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Characterization of selected Gram-negative non-fermenting bacteria isolated from honey bees (*Apis mellifera carnica*)

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Abstract – This study was conducted to improve the knowledge about bacteria associated with honey bees, *Apis mellifera carnica*. In this survey, the diversity of Gram-negative non-fermenting bacteria isolated and cultivated from pollen loads, honey sac, freshly stored nectar, and honey was investigated. Bacteria were characterized by a polyphasic approach. Based on morphological and physiological characteristics and comparison of isolates protein patterns after sodium dodecyl sulfate–polyacrylamide gel electrophoresis, 11 protein similarity groups were established and confirmed by enterobacterial repetitive intergenic consensus PCR. One isolate, representing a protein similarity group (representative strain), was characterized in more detail by analysis of respiratory quinones, amplified ribosomal DNA restriction analysis, and 16S rDNA sequence analysis. Based on the results of these examinations, seven representative strains were identified as members of the genus *Pseudomonas*. The remaining representative strains were allocated to the genera *Acinetobacter*, *Chryseobacterium*, *Stenotrophomonas*, and *Commamonas*.

bacterial diversity / PCR / honey / honey bees / 16S rDNA gene

1. INTRODUCTION

Honey bees, like other social insects, harbor a wide variety of bacteria, due to the high density of individuals within the colony, the sharing of food, and the coexistence of colony members from multiple generations (Evans and Armstrong 2006; Loncaric et al. 2009; Kačániová et al. 2009).

Until the present, there are only a few reports about the non-pathogenic bacterial flora associated with honey bees and honey. Snowdon and Cliver (1996) and Gilliam (1997) reviewed about microorganisms in honey and the role of non-pathogenic microbiota, respectively. Rada et al. (1997) reported about the microorganism in the honey bee digestive tract. In that study, the microorganisms were enumerated and characterized by means of classical phenotyping. Anaerobic and aerobic microorganisms, lactobacilli, coliforms, staphylococci, *Bacillus* sp, and yeast

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were found. Only one out of 31 isolates was identified (*Bifidobacterium asteroides*). Comparable results were published by Kačániová et al. (2004). In contrast to the study of Rada et al. (1997), Kačániová et al. (2004) did not detect lactobacilli and staphylococci. Piccini et al. (2004) described an approach for the characterization of the cultivable heterotrophic bacterial flora of honey bee hives. Lyapunov et al. (2008) studied enterobacteria of normal intestinal microbiota in *Apis mellifera mellifera* L. bees hibernating under snow in the Western Urals, whereas several bacterial groups were identified and enumerated. Very recently, Kačániová et al. (2009) isolated and enumerated different bacterial groups from gastrointestinal tract, honey, and pollen. Characterization of bacteria in that study was carried out using classical phenotyping. Molecular tools were used only for detection of *Clostridium botulinum* and *Paenibacillus larvae*. Some other investigations dealing with bacterial diversity in honey bee colonies were obtained using molecular tools or its combination with classical phenotyping. Jeyaprakash et al. (2003) reported about bacterial diversity in worker adults of *A. mellifera capensis* and *A. mellifera scutellata* in South Africa using 16S rRNA sequence analysis, but without cultivation of any bacteria. Babendreier et al. (2007) investigated the effects of the Bt-toxin Cry1Ab and a soybean trypsin inhibitor on intestinal bacterial communities of adult honey bees applying culture-independent terminal restriction fragment length polymorphism followed by 16S rRNA gene sequencing. Recently, Mohr and Tebbe (2006, 2007) compared bacterial diversity in the guts of *A. mellifera* ssp. *carnica* and *Bombus bicornis* (bumblebee) at an oilseed rape field and risk of a horizontal gene transfer mediated by transport of transgenic herbicide-resistant oilseed rape pollen in the gut bacteria of *A. mellifera carnica*, *B. bicornis*, and *Osmia bicornis* (red mason bee).

Evans and Armstrong (2006) reported about antagonistic interactions between honey bee bacterial symbionts and possible implications for disease tolerance and resistance, respectively. Based on sequence similarity, using 16S rRNA sequence analysis, isolates ($n=61$) from

honey bee larvae were placed into four bacterial genera: *Acinetobacter*, *Bacillus*, *Brevibacillus*, and *Stenotrophomonas*.

