Enterococcus faecalis OG1RF:pMV158 Survives and Proliferates in the House Fly Digestive Tract

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ABSTRACT *Enterococcus faecalis* is an important nosocomial pathogen and house flies have been implicated in the dissemination of this bacterium. In this study, GFP-expressing *E. faecalis* OG1RF:pMV158 was used to track the fate of the bacterium in the digestive tract of the house fly, *Musca domestica* (L.) to assess the vector potential of this insect for *E. faecalis*. Colony forming unit (CFU) counts were obtained from viable fluorescing *E. faecalis* recovered from mouthparts and digestive tract regions (labelum, foregut, midgut, and hindgut) at 1, 4, 8, 24, 48, 72, and 96 h after the bacterial exposure. Bacterial counts were significantly highest in the midgut at 1 h and 4 h and declined during the first 24 h. In the labelum, *E. faecalis* concentrations were low within the first 24 h and then greatly increased. Bacterial counts and direct observations of the digestive tract under a dissecting microscope with ultra violet light revealed that *E. faecalis* peaked in the crop after 48 h and remained high until the end of the experiment. Concentrations of *E. faecalis* in the hindgut were low when compared with other parts of the digestive tract. Microscopy and CFU counts suggest that *E. faecalis* was digested in the midgut but proliferated in the crop. Both drinking water and feed (flaked corn) sampled at the end of the assay (96 h) were contaminated by fluorescing *E. faecalis*, demonstrating that the flies disseminated *E. faecalis*. Our data support the notion that house flies can act as a bioenhanced vector for bacteria.

KEY WORDS *Musca domestica*, enterococci, digestive tract, proliferation, crop

The house fly, *Musca domestica* (L.) is an important nuisance pest because of its abundant populations worldwide and synanthropic nature. In addition, house flies are recognized as mechanical vectors of a number of parasites and pathogens including protozoa, viruses, fungi, and bacteria (Graczyk et al. 2001, Zurek and Gorham, 2008). Several house fly attributes contribute to its ability to function as a mechanical vector, including larval developmental habitats (manure and other decaying organic substrates), mode of feeding (regurgitation), unrestricted movement, and close association with humans. House flies ingest microbes with their food and microbes reside transiently in the fly digestive tract but can also proliferate and be disseminated and deposited to various sites through fly feeding and defecation (Sasaki et al., 2000, Kobayashi et al. 2002). Kobayashi et al. (1999) proposed the term "bioenhanced transmission" to describe this phenomenon, which is more than simple mechanical transmission and reflects the microbial growth in the house fly digestive tract.

Enterococcus faecalis is a commensal bacterium in the digestive tract of many animals including humans. Enterococci are also the third most important bacterial group responsible for human nosocomial infections and *E. faecalis* causes the majority of these infections (Tannock and Cook 2002, Fisher and Phillips 2009). Furthermore, *E. faecalis* frequently harbors a variety of antibiotic resistance genes and is capable of their intra- and interspecific horizontal gene transfer (Huycke et al. 1998, Fisher and Phillips 2009). Enterococci have been detected in the gut of house flies associated with food animal production systems (Graham et al. 2009, Ahmad et al. 2011) as well as in urban locations including restaurants (Macovei and Zurek 2006, Chakrabarti et al. 2010). Moreover, there is indirect and direct evidence that the flies can readily contaminate human food with antibiotic resistant enterococci (Macovei and Zurek 2007, Macovei et al. 2008).

An important aspect of understanding the role of house flies in the ecology of *E. faecalis* is determining the ability of the fly to ingest and harbor these bacteria. The aim of this study was to track the fate of *E. faecalis* OG1RF:pMV158GFP within the digestive tract for up to 96 h in flies that maintained their acquired natural gut microbiota and continually gained an influx of microbes from a natural food source.

