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Use of Genetically Engineered *Escherichia coli* to Monitor Ingestion, Loss, and Transfer of Bacteria in Termites

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Abstract. *Escherichia coli* was transformed with a recombinant plasmid (pEGFP) containing the genes for ampicillin resistance and Green Fluorescent Protein (GFP). *Escherichia coli* expressing GFP (*E. coli/GFP+*) was then fed to workers of the termite *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). The transformed bacteria in the termite guts were detected by growing the gut flora under selective conditions and then checking the cultures for fluorescence. Recombinant plasmids in the termite gut were detected by plasmid extraction with subsequent restriction enzyme digest. The presence of the GFP gene in the gut of termites fed with *E. coli/GFP+* was verified by PCR amplification. Transformed *E. coli* were ingested rapidly when workers fed on filter paper inoculated with *E. coli/GFP+*. After 1 day, 42% of termite guts harbored *E. coli/GFP+*. Transfer of *E. coli/GFP+* from donor termites (fed with *E. coli/GFP+*) to recipients (fed with moist filter paper) occurred within 1 day. However, without continuous inoculation, termites lost the transformed bacteria within 1 week.

Introduction

Paratransgenesis, a novel approach to study microbe–host interactions, harnesses genetically engineered microorganisms that reside within the host for use as "shuttles" or "Trojan horses" to deliver and express foreign genes in a host [5]. For example, this technique has been used successfully to monitor uptake of genetically marked bacteria and colonization of the digestive tract in insects [4, 10, 14]. Since it has been suggested that genetically engineered symbionts can be used for insect pest control [13], an efficient shuttle system can be an especially valuable tool for improving methods of control in social insects, such as termites.

Termites are known to depend on microbial symbionts for survival [6]. The so-called lower termites, including the pest species in the genera *Reticulitermes* and *Coptotermes*, harbor a great diversity of protozoa and bacteria in their gut, which play important ecological and physiological roles [2, 15, 17]. Utilization of genetically modified bacteria carrying marker genes could be used to study the spatial and functional interactions of various trophic levels of microbes within the termite gut and also to introduce detrimental genes into a termite colony in order to suppress the colony.

Development of a bacterial system that expresses foreign genes in a termite colony allows monitoring of efficiency of ingestion, long-term stability, and transfer of bacteria between individuals within a colony. For this study, an *E. coli* DH5α laboratory strain was transformed with a recombinant plasmid (pEGFP) containing ampicillin resistance genes as a selectable marker and the Green Fluorescent Protein (GFP) gene as a reporter gene. The transformed bacteria were fed to *C. formosanus* workers, and the usefulness of this monitoring system was assessed by determining (1) if it is possible to introduce transformed *E. coli* bacteria into individual termites by feeding, (2) if transformed bacteria form a stable population in the termite gut to guarantee continuous expression of the genes in the termites over time, and (3) if transformed bacteria are transferred between individual termites and thus spread throughout a laboratory colony.

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Materials and Methods

Termites. *C. formosanus* workers were collected from aggregation traps near Gilmore Hall (G), Miller Hall (M), and the Hale Halawai Residence Hall (H) at the University of Hawaii at Manoa campus [8].

Host bacteria and expression system. We introduced marker genes into *E. coli* DH5α by plasmid-mediated transformation [1]. The 3355-bp vector plasmid (pEGFP; Clontech, Palo Alto, CA) replicated in a relaxed fashion (pMB1 replicon) and contained genes for ampicillin resistance and GFP under the control of a lacZ promoter. This plasmid with a narrow host range (including Enterobacteriaceae) and no mobilizing (mob-, nic-, bcm-) or conjugation functions (tra-) was chosen because the goal of this study was to use *E. coli* to spread and express foreign genes in a termite colony with a low likelihood of gene transfer to other bacteria in the termite gut or the environment. Plasmid and GFP expression in the host bacteria (*E. coli/GFP+*) was stable and comparable to that in other studies [e.g., Refs. 13 and 17].

Feeding. *E. coli/GFP+* was grown in LB broth (Fisher Scientific, Pittsburgh, PA.) in nonselective overnight cultures because of the potential toxicity of antibiotics to termites and to their gut flora [20]. Overnight cultures contained on the average 2.75×10^8 bacteria (SD = 0.09×10^8; $n = 6$). Filter paper was inoculated with 400 µl *E. coli/GFP+* diluted 1:2 and then fed to the termites.