During the last two decades (Klingauf 2006), there has been a growing interest to use antagonistic microorganisms (e.g., *Pantoea agglomerans*, *Pseudomonas fluorescens*, and fungus *Aureobasidium pullulans* (Cañamás et al. 2007; Pujol et al. 2007; Lončarić et al. 2008)) for the biological control of some plant pathogenic bacteria (e.g., *Erwinia amylovora*, fire blight). The honey bees have been used as a vector for establishment of these three antagonistic of *E. amylovora* in apple and pear orchards (Thomson et al. 1992; Lončarić et al. 2008). *A. pullulans* and *P. agglomerans* are commonly found in honey bee's environment (Lončarić et al. 2008, 2009). There are also other bacteria found in the honey bee environment including members of *Burkholderia* (Palumbo et al. 2007), *Acinetobacter* (Evans and Armstrong 2006; Liu et al. 2007), *Pseudomonas* (de Werra et al. 2008), and *Stenotrophomonas* (Evans and Armstrong 2006; Romanenko et al. 2008) that exhibit antagonistic activity against several different pathogens such as mycotoxicogenic fungi or *P. larvae*.

Some of these antagonists produce natural antibiotics such as *P. agglomerans* (Giddens et al. 2003). With respect to the possible role of the natural microflora and the discussion about possible risks of biological plant protection agents for bee health, it is necessary to gather more basic knowledge about the natural microflora of honey bees and beehives. To the best of our knowledge, there are no reports dealing with the characterization of Gram-negative non-fermenting bacteria, which were isolated from pollen loads, honey sac, freshly stored nectar, and honey.

The aim of our study was to contribute to the knowledge of diversity of Gram-negative non-fermenting bacterial isolates from the aforementioned sources. Macromorphological and physiological characteristics, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins, analyses of respiratory quinones, amplified ribosomal

DNA restriction analysis (ARDRA), and 16S rDNA sequence analysis were used to characterize selected isolates from Austrian apiaries.

2. MATERIAL AND METHODS

2.1 Bacterial isolates

Honey bees (*A. mellifera carnica*) were collected from two bee hives (hives 169 and 186) of the Institute for Apiculture, Austrian Agency for Health and Food Safety, from April to October 2003. Freshly stored nectar (FSN) was collected during the same period. Adult honey bee workers were collected at the hive entrance and killed by freezing at -25°C for at least 20 min.

For preparation of honey sacs, the abdomen was separated from the thorax of the frozen bees. After thawing, the first and second abdominal segments were removed using two pairs of #5 Dumont forceps (A. Dumont & Fils, Autils, Switzerland) to expose the honey sac. Only full honey sacs were tested. The honey sac was then removed and placed in a sterile 15-mL glass tube. The bulk sample of 40 full honey sacs was mixed with 3 mL of 0.9% NaCl and vortexed for 60 s. Two hundred microliters of this mixture was transferred on blood agar (Columbia blood agar base (Oxoid), supplemented with 5% defibrinated sheep blood) and peptone–yeast–extract (PYE) agar (per liter: 3.0 g pepton from casein (ROTH), 3.0 g yeast extract (Merck), 15.0 g agar (Oxoid), pH 7.2) and incubated at 28°C for 48 h. For the isolation of bacteria from pollen loads, honey bees were collected at the same time as for extraction of honey sacs. Thirty pollen loads were mixed with 3 mL of 0.9% NaCl and vortexed for 60 s. One hundred microliters of this mixture was diluted with 900 μL of 0.9% NaCl, and 100 μL of dilution was plated and incubated as described for honey sacs. Isolation of bacteria from FSN: Before sampling, sweetness test of FSN was performed by testing of three drops of collected fluid. Positive reaction was characterized if the drops were sweet and negative if the drops were not sweet. If sweetness test was positive, one sample per hive was collected and monitored for bacteria. A total of 5 mL of FSN was sampled from nectar-filled cells from the brood nest area, or from cells of the honey stores surrounding

the brood area of a comb. All samples were taken from different combs. Five milliliters of collected nectar was mixed with 5 mL of sterile water and vortexed for 30 s. Two hundred microliters of diluted nectar was directly inoculated on blood agar plates and on PYE agar and incubated at 28°C for 48 h. For the isolation of bacteria from honey, the method described by Bakonyi et al. (2003) to detect the causative agent of American foulbrood (*P. larvae*) was slightly modified to keep non-spore-forming bacteria alive and germinable. Two hundred microliters of diluted honey was directly inoculated on two blood agar plates. The plates were incubated as described for honey sacs.

