SESSION 2. MICROBIAL DIVERSITY: ACCOMMODATING NEWCOMERS

TAXONOMY OF THE GENUS *RATHAYIBACTER*

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Fig. 1. Bacterial (left) and nematode (right) galls (Murray et al., 2017)

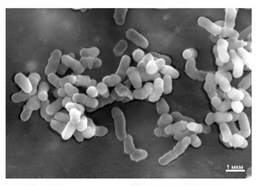
INTRODUCTION

Actinobacteria of the genus *Rathayibacter* Zgurskaya *et al.* 1993 (family *Microbacteriaceae*) are characterized by 2,4-diaminobutyric acid (L-isomer) in the cell-wall peptidoglycan and predominant menaquinone MK-10. Until 2018, the genus included six species with validly published names: *R. rathayi*, *R. iranicus*, *R. tritici*, *R. toxicus*, *R. caricis* and *R. festucae*. More recently, another new species, *R. agropyri*, has been described. The species *R. rathayi*, *R. iranicus*, *R. tritici*, *R. toxicus* and *R. agropyri* are plant pathogens causing gumming disease of wheat and cereal grasses (Poaceae) (Fig. 1) and are transmitted to their host plants by seed gall nematodes of the genus *Anguina*. *R. toxicus* is also responsible for toxicity of ryegrass (*Lolium rigidum*) and some other grasses, which often results in fatal poisoning of grazing animals in Australia and some other countries. The type strain of *R. festucae* was isolated from *Festuca rubra* infected by *Anguina graminis* and the type strain of *R. caricis* was recovered from *Carex* sp. (Cyperaceae) without any symptoms of bacterial diseases or nematode infestation.

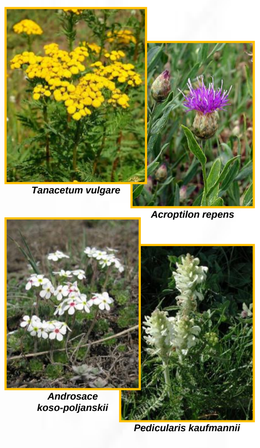
Here we provide the results of comparative study of *Rathayibacter* strains preserved in the All-Russian Collection of Microorganisms (VKM). The set of 26 strains used in this study (Table 1) contained members of six recognized species (described before 2018) and several novel strains, including those isolated from the nematode galls on *Acroptilon repens* (Asteraceae) and from necrotic lesion on *Tanacetum vulgare* (Asteraceae), as well as from plants without any symptoms of bacterial and nematode infestation, such as *Pedicularis kaufmannii* (Orobanchaceae) and *Androsace koso-poljanskii* (Primulaceae).

Table 1. Overview of strains of the genus Rathayibacter used in this study

Organism	VKM strain number	Isolation source
Rathayibacter rathayi	**Ac-1601^T**	Seeds of *Dactylis glomerata*, nematode *Anguina agrostis*, New Zealand, 1968
	Ac-2581	*Dactylis glomerata*, England, 1934
	Ac-2582	*Dactylis glomerata*, New Zealand, 1968
	Ac-2583	*Dactylis glomerata*, New Zealand, 1968
	Ac-2584	*Dactylis glomerata*, Scotland
Rathayibacter tritici	**Ac-1603^T**	Seeds of *Triticum aestivum*, nematode *Anguina tritici*, Egypt
	Ac-2585	*Triticum aestivum*, India
	Ac-2586	*Triticum aestivum*, Egypt, 1941
	Ac-2587	*Triticum aestivum*, India
	Ac-2588	*Triticum aestivum*, India
	Ac-2589	*Triticum aestivum*, Iran, 1966
R. iranicus	**Ac-1602^T**	Seeds of *Triticum aestivum*, nematode *Anguina tritici*, Iran, 1966
Rathayibacter toxicus	Ac-1600	*Phalaris minor*, Australia, 1978
	Ac-2590	*Lolium rigidum*, Australia, 1977
	Ac-2591	*Lolium rigidum*, Australia, 1978
	Ac-2592	*Phalaris minor*, Australia, 1978
	Ac-2593	*Phalaris paradoxa*, Australia, 1978
	Ac-2594	*Avena fatua*, Australia, 1978
R. festucae	**Ac-1390^T**	Seeds of *Festuca rubra*, nematode *Anguina graminis*, Moscow region, 1991
R. caricis	**Ac-1799^T**	Phyllosphere of *Carex* sp., nematode not detected, Belgorod region, 1996
Rathayibacter sp.	**Ac-2595**	Leaf of *Tanacetum vulgare*, nematode *Aphelenchoides fragariae*, Moscow region, 2000
	Ac-2596^T	
	Ac-2597	Leaf of *Acroptilon repens*, nematode *Mesoanguina picridis*, Uzbekistan, 1992
	Ac-2630^T	
	Ac-2121^T	*Androsace koso-poljanskii*, nematode not detected, Belgorod region, 1996
	Ac-2629	Seeds of *Pedicularis kaufmanii*, nematode not detected, Moscow region, 1995



