Bacterial community structures in honeybee intestines
and their response to two insecticidal proteins

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Key words: transgenic plants, risk assessment, bacteria, Apis mellifera, T-RFLP, Bt-maize

Running title: Bacterial community of honeybee guts

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Abstract In this study, effects of the Bt-toxin Cry1Ab and a soybean trypsin inhibitor (SBTI) on intestinal bacterial communities of adult honeybees (Apis mellifera) were investigated. It was hypothesised that changes in intestinal bacterial communities of honeybees may represent a sensitive indicator for altered intestinal physiology. Honeybees were fed in a laboratory-setup with maize pollen from the Bt-transgenic cultivar MON810 or from the non-transgenic near isoline. Purified Cry1Ab (0.0014% w:v) and SBTI (0.1% or 1% w:v) represented supplementary treatments. For comparison, free-flying honeybees from two locations in Switzerland were analyzed. PCR-amplification of bacterial 16S rRNA gene fragments and terminal restriction fragment length polymorphism analyses revealed a total of 17 different terminal restriction fragments (T-RFs), which were highly consistent between laboratory-reared and free-flying honeybees. The T-RFs were affiliated to α-, β-, and γ-Proteobacteria, to Firmicutes, and to Bacteriodetes. Neither Bt-maize pollen nor high concentrations of Cry1Ab significantly affected bacterial communities in honeybee intestines. Only the high concentration of SBTI significantly reduced the number of T-RFs detected in honeybee midguts, a concentration that also increases bee mortality. Therefore, total bacterial community structures may not be a sensitive indicator providing evidence for impact of insecticidal proteins on honeybees already at sub-lethal levels.

1. Introduction

Agricultural application of transgenic plants that are engineered for improved insect resistance has gained importance world-wide, however, field releases have stimulated considerable discussion on potential detrimental effects on the environment (Hails, 2000). One particular concern is that insect resistant transgenic plants may pose risks for non-target organisms (Conner et al., 2003). The honeybee, Apis mellifera, is generally considered as a key non-target species because of its ecological and economic importance as pollinator of many crops and wild plants (Free, 1993).
Therefore, novel plant protection strategies must be evaluated for potential detrimental effects on this beneficial insect (US EPA, 1996; EPPO, 2001).

Many studies have been performed to assess effects of transgenic plants on *A. mellifera* but mainly focussed on survival of bees exposed to transgenic plants or their insecticidal proteins (for review see Malone and Pham-Delegue, 2001). There are, however, other factors than survival that have the potential to compromise the development of entire colonies as for instance effects of insecticidal proteins on the development of the hypopharyngeal gland of adult worker bees (Malone *et al.*, 2004, Babendreier *et al.*, 2005). In addition, the microbial community of the alimentary tract may be an important factor for the health of honeybees at the individual and colony level. So far, most studies on honeybee microflora have focussed on disease-causing microorganisms (e.g. Alippi *et al.*, 2002), while much less emphasis has been given to non-pathogenic microorganisms and their potential benefit for individual bees or whole colonies. However, there is growing awareness of the importance of the composition of the intestinal micro-flora for health and growth of honeybees (Gilliam, 1979; Gilliam *et al.*, 1988a; Gilliam, 1997; Dillon & Dillon, 2004).

Microbial communities in honeybee intestines have been studied mainly with cultivation-dependent techniques (Gilliam & Valentine, 1976; Gilliam & Morton, 1978; Gilliam *et al.*, 1990; Gilliam, 1997). These methods, however, are known to be biased by selectivity for culturable microorganisms and therefore do not reflect entire microbial communities. With the development of molecular methods, possibilities for analysing microbial communities have greatly increased. Few studies have applied these new techniques to analyse microbial communities in insect guts but mainly focussed on termites (e.g. Tokura *et al.*, 2000). Recently, Jeyaprakash *et al.* (2003) have investigated the bacterial communities in the intestinal tract of South African honeybees and Mohr & Tebbe (2006) have studied the bacterial communities in the intestinal tract of honeybees, a bumble bee and a solitary bee species in Germany. Both studies were based on molecular genetic analyses and revealed a relatively low diversity of bacteria. However, except for a single study
conducted by Deml et al. (1999), effects of insect resistant transgenic plants or their expressed products on arthropod intestinal microflora have not been assessed.

Several different Bt-maize events are available that express Bt-toxins for the specific control of certain pest insects when feeding on these plants. Upon ingestion, the Bt-toxin forms pores in intestinal epithelial cells and thereby disrupts intestinal function (Schnepf et al., 1998). The lepidopteran-specific Cry1Ab toxin is one of the most important Bt-toxins commercially used and is for instance expressed in the Bt-maize event Mon810 (Shelton et al., 2002). In addition to Bt-toxins, other proteins may be used to protect plants from insect pests. Proteinase inhibitors (PIs) for example are known to affect protein digestion of insects by blocking their digestive proteinases thus reducing the insect's digestive capacity (Laskowski & Kato, 1980). Many plants have been successfully engineered to produce PIs and have been shown to reduce growth and survival of a range of pest insects when added to their food (Jouanin et al., 1998; Lawrence & Koundal, 2002). A number of studies have been conducted to evaluate effects of insect-resistant transgenic plants on bees and none of them revealed negative effects of Bt-toxins or Bt-pollen (for review see Malone & Pham-Delegue, 2001). In contrast, increased mortality occurred when bees were fed with high concentrations of serine type PIs, e.g. the Kunitz Soybean trypsin inhibitor (SBTI) (Malone & Pham-Delegue, 2001).

