THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

DO MANDUCA SEXTA HAVE A RESIDENT GUT MICROBIOME?

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SPRING 2019

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Toxicology
with honors in Toxicology

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ABSTRACT

It is unknown whether caterpillars (specifically *Manduca sexta*) have a resident microbiome or if the bacteria are transient. A resident microbiome would play a more significant role in aiding caterpillars with the metabolism of nutrients and toxic compounds. The current literature shows conflicting results. We hypothesized that caterpillars do indeed have a resident microbiome. To test our hypothesis, we developed three transgenic bacteria (*P. putida, B. mega,* and *E. coli*) and designed a pulse-chase feeding assay for caterpillars to determine how long bacteria can persist within *Manduca sexta*. We showed that the pulse-chase assay was capable of tracking transgenic bacterial persistence by fluorescent microscopy, PCR or spreading the frass on selective plates. The first series of pulse chase experiments following each strain of bacteria separately showed that it is possible for endemic bacteria to persist within caterpillars for over a week, through multiple molting events. Additionally, the bacteria persisted within the *Manduca sexta* colony between experiments and multiplied within the caterpillars' guts. The second series of pulse chase experiments showed that if caterpillars molt before the bacteria have a chance to colonize the caterpillars fully, the bacteria is much less likely to persist within the caterpillar gut and can be cleared from the system. The answer to the question of whether or not bacteria can persist within caterpillars is a definitive yes. The question of whether *Manduca sexta* have a resident microbiome remains undecided since it is still unclear whether persistence or transiency is normal in environmental conditions.

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ACKNOWLEDGEMENTS

I would like to thank my mentors Dr. James Tumlinson and Dr. Curtis Omiecinski for all their help and support over the years, both inside and outside the lab.

Special thanks goes to Dr. Irmgard Seidl-Adams who was a tremendous help during my time in the lab and was a major source of insight while designing the experiments that became this thesis and a huge support in carrying them out.

I would like to thank Tracey Baumgartner for helping to isolate and identify strains of bacteria for this project and for working with me day to day when I first joined the lab.

Thanks to summer student researcher Bobbi Dunton for her help with transforming the bacteria, performing the proof of concept pulse-chase experiments and conducting the passaging experiments.

I would like to thank Anne Jones, Tristan Cofer, Arash Maleki and Bipana Paudel for all their help during my time in the lab.

Finally, I would like to thank the Schreyer Honors College for providing me the opportunity to write this thesis.

Chapter 1

Introduction

Symbiosis is nearly ubiquitous in nature. Mitochondria and chloroplasts originated as symbiotic organisms, and it exists on every scale of life, from plants and insects during pollination, to the more recent discovery of gut microbiomes in humans and other animals (Margulis Lynn, 1981; Wilson & Nicholson, 2017). Bacteria living within the gut of a host organism are primarily obtained through the diet, although live birth animals (including humans) obtain their initial microbiome while passing through the birth canal (Knight et al., 2010). Bacterial interactions in the microbiome can encompass mutualistic, commensalistic, opportunistic and even parasitic relationships. In general, the bacteria obtain nutrients and a living environment from their host. In return, bacteria can break down complex nutrients into simpler forms of energy such as *Lactobacillus* in the human digestive tract which breaks down polysaccharides into glucose (Ramakrishna, 2013). Bacteria can impact behavior, alter development and provide defenses against pathogens or toxic compounds (Chen et al., 2016; Heerman, Weng, Hurwitz, Durvasula, & Ramalho-Ortigao, 2015; Mason, Jones, & Felton, 2018; Voirol, Frago, Kaltenpoth, Hilker, & Fatouros, 2018; Wilson & Nicholson, 2017). In humans, the gut microbiome has been shown to directly metabolize a variety of drugs and certain species have even been identified in relation to the reactions they perform. *Clostridia* and *Eubacteria*, for example, have the ability to reduce prontosil and neoprontosil to 5-aminosalicylic acid, their active metabolite (Wilson & Nicholson, 2017). In mammals, the gut microbiome community also plays a major role in the variety and concentrations of the metabolites present in the blood

