Heterotrophic bacteria associated with Varroa destructor mite

Slavomira Vanikova, Alzbeta Noskova, Peter Pristas, Jana Judova, Peter Javorsky

► To cite this version:

Slavomira Vanikova, Alzbeta Noskova, Peter Pristas, Jana Judova, Peter Javorsky. Heterotrophic bacteria associated with Varroa destructor mite. *Apidologie*, 2015, 46 (3), pp.369-379. 10.1007/s13592-014-0327-9 . hal-01284451

HAL Id: hal-01284451

<https://hal.science/hal-01284451>

Submitted on 7 Mar 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Heterotrophic bacteria associated with *Varroa destructor* mite

Slavomira VANIKOVA^{1,2}, Alzbeta NOSKOVA³, Peter PRISTAS^{1,2}, Jana JUDOVA³,
Peter JAVORSKY²

¹Department of Genetics, Institute of Biology and Ecology, Pavol Jozef Šafárik University in Košice, Mánesova 23, 040 01, Košice, Slovakia

²Institute of Animal Physiology, Slovak Academy of Science, Šoltésovej 4-6, 040 01, Košice, Slovakia

³Department of Biology and Ecology, Matej Bel University, Tajovského 40, 974 01, Banská Bystrica, Slovakia

Received 26 December 2013 – Revised 13 August 2014 – Accepted 9 October 2014

Abstract – *Varroa* bee hive attack is a serious and common problem in bee keeping. In our work, an ecto-microflora of *Varroa destructor* mites was characterised as a potential source of bacterial bee diseases. Using a cultivation approach, a variable population of bacteria was isolated from the body surface of *Varroa* mites with frequency of about 150 cfu per mite individual. Nine studied isolates were classified to four genera and six species by a combination of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)- and 16S ribosomal RNA (rRNA)-based methods, suggesting relatively low diversity of *Varroa* mite-associated ecto-microflora. The *Varroa* mite-associated bacterial population was found to be dominated by Gram-positive bacteria of *Bacillus* and *Microbacterium* genera. Gram-negative bacteria were represented by members of *Brevundimonas* and *Rhizobium* genera. Most of the identified species are not known to be associated with *Varroa* mite, either honey bee or honey up until now and some of them are probably representatives of new bacterial taxa.

Varroa destructor / ecto-microflora / heterotrophic bacteria / *Microbacterium*

1. INTRODUCTION

Insect pollinators are important for the sexual reproduction of many crops, fruits and the majority of wild plants. Among the insect, bees play the main role. Honey bees (mainly *Apis mellifera*) are economically important pollinators of monocultures worldwide. However, nowadays, deterioration of hives' health and their increase in mortality is observed. Cause of these decreases could be numerous pathogens like viruses, bacteria, fungi and parasites which attack honey bees (Genersch 2010). Recent studies support the view that increased mortality is caused by the interaction of a variety of pathogens with additional stress factors

(Nazzi et al. 2012), including the *Varroa* mite (*Varroa destructor*), which is considered as the main factor causing the decline of bee colonies (Rosenkranz et al. 2009).

The *V. destructor* is a haemophagous parasite of honey bees. The natural host of *Varroa* mite is the Eastern honey bee (*Apis cerana*) living in Asia, from where the mite has been introduced to Europe (Le Conte et al. 2010). The first infestation in Germany occurred in the 1970s and since then, the mite has spread throughout Europe to most continents of the world, except Australia and New Zealand, most likely by bee shipments and imports, within a short time period (Boecking and Genersch 2008). The mite gets attached to the body of the bee and brood and weakens the bee by repeated sucking of haemolymph. The bee can lose up to 3 % of its body water for every female mite present during the bee's development. The presence of mites decreases the concentration of

Corresponding author: P. Pristas, pristas@saske.sk
Manuscript editor: Yves Le Conte

saccharides and proteins. As a result, the bees are more susceptible to ailment caused by viruses, bacteria or are weakened by intensive use of pesticides (Bowen-Walker and Gunn 2001). The life cycle of *Varroa* begins by entering of a mature fertilised female into the cell before it is capped. The mother mite lays eggs on the wall of the cell and adult mites escape from the brood cell when the young adult worker bees emerge. Usually, depending on the infestation rate, the mite does not kill the host (Oldroyd 1999) but the invasion of the *V. destructor* mite is the greatest threat for apiculture around the world. There is no chemical treatment against *Varroa* mite with 100 % effectiveness. Use of treatments leaves the more resistant mites and the next generation becomes increasingly resistant (Le Conte et al. 2010). Without periodic treatment, most of honey bee colonies could collapse within 2–3-year periods. Chemical treatments can also increase the risk of chemical residues in bee products (De la Rúa et al. 2009). *V. destructor* is also known as a vector of several viruses (Chen and Siede 2007), bacteria (Ball 1997) and fungi (Benoit et al. 2004) that cause diseases of bees. The studies focus on viruses transmitted with *Varroa* mite like deformed wing virus (Bowen-Walker et al. 1999), Kashmir bee virus (Chen et al. 2004), sacbrood virus (Shen et al. 2005), acute bee paralysis virus (Ball 1985) or chronic bee paralysis virus (Berényi et al. 2006), but there are limited data on transmission of bacteria by *Varroa* mite.