Since both Bt-toxin and SBTI do affect the digestion processes of sensitive insects, we hypothesized that the insect’s intestinal bacterial communities may be indirectly affected by altered gut physiology. Consequently, the bacterial community may be an indicator showing changes already when sub-lethal quantities of transgene products are provided. Therefore, the bacterial communities in honeybee intestines were studied based on bacterial 16S rRNA gene profiles determined by specific PCR amplification and terminal restriction fragment length polymorphism (T-RFLP) analyses. The honeybees tested originated from a laboratory experiment in which they were fed with Bt-maize pollen, Bt-toxin or SBTI. In order to assess the ecological relevance of
potential effects on the bacterial communities of the laboratory specimens, bacterial communities from the intestine of free-living honeybees from two locations in Switzerland were also analyzed.

2. Material and Methods

2.1. Honeybees

Honeybee (Apis mellifera mellifera; Hymenoptera: Apidae) colonies for experiments in the laboratory were provided by a beekeeper in Zurich. Free-flying honeybees were sampled at two different locations in Switzerland, i.e. in Weiningen (close to Zurich) and in Bern-Liebefeld, ca. 100 km apart. Honeybees were colour-coded after emergence, put back into their hives and removed at an age of ten days. Eight bees from each of three colonies were taken at the two locations and at two different times each. The first collection was performed between May 23 and 26, 2003 and the second collection date was August 15, 2003. Bees were stored at −80°C until further processing.

2.2. Diets

Bt-maize pollen was harvested manually from a field near Freiburg in southern Germany, from a field trial with the transgenic maize variety MON 810. This variety contained a truncated synthetic version of the gene coding for the insecticidal δ-endotoxin Cry1Ab from Bacillus thuringiensis var. kurstaki. The concentration of Cry1Ab in the pollen was below the quantification level of 5 ng g⁻¹ dry weight (Babendreier et al., 2005). Pollen of the non-transformed near isoline was harvested from a maize field close to Zurich. All pollen grains were air-dried in the laboratory for 24 h, which reduced weight of the pollen by 50%, and then stored at −80°C. Bt-toxin used in the experiments was provided by Marianne P. Carey (Cleveland, Ohio, USA). The Kunitz soybean trypsin inhibitor (SBTI) was purchased from Sigma-Aldrich, Buchs, Switzerland.
2.3. Experimental design

For each replicate, one comb (10 x 10 cm) that was free of pollen but contained eggs and capped brood cells was transferred to a wooden cage (14 x 16 x 4.5 cm internal dimensions) together with the queen and approximately 250 worker bees (Babendreier et al., 2005). Each of the cages was placed into a gauze cage (40 x 40 x 35 cm size) in a climate chamber at 34 ± 0.5°C, 60 ± 5% relative humidity and a L15:D9 h light regime. The bees could leave the wooden cage and fly inside the gauze cage. Another 50 newly emerged bees from a different hive were tagged with dots of correction fluid on their backs and introduced into the cages at the start of the experiment. The honeybees were maintained for ten days in this system and fed with the defined diets described below. After this time the experiment was terminated, tagged bees were put into liquid nitrogen and stored at –80°C until further analysis. The experiment was repeated three times resulting in a total of 15 bee hives analyzed. From each of these 15 replicates, eight bees were randomly selected for subsequent analysis, i.e. 24 bees for each treatment.

2.4. Treatments

Five defined diets (treatments) including a control were tested in this study. In order to provide food ad libitum, bees were offered 4 g of maize pollen and 25 ml of a 1:1 (sucrose : water) solution, freshly supplied every two days on a pillar inside the gauze cage (for more details on the experimental design, see Babendreier et al., 2005). In one treatment, bees were provided with pure sucrose solution and transgenic maize pollen. In three other treatments, bees were fed pollen of the non-transformed near isiline together with the insecticidal proteins mixed into the sucrose solution. This included the Bt-toxin Cry1Ab (0.0014% w:v) and the proteinase inhibitor SBTI in a high (1% w:v) and a ten-fold lower concentration (0.1% w:v). Taking into account the different amount of pollen and nectar (sucrose) consumed by bees, Bt-toxin concentration in this study is at least 10 times higher than that of maize events expressing the Bt toxin in the pollen such as Bt 176 (Koziel
et al., 1993). Though little information is available on the content of PIs in pollen, McManus et al. (1994) found that plants can be protected from pests when protease inhibitors are expressed at c. 1% of total soluble leaf protein. Thus the Bt-toxin treatment and the high dose SBTI treatment are representing worst case scenarios. Control bees were fed with pollen from the non-transformed near isoline and pure sucrose solution.