2.2 Phenotyping of bacterial isolates

To identify significant groups, isolates showing obvious similarities in colony morphology and pigmentation were picked from blood agar plates, subcultivated on PYE, and incubated at 28°C for 48 h and subjected to the following tests for preliminary selection of Gram-negative non-fermenting bacteria from other bacteria: Gram reaction was performed as described by Gerhard et al. (1994); cell morphology was studied under a light microscope ($\times 1,000$ magnification); to identify the bacteria by their ability to ferment or oxidize glucose, glucose oxidation–fermentation test was conducted in Cap-o-test (Milian) tubes containing 2 mL of Hottinger bouillon (per liter: 10.0 g pepton from casein (ROTH), 2.5 g NaCl, 1.25 g K_2HPO_4 , 40 ml bromothymol blue 0.1%, pH 8.0) and 1 mL of 1% glucose. For confirming whether isolated bacteria are non-spore-forming, a medium was applied to induce sporulation (per liter: 5.0 g pepton from casein (ROTH), 3.0 g yeast extract (Merck), 3.0 g meat extract (Merck), 0.1 g MnSO_4 , 10 g NaCl, 15.0 g agar (Oxoid), pH 7.0). Bacteria were incubated at 28°C for 120 h. The staining technique employed was the Schaeffer–Fulton method as described by Gerhard et al. (1994). Isolates, which are non-spore-forming rods, stained Gram-negative, and could not produce acid from glucose, were further examined: Oxidase activity by oxidase activity test (Bactident-Oxidase test strips (Merck)), which was performed according to the manufacturer instructions; catalase activity test was conducted using 3% H_2O_2 ; reduction of nitrate to nitrite test

was carried out in nitrate broth (Merck) and incubated, after incubation equal volumes of API test reagents (Bio Merieux) NIT1 and NIT2 were added and evaluated; tests for bacterial growth on various media included trypticase soy agar (Oxoid), Nutrient agar (Difco), and *Pseudomonas* F (Difco).

2.3 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of whole-cell proteins

The SDS-PAGE was performed as described by Loncaric et al. 2009. Groups with similar protein patterns consisting of at least three isolates were subjected to analyses of respiratory quinones (representative strains only) and further genotypic characterization (enterobacterial repetitive intergenic consensus (ERIC)-PCR, ARDRA, 16S rDNA sequence analysis).

2.4 Analyses of respiratory quinones

Analyses of respiratory quinones of representative strains were done as described by Tindall (1990).

2.5 Genetic characterization

Bacterial DNA extraction was as described by Loncaric et al. 2009. Primers used for ERIC-PCR were as described by Versalovic et al. (1991). The PCR reaction was set up in a GeneAmp 2400 PCR System Thermocycler (Applied Biosystem) in a 15- μ L reaction volume containing 7.5 μ L of a REDTaq ReadyMix PCR Reaction Mix (20 mM Tris/HCl, 100 mM KCl, 3 mM MgCl₂, 0.4 mM dNTP), with 0.45 U of Taq DNA polymerase (Sigma), 10–20 ng of template DNA, and 10 pmol of each primer for ERIC-PCR. DNA amplifications were performed with an initial denaturation (7 min at 95°C) followed by 30 cycles of denaturation (1 min at 94°C), annealing 1 min at 52°C, and extension 8 min at 65°C with a final extension 15 min at 65°C. PCR products were analyzed by electrophoresis in 1.5% agarose gel in TAE buffer run at 5 V/cm with 100-bp ladder and detected by staining with ethidium bromide (0.5 μ g mL⁻¹) under UV light and photographed.

The 16S rRNA genes of representative strains were amplified with universal primers 27f and 1492r

(Lane 1991). Amplification reactions were performed in a total volume of 20 μ L containing 10 μ L of a REDTaq ReadyMix PCR Reaction Mix (20 mM Tris/HCl, 100 mM KCl, 3 mM MgCl₂, 0.4 mM dNTP), with 0.6 U of Taq DNA polymerase (Sigma), 15–20 ng of template DNA, and 10 pmol of each primer. All reaction mixtures were amplified in a thermal cycler (Applied Biosystems GeneAmp PCR System 2700) for 5 min at 95°C and then subjected to 30 amplification cycles of 1 min at 94°C, annealing 1 min at 50°C, and 1.5 min at 72°C, followed by a final extension step of 7 min at 72°C. PCR products were digested with restriction enzymes *Hha* I and *Msp* I as recommended by the manufacturer (Promega). After restriction digestion, the DNA products were separated on a 2.5% agarose gel in TAE buffer and detected as described for the ERIC-PCR. For sequence analysis, the 16S rRNA was amplified in a total volume of 100 μ L. The DNA sequencing was carried out as described by Ruppitsch et al. (2007). Similarity searches with the obtained sequence were done in the EMBL database using the program FASTA (Pearson and Lipman 1988). The sequences were aligned with the most similar sequences of established bacterial species from the database. Phylogenetic analyses were conducted using the PHYLIP software package, version 3.68 (Felsenstein 2005) after multiple alignment data employing CLUSTALW (Chenna et al. 2003). Aligned sequences were corrected manually and nucleotide positions that contained ambiguities were removed from further analyses. The data were examined using maximum likelihood analysis (DNAML in PHYLIP). Bootstrap values were obtained for a consensus based on 100 randomly generated trees using SEQBOOT and CONSENSE. Tree figures were generated using Molecular Evolutionary Genetics Analysis version 4.0 software (Tamura et al. 2007).