R. oskolensis VKM Ac-2121^T



RESULTS AND DISSCUTION

1. GENETIC CHARACTERIZATION

1.1. Phylogenetic analysis based on 16S rRNA and *gyr*B gene sequences

Phylogenetic analyses based on 16S rRNA gene sequences indicated that all studied strains belonged to the genus *Rathayibacter* and they have high levels of 16S rRNA gene similarity with validly described species (Fig. 2).

The strain VKM Ac-2121^T was most closely related to *R. tritici* (98.9%).

The strain VKM Ac-2596^T was most closely related to *R. rathayi* and *R. iranicus* (99.7%).

The strains VKM Ac-2630^T and VKM Ac-2597 were most closely related to *R. caricis* (99.8%).

The strains VKM 2595 and VKM Ac-2629 were most closely related to *R. festucae* (99.9%).

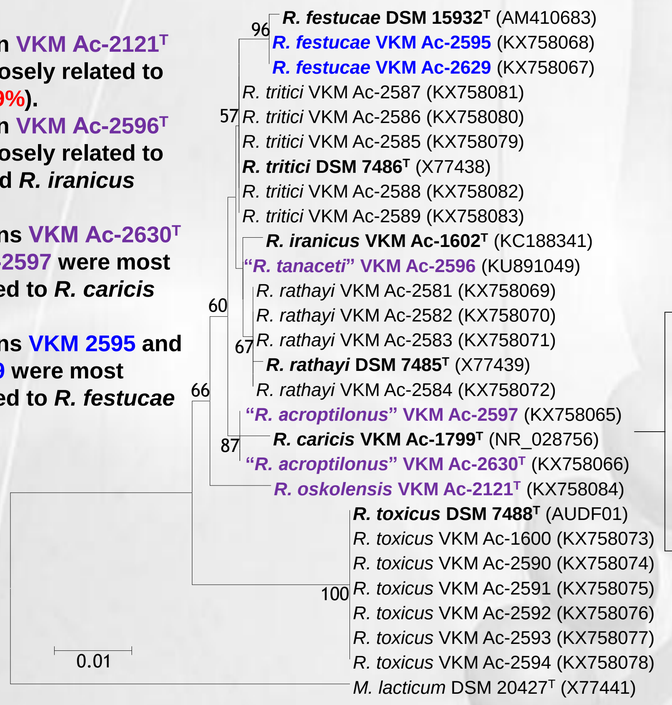


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence

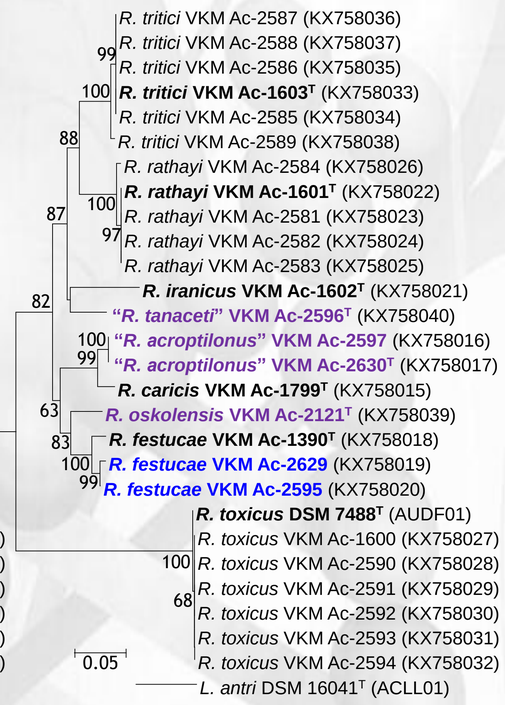


Fig. 3. Tree of the GyrB gene

A fragment of the *gyr*B (765 bp) was amplified with the designed primers 5'-GAGGTSAAGCGYCAGGGTGC-3' and 5'-CTGGCAGTCCTTRAGCTTGCC-3' and previously described primers *gyr*B-F and *gyr*B-R (Jacques *et al.*, 2012).

Analysis of partial *gyr*B gene sequences (Fig. 3) and MALDI mass-spectra (Fig. 6, Table 2) showed that cultures under study were affiliated with both known and new species of the genus *Rathayibacter* (*R. oskolensis*, "*R. tanaceti*" and "*R. acroptilonus*").