Detection of *E. coli/GEP+* in termite guts. Termites were surface sterilized with 70% ethanol, and fluid was squeezed out of the termites' hindgut to determine if transformed bacteria were present there. Then the whole gut was extirpated under sterile conditions. Selective LB broth (100 µg/ml ampicillin; Sigma Scientific, St. Louis, MO) was inoculated with hindgut fluid or homogenized whole guts and incubated at 37°C overnight. Growth of ampicillin-resistant bacteria and green fluorescence under UV light (365 nm) indicated that *E. coli/GFP+* was present in the hindgut fluid or the digestive tract. For additional confirmation, overnight cultures were streaked on selective LB agar plates (1.5% agar; Fisher Scientific, Pittsburgh, PA; 100 µg/ml ampicillin) and checked for growth and fluorescence. Whenever fluorescing bacteria were recovered from termite guts, we verified the species (*E. coli*) with standard morphological and biochemical techniques, including API 20E kits (bioMerieux, Hazelwood, MO), and the presence of the pEGFP plasmid was confirmed by plasmid extraction (High Pure Plasmid Isolation Kit; Roche Applied Science, Indianapolis, IN). Extracted plasmid was digested with *Eco*RI plus *Bam*HI and *Eco*RI plus *Hind*III (Fermentas Inc. Hanover, MD) and the restriction fragment length pattern was compared to the original pEGFP plasmid (Fig. 1). In addition, we designed PCR primers to amplify a 363-bp fragment of the GFP gene (forward, 5′-AAGTTCATCTGCACCACC-3′; reverse, 5′-GAAGTTCACCTTGATGCC-3′) using VectorNTI 6.0. (Informax Technologies, Bethesda, MD). The 50-µl PCR reactions contained 10–30 ng plasmid DNA, dNTPs (10 m*M* each), 2.5 m*M* $MgCl_2$, a 100 µ*M* concentration of each primer and 5 U *Taq* polymerase (BIOLASE Technology Inc., San Clemente, CA). Amplification was achieved using a PTC 200 DNA Engine (MJ Research Inc., Reno, NV) programmed with the following PCR conditions: initial denaturation at 94°C (3 min), 25 cycles of denaturation at 94°C (30 s), annealing at 60°C (45 s), and extension at 72°C (60 s). Final extension was at 72°C for 7 min.

Experimental design to monitor uptake and loss of transformed bacteria. Four experimental treatments and one control, each with three replications, were set up for each of the three termite colonies as shown in Fig. 2. In each of the petri dishes, 150 workers were fed daily with filter paper inoculated with 400 µl overnight culture of *E. coli/GFP+*. Termites were fed with *E. coli/GFP+* for 1 to 4 days to introduce transformed bacteria into the gut. They were then fed water-treated filter paper for a week to determine the rate of loss of introduced bacteria. Guts were removed from 10 termites per treatment daily and screened for *E. coli/GFP+*.

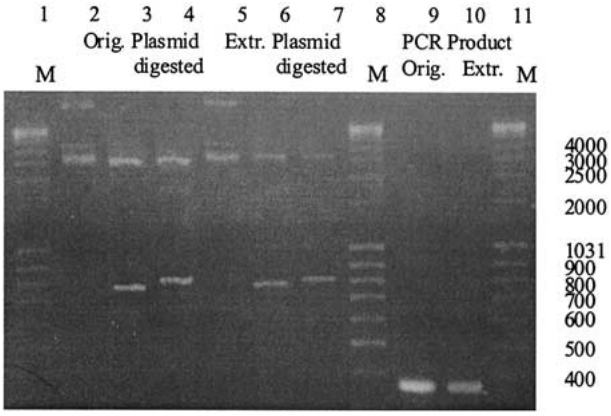

Fig. 1. Identification of the GFP plasmid and the GFP gene. The plasmid extracted from fluorescent bacteria recovered from the termite gut in its uncut form (lane 5) and digested with *Eco*RI + *Bam*HI and *Eco*RI + *Hind*III (Fermentas Inc. Hanover, MD; lanes 6 and 7) is identical to the original plasmid (pEGFP; Clontech, Palo Alto, CA; lanes 2–4). The PCR product using the extracted plasmid as a template (lane 10) is identical to the PCR product from the original plasmid (lane 9). A MassRuler DNA Ladder (Fermentas Inc.) was used as a size standard (kb) in lanes 1, 8, and 11.

Experimental design to monitor transfer of *E. coli/GFP+* between termites. To distinguish termites that transfer bacteria (donors) from termites that receive bacteria (recipients), filter paper containing 1% (wt/wt) Sudan Red 7B (Aldrich, Milwaukee, WI) was used to feed either group for 7 days (experimental setup is shown in Fig. 3). Fifty donors were fed with *E. coli/GFP+* for 2 days prior to being combined with 50 recipients that were fed with moist filter paper only. Guts were removed from five donors and five recipients daily to determine if *E. coli/GFP+* was transferred. Each experiment was replicated four times for each of the three colonies. *E. coli/GFP+* was ingested by donors and transferred to recipients regardless of which of the groups were dyed.