3. RESULTS

3.1 Characteristics and analysis of whole-cell protein profiles by SDS-PAGE

Overall, 724 bacterial isolates were recovered from pollen loads, honey sac, freshly stored nectar, and honey. Only isolates ($n=104$) show

to be non-spore-forming rods, which stained Gram-negative and could not produce acid from glucose were studied in more details by SDS-PAGE, analyses of respiratory quinones, and genetic characterization. After comparison of protein profiles using SDS-PAGE, 51 isolates were grouped in 11 protein similarity groups (PSG1–PSG11; Figure 1). Each PSG consists of at least three isolates. Details of their characteristics are given in Table I.

3.2 Analyses of respiratory quinones

The results of the quinone analyses are shown in Table I. Eight representative strains contained ubiquinone 9 (Q-9), two representative strains contained ubiquinone 8 (Q-8), and one representative strain contained menaquinone MK-6.

3.3 ERIC-PCR analysis

Genomic fingerprinting of the strains after ERIC-PCR (Figure 2) provided profiles which

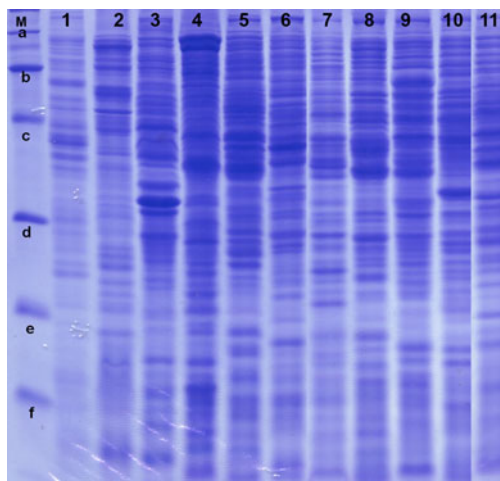


Figure 1. SDS-PAGE of the whole-cell proteins of the 11 representative strain of each protein similarity group: lane M molecular weight marker. Letters indicate molecular weight in kilodaltons: a (191), b (66), c (43), d (29), e (20), f (14.3). lane 1 NF4, 2 549, 3 NF27, 4 125, 5 NF1a, 6 323, 7 607, 8 NF20, 9 NF30, 10 477, 11 1043.

consisted of bands ranging from 0.2 to 5 kb. All strains of a protein similarity group shared several mutual bands or an identical fingerprint with the corresponding reference strain, confirming the grouping according to the protein profiles and indicating relatedness at the species level.

3.4 Amplified ribosomal DNA restriction analysis

After restriction endonuclease analysis of amplified 16S rRNA coding genes of representatives of each protein similarity groups using the enzyme *Hha* I six different profiles (1–6) were detected. Representative strains 477, NF30, NF20, 607, 323, and 1043 showed identical profiles consisting of bands at approximately 400, 350, 280, 250, and 200 bp. Representatives of the other five different PSG were characterized by a unique ARDRA pattern. ARDRA analysis using the enzyme *Msp* I did not discriminate strains 477, NF30, NF20, 607, 323, 1043, and NF1a (profile 1) but also enabled differentiation amongst isolates NF4, 549, NF27, and 125 (profiles 2–5; Table I).

3.5 16S rDNA sequence analysis

Sequence analysis of the 16S rRNA gene of the 11 representative strains of this study indicated similarities from 95.5% to 99.0% with corresponding sequences of type strains of established bacterial species. The majority of strains shared highest similarity scores with type strains of certain *Pseudomonas* species: strain 477 with *Pseudomonas parafulva* AJ 2129^T (99.5%), NF30 with *Pseudomonas poae* DSM 14936^T (99.4%), NF20 with *Pseudomonas graminis* DSM 11363^T (99.3%), 607 with *P. parafulva* AJ 2129^T (99.0%), 323 with *Pseudomonas argentinensis* LMG 22563^T (98.2%), NF1a with *Pseudomonas oryzihabitans* IAM 1568^T (98.7%), and 1043 with *Pseudomonas koreensis* KACC 10848^T (98.2%). Other representative strains were most closely related with members of genera *Chryseobacterium*, *Comamonas*, *Stenotrophomonas*, and *Acinetobacter*.