1.2. Phylogenetic analysis based on MLSA with concatenated sequences

In order to obtain the gene sequences, gene specific PCR primers were designed:

- DNA gyrase subunit B (765 bp),
- recombinase A (600 bp),
- RNA polymerase subunit B (1042 bp),
- polyphosphate kinase (1060 bp).

Multilocus sequence analysis of the concatenated genes *gyr*B, *rec*A, *rpo*B and *ppk* showed that the recognized and new species formed separate, well-defined clades within the genus *Rathayibacter* with high bootstrap values (Fig. 4).

Using the results of this study and the published data, we propose a cut-off value of similarity for four concatenated genes being 96%, the similarity of genes *gyr*B – 94%, *rec*A – 95%, *rpo*B – 97% and *ppk* – 95.5% to distinguish between known and new species of the genus *Rathayibacter*.

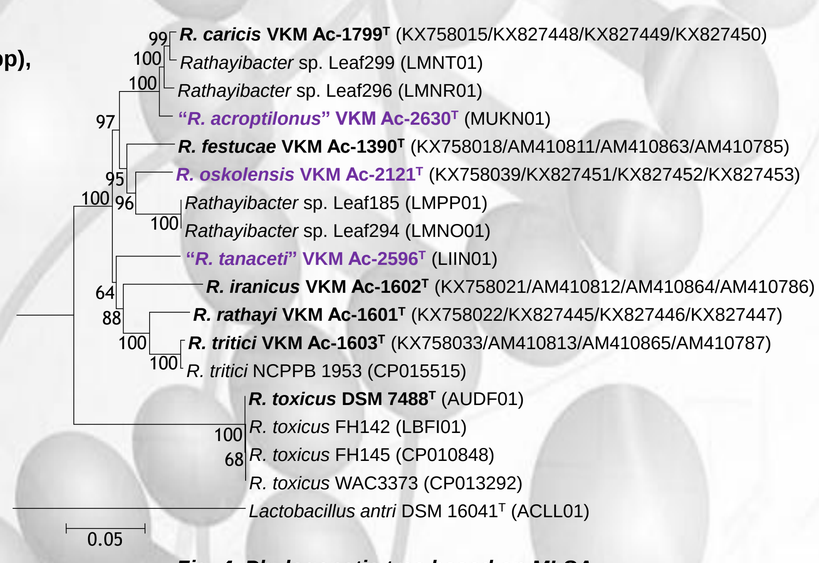


Fig. 4. Phylogenetic tree based on MLSA

1.3. Phylogenetic analysis based on whole genome sequences using ANI, dDDH and TETRA

The proposed and generally accepted species boundaries for ANI and digital DDH values are 95–96% and 70%, respectively (Chun *et al.*, 2018; Goris *et al.*, 2007; Richter, Rosselló-Móra, 2009).

