and all halotolerant strains from the salt-mining area formed a tight MALDI cluster and distinctly separated from all other *Arthrobacter* species included in the study (Fig. 1). In the *"A. crystallopoietes"* MALDI cluster, the halotolerant strains divided into two groups. Group 1 comprised two strains (VKM Ac-2063 and VKM Ac-2064) and group 2, five strains (VKM Ac-2548, VKM Ac-2549, VKM Ac-2550, VKM Ac-2551, and VKM Ac-2552) (Fig. 1).

On the whole, the data obtained were in agreement with the results of phylogenetic analysis (16S rRNA) (Fig. 2), as well as with the grouping revealed by BOX-PCR (Fig. 3) and DNA–DNA hybridization. The 16S rRNA gene sequence similarities among the strains comprising the *"A. crystallopoietes"* phylogenetic group (*A. crystallopoietes* VKM Ac-1107^T and seven strains from the salt-mining area) were 99.6–100% (as determined from approximately 1400 bp). The levels of DNA–DNA hybridization between *A. crystallopoietes* VKM Ac-1107^T and strains of the first

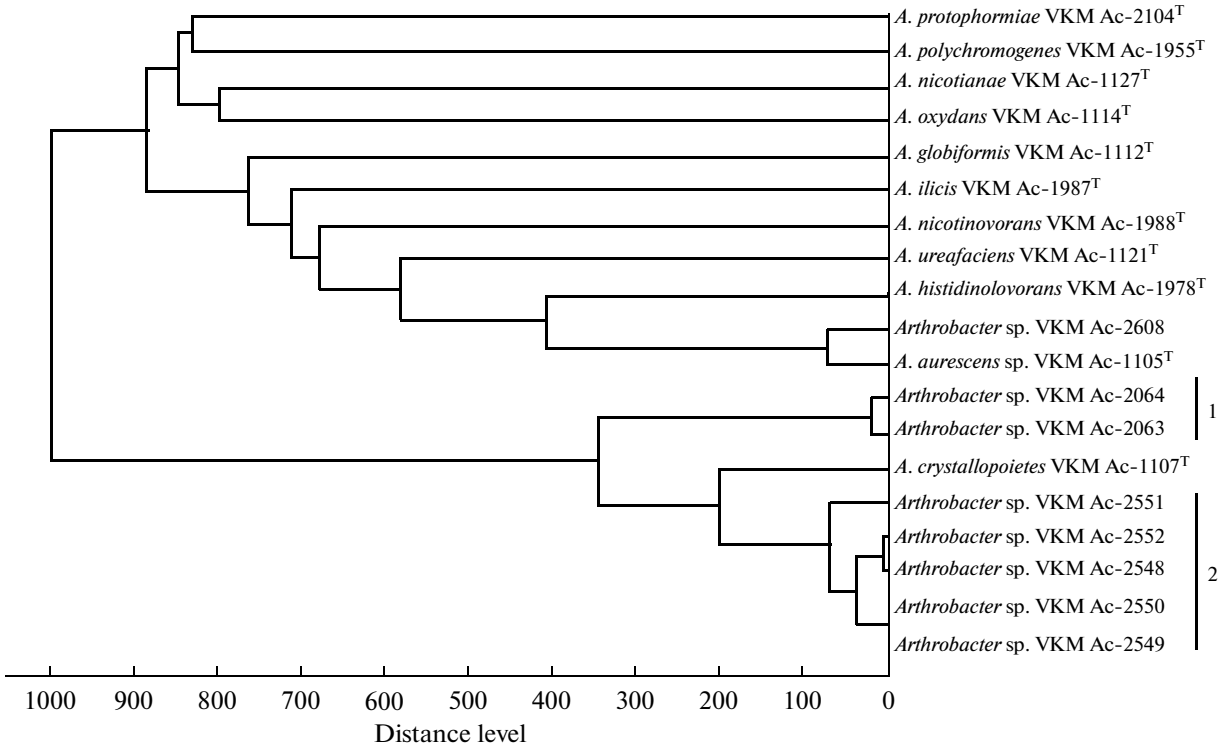

Fig. 1. Score-oriented dendrogram of MALDI-TOF mass spectral profiles of bacteria of the genus *Arthrobacter* generated by Bio-Typer 2.0 software (Bruker Daltonics). The numerals indicate MALDI groups 1 and 2, respectively.