In our work, we focused on the characterisation of ecto-microflora of *Varroa* mite as a potential vector of bacterial bee diseases.

2. MATERIALS AND METHODS

2.1. Samples, culturing and identifications of bacteria

The mite individuals came from hives near Bacuch (48° 51' 23" N, 19° 48' 28" E), in Central Slovakia. Adult female mites were collected from adult bees from two beehives in spring time (20 individuals per beehive). The mite bodies were washed in PBS solution for 15 min and serial dilutions were spread on nutrient agar no. 2 (Difco). After culturing on the agar plates for 40 h at 37 ° C, the nine different bacterial isolates were

chosen based on colony morphology for further identification. For identification of bacteria basic microbiological methods (Gram stain, catalase test, oxidase test) (Reddy et al. 2007) combined with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, and 16S ribosomal RNA (rRNA) analysis were used.

For MALDI-TOF analysis, a small amount of a single freshly grown overnight colony was applied directly onto a polished steel MALDI target plate, as a thin film. Alternatively, the biological material (one bacterial colony) was resuspended in 300-μL distilled water. Then, 900 μL of absolute ethanol was added, and the mixture was centrifuged at 13,000g for 2 min, after which the supernatant was discarded. Thirty microlitres of formic acid (70 % v/v) was added to the pellet and thoroughly mixed by pipetting before the addition of 30 μL of acetonitrile to the mixture. The mixture was centrifuged again at 13,000g for 2 min. One microlitre of the supernatant was placed onto a spot of steel target plate and air-dried at the room temperature. Both the microbial film and the supernatant of the extracted proteins were overlaid with 1 μL of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in organic solvent (50 % acetonitrile and 2.5 % trifluoroacetic acid)) and air-dried (Ferreira et al. 2011).

MALDI-TOF was performed on Microflex LT instrument (Bruker Daltonics GmbH, Leipzig, Germany) using FlexControl software (version 3.0). Spectra were recorded in the positive linear mode. For each spectrum, 240 shots in 40-shot steps from different positions of the target spot (in automatic mode) were collected and analysed.

The raw spectra obtained from each isolate were imported into Biotyper software—version 3.0 (Bruker Daltonics GmbH, Leipzig, Germany, database version 3.3.1.0) and analysed by standard pattern matching with default settings without any user intervention. Scores of ≥ 2.0 were considered high-confidence (secure species) identification, scores between 1.7 and 2.0 were considered intermediate confidence (genus only) identification, and scores < 1.7 were considered unacceptable identification.

2.2. Isolation of DNA and amplification

The DNA was isolated from bacterial culture incubated in nutrient broth no. 2 at room temperature,

overnight. For the extraction of DNA the method of Pospiech and Neumann (1995) was used.

Polymerase chain reaction (PCR) was performed in an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Richmond, USA). Of about 1,500-bp amplicon of the 16S rRNA gene was amplified by PCR with universal primers fD1 (5'-AGAGTTTGAT CCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTG TTACGACTT-3') (Weisburg et al. 1991). The PCR mixture (50- μ L final volume) contained 1 $\times$ PCR buffer, 20 pmol of each primer, 2 mmol/L $MgCl_2$, 0.5 μ L of a 200 μ mol/L of each dNTP, 0.5 U Taq DNA polymerase (Invitrogen, UK) and 1 μ L total DNA. The PCR cycling conditions included an initial denaturation at 94 $^{\circ}$ C for 5 min, followed by 30 cycles of 94 $^{\circ}$ C for 1 min, 54 $^{\circ}$ C for 1 min, 72 $^{\circ}$ C for 1.30 min, and a final extension at 72 $^{\circ}$ C for 10 min. The PCR products were subjected to electrophoresis through 0.8 % (wt/vol) agarose gel which contained ethidium bromide and then visualised under UV light and recorded using Carestream Gel Logic 212 Pro (Carestream Health, NY, USA) documentation system.

2.3. RFLP analysis

Ten microlitres of PCR products were digested with selected restriction enzyme (*Bsp* 143I, *Bsu* RI or *Taq* I) for 1 h under conditions recommended by manufacturer (Fermentas, Germany). The DNA fragments were separated by electrophoresis through 1.5 % agarose gels and recorded as above.

2.4. Recombinant DNA techniques, 16S rRNA gene sequencing, sequences and statistical analyses

Amplified 16S rRNA gene fragments were purified by Wizard SV Gel and PCR Clean-Up purification kit (Promega) according to the manufacturer's instruction. Purified PCR products were ligated into pTZ57R/T plasmid (Fermentas, Germany), and *Escherichia coli* MC1061 (F⁻ araD139 Δ (ara-leu)7696 galE15 galK16 Δ (lac)X74 rpsL (Str^R) hsdR2 (rK⁻ mK⁺) mcrA mcrB1) competent cells were transformed with the ligation mixture. Transformation was carried out by the calcium chloride procedure as described by Maniatis et al. (1982). Recombinant plasmids were isolated with GenElute Plasmid Miniprep kit (Sigma-Aldrich), and the inserted DNA fragments were sequenced by

dideoxy chain termination sequencing method at GATC Biotech sequencing facility (GATC Biotech AG, Konstanz, Germany). The sequences obtained were deposited in the GenBank database under accession numbers KF975411-KF975415 (see Table I).

