SYMPOSIUM

Paratransgenesis: An Approach to Improve Colony Health and Molecular Insight in Honey Bees (Apis mellifera)?

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Synopsis The honey bee (Apis mellifera) is highly valued as a commercial crop pollinator and a model animal in research. Over the past several years, governments, beekeepers, and the general public in the United States and Europe have become concerned by increased losses of honey bee colonies, calling for more research on how to keep colonies healthy while still employing them extensively in agriculture. The honey bee, like virtually all multicellular organisms, has a mutually beneficial relationship with specific microbes. The microbiota of the gut can contribute essential nutrients and vitamins and prevent colonization by non-indigenous and potentially harmful species. The gut microbiota is also of interest as a resource for paratransgenesis; a Trojan horse strategy based on genetically modified symbiotic microbes that express effector molecules antagonizing development or transmission of pathogens. Paratransgenesis was originally engineered to combat human diseases and agricultural pests that are vectored by insects. We suggest an alternative use, as a method to promote health of honey bees and to expand the molecular toolbox for research on this beneficial social insect. The honey bees’ gut microbiota contains lactic acid bacteria including the genus Lactobacillus that has paratransgenic potential. We present a strategy for transforming one Lactobacillus species, L. kunkeei, for use as a vector to promote health of honey bees and functional genetic research.

Introduction

Insects vector several disease-causing pathogens, creating a global challenge through their negative impact on human health and agriculture (Robinson et al. 2004; Coutinho-Abreu et al. 2010). To halt the spread of these pathogens, scientists have attempted to genetically transform insect-associated symbiotic microorganisms, a process called the paratransgenic method (Durvasula et al. 1997). Paratransgenesis is a Trojan-horse approach in which symbiotic bacteria, fungi, or viruses of the vector insect are genetically manipulated to deliver effector proteins that block development or transmission of the pathogen (Fig. 1). The ultimate goal of paratransgenesis is to combat the disease vectored by the insect, thereby reducing its ability to damage human health or economic interests (Robinson et al. 2004; Coutinho-Abreu et al. 2010).

Although general, in principle, the paratransgenic method has so far been exclusively applied to insect systems, starting with the kissing bug Rhodnius prolixus and its endosymbiotic bacterium Rhodococcus rhodnii (Durvasula et al. 1997). R. rhodnii was transformed to express cecropin A, a peptide lethal to the R. prolixus parasite Trypanosoma cruzi, which is responsible for Chagas disease. Malaria is another insect-vectored disease for which paratransgenic control has been tested. The genus Plasmodium contains four species of parasitic protozoa that cause malaria, and the bacterium Pantoea agglomerans, a symbiont of the insect vector Anopheles mosquitoes, was recently engineered to secrete anti-Plasmodium effector proteins (Bisi and Lampe 2011). Another example is the transformed fungus Mucorizum anisopliae that carries effector genes against Plasmodium development (Fang et al. 2011). Symbiotic denso...
viruses (or densoviruses) are also attractive agents for paratransgenic malaria control (Ren et al. 2008). In protection of economically important agricultural crops, transgenic *Enterobacter* strains have been developed into vehicles that target populations of the larvae of cane beetles (*Dermolepida albomirtum*), corn borers (*Pyrausta nubialis*), and cotton bollworms (*Helicoverpa armiger*) (reviewed by Pittman et al. 2008).

Developing a paratransgenic strategy involves several steps: the isolation of suitable microorganisms; identification of effector proteins; transformation of a bacterium, fungus, or virus; effective delivery of the transformed microorganism (transformant) back into the insect host; and when desirable, the spread of the transformant within and between host populations (Fig. 1). Overall, and so far, paratransgenesis has not been a widely successful method against insect-vectored disease or against the decimation of crops (Coutinho-Abreu et al. 2010). A recurrent issue is implicit negative effects on host fitness that impair the spread of the paratransgenic control mechanism in the target insect population (Fang et al. 2011). Due to these negative effects on the host’s health, the potential of this method to further human interests has thus far failed to meet expectations.

Here, we propose an alternative scope of paratransgenesis: the genetic manipulation of symbiotic microorganisms with the goal of enhancing a host organism’s positive effects on human health and economic interests. We propose using the honey bee, *Apis mellifera*, and its symbiotic gut microbes. Several of these microorganisms are potential candidates for genetic transformation and can, in theory, be modified into delivery vehicles for effector proteins against honey bees’ pathogens. Furthermore, the microbes may be engineered to secrete active metabolites, peptides, or enzymes that can influence the physiology or behavior of honeybees, with the purpose of promoting their health or advancing the understanding of their biology. The latter use would expand the molecular genetic toolbox of honey bees to include functional paratransgenetics.

