Bacterial Probiotics Induce an Immune Response in the Honey Bee (Hymenoptera: Apidae)

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ABSTRACT To explore immune system activation in the honey bee, *Apis mellifera* L., larvae of four ages were exposed through feeding to spores of a natural pathogen, *Paenibacillus larvae larvae*, to cells of a diverse set of related nonpathogenic bacteria, and to bacterial coat components. These larvae were then assayed for RNA levels of genes encoding two antibacterial peptides, abaecin and defensin. Larvae exposed to either *P. l. larvae* or a mix of nonpathogenic bacteria showed high RNA levels for the abaecin gene relative to controls. First instars responded significantly to the presence of the nonpathogenic mix within 12 h after exposure, a time when they remain highly susceptible to bacterial invasion. This response was sustained for two successive instars, eventually becoming 21-fold higher in larvae exposed to probiotic spores versus control larvae. The mixture of nonpathogenic bacteria is therefore presented as a potential surrogate for assaying the immune responses of different honey bee lineages. It also is proposed that nonpathogenic bacteria can be used as a probiotic to enhance honey bee immunity, helping bee larvae, and other life stages, survive attacks from pathogens in the field.

KEY WORDS innate immunity, antimicrobial peptide, American foulbrood

HONEY BEES, *Apis mellifera* L. (Hymenoptera: Apidae), face numerous pests and pathogens, costing millions of dollars annually to the important pollination and honey industries (Morse and Flottum 1997). A primary goal of honey bee research remains to breed bees that resist or tolerate pests and pathogens. Our work is focused on the abilities of individual bees to inhibit pathogens through internal immune defenses. We are particularly interested in immune responses toward *Paenbacillus larvae larvae* (Heyndrickx et al. 1996), a gram-positive bacterium responsible for the widespread honey bee disease American foulbrood (AFB). Bees and other arthropods possess an innate immune response that is predicted to limit disease (Hoffmann 2003). One branch of this response includes a diverse set of short antimicrobial peptides that show activity against fungi, bacteria, and eukaryotic pathogens. Although the major antimicrobial peptides of honey bees have been known for some time (Casteels et al. 1989, 1990; Casteels-Josson et al. 1994), their effects on natural pathogens remain obscure. Nevertheless, initial evidence suggests that these peptides play a role against *P. l. larvae* and other pathogens. First, four antimicrobial peptides in bees; abaecin, apidaecin, hymenoptaecin, and defensin, all show inhibitory activity in vitro against bacteria (Casteels-Josson et al. 1994), and two of these are up-regulated after natural exposure to *P. l. larvae* (Evans 2004). Also, unidentified heat-stable substances in the hemolymph of larval and adult bees show inhibitory activity against *P. l. larvae* (Riessberger-Galle et al. 2001, Wedenig et al. 2003), suggesting that antimicrobial peptides, or other chemical components of hemolymph, are effective against this pathogen. Finally, orthologs to these peptides (e.g., defensin; Shin et al. 2003) are involved with disease resistance in other insect species.

Spores of *P. l. larvae* can be transferred to young larvae during feeding by adult nurse bees. After germination, these spores invade the honey bee gut epithelium and replicate to sufficient levels to kill developing bees before pupation. Larval bees are highly susceptible to *P. l. larvae* spores, with fewer than 10 spores needed to cause mortality when fed to first instars (Shimanuki 1997). Because this pathogen is fairly widespread in bee populations (≈10% of colonies contained measurable spore levels in one study reviewed by Hansen and Brodsgaard 1999), we anticipate that larvae often are exposed to low levels of *P. l. larvae* spores but avoid disease symptoms in part through an inhibitory immune response. Survival of these individual larvae becomes critical at the colony level, because bees that succumb to AFB act as a tremendous source of spores with the potential to infect additional colony members (2×10^9 spores per deceased bee; Shimanuki 1997).