Isolates were identified using the EzTaxon-e server available at <http://eztaxon-e.ezbiocloud.net> (Kim et al. 2012). To analyse the phylogenetic relatedness of isolated bacteria, 16S rRNA sequences obtained were compared against the database containing 16S rRNA sequences of type strains with validly published prokaryotic names and representatives of uncultured phylotypes (Kim et al. 2012). The 10 sequences with the highest scores were then downloaded from EzTaxon-e server, compared using ClustalW alignment algorithm and phylogenetic trees were constructed using maximum parsimony algorithm implemented in MEGA 5 software (Tamura et al. 2011).

For statistical analyses, Species diversity and richness software version 4.1.2. (Pisces Conservation Ltd., UK) was used.

3. RESULTS

Relatively low variable population of bacteria was isolated from the surface of the body of *Varroa* mites. Cultivable bacteria were detected with frequency of about 150 cfu per mite individual. Very limited morphological variability of cultivable bacteria was observed when majority of the colonies had light yellow colour with round shape of the colony. The colour of the rest of the colonies was red when cultivated on nutrient agar (KK6 and KK8). Nine different bacterial colonies were chosen based on colony and cell morphology for further identification. Only three bacterial strains were identified reliably by MALDI-TOF analysis (using database version 3.3.1.0): KK3 as *Bacillus cereus*, KK8 as *Brevundimonas vesicularis* and KK7 as *Microbacterium* sp. Other isolates showed a lower score of reliability (Table I). Two isolates, KK1 and KK4, produced high-quality MALDI-TOF spectra but could be not identified by this method. Further identification showed that KK1 isolate is an oxidase negative Gram-positive coccus while KK4 isolate is an oxidase positive Gram-positive bacillus. Both isolates were positive for the catalase.

Table 1. Overview of *Varroa destructor* ecto-microflora identification.

Isolate	MALDI	RFLP type				16S rRNA		EzTaxon-e identification (best hit)	Similarity (%)	
		Biotype	Organism (best match)	Score Value	Bsp143I	BsuRI	TaqI			GenBank Accession Number
KK1	I		Not reliable identification	1.664	1	1	1	KF975411	<i>Microbacterium oxydans</i>	99.7
KK2	I		<i>Microbacterium maritpicum</i>	1.839	1	1	1	–	–	–
KK3	II		<i>Bacillus cereus</i>	2.347	2	2	2	–	–	–
KK4	III		Not reliable identification	1.486	3	3	3	KF975412	<i>Bacillus altitudinis</i>	99.8
KK5	IV		<i>Rhizobium radiobacter</i>	1.958	4	4	4	KF975413	<i>Agrobacterium tumefaciens</i>	99.7
KK6	V		<i>Brevundimonas vesicularis</i>	1.794	5	5	5	KF975414	<i>Brevundimonas vesicularis</i>	99.0
KK7	I		<i>Microbacterium</i> sp.	2.541	6	1	1	KF975415	<i>Microbacterium paraoxydans</i>	99.5
KK8	V		<i>Brevundimonas vesicularis</i>	2.035	5	5	5	–	–	–
KK9	I		<i>Microbacterium liquefaciens</i>	1.807	1	1	1	–	–	–

On the base of the similarity of protein profiles obtained by MALDI-TOF analysis, a dendrogram showing the relatedness of bacterial strains was constructed (Figure 1). Using this approach, five different biotypes of *Varroa* isolates were detected with a distance level over 500. This distance level is set arbitrary as a discriminatory level for the isolates of the same species. Three well-separated biotypes are represented by single isolate (KK3, KK4, KK5, resp.), another biotype was represented by two isolates identified as *Brevundimonas vesicularis*, and the last biotype included the group of four isolates belonging to the *Microbacterium* genus. Based on MALDI profile comparison isolates KK1, KK2, and KK9 produced practically identical protein profiles but were identified to three different species, although in all cases with low similarity score, suggesting secure genus identification only. The isolate KK7 was placed outside this branch with a relatively low distance suggesting that KK1, KK2, KK7 and KK9 isolates belong to the same genus.

RFLP analysis of amplified 16S rRNA gene was used for further analysis of phylogenetic relatedness of all isolates. 16S rRNA PCR products were digested with three different restriction enzymes and banding patterns were compared. Isolate-specific banding pattern was observed for KK3, KK4 and KK5 isolates. Similarly, KK6 and KK8 isolates produced identical profiles, different from all other isolates. RFLP analysis showed that the group of *Microbacterium*-related isolates could be split in two groups in consistency with the MALDI data. The RFLP analysis of KK7 isolate yielded identical profiles like KK1, KK2, and KK9 isolates using BsuRI and TaqI endonucleases, but produced a different profile after digestion with restriction enzyme BspI43I (Table I, Figure 2).

