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To cite this version:

Igor Loncaric, Helmut Heigl, Elisabeth Licek, Rudolf Moosbeckhofer, Hans-Jürgen Busse, et al.. Typing of Pantoea agglomerans isolated from colonies of honey bees (Apis mellifera) and culturability of selected strains from honey. Apidologie, 2009, 40 (1), pp.40-54. hal-00891952

HAL Id: hal-00891952

https://hal.science/hal-00891952v1

Submitted on 11 May 2020

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Apidologie 40 (2009) 40–54
© INRA/DIB-AGIB/ EDP Sciences, 2009
DOI: 10.1051/apido/2008062

Available online at:
www.apidologie.org

Original article

Typing of *Pantoea agglomerans* isolated from colonies of honey bees (*Apis mellifera*) and culturability of selected strains from honey*

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Received 6 December 2007 – Revised 15 September 2008 – Accepted 1 October 2008

Abstract – *Pantoea agglomerans* is a possible biocontrol agent against fire blight (*Erwinia amylovora*) and a facultative pathogen of humans. Isolates were gathered from flowers, pollen loads, honey sacs, and freshly stored nectar (FSN). Under artificial inoculation conditions, strains completely lost their culturability at 24 °C after 120 h of incubation in honey and 156 h in honey solution, respectively. None of tested strains could be cultivated from FSN, honey, or honey solution after 48 h at temperatures higher then 28 °C. At different time intervals, culturable population levels at 35 °C and 24 °C were significantly higher in blossom honey or its solution than in blossom and honeydew honey or its solution. Our results indicated that *P. agglomerans* is widely spread throughout honey bee's environment. Strains lost culturability after short periods of incubation in honey or honey solution. In samples of honey and royal jelly from test colonies, no culturable *P. agglomerans* isolates could be detected.

bacterial diversity / strain traceability / pollen analysis / genomic fingerprinting / *Erwinia amylovora*

1. INTRODUCTION

Pantoea agglomerans, previously named as *Erwinia herbicola* or *Enterobacter agglomerans* (Gavini et al., 1989) is a member of the *Enterobacteriaceae* and ubiquitous in nature. It was isolated from plant surfaces, seeds, water, animals, and humans (Gavini et al., 1989). *P. agglomerans* is of commercial interest as a biological control agent of the major postharvest pathogens on pome and citrus fruits, especially against fire blight (Costa et al., 2002). *P. agglomerans* is harmless to apple and pear trees and is able to protect them against invasion of the *Erwinia amylovora* (Wright et al., 2001). Fire blight (*E. amylovora*) and its occurrence has been reported in more than 40 countries around the world (Ordax et al., 2006). It is a very serious and destructive disease of pome fruits and many ornamental plants from the *Rosaceae* family (Ordax et al., 2006). Several strains of *P. agglomerans* have been selected as a biocontrol agent against *E. amylovora* (Ishimaru et al., 1988; Wilson et al., 1990; Vanneste et al., 1992; Wodzinski et al., 1994; Wright and Beer, 1996; Kearns and Hale, 1996; Pusey, 1997; Vanneste et al., 2002; Özaktan and Bora, 2004). Control of fire blight with antagonistic strains has

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been achieved either by spraying a suspension of the antagonistic strains onto apple, pear, Asian pear, and hawthorn blossoms, or by using honey bees (*Apis mellifera*) as a vector (Vanneste, 1996). Honeybees also play an important role as a secondary colonizer of biocontrol agents in orchards and to surrounding plants (Nuclo et al., 1998).

As *P. agglomerans* is also considered to be an opportunistic pathogen of humans and animals, there is additional current interest in this bacterium. The most common infection caused by *P. agglomerans* is septic arthritis or synovitis (Kratz et al., 2003). Other reported infections caused by *P. agglomerans* include ostitis (Laporte et al., 2002), polymicrobial peritonitis (Lau et al., 2005), peritonitis (Lim et al., 2006), and sepsis after rotavirus gastroenteritis (Cicchetti et al., 2006), and it is also considered to be a strong allergen (Dutkiewicz, 1997). Recently, Cruz et al. (2007) reported about 53 pediatric cases of *P. agglomerans* infections. However, until present there are no easy and reliable tests to determine whether any particular strain of *P. agglomerans* has pathogenic or non-pathogenic characteristics (Wright and Beer, 2006). Therefore, there are controversial opinions about the use of *P. agglomerans* strains for biocontrol purposes.

One of the crucial points in this context is to avoid any risks for the quality of hive products and their consumers. So, in the run-up to a potential application of selected *P. agglomerans* strains from abroad for fireblight control in Austria, information about the natural occurrence of *P. agglomerans* on bee forage plants,diversity of strains, their traceability from blossoms to beehive, and their culturability from bee products (e.g. honey) is needed to support risk assessment.

Heissenberger (2004) and Heissenberger et al. (2006) reported about isolation and characterization of different *P. agglomerans* strains from different fire blight host plants in Austria. But until present there are no Austrian reports about intraspecies diversity of *P. agglomerans* strains isolated from honeybees, honey, or non-host plant blossoms visited by honeybees after the blooming period of apple and pear. There are also no reports about the culturability of *P. agglomerans* from bee forage and bee products.

The focus of this study was therefore to investigate (i) the intraspecies diversity of *P. agglomerans* from pollen loads, honey sacs, freshly stored nectar, royal jelly, and honey, and (ii) the traceability of particular *P. agglomerans* strains from collected blossoms to colonies of honeybees by means of pollen analysis. Because of the importance of honey as a natural product for human consumption, the time dependent culturability of selected *P. agglomerans* strains from artificially spiked samples of honey and honey solution was also evaluated.

2. MATERIALS AND METHODS

2.1. Bacterial isolates

Adult honey bee workers (*Apis mellifera* L.) were collected from two bee hives (hives 169 and 186) of the Institute for Apiculture, Austrian Agency for Health and Food Safety (AGES), 13 times between June 18th and October 2nd. Freshly stored nectar was collected 6 times during the same period when found present in the comb cells. Honey bees were killed by freezing at –25 °C for at least 20 minutes.

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. 200 μL of this mixture were transferred on blood agar (Columbia blood agar base (Oxoid), supplemented with 5% defibrinated sheep blood) and Peptone-Yeast-Extract agar (PYE) (l^{-1}: 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. Additionally, pollen analysis was performed from 15 honeysac contents.