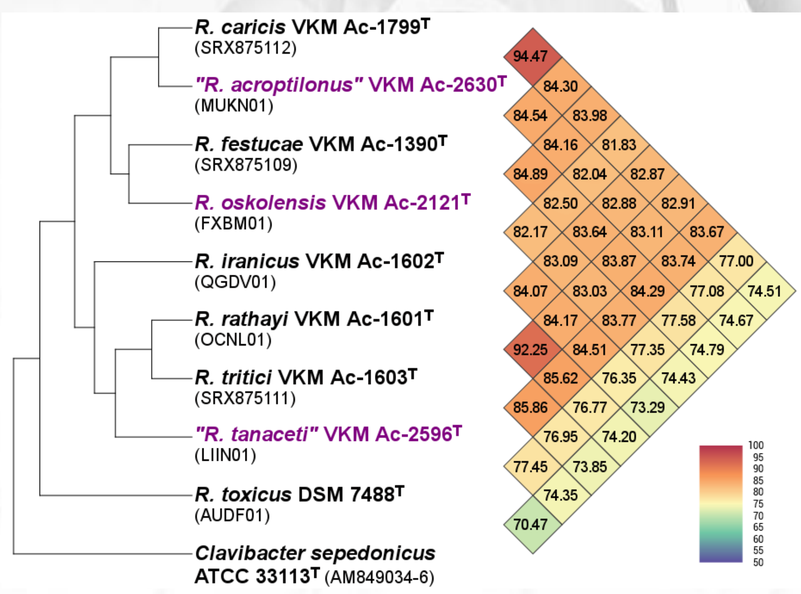


Fig. 5. Phylogenetic tree based on OrthoANI (data are shown for 10 strains)

Complete genomes of *R. rathayi* VKM Ac-1601^T (the type strain of the type species of *Rathayibacter*), *R. iranicus* VKM Ac-1602^T and representatives of the recently proposed or revealed new species, *R. oskolensis* VKM Ac-2121^T, "*R. tanaceti*" VKM Ac-2596^T and "*R. acroptilonus*" VKM Ac-2630^T, were sequenced and annotated (Starodumova *et al.*, 2017; Vasilenko *et al.*, 2016, 2018).

Comparative analysis was performed with genomes of 17 *Rathayibacter* strains which included the above and other strains whose genome sequences were available from data bases. Calculated genome relatedness indices, such as average nucleotide identity (ANI), tetranucleotide signature frequency correlation coefficient (TETRA) and digital DNA-DNA hybridization (dDDH), provided clear evidence in support of separate species status of strains VKM Ac-2121^T, VKM Ac-2596^T and VKM Ac-2630^T.

2. PHENOTYPIC CHARACTERIZATION

2.1. MALDI-TOF mass spectrometry

For all 26 strains MALDI mass-spectra (including up to 120 peaks) were obtained. The results of the MALDI MS showed that the majority of strains were affiliated with 4 known species of the genus *Rathayibacter* (*toxicus*, *tritici*, *festucae* and *rathayi*) and, most likely, belonged to these species. Four strains (VKM Ac-2121^T, VKM Ac-2596^T, VKM Ac-2597 and VKM Ac-2630^T) were isolated from clusters of the described species and could relate to new species.

Common components of MALDI mass-spectra (new chemotaxonomic markers of the genus) were found for all studied strains of *Rathayibacter* (3954, 4428 и 6458 m/z); specific mass values for each species were also detected (Table 2).