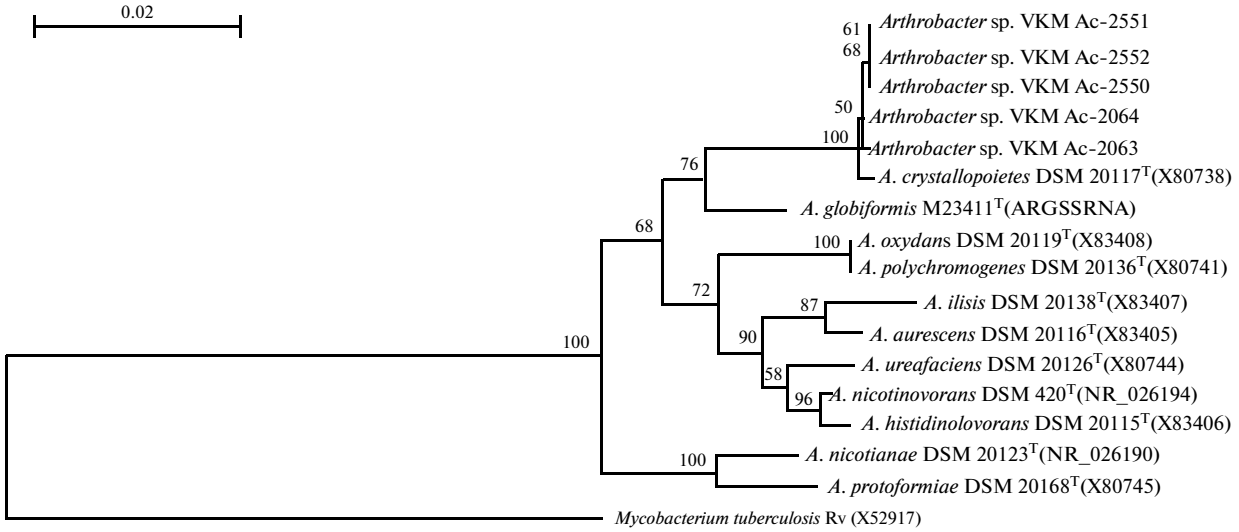

Fig. 2. Neighbor-joining phylogenetic tree built on the basis of 16S rRNA gene nucleotide sequence analysis [27], demonstrating the position of the strains. Scale bar, two substitutions per 100 nucleotides. Figures show the statistical significance of branching determined by bootstrap analysis with 1000 iterations (values above 50% are presented).

(VKM Ac-2063 and VKM Ac-2064) and second (VKM Ac-2550 and VKM Ac-2552) MALDI groups were 40–46% and 36–37%, respectively; the DNA similarity levels between representatives of groups 1 and 2 was 48–54%.

Comparison of the mass spectra of bacteria forming the *"A. crystallopoietes"* MALDI cluster revealed 15 peaks common to all strains of the cluster (see table and Fig. 4). Nine of the peaks (indicated by boldface in the table) were exclusively characteristic of strains

belonging to this cluster and were not found in other type and reference strains studied in this work. Mass spectra of strains VKM Ac-2063 and VKM Ac-2064 (MALDI group 1) had 30 common peaks, 15 of them were identified only in spectra of these two strains (table). In the strains of MALDI-group 2, 29 common peaks were revealed, eight of which were unique for the group (species), while five peaks were present also in the spectrum of the type strain *A. crystallopoietes* VKM Ac-1107^T.

The obtained values of DNA–DNA hybridization, indicating that the halotolerant strains belong to two genomospecies, and also the differences between these genomospecies in the mass spectra (table), the ability to degrade phenanthrene and its derivatives, and the catabolic pathways of naphthalene [20], provide evidence that these bacteria represent two different (new) species [4, 5].The revealed specific patterns of components of their mass spectra may be used as chemotaxonomic markers for the newly revealed species and *A. crystallopoietes*.