Table I. (continued).

PSG	Strain	Origin	Colony F/E/M/P	O	C	N	F	Q	ARDRA		GenBank accession no	Highest 16S rRNA gene similarity (%)
									<i>Hha I</i>	<i>Msp I</i>		
7	704	P 186										
	725	HB 186										
	323	P 169	c/c/e/y	+	+	+	(+)	Q-9	1	1	EF565941	<i>Pseudomonas argentinensis</i> LMG 22563^T (98.2%)
8	329	HB 186										
	1019	P 186										
	607	P 169	c/c/e/y	+	+	-	-	Q-9	1	1	EF565942	<i>Pseudomonas parafulva</i> AJ 2129^T (99.0%)
9	606	P 186										
	NF67	N 169										
	NF20	P 169	c/c/e/w	-	+	-	-	Q-9	1	1	EF565943	<i>Pseudomonas graminis</i> DSM 11363^T (99.3%)
10	1476	P 169										
	1521	P 186										
	NF30	N 186	c/c/e/y	(+)	+	-	+	Q-9	1	1	EF565944	<i>Pseudomonas poae</i> DSM 14936^T (99.4%)
11	NF212	P 186										
	NF507	P169										
	1043	P 169	c/c/e/y	+	+	-	+	Q-9	1	1	EF565945	<i>Pseudomonas koreensis</i> KACC 10848^T (98.2%)
	1042	P 186										
	1058	P 186										
	1125	N 186										
	1127	N 169										
	1367	P 169										

GenBank accession number for the 16S rRNA gene sequence. Data are given for strains only if they differ from representing strain within similarity group. The representative strain of each protein similarity group is shown in bold face

P pollen loads, *HB* honey sac, *N* freshly stored nectar, *H* honey (origin with hive identification number); *F* form, *E* elevation, *M* margin, *P* pigmentation (colony morphology and pigmentation); *O* oxidase activity, *C* catalase activity, *N* reduction nitrate to nitrite, *F* production of fluorescence on *Pseudomonas* F agar (physiological characteristics); + positive, (+) weakly positive, - negative

^a Form—circular

^b Elevation—convex

^c Margin—entire

^d Pigmentation—gray

^e Pigmentation—yellow

^f Pigmentation—white

^g Form—irregular

^h Elevation—raised

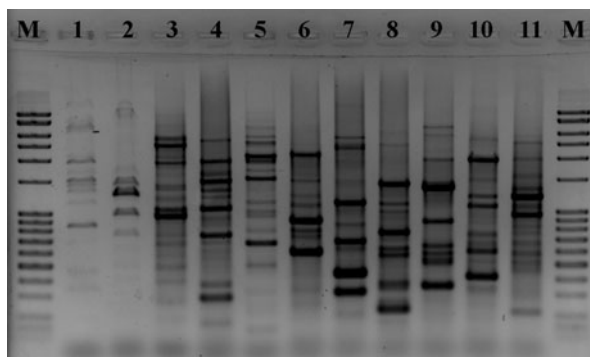


Figure 2. Electrophoretic profiles of representative strain of each protein similarity group obtained by ERIC-PCR: lane: M 1 kbp DNA ladder, lane 1 NF4, 2 549, 3 NF27, 4 125, 5 NF1a, 6 323, 7 607, 8 NF20, 9 NF30, 10 477, 11 1,043, M 1 kbp DNA ladder.

Isolate 549 shared 97.8% similarity with *Chryseobacterium taiwanensis* BCRC 17412^T, isolate NF27 shared 97.5% with *Comamonas koreensis* KCTC 12005^T, isolate 125 shared 98.4% similarity with *Stenotrophomonas maltophilia* LMG 958^T, and isolate NF4 shared 96.0% similarity with *Acinetobacter baumannii* ATCC 19606^T. These indicated relationships are very well reflected by phylogenetic analysis (Figure 3).

4. DISCUSSION

In our study, the majority of bacterial isolates, 297 (= 41%) were Gram-positive, aerobic, spore-forming rods (results not shown). Microorganisms of that type, mostly *Bacillus* spp. are commonly found on honey bees, honey bee larvae, in honey bee gastrointestinal tract as well as in honey and the honey bees environment (Smolska-Szymczewska 1989; Snowden and Cliver 1996; Gilliam 1997; Rada et al. 1997; Kačániová et al. 2004; Piccini et al. 2004; Iurlina and Fritz 2005; Mohr and Tebbe 2006; Evans and Armstrong 2006; Mohr and Tebbe 2007; Babendreier et al. 2007; López and Alippi 2007; Kačániová et al. 2009).

