Original research paper

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

Dirk Babendreier, David Joller, Jörg Romeis, Franz Bigler and Franco Widmer

Agroscope Reckenholz-Tänikon Research Station ART, Reckenholzstr. 191, 8046 Zürich, Switzerland

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

Running title: Bacterial community of honeybee guts

Dr. Dirk Babendreier
Agroscope Reckenholz-Tänikon Research Station ART
Reckenholzstr. 191
CH - 8046 Zürich, Switzerland
E-mail: dirk.babendreier@art.admin.ch
Tel: +41 44 377 72 17
Fax: +41 44 377 72 01

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).

52 Therefore, novel plant protection strategies must be evaluated for potential detrimental effects on
53 this beneficial insect (US EPA, 1996; EPPO, 2001).

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

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

77 conducted by Deml et al. (1999), effects of insect resistant transgenic plants or their expressed
78 products on arthropod intestinal microflora have not been assessed.

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

94 Since both Bt-toxin and SBTI do affect the digestion processes of sensitive insects, we
95 hypothesized that the insect's intestinal bacterial communities may be indirectly affected by altered
96 gut physiology. Consequently, the bacterial community may be an indicator showing changes
97 already when sub-lethal quantities of transgene products are provided. Therefore, the bacterial
98 communities in honeybee intestines were studied based on bacterial 16S rRNA gene profiles
99 determined by specific PCR amplification and terminal restriction fragment length polymorphism
100 (T-RFLP) analyses. The honeybees tested originated from a laboratory experiment in which they
101 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 perfomed 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.

126 *2.3. Experimental design*

127 For each replicate, one comb (10 x 10 cm) that was free of pollen but contained eggs and
128 capped brood cells was transferred to a wooden cage (14 x 16 x 4.5 cm internal dimensions)
129 together with the queen and approximately 250 worker bees (Babendreier *et al.,* 2005). Each of the
130 cages was placed into a gauze cage (40 x 40 x 35 cm size) in a climate chamber at 34 ± 0.5°C, 60 ±
131 5% relative humidity and a L15:D9 h light regime. The bees could leave the wooden cage and fly
132 inside the gauze cage. Another 50 newly emerged bees from a different hive were tagged with dots
133 of correction fluid on their backs and introduced into the cages at the start of the experiment. The
134 honeybees were maintained for ten days in this system and fed with the defined diets described
135 below. After this time the experiment was terminated, tagged bees were put into liquid nitrogen and
136 stored at –80°C until further analysis. The experiment was repeated three times resulting in a total
137 of 15 bee hives analyzed. From each of these 15 replicates, eight bees were randomly selected for
138 subsequent analysis, i.e. 24 bees for each treatment.
139
140 *2.4. Treatments*

141 Five defined diets (treatments) including a control were tested in this study. In order to
142 provide food *ad libitum*, bees were offered 4 g of maize pollen and 25 ml of a 1:1 (sucrose : water)
143 solution, freshly supplied every two days on a pillar inside the gauze cage (for more details on the
144 experimental design, see Babendreier *et al.,* 2005). In one treatment, bees were provided with pure
145 sucrose solution and transgenic maize pollen. In three other treatments, bees were fed pollen of the
146 non-transformed near isoline together with the insecticidal proteins mixed into the sucrose solution.
147 This included the Bt-toxin Cry1Ab (0.0014% w:v) and the proteinase inhibitor SBTI in a high (1%
148 w:v) and a ten-fold lower concentration (0.1% w:v). Taking into account the different amount of
149 pollen and nectar (sucrose) consumed by bees, Bt-toxin concentration in this study is at least 10
150 times higher than that of maize events expressing the Bt toxin in the pollen such as Bt 176 (Koziel

151 *et al.*, 1993). Though little information is available on the content of PIs in pollen, McManus *et al.*
152 (1994) found that plants can be protected from pests when protease inhibitors are expressed at c. 1%
153 of total soluble leaf protein. Thus the Bt-toxin treatment and the high dose SBTI treatment are
154 representing worst case scenarios. Control bees were fed with pollen from the non-transformed near
155 isoline and pure sucrose solution.
156
157 *2.5. Dissection of bees*
158 The intestine consisting of midgut (ventriculus) and hindgut (rectum) was isolated on ice from
159 frozen bees by clipping the stinger and the posterior segment of the abdomen with sterile forceps
160 and carefully removing the intestine. The two intestinal sections were separated with a sterile
161 scalpel, immediately frozen in liquid nitrogen, and stored at –80°C.
162
163 *2.6. DNA extraction*
164 DNA extraction was performed according to a protocol for soil DNA extraction (Bürgmann *et*
165 *al.,* 2001). A total of 300 µl lysis buffer pH 7.5 [200 mM Tris-HCl pH 7.5, 2 M NaCl, 50 mM
166 EDTA, 2% CTAB (hexacetyltrimethylammonium bromide)] and approximately 0.4 g silica beads
167 (diameter 1 mm, Braun Biotech International GmbH, Melsungen, Germany) were added to the tube
168 containing the still frozen intestine. Cells were lysed by processing the samples for 30 s at 5.5 m s^{-1}
169 in a FP120 FastPrep beat beater (Bio101 Savant, Inc. Holbrook, NY, USA). After centrifugation for
170 5 min at 13000 x g, the supernatant was collected. Each sample was extracted two more times by
171 resuspension in 300 µl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA pH 8.0) and
172 centrifugation at 13000 x g. Combined supernatants were extracted with one volume
173 chloroform:isoamylalcohol (24:1) followed by centrifugation for 1 min. Nucleic acids were
174 precipitated by adding 1 volume of the precipitation solution (20% PEG 6000, 2.5 M NaCl),
175 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 $MgCl_2$ (Qiagen,), 0.2 µM of each primer (Microsynth, Balgach, Switzerland), 0.4 mM 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 Laboratrories, 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, $MgCl_2$ 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