Five strains (KK1, KK4, KK5, KK6 and KK7) were selected based on their MALDI biotype, so that each biotype was represented, to perform a 16S rRNA sequence analysis. The obtained partial 16S rRNA gene sequences were subjected to EzTaxon-e comparisons to identify sequences of the highest similarity. Molecular analysis based on 16S rRNA sequence comparisons allowed identification of all analysed bacteria (Table I). In case of KK6 isolate, 16S RNA analysis identification

confirmed an identification obtained by MALDI approach. Both methods identified the isolate as a *Brevundimonas vesicularis*. The KK5 isolate identified as a *Rhizobium radiobacter* using both MALDI-TOF analysis and 16S rRNA analysis. The KK4 isolate showed 99.8 % similarity at 16S rRNA level to several species of *Bacillus* genus—*Bacillus altitudinis*, *Bacillus aerophilus*, and *Bacillus stratosphericus*; and over 99 % of similarity to *Bacillus pumillus* and *Bacillus safensis*. To evaluate phylogenetic relatedness of KK4 isolate to other members of *Bacillus* genus, a phylogenetic tree was constructed (Figure 3a). The analysis showed that 16S rRNA sequence of KK4 isolate is placed within branch of *Bacillus altitudinis*, *Bacillus aerophilus* and *Bacillus stratosphericus* isolates. However, 16S rRNA sequence of KK4 isolate formed a well supported sub-branch and KK4 isolate could be possibly better described as a new species of *Bacillus* genus.

Isolate KK1 non-identifiable by MALDI-TOF analysis showed the highest similarity at 16S rRNA level to a group of cross-related *Microbacterium* species: 99.7 % to *Microbacterium maritropicum*, 99.7 % to *Microbacterium oxydans*, 99.4 % to *Microbacterium liquefaciens*, *Microbacterium paraoxydans* and *Microbacterium luteolum* and 99.3 % to *Microbacterium saperdae*. Multiple sequence comparison placed KK1 isolate to the well-separated branch formed by *Microbacterium maritropicum*, *Microbacterium oxydans*, and *Microbacterium liquefaciens* species (Figure 3b). Similarly to the KK4 isolate, the 16S rRNA sequence of KK1 isolate is separated from all three species forming a specific sub-branch, thus KK1 isolate possibly represents a new species of *Microbacterium* spp. The KK7 isolate identified at a genus level by MALDI-TOF only, showed the highest similarity (99.5 % at 16S rRNA level) to *Microbacterium paraoxydans* species and phylogenetic analysis confirmed this relatedness.

4. DISCUSSION

For the first time, the ecto-microflora associated with *Varroa* mite was analysed using

Figure 1. Dendrogram showing the relatedness of isolates from the body surface of *Varroa* mite based on comparisons of the mass spectra obtained with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The dendrogram was constructed with Biotyper 2.0 software (Bruker Daltonics, Bremen, Germany) using Euclidean distance.

cultivation approach. Several species of Gram-positive and Gram-negative bacteria occurring on mite's body surface were isolated and most of them are reported for the first time in connection with *Varroa* or with honey bees. Shannon diversity index of the heterotrophic bacteria population

associated with *Varroa* mite was 1.68, suggesting relatively low diversity. Small bodies of the insect usually host a limited number of species (Shi et al. 2010). However, our results probably do not cover complete variability of *Varroa* mite ecto-microflora, as estimated number of species is up

Figure 2. The most parsimonious non-rooted consensus tree showing relatedness of selected isolates from the body surface of *Varroa* mite. The percentage of parsimonious trees in which the associated taxa clustered together are shown next to the branches (only values over 60 are shown). The sequences obtained in this study are shown in **bold**. **a** Phylogenetic relatedness of KK4 isolate. **b** Phylogenetic relatedness of KK1 and KK7 isolates.

Figure 3. Restriction fragment length of amplified 16S rRNA of *Varroa* mite isolates belonging to the *Microbacterium* genus. **a** Cleavage by TaqI restriction endonuclease. **b** Cleavage by Bsp143I restriction endonuclease. Lane M marker of molecular weight.

to 14. Also, the sampling was done only on two different colonies of honeybees, which represent a limited picture of variability of *Varroa* mite-associated heterotrophic microflora.

Nine selected isolates were classified to four genera and six species by combination of MALDI-TOF and 16S rRNA analysis. MALDI-TOF protein spectroscopy is a modern, powerful, rapid, precise and cost-effective method for identification of bacteria, based on comparison of protein profiles to the appropriate database (van Belkum et al. 2012). As with other identification systems, the reliability of identification is based on the availability of comprehensive reference spectra databases. The MALDI-TOF MS was originally applied for rapid identification of clinically important bacteria and is still used mainly for this purpose, but many environmental strains of bacteria are absent from MALDI Biotyper database. 16S rRNA-based identification confirmed that bacteria non-identifiable by MALDI-TOF approach are not included in MALDI Biotyper database version 3.3.1.0, used for identification of isolates, which would explain why no reliable identification was obtained.

The *Varroa* mite ecto-bacterial population was found to be dominated by Gram-positive bacteria of *Bacillus* and *Microbacterium* genera. Gram-negative bacteria were represented by members of *Brevundimonas* and *Rhizobium* genera. Prevalence of Gram-positive bacteria could be possibly caused by selected cultivation medium nutrient agar no. 2, composition of this medium, cultivation conditions or other factors.