Concerning Gram-negative, non-fermenting bacteria much less information is available. To add some knowledge about presence of this group of bacteria in the honey bee environment, we examined a total of 51 Gram-negative, non-fermenting bacterial isolates. Only species, of

which several representatives can be isolated from the same source, can be also considered to have the potential to play a role in a certain habitat whereas those represented by a single isolate only may be an arbitrary result. Hence, we aimed this study on those potential bacterial species of which at least three strains could be isolated. With such a high number of strains to be examined, a method for identification at the species level has to be employed able to compare several strains at the same time. Among others comparison of protein patterns after SDS-PAGE is a cost-efficient method for the comparison of large groups of bacteria, and it can be used for an initial step in polyphasic characterization (Kerstens 1985; de Vos 2002). Applying this approach, 11 protein similarity groups could be identified and a representative of each group was defined (Table I).

For evaluation of SDS-PAGE-based phenotyping, further characterization by ERIC-PCR-based fingerprinting was carried out as well, an approach which has been applied for classification of diverse groups of Gram-negative bacteria (Versalovic et al. 1991; Goncalves and Rosato 2000; Buczolits et al. 2002; Busse et al. 2003; Kardos et al. 2007; Loncaric et al. 2009). ERIC-PCR generated reproducible fingerprints and genetic homogeneity between isolates within a certain protein similarity group was observed. Hence, ERIC-PCR generated fingerprints were in agreement with the SDS-