(Wikoff et al., 2009). The gut microbiome is currently a topic of great interest in human medicine as well as in other organisms. There is still much research left undone on both fronts, but the gaps in knowledge are especially large in regards to Lepidopterans. It is suspected that midgut bacteria help *Manduca sexta* to counteract the defensive mechanisms employed by their major food source, *Nicotiana attenuata* by decreasing green leaf volatile (GLV) emissions (Hammer & Bowers, 2015; A. C. Jones et al., 2018; A. G. Jones, Mason, Felton, & Hoover, 2019; Mason et al., 2018; Spiteller, Dettner, & Boland, 2000). GLVs may cause direct harm to *Manduca sexta*, or act as signaling molecules that prime plants to activate their defenses or attract predatory insects (Scala, Allmann, Mirabella, Haring, & Schuurink, 2013; Whitman & Eller, 1990). *Manduca sexta*'s oral secretion has the ability to suppress GLVs by isomerizing the (Z)-3-hexenal to (E)-2-hexenal, but it is unclear whether the enzyme responsible for this reaction is produced by the caterpillars or by their microbiome (A. C. Jones et al., 2018). It is still unclear whether the bacteria endemic to *Manduca sexta* colonize part of their gut, or if the bacteria are merely transient as shown by Hammer et al., 2017 who analyzed 16S rRNA genes in wild caterpillars but primarily found chloroplast rRNA. The low levels of microbial rRNA could potentially be due to the volume of plant material ingested drowning out the microbial 16S signals, rather than a lack of resident microbes. The same study found that suppressing bacteria with an antibiotic treatment had no detectable effect on the survival of *Manduca sexta* caterpillars (Hammer et al., 2017). A study on Gypsy Moth midgut bacterial communities found that initial egg mass-associated midgut bacteria varied a great deal, but as larvae developed, their midgut communities became relatively characteristic. The midgut bacteria community was reflective of the bacterial composition of the foliage diet but varied enough to be distinct from the diet (Mason & Raffa, 2014). In a paper by Chen et al., gut microbiota composition in *S.*

littoralis was shown to change over the life cycle and functional analysis showed increased transcription of bacterial genes advantageous for each life stage (Chen et al., 2016). If the bacteria have the ability to colonize the caterpillars' midgut, it is unknown whether they can persist across molting events, through metamorphosis or even through multiple generations.

Manduca sexta

The life cycle of a *Manduca sexta* (Tobacco Hornworm) occurs in five larval instars, a pupal stage and an adult moth phase. An instar is a phase of development in insects that occurs between periods of molting. Tobacco hornworm eggs are transparent green and about 2mm in length. The eggs hatch after three to five days. Each of the first three instars lasts for 2-3 days, the fourth instar lasts for four days and the fifth instar lasts for five days. When a caterpillar molts after the fifth instar, it will begin to pupate. The pupal stage lasts 19 to 23 days until the pupa becomes an adult moth (Berkley, n.d.). "During metamorphosis, the overall body organization of the larva changes completely: most organs undergo deep remodeling or even completely degenerate…. (Chen et al., 2016)."

Bacteria

Psuedomonas putida (*P. putida*) is a member of the Pseudomonoadacaeae family and is a rod-shaped, Gram-negative soil bacterium. It is one of the common family and genera found within the gut microbiome of caterpillars (Voirol et al., 2018). *P. putida* colonies grew over the course of 1-2 days and appeared small, circular, beige colored and slightly glossy.

Bacillus megaterium (*B. mega*) from the Bacillaceae family is a rod-shaped, Gram-positive, spore forming, aerobic bacterium that primarily lives in soil. Bacillus is a common genera within the gut microbiome of caterpillars (Voirol et al., 2018). Its colonies grew over the course of 1-2 days and appeared white and fuzzy.

Escherichia coli (*E. coli*) is a rod-shaped, Gram-negative, facultative anaerobe from the Enterobacteriaceae family. It is common within the human gut microbiome and some serotypes can cause food poisoning while others are symbiotic. In general, *E. coli* does not persist within *Manduca sexta*, instead it is encapsulated by the caterpillars' hemocytes which clear the infection from the caterpillar (Daborn et al., 2002). Its colonies grew into circular colonies and appeared greenish-white on the plate due to the GFP plasmid.