Materials and Methods

House Flies. House flies used for the study were obtained from the laboratory colony, Department of Entomology, Kansas State University. The colony is

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maintained at 25 ± 2°C, 70 ± 10% RH, and an 18L:6D cycle.

E. faecalis. *E. faecalis* strain OG1RF with plasmid pMV158GFP (Nieto and Espinosa 2003) was used in the assays. The strain was maintained on trypticase soy agar (TSB) (BD, Sparks, MD) and streaked on fresh TSB plates and incubated at 37°C for 24 h before use.

Assay. We used 2 to 5 d old mixed sex house flies that were starved for 12 h before use in the assays. The flies were then placed individually into 60 × 15 mm sterile petri dishes with the inoculum of *E. faecalis* OG1RF: pMV158GFP in 4 μl of phosphate buffered saline (PBS) (MP Biomedicals, Solon, OH). *E. faecalis* was streaked from the stock on TSB and incubated at 37°C for 24 h before the assay to obtain fresh cells for the inoculum. Bacteria were transfer by a loop to PBS to a concentration of 3.1 (assay 1) to 7.8 (assay 2) × 10^6 CFU per inoculum (4 μl). Control flies were placed in dishes with 4 μl of sterile PBS alone. The flies were observed to verify uptake of the solution for 20 min, then were transferred individually to 60 × 15 mm petri dishes with 0.2 g of steam flaked corn from a cattle feedlot and 500 μl of sterile tap water. The corn was intentionally not sterilized to mimic natural field conditions and preserve influx of microbes from the food source. The flies were maintained at 25 ± 2°C and 16L:8D light regime during the assay. Every 24 h, flies were moved to new sterile petri dishes with fresh water and food to limit self-inoculation from contaminated food and water. The flaked corn and house flies were screened before the assay to ensure no fluorescing enterococci were present.

Enumeration of Bacteria From the House Fly Gut. Three treated flies were randomly selected for dissection at each time interval (1, 4, 8, 24, 48, 72, and 96 h after bacterial exposure) and placed in −20°C for 5 min for immobilization before dissection. One control fly was also randomly selected for dissection at all time intervals. The labelum was removed and placed in 100 μl sterile PBS. The fly was then surface sterilized (Zurek et al. 2000) and dissected to remove foregut (with the crop), midgut, and hindgut. Each alimentary canal region (including labelum) was homogenized in 100 μl of PBS, serially diluted, and spread plated on TSB agar. All plates were incubated at 37°C for 48 h. After incubation, fluorescing colonies under transmission ultra violet (UV) light were counted to obtain the number of colony forming units (CFUs) per the respective digestive tract region. Only fluorescence was used as detection marker for *E. faecalis* OG1RF: pMV158GFP when taking CFU counts on TSB media. Although tetracycline resistance was another marker for the pMV158 plasmid, it could not be used because preliminary screening of the house fly colony revealed presence of tetracycline-resistant enterococci. The assay was conducted twice resulting in two biological replicates and three technical replicates with a total of 49 flies analyzed (six treatment and one control group for each time period).

Microscopic Observation of the House Fly Gut. The dissected digestive tract of three treated flies and one control fly for each time interval was viewed under a dissecting scope with epifluorescent UV light (model Nikon SMZ 1500; UV filter, Ex 470/40, dm 495, ba 525/50) (Nikon Corporation, Tokyo, Japan). Photographs were taken using camera model Leica DFC 400 with dissecting scope Lecia M205 FA and GFP2 filter (ex 460–500 nm, dm 510 pl) (Leica Microsystems AG, Wetzlar, Germany). Images were enhanced by adjusting contrast and brightness in the software Canvas nine Professional Edition (ACD Systems International Inc., Victoria, British Columbia, Canada). No other enhancements/alterations were made to the photographs.