2.5. Dissection of bees

The intestine consisting of midgut (ventriculus) and hindgut (rectum) was isolated on ice from frozen bees by clipping the stinger and the posterior segment of the abdomen with sterile forceps and carefully removing the intestine. The two intestinal sections were separated with a sterile scalpel, immediately frozen in liquid nitrogen, and stored at –80°C.

2.6. DNA extraction

DNA extraction was performed according to a protocol for soil DNA extraction (Bürgmann et al., 2001). A total of 300 µl lysis buffer pH 7.5 [200 mM Tris-HCl pH 7.5, 2 M NaCl, 50 mM EDTA, 2% CTAB (hexacyetyltrimethylammonium bromide)] and approximately 0.4 g silica beads (diameter 1 mm, Braun Biotech International GmbH, Melsungen, Germany) were added to the tube containing the still frozen intestine. Cells were lysed by processing the samples for 30 s at 5.5 m s⁻¹ in a FP120 FastPrep beat beater (Bio101 Savant, Inc. Holbrook, NY, USA). After centrifugation for 5 min at 13000 x g, the supernatant was collected. Each sample was extracted two more times by resuspension in 300 µl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA pH 8.0) and centrifugation at 13000 x g. Combined supernatants were extracted with one volume chloroform:isoamylalcohol (24:1) followed by centrifugation for 1 min. Nucleic acids were precipitated by adding 1 volume of the precipitation solution (20% PEG 6000, 2.5 M NaCl), incubation for 1 h at 37°C and centrifugation for 15 min at 13000 x g. After washing the pellet with
70% ethanol and air drying, DNA was resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). RNA was removed by RNase A (1 mg ml⁻¹; Qiagen Hilden, Germany) treatment and incubation for 30 min at 37°C. DNA was quantified using PicoGreen (Molecular Probes, Eugene, OR, USA) and adjusted to 10 ng µl⁻¹ (Bürgmann et al., 2001).

2.7. Terminal-Restriction Fragment Length Polymorphism analysis

For PCR amplification of partial bacterial 16S rRNA genes, primer 27F (FAM-labelled) and primer 1378R (Table 1) were used. PCR reactions were performed in a volume of 50 µl containing 1 ng µl⁻¹ DNA or transformed E. coli cells from gene libraries, 1x PCR buffer, 0.5 mM additional MgCl₂ (Qiagen,), 0.2 µM of each primer (Microsynth, Balgach, Switzerland), 0.4 µM of each dNTP (Invitrogen, Carlsbad, CA, USA), 0.6 mg ml⁻¹ bovine serum albumin (Sigma, Aldrich) and 2 U of HotStar Taq DNA polymerase (Qiagen). PCR was performed in an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) with 35 cycles for community intestinal analysis and 27 cycles for gene library screening. After initial denaturation and enzyme activation for 15 min at 95°C, cycles of denaturation for 45 s at 94°C, annealing for 45 s at 48°C, and extension for 2 min at 72°C were performed followed by a final extension step for 5 min at 72°C. Quality of PCR products was inspected by electrophoresis in agarose gels (1% w:v) and ethidium bromide-staining.

Amplified PCR products were diluted in MspI restriction enzyme conversion buffer (Tris pH3 0.4 mM, NaCl 5 mM, MgCl₂ 0.8 mM; (Hartmann et al., 2005)) in a ratio of 1:2 and digested overnight at 37°C using MspI (Promega Corporation, Madison, WI, USA). Quality of digestion was inspected by gel electrophoresis in MetaPhor gels (3% w:v, FMC BioProducts, Rockland, ME, USA) and ethidium bromide-staining. For T-RFLP analysis, 2 µl restriction products were mixed with 12 µl HiDi-formamid (Applied Biosystems, Foster City, CA) and 0.2 µl internal 500 bp size
standard ROX500 (Applied Biosystems, Foster City, CA, USA). DNA samples were denatured for 2 min at 92°C and then chilled on ice.

Restriction fragments were analyzed on a genetic analyzer ABI3100 (Applied Biosystems) equipped with 36 cm capillaries filled with performance optimized polymer POP-4 (Applied Biosystems). Sizes of FAM-labelled terminal restriction fragments (T-RFs) were detected automatically relative to the internal size standard using the GenScan V3.1 software (Applied Biosystems). Conversion of T-RF signals into numeric data of fragment size (relative migration units, rmu) and T-RF signal heights (relative fluorescence units, rfu) was performed using the Genotyper V3.7 NT software (Applied Biosystems). The baseline threshold for signal detection was set to 50 rfu. Compiled data were exported to Excel (Microsoft Corporation, Redmond, WA, USA) for standardization. Peak heights of each sample were divided by the sum of all peak height values from the corresponding sample. This step compensated for differences in PCR product quantity and T-RFLP profile intensity among samples.