For the isolation of *P. agglomerans* from pollen loads, honey bees were collected at the same time as for extraction of honey sacs. 30 pollen loads, showing the same color, were mixed with 3 mL of 0.9% NaCl and vortexed for 60 s. 100 μL 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. Additionally, pollen analysis was performed from 5 pollen loads, showing identical color.

Isolation of *P. agglomerans* from FSN: before sampling, a sweetness test of FSN was performed by testing 3 drops of collected fluid. Positive reaction was characterized if the drops were sweet and negative if the drops were not sweet. If the 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 mL of collected nectar was mixed with 5 mL of sterile water and vortexed for 30 s 200 μL of diluted nectar was directly inoculated on blood agar plates as well as on PYE agar and incubated at 28 °C for 48 h. One mL of freshly stored nectar was used for pollen analysis (see below).

For the isolation of bacteria from honey, the method described by Bakonyi et al. (2003) to detect the causative agent of American foulbrood (*Paenibacillus larvae*) was slightly modified to keep non-spore forming bacteria alive and viable. 200 μL of diluted honey was directly inoculated on two blood agar plates. The plates were incubated as described for honey sacs. Additionally honey was screened for pollen (see below).

During the sampling period, a total of 13 queen cells were collected, from which the royal jelly was obtained and tested for the presence of *P. agglomerans*. Royal jelly was collected from each individual queen cell and mixed with 1 mL of 0.9% NaCl. 200 μL of this dilution was plated and incubated as described above. In all, 13 queen cells were collected from both bee hives.

In order to isolate bacteria from blossoms, a total of 21 different plants were collected simultaneously with honey bee probes. Their blossoms were examined for presence of *P. agglomerans*. Blossoms used in this study were collected within a radius of 150 m from hives. The sampling took place from different plants in a meadow and a small plot of *Phacelia tanacetifolia* on which honey bees had been seen or pollen have been found either in honey sacs, pollen loads, or freshly stored nectar during the last honey bees sampling (usually a week before). Blossoms were collected every time from the same place with the exception of *Taraxacum officinale*, which were sampled from different spots of the meadow. Blossoms were washed in 20 mL of 0.9% NaCl and mixed under agitation 200 rpm for 30 min 100 μL of washing solution was transferred to agars and incubated as described above.

2.2. Pollen analysis

Preparation of FSN and honey sac content for pollen analyses: 0.5 mL of freshly stored nectar or diluted honey sac content was filled into a centrifuge tube (capacity 50 mL) and 15 mL of distilled water was added. The solution was agitated until homogeneity and centrifuged for 10 min at 1000 *g*. The supernatant liquid was decanted, and the sediment with some remaining water transferred with a micropipette onto an object holder covering an area of 15 × 15 mm. After drying on a heating plate at 40 °C, a drop of liquified glycerine jelly was added and the preparation covered with a coverslip. After hardening, microscopic examination was carried out under 1000 × magnification.

Preparation of pollen loads: five pollen loads were mixed with a couple drops of distilled water. Using a micropipette, the suspension was transferred to object holder. The following steps were as described for FSN.

Pollen analysis from honey was performed as described by Louveaux et al. (1970). For pollen identification, reference material as well as the data set from the pollen database (PONET) of the Institute for Apiculture was used (URL: http://www15.ages.at:7778/pls/pollen/pollen_suche).

2.3. Preselection of *P. agglomerans* isolates

Preselection was performed based on some differential characteristics for *P. agglomerans* as described by Gavini et al. (1989). After incubation, 3 yellow pigmented colony forming units (CFUs) were randomly picked from blood agar plate. The isolates were subcultivated on PYE and McConkey agar (Oxoid) and incubated at 28 °C for 48 h for further analyses. After incubation isolates that had grown well and showed characteristic colony morphology on McConkey agar were tested as following: the Gram reaction, as described by Gerhard et al. (1994); cell morphology was observed under a light microscope (1000 × magnification); oxidase activity, using Bactident-Oxidase test strips (Merck) according to the manufacturer instructions; catalase activity, using 3%

H_2O_2; reduction nitrate to nitrite test was performed in nitrate broth (Merck). After incubation at 28 °C for 48 h equal volumes of API test reagents (Bio Merieux) NIT1 and NIT2 were added and evaluated; indole production, using DMACA Indole reagent (BBL) according to the manufacturer instructions; the glucose oxidation-fermentation test was done in Cap-o-test (Milian) tubes containing 2 mL of Hottinger bouillon (l^{-1}: 10.0 g pepton from casein (ROTH), 2.5 g NaCl, 1.25 g K_2HPO_4, 40 mL bromthymolblue 0.1%, pH 8.0) and 1 mL of 1% glucose; Voges-Proskauer reaction (MR-VP medium). All isolates, which showed yellow or white colony morphology, Gram-negative behavior, non-spore-forming rods, no oxidase activity but catalase activity, reduction of nitrate to nitrite, no indole formation, production of acid from glucose by oxidation as well as by fermentation and positive Voges-Proskauer reaction, were subjected to comparison of their protein patterns after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins

For SDS-PAGE analyses (Laemmli, 1970), isolates were grown on PYE agar at 28 °C for 48 h. One loop of biomass was scraped off of agar plates and suspended in 55 μL sterile water. 1 mL of 10% trichloracetic acid (TCA) was added to biomass, vortexed and incubated for 5 min at room temperature, centrifuged at 10000 rpm for 1 min, then washed in 1 mL of 90% ice cold acetone containing 20 mM HCl, incubated for 30 min at –25 °C and washed for one more time without incubation. After centrifugation at 10000 rpm for 1 min, the final acetone supernatant was removed and the pellets were air dried. 0.2 mL of sample buffer containing 1% SDS, 9 M urea, 25 mM Tris-HCl pH 6.8, 1 mM EDTA, 0.7 M 2-mercaptoethanol, 0.01% bromphenol blue was mixed with the pellets, boiled for 4 min in thermo mixer under agitation of 1300 rpm, and centrifuged at 10000 rpm for 10 minutes. Once it had completely cooled, 4–6 μL of the boiled sample was loaded onto a gel, 0.75 mm in thickness (4% stacking gel and 12.5% running gel), and electrophoresed overnight at 6 mA/gel using Protean II (Bio-Rad) electrophoresis units. The gel was stained with Coomassie brilliant blue R-250 and destained in a solution containing 10% acetic acid in distilled water until the bands were clearly visible to the naked eye. The molecular size marker Roti®-Mark Standard (Roth) was included in each run. The gel was dried in a gel dryer, and the bands were visually compared. Isolates showing significant similarities with the reference strains of *P. agglomerans* were selected for genomic analysis.