199 standard ROX500 (Applied Biosystems, Foster City, CA, USA). DNA samples were denatured for
200 2 min at 92°C and then chilled on ice.

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

212

213 *2.8. Screening and DNA sequence analysis*

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

223 distinguish them from the experimentally determined T-RF sizes shown in Figure 1. The relation of
224 T-RF size group (in rmu) to the OTUs (in bp) is shown in Table 2.
225
226 *2.9. Phylogenetic affiliation*
227 For DNA sequence comparison of honeybee intestine-derived bacterial 16S rRNA gene
228 clones, the ribosomal database Project II was used (Cole *et al.,* 2003). For each clone, best-
229 matching database entries were retrieved in the aligned format. Clone sequences were aligned to
230 RDP-derived sequences using the BioEdit sequence alignment editor (Hall *et al.,* 1999). Additional
231 matching control sequences were searched and retrieved from the GenBank database. For
232 phylogenetic inference, PCR primer sequences 27F and 1378R were excluded. A UPGMA and a
233 rooted neighbour-joining phylogenetic tree were constructed with Treecon 1.3 for windows (Van de
234 Peer & Dewachter, 1994) using Jukes & Cantor distance estimation and 100 bootstrap resamplings.
235
236 *2.10. Statistical analyses*
237 Generalized linear models (GLM) on Poisson distributed data and applying the log-link
238 function were used to test for effects of the treatments on the number of OTUs. To better fit the
239 variance of the response variable, it was assumed that our data were overdispersed (Sokal & Rohlf,
240 1995). A maximum likelihood test procedure was applied to investigate treatment effects (Sokal &
241 Rohlf, 1995). The Bonferroni-Holmes correction procedure was applied for pairwise comparisons
242 of the control and the four treatments (Sokal & Rohlf, 1995). Spearman rank correlation was used to
243 test for correlations in the data sets. These analyses were conducted with the program Statistica
244 (StatSoft, Inc., Tulsa, USA).
245 In addition, the data were subjected to multivariate analysis using the program CANOCO (Ter
246 Braak, 1996). T-RF intensity data were z-transformed to average 0 and standard deviation 1. This
247 served to give each T-RF the same relative weight in statistical analysis. Data were then ordinated

248 by principal component analysis (PCA) and redundancy analysis (RDA). In RDA, the significance
249 of treatments was assessed by Monte Carlo permutation. Both PCA and RDA assume a linear
250 model for the relationship between the response of OTUs and the ordination axis. Such a linear
251 model was found to be appropriate for the data of this study because a preliminary detrended
252 analysis showed short gradient lenghts (< 3 SD). The three replicate colonies used per treatment
253 were included in the model as a covariable.
254
255 *2.11 Nucleotide sequence accession numbers*
256 The nucleotide sequences of the clones retrieved in this study have been deposited in
257 GenBank (Accession numbers DQ837602 to DQ837639).
258
259 **3. Results**
260 *3.1. T-RFLP profiles of intestinal bacterial communities from experimental bee populations*
261 Bacterial T-RFLP profiles of midguts from 97 individual bees derived from all 15
262 experimental bee populations studied under laboratory conditions revealed 1 to 7 T-RFs for each
263 sample with an average of 3.37 ± 0.99 (SE). Bacterial T-RFLP profiles of hindguts from 39 bees
264 from five different experimental bee populations revealed 2 to 8 T-RFs per sample with an average
265 of 4.38 ± 1.48. Three T-RFs occurred in more than 65% of the samples taken from the midgut while
266 8 T-RFs were detected in less than 20% of the samples (Figure 1A). In total, 12 different T-RFs
267 ranging from 79 to 491 rmu in size were identified in both midgut and hindgut. For individual bee
268 samples, however, a significantly lower number of peaks was found in the midgut as compared to
269 the hindgut regardless of whether all 15 populations were included in the analysis (GLM, $\chi^2 = 21.4$;
270 df = 1,134; $P < 0.001$) or only those five populations from which the hindgut samples were
271 collected (GLM, $\chi^2 = 12.5$; 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 ± 1.49 (n = 15) and 2 to 9 T-RFs for hindguts with an average of 5.73 ± 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 ± 1.98 (n = 37) and 3 to 9 T-RFs for hindguts with an average of 7.29 ± 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