Pulse-Chase Assay and Transformation

Persistence is the continued inhabitance of bacteria within its host organism. In order to determine whether bacteria are persistent within *Manduca sexta*, it was necessary to find a method of tracking them. The bacteria were transformed with green fluorescent protein (GFP) in order to make them trackable. A pulse-chase feeding assay was developed to investigate whether bacteria are persistent within the caterpillar, or simply pass through with the food bolus.

The gene for green fluorescent protein originated in the Pacific Northwest *Aequorea Victoria* jellyfish (Cubitt, Woollenweber, & Heim, 1999). The protein has seen widespread use as a marker for gene expression and in the localization of gene products (Ormo et al., 1996). One usage method involves inserting the GFP gene into a plasmid that contains a gene for antibiotic resistance. Researchers can then expose bacteria to the antibiotic that the gene provides

resistance against and select for the transgenic bacteria. GFP has been expressed in bacteria such as *E. coli* (Chavshin et al., 2013) and *P. putida* (Chavshin et al., 2015). Green fluorescent protein expressed by bacteria makes them emit green light with an emission wavelength of 509nm under UV light and allows for direct visualization of the transgenic bacteria. Therefore, transformation of bacteria with a plasmid encoding GFP and antibiotic resistance allows tracking with fluorescence microscopy, PCR amplification of the GFP gene, or growth of the bacteria on selective media.

Pulse-chase analysis is a method used to examine processes that occur over time. A cell or organism is first exposed to a labeled compound (pulse) which is then followed by an unlabeled form of the compound (chase). Pulse-chase analysis is frequently used to illuminate biochemical pathways and mechanisms of action. Generally, the pulse consists of a fluorescently labeled or radiolabeled compound. In the case of this set of pulse-chase feeding experiments, diet cubes were used in place of a compound. Labeling took the form of blue food dye so that the caterpillars' frass would appear blue during inoculation (pulse) and brown post-inoculation (chase). The caterpillar's gut does not mix the food, making it easy to track a food bolus by feeding the caterpillar on colored diet for a specified amount of time before replacing it with uncolored diet.

We investigated the following questions:

- Do bacteria colonize the gut of *Manduca sexta* or pass through transiently?
- If the bacteria persist, do they persist through molting events, pupation and/or across generations?
- If the bacteria persist, what part of the caterpillar do they inhabit?

Chapter 2

Materials and Methods

Caring for Caterpillars

Manduca sexta were sourced from the lab's colony. The caterpillars' normal diet consisted of 81g Southland Products Incorporated Tobacco Hornworm powdered diet added to 465mL boiling water and mixed for a minute with a hand mixer on the highest speed. The mixture solidified and was stored in the refrigerator.

Isolation of Bacteria

Bacteria were isolated from the regurgitant and frass of *Manduca sexta* (Tobacco Hornworm) caterpillars fed exclusively on *Nicotiana benthamiana*. Genomic DNA was extracted with the PrepGEM-bacteria kit from Zygem from isolated colonies. The V5 through V8 regions of the 16S rRNA gene were amplified and sequenced with 799F-Mod6: CMGGATTAGATACCCKGGT and 1392R: ACGGGCGGTGTGTRC to allow identification of the strain of each isolate (Hanshew, Mason, Raffa, & Currie, 2013). Glycerol stocks of the isolates with a final concentration of 20% glycerol were stored at -80˚C.

Transformation of Bacteria

P. putida, *B. megaterium, E. cloacae* and *E. coli* were transformed with the pfdC4Z'-gfp (Bogs, Bruchmüller, Erbar, & Geider, 1998; Geider, Baldes, Bellemann, Metzger, & Schwartz, 1995; Spinelli, Ciampolini, Cresti, Geider, & Costa, 2005) plasmid containing a truncated version of the gene for Green Fluorescent Protein (GFP) and a gene for Chloramphenicol resistance. Successful transformations of *P. putida, B. megaterium* and *E. cloacae* were performed following the methods described by Aune & Aachmann, (2010) and modified to match the methods in Iwasaki et al., (1994).

P. putida (and *E. cloacae* and *B. megaterium)* was grown as a starter culture overnight in 3ml LB Medium with reduced (50%) NaCl content. The starter culture was used to inoculate 25ml of NaCL reduced LB liquid broth. It was incubated at 28°C, and grown to mid-log phase (A_{600} = 0.26-0.38).