Determination of House Fly Food and Water Contamination. Samples of flaked corn and water were taken at the end of the assay (96 h) to determine the contamination with *E. faecalis.* Four samples of water (100 μl) and flaked corn (100 μl from the solution of 0.2 g corn in 10 ml PBS) that the flies had used during the assay were sampled and spread onto TSB agar plates and incubated at 37°C as described above to determine the presence of the GFP-labeled *E. faecalis.*

Statistical Analysis. Analysis of variance (ANOVA) of CFU counts within the labelum and each digestive tract region across the time intervals was performed using SAS (PROC GLM, SAS Institute 2003). Counts were log transformed (Log_{10}) to meet assumptions of equal variance. If ANOVA revealed significantly different ($P < 0.05$) counts for a digestive tract region, pairwise comparisons were conducted using the least significant difference (LSD) method in SAS to assign groupings.

Results

Viable fluorescent *E. faecalis* were recovered from each region of the fly digestive tract at every time period although the counts varied greatly (Figs. 1 and 2). Differences in mean CFU bacteria recovered from each gut region, across time, revealed significant effects of time on bacterial recovery from the foregut ($F = 2.37$; df = 13, 28; $P = 0.0273$) and midgut ($F = 3.0$; df = 13, 28; $P = 0.0072$) but not the labelum ($F = 1.13$; df = 13, 28; $P = 0.3789$) and hindgut ($F = 1.46$; df = 13, 28; $P = 0.1953$). The numbers of *E. faecalis* from the labelum were relatively low at 1, 4, 8, and 24 h and increased at 48, 72, and 96 h (Fig. 2). Foregut CFU counts ranged from 9.4 ± 6.2 × 10^3 at 24 h to 1.8 ± 0.8 × 10^6 at 72 h and increased during the latter part of the assay. The highest midgut CFU count (2.8 ± 1.3 × 10^6) was obtained at 1 h and declined at 4, 8, and 24 h to a mean of 9.8 ± 4.0 × 10^4. The midgut CFU count remained relatively constant at 48 h, rose to a mean of 1.3 ± 1.1 × 10^6 at 72 h and fell to 1.7 ± 1.3 × 10^4 at 96 h (Fig. 1). Mean hindgut CFU counts were variable, ranging from 2.0 ± 1.1 × 10^3 at 24 h to 2.5 ± 1.2 × 10^5 at 72 h with no apparent trends across the time periods (Fig. 1). However, hindgut counts were consistently lower than that of the foregut and midgut. Three samples of drinking water and corn were sampled at the end of the assay at 96 h and resulted in CFU counts of water and corn of 5.5 ± 4.6 × 10^6 per milliliter and 9.0 ± 1.8 × 10^4 per g, respectively.

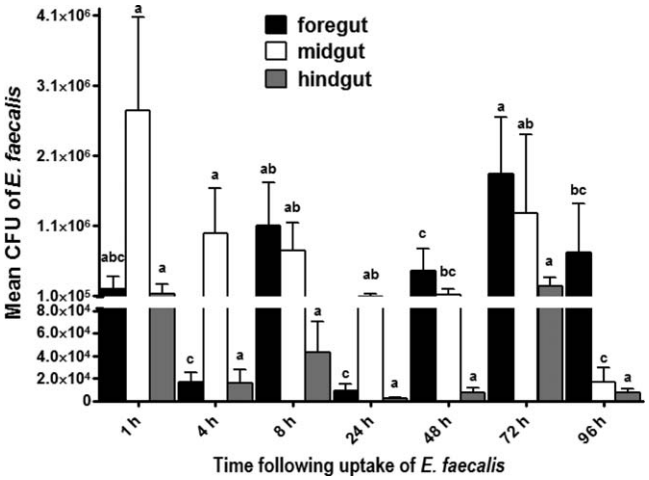

Fig. 1. Count of *E. faecalis* OG1RF:pMV158 in the foregut, midgut, and hindgut of house flies ($n = 6$ for each time period). CFU = colony forming units. Different letters above error bars (SEM) indicate a statistically significant difference ($P < 0.05$) among different sampling time periods within the same digestive tract region.