The current study had two goals. Our primary goal was to design and test safe assays that may be used by bee breeders and researchers to select for immunocompetence in honey bee populations, without the use of dangerous pathogens. These assays build upon a successful in vitro rearing method for honey bee larvae (Brodsgaard et al. 2000). Three recent suc-

cesses in breeding bees with desired traits underscore the importance of experiments aimed at artificially selecting disease-resistance traits. First, honey bees from far-east Russia were successfully screened and selected for traits that confer resistance to the primary arthropod pest of bees, the mite *Varroa destructor* (Anderson & Trueman) (Rinderer et al. 2001). Second, the ability of developing bees to suppress mite reproduction was shown to be a robust breeding tool (Harbo and Harris 2001). Finally, bees from diverse lineages have been bred for enhanced hygienic behavior as adults, a trait that may improve survival against both pests and pathogens (Spivak and Reuter 2001). Programs to breed bees with these traits could benefit from screens for additional desired traits, such as enhanced immunity.

While developing protocols to test the immune responses of bees, we found that nonpathogenic bacteria can generate a sustained increase in levels of the antibacterial peptide abaecin during honey bee development. Accordingly, a second goal of this project became to explore ways to stimulate the honey bee immune system through the feeding of nonpathogenic surrogates. We anticipate that such methods can be used, either as a prophylactic or a therapeutic treatment, to help honey bees survive natural attacks from diverse pathogens. Similar "probiotic" (Schrezenmeir and de Vrese, 2001) strategies are widely used in the raising of livestock and poultry (Dalloul et al. 2003), and they have been used successfully to reduce disease in at least one invertebrate, the tiger shrimp, *Penaeus monodon* (Rengpipat et al. 2003).

Materials and Methods

Insect Material and Rearing. Larvae for all trials were the progeny of a single wild-mated honey bee queen (*Apis mellifera ligustica*) maintained in a disease-free apiary at the USDA Bee Research Lab (Beltsville, MD). Larvae were collected by transferring them manually using a fine spatula (Laidlaw and Page 1997) into plastic microtiter trays used for rearing. These trays consisted of 96 U-bottomed wells each of which was 8 mm in diameter and ≈6 mm in depth at center (ISC BioExpress, Kaysville, UT). Larvae were fed an excess of a liquid diet consisting of royal jelly, glucose, honey, and yeast extract (Vandenberg and Shimanuki 1988). Trays were covered with wet paper towels and maintained in a humid incubator at 34°C.

At the start of each trial, worker-destined larvae of different ages were placed randomly into control and experimental groups. Larval age (instar) was determined by morphological and size-based comparisons with known landmarks of larval development. We estimate that these age estimates varied by ±6 h and were never outside the predicted instar stage. First instars, in particular, were chosen early in the instar, when larvae were transparent and only slightly larger than prehatching embryos. Control larvae were fed only the artificial diet. One experimental group in each trial was fed the above-mentioned diet plus a final concentration of 100 spores per microliter from a pathogenic isolate of *P. l. larvae* (isolate BRL230010, Berkeley, CA). For inoculation with species unlikely to be pathogenic for bees, an initial trial involved feeding first instars a mix of bacterial spores from species in the genera *Bifidobacterium* and *Lactobacillus* (*B. infantis, B. longum, L. rhamnosus, L. acidophilus,* and *L. reuteri;* Nature's Way, Springville, UT). This mixture was fed to bees at a cumulative concentration of 2×10^6 cells per microliter of food. All subsequent trials used a similar mix containing *L. rhamnosus* (20%), *L. casei* (20%), *L. plantarum* (10%), *L. acidophilus* (20%), *B. longum* (20%), and *B. breve* (10%; Jarrow Formulas, Los Angeles, CA), fed at a cumulative concentration of 5×10^5 cells per microliter of bee food. Additional inoculations were carried out with the bacterial coat components lipopolysaccharide (LPS; 0.2 μg/μl final concentration, Sigma, St. Louis, MO) and peptidoglycan (1 μg/μl, Sigma) dissolved directly in the artificial diet.

Food was refreshed every 24 h. Larvae continued to receive the same diet, with added bacteria as appropriate, throughout the trials. Trials were initiated when larvae were 12 h (first instar), 36 h (second instar), 60 h (third instar), and 80 h (fourth instar) old. After incubations of 12, 24, or 48 h, trays of inoculated and control larvae were placed at −80°C for storage before immune-gene analyses.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from individual larvae by using the RNAqueous protocol (Ambion, Austin, TX). DNA was removed from this extraction by using a 45-min DNase incubation at 37°C (5 U of DNase I in appropriate buffer, Roche Diagnostics, Indianapolis, IN, with the RNase inhibitor RNAsin, Roche Diagnostics). Next, first-strand cDNAs were generated from ≈2 μg of total RNA by using a mix of 50 U of Superscript II (Invitrogen, Carlsbad, CA), 2 nmol of DNTP mix, and a composite of 2 nmol of poly dT-18 and 0.1 nmol of poly dT(12–18). Synthesis was carried out at 42°C for 1 h.