Two isolates were identified as *Bacillus* spp. Representatives of this genus are Gram-positive spore-forming bacilli, widespread in nature. The presence of *Bacillus* spp. in connection with the bees and their products such as honey and bee bread have already been reported (Gilliam and Valentine 1976; Gilliam 1978, 1979; Gilliam and Prest 1987; Alippi 1995; Alippi et al. 2004). Similarly to our study, the most frequently *B. cereus* species was detected. This species is known as a food-borne pathogen for human that produces various enterotoxins (Kotiranta et al. 2000).

Another isolate classified to the *Bacillus* genus was KK4, non-identifiable using MALDI-TOF analysis. Based on 16S rRNA gene sequence analysis this isolate is cross-related with species *Bacillus altitudinis*, *Bacillus aerophilus* and *Bacillus stratosphericus* with overall similarity more than 99 % at 16S rRNA level. The *Bacillus altitudinis*, *Bacillus aerophilus* and *Bacillus stratosphericus* species were recently isolated from cryogenic tubes used for collecting air samples from high altitudes (Shivaji et al. 2006). Multiple sequence comparison indicates that the KK4 isolate could be possibly a representative of new species.

The KK6 and KK8 strains were identified as *Brevundimonas vesicularis*. *Brevundimonas vesicularis* (formerly known as *Pseudomonas vesicularis* and *Corynebacterium vesiculare*) is an aerobic, non-spore-forming, Gram-negative bacillus. The representatives of this species had been isolated from the external environment (Davis et al. 1997; Morais and da Costa 1990; Beilstein and Dreiseikelmann 2006) and are also part of the skin microflora of man and may cause various infectious diseases (e.g., meningitis) (Yang et al. 2006; Shang et al. 2012). The presence of representatives of the genus *Pseudomonas* in honey has already been noted (Iurlina and Fritz 2005), but pseudomonads are not usual honey colonisers. More often,

they have been detected in the digestive tract of honey bees (Snowdon and Cliver 1996; Kacaniova et al. 2004), but never in connection with *Varroa* mite up to now.

The species *R. radiobacter* (formerly known as *Agrobacterium tumefaciens*, Young et al. (2001)) was represented by single isolate, KK5. It is aerobic, non-spore-forming, Gram-negative bacterium. *R. radiobacter* is a well-known soil-borne bacterium. In the environment, it occurs mainly as significant plant pathogens infecting a wide range of plants and causing crown gall disease (Bouzar et al. 1993; Escobar and Dandekar 2003), but is also known as the human pathogen (Egemen et al. 2012). The representatives of this species occur often on different kinds of plants so it's presumable they are transmitted on worker bee during pollination and subsequently transmitted onto mite. *R. radiobacter* has not yet been isolated from bees or *Varroa* mite.

Among *Varroa* mite ecto-microflora, members of the *Microbacterium* genus were most frequently detected. Microbacteria are Gram-positive, non-spore-forming bacteria which have been isolated from a variety of habitats, such as plants, soil as well as digestive tract of insects and animals or from various foods (Shivaji et al. 2004; Dworkin et al. 2006; Mounier et al. 2007; Gutiérrez et al. 2010; Hung et al. 2011). Members of the genus *Microbacterium* have been isolated especially from the digestive tract of fish (Ringø et al. 2008) and gut of larvae of various insect orders, such as Coleoptera, Diptera, Hemiptera and Lepidoptera (Indiragandhi et al. 2010; Apte-Deshpande et al. 2012; Vilanova et al. 2012) but not from Acarina. Strains belonging to the genus *Microbacterium* are also known as pathogens in humans (Funke et al. 1997).

In our work, four strains of *Microbacterium* spp. were identified. In difference to all other strains isolated from *Varroa* mite a clear discrepancy was observed between identification by MALDI-TOF and 16S rRNA-based analysis for these isolates. Three isolates were identified by MALDI-TOF analysis as *Microbacterium liquefaciens*, *Microbacterium maritopicum* and *Microbacterium* sp., respectively, the KK1 isolate was not reliably identified by this method. However, comparison of protein profiles indicated that three isolates form a separate group with

practically identical spectra, despite being identified to three different species. RFLP analysis confirmed grouping based on MALDI-TOF protein spectra comparison. KK1, KK2 and KK9 isolates produced identical restriction profiles different from KK7 isolate profile. On the bases of nearly complete 16S rRNA sequence comparisons of two isolates (KK1 and KK7) the KK7 was found to be closely related to a group consisting of *Microbacterium oxydans*, *Microbacterium maritopicum* and *Microbacterium liquefaciens* with similarity level over 99.4 % at 16S rRNA level. However, multiple sequence comparison indicated that KK1 isolate is possibly a new species of *Microbacterium* genus.