Results and Discussion

The purpose of this study was to develop a functional shuttle system to deliver and express foreign genes within members of a termite colony. We used *E. coli* as host bacteria and GFP as reporter gene, and assessed the functionality of the system by monitoring (1) if genetically modified bacteria were successfully ingested by termites, (2) if they established a stable population in the termite gut over time, and (3) if bacteria were efficiently transferred among termites.

Detection of *E. coli/GFP+* in termite guts. The different screening methods yielded comparable results. Whenever fluorescent bacteria could be grown under

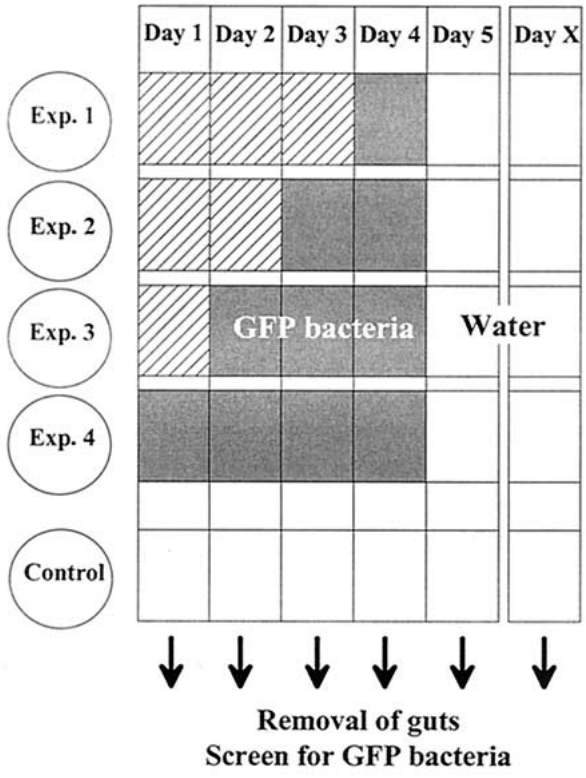

Fig. 2. Experimental design for measuring ingestion and loss of *E. coli/GFP+* in termites. Each of the four experiments and the control consisted of three replications. One hundred fifty workers were placed in each of the 15 petri dishes. Workers were fed with *E. coli/GFP+* from 1 to 4 days (shaded cells) and then with water-treated filter paper only (white cells). The control termites were fed with water-treated filter paper. Each day guts were extracted from 10 termites in each petri dish and screened for *E. coli/GFP+*. For measuring ingestion of *E. coli/GFP+*, results for 1 day of feeding were averaged over all four experiments and their replications ($n = 12$), results for 2 days were averaged over three experiments and replications ($n = 9$), results for 3 days were averaged over two experiments and their replications ($n = 6$), and results for 4 days of feeding were averaged over the three replications of experiment 4 ($n = 3$). The loss of *E. coli/GFP+* was averaged over all four experiments and their replications ($n = 12$; see also Fig. 4).

selective conditions, we were able to extract the 3.4-kb plasmid and confirm the identity of the plasmid by comparing its restriction fragment length patterns to that of the original pEGFP plasmid. In addition, the presence of the GFP gene was confirmed by PCR (Fig. 1). Whenever *E. coli/GFP+* were detected in whole-gut assays, *E. coli/GFP+* was also found in the hindgut fluid.

Ingestion and loss of *E. coli/GFP+*. Uptake of engineered bacteria through feeding and colonization of the alimentary canal has been reported for other insects [14]. As for this study, rapid ingestion of *E. coli/GFP+* was

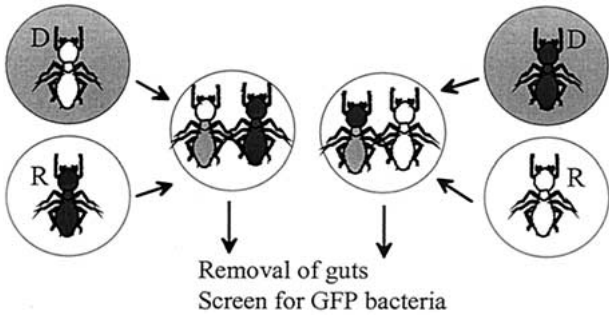

Fig. 3. Experimental design for monitoring transfer of *E. coli/GFP+* between termites. In two separate sets donors (upper right) and recipients (lower left) were dyed with Sudan Red 7B (black termites). Donors (D; upper left and right) were fed for 2 days on filter paper soaked with *E. coli/GFP+* (gray). Recipients (R; lower left and right) were fed with water-treated filter paper only. After 24 h donors and recipients were combined (middle). Each day guts of five donors and five recipients were pulled to be screened for *E. coli/GFP+*.