**Honey bees**

Honey bees are highly social insects (Fig. 2). They live in colonies of about 10,000–50,000 females and a few hundred males (Wilson 1971). Each colony has one reproductive queen (Fig. 2A), which lays eggs and produces several pheromones that are essential for the integrity and functioning of the colony. The remaining females are functionally sterile helpers, called workers. These workers perform many different tasks during their lifetimes, including rearing of larvae (Fig. 2B), colony defense (Fig. 2E), and foraging (Fig. 2F). Foraging workers utilize advanced navigation, communication, learning, and memory skills to ensure optimal influx of resources to the colony (Frisch 1967; Seeley 1995; Menzel et al. 2006). Through the domestication of honey bees, these skills are exploited by human societies for pollination of crops (Fig. 2G), as well as for basic research (Fig. 2H).

Honey bees are of considerable value to agriculture, with a yearly impact estimated to be worth more than $15 billion in the United States and at least €15 billion in Europe (van Engelsdorp and Meixner 2010; European Parliament 2011). Several
varieties of crops such as almonds, apples, blueberries, oranges, strawberries, sunflower, and canola are dependent on pollination by honey bees to give economically valuable yields. In consequence, massive mortality of honey bees due to colony collapse disorder (CCD) can lead to dramatic crop failures (Cox-Foster et al. 2007). Although CCD is not fully understood, several explanatory models involve gut pathogens such as the microsporidium *Nosema cerana* (Tokarz et al. 2011). These models are debated and more knowledge is needed, for instance, on *N. cerana*’s cycle of infection in CCD (Traver and Fell 2011; Dainat et al. 2012). *Paenibacillus larvae* is another pathogen that has severe effects on honey bees’ health. This bacterium invades the guts of larvae causing American Foulbrood disease, which can lead to the demise of the colony (Chan et al. 2011).

The use of a paratransgenic method to promote honey bee health requires knowledge of the bees’ “microbiota.” The microbiota is the complete assembly of microorganisms in a particular organ or region. Until recently, the microbiota had been considered a black box, especially regarding how microbial species in the gut affect their host, and vice versa. This lack of data was primarily due to limited availability of tools for assessing microbial diversity (Hamady and Knight 2009). Recent technological advancements have shed light on previously unknown microbiota, revealing an important interplay between microorganisms and their hosts (Shi et al. 2010).

**The microbiota**
A microbiota is defined as a collection of microorganisms living in a limited region or habitat. For example, all microbes inhabiting the gastrointestinal (GI)-tract of an organism are referred to as a gut microbiota (Fig. 3). The composition of the gut microbiota in its eukaryotic host is not static but subject to dynamic changes during the course of
the host’s development or the host’s physiological status or health. For instance, *Bifidobacterium* (often referred to as a probiotic) is a predominant bacterium in newborn infants that is found in low numbers in elderly people (Tannock 2010). Antibiotic-associated diarrhea is often caused by compromised gut microflora leading to altered carbohydrate metabolism and an osmotic imbalance in the gut. A disturbed gut microflora is also implicated in heart disease, obesity, and food allergy in humans. Research reveals that the human gut microbiota can interfere with the host’s metabolism (Ley et al. 2006); healthy gut microbiota are absent or distorted in many disease-states (Arumugam et al. 2011), and some chronic inflammatory bowel diseases such as Crohn’s diseases and ulcerative colitis are associated with distinct changes in the biostructure of the microbiota (Qin et al. 2010).

Animals, including humans (Fig. 3A), are largely dependent on gut microbes to extract nutrients from food and to produce some essential vitamins. Ruminants, for example, rely entirely on their microflora of bacteria, archaea, protozoa, and fungi to digest cellulose. Invertebrates like honey bees (Figs. 3B and 3C) also rely on their microflora. Insects with highly restricted diets containing limited nutrition (e.g., plant sap, nectar, wood, chitin-rich shell) use symbiotic microbiota to assimilate nitrogen and phosphate as well as to produce fatty acids, digestive enzymes, amino acids, and other necessary nutrients (Akman Gunduz and Douglas 2009). The microbiota of insects is commonly less complex than that of vertebrate animals (Dillon and Dillon 2004) and perhaps this is due to the lack of immunological memory (acquired immunity) in insects, leading to an inability to host a complex microbiota (McFall-Ngai 2007). However, such differences between vertebrates and invertebrates could also be partly caused by technical artifacts stemming from greater sampling efforts for vertebrates, especially mammals.

**The microbiota of honey bees**

The sugar-loving bacterium *Gluconobacter* was identified as a significant component of the gut microbiota of honey bees as early as the beginning of the 20th century (White 1921). In the 1980s, the principal microbiota of this insect was described as Gram-negative, Gram-positive, and Gram-variable bacteria, and molds and yeast (Gilliam 1997). Up until the turn of the century, however, almost all identifications and quantifications of the honey bee gut microbial assemblage were determined by cultivation-dependent techniques, including selective and specially formulated culture media, different atmospheric conditions, and different taxonomic keys (for an example, see Rada et al. [1997]). Unfortunately, these methods are biased toward culturable microorganisms and thus fail to capture the microbial assemblage accurately.