2.5. DNA extraction (supplementary data Appendix I)

After incubation of bacteria for 48 h at 28 °C on PYE agar, bacterial DNA was extracted as described by Loncaric et al. (2008), except that we used a shorter step of heating cells (20 min instead of 45 min) after adding MD1 solution.

2.6. Random amplification of polymorphic DNA (RAPD) analysis and PCR conditions

For RAPD-PCR (Williams et al., 1990), nine 10-nucleotide random primers were tested in order to obtain a specific fingerprint profile of each *P. agglomerans* isolate. A preliminary screening was conducted using eight *P. agglomerans* strains, which showed dissimilarities in their protein profiles. Primer Opl-11 (5'-ACGATGAGCC-3') (Operon Technologies) was selected, producing a robust, reproducible, and unique profile of each strain tested and applied for genomic fingerprinting of all isolates. Amplification reactions with single RAPD primers were performed in a GeneAmp PCR System Thermocycler (Perkin Elmer) in a 15 μL reaction volume containing 7.5 μL of a REDTaq ReadyMix PCR Reaction Mix (20 mM Tris/HCl, 100 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTP), with 0.45 units of Taq DNA polymerase (Sigma), 1–3 ng of template DNA and 12.5 pmol of primer. Thermal cycling parameters were as follows: initial denaturation at 94 °C for 5 min; 45 cycles of denaturation at 94 °C for 1 min annealing at 36 °C for 1 min, and extension at 72 °C for 2 min and a final extension step at 72 °C for 5 min. Each RAPD assay was performed twice to check the consistency of the method. PCR products were analyzed by electrophoresis in 1.5% agarose gel in TAE buffer run at 5 V/cm with 100 bp ladder (ROTH, Invitrogen) and detected by staining with ethidium bromide (0.5 μg mL^{-1}) under UV light and photographed.

2.7. Characterization of strains by the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

The primers used for ERIC-PCR were described by Versalovic et al. (1991). The PCR reaction was set up in a GeneAmp PCR System Thermocycler (Applied Biosystem) in a 15 μL reaction volume containing 7.5 μL of a REDTaq ReadyMix PCR Reaction Mix (20 mM Tris/HCl, 100 mM KCl, 3 mM MgCl2, 0.4 mM dNTP), with 0.45 units 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. Amplification reactions were determined twice to corroborate the reproducibility of the method. PCR products were analyzed and visualized as described for RAPD-PCR.

2.8. Cluster analysis

Relationships were estimated on the basis of patterns of all isolates obtained after RAPD-PCR with Opl-11 primer and ERIC-PCR. Data were analyzed considering the presence (1) or the absence (0) of bands for every isolate. All bands were analyzed. Variations in band intensity were not considered to be different. The binary data sets were fed into DendroUPGMA, a dendrogram construction utility (DendroUPGMA, S. Garcia- Vallvé, Biochemistry and Biotechnology Department, Universitat Rovira i Virgili, Tarragona, Spain, [http://genomes.urv.es/UPGMA/], Garcia-Vallvé et al., 1999). Using DendroUPGMA program Jaccard's coefficient (S) similarity matrixes were then calculated. The distance (d) between two strains is calculated with the formula $d = 1 - S$. A d value of 0 indicates that the two isolates have identical RAPD- or ERIC-PCR products, and a value of 1 indicates that the two isolates have no markers in common. A distance matrix of pair-wise comparisons between isolates was constructed. The relationship between isolates was analyzed with the unweighted pair group method with arithmetic averages (UPGMA) using the same program. For each dendrogram, the cophenetic correlation coefficient (CCC) was calculated. The CCC measures the agreement between the similarity values implied by the dendrogram and those of the original similarity matrix. The adequacy of a dendrogram is indicated by high CCC, and if CCC is over 0.8 the dendrogram is likely to be fairly satisfactory in this respect (Sneath and Sokal, 1973). The dendrogram tree was viewed and drawn using the TREEVIEW program (Page, 1996).

2.9. Diversity analysis

Two different statistical analyses were used to evaluate whether total diversity was covered by screening 301 isolates using RAPD-PCR method. The coverage (C) [$C = 1 - (n1/N) \times 100$, where $n1$ is the number of strains which occurred only once in our culture collection and N is the total number of isolates (Ravenschlag et al., 1999). In addition, a rarefaction analysis was performed to determine the number of unique OTUs as a proportion of the estimated total diversity. Calculations were performed using the freeware program Analytic Rarefaction version 1.3 (Holland, 2003), [http://www.uga.edu/strata/software/Software.html]. The program uses the rarefaction equations described by Hurlbert (1971) and Heck et al. (1975).

2.10. Estimation of culturable population levels (CPL) of selected *P. agglomerans* strains in honey

In order to test culturability in honey in vitro, four strains of *P. agglomerans* were selected based on their interaction with the growth of the Austrian local strain *Erwinia amylovora* 295/93 in vitro. Selected *P. agglomerans* strains 64b and 280b could suppress *E. amylovora* growth in vitro and strains 700b and 1376b were ineffective (Heissenberger, 2004). Type strain of *P. agglomerans* DSM 3493^{T} was also included in the study. Examinations were performed with two types of honey not originated from tested beehives: blossom honey (BH – moisture 17.2%, pH 3.6, dominant pollen group: *Cardamine pratensis*, *Tilia platyphyllos*, *Allium* sp.) or blossom and honeydew honey (BH/HD – moisture 17.4%, pH 3.1, dominant pollen group: *Prunus* sp. *Malus* sp. – details see supplementary data Appendix I). Two types of tests were conducted: (a) with pure honeys; (b) with honey solutions. Culturability from pure honey and solutions was tested after artificial inoculation with selected *P. agglomerans* strains.