demonstrated by termites feeding on filter paper soaked with bacterial overnight cultures. Although termites will digest microbes for nutrition [7], some of the *E. coli/GFP+* successfully reached the hindgut, as we detected *E. coli/GFP+* in the hindgut fluid by culturing hindgut fluid under selective conditions. This was recently confirmed by fluorescence microscopy, which allowed detection of *E. coli/GFP+* in the lumen of dissected guts (C. Husseneder, unpublished data).

After 1 day of feeding workers with transformed bacteria, 42 to 45% of termites from the three colonies contained *E. coli/GFP+* in their guts (range, 17–83%; $n = 12$). After 2 or 3 days of feeding the percentage of GFP-positive termites stabilized between 47% (colony H) and 72% (colony G) as shown in Fig. 4. During the course of the experiment, no *E. coli/GFP+* could be detected in control termites fed with water-treated filter paper only.

No significant difference was found in the fraction of termites containing *E. coli/GFP+* whether they were fed for 1, 2, 3, or 4 consecutive days (Friedman test; $p > 0.20$, $N = 3$, df = 3). Nor was there any significant difference in uptake among the colonies, due to the large standard deviations (day 1, $p > 0.20$, $N = 12$, df = 2; day 2, $p = 0.18$, $N = 9$, df = 2; day 3, $p > 0.20$, $N = 6$, df = 2; day 4, $p > 0.20$, $N = 3$, df = 2). Thus, ingestion of transformed bacteria by feeding occurs quickly, i.e., within a single day. However, even after 4 days of continuous exposure to *E. coli/GFP+*, the highest percentage of GFP-positive termites did not exceed 72% (colony G).

After feeding with *E. coli/GFP+* was discontinued, and termites were fed with water-treated filter paper only, the fraction of termites with fluorescent bacteria in

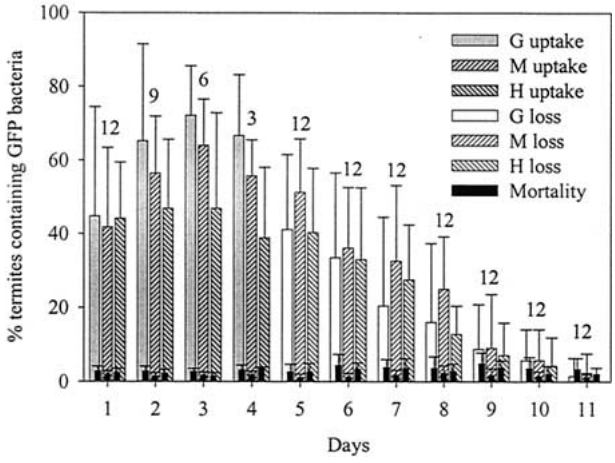

Fig. 4. Uptake and loss of *E. coli/GFP+* in three lab termite colonies (G, M, H). The experimental setup followed Fig. 2. Workers were fed 1 to 4 days with *E. coli/GFP+* (gray columns) and then with water-treated filter paper only (white columns). Each day guts from 10 workers were screened for *E. coli/GFP+*. Average percentage of GFP-positive termites and standard deviations were calculated. Dead termites were counted and removed daily to determine mortality (black columns). At day 12, i.e., 1 week after feeding with *E. coli/GFP+* was discontinued, no more fluorescent bacteria were found in the screened guts.

their guts continuously decreased. After 1 week, fluorescence was no longer detected in the selective cultures of the gut flora (Fig. 4). Plasmid extractions did not produce pEGFP in traceable amounts, and attempts to amplify the GFP gene using the DNA of the whole-gut flora as a template failed. Furthermore, we could not detect *E. coli* among isolated Enterobacteriaceae from the bacterial gut community. Thus, *E. coli/GFP+* was not able to survive and form a stable population in the termite gut. Expression of GFP may be costly for the host bacterium and thus impact its fitness and survival, though studies have shown otherwise for *E. coli* and other bacteria [11, 18]. Also, some bacteria species lose the ability to be cultured over time [16]. However, we could not detect GFP culture by microscopic examination of gut contents without previous culture. Most likely, the bacteria we used in our experiments were not adapted to the selective pressures of the microbial community in the termite gut. *E. coli* was described by Mannesmann and Piechowski [12] as a naturally occurring bacteria species in termite guts; however, the bacterial strain we used in this study (DH5α) was not isolated from the termite gut. Veivers et al. [19] showed the possibility that the microbial community protects the termite host from invasion by foreign bacteria. Similarly, Leff and Leff [11] found that introduced *E. coli* was not able to survive in stream water but survived longer in autoclaved water. The authors concluded that