Over the past decade, some new molecular methods (reviewed by Shi et al. [2010]) have increased our ability to correctly describe microbial assemblages, including that of the honey bee gut. By utilizing
16S rDNA (a gene encoding the rRNA involved in the small subunit of the bacterial ribosome), researchers have identified species belonging to the genera *Bifidobacterium* and *Lactobacillus* in honey bee gut extracts (Jeyaprakash et al. 2003; Mohr and Tebbe 2006; Babendreier et al. 2007; Cox-Foster et al. 2007; Olofsson and Vasquez 2008). Despite environmental, geographic, and subspecies-specific differences among honey bees, 95% of the *A. mellifera* microbiota can be assigned to eight characteristic bacterial phylotypes, representing five bacterial classes; Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Actinobacteria, and Firmicutes (Martinson et al. 2011), see Table 1 for an overview.

The exact functions of the microbiota of the honey bee gut remain largely unknown, including whether some of the microbiota is indigenous and permanently established or transient and only found for a limited period of time. It is also unclear whether the dominant phylotypes are commensal, parasitic, or mutualistic. The consistent association with a limited number of phylotypes, however, may point toward mutualistic symbiosis (Martinson et al. 2011), see Table 1 for an overview.

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Honey bee paratransgenesis—a feasible approach

A project that aims to succeed with honey bee paratransgenesis could unfold in three phases. First, one must identify and describe bacteria from the gut that have substantial potential to serve as a transgenic vehicle. This potential must include compatibility with the gut microbiota of honey bees in major ecogeographic regions such as Europe and the United States. During the next phase, the bacterium is transformed with an inert reporter protein, tested, and described for its performance. Measures of performance should include efficient return to the honey bee, appropriate retention time in the gut, lack of interference with the established microflora of the gut or with the health, and survival of the bees. In the final phase, the selected bacterium is transformed with effector genes against honey bee pathogens or used in a functional (paratrans-)genetic approach to produce active metabolites, peptides, or enzymes that promote healthy honey bee physiology or that dissect the phenotypic effects of specific
honey bee genes (Fig. 4). The full potential of these methods remains to be discovered, but recombinant gut bacteria should be generally well-suited to produce factors that can act in the mid-gut lumen or in the columnar cells that line the lumen of the gut.

Members of the lactic acid bacterial group are appropriate candidates for paratransgenesis. These bacteria are common inhabitants of guts where many of them can have probiotic properties. They are also found in diverse vegetables, meat products, and especially in fermented dairy products, such as yogurt and cheese, where they play a key role in the formation of texture and exert preservative effects, the latter being due to the production of organic acids (lowering pH) and of antimicrobial peptides such as bacteriocins. Lactic acid bacteria are, therefore, “generally regarded as safe” (GRAS) for human consumption (Salminen et al. 1998).

Due to their GRAS status and a sizable molecular tool box available for these organisms, many recent studies have attempted to use lactic acid bacteria as delivery vehicles for therapeutic proteins and a variety of vaccines (Seegers 2002; Bermudez-Humaran et al. 2011). Amongst lactic acid bacteria, the genus Lactobacillus has been a preferred delivery vehicle because it is mostly nonpathogenic and robust. The discovery of lactic acid bacteria, including Lactobacillus, in the honey bee gut (Olofsson and Vasquez 2008; Audisio et al. 2011), therefore, encourage us to assess them for use as delivery vehicles.

**Development of a Lactobacillus vehicle system**

The mid-gut (Fig. 3) is the primary site of enzymatic digestion and uptake of nutrients into the hemolymph (blood) that circulates throughout the insect...
body. We performed a screening of samples from honey bee mid-guts with growth conditions selective for *Lactobacillus*. Over a period of about 4 months (September to December 2010), more than 200 isolates were genotyped by 16S rDNA sequencing, and about 75% of them were identified as *Lactobacillus kunkeei*. To examine whether the *L. kunkeei* isolates were clonally related or diverse, 40 isolates were randomly chosen for plasmid profiling. We found that the number and size of these plasmids varied greatly. Some isolates contained none, while others carried 5–6 plasmids (Fig. 5). This pattern suggests that the *L. kunkeei* of the honey bee gut represents a relatively heterogeneous group.