297 factor. The T-RFs at 167.5 rmu and 87.7 rmu were exclusively found at the second sampling date in
298 late summer resulting in significantly different bacterial communities in the midguts between the
299 two sampling dates (Monte Carlo permutation test, F = 7.13; $P < 0.001$; n = 52). Looking at
300 replicate colony variation, there was no difference in mid-gut bacterial communities neither for the
301 colonies located in Zurich ($P = 0.329$; n = 37) nor for those located in Berne ($P = 0.604$; n = 15).
302 The bacterial T-RFLP profiles of bee intestines showed comparable patterns between free-
303 flying bees and experimental populations as underlined by the significant correlation between the
304 signal intensities of T-RFs from free-flying bees and laboratory-reared populations (Spearman rank
305 correlation; r = 0.681; $P < 0.05$; dots in Figure 1). Similarly, T-RFs that were frequently found in
306 experimental populations were generally also detected at high frequencies in free-flying bees,
307 resulting in a significant correlation (r = 0.818; $P < 0.05$; bars in Figure 1). Furthermore, a
308 significant correlation was found between T-RF signal intensities (the dots in Fig. 1) and
309 frequencies (the bars in Fig. 1) for the experimental population (r = 0.608; $P < 0.05$) but not for the
310 free-flying bee population (r = 0.463; $P > 0.05$).
311
312 *3.3. Sequence analyses*
313 Overall 13 different T-RFs were identified in the populations analysed (Figure1). In order to
314 obtain sequences representing these specific T-RFs, PCR products from 13 selected samples were
315 cloned, and resulting gene libraries were screened for clones encoding these T-RFs. Screening
316 yielded 17 different T-RFs, including the 13 T-RFs that were detected with community T-RFLP
317 analyses. Four T-RFs, i.e. at 124.0 rmu, 280.0 rmu, 297.0 rmu and >500 rmu, were not detected
318 with community T-RFLP analyses (Table 2).
319 DNA sequences of 38 selected clones were determined, i.e. 35 clones from the experimental
320 populations and for 3 clones from the free-flying bees. Comparison of T-RFLP and sequence data
321 indicated a maximal difference of 3 bp between relative migration units determined for T-RFs and

322 the actual sequence length (Table 2). Chimera check analysis in RDP identified no chimeric
323 sequences.
324
325 *3.4. Phylogenetic analyses*
326 For phylogenetic affiliation, the 38 clone sequences were aligned with 43 sequences identified
327 as closely related reference sequences in RDPII or GenBank and the sequence of *Saccharomyces*
328 *cerevisiae* (GenBank accession J01353 as an outgroup, which resulted in an alignment length of
329 1483 homologous positions. Phylogenetic analysis based on Jukes & Cantor distances calculation
330 and UPGMA cluster analysis with 100 bootstrap re-samplings identified five clusters, which
331 contained honey bee gut (HPG) clones, i.e. α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria,
332 Firmicutes and Bacteriodetes (Figure 2). Among the α-Proteobacteria two sub-clusters (α-1 and α-2)
333 were found. Sub-cluster α-1 contained all three clones from OTU-403bp and reference sequences
334 from *Bartonella* sp. (Figure 2). Sub-cluster α-2 contained both clones from OTU-339bp and the
335 reference sequences of *Acetobacter* sp. and *Gluconobacter* sp. as well as an uncultured clone
336 labeled as *Gluconacetobacter* clone, which was derived from a study on bee intestinal bacteria
337 (Jeyaprakash *et al.,* 2003). For the β-Proteobacteria, the sub-cluster β-1 contained all six clones
338 obtained from our study, which belonged to OTU-447/448bp and associated most closely with a
339 *Simonsiella* clone again derived from a study on bee intestinal bacteria (Jeyaprakash *et al.,* 2003).
340 Among the four sub-clusters of the γ-Proteobacteria (γ-1 to γ-4), sub-cluster γ-1 was heterogeneous
341 and contained OTU-124bp, OTU-279bp, OTU-296bp, and OTU-492bp, which all associated most
342 closely to clones derived from bee intestinal bacteria that were identified as *Serratia* spp.
343 (Jeyaprakash *et al.,* 2003). Sub-cluster γ-2 contained OTU-488bp, which separated from the γ-1
344 cluster with a 100% support from the bootstrap analysis but for which no other close relative was
345 found. Sub-cluster γ-3 contained OTU-88bp, which associated with *Arsenophonus nasoniae* SKI4
346 and sub-cluster γ-4 contained OTU-480bp and most closely clustered with *Pseudomonas* spp..