2.8. Screening and DNA sequence analysis

Gene libraries of PCR amplified 16S rDNA were constructed with the pGEM-T Easy kit (Promega) and PCR products amplified with unlabelled primers 27F and 1378R as described above. For screening of these libraries, cloned 16S rDNA of randomly-picked white colonies were amplified and analyzed with T-RFLP analysis as described above. Plasmids were isolated using Wizard Plus SV Minipreps (Promega). DNA sequencing was done using the BigDye terminator cycle sequencing ready reactions kit (Applied Biosystems) and the primers shown in Table 1. DNA sequences of both strands were determined for all inserts using an ABI 3100 genetic analyzer. Sequences were assembled using the Auto Assembler V2.1 (Applied Biosystems). In the following we will refer to the sequence size defined groups as operational taxonomic units (OTU) in order to
distinguish them from the experimentally determined T-RF sizes shown in Figure 1. The relation of T-RF size group (in rmu) to the OTUs (in bp) is shown in Table 2.

2.9. Phylogenetic affiliation

For DNA sequence comparison of honeybee intestine-derived bacterial 16S rRNA gene clones, the ribosomal database Project II was used (Cole et al., 2003). For each clone, best-matching database entries were retrieved in the aligned format. Clone sequences were aligned to RDP-derived sequences using the BioEdit sequence alignment editor (Hall et al., 1999). Additional matching control sequences were searched and retrieved from the GenBank database. For phylogenetic inference, PCR primer sequences 27F and 1378R were excluded. A UPGMA and a rooted neighbour-joining phylogenetic tree were constructed with Treecon 1.3 for windows (Van de Peer & Dewachter, 1994) using Jukes & Cantor distance estimation and 100 bootstrap resamplings.

2.10. Statistical analyses

Generalized linear models (GLM) on Poisson distributed data and applying the log-link function were used to test for effects of the treatments on the number of OTUs. To better fit the variance of the response variable, it was assumed that our data were overdispersed (Sokal & Rohlf, 1995). A maximum likelihood test procedure was applied to investigate treatment effects (Sokal & Rohlf, 1995). The Bonferroni-Holmes correction procedure was applied for pairwise comparisons of the control and the four treatments (Sokal & Rohlf, 1995). Spearman rank correlation was used to test for correlations in the data sets. These analyses were conducted with the program Statistica (StatSoft, Inc., Tulsa, USA).

In addition, the data were subjected to multivariate analysis using the program CANOCO (Ter Braak, 1996). T-RF intensity data were z-transformed to average 0 and standard deviation 1. This served to give each T-RF the same relative weight in statistical analysis. Data were then ordinated
by principal component analysis (PCA) and redundancy analysis (RDA). In RDA, the significance
of treatments was assessed by Monte Carlo permutation. Both PCA and RDA assume a linear
model for the relationship between the response of OTUs and the ordination axis. Such a linear
model was found to be appropriate for the data of this study because a preliminary detrended
analysis showed short gradient lengths (< 3 SD). The three replicate colonies used per treatment
were included in the model as a covariable.

2.11 Nucleotide sequence accession numbers

The nucleotide sequences of the clones retrieved in this study have been deposited in
GenBank (Accession numbers DQ837602 to DQ837639).

3. Results

3.1. T-RFLP profiles of intestinal bacterial communities from experimental bee populations

Bacterial T-RFLP profiles of midguts from 97 individual bees derived from all 15
experimental bee populations studied under laboratory conditions revealed 1 to 7 T-RFs for each
sample with an average of 3.37 ± 0.99 (SE). Bacterial T-RFLP profiles of hindguts from 39 bees
from five different experimental bee populations revealed 2 to 8 T-RFs per sample with an average
of 4.38 ± 1.48. Three T-RFs occurred in more than 65% of the samples taken from the midgut while
8 T-RFs were detected in less than 20% of the samples (Figure 1A). In total, 12 different T-RFs
ranging from 79 to 491 rnu in size were identified in both midgut and hindgut. For individual bee
samples, however, a significantly lower number of peaks was found in the midgut as compared to
the hindgut regardless of whether all 15 populations were included in the analysis (GLM, \( \chi^2 = 21.4; \\
\text{df} = 1,134; P < 0.001 \)) or only those five populations from which the hindgut samples were
collected (GLM, \( \chi^2 = 12.5; \text{df} = 1,69; P < 0.001 \)). Furthermore, a multivariate analysis
demonstrated a significant difference of bacterial community structure between midgut and hindgut (Monte Carlo permutation test; $F = 53.2; P < 0.001; n = 136$). The sum of all canonical eigenvalues showed that 28% of total variance in the data was explained by the differences observed between mid- and hindgut. This difference was particularly prominent for the T-RF at 322.2 rmu, which was found in 97.4% of all hindgut samples, while it was only observed in 13.4% of all midgut samples. All other T-RFs occurred in comparable frequencies in midgut and hindgut samples with differences in the range of 0 to 24%.