The group of three isolates represented by the KK1 isolate showed the highest similarity to the 16S rRNA sequence of *Microbacterium paraoxydans* (99.5 %). Phenotypic, chemotaxonomic and genetic studies suggest that *Microbacterium paraoxydans* is closely related to *Microbacterium oxydans*, *Microbacterium saperdae*, *Microbacterium luteolum* and *Microbacterium liquefaciens* (Laffineur et al. 2003). Both *Microbacterium oxydans* and *Microbacterium paraoxydans* belong to the most frequently isolated microbacteria (Laffineur et al. 2003) and were isolated from contaminated hospital material, but neither of the species was found to be associated with honey, honey bees or *V. destructor*, despite being common in environment.

This work was focused on *Varroa* mite as a possible vector of bee pathogens, but no recognised bee pathogens were detected. The mite is mainly considered as a transmitter of viruses. The transmission of other microorganisms (bacteria, fungi) by *Varroa* mite has not been established, although several investigations have been carried out (Alippi et al. 1995; De Rycke et al. 2002; Benoit et al. 2004). The transmission, however, was observed in artificially infected *Varroa* mite that transmitted bacterial infections to recipient bees (Gliński and Jarosz 1992). Several pathogenic bacteria were isolated from the surface of mite, e.g. representatives of *Melissococcus pluton*, causal agent of European foulbrood, spores of *Paenibacillus larvae*, causal agent of American foulbrood (AFB), *Serratia marcescens*, *Pseudomonas apiseptica*, and *Pseudomonas aeruginosa* are involved in septicemic

infections of both adult bees and brood (Ball 1997). However, Alippi et al. (1995) found spores of *Paenibacillus larvae* on *Varroa jacobsoni* but authors did not consider that the mite can be responsible for AFB transmission from infected to healthy colonies. They presumed that the number of spores transmitted is not sufficient to cause infection, because there is no probability that the mite can contaminate the larval food by contact. De Rycke et al. (2002), on other hand, argued that mites enter larval cells and swim in the worker jelly and this way, can transmit AFB; whether *Varroa* mite is a carrier of pathogenic bacteria or not, the effect of its parasitism is detrimental on infected hives.

The body surface of the mite is not the only place where bacteria could reside. Salivary glands, intestine and haemolymph are also colonised by bacteria (Ball 1997). For that reason, other investigations targeted to endo-microflora of *Varroa destructor* are necessary to completely understand the *Varroa* mite role in the spreading of bacteria in honey bee populations.

ACKNOWLEDGMENTS

This work was supported by the Slovak Grant Agency VEGA, grant no. VEGA 2/0016/12.

Bactéries hétérotrophes associées à *Varroa destructor*

Varroa destructor / Acari / *Microbacterium* / microflora externe / hétérotrophie

Die Assoziation heterotropher Bakterien mit der Milbe *Varroa destructor*

Varroa destructor / Ekto-Mikroflora / *Microbacterium* / heterotrophe Bakterien

REFERENCES

- Alippi, A.M. (1995) Detection of *Bacillus larvae* spores in Argentinian honeys by using a semi-selective medium. *Microbiologia*. **11** (3), 343–350
- Alippi, A.M., Albo, G.N., Marcangeli, J., Leniz, D., Noriega, A. (1995) The mite *Varroa jacobsoni* does not transmit American foulbrood from infected to healthy colonies. *Exp. Appl. Acarol.* **19** (10), 607–613
- Alippi, A.M., Reynaldi, F.J., López, A.C., De Giusti, M.R., Aguilar, O.M. (2004) Molecular epidemiology of *Paenibacillus larvae larvae* and incidence of American foulbrood in Argentinean honeys from Buenos Aires Province. *J. Apic. Res.* **43** (3), 135–143
- Apte-Deshpande, A., Paingankar, M., Gokhale, M.D., Deobagkar, D.N. (2012) *Serratia odorifera* a midgut inhabitant of *Aedes aegypti* mosquito enhances its susceptibility to dengue-2 virus. *PLoS ONE*. doi:10.1371/journal.pone.0040401
- Ball, B.V. (1985) Acute paralysis virus isolated from honeybee colonies infested with *Varroa jacobsoni*. *J. Apic. Res.* **24**, 115–119
- Ball, B.V. (1997) Secondary infections and diseases associated with *Varroa jacobsoni*. *Options Méditerranéennes*. **21**, 49–58
- Beilstein, F., Dreiseikelmann, B. (2006) Bacteriophages of freshwater *Brevundimonas vesicularis* isolates. *Res. Microbiol.* **157** (3), 213–219
- Benoit, J.B., Yoder, J.A., Sammataro, D., Zettler, L.W. (2004) Mycoflora and fungal vector capacity of the parasitic mite *Varroa destructor* (Mesostigmata: Varroidae) in honey bee (Hymenoptera: Apidae) colonies. *Int. J. Acarol.* **30** (2), 103–106
- Berényi, O., Bakonyi, T., Derakhshifar, I., Köglberger, H., Nowotny, N. (2006) Occurrence of six honeybee viruses in diseased Austrian apiaries. *Appl. Environ. Microbiol.* **72** (4), 2414–2420
- Boecking, O., Genersch, E. (2008) Varroosis - the ongoing crisis in bee keeping. *J. Verbr. Lebensm.* **3** (2), 221–228
- Bouzar, H., Jones, J.B., Hodge, N.C. (1993) Differential characterization of *Agrobacterium* species using carbon source utilization pattern and fatty acid profiles. *Phytopathology* **83** (7), 733–739
- Bowen-Walker, P.L., Gunn, A. (2001) The effect of the ectoparasitic mite, *Varroa destructor* on adult worker honeybee (*Apis mellifera*) emergence weights, water, protein, carbohydrate, and lipid levels. *Entomol. Exp. Appl.* **101**, 207–217
- Bowen-Walker, P.L., Martin, S.J., Gunn, A. (1999) The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. *J. Invertebr. Pathol.* **73** (1), 101–106
- Chen, Y.P., Siede, R. (2007) Honey bee viruses. *Adv. Virus Res.* **70**, 33–80
- Chen, Y.P., Pettis, J.S., Evans, J.D., Kramer, M., Feldlaufer, M.F. (2004) Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. *Apidologie*. **35** (4), 441–448
- Davis, E.P., Boopathy, R., Manning, J. (1997) Use of trinitrobenzene as a nitrogen source by *Pseudomonas vesicularis* isolated from soil. *Curr. Microbiol.* **34** (3), 192–197
- De la Rúa, P., Jaffé, R., Dall'Olio, R., Muñoz, I., Serrano, J. (2009) Biodiversity, conservation and current threats to European honeybees. *Apidologie* **40** (3), 263–284
- De Rycke, P.H., Joubert, J.J., Hosseini, S.H., Jacobs, F.J. (2002) The possible role of *Varroa destructor* in the