Culturability in pure honey was tested in a sterile 50 mL centrifuge tube by mixing 20 g of each honey

with 1 mL of bacterial water suspension (1 OD600 approx. 2 × 109 CFU mL^{-1}) of each strain which was incubated for 48 h at 28 °C on PYE. Tests were performed at 24 °C, the temperature in the outer regions of a comb (Ritter, 1982), and 35 °C, the central colony temperature if brood is present (Southwick, 1991). The temperature intervals were based on results of pretests performed with *P. agglomerans* DSM 3493^T and BH. The honey and honey solution samples incubated at 24 °C and at 35 °C were plated every 12 h and 4 h, respectively. After mixing 0.1 mL of solution were serial diluted (1:10) and plated in triplicate on PYE agar and incubated for 72 h at 28 °C.

For the honey solution, 20 g of honey was vortexed thoroughly with 2 mL of sterile water until homogeneity was obtained, then 1 mL of the bacterial suspension was added, incubated, and plated as described above. Initial number of CFU mL^{-1} of honey or honey solution was counted after mixing bacterial suspension with honey or honey solution by plating each sample tested in serial dilutions, where 0.1 mL was plated on 6 PYE plates. Initial number of CFU mL^{-1} of honey or honey solution was estimated by dividing obtained CFUs with 15.2 for honey and 17.2 for honey solution, which are the total amounts of mL in artificially contaminated honey or honey solution. Specific gravity of honey is about 1.4 (Krell, 1996), 20 g ≈ 14.2 mL of honey + 1 mL of bacterial suspension for culturability test in pure honey or 1 mL of bacterial suspension + 2 mL of sterile H_2O.

2.11. Estimation of culturable population levels (CPL) of selected *P. agglomerans* strains in FSN

FSN was collected from two beehives used for this study. Culturability in FSN was tested by mixing 5 mL of nectar (characteristics: pH < 5, dominant pollen content: *Trifolium repens*) with 0.2 mL OD_{600} = 1 (approx. 2×10^9 CFU/mL) of each strain used for CPL-experiments in this study in sterile 15 mL centrifuge tubes. Tests were performed at 28 °C in two independent experiments and samples were prepared as described for honey experiments and plated in triplicate on PYE agar every 4 h. The bacterial growth on PYE agar was controlled after 72 h of incubation at 28 °C. CFUs were not counted.

2.12. Statistical analysis

Numbers of CFU ml^{-1} were logarithm transformed to achieve a normal distribution, and all results were expressed as log (CFU). Data were subjected to statistical analysis using SPSS for Windows. The differences among the strains and among two honey types incubated were analyzed using ANOVA when the mean counts were greater than 0.5 log CFU mL^{-1}. In order to compare the difference between each group, Bonferroni multiple comparison was applied.

3. RESULTS

3.1. *P. agglomerans* isolates and pollen analysis

A total of 307 *P. agglomerans* like CFUs were recovered and undertaken further examinations. Origin and frequencies of isolates are given in Table I. Detailed characteristics of isolates and results of pollen analysis are summarized in Appendix II (supplementary data). No culturable bacteria could be detected in royal jelly and two honey samples originating from test hives.

3.2. Analysis of whole-cell protein profiles by SDS-PAGE

With this technique, 50 different profiles relating to *P. agglomerans* reference strains could be identified within wild isolates (Appendix III (supplementary data)). Comparison of the protein patterns demonstrated that 301 isolates that showed obvious similarities with the protein profiles of two reference strains confirmed the preliminary identification based on physiological characteristics. Differences between protein profiles of *P. agglomerans* strains were primarily observed approximately in the range 35 and 66 kD. Six *P. agglomerans*-like CFUs, showing two different protein profiles, had no similarities with reference strains and were therefore excluded of further studies.

Table I. Origin of *Pantoea agglomerans* strains utilized in this work, groups based on ERIC-PCR pattern with frequency of RAPD-PCR profiles and isolates within a group.

ERIC Group	No. of RAPD profiles	No. of isolates	Origin
1	1	1	Pl
2	12	96*	B, Hs, Pl, N
3	2	9	B, Hs, Pl
4	1	4	B, Hs, Pl
5	1	1	Pl
6	1	1	B
7	1	3	B, Hs, Pl
8	3	15	B, Hs, Pl
9	1	3	B, Hs, Pl
10	1	4	B, Pl
11*			
12	1	5	B, Hs, Pl
13	1	16	B, Hs, Pl, N
14	1	3	B, Pl
15	1	4	B
16	1	3	B, N, Pl
17	2	14	B
18	1	2	B
19	1	3	B, Pl
20	4	33	B, Hs, Pl
21	3	4	B, Hs, Pl
22	1	13	B, Hs, Pl
23	1	7	B, Hs, Pl
24	1	1	Hs
25	1	2	Pl
26	1	3	B, Pl
27	2	2	B
28	1	2	B, Pl
29	1	2	Pl
30	1	44	B, Hs, Pl, N
31	1	1	Pl

B: blossom; Hs: honey sac; Pl: pollen loads; N: nectar.
* Excl. DSM 3493^{T}.
** DSM 1619.

3.3. RAPD PCR and cluster analysis

Employing RAPD primer Opl-11 each of the 50 isolates exhibited a unique genomic fingerprint. Bands with identical electrophoretic behavior were only rarely observed, demonstrating that the employed primer is most useful for differentiation among strains of *P. agglomerans* and may be also useful for epidemiological studies. The number of RAPD-PCR generated banding patterns employing Opl-11 was identical that obtained by SDS-PAGE (n = 50) (Fig. 1). From each group of isolates sharing the same fingerprints obtained after SDS-PAGE and RAPD PCR with Opl-11, one isolate was selected as a representative strain for cluster analysis. Similarities of the isolates in the RAPD-PCR banding patterns with the pattern of *P. agglomerans* DSM 3493T were in the range between 0 and 50%. Between *E. amylovora* and all *P. agglomerans* strains, there were no genetic similarities. The genetic distance between all strains of *P. agglomerans* was > 0.17. The dendrogram has a cophenetic correlation r = 0.754 (data not shown). Diversity coverage reaches a value of