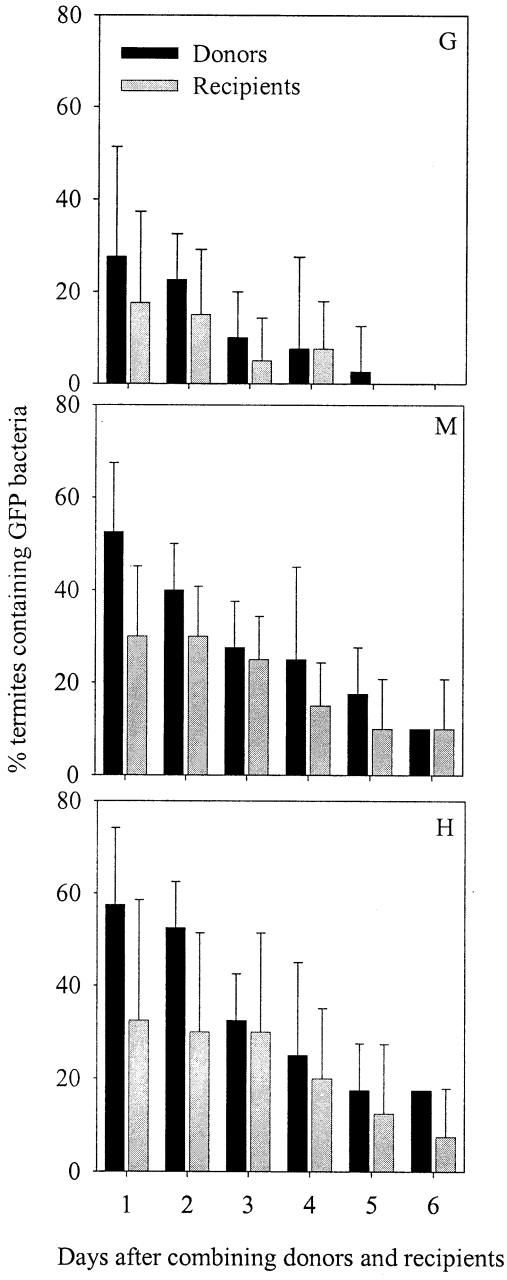

Fig. 5. Transfer of *E. coli/GFP+* between termites. For each of the three colonies (G, M, H) 50 donors (fed with *E. coli/GFP+*; black columns) and 50 recipients (fed with water-treated filter paper only; gray columns) were combined. Each day five guts from donors and recipients were screened for the presence of *E. coli/GFP+* and averages and standard deviations were calculated.

"its survival in nature is limited by biological interactions."

Transfer of *E. coli/GFP+* between workers. *E. coli/GFP+* were transferred between individual termites, i.e.,

from donors, which were originally fed with *E. coli/GFP+*, to recipients, which were not previously exposed to *E. coli/GFP+* (Fig. 3). Initially, around 28–58% of the donors harbored *E. coli/GFP+* in their guts. Even on the first day after combining donors and recipients, *E. coli/GFP+* were transferred to a considerable percentage of the recipients (18–33%; Fig. 5). As shown in the previous experiment, termites lost their *E. coli/GFP+* within a week. Nevertheless, bacteria were transferred efficiently as long as they were present. Although the experimental design did not measure the modes of transfer among termites, most likely bacteria were transferred by grooming, trophallaxis, and/or coprophagy [9].

Use of transformed bacteria that occur naturally in the insect gut may increase the percentage of termites containing and retaining genetically marked bacteria. For example, Chapco and Kelln [4] fed grasshoppers with genetically modified *Enterobacter agglomerans* originally derived from the grasshoppers' own gut. These authors reported that the bacteria established residency in the digestive system and persisted for at least 22 days without continuous feeding.

Introduction and monitoring of genetically engineered microbes will make it possible to investigate in detail the role of certain bacteria in termite biology, for example, the specific location of bacteria groups, the interaction between trophic levels, and the vertical and horizontal transfer of gut symbionts in termites. In addition, such a shuttle system would be useful to deliver and express genes in a termite colony that produce detrimental substances against the gut flora or the termites themselves.

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