Generally, only a dim glow of fluorescence was observed from the foregut and midgut of the flies for the first 24 h of the assay. However, beginning at 48 h and peaking at 72 h, several flies exhibited a marked increase in fluorescence, mostly associated with the crop (Fig. 3). At 72 h, the fluorescing bacteria could be observed in the crop lumen, along the duct of the crop and into the midgut. Fluorescence in the midgut was generally greater in the anterior portion and declined until it was no longer detectable in the posterior midgut. Comparison of treatment and control flies revealed that the hindgut exhibited a significant amount of auto-fluorescence. Therefore, limited observational data could be obtained for this region of the digestive tract.

Discussion

Better understanding of the fate of bacteria in the house fly alimentary canal is important when considering this insect's role in dissemination of food-borne and other pathogens. Several recent studies have highlighted the efficiency at which house flies can acquire and disperse antibiotic resistant enterococci (Graham et al. 2009, Chakrabarti et al. 2010, Ahmad et al. 2011). Further, the ability of various microbes to proliferate and possibly spread antibiotic resistance genes horizontally in the house fly digestive tract underscores the potential of house flies to amplify bacterial pathogens (Kobayashi et al. 1999, 2002; Sasaki et al. 2000; Petridis et al. 2006; Akhtar et al. 2009; McGaughey and Nayduch 2009). This current study adds to our understanding of the fate of *E. faecalis* in the house fly digestive tract.

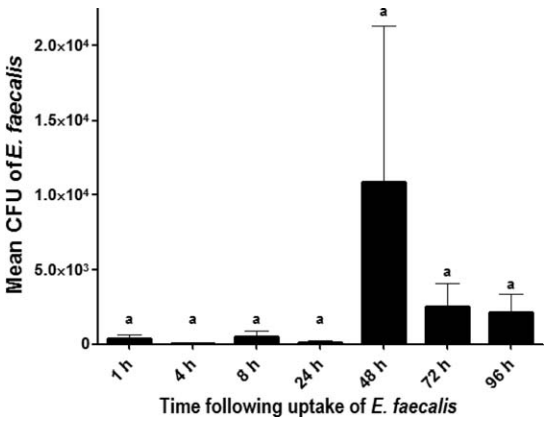

Fig. 2. Count of *E. faecalis* OG1RF:pMV158 in the labellum of house flies ($n = 6$ for each time period). CFU = colony forming units, error bars represent SEM.

Concentration of *E. faecalis* OG1RF:pMV158 in the midgut trended, as expected, from higher to lower within the first 24 h of the assay. Having been starved for 12 h, the flies were dehydrated and readily ingested the saline solution with inoculum, most of which was likely moved directly to the midgut resulting in the initial high bacterial counts. The subsequent reduction in *E. faecalis* concentration during the first 24 h was likely the result of digestion of bacteria in the midgut. The house fly midgut provides appropriate pH and enzymes suitable for digestion of bacteria (Espinosa-Fuentes and Terra 1987). The hindgut CFU counts were lower relative to that of the foregut and midgut throughout the study, further suggesting bacterial digestion in the midgut. This trend was also observed when viewing the intact digestive tract under UV light; fluorescing bacteria were more apparent in the anterior midgut as compared with that in the posterior midgut. The pattern of CFU counts in the foregut was less predictable but the relatively high counts in the latter part of the assay correspond with the observation of greater fluorescence seen in the crop. It is likely that some ingested bacteria were shunted to the crop and after 48–72 h the bacteria prolifer-