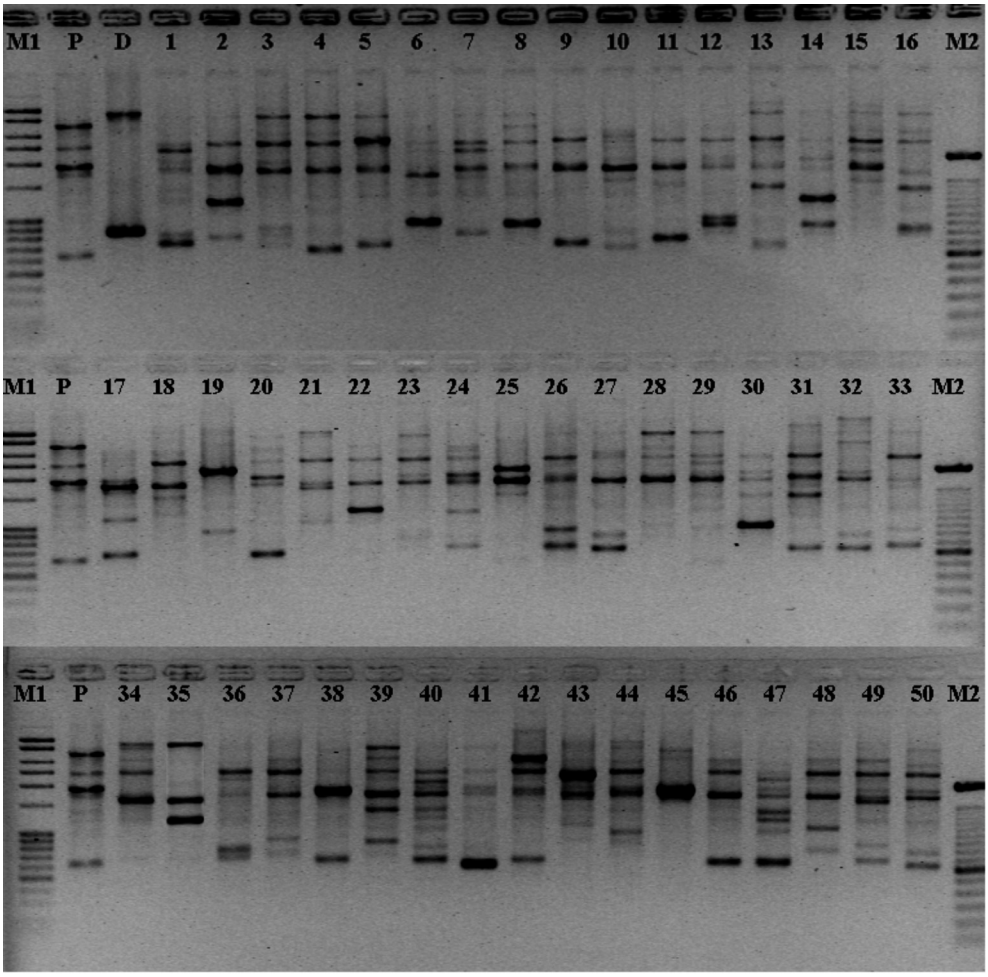

Figure 1. RAPD fingerprints of the 52 *Pantoea agglomerans* strains. Lane: P corresponds to *P. agglomerans* DSM 3493^{T}, D to *P. agglomerans* DSM 1619. The lane numbers correspond to the isolate numbers shown in Appendix II (supplementary data); M1 1 kb DNA leader; M2 1 kb DNA leader.

98.34%, indicating that nearly the total diversity was covered. Rarefaction analysis (Fig. 2) also revealed that continuing sampling of isolates would have yielded very few new strains, as documented by the modest slope of rarefaction curve.

3.4. ERIC PCR analysis of *P. agglomerans* strains

A total of 31 different ERIC banding patterns were observed among 52 strains examined using ERIC PCR (Fig. 3). All strains shared at least 3 of the 6 major bands of approximately 0.5, 0.7 and 2.6 kb in size, which indicates a high degree of relatedness and confirms the identification of the isolates as members of *P. agglomerans*. No similarities were observed between patterns of *P. agglomerans* strains tested and *E. amylovora* 295/93. All *P. agglomerans* isolates were grouped at a level higher than 57% using UPGMA method. The dendrogram has a cophenetic correlation r = 0.820 (Appendix IV). All selected strains grouped based on their ERIC-PCR profile with

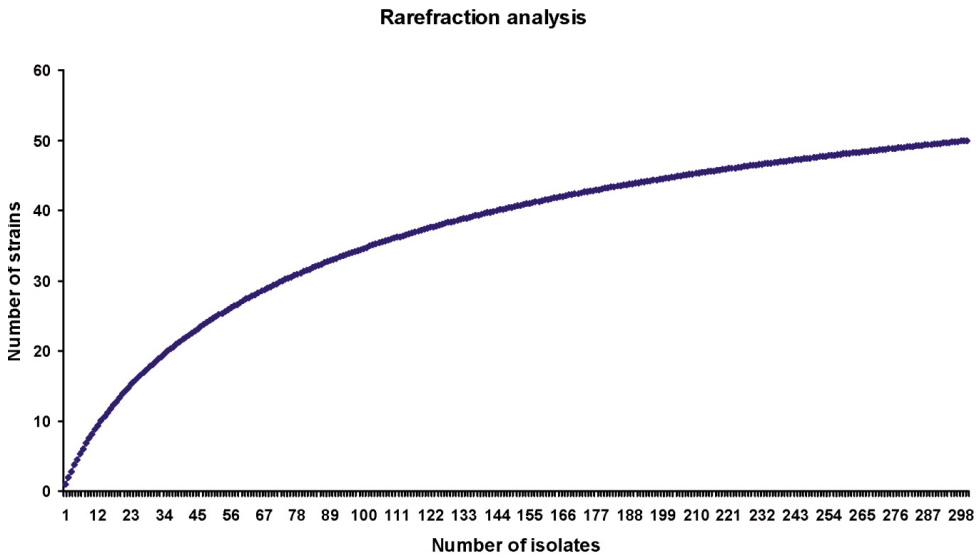

Figure 2. Rarefaction curve for the different RAPD patterns (strains). The line represents 95% confidence intervals.

frequencies of RAPD profiles and isolation are summarized in Table I.