Quantitative Real-Time Polymerase Chain Reaction (PCR). Specific genes were amplified by PCR by using an Icycler real-time PCR thermal cycler (Bio-Rad, Hercules, CA). Fifty-microliter reaction mixes consisted of 2 U of *Taq*DNA polymerase with suggested buffer (Roche Diagnostics), 0.2 μM fluorescein, 1 mM DNTP mix, 2 mM $MgCl_2$, 0.2 μM of each gene-specific primer, and a final concentration of 2.5× SYBR Green 1 (Applied Biosystems, Foster City, CA). Abaecin and defensin primers were designed from precursor sequences for these genes (Casteels-Josson et al. 1994), GenBank entries U15954 and U15955, respectively. The sequence for primer abaecin.F was 5′-CAG CAT TCG CAT ACG TAC CA-3′ and abaecin.R was GAC CAG GAA ACG TTG GAA AC, whereas defensin.F was TGC GCT GCT AAC TGT CTC AG and defensin.R was AAT GGC ACT TAA CCG AAA CG. Transcript levels for a gene whose activity is closely tied with mRNA concentration (ribosomal protein S5, GenBank BG101562, Evans and Wheeler 2000) were used to normalize against vari-

Table 1. RNA levels for abaecin and defensin in honey bee larvae after exposure to spores of *P. l. larvae* or a probiotic mix (see text)

Age	Inoculation (h)	Abaecin			Defensin		
		Sample size	*P. l. larvae*	Probiotic	Sample size	*P. l. larvae*	Probiotic
1st Instar	3	8	**7.80**	**2.90**	8	0.20	**6.79**
	6	8	0.20	0.58	8	**1.50**	0.54
	12	8	**3.33**	**5.86***	8	0.52	0.68
	24	60	**2.69**	**8.22***	54	**1.21**	0.98
	48	16	**28.38****	**21.1****	12	**4.63**	**2.34**
2nd Instar	24	16	**2.99**	**2.76**	12	0.85	0.61
	48	16	**9.67**	**4.75**	12	**4.14**	**1.09**
3rd Instar	24	16	**2.56**	0.78	12	**1.94**	0.53
	48	16	0.23	**3.66**	12	0.23	**2.46**
4th Instar	24	16	**3.45**	**3.80**	12	**3.44**	0.31
	48	16	**1.47**	**1.53**	12	**1.22**	**2.91**

Values refer to fold-changes relative to untreated control bees, within each age × inoculation time trial. Comparisons estimated to have an increased RNA level in treatment larvae shown in bold. Sample sizes are for each of three categories (controls, treated with *P. l. larvae* and treated with probiotic bacteria.

Significance by one-tailed ANOVA, * $P < 0.05$, ** $P < 0.01$.

able mRNA levels. Primers for this gene were AmRPS5.F AAT TAT TTG GTC GCT GGA ATT G and AmRPS5.R TAA CGT CCA GCA GAA TGT GGT A. All reactions were carried out using a thermal program of 95°C for 3 min followed by 40 cycles of (denaturing at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min 30 s). Fluorescence was measured during the annealing step. Melt-curve analyses were used after amplification to confirm that fluorescence was the result of amplified products of the predicted size.

Data Analyses. Fluorescence levels during PCR were normalized within wells by using average fluorescence during cycles 2–10. We then measured, for each sample, the number of PCR cycles at which fluorescence from amplified PCR products reached a specific threshold. This threshold was defined as the point when well fluorescence became >10 times the mean standard deviation across all samples. Threshold cycle numbers for defensin and abaecin were then subtracted from the ribosomal protein S5 (RPS5) threshold for each sample. This value was then scaled as a power of 2 to produce an estimate of relative cDNA abundance for each gene, in each sample. Analyses of variance were carried out for each age and incubation time, by using treatment as a factor and the controlled threshold cycle as a response. Values are presented as both fold-differences relative to expression found in control larvae and as predicted values relative to the control gene RPS5.