347 Beside the Proteobacteria-associated clones a complex group of Firmicutes-associated clones and
348 two Bacteriodetes associated clones were found, which could be separated into five and one
349 different sub-clusters respectively (F-1 to F-5 and B1, see Figure 2). Sub-cluster F-1 contained
350 OTU-146bp, which associated with an uncultured rumen clone (Tajima *et al.*, 2000). Sub-cluster F-
351 2 contained OTU-168-bp, which associated with *Leuconostoc* sp.. Sub-clusters F-3 and F-4
352 contained OTU-177bp and OTU-81bp respectively, without close associations to known sequences.
353 Sub-cluster F-5 contained OTU-321/322bp, and OTU-570/571bp, which were most closely
354 associated with a *Lactobacillus* clone, previously isolated from bee intestine (Jeyaprakash *et al.,*
355 2003). Cluster B1 contained OTU-89bp and was associated with Bacteriodetes without close
356 association with known sequences.
357
358 *3.5. Effects of two insecticidal proteins on bee midgut bacterial community structures*
359 No significant differences in the number of T-RFs (GLM, $\chi^2 = 0.17$; df = 2,92; $P = 0.92$) or
360 the bacterial community structure (Monte Carlo permutation testing, F = 0.64, $P = 0.190$; n = 97)
361 were found among the three different experiments that were replicated over time. In contrast, highly
362 significant differences in the number of T-RFs in honeybee midguts were observed among
363 treatments (GLM, $\chi^2 = 51.4$, df = 4,92, $P < 0.001$; Figure 3). Pairwise comparisons showed that
364 only the high concentration of SBTI significantly reduced the number of T-RFs as compared to the
365 control (GLM, $\chi^2 = 41.2$, $P < 0.001$, see Figure 3). The comparison of the control with the treatment
366 where bees were fed Cry1Ab in high concentrations (0.0014% w:v) was not significant based on the
367 adjusted significance level (GLM, $\chi^2 = 5.9$, $P = 0.0151$). A multivariate analysis further showed
368 that bacterial community structures in honeybee midguts significantly differed among the
369 treatments (Monte Carlo permutation test, F = 1.78; $P = 0.002$; n = 97) although only 7% of the
370 variance could be explained by the treatments. These differences observed among treatments could
371 not be attributed to any specific T-RF (GLM, $P > 0.05$ for all pairwise comparisons).

372

373 **4. Discussion**

374 The present study investigated the intestinal bacterial communities of honeybee populations
375 and evaluated whether consumption of two insecticidal proteins could affect the structural diversity
376 of these communities. The diversity found was relatively low as compared with other insects such
377 as termites (Hongoh *et al.,* 2003). However, in comparison to previous studies on honeybees using
378 cultivation-dependent methods (Gilliam, 1997), diversity was much higher in our study.
379 Cultivation-dependent analyses have regularly demonstrated a high prevalence of *Bacillus* spp.,
380 which could not be confirmed in the present study. Concordance with other groups of bacteria was
381 better, e.g. *Lactobacillus* spp., which were found with either approach. The present study revealed
382 several T-RFs, which clustered with *Lactobacillus* although each T-RF was only found in some of
383 the bees. Furthermore, members of the γ-Proteobacteria, i.e. sub-clusters γ-1 (T-RF at 490.8 rmu)
384 and γ-2 (T-RF at 486.0 rmu) were found to represent the most common group of bacteria in
385 honeybee intestines (Figure 1). These sub-clusters were components of a large γ-proteobacterial
386 cluster, which also included two cloned sequences isolated from bee guts (Jeyaprakash *et al.,* 2003).
387 Recently, Mohr & Tebbe (2006) reported on the bacterial communities of bee intestines with
388 phylogenetic analyses based on partial sequences (370 bp) of 16S rRNA genes. The phylogenetic
389 analysis performed in this study was based on approximately 1.4 kb long sequences (Figure 2),
390 extending the potential to assign these sequences to other bacterial sequences deposited in public
391 databases. The honeybee intestinal bacterial 16S rRNA gene sequences reported by Jeyaprakash *et*
392 *al.* (2003) (underlined in Figure 2) show strong similarities to the honeybee intestinal bacterial
393 communities reported here. In accordance with both studies (Jeyaprakash *et al.* 2003; Mohr &
394 Tebbe 2006) our results indicate a relatively low diversity of bacterial groups in honeybee guts. The
395 high consistency of bacterial community compositions in the guts of honeybees found in studies
396 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, γ-Proteobacteria from subclusters γ-1 and γ-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 γ-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

422 of the pollen source and indicated that experimental systems can be representative for the field
423 situation. However, a conclusive assessment of the severity of effects detected will depend on a
424 more profound knowledge of essential bacteria in the honeybee gut and on a more detailed
425 understanding of their specific physiological functions.

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

442 What ever the causal factors of the observed changes in the bacterial microflora were, data
443 suggest that honeybees might be able to tolerate these changes because those related to the high-
444 dose SBTI treatment were not qualitatively different from those related to other environmental
445 factors, including season, location or pollen source. In contrast to SBTI, Cry1Ab-expressing Bt-
446 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.

References

Amann RI, Ludwig W & Schleifer KH (1995) Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microb Rev* **59**: 143-169.

Alippi AM, Lopez AC & Aguilar OM (2002) Differentiation of *Paenibacillus larvae* subsp. *larvae*, the cause of American foulbrood of honeybees, by using PCR and Restriction Fragment Analysis of genes encoding 16s R-rna. *Appl Environ Microbiol* **68**: 3655-3660.

Babendreier D, Kalberer N, Romeis J, Fluri P, Mulligan, E & Bigler F (2005) Influence of transgenic Bt-Pollen, Bt-Toxin and Proteinase Inhibitor (SBTI) ingestion on development of the hypopharyngeal gland in honeybees. *Apidologie* **36**: 585-594.