3.2. Bacterial T-RFLP profiles from intestinal communities of free-flying bees

Bacterial T-RFLP profiles derived from honeybee intestines obtained from colonies located in Berne revealed 1 to 6 T-RFs for midguts with an average of $3.27 \pm 1.49$ (n = 15) and 2 to 9 T-RFs for hindguts with an average of $5.73 \pm 1.83$ (n = 15). Bacterial T-RFLP profiles derived from the colonies located in Zurich revealed 1 to 9 T-RFs for midguts with an average of $4.65 \pm 1.98$ (n = 37) and 3 to 9 T-RFs for hindguts with an average of $7.29 \pm 1.68$ (n = 21). From both locations and both sampling dates together a total of 10 different T-RF sizes was identified ranging from 85 to 491 rmu in size (Figure 1B). Two T-RFs occurred in more than 65% of the samples taken from the midgut while 4 T-RFs were detected in less than 20% of the samples (Figure 1B). Four T-RFs were detected in the midgut samples with an intermediate frequency in the free-flying bees. Multivariate analysis confirmed that there was a highly significant difference in the bacterial community structures between midgut and hindgut at both locations (Monte Carlo permutation test, $F = 8.58; p = 0.002; n = 88$). The T-RF at 322.2 rmu was found in 97% of the hindgut samples but only in 5.7% of the mid-gut samples of free-flying bees while differences in frequencies were generally small for the other T-RFs (range 0 to 43%).

A significant difference in the midgut bacterial communities was detected between the two locations ($F = 1.89; P = 0.033; n = 52$) although only 3.6% of the variance was explained by this
factor. The T-RFs at 167.5 rmu and 87.7 rmu were exclusively found at the second sampling date in late summer resulting in significantly different bacterial communities in the midguts between the two sampling dates (Monte Carlo permutation test, $F = 7.13; P < 0.001; n = 52$). Looking at replicate colony variation, there was no difference in mid-gut bacterial communities neither for the colonies located in Zurich ($P = 0.329; n = 37$) nor for those located in Berne ($P = 0.604; n = 15$).

The bacterial T-RFLP profiles of bee intestines showed comparable patterns between free-flying bees and experimental populations as underlined by the significant correlation between the signal intensities of T-RFs from free-flying bees and laboratory-reared populations (Spearman rank correlation; $r = 0.681; P < 0.05$; dots in Figure 1). Similarly, T-RFs that were frequently found in experimental populations were generally also detected at high frequencies in free-flying bees, resulting in a significant correlation ($r = 0.818; P < 0.05$; bars in Figure 1). Furthermore, a significant correlation was found between T-RF signal intensities (the dots in Fig. 1) and frequencies (the bars in Fig. 1) for the experimental population ($r = 0.608; P < 0.05$) but not for the free-flying bee population ($r = 0.463; P > 0.05$).

3.3. Sequence analyses

Overall 13 different T-RFs were identified in the populations analysed (Figure 1). In order to obtain sequences representing these specific T-RFs, PCR products from 13 selected samples were cloned, and resulting gene libraries were screened for clones encoding these T-RFs. Screening yielded 17 different T-RFs, including the 13 T-RFs that were detected with community T-RFLP analyses. Four T-RFs, i.e. at 124.0 rmu, 280.0 rmu, 297.0 rmu and >500 rmu, were not detected with community T-RFLP analyses (Table 2).

DNA sequences of 38 selected clones were determined, i.e. 35 clones from the experimental populations and for 3 clones from the free-flying bees. Comparison of T-RFLP and sequence data indicated a maximal difference of 3 bp between relative migration units determined for T-RFs and
the actual sequence length (Table 2). Chimera check analysis in RDP identified no chimeric sequences.

3.4. Phylogenetic analyses

For phylogenetic affiliation, the 38 clone sequences were aligned with 43 sequences identified as closely related reference sequences in RDPII or GenBank and the sequence of *Saccharomyces cerevisiae* (GenBank accession J01353 as an outgroup, which resulted in an alignment length of 1483 homologous positions. Phylogenetic analysis based on Jukes & Cantor distances calculation and UPGMA cluster analysis with 100 bootstrap re-samplings identified five clusters, which contained honey bee gut (HPG) clones, i.e. α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Firmicutes and Bacteriodetes (Figure 2). Among the α-Proteobacteria two sub-clusters (α-1 and α-2) were found. Sub-cluster α-1 contained all three clones from OTU-403bp and reference sequences from *Bartonella* sp. (Figure 2). Sub-cluster α-2 contained both clones from OTU-339bp and the reference sequences of *Acetobacter* sp. and *Gluconobacter* sp. as well as an uncultured clone labeled as *Gluconacetobacter* clone, which was derived from a study on bee intestinal bacteria (Jeyaprakash *et al.*, 2003). For the β-Proteobacteria, the sub-cluster β-1 contained all six clones obtained from our study, which belonged to OTU-447/448bp and associated most closely with a *Simonsiella* clone again derived from a study on bee intestinal bacteria (Jeyaprakash *et al.*, 2003). Among the four sub-clusters of the γ-Proteobacteria (γ-1 to γ-4), sub-cluster γ-1 was heterogeneous and contained OTU-124bp, OTU-279bp, OTU-296bp, and OTU-492bp, which all associated most closely to clones derived from bee intestinal bacteria that were identified as *Serratia* spp. (Jeyaprakash *et al.*, 2003). Sub-cluster γ-2 contained OTU-488bp, which separated from the γ-1 cluster with a 100% support from the bootstrap analysis but for which no other close relative was found. Sub-cluster γ-3 contained OTU-88bp, which associated with *Arsenophonus nasoniae* SK14 and sub-cluster γ-4 contained OTU-480bp and most closely clustered with *Pseudomonas* spp..
Beside the Proteobacteria-associated clones a complex group of Firmicutes-associated clones and two Bacteriodetes associated clones were found, which could be separated into five and one different sub-clusters respectively (F-1 to F-5 and B1, see Figure 2). Sub-cluster F-1 contained OTU-146bp, which associated with an uncultured rumen clone (Tajima et al., 2000). Sub-cluster F-2 contained OTU-168-bp, which associated with *Leuconostoc* sp.. Sub-clusters F-3 and F-4 contained OTU-177bp and OTU-81bp respectively, without close associations to known sequences. Sub-cluster F-5 contained OTU-321/322bp, and OTU-570/571bp, which were most closely associated with a *Lactobacillus* clone, previously isolated from bee intestine (Jeyaprakash et al., 2003). Cluster B1 contained OTU-89bp and was associated with Bacteriodetes without close association with known sequences.