Results

Samples for which RPS5 levels were not detected were dropped from the analysis, leaving a total of 660 and 588 larvae sampled for abaecin and defensin, respectively. Transcript levels for abaecin increased significantly upon exposure of first instars both to spores of *P. l. larvae* and to cells of the mixture of gram-positive bacteria (Table 1). The effect of the bacterial exposure was particularly strong 48 h after the inoculation of first instars, where the natural pathogen and the probiotic mix generated increases of 28- and 21-fold over the control larvae, respectively (Table 1; Fig. 1). Overall, nine of 11 experimental trials showed increased abaecin expression both when *P. l. larvae* and probiotic spores were added (one-tailed sign test, $P < 0.05$). Two bacterial coat components, LPS and peptidoglycan, did not cause a significant change in expression of abaecin in either of the two ages or incubation times for which they were tested.

Defensin transcripts did not change significantly in response to either the natural pathogen or the probiotic mixes. There were, however, borderline effects of the nonliving bacterial components for defensin expression (Table 2), including a nonsignificant *decrease* in defensin expression in first instars, after 48-h incubation ($P = 0.07$).

There was substantial variation among individuals for immune-gene expression, even within treatments and ages, a result also seen in an earlier study with only the natural pathogen. This variation was not significantly different under the different rearing conditions, although it may have been highest for larvae exposed to bacteria. For abaecin measurements on first instars incubated for 24 h, for example, the coefficient of variation was 64% for control larvae, 91% for larvae treated with *P. l. larvae*, and 83% for the probiotic mix.

Discussion

These experiments show diet-based changes in RNA levels of the gene encoding abaecin, an antimicrobial peptide implicated in the honey bee disease response. Although it is arguably not surprising that abaecin transcripts increased in response to a primary natural pathogen (*P. larvae larvae*), it is intriguing that bacteria normally foreign to bees are capable of inducing an equally strong immune response when ingested. This suggests that the honey bee immune response is fairly nonspecific, something not yet shown for ingested bacteria. It also indicates that the probiotic bacteria described here could function as surrogates for *P. l. larvae* in trials aimed at assessing the immune responses of bees. Because *P. l. larvae* out-

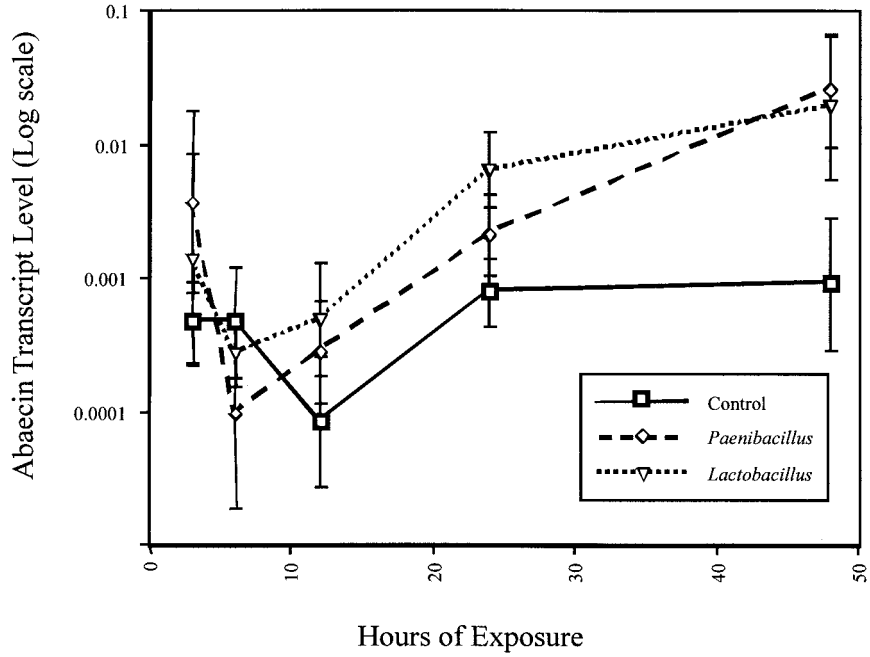

Fig. 1. Transcript levels for the antibacterial peptide abaecin during development of honey bee larvae. First instars were fed in vitro for, 3, 6, 12, 24, and 48 h before analyses ($n = 8, 8, 8, 60$, and 16 per treatment for these sampling times, respectively). Treatments were 100 spores per μl of *P. l. larvae* or 10^5–10^6 cells per μl of the probiotic *Lactobacillus*-based mixture described in the text. Standard error bars are shown.