- spreading of American foulbrood among apiaries. *Exp. Appl. Acarol.* **27** (4), 313–318
- Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E. (2006) *The Prokaryotes: A Handbook on the Biology of Bacteria*. Springer, Berlin
- Egemen, O., Ozkaya, O., Bingol, D., Akan, M. (2012) *Rhizobium radiobacter*: an unusual pathogen isolated on an active chronic ulcerous inflammation. *J. Plast. Reconstr. Aes.* **65** (8), 233–235
- Escobar, M.A., Dandekar, A.M. (2003) *Agrobacterium tumefaciens* as an agent of disease. *Trends Plant Sci.* **8** (8), 380–386
- Ferreira, L., Sánchez-Juanes, F., García-Fraile, P., Rivas, R., Mateos, P.F., et al. (2011) MALDI-TOF Mass Spectrometry Is a Fast and Reliable Platform for Identification and Ecological Studies of Species from Family Rhizobiaceae. *PLoS ONE* **6** (5), e20223
- Funke, G., Haase, G., Schnitzler, N., Schrage, N., Reinert, R.R. (1997) Endophthalmitis due to *Microbacterium* species: case report and review of microbacterium infections. *Clin. Infect. Dis.* **24** (4), 713–716
- Genersch, E. (2010) American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *J. Invertebr. Pathol.* **103** (suppl. 1), 10–19
- Gilliam, M. (1978) Bacteria belonging to the genus *Bacillus* isolated from selected organs of queen honey bees, *Apis mellifera*. *J. Invertebr. Pathol.* **31** (3), 389–391
- Gilliam, M. (1979) Microbiology of pollen and bee bread: the genus *Bacillus*. *Apidologie* **10** (3), 269–274
- Gilliam, M., Prest, D.B. (1987) Microbiology of feces of the larval honey bee, *Apis mellifera*. *J. Invertebr. Pathol.* **41** (1), 70–75
- Gilliam, M., Valentine, D.K. (1976) Bacteria isolated from the intestinal contents of foraging worker honey bees, *Apis mellifera*: the genus *Bacillus*. *J. Invertebr. Pathol.* **28** (2), 275–276
- Gliński, Z., Jarosz, J. (1992) *Varroa jacobsoni* as a carrier of bacterial infections to a recipient bee host. *Apidologie* **23** (1), 25–31
- Gutiérrez, A.M., Cabrales, J.J.P., Vega, M.M. (2010) Isolation and characterization of hexavalent chromium-reducing rhizospheric bacteria from a wetland. *Int. J. Phytoremediation* **12** (4), 317–334
- Hung, W.L., Wade, W.G., Boden, R., Kelly, D.P., Wood, A.P. (2011) Facultative methylotrophs from the human oral cavity and methylotrophy in strains of *Gordonia*, *Leifsonia*, and *Microbacterium*. *Arch. Microbiol.* **193** (6), 407–417
- Indiragandhi, P., Yoon, C., Yang, J.O., Cho, S., Sa, T.M., Kim, G.H. (2010) Microbial communities in the developmental stages of B and Q biotypes of sweet potato whitefly, *Bemisia tabaci* (hemiptera: Aleyrodidae). *J. Korean Soc. Appl. Biol. Chem.* **53** (5), 605–617
- Iurlina, M.O., Fritz, R. (2005) Characterization of microorganisms in Argentinean honeys from different sources. *Int. J. Food Microbiol.* **105** (3), 297–304
- Kacaniova, M., Chlebo, R., Kopernicky, M., Trakovicka, A. (2004) Microflora of the honeybee gastrointestinal tract. *Folia Microbiol.* **49** (2), 169–171
- Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., et al. (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* **62** (3), 716–721
- Kotiranta, A., Lounatmaa, K., Haapasalo, M. (2000) Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infect.* **2** (2), 189–198
- Laffineur, K., Avesani, V., Cornu, G., Charlier, J., Janssens, M., Wauters, G., Delmée, M. (2003) Bacteremia due to a novel *Microbacterium* species in a patient with leukemia and description of *Microbacterium paraoxydans* sp. nov. *J. Clin. Microbiol.* **41** (5), 2242–2246
- Le Conte, Y., Ellis, M., Ritter, W. (2010) *Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses? *Apidologie* **41** (3), 353–363
- Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York
- Morais, P.V., da Costa, M.S. (1990) Alterations in the major heterotrophic bacterial populations isolated from a still bottled mineral water. *J. Appl. Bacteriol.* **69** (5), 750–757
- Mounier, J., Rea, M.C., O'Connor, P.M., Fitzgerald, G.F., Cogan, T.M. (2007) Growth characteristics of *Brevibacterium*, *Corynebacterium*, *Microbacterium*, and *Staphylococcus* spp. isolated from surface-ripened cheese. *Appl. Environ. Microbiol.* **73** (23), 7732–7739
- Nazzi, F., Brown, S.P., Annoscia, D., Piccolo, F.D., Prisco, G.D., Varricchio, P., Vedova, G.D., Cattonaro, F., Caprio, E., Pennacchio, F. (2012) Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathog.* doi:10.1371/journal.ppat.1002735
- Oldroyd, B.P. (1999) Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees. *Trends Ecol. Evol.* **14** (8), 312–315
- Pospiech, A., Neumann, B. (1995) A versatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends Genet.* **11** (6), 217–218
- Reddy, C.A., Beveridge, T.J., Breznak, J.A., Marzluf, G.A., Schmidt, T.M., Snyder, L.R. (2007) *Methods for General and Molecular Microbiology*. ASM Press, Washington DC. ISBN: 1555812236
- Ringø, E., Sperstad, S., Kraugerud, O.F., Kroghdahl, Å. (2008) Use of 16S rRNA gene sequencing analysis to characterize culturable intestinal bacteria in Atlantic salmon (*Salmo salar*) fed diets with cellulose or non-starch polysaccharides from soy. *Aquat. Res.* **39** (10), 1087–1100
- Rosenkranz, P., Aumeier, P., Ziegelmann, B. (2009) Biology and control of *Varroa destructor*. *J. Invertebr. Pathol.* **103** (suppl. 1), 96–119