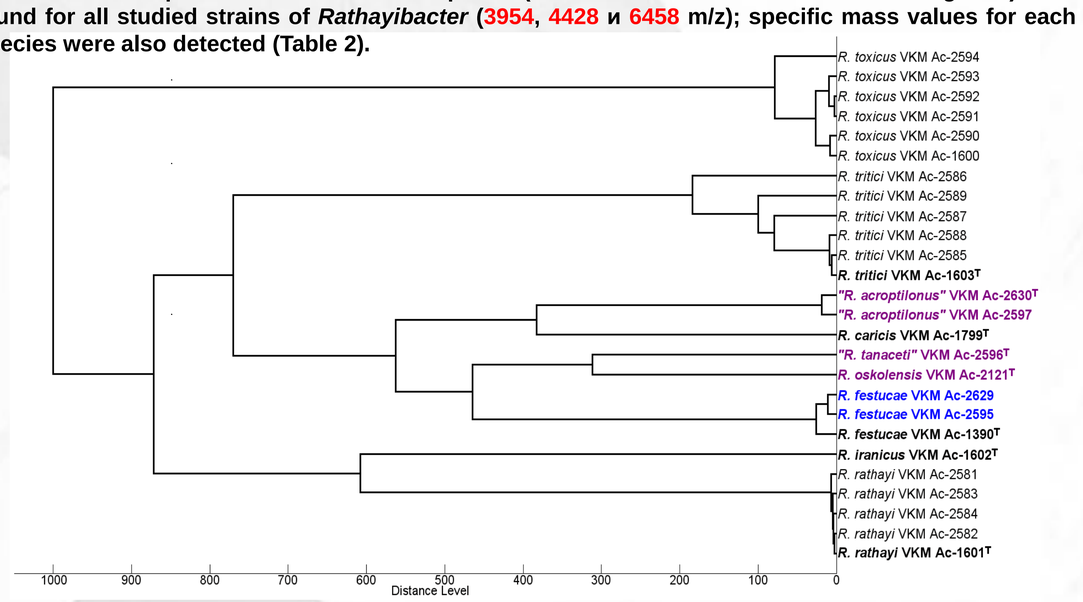


Fig. 6. Dendrogram derived from MALDI-TOF MS protein profiling

2.2. Chemotaxonomy, physiology and biochemistry

The revealed physiological and chemotaxonomic characteristics, including cell-wall sugars, cell-wall glycopolymers and polar lipid profiles, allowed clear phenotypic differentiation of strains VKM Ac-2121^T, VKM Ac-2596^T and VKM Ac-2630^T from each other and from the recognized *Rathayibacter* species.