### 3.5. Effects of two insecticidal proteins on bee midgut bacterial community structures

No significant differences in the number of T-RFs (GLM, $\chi^2 = 0.17$; df = 2,92; $P = 0.92$) or the bacterial community structure (Monte Carlo permutation testing, $F = 0.64$, $P = 0.190$; n = 97) were found among the three different experiments that were replicated over time. In contrast, highly significant differences in the number of T-RFs in honeybee midguts were observed among treatments (GLM, $\chi^2 = 51.4$, df = 4,92, $P < 0.001$; Figure 3). Pairwise comparisons showed that only the high concentration of SBTI significantly reduced the number of T-RFs as compared to the control (GLM, $\chi^2 = 41.2$, $P < 0.001$, see Figure 3). The comparison of the control with the treatment where bees were fed Cry1Ab in high concentrations (0.0014% w:v) was not significant based on the adjusted significance level (GLM, $\chi^2 = 5.9$, $P = 0.0151$). A multivariate analysis further showed that bacterial community structures in honeybee midguts significantly differed among the treatments (Monte Carlo permutation test, $F = 1.78$; $P = 0.002$; n = 97) although only 7% of the variance could be explained by the treatments. These differences observed among treatments could not be attributed to any specific T-RF (GLM, $P > 0.05$ for all pairwise comparisons).
4. Discussion

The present study investigated the intestinal bacterial communities of honeybee populations and evaluated whether consumption of two insecticidal proteins could affect the structural diversity of these communities. The diversity found was relatively low as compared with other insects such as termites (Hongoh et al., 2003). However, in comparison to previous studies on honeybees using cultivation-dependent methods (Gilliam, 1997), diversity was much higher in our study. Cultivation-dependent analyses have regularly demonstrated a high prevalence of Bacillus spp., which could not be confirmed in the present study. Concordance with other groups of bacteria was better, e.g. Lactobacillus spp., which were found with either approach. The present study revealed several T-RFs, which clustered with Lactobacillus although each T-RF was only found in some of the bees. Furthermore, members of the γ-Proteobacteria, i.e. sub-clusters γ-1 (T-RF at 490.8 rmu) and γ-2 (T-RF at 486.0 rmu) were found to represent the most common group of bacteria in honeybee intestines (Figure 1). These sub-clusters were components of a large γ-proteobacterial cluster, which also included two cloned sequences isolated from bee guts (Jeyaprakash et al., 2003).

Recently, Mohr & Tebbe (2006) reported on the bacterial communities of bee intestines with phylogenetic analyses based on partial sequences (370 bp) of 16S rRNA genes. The phylogenetic analysis performed in this study was based on approximately 1.4 kb long sequences (Figure 2), extending the potential to assign these sequences to other bacterial sequences deposited in public databases. The honeybee intestinal bacterial 16S rRNA gene sequences reported by Jeyaprakash et al. (2003) (underlined in Figure 2) show strong similarities to the honeybee intestinal bacterial communities reported here. In accordance with both studies (Jeyaprakash et al. 2003; Mohr & Tebbe 2006) our results indicate a relatively low diversity of bacterial groups in honeybee guts. The high consistency of bacterial community compositions in the guts of honeybees found in studies conducted on several subspecies in different parts of the world suggests the existence of bacteria
well adapted to this specific habitat. In the present study, $\gamma$-Proteobacteria from subclusters $\gamma$-1 and $\gamma$-2 together represented the most frequent and abundant bacteria detected in experimental and natural honeybee populations. This may be an indication of their importance in honeybee gut physiology, especially as together they appear to represent a resident honeybee gut bacterial population. However, no sequences of cultured isolates were deposited in public databases and therefore the potential function of these $\gamma$-Proteobacteria remains unresolved.