Electrocompetent cells were made by pelleting bacterial cells and replacing the growth media with a medium containing no (low) ionic strength. Bacteria were centrifuged at 5000g for 10 minutes at 4˚C, the supernatant was discarded and washed twice with 2.5 ml of sterile ice cold 300mM sucrose solution followed by a wash with 1ml of 300mM sucrose, 10% glycerol solution and finally resuspended in 500µl of 300mM sucrose-10% glycerol solution. Each centrifugation step occurred at 4˚C and 5000g for 10 minutes. 40µl aliquots were used either directly for electroporation or were stored at -80˚C.

40µl of electrocompetent cell solution and 1µl of the plasmid (at 10ng/µl) were incubated on ice for 10 minutes in a pre-chilled electroporation cuvette (2mm gap). The cuvette was placed in the electroporator (ECM399 by BTX) set to 2500V. The actual delivered pulse was 2460V for 5ms. Immediately after electroporation, 500µl of the LB Broth (low salt, no chloramphenicol)

was added to the cuvette. Cells and media in the cuvette were transferred to a 1.5ml Eppendorf tube and incubated at 28°C, shaking at 140 rpm for 1 hour. An aliquot of 150µl was spread on each chloramphenicol (20µg/ml) containing selective LB plates. The plates were covered with tinfoil and incubated at 28°C for 1-2 days. Visible colonies were screened for plasmid insertion by PCR with GFP specific primers GFPL-502: CTGGGTATCTCGCAAAGCAT and GFPR-670: GGTGATGTTAATGGGCACAA. All tested colonies carried the plasmid. Colonies that grew on the selective plates were also imaged using fluorescence microscopy on the default GFP settings and all were found to fluoresce. Two glycerol stocks were prepared using LB broth (low salt), 20µg/ml chloramphenicol 20% Glycerol and frozen at -80˚C.

E. coli One Shot Top 10 (Invitrogen, USA) chemically competent cells were successfully transformed by heat shock; 40µl of competent cells and 1µl of plasmid were combined and incubated on ice for 30 minutes. The mixture was heated in a 42°C water bath for 2 minutes, then 300µl of LB Broth was added and the solution was incubated for 1 hour at 28°C while shaking at 140rpm in order to recover. After an hour of recovery, the heat-shock transformed *E. coli* followed the same post-recovery procedure used with the electroporated cells.

Pulse-Chase

Manduca sexta caterpillars (second instar) were collected and placed in separate transparent portion cups with air holes poked into the lids using sterilized tweezers. Caterpillars at this life stage were chosen so that they would have the chance to molt multiple times. The caterpillars fed on bacterial diet for a variable number of days (three days in the proof of concept experiment, two days in the group experiment and one day in the individual experiments). In

order to create the bacterial diet, eleven drops of blue food dye were added to the caterpillars' normal diet and stirred until the entire mixture was blue. The blue diet was cut into 1cm x 1cm x 1cm cubes and immersed in overnight bacterial LB suspensions grown to OD 1.20 (Abs. 600nm) for one minute. Excess solution was shaken off and one cube was placed in each feeding cup. After feeding on bacterial diet, the caterpillars were moved to new transparent cups that contained normal diet. The caterpillars remained in these cups until the end of each experiment.

Frass was collected daily and both the number and size of the pellets was recorded for each caterpillar. Size was categorized by length of the pellets: small (0.1cm or less), medium (0.1-0.3cm), large (0.3-0.4cm), or extra-large (0.5cm or more). Only the blue pellets not touching the food source were collected when the caterpillars had been fed bacterial diet. Once the caterpillars were switched to normal diet, only the non-blue pellets were collected for analysis. In both cases, any remaining pellets were removed from the container. Molting behavior was also recorded each day based upon the presence of horn or skin fragments or visible molting behavior.

The frass was suspended in just enough LB broth (low NaCl content) to make it easy to pipette. For small pellets, 10µl was added per pellet. For medium pellets, 15µl was added per pellet. For large pellets, 20µl was added per pellet. For extra-large pellets, 50-100µl was added per pellet. A minimum of 30µl was added if there was only 1 pellet. The frass and broth were mixed using a pipette tip to create a slurry.