Table 2. Characteristics that differentiate Rathayibacter species

Characteristic \ Strain	*R. caricis*	*R. festucae*	*R. iranicus*	*R. rathayi*	*R. toxicus*	*R. tritici*	*R. oskolensis*	«*R. tanaceti*»	«*R. acroptilonus*»
Colony color	Yellow	Rose-orange	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Oxidase	+	+	+	+	-	+	+	+	+
Methyl red test	+	+	-	-	-	-	+	nd	nd
Voges–Proskauer	+	+	-	-	-	-	-	nd	nd
Cell-wall sugars:									
Galactose	-	-	+	+	-	+	+	+	+
Xylose	+	+	-	+	-	+	+	+	-
Fucose	+	-	-	-	-	-	-	-	+
Glu, Man, Rha	+	+	+	+	+	+	+	+	+
Polar lipids:									
Phospholipids	PL1	PL2	-	-	-	PL1, PL5	PL1, PL3, PL4	PL2, PL3-1, PL6	PL1, PL7
Glycolipids	G, G1, G2, G4, G5, G6	G, G1, G2, G2-1, G3, G4	G, G1, G2, G3	G2, G3, G5	G1, G2-1, G3, G4, G5	G2, G3	G1, G2, G2-1, G4	G1, G2, G2-1	G1, G2, G2-1, G4
Unique peaks in MALDI mass-spectrum (m/z)	2410, 2706, 2822, 2949, 3025, 3307, 3329, 4046, 5699, 6664, 6966, 9407, 11219, 13905	3004, 3270, 3627, 3858, 4761, 6583, 6612	2659, 3941, 4083, 4373, 4647, 4687, 4824, 6046, 6108, 6169, 7458	2890, 3344, 3410, 4153, 4469, 4843, 4901, 5264, 5410, 5615, 5728, 5785, 6236, 6506, 6692, 7059, 7088, 8675, 9664, 11570, 17354	2201, 2799, 3200, 3727, 3792, 4162, 4525, 5023, 5882, 6588, 11763	955, 3900, 4932, 5581, 5694	2659, 3941, 4083, 4373, 4647, 4687, 4824, 6046, 6108, 6169, 7458	2010, 2300, 2400, 2470, 2631, 2899, 3015, 3046, 4131, 4246, 4420, 4563, 5006, 5225, 5443, 5797, 5824, 5860, 6031, 6093, 6637, 6836, 7931, 8084, 8448, 9551, 9638, 11290	2578, 3035, 3079, 3193, 3522, 3538, 3548, 4323, 5395, 5803, 5885
Utilization of:									
Adonitol	+	+	-	-	-	-	-	-	-
Dulcitol	+	+	-	-	-	-	+	-	-
meso-Erytritol	-	+	-	-	-	-	-	-	-
meso-Inositol	+	+	-	v	-	-	+	+	+
Inuline	+	+	v	-	-	+	+	+	+
Lyxose	-	-	-	-	-	-	+	-	-
Melibiose	+	+	-	-	-	-	+	+	+
Mannitol	+	+	+	+	-	+	+	+	+
L-Rhamnose	+	+	-	-	-	-	+	+	+
Salicin	+	+	+	-	-	+	+	+	+
Sorbitol	+	+	v	v	-	v	+	-	-
Hydrolysis of:									
Tween 40	+	+	+	+	-	+	-	+	+
Tween 60	-	+	v	+	-	+	-	+	-
Tween 80	-	+	v	-	-	v	-	-	-
H_2S production from peptone	+	+	+	+	-	+	-	nd	nd
Growth at 7°C	-	-	-	-	-	-	-	-	+
Growth at 5% NaCl	+	-	-	-	-	v	-	-	-

+, Positive; – , negative; v, variable between experiments or test methods.

CONCLUSION

Based on the obtained data, a novel species, *R. oskolensis* have been described (Dorofeeva *et al.*, 2018), while strains VKM Ac-2596^T from *Tanacetum vulgare* and VKM Ac-2630^T from *Acroptilon repens* are still waiting for their descriptions as two novel species.

Our work also resulted in creating reference MALDI mass-spectral database for the genus *Rathayibacter*, comprising validly described and putative new species, which allows fast and reliable identification of strains. Genus-specific (3954, 4428 и 6458 m/z) and several species-specific components of mass-spectra were detected which can serve as chemotaxonomic markers of the genus *Rathayibacter* and its species. And finally, borderline similarity values for housekeeping genes were proposed that allows one to distinguish strains of the recognized and novel species of this genus: *gyr*B – 94%, *rec*A – 95%, *rpo*B – 97% and *ppk* – 95.5%.

Acknowledgements: The authors are grateful to Dr. Vladimir Chizhov (Center of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Moscow, Russia) and Dr. Sergei Subbotin, California Department of Food and Agriculture, Sacramento, California, USA) who provided samples of nematode-infested plants.

Funding information: This work was financially supported by the Russian Foundation for Basic Research (grant No. 16-34-01048 mol_a) and the Russian Federation Federal Agency of Scientific Organizations (the Program "Bioresource collections").

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