3.5. Estimation of CPL of selected *P.agglomerans* strains in honey solution, honey and FSN

Initial means of tested strains in honey were 8.11 ± 0.19 log CFU mL^{-1} and 8.06 ± 0.19 log CFU mL^{-1} in honey solution without any significant difference between honey types or strains. CPLs of all strains tested were significantly greater ($P < 0.05$) for BH and BH solution than for BH/HD and BH/HD solution. Significant differences between strains could not be detected except examinations at 35 °C in solution of BH/HD. CPLS of one representative strain are shown in Figure 4. None of *P. agglomerans* strains were culturable after incubation for 48 h at 28 °C in FSN.

4. DISCUSSION

So far there are only few studies involving the nonpathogenic bacteria associated with honey bees (*Apis mellifera*). Gilliam (1997) reviewed identification results and roles of nonpathogenic microflora and some details about *Enterobacteriaceae*, especially *Enterobacter cloacae*, *E. aerogenes* and *Klebsiella pneumoniae*. Rada et al. (1997) reported about gut microflora of honey bee and described 31 isolates of which one was determined as *Bifidobacterium asteroides*. Jeyaprakash et al. (2003) did not detect *P. agglomerans* using 16S rRNA sequence analysis in worker adults of *A. m. capensis* and *A. m. scutellata* in South Africa. Very recently, Mohr and Tebbe (2006, 2007) compared bacterial diversity in the guts of *A. m. carnica* and *Bombus bicornis* (bumblebee) at an oilseed rape field, as well as reported about the probability and risk of a horizontal gene transfer from transgenic herbicide-resistant oilseed rape pollen to gut bacteria of *A. m. carnica*, *B. bicornis*, and *Osmia bicornis* (red mason bee). In gut flora of *A. m. carnica*, they did not isolate bacteria related to *P. agglomerans*. Best to our knowledge there is no report about isolation of *P. agglomerans* from honey.

To obtain data of *P. agglomerans* spreading in honey bee nutrition pathway, the whole path from flowers to honey as the final product was examined. Best to our knowledge, no studies have reported showing isolation of *P. agglomerans* from honey or royal jelly. Snowdon and

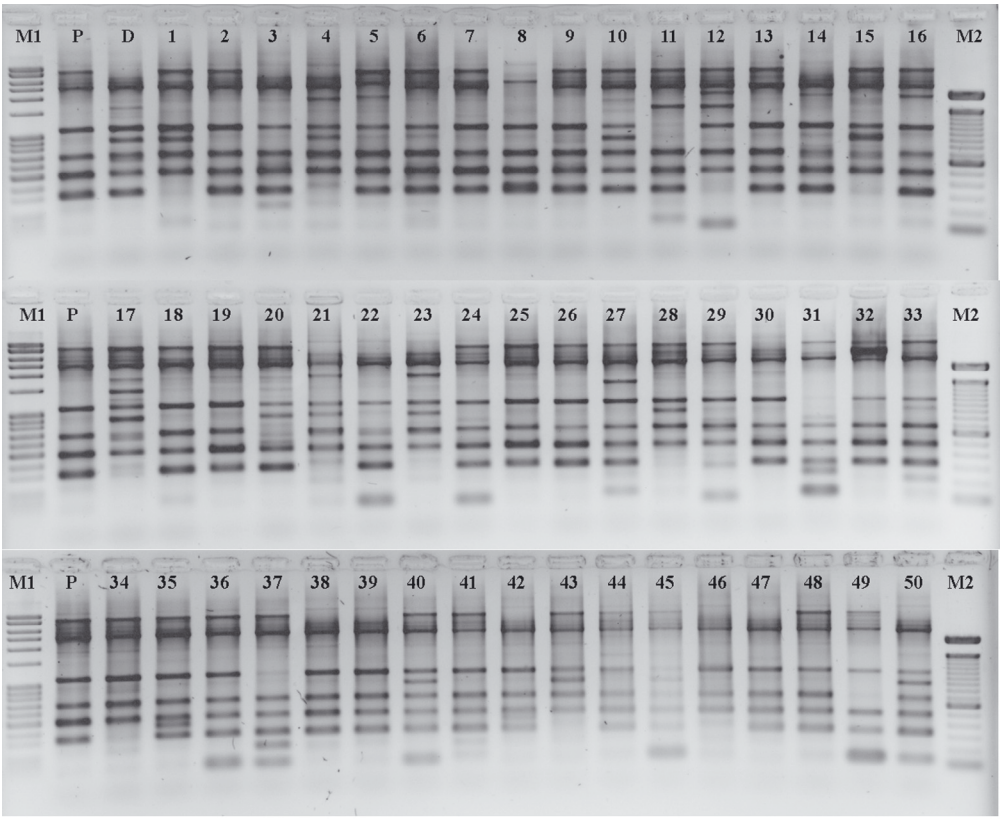

Figure 3. ERIC fingerprints of the 52 *Pantoea agglomerans* strains. Lane: P corresponds to *P. agglomerans* DSM 3493^T; D to *P. agglomerans* DSM 1619. The lane numbers correspond to the isolate numbers shown in Appendix II (supplementary data); M1 1 kb DNA leader; M2 1 kb DNA leader.

Cliver (1996), and results described by Rall et al. (2003), indicated that no vegetative forms of causative agents of human bacterial diseases have been found in honey.

The technique of SDS PAGE has proven to be a rapid and cost-efficient method for the comparison of large groups of bacteria and it can be used for an initial step in polyphasic characterization (de Vos, 2002). The comparison of protein fingerprints obtained by SDS-PAGE is suitable for identification and differentiation of closely related bacteria (Pot et al., 1994; Lyra et al., 1997; Esteban et al., 2003; de Vos, 2002). Therefore, considering the fact that all our isolates have almost identical protein fingerprints with the type strain of *P. agglomerans* DSM 3493T and reference strain DSM 1619, it can be deduced that they belong to this species.