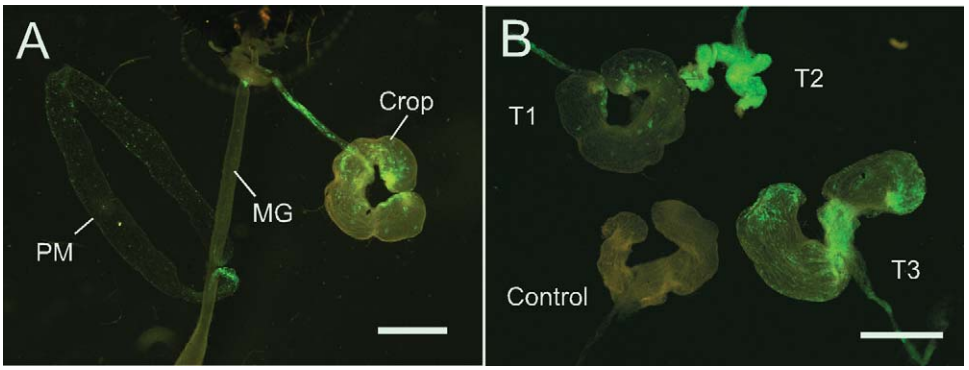

Fig. 3. GFP-expressing *E. faecalis* in house fly digestive tract at 72 h after ingestion. (A) Crop, midgut (MG), and peritrophic matrix (PM) exhibiting granular pockets of fluorescing bacteria. Scale bar, 1 mm. (B) Control crop and three treated crops (T1, T2, T3). Variation in fluorescence and crop distention are evident among the three treatments. Scale bar, 1 mm. (Online figure in color.)

ated to the extent that they could be easily viewed in the crop lumen and resulted in the granular pockets of fluorescence (Fig. 3). Whereas the initial high counts observed in the midgut were because of the initial influx of the inoculum, higher counts, in some cases in the range of 10^6 CFU later in the assay, are likely the result of bacterial proliferation in the crop and their subsequent movement to the midgut. Furthermore, it is probable that the bacteria that multiplied in the crop were not only periodically transferred to the midgut but were also regurgitated and led to *E. faecalis* recovered in the labelum after the first 24 h. Additionally, while the relative bacterial counts in the midgut and hindgut were lower at 96 h, the foregut retained a mean CFU count of $7.2 \pm 7.0 \times 10^5$, suggesting that the bacterium continued to proliferate in the crop/foregut. Kobayashi et al. (1999) found the pseudotracheae of the labelum to be important for proliferation of *E. coli* O157: H7. Concentrations of *E. faecalis* in the labelum in our study were variable but did reveal a trend of higher counts during the latter part of the assay indicating that *E. faecalis* either multiplied on the surface of the labelum or propagated in the foregut/crop and were regurgitated during feeding.

In general, the concentrations of *E. faecalis* were marked by considerable variation among individual flies. Though a number of variables can influence this, the most important contributor was probably different amounts of inoculum initially ingested by individual flies. Based on visual observation of fly exposure to the inoculum, it was noted that some flies ingested all 4 μl of the inoculum while others had taken lesser amounts. This varying amount of consumed inoculum had an impact on bacteria ingested and subsequently the amount available to recover. Another likely factor contributing to CFU variance involved the amount of subsequent feeding of individual flies after uptake of the inoculum as well as a phase of food and bacterial digestion in the midgut at the time when the individual flies were selected for dissection.

The experiment was designed to allow interaction and competition between *E. faecalis* and the other fly gut microbiota. Therefore, no attempts were made to modify or reduce the resident fly gut microbes and the flies were provided with a nonsterile food throughout the assay to better estimate how the *E. faecalis* population would compete with a steady influx of other bacteria from food. Because of ad libitum availability of food and water and a confined space during the assay, *E. faecalis* were probably deposited on surfaces and food and water and the fly likely reacquired the bacteria during grooming and feeding. In fact, direct evidence of food and water contamination was observed at the end of the assay (96 h). Although it is possible that the bacterium proliferated to some extent on the food source, because the corn was maintained dry and drinking water did not contain any nutrients, the majority of bacterial proliferation likely took place in the crop of the flies. To lower the extent of reintroduction of *E. faecalis* over the course of the study, the flies were moved to new plates daily with fresh food and water. Therefore, 24 h was the extent of time available for *E. faecalis* to multiply in the dish. If this had been the major contributor to *E. faecalis* in the fly alimentary canal, much more consistent increases in CFU counts would be expected across the four days flies were sampled. Furthermore, the highest CFU counts and observed fluorescence in the fly digestive tract would be expected during the first 24 h. As it has been shown, with the exception of the midgut, higher counts were generally observed after the first 24 h and across the three gut regions (foregut, midgut, and hindgut) and the highest combined CFU counts occurred at 72 h.