The type strains of other species studied in the work (*A. ilicis, A. nicotinovorans, A. ureafaciens, A. histidinolovorans*, and *A. aurescens*) forming a common phylogenetic group (Fig. 2) with 97.6–99.7% 16S rRNA sequence similarity, also grouped together according to MALDI mass spectra (Fig. 1). It's worth noting that the above organisms also possess identical structure of the cell wall peptidoglycan (type A3α; Lys-Ala-Thr-Ala) and the same type of respiratory menaquinones, MK-9(H_2) (predominates menaquinone with monounsaturated nine-unit isoprenoid side chain) [19]. The type strain of *A. globiformis*, adjacent to this MALDI group (Fig. 1), forms a separate branch on the phylogenetic tree (Fig. 2) and differs in the peptidoglycan structure (type A3α; Lys-Ala-Ala-Ala) [19]. The remaining species (*A. polychromogenes, A. oxydans, A. nicotianae*, and *A. protoformiae*,) are phylogenetically distant from the above group and occupy a farther position on the mass spectral dendrogram (Fig. 1). These also differ in the structure of peptidoglycans and/or the composition of menaquinones

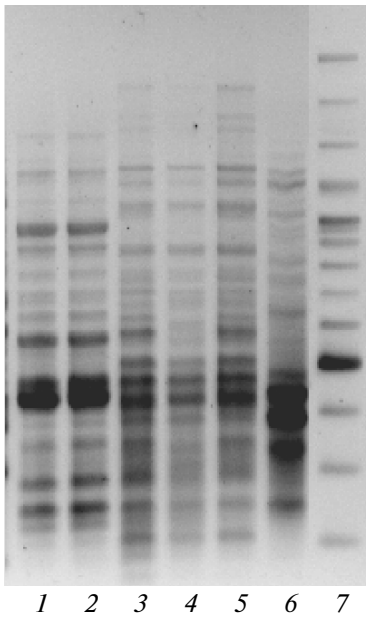

Fig. 3. Electrophoregrams of amplified boxA fragments (BOX-PCR) from DNA of *Arthrobacter* strains: *Arthrobacter*. sp. VKM Ac-2063 (*1*), *Arthrobacter*. sp. VKM Ac-2064 (*2*), *Arthrobacter*. sp. VKM Ac-2548 (identical to the *boxA* fragment profiles of strains VKM Ac-2549 and VKM Ac-2550 [20]) (*3*), *Arthrobacter*. sp. VKM Ac-2552 (*4*), *Arthrobacter*. sp. VKM Ac-2551 (*5*), *A. crystallopoietes* VKM Ac-1107^T (*6*), and O'Gene Ruller 100 bp (Fermentas, Lithuania) molecular weight marker (*7*).

both from the above-mentioned group and among themselves [19]. It should be also noted that, according to MALDI mass spectral analysis, several of the previously studied representatives of the genus *Arhtrobacter* and members of other genera formed a common MALDI group but were separated from other genera of actinobacteria (*Microbacterium, Gordonia, Rhodococcus, Oerskovia*) and proteobacteria [22].

Thus, it was demonstrated by the example of the *"A. crystallopoietes"* group that the method of MALDI mass spectrometry can be successfully used to differ-

Mass values (*m/z*) specific for the "*A. crystallopoietes*" MALDI cluster and the comprising groups (species)

<table>
<tr><th colspan="3">Experimental mass (average values)*, m/z</th></tr>
<tr><td>VKM Ac-2063,
VKM Ac-2064
(MALDI group 1)</td><td>VKM Ac-2548, VKM Ac-2549, VKM Ac-2550,
VKM Ac-2551, VKM Ac-2552 (MALDI group 2)</td><td>A. crystallopoietes
VKM Ac-1107^T</td></tr>
<tr><td colspan="3">6020, 6704, 7293, 7344, 7524, 7652, 7998, 8516, 9472, 3242, 3544, 6494, 7091, 7108, 7206</td></tr>
<tr><td rowspan="2">3788, 3942, 4327, 4518, 5054, 5069, 5089, 5878, 6030, 6359, 6518, 7147, 7890, 8658, 9044</td><td colspan="2">4320, 4536, 7242, 8644, 9074</td></tr>
<tr><td>3328, 3620, 3958, 5850, 5943, 6660, 7743, 8276</td><td>2954, 3966, 4075, 4992, 5906, 5967, 6809, 7189, 8157, 8377, 9556, 9989</td></tr>
</table>

Note: Boldface indicates mass values revealed in mass-spectra of all studied strains of the "*A. crystallopoietes*" MALDI cluster without exception.