Bürgmann H, Pesaro M, Widmer F & Zeyer J (2001) A strategy for optimizing quality and quantity of DNA extracted from soil. *J Microbiol Methods* **45**: 7-20.

Burgess EPJ, Malone LA & Christeller JT (1996) Effects of two proteinase inhibitors on the digestive enzymes and survival of honey bees (*Apis mellifera*). *J Insect Physiol* **42**: 823-828.

Cole JR, Chai B, Marsh TL *et al.* (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**: 442-443.

Conner AJ, Glare TR & Nap JP (2003) The release of genetically modified crops into the

environment - part II. Overview of ecological risk assessment. *Plant J* **33**: 19-46.

Deml R, Meise M & Dettner K (1999) Effects of *Bacillus thuringiensis* delta-endotoxins on food utilization, growth, and survival of selected phytophagous insects. *J Appl Entomol* **123**: 55-64.

Dillon RJV & Dillon M (2004). The gut bacteria of insects: Nonpathogenic interactions. *Annu Rev Entomol* **49**: 71-92.

EPPO (2001) Revised draft of EPPO Guidelines PP 1/170: Guidelines for the efficacy evaluation of plant protection products: side effects on honey bees. Appendix I, (Belzunces LP, Pélissier C & Lewis GB, eds.), pp. 279-286, Hazards of pesticides to bees, Les Colloques de l'INRA 98, INRA, Paris.

Free JB (1993) *Insect pollination of crops*. Academic Press, London, UK.

Gilliam M (1979) Microbiology of pollen and beebread: the genus *Bacillus*. *Apidologie* **10**: 269-274.

Gilliam M (1997) Identification and roles of non-pathogenic microflora associated with honey bees. *Fems Microbiol Lett* **155**: 1-10.

Gilliam M & Valentine DK (1976) Bacteria isolated from the intestinal contents of foraging worker honey bees, *Apis mellifera*: The genus *Bacillus*. *J Invertebr Pathol* **28**: 275-276.

Gilliam M & Morton HL (1978) Bacteria belonging to genus *Bacillus* isolated from honey bees, *Apis mellifera*, fed 2,4-d and antibiotics (1). *Apidologie* **9**: 213-221.

Gilliam M, Taber S, Lorenz BJ & Prest DB (1988a) Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. *J Invertebr Pathol* **52**: 314-325.

Gilliam M, Lorenz BJ & Richardson GV (1988b) Digestive enzymes and microorganisms in honey bees, *Apis mellifera* - Influence of Streptomycin, age, season and pollen. *Microbios* **55**: 95-114.

Gilliam M, Buchmann SL, Lorenz BJ & Schmalzel RJ (1990) Bacteria belonging to the genus *Bacillus* associated with 3 species of solitary bees. *Apidologie* **21**: 99-105.

497 Hails RS (2000) Genetically modified plants - the debate continues. *Trends Ecol Evol* **15**: 14-18.
498 Hall TA (1999) Bio Edit: a user-friendly biological sequence alignment editor and analysis program
499 for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**: 95-98.
500 Hartmann M, Frey B, Kölliker R & Widmer F (2005) Semi-automated genetic analyses of soil
501 microbial communities: comparison of T-RFLP and RISA based on descriptive and
502 discriminative statistical approaches. *J Microbiol Methods* **61**: 349-360.
503 Heuer H, Krsek M, Baker P, Smalla K & Wellington EMH (1997) Analysis of actinomycete
504 communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic
505 separation in denaturing gradients. *Appl Environ Microbiol* **63**: 3233-3241.
506 Hongoh Y, Ohkuma M & Kudo T (2003) Molecular analysis of bacterial microbiota in the gut of
507 the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). *Fems Microbiol Ecol* **44**: 231-
508 242.
509 Jeyaprakash A, Hoy MA & Allsopp MH (2003) Bacterial diversity in worker adults of *Apis*
510 *mellifera capensis* and *Apis mellifera scutellata* (Insecta: Hymenoptera) assessed using 16S
511 rRNA sequences. *J Invertebr Pathol* **84**: 96-103.
512 Jouanin L, Bonade-Bottino M, Girard C, Morrot G & Giband M (1998) Transgenic plants for insect
513 resistance. *Plant Sci* **131**: 1-11.
514 Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson H, Desai N,
515 Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E,
516 Rhodes R, Warren GW Wright M & Evola SV (1993) Field performance of elite transgenic
517 maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Biotechn.
518 **11**: 194-200.
519 Laskowski M & Kato L (1980) Protein inhibitors of proteinases. *Annu Rev Biochem* **49**: 593-626.
520 Lawrence PK, Koundal KR (2002) Plant protease inhibitors in control of phytophagous insects.
521 *Electr J Biotechnol* **5**: U122-U138.