Only fluorescing colonies were counted when taking CFU counts on TSB media. No direct study on pMV158GFP stability has been done for *E. faecalis;* however, Lakticova et al. (2006) used the same plasmid in *E. faecium* D344SRF and tracked its fate in the mouse digestive tract. They found the plasmid was unstable in this environment with only 1% of viable *E. faecium* D344SRF in the feces fluorescing. Estimates were not made of the rate of plasmid loss

in the current study, however, it was likely not as dramatic based on the relatively high CFU counts observed throughout the study. However, the potential of *E. faecalis* to lose the plasmid over the 96 h of the assay is worth considering and possibly led to some underestimates of the actual *E. faecalis* OG1RF concentration.

This study has implications regarding the role of house flies in the ecology of this clinically significant bacterium. Because of the ability of house flies to disperse up to 12 km (Quarterman et al. 1954, Broce 1993), the fly could acquire virulent and antibiotic resistant *E. faecalis* from decaying organic substrates (e.g., animal manure/feces, human waste at wastewater treatment facilities) and successfully deposit the bacteria to a number of substrates (human food/drinks) remote from the area they were acquired.

In conclusion, we show that house flies serve as a bioenhanced vector of *E. faecalis* under laboratory conditions. Viable *E. faecalis* were recovered from the digestive tract of all treated flies throughout the assay up to the end of the assay at 96 h as well as from food and water at 96 h sampling period. The crop is an important site for proliferation of *E. faecalis* and fly regurgitation is likely the main mode of surface contamination with enterococci. Incorporation of integrated pest management (IPM) strategies for house flies into pre- and postharvest food safety programs is suggested.

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References Cited

Ahmad, A., A. Ghosh, C. Schal, and L. Zurek. 2011. Insects in confined swine operations carry a large antibiotic resistant and potentially virulent enterococcal community. BMC Microbiol. 11: 23.

Akhtar, M., H. Hirt, and L. Zurek. 2009. Horizontal transfer of the tetracycline resistance gene tetM mediated by pCF10 among *Enterococcus faecalis* in the house fly (*Musca domestica* L.) alimentary canal. Microb. Ecol. 58: 509–518.

Broce, A. 1993. Dispersal of houseflies and stable flies, pp. 50–60. *In* G. Thomas and S. Skoda (eds.), Rural flies in the urban environment? vol. 317. University of Nebraska Press, Lincoln, NE.

Chakrabarti, S., S. Kambhampati, and L. Zurek. 2010. Assessment of house fly dispersal between rural and urban habitats in Kansas, USA. J. Kans. Entomol. Soc. 83: 172–188.

Espinosa-Fuentes, F. P., and W. R. Terra. 1987. Physiological adaptations for digestion bacteria. Water ßuxes and distribution of digestive enzymes in *Musca domestica* larval midgut. Insect. Biochem. 17: 809–817.

Fisher, K., and C. Phillips. 2009. The ecology, epidemiology and virulence of *Enterococcus.* Microbiology 155: 1749–1757.

Graczyk, T. K., R. Knight, R. H. Gilman, and M. R. Cranfield. 2001. The role of non-biting flies in the epidemiology of human infectious diseases. Microbes Infect. 3: 231–235.