A 30µl aliquot of suspended frass was plated on one half of a chloramphenicol selective LB plate. The plates were covered with aluminum foil to protect the chloramphenicol from photodegradation and incubated at 28°C for one day. After one day, the plates were checked for the presence of colonies. The strains of bacteria present on each plate were recorded. If none

grew, the plates were returned to the incubator and checked again the next day. If nothing grew again, the plate was stored at room temperature and monitored for any growth.

In the proof of concept set of experiments performed by Bobbi Dunton, 10 caterpillars from the third instar were assigned to two groups. The control group was fed blue diet immersed in LB media for one minute. The experimental group was fed diet that was immersed in bacterial suspensions of either *E. coli* or *P. putida*. Both groups were switched to normal diet after 3 days. The experiment was concluded on day 6. These experiments amplified GFP gene fragments from the frass instead of relying on chloramphenicol selective plates.

In the group experiment that lasted for five days, 24 *Manduca sexta* caterpillars in the second instar were collected and assigned to four cohorts of six caterpillars. Each cohort was assigned a bacterial strain transformed with the GFP plasmid (*E. coli, B. megaterium, P. putida* and *E. cloacae*). They were fed on bacterial diet for 2 days before being switched back to normal diet.

In the individual bacteria experiments, the blue diet was inoculated with one strain of bacteria (*P. putida, B. megaterium,* or *E. coli*) and then fed to a cohort of ten caterpillars for one day before they were switched over to normal diet. These experiments were concluded after 7-10 days after the strain of choice was no longer present or multiple molting events were recorded.

Dissection of *Manduca Sexta*

Following the second round of individual experiments, *Manduca sexta* caterpillars were dissected. The caterpillars were covered with ice for 15 minutes. The frozen caterpillars were placed on their backs and pinned down through the head and anal prolegs. An incision was made

in the center of the abdomen, closest to the anal prolegs using microdissection scissors. A micropipette was used to remove hemolymph from the incision. The incision was extended up the abdomen without puncturing the gut. When the incision extended from the anal prolegs to the abdomen, four more pins were used to spread the caterpillar open. Two pins were placed, one on each side between the first and second true legs. The other two pins were placed one on each side between the second and third abdominal prolegs. The white strands of muscle tissue holding the gut in place were then cut away. The gut was severed at the head and the rear. It was then removed with microdissection forceps. The guts, skin, molted skin if any, and hemolymph were stored in separate 1.5ml tubes. 200µl of LB broth (low NaCl content) was added to each of the tubes containing guts, skin or molted skin and the tubes were vortexed. 25µl of broth from each tube and 25µl of hemolymph were spread onto chloramphenicol selective plates or nonselective plates and incubated for two days at 28°C.

DNA Extraction and PCR Clean-Up

DNA was extracted using the prepGem bacteria kit from ZyGem. 100µl of *P. putida* was centrifuged at 11,000 rpm for 6 minutes using a tabletop centrifuge. The supernatant was pipetted away. Three frass pellets from *P. putida* fed caterpillars were added to a separate tube. 89µl of DNA-free water, 10µl of 10x GREEN buffer and 1µl of *prep*GEM were each added to the tube containing the three frass pellets and the tube containing the *P. putida* pellet. The mixtures were vortexed twice and placed in the PCR*Sprint* thermal cycler set to 37°C for 15 minutes, 75°C for 10 minutes and 95°C for 2 minutes. Mo Bio Laboratories, Incorporated PCR clean-up kit was used for initial DNA extractions to remove any contaminants within the DNA

by adding 500µl of SpinBind to 100µl of DNA. The DNA and SpinBind mixture was transferred to a Spin Filter unit and centrifuged for 30 seconds at 13,000 rpm in a tabletop centrifuge (MicroMax from Thermo IEC). The Spin Filter basket was removed to discard the liquid and put back into the original tube, and then 300µl of SpinClean Buffer was added to the Spin Filter and centrifuged for 30 seconds at 13,000rpm. The flow through was discarded and the filter was centrifuged again to remove any remaining liquid. The filter was transferred to a clean collection tube. 50µl of Elution buffer (10mM Tris) was added directly to the center of the Spin Filter membrane and centrifuged for 60 seconds at 13,000rpm. The Spin Filter basket was discarded, and the flow through DNA was stored at -20°C.