*Lactobacillus kunkeei* is described as a fructophilic bacterium (i.e., fructose is its preferred carbon source, Endo et al. [2012]). This bacterium has been identified in honey bees before (Olofsson and Vasquez 2008) and is also found during wine fermentation and on flowers (Edwards et al. 1998; Endo et al. 2009; Endo et al. 2012). Our *L. kunkeei* isolates may therefore originate from nectar and/or pollen grains collected and ingested by the bees. Pollen is the bee’s major source of proteins, lipids, vitamins, and minerals, whereas nectar is their main source of energy. Most nectars are dominated by a limited number of sugars: sucrose, glucose, and fructose (Nicolson 2011). Using the API50 CHL system from BioMérieux that assesses the fermentation of 49 different sugars, we found that the 40 *L. kunkeei* isolates (Fig. 5) fermented only 4 to 7 carbohydrates (Table 2). The strongest activity was measured on fructose and disaccharide sucrose that is composed of one unit glucose and one unit fructose, corresponding to major constituents in nectar.

To our knowledge, genetic manipulation on *L. kunkeei* has not been attempted before. For transformation, several isolates of *L. kunkeei* were made electro-competent using a growth medium containing a higher concentration of glycine to weaken the bacterial cell wall, a protocol that has been applied successfully to other lactic acid bacteria (Aukrust et al. 1995). A set of isolates were transformed with a broad-spectrum plasmid containing a replicon derived from the lactococcal plasmid pSH71 (de Vos 1987; Sorvig et al. 2005). For heterologous gene expression, we used the secretion signal of a protein (annotated as lp_3050) from the genome-sequenced *L. plantarum* WCFSI strain to drive secretion of the reporter protein nuclease A (encoded by *nucA*) (Mathiesen et al. 2009). Our transformed *L. kunkeei* secreted this reporter protein in both low and high sugar containing growth media. Transformed bacteria were fed back to individual bees in the laboratory, and we could recover a substantial number of transformants from mid-gut samples 24 h after feeding. Presence of the transformed *nucA* plasmid was confirmed by PCR. These observations suggest that *L. kunkeei* provides a basis for the development of paratransgenic methods in honey bees (Fig. 4, detailed data to be reported elsewhere).

**Future work**

The genetic tool box for *L. kunkeei* must be expanded. This work will include more effective systems for plasmid replication and the construction of new reporter systems, including visual markers such as green fluorescent protein or luciferase that can be
Table 2: Metabolic profiling of 40 L. kunkeei isolates using the API50 CHL system from BioMérieux

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This panel tests 49 different carbohydrates, but only seven of these produced positive fermentation reactions in our experiment (listed): “++” indicates strong positive reaction, “+” intermediate, “w” weak, and “–” no observable reaction.
monitored through the gut wall (Fig. 6). These aids are requirements for future work with effector genes.

Moreover, although the honey bee is an increasingly important model for studies of behavior, neurobiology, and aging, few methods are available for studying its functional genetics. RNA interference technology is currently the only tool for gene silencing, and no method is available for gene over-expression (reviewed by Amdam [2011]). In principle, a transformed gut bacterium may not only provide a system for fighting disease but it can also deliver proteins and enzymes that test functional genetic hypotheses. This makes the development of honey bee paratransgenesis all the more important (Fig. 4).

Finally, a field that has not yet been widely explored is the transmission and persistence of the microbiota within populations. For humans, it has been suggested that the increase in immunological disorders such as asthma and allergy can partly be attributed to the loss of beneficial bacteria within populations, although specific mechanisms have not been derived (Blaser and Falkow 2009). Social insects such as honey bees represent a particularly attractive model for investigating the dispersal of the microbiota within populations in relation to health and disease. Like humans, honey bees have evolved hygienic behavior that limit and prevent the spread of pathogens (Evans and Spivak 2010), but unlike humans, honey bees may have evolved mechanisms that secure the selective transmission of beneficial bacteria (Evans and Armstrong 2006). Understanding these mechanisms in honey bees could facilitate the development of strategies for preventing the loss of beneficial bacteria in human populations.

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References


Fig. 6 Transformed bacteria imaged through the wall of the honey bee gut. We used Enterococcus transformed with a plasmid containing the luxABCDE operon, which encodes luminescent luciferase, to determine the reporter potential of luminescence light. The bacterium was grown in GM17 medium until the optical density of ~0.5 was reached. Ten microliters of the bacterial suspension was subsequently fed to individual honey bees. Bees fed with 10 μl GM17 medium only, i.e., without luminescent bacteria, were included as a control. Enterococcus was not originally isolated from the honey bee gut, and its retention time is short in bees. Thus, the gut was removed after 1 h and assayed for bioluminescence using the IVIS® Lumina Imaging System from Caliper (Binding: M, exposure time: Auto, f/stop: 1). Emitted light was detected through the gut wall of bees fed with transformed Enterococcus (A) and was absent in the control (B). We conclude that luminescent light has reporter potential in the honey bee gut. It can be worthwhile to transfer this system to a more appropriate vector, such as L. kunkeei.


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