- Shang, S.T., Chiu, S.K., Chan, M.C., Wang, N.C., Yang, Y.S., Lin, J.C., Chang, F.Y. (2012) Invasive *Brevundimonas vesicularis* bacteremia: Two case reports and review of the literature. *J. Microbiol. Immunol. Infect.* **45** (6), 468–472
- Shen, M.Q., Cui, L.W., Ostiguy, N., Cox-Foster, D. (2005) Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. *J. Gen. Virol.* **86** (8), 2281–2289
- Shi, W., Syrenne, R., Sun, J.Z., Yuan, J.S. (2010) Molecular approaches to study the insect gut symbiotic microbiota at the ‘omics’ age. *Insect Sci.* **17** (3), 199–219
- Shivaji, S., Reddy, G.S., Aduri, R.P., Kutty, R., Ravenschlag, K. (2004) Bacterial diversity of a soil sample from Schirmacher Oasis, Antarctica. *Cell Mol. Biol.* **50** (5), 525–536
- Shivaji, S., Chaturvedi, P., Suresh, K., Reddy, G.S.N., Dutt, C.B.S., Wainwright, M., Narlikar, J.V., Bhargava, P.M. (2006) *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. *Int. J. Syst. Evol. Microbiol.* **56** (7), 1465–1473
- Snowdon, A.J., Cliver, D.O. (1996) Microorganisms in honey. *Int. J. Food Microbiol.* **31** (1–3), 1–26
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nie, M., Kumar, S. (2011) MEGA5: molecular evolution-ary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28** (10), 2731–2739
- van Belkum, A., Welker, M., Erhard, M., Chatellier, S. (2012) Biomedical mass spectrometry in today’s and tomorrow’s clinical microbiology laboratories. *J. Clin. Microbiol.* **50** (5), 1513–1517
- Vilanova, C., Marco, G., Domínguez-Escribà, L., Genovés, S., Sentandreu, V., Bataller, E., Ramón, D., Porcar, M. (2012) Bacteria from acidic to strongly alkaline insect midguts: potential sources of extreme cellulolytic enzymes. *Biomass Bioenerg.* **45**, 288–294
- Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173** (2), 697–703
- Yang, M.L., Chen, I.H., Chen, T.C., Lin, W.R., Lin, C.Y., Lu, P.L. (2006) Case report: infective endocarditis caused by *Brevundimonas vesicularis*. *BMC Infect. Dis.* **6**, 179
- Young, J.M., Kuykendall, L.D., Martinez-Romero, E., Kerr, A., Sawada, H. (2001) A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *Int. J. Syst. Evol. Microbiol.* **51** (1), 89–103