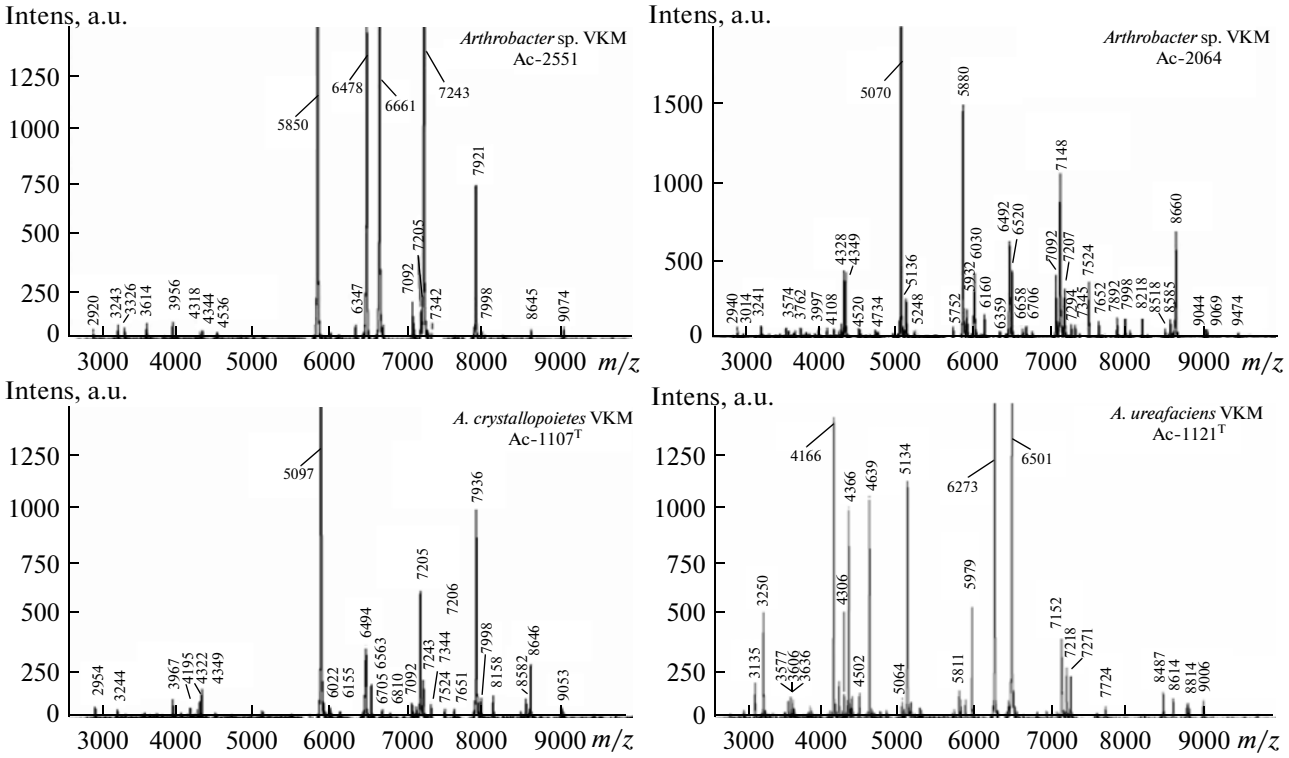

Fig. 4. MALDI mass spectra of bacteria of the genus *Arthrobacter*. The numerals indicate masses corresponding to the peaks (m/z).

entiate between phylogenetically closely related species and to reveal potential representatives of the new species. Moreover, the method makes it possible to determine a set of specific components of mass spectra to distinguish closely related species (genomospecies). The results of our work, together with the previously published data [22], provide evidence that MALDI mass spectrometry is also very useful for preliminary identification of new isolates within groups of other species of the genus *Arthrobacter*.

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