In order to assess potential effects of insecticidal proteins on bacterial communities in the honeybee gut, bees were fed Bt-toxin (Cry1Ab) and Kunitz soybean trypsin inhibitor (SBTI), in small flight cages in the laboratory (see Babendreier et al., 2005). While Cry1Ab has previously been shown to have no negative effects on bees (Malone & Pham-Delegue, 2001, Babendreier et al., 2005), SBTI is known to have high in vitro binding affinities for the major honeybee digestive endopeptidases and has been shown to increase mortality when fed in sugar solution to adult honeybees (Malone et al., 1995; Burgess et al., 1996; Babendreier et al., 2005). The present study revealed that consumption of SBTI for the first 10 days of adult life at high concentrations (1% w:v) significantly affected the bacterial communities of honeybee midguts. However, no specific individual T-RF or group of T-RFs could be correlated with these differences. The six most common OTUs found in the present study were all detected in each of the treatments. For the six less frequent OTUs, stochastic effects did not allow to link them to specific treatments. Similarly, considerable variability of intestinal bacterial communities in bees from the same colony was found by Gilliam & Valentine (1976) and Mohr & Tebbe (2006). This variability may be induced by several factors such as season and the type of pollen ingested (Gilliam & Morton, 1978; Gilliam et al., 1988b; Mohr & Tebbe, 2005). The results of the present study showed differences in intestinal bacterial communities between honeybees from spring and summer but only small differences between colonies and locations. In addition, a high similarity between free-flying bees and laboratory-reared bees that were exclusively fed with pollen from maize suggested a small influence
of the pollen source and indicated that experimental systems can be representative for the field situation. However, a conclusive assessment of the severity of effects detected will depend on a more profound knowledge of essential bacteria in the honeybee gut and on a more detailed understanding of their specific physiological functions.

Effects of transgene products on the honeybee intestinal microflora may be the result of direct bacterial toxicity or may be induced by an altered gut physiology such as reduced proteinase activity or lesions in the gut epithelium. Deml et al. (1999) analyzed the intestinal microflora of four lepidopteran species and one beetle that were fed with either of two Bt-toxins [Cry1A(c), CryIIIA]. Even though no quantitative measurements have been performed with the cultivation-dependent approach chosen, evidence for qualitative changes in the pattern of aerobic bacterial populations due to the Bt-toxins was found. However, the insects tested were susceptible to at least one of the toxins consumed, suggesting that the effects observed were related to altered gut physiologies rather than to direct toxic effects of the Cry toxins on gut bacteria. Similarly, in the present study the significant SBTI related changes in bacterial communities occurred only at the high concentration of SBTI (1% w:v), a treatment that also induced increased mortality (48.5% within 10 days) in the same honeybee population (Babendreier et al., 2005). This suggests that the observed effects on the bacterial gut microflora were indirect, i.e. in response to SBTI-induced physiological changes of the honeybee gut. It further suggests that total bacterial community structures may not be a sensitive indicator for direct impact of insecticidal proteins on honeybees already at sub-lethal levels.

Whatever the causal factors of the observed changes in the bacterial microflora were, data suggest that honeybees might be able to tolerate these changes because those related to the high-dose SBTI treatment were not qualitatively different from those related to other environmental factors, including season, location or pollen source. In contrast to SBTI, Cry1Ab-expressing Bt-plants do not appear to affect the intestinal bacterial diversity of honeybees at all.
Acknowledgements

Many thanks go to Peter Fluri and Hansueli Thomas for collecting the free-flying bees from the two locations. We appreciated the technical assistance of Martin Hartmann and the statistical advice of Philippe Jeanneret regarding Canoco analysis. We also like to thank Douglas Inglis for very helpful comments on an earlier version of the manuscript. This study was funded by the Federal Office for the Environment, project number 810.3189.004.

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Tokura M, Ohkuma M & Kudo T (2000) Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. Fems Microbiol Ecol 33:

Table 1: Primers used for PCR amplification and sequencing

<table>
<thead>
<tr>
<th>Primer sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>27f AGA GTT TGA TCM TGG CTC AG</td>
<td>Heuer et al. (1997)</td>
</tr>
<tr>
<td>1378r CGG TGT GTA CAA GGC CCG GGA AGG</td>
<td>Heuer et al. (1997)</td>
</tr>
<tr>
<td>927f GGG CCC GCA CAA GCG GT</td>
<td>Amann et al. (1995)</td>
</tr>
<tr>
<td>927r ACC GCT TGT GCG GGC CC</td>
<td>Amann et al. (1995)</td>
</tr>
<tr>
<td>1055f ATG GCT GTC GTC AGC TCG TG</td>
<td>Amann et al. (1995)</td>
</tr>
<tr>
<td>1055r CAC GAG CTG ACG ACA GCC AT</td>
<td>Amann et al. (1995)</td>
</tr>
<tr>
<td>uni-b-for TGC CAG CMG CCG CGG TA</td>
<td>modified from Amann et al. (1995)</td>
</tr>
<tr>
<td>T7 (-77)b TAA TAC GAC TCA CTA TAG GG</td>
<td>Promega</td>
</tr>
<tr>
<td>Sp6 (+98)b ATT TAG GTG ACA CTA TAG</td>
<td>Promega</td>
</tr>
</tbody>
</table>

a M represents the degeneracy A/C.

b Position of the 5'-end of the primer relative to the cloning site in the pGEM-T Easy Vector (Promega).
Table 2: List of terminal restriction fragment (T-RF) sizes and corresponding 16S rRNA gene clones derived from honeybee intestines.