breaks are highly risky for the apiaries of both queen breeders and researchers, having a safer alternative for screening bees seems useful. The rearing technique described here, a scaled-up version of an in vitro protocol first proposed by Brodsgaard et al. (1998), can generate immune-response data for large numbers of bees, which would be critical for assessing diverse genetic lineages for desired responses. Indeed, exposure to nonpathogenic bacteria reveals substantial variation among individuals for immune-gene expression, mirroring a previous study by using the pathogen *P. l. larvae* alone (Evans 2004).

We feel the evidence presented here also supports the potential of using nonpathogenic bacteria as probiotic diet additives to spur the immune responses of bees. First instars showed a substantial increase in abaecin transcript levels beginning 12 h after exposure to food containing the probiotic mix relative to controls. This increase reached a 20-fold difference after 48 h of feeding, when larvae were in the third instar, indicating that the response can be maintained for the long term. Although caution should be taken in any field assays, the history of known bee pathologies suggests that these bacteria pose little risk for honey bee (or human) health and that they are good candidates as field treatments for bee colonies. The probiotic bacterial spores used are readily available as veterinary and human dietary supplements, suggesting that it would be relatively easy to generate a supply of probiotic treatments for bees. Nevertheless, it might be helpful to test and develop bee-specific probiotics, perhaps by relying on nonpathogenic bacteria endogenous to bees (including other species of *Paenibacillus;* Gilliam 1997). We were unable to elicit an immune response by using the nonliving bacterial surrogates peptidoglycan and LPS.

Table 2. RNA levels for abaecin and defensin in honey bee larvae exposed to bacterial coat components LPS and peptidoglycan

	Age	Incubation (h)	Sample size	LPS	Peptidoglycan
Abaecin	1st Instar	48	16	0.69	0.13
	2nd Instar	12	12	**1.62**	0.90
Defensin	1st Instar	48	12	**2.42**	0.26
	2nd Instar	12	8	**7.93**	**3.70**

Values refer to fold-changes relative to untreated control bees; those in bold had more predicted transcripts than control bees, although none were significant by ANOVA.

A potential cost to activating the immune system prophylactically could be in the extra energy or resources used in mounting the immune response. Although a slight growth cost from immunopeptide production has been found in the beetle *Tenebrio molitor* L. (Armitage et al. 2003), we have no evidence for such a cost in bees (J.D.E., unpublished data). Further field or laboratory trials with bees raised to adulthood are needed to determine any growth or survival costs of immune system activation. A second potential cost would be the elimination of commensal or beneficial

bacteria in the digestive tracts of bees (Gilliam 1997). We anticipate, however, that the primary antibacterial activity of the induced genes will occur in the hemolymph (Casteels-Josson et al. 1994) and hence will be of importance only for those bacteria, presumably pathogenic, that transgress the gut wall.

Although both the colony level impacts of probiotic feeding on disease and the mechanisms by which large numbers of bees could be exposed to probiotics remain to be determined, a strategy of diet-induced immune response seems viable for two reasons. First, many of the critical larval and adult bee pathogens gain entry into the body by this route, including fungi, bacteria, and protozoal pathogens (Morse and Flottum 1997). It seems likely that bees therefore have responses focused on detecting potential pathogens in the gut. Second, beekeepers currently use various feeding mechanisms to introduce antidisease agents to the colonies. These include oil patties with the antibiotic oxytetracycline to combat foulbrood, liquid applications of antibiotics against *P. l. larvae* or *Nosema* disease, and powdered sugar or similar compounds to reduce mite levels on adult bees. Any one of these systems might be co-opted to introduce probiotic cells to honey bee larvae via nurse bee intermediaries.

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