The RAPD fingerprinting technique has been described to be a powerful typing method for many bacterial species (Mbwana et al., 2006). In our work, the use of the RAPD technique allowed discrimination among different strains of *P. agglomerans* and yielded reproducible results in different assays. The results presented here allow us to conclude that RAPD analysis can be a useful tool for the genotyping of *P. agglomerans*. Our results suggest that the strains circulating in a honey bee's environment are unevenly distributed on plants, bees, and two hives tested. Eleven strains, which were isolated three or more times from blossoms, honey sacs, pollen loads, and/or FSN during sampling period showed

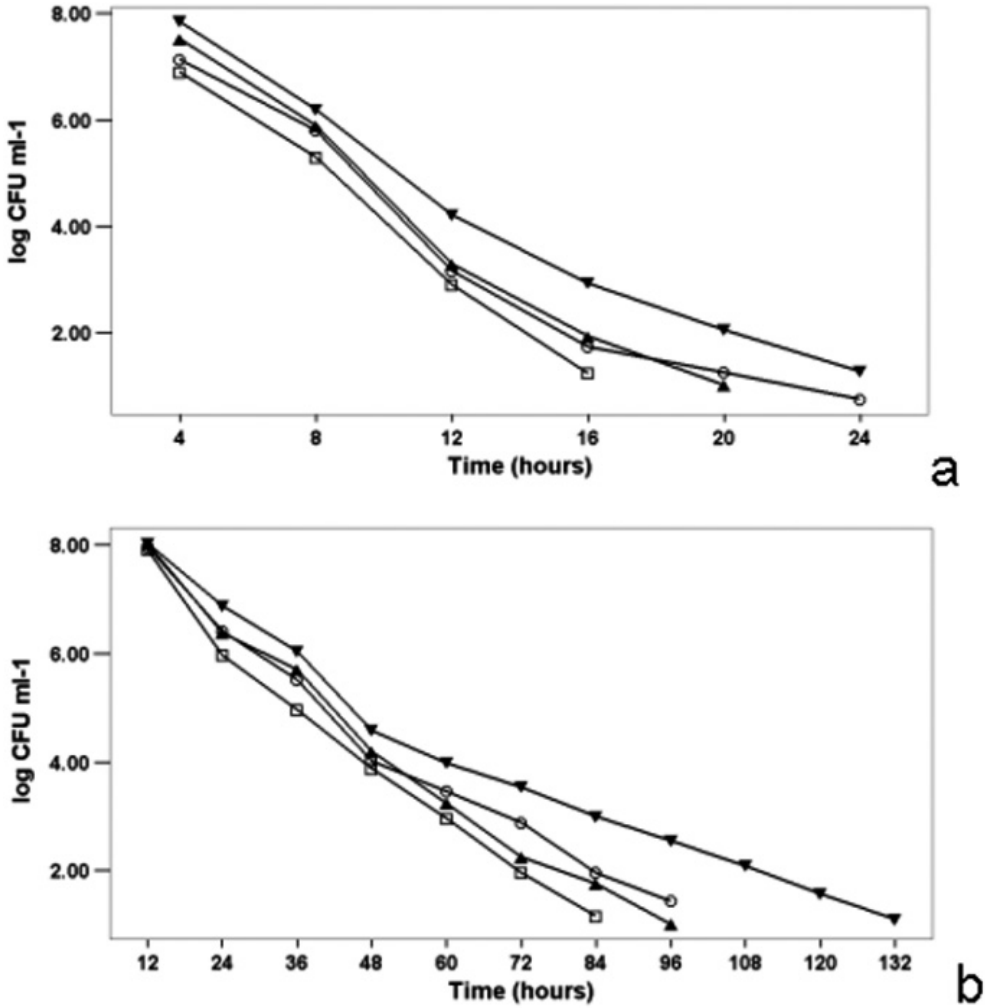

Figure 4. Log10 reductions in the culturable levels of *P. agglomerans* strain 64b in artificially contaminated blossom honey (▲), blossom and honeydew honey (□), blossom honey solution (▼), blossom and honeydew honey solution (○) incubated at 35 °C (a) or 24 °C (b). Dots show means.

identical protein and RAPD banding patterns that could be assumed as plant specific. Origin of isolates from a certain plant was confirmed by means of pollen analysis.

For evaluation of SDS-PAGE results, ERIC-PCR was employed. ERIC-PCR fingerprinting technique has been described as a useful tool for typing and identification of diverse Gram-negative bacteria (Versalovic et al., 1991; Kardos et al., 2007) as well as gram-positive bacteria (Wieser and Busse, 2000; de Vos, 2002; Genersch et al., 2006). In a recently published paper, the same method was used for genotyping of *Pantoea* strains isolated from clinical human samples (Koo et al., 2006). ERIC-PCR generated reproducible fingerprints and the genetic homogeneity between isolates and reference strains was observed. ERIC-PCR-generated banding patterns showed some common bands for all isolates. This indicates that this method could be used for confirmation of the identity of *P. agglomerans* strains by the presence of these common bands. ERIC-PCR-generated

fingerprints are in agreement with the SDS-PAGE results and indicate that the isolates in fact are members of *P. agglomerans*. The dendrograms obtained in the present study showed no association between isolates with regard to time of isolation or origin. A possible explanation for that is that the foraging radius of honey bees can cover distances up to 2000 m from hives (Seeley, 1997). Considering this fact and result of our study, which proved that almost all tested plants were contaminated with *P. agglomerans*, it could be assumed that honey bees can come into contact with the most *P. aggomerans* strains present on bee-visited plants in the foraging area. As honey is known to have a variety of antimicrobial properties (Molan, 1992a,b), several tests were performed during this study to evaluate the CPL of selected *P. agglomerans* strains in FSN and ripe, extracted honey. Until present, no information is available concerning the estimation of CPL of *P. agglomerans* in freshly stored nectar (FSN) and honey. However, several authors describe the given survival rates of some other bacterial species (Tysset and Durand, 1973; Beyme et al., 1975; Tysset et al., 1979; de Wael et al., 1990; Alexandrova et al., 2002). Because of differences in species tested, incubation temperatures and bacterial concentration, a direct comparison with those reports was not possible. Based on our in vitro studies, it can be concluded that the time within which *P. agglomerans* could be recovered – expressed by the culturability on PYE agar – is considerably shorter than the period between nectar collection and honey harvesting. According to Horn (1992), honey ripening takes at least 1–3 days and the honey is usually harvested at the end of blooming period, which could last at least one week or more. It is important to mention that some bacteria under stress may enter into the viable-but-nonculturable (VBNC) state (Ordax et al., 2006). Honey with its antimicrobial properties (e.g., high osmolarity) could probably induce a VBNC state. Thus, to obtain exact survival rates and to avoid false negative results, further investigations are necessary to clarify whether or not strains of *P. agglomerans* could enter into the VBNC state.