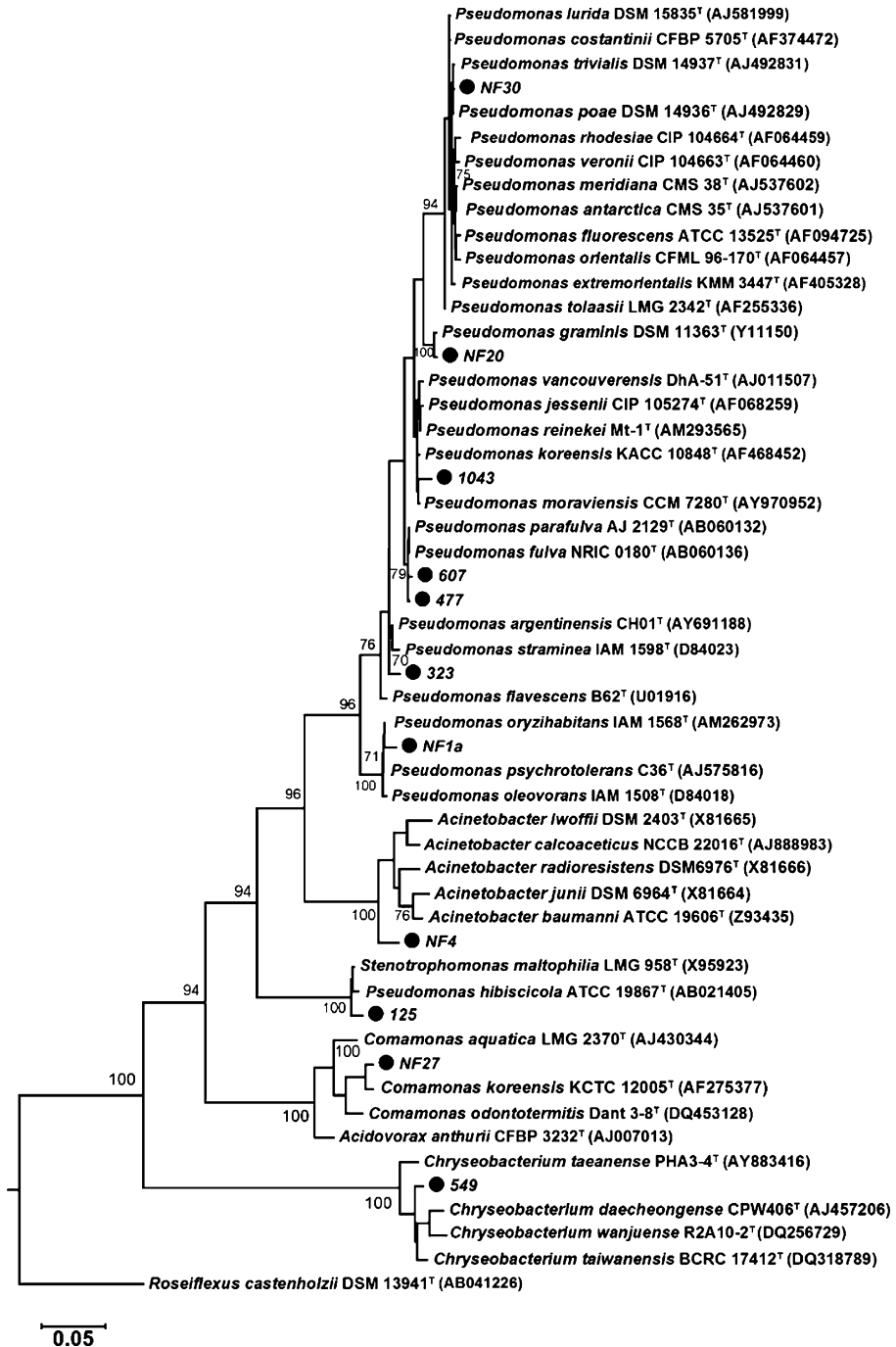


Figure 3. Maximum likelihood phylogenetic tree generated of 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 100 replications) greater than 75% are shown at branching points. The tree was rooted using *Roseiflexus castenholzii* DSM 13941^T as an outgroup. The sequences generated during this study were labeled (circle).

PAGE results and indicated that the isolates within a group are in fact related at the species level.

Representative strains of PSG1 and PSG3 were placed by 16S rRNA gene sequence similarities (Table I) in the genera *Acinetobacter* and *Comamonas*, respectively, but affiliation with an established species was not suggested by these data. Reports on the presence of *Acinetobacter* and *Comamonas* in the honey bee environment are scarce. The finding of *Acinetobacter* spp. as symbionts of honey bee larvae was described by Evans and Armstrong (2006), and the presence of *Comamonas* spp. in the gut of bees was reported by Mohr and Tebbe (2006).

Based on the highest 16S rRNA gene sequence similarity of strain 545 with *C. taiwanensis* BCRC 17412^T, strains of PSG2, which mainly were isolated from honey samples, could be placed in the genus *Chryseobacterium*. To the best of our knowledge, this is the first report describing the isolation of chryseobacteria from honey. This is remarkable because honey harbors very low numbers of viable bacteria (Tysset et al. 1980; Snowdon and Cliver 1996; Gilliam 1997; Iurlina and Fritz 2005; López and Alippi 2007; Loncaric et al. 2009; Kačániová et al. 2009) due to its antimicrobial properties (White et al. 1963; Molan 1992a, b, 2001; Taormina et al. 2001; Lusby et al. 2005; Gomes et al. 2010; Kwakman et al. 2010). Exceptions are spore-forming bacteria like *P. larvae*, causative agent of American Foulbrood (Genersch 2010); *C. botulinum*, facultative pathogen for humans (Brook 2007); and other spore-forming bacteria primarily *Bacillus* and relatives (Iurlina and Fritz 2005), which is not inactivated by the inhibitory properties of honey. The representative of PSG4, strain 125, exhibited the highest 16S rRNA gene sequence similarity (98.4%) with the type strain of *Stenotrophomonas maltophilia* (X95923), and it can be considered as a representative of the genus *Stenotrophomonas*. However, the similarity value seems too low to suggest a relationship at the species level, and hence, strains of PSG4 may represent

a novel species of the genus *Stenotrophomonas*. Isolation of *Stenotrophomonas* strains from the honey bee environment is not a new finding. Evans and Armstrong (2006) found ten isolates in honey bee larvae, six of them showing $\geq 99.0\%$ sequence similarity to *S. maltophilia*. From guts of *A. mellifera* and *O. bicornis* larvae, two 16S rRNA gene sequences were obtained which showed the highest similarity values with *S. maltophilia* as well (Mohr and Tebbe 2006). Representatives of PSG5–PSG11 showed the highest 16S rRNA gene sequence similarities ($>98\%$) with certain type strains of the genus *Pseudomonas* such as *P. graminis*, *P. parafulva*, *P. argentinensis*, and *P. koreensis*, demonstrating unequivocally the affiliation with the genus *Pseudomonas*.

In accordance with these results is the isolation of *Pseudomonas* spp. from honey (Snowdon and Cliver 1996). Kačániová et al. (2004) detected *Pseudomonas* strains in the gastrointestinal tract of honey bees. Unfortunately, the above-mentioned studies did not provide detailed characterization to demonstrate affiliation with an established species nor were sequence data provided. Very recently, Babendreier et al. (2007) analyzed the bacterial community structures in honey bee intestines and their response to two insecticidal proteins. In this study, two derived sequences originating from uncultured bacteria clustered most closely with *Pseudomonas indica* IMT37^T and *Pseudomonas aeruginosa* strain 796.