522 Malone LA & Pham-Delegue MH (2001) Effects of transgene products on honey bees (*Apis*
523 *mellifera*) and bumblebees (*Bombus* sp.). *Apidologie* **32**: 287-304.
524 Malone LA, Giacon HA, Burgess EPJ, Maxwell JZ, Christeller JT & Laing WA (1995) Toxicity of
525 Trypsin endopeptidase inhibitors to honeybees (Hymenoptera, Apidae). *J Econ Entomol* **88**:
526 46-50.
527 Malone LA, Todd JH, Burgess EPJ & Christeller JT (2004) Development of hypopharyngeal glands
528 in adult honey bees fed with a Bt toxin, a biotin binding protein and a protease inhibitor.
529 *Apidologie* **35**: 655-664.
530 McManus MT, White DWR & McGregor PG (1994) Accumulation of a chymotrypsin inhibitor in
531 transgenic tobacco can affect the growth of insect pests. *Transgenic Research* **3**: 50-58.
532 Mohr KI & Tebbe CC (2006) Diversity and phylotype consistency of bacteria in the guts of three
533 bee species (Apoidea) at an oilseed rape field. *Environ Microbiol* **8**: 258-272.
534 Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR & Dean DH
535 (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Molec Biol Rev* **62**:
536 775- 806.
537 Shelton AM, Zhao JZ & Roush RT (2002) Economic, ecological, food safety, and social
538 consequences of the deployment of Bt transgenic plants. *Annu Rev Entomol* **47**: 845-881.
539 Sokal RR & Rohlf FJ (1995) Biometry. W.H. Freeman and Company, New York, USA.
540 Tajima K, Arai S, Ogata K, Nagamine T, Matsui H, Namakura M, Aminov RI & Benno Y (2000)
541 Rumen bacterial community transition during adaptation to high-grain diet. Anaerobe **6**: 273-
542 284.
543 Ter Braak (1996) Unimodal models to relate species to environment. PhD thesis, Agricultural
544 Mathematics Group-DLO: Wageningen, the Netherlands, 266 pp.
545 Tokura M, Ohkuma M & Kudo T (2000) Molecular phylogeny of methanogens associated with
546 flagellated protists in the gut and with the gut epithelium of termites. *Fems Microbiol Ecol* **33**:

233-240.

US EPA (1996) Ecological effects test guidelines OPPTS 850.3020 Honeybee acute contact toxicity test, EPA 712-C-96-147, Washington, DC.

Van de Peer Y & Dewachter R (1994) Treecon for Windows - a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computer Appl Biosci* **10**: 569-570.

554 Table 1: Primers used for PCR amplification and sequencing

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

555 [a] M represents the degeneracy A/C.

556 [b] Position of the 5'-end of the primer relative to the cloning site in the pGEM-T Easy Vector (Promega).

557

Table 2: List of terminal restriction fragment (T-RF) sizes and corresponding 16S rRNA gene clones derived from honeybee intestines.

T-RF size		Phylogenetic affiliation[b]	Clone names		
T-RLFP analysis (rmu)	sequence analysis (bp)				
Sequences corresponding to detected T-RFs					
79.2 ± 0.5	81	F-4	A5R3-3	A5R3-1	
85.0 ± 1.0	88	γ-3	A4R5-3	B4V1-1	
87.7 ± 0.5	89	B-1	A2V3-2	A2V3-4	
144.6 ± 2.1	146	F-1	A4R5-4	A2R6-2	
167.5 ± 1.0	168	F-2	D2V2-1		
178.0 ± 0.5	177	F-3	B1V3-2	B1V3-3	
322.2 ± 0.5	321	F-5	A5R3-2		
	322	F-5	B1V1-3		
402.3 ± 0.5	403	α-1	A2R6-1	A2V3-3	A4R5-2
442.0 ± 0.5	439	α-2	B2V6-3		
	439	α-2	B2V6-2		
446.8 ± 1.5	447	β-1	B1V1-2	A5V8-2	A5V8-4
	448	β-1	B2V6-4		
	448	β-1	B2V6-1	D2V2-3	
478.8 ± 1.3	480	γ-4	A2V5-1	B1V3-4	
486.0 ± 2.1	488	γ-2	B1V3-1	C1V8-1	
490.8 ± 2.6	492	γ-1	A5V8-1	A1R2-1	D2V2-2
Sequences isolated from gene libraries					
124.0 ± 0.5	124	γ-1	A2V5-2	A2V5-3	
280.0 ± 0.5	279	γ-1	A2V3-1	A5V8-3	
297.0 ± 0.5	296	γ-1	A5V8-5		
nd[c]	569	F-5	B1V1-1		
	570	F-5	A4R5-1		

[a] based on MspI digestion

[b] α = α-Proteobacteria; β = β- Proteobacteria, γ = γ-Proteobacteria; B = Bacteroidetes; F = Firmicutes; for details see Figure 2