In this study, we demonstrated that *P. agglomerans* strains are widely spread in a honey bee's environment and on flowers visited by bees. Strains were highly genetically diverse with good diversity coverage. From all samples of bee visited flowers, at least one isolate of *P. agglomerans* could be detected. Some obtained strains seem to be plant-specific. Because of relatively small numbers of isolates in our study, further studies about this issue are necessary to confirm or reject the hypothesis of origin specificity to certain plant species. We also demonstrated that *P. agglomerans* strains completely lost their culturability in artificially contaminated FSN and honey within 48 h at temperatures higher than 28 °C as well as 120 h in honey and 156 h in honey solution at 24 °C, respectively. Further studies under natural conditions are necessary to clarify the possible role of *P. agglomerans* as a food contaminant, prior to its use for biocontrol in the field.

ACKNOWLEDGEMENTS

This work was supported in part by internal funds of the Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine Vienna, Institute for Apiculture, Austrian Agency for Health and Food Safety (AGES) as well as Clinic for Avian, Reptile and Fish Medicine, University of Veterinary Medicine Vienna. We are grateful to Marianne Keck for providing DNA of *Erwinia amylovora* strain 295/93 used in this study and to Ewald B. M. Denner and Birgit Heissenberger for providing reference strains of *Pantoea agglomerans*. We thank Sandra Buczolits, Gabriele Rothmüller, Irmgard Derakhshifar and Hermann Pechhacker for helpful comments and suggestions, and Christine Schramm and Katharina Etter for technical assistance.

Caractérisation de la diversité des bactéries *Pantoea agglomerans* isolées de colonies d'abeilles domestiques (*Apis mellifera*) et possibilité de croissance dans le miel de lignées sélectionnées.

diversité bactérienne / traçabilité des souches / analyse pollinique / empreinte génomique / *Erwinia amylovora*

Zusammenfassung – Typisierungen von *Pantoea agglomerans* isoliert aus Kolonien von Honig-Bienen (*Apis mellifera*), sowie Untersuchung der Kultivierbarkeit ausgewählter Stämme in Honig. Ziel dieser Studie war die Untersuchung der Diversität von *Pantoea agglomerans* und seiner Rückverfolgbarkeit von Trachtpflanzen zum Bienenvolk, sowie die Abschätzung der kultivierbaren Keimzahl (CPL) dieses Bakteriums in Honig, Honiglösung und frisch eingelagertem Nektar (FSN).
P. agglomerans ist ein möglicher Kandidat zur biologischen Bekämpfung von Feuerbrand (*Erwinia amylovora*),wurde aber auch als fakultativ humanpathogener Keim beschrieben.
Blüten verschiedener Pflanzen, Pollenhöschen, Honigblaseninhalt und frisch eingelagerter Nektar wurden gesammelt, aufbereitet und das gewonnene Probenmaterial auf Agarplatten inkubiert, um Isolate von *P. agglomerans* zu gewinnen. Zur Bewertung der Diversität wurden SDS-PAGE, RAPD- and ERIC-PCR eingesetzt. Die Abschätzung der Verwandtschaft der Isolate erfolgte mittels Cluster-Analyse.
Zur Untersuchung der Kultivierbarkeit von *P. agglomerans* in Honig, Honiglösung und frisch eingelagertem Nektar wurden diese mit einer wässrigen Suspension von 5 ausgewählten Stämmen des Bakteriums künstlich inokuliert und sorgfältig durchmischt. In bestimmten Intervallen wurden Proben entnommen, auf Agarplatten ausplattiert und diese bebrütet. Für jeden Stamm wurde die Zahl der kultivierbaren koloniebildenden Einheiten (KBE) ermittelt.
Von Blüten verschiedener Pflanzenarten, Pollenhöschen, Honigblaseninhalten und frisch eingelagertem Nektar konnten einzelne Isolate von *P. agglomerans* gewonnen werden, nicht aber aus Honig- und Weiselfuttersaftproben der Testvölker. Auf Basis der Koloniemorphologie, Pigmentierung, biochemischen Eigenschaften und dem Vergleich der Proteinmuster mit Referenzstämmen nach Natriumsulfat-Polyacrylamid Gel Elektrophorese (SDS-PAGE) wurden 301 Isolate ausgewählt. Von diesen zeigten 50 Stämme unterschiedliche Eiweißprofile. Die Analyse mittels RAPD-PCR erbrachte die gleiche Profilanzahl PCR (Abb. 1). Eine Identifizierung der isolierten Stämme erfolgte mit ERIC-PCR (Abb. 2).
Aus den künstlich inokulierten Proben konnte auf PYE Agar bei 24 °C und einer Inkubationsdauer von mehr als 120 h (Honig) bzw. 156 h (Honiglösung) keiner der Teststämme reisoliert werden. Bei Temperaturen über 28 °C war bereits nach 48 h Bebrütungsdauer keine Rückisolation der Teststämme aus Honig, Honiglösung oder frisch eingelagertem Nektar mehr möglich.
Sowohl bei 35 °C als auch bei 24 °C war die kultivierbare Keimzahl der Teststämme zu verschiedenen Zeitintervallen in „Blütenhonig“ (BH) signifikant höher ($P < 0,05$) als in „Blüten- mit Waldhonig“, oder ihren Lösungen (Abb. 4).
Gestützt auf diese Ergebnisse kann geschlossen werden, dass die Zeit, in der die getesteten *P. agglomerans* Stämme aus Honig kultiviert werden konnten, beträchtlich kürzer ist, als die Zeitspanne, die in der imkerlichen Praxis zwischen dem Sammeln des Nektars und der Ernte des Honigs üblicherweise eingehalten wird. Im Falle eines Einsatzes von *P. agglomerans* als biologisches Mittel zur Feuerbrandbekämpfung in blühenden Obstanlagen kann dies ein wichtiger Punkt sein.

Bakterielle Vielfalt / Stamm-Rückverfolgbarkeit / Pollenanalyse / genetischer Fingerabdruck / *Erwinia amylovora*

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