Results from ARDRA analyses were in good accordance with classification of strains based on 16S rRNA gene sequences. For every genus, a unique restriction pattern was obtained with the two restriction enzymes, but one strain of the genus *Pseudomonas*, NF1a, exhibited a unique pattern after restriction with *Hha* I (Table I). This observation might reflect the deep branching of this strain and its relatives in the phylogenetic tree (Figure 3).

The quinone systems of the representative strains, ubiquinone Q-8 of the strains affiliated with *Comamonas* or *Stenotrophomonas*, menaquinone MK-6 of the *Chryseobacterium* strain, and ubiquinone Q-9 of the *Acinetobacter* or

Pseudomonas affiliated strains were in complete agreement with the traits reported for representatives of the genus (Collins and Jones 1981; Oyaizu and Komagata 1983; Busse et al. 1989; Yokota et al. 1992; Kämpfer et al. 2003; Hauser et al. 2004; Stolz et al. 2007).

In colonies of *A. mellifera*, workers have mouth-to-mouth contact with larvae and each other, the latter by reciprocal feeding (trophallaxis; Beetsma 1985). Therefore, it is not possible to trace bacteria exactly to their origin, i.e., pollen loads, honey sac, freshly stored nectar, or honey. What has been clearly shown in the present study was that the bacteria were not isolated only from a single source.

In summary, in this study we demonstrated that Gram-negative non-fermenting bacteria are widely spread in the honey bee's environment. The 16S sequences of representatives of protein similarity groups recovered that some of these bacteria were related to previously identify bacterial genera from the honey bee's environment. One group of bacteria could be classified as members of the genus *Chryseobacterium* which was not previously reported to be present in the honey bee environment, and further elucidation of its role in this habitat has to be assessed.

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Caractérisation de bactéries sélectionnées, à Gram-négatif, non fermentantes, associées aux abeilles (*Apis mellifera carnica*)

Diversité bactérienne / PCR / miel / abeille / gène 16S rADN

Zusammenfassung – Charakterisierung Gram-negativer, nicht fermentierender Bakterien, die aus Honigbienen (*Apis mellifera carnica*) isoliert worden waren. Die vorliegende Studie wurde durchgeführt, um die Diversität der ausgewählten Gram-negativen, nicht fermentierenden Bakterien, die von Honigbienen (*Apis mellifera carnica*) gesammelten Pollenhörschen, dem Honigblaseninhalte, frisch eingetragenen Nektar (FSN) und Honig zu untersuchen. Die Diversität der Gram-negativen, nicht fermentierenden Bakterien wurde durch einen polyphasischen Ansatz (Kulturbedingungen, morphologische und biochemische Charakteristika; Vergleich der Proteinmuster der Isolate nach SDS-PAGE, ERIC-PCR; Analyse der respiratorischen Chinone, ARDRA und 16S rDNA Sequenzanalyse) charakterisiert. Dabei wurden aus den insgesamt 724 gewonnenen Bakterienisolate 104 Isolate als nicht-sporenbildende gram-negative Stäbchen charakterisiert, die aus Glucose keine Säure durch Gärung bilden konnten. Diese 104 Isolate wurden dann weiter untersucht. Nach dem Vergleich der mittels SDS-PAGE erhaltenen Proteinmuster wurden 11 Gruppen mit ähnlichem Proteinmuster gebildet, die durch ERIC-PCR bestätigt werden konnten. Aus jeder Gruppe mit ähnlichem Proteinmuster wurde je ein Isolat ausgewählt (= repräsentativer Stamm) und durch die Analyse der respiratorischen Chinone, ARDRA und 16S rDNA Sequenzanalyse näher untersucht. Ausgehend von den Ergebnissen dieser Untersuchungen konnten 7 der repräsentativen Stämme als Mitglieder der Gattung *Pseudomonas* identifiziert werden. Die verbleibenden repräsentativen Stämme wurden den Gattungen *Acinetobacter*, *Chryseobacterium*, *Stenotrophomonas* und *Comamonas* zugeordnet. Wie gezeigt werden konnte, sind Gram-negative nicht fermentierende Bakterien im Umfeld eines Bienenvolkes weit verbreitet. Somit könnten weiterführende Untersuchungen hinsichtlich des antagonistischen Potenzials dieser Bakterien gegenüber verschiedenen Krankheitserregern bei Bienen vielversprechend sein.

Bakteriendiversität / PCR / Honig / Honigbienen / 16S rDNA Gen

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