[c] not determined as fragment size analysis was restricted to 500 bp

565 **Captions to Figures**

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

572

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

578

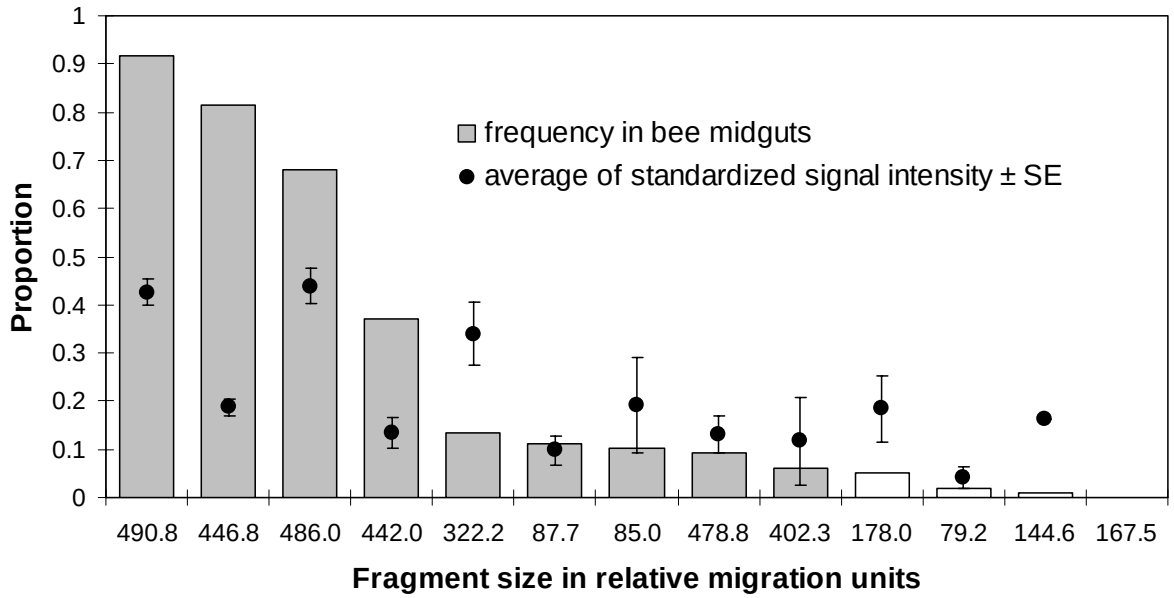
579 Figure 3: Mean number of operational taxonomic units (OTUs) found in the guts of honeybees fed
580 with Bt-maize pollen (event Mon 810) or insecticidal proteins (the Bt-toxin Cry1Ab at 0.0014% and
581 the Kunitz soybean trypsin inhibitor (SBTI) at 0.1% and 1%) dissolved in sugar solution. The only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