Graham, J. P., L. B. Price, S. L. Evans, T. K. Graczyk, and E. K. Silbergeld. 2009. Antibiotic resistant enterococci and staphylococci isolated from flies collected near confined poultry feeding operations. Sci. Total Environ. 407: 2701–2710.

Huycke, M. M., D. F. Sahm, and M. S. Gilmore. 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg. Infect. Dis. 4: 239–249.

Kobayashi, M., T. Sasaki, and N. Agui. 2002. Possible food contamination with the excreta of housefly with enterohemorrhagic *Escherichia coli* O157: H7. Med. Entomol. Zool. 53: 83–87.

Kobayashi, M., T. Sasaki, N. Saito, K. Tamura, K. Suzuki, H. Watanabe, and N. Agui. 1999. Houseflies: not simple mechanical vectors of enterohemorrhagic *Escherichia coli* O157:H7. Am. J. Trop. Med. Hyg. 61: 625–629.

Lakticova, V., R. Hutton-Thomas, M. Meyer, E. Gurkan, and L. B. Rice. 2006. Antibiotic-induced enterococcal expansion in the mouse intestine occurs throughout the small bowel and correlates poorly with suppression of competing flora. Antimicrob. Agents Chemother. 50: 3117–3123.

Macovei, L., and L. Zurek. 2006. Ecology of antibiotic resistance genes: characterization of enterococci from houseflies collected in food settings. Appl. Environ. Microbiol. 72: 4028–4035.

Macovei, L., and L. Zurek. 2007. Influx of enterococci and associated antibiotic resistance and virulence genes from ready-to-eat food to the human digestive tract. Appl. Environ. Microbiol. 73: 6740–6747.

Macovei, L., B. Miles, and L. Zurek. 2008. The potential of house flies to contaminate ready-to-eat food with antibiotic resistant enterococci. J. Food Prot. 71: 432–439.

McGaughey, J., and D. Nayduch. 2009. Temporal and spatial fate of GFP-expressing motile and nonmotile *Aeromonas hydrophila* in the house fly digestive tract. J. Med. Entomol. 46: 123–130.

Nieto, C., and M. Espinosa. 2003. Construction of the mobilizable plasmid pMV158GFP, a derivative of pMV158 that carries the gene encoding the green fluorescent protein. Plasmid 49: 281–285.

Petridis, M., M. Bagdasarian, M. K. Waldor, and E. Walker. 2006. Horizontal transfer of Shiga toxin and antibiotic resistance genes among *Escherichia coli* strains in house fly (Diptera: Muscidae) gut. J. Med. Entomol. 43: 288–295.

Quarterman, K. D., W. Mathis, and J. W. Kilpatrick. 1954. Urban fly dispersal in the area of Savannah, Georgia. J. Econ. Entomol. 47: 405–412.

SAS Institute. 2003. SAS online 9.1. SAS Institute, Cary, NC.

Sasaki, T., M. Kobayashi, and N. Agui. 2000. Epidemiological potential of excretion and regurgitation by *Musca domestica* (Diptera: Muscidae) in the dissemination of *Escherichia coli* O157: H7 to food. J. Med. Entomol. 37: 945–949.

Tannock, G. W., and G. Cook. 2002. Enterococci as members of the intestinal microflora of humans, pp. 101–132. *In* M. S. Gilmore (ed.), The enterococci: pathogenesis,

molecular biology, and antibiotic resistance, 1st ed. ASM Press, Washington DC.

Zurek, L., C. Schal, and D. W. Watson. 2000. Diversity and contribution of the gastrointestinal bacterial community to the development of *Musca domestica* (Diptera: Muscidae) larvae. J. Med. Entomol. 37: 924–928.

Zurek, L., and J. R. Gorham. 2008. Insects as vectors of foodborne pathogens, pp. 1–16. *In* J. G. Voeller (ed.), Wiley handbook of science and technology for homeland security. Wiley Inc., Hoboken, NJ.

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