<table>
<thead>
<tr>
<th>T-RF size</th>
<th>Clone names</th>
<th>Phylogenetic affiliation</th>
<th>T-RLFP analysis (rmu)</th>
<th>sequence analysis (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>79.2 ± 0.5</td>
<td>A5R3-3 A5R3-1</td>
<td>F-4</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>85.0 ± 1.0</td>
<td>A4R5-3 B4V1-1</td>
<td>γ-3</td>
<td>88</td>
<td></td>
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<tr>
<td>87.7 ± 0.5</td>
<td>A2V3-2 A2V3-4</td>
<td>B-1</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>144.6 ± 2.1</td>
<td>A4R5-4 A2R6-2</td>
<td>F-1</td>
<td>146</td>
<td></td>
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<tr>
<td>167.5 ± 1.0</td>
<td>D2V2-1</td>
<td>F-2</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td>178.0 ± 0.5</td>
<td>B1V3-2 B1V3-3</td>
<td>F-3</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td>322.2 ± 0.5</td>
<td>A5R3-2</td>
<td>F-5</td>
<td>321</td>
<td></td>
</tr>
<tr>
<td>402.3 ± 0.5</td>
<td>A2R6-1 A2V3-3 A4R5-2</td>
<td>α-1</td>
<td>403</td>
<td></td>
</tr>
<tr>
<td>442.0 ± 0.5</td>
<td>B2V6-3</td>
<td>α-2</td>
<td>439</td>
<td></td>
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<tr>
<td>446.8 ± 1.5</td>
<td>A5V8-2 A5V8-4</td>
<td>β-1</td>
<td>447</td>
<td></td>
</tr>
<tr>
<td>480.0 ± 1.3</td>
<td>A2V5-1 B1V3-4</td>
<td>γ-4</td>
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</tr>
<tr>
<td>486.0 ± 2.1</td>
<td>B2V6-4</td>
<td>β-1</td>
<td>488</td>
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<tr>
<td>490.8 ± 2.6</td>
<td>A5V8-1 A1R2-1 D2V2-2</td>
<td>γ-1</td>
<td>492</td>
<td></td>
</tr>
</tbody>
</table>

Sequences isolated from gene libraries

<table>
<thead>
<tr>
<th>T-RF size</th>
<th>Clone names</th>
<th>Phylogenetic affiliation</th>
<th>T-RLFP analysis (rmu)</th>
<th>sequence analysis (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>124.0 ± 0.5</td>
<td>A2V5-2 A2V5-3</td>
<td>γ-1</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>280.0 ± 0.5</td>
<td>A2V3-1 A5V8-3</td>
<td>γ-1</td>
<td>279</td>
<td></td>
</tr>
<tr>
<td>297.0 ± 0.5</td>
<td>A5V8-5</td>
<td>γ-1</td>
<td>296</td>
<td></td>
</tr>
<tr>
<td>nd&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A4R5-1</td>
<td>F-5</td>
<td>569</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B1V1-1</td>
<td>F-5</td>
<td>570</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> based on MspI digestion

<sup>b</sup> α = α-Proteobacteria; β = β-Proteobacteria, γ = γ-Proteobacteria; B = Bacteroidetes; F = Firmicutes; for details see Figure 2

<sup>c</sup> not determined as fragment size analysis was restricted to 500 bp
Captions to Figures

Figure 1: Frequencies of specific terminal restriction fragments (T-RFs) resulting from T-RFLP analysis in honeybee midguts (bars) and average standardized signal intensity of specific T-RFs within bee guts where total peak intensity has been set to one (dots). A) From experimental population (n = 97 samples including all treatments), B) from free-flying population (n = 52 samples including both locations and both dates). The open bars represent operational taxonomic units (OTUs) that were only found in either the experimental (A) or the free-flying population (B).

Figure 2: UPGMA dendrogram based on 16S rRNA sequences from clone sequences and closely related reference sequences using Jukes & Cantor distance calculation of 100 bootstrap resamplings. Only bootstrap values higher than 50 are indicated at the nodes. Clone sequences are indicated as “clone HBG” for honeybee gut. Clone sequences were clustered and labelled according to their phylogenetic affiliation (α-1, β-1 etc.).

Figure 3: Mean number of operational taxonomic units (OTUs) found in the guts of honeybees fed with Bt-maize pollen (event Mon 810) or insecticidal proteins (the Bt-toxin Cry1Ab at 0.0014% and the Kunitz soybean trypsin inhibitor (SBTI) at 0.1% and 1%) dissolved in sugar solution. The only significant difference was found between the high dose SBTI treatment and the control (indicated by an asterisk).
Figure 1
Figure 3