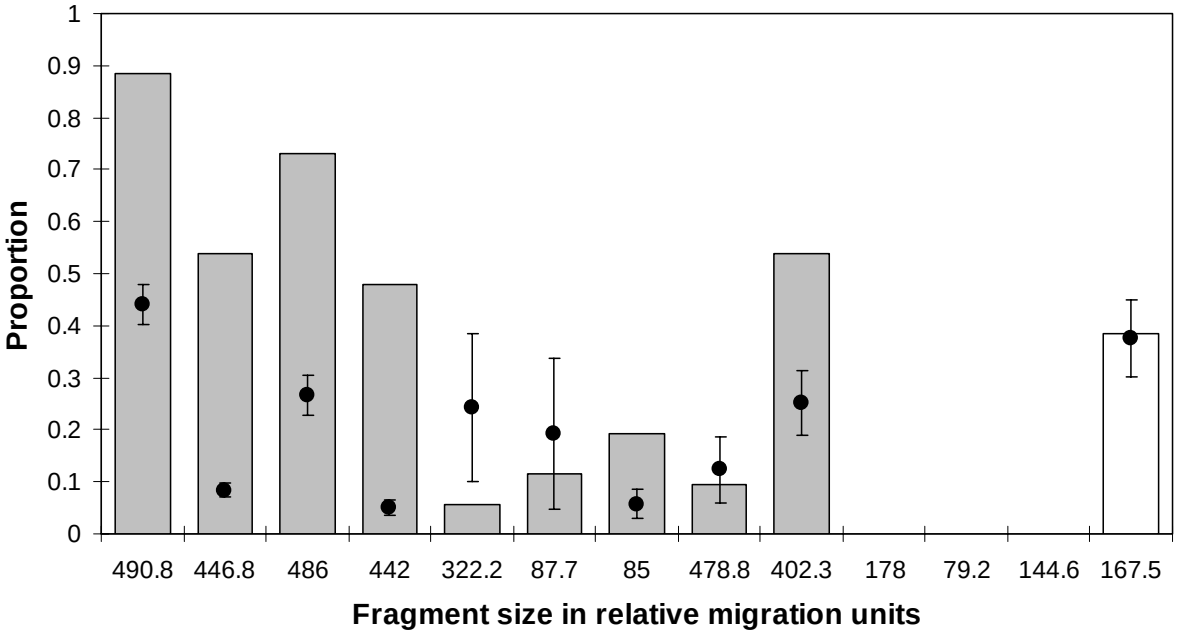
584

585

586

587

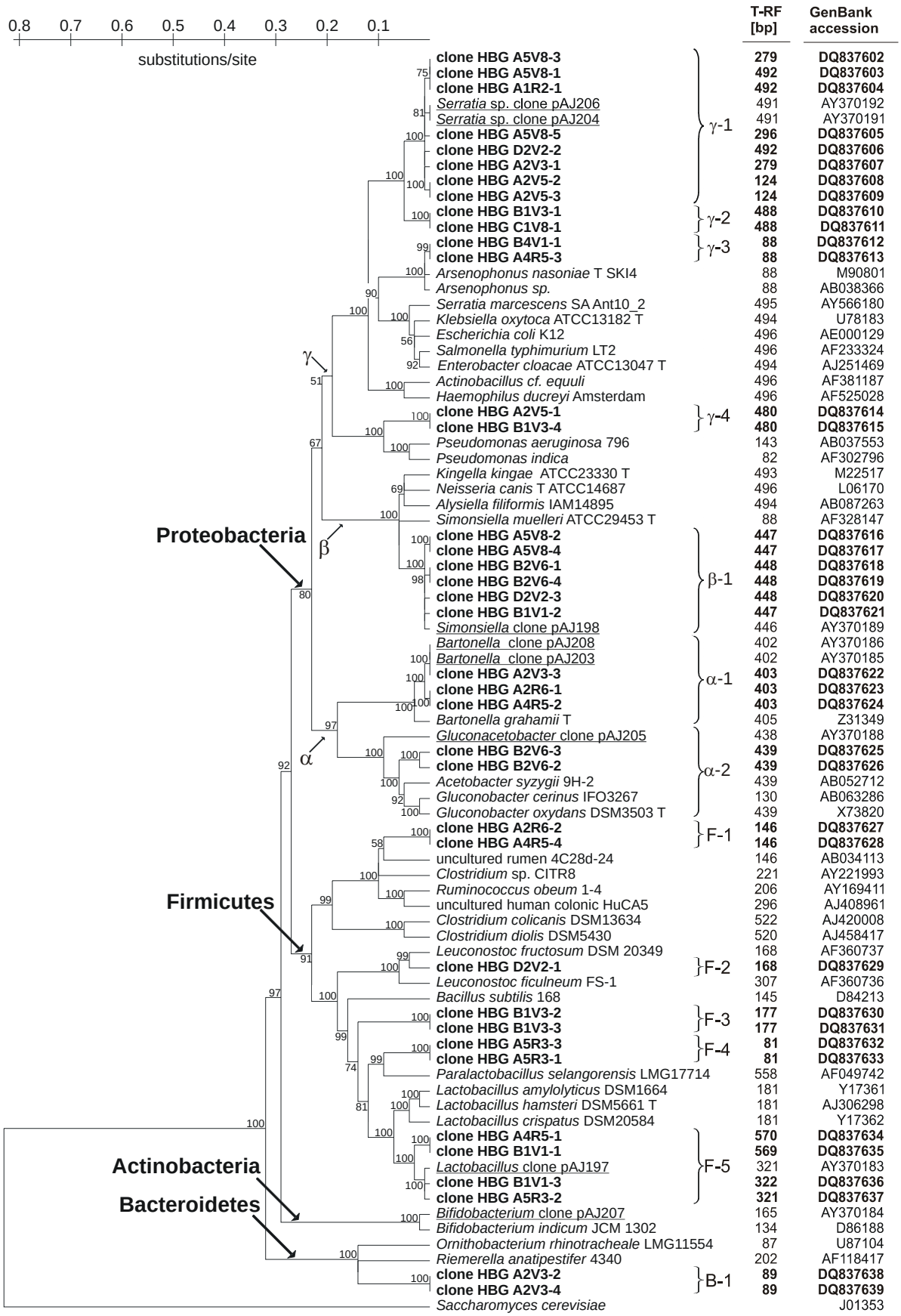




588

589 Figure 1

590

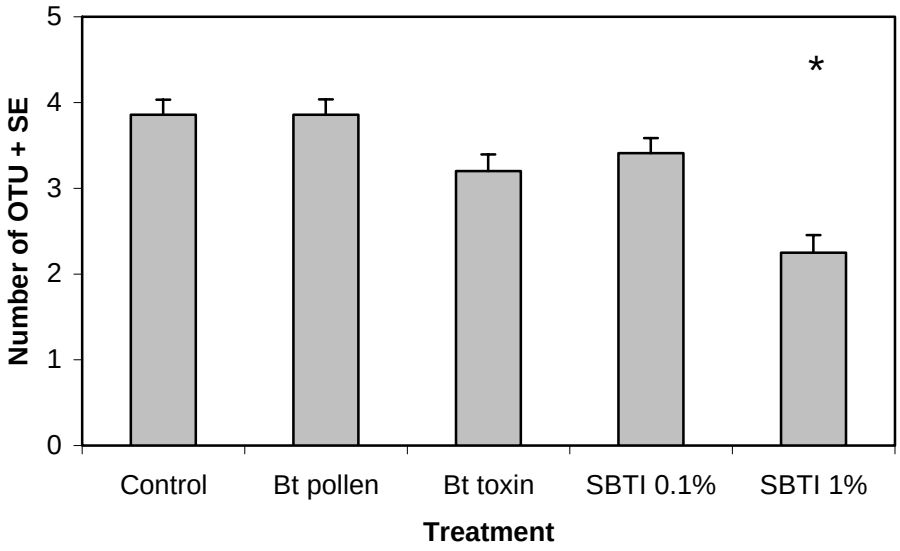


591

592 Figure 2

593

594



595

596 Figure 3

597

598