Polymerase Chain Reaction

A MasterMix was created in two separate tubes, which include water, GreenGOTAQ 2x Master Mix (10µl), 0.5µl GFP primer mix (10µM) and cleaned up DNA (5µl). Fifteen microliters of reaction master mix were aliquoted into the PCR tube strip and 5µl of DNA or water were added individually. The PCR tube strip was centrifuged briefly to collect the reaction mix at the bottom of the tubes and placed into the Bio-Rad thermocycler (iQ5 Cycler). The PCR (polymerase chain reaction) program consisted of an initial denaturation step at 95°C for 2 minutes followed by 40 cycles consisting of three steps: denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72 °C for 30 seconds and ended with a final extension at 72°C for 5 minutes. The samples were held at 4°C after completion of the 40 cycles. Quantitative PCR used a 2x Mastermix that contained SsoFastEvaGreen, and the template was 1µl of DNA from the ZyGem DNA extraction kit procedure. The cycling program was 2 min at

95˚C, followed by 40 cycles of 15 sec incubations at 95˚C, 60˚C, and 72˚C each, a final extension at 60˚C for 5 minutes and a melting curve in 0.3˚C increases from 60˚C to 94.5˚C.

Gel Electrophoresis and Gel Doc XRS

An agarose gel was prepared using 40mL 1x TBE (Tris/Borate/EDTA) buffer and 0.55g of Agarose Type 1 powder, which were boiled together for two minutes in a microwave to dissolve the agarose in the buffer. After some cooling 17.5µl of Ethidium Bromide (0.5 mg/ml) was added to the mixture. The mixture was poured into the gel-casting tray and a comb was added to create wells. The comb was removed after the gel solidified. 1x TBE buffer was added to the gel rig until the gel was completely covered. 10µl of PCR sample was added per well. The electrophoresis apparatus was set to 120 Volts for 30 minutes. The gel was removed from the casting tray and placed on the Gel Doc XRS Imager. UV light was used during imaging of the gel.

Preparation of Luria Bertani (LB) Medium (Reduced NaCl)

Table 1: LB Broth Media

LB Broth Media			
	1L Solution	500ml Solution	250ml Solution
Tryptone	10g	5g	2.5g
Yeast	5g	2.5g	1.25g
NaCl	5g	2.5g	1.25g
Water	1000ml	500ml	250ml

** Normal LB Broth has double the NaCl listed here*

Table 2: LB Agar Plate Media

LB Agar Plate Media			
	1L Solution	500ml Solution	250ml Solution
Tryptone	10g	5g	2.5g
Yeast	5g	2.5g	1.25g
NaCl	5g	2.5g	1.25g
Water	1000ml	500ml	250ml
Agar	14g	7g	3.5g

Tryptone, Yeast and NaCl were weighed out separately and combined in a media bottle following the quantities in Table 1. The appropriate volume of water was added and the bottle was capped, then swirled and shaken to mix. The cap was loosened almost all of the way, and then the bottle was microwaved until it boiled, then taken out and swirled until a uniform solution was achieved. After microwaving, the solution was autoclaved on the liquid cycle. The media was then cooled.

The same steps were followed to create LB Agar Plates using Table 2, with the addition of Agar being weighed out and combined in the media bottle before the water was added. Once the media cooled to 50˚C, it could be poured or pipetted into plates. Larger plates received 20-25ml of media to cover the entire plate.

Preparation of Chloramphenicol LB Medium (Reduced NaCl)

Chloramphenicol was used as a selective agent to ensure that only the successfully transformed bacteria survived. Normally *P. putida, E. coli* and *B. megatarium* are susceptible to chloramphenicol. The plasmid encodes both a GFP gene and a chloramphenicol resistance gene, so the *P. putida, E. coli* and *B. megatarium* that survive the electroporation or heat shock, but were not transformed will die off, while the transgenic bacteria would survive.

The preparation of chloramphenicol LB Medium (Reduced NaCl) follows the same procedure as above, but chloramphenicol was added at a final concentration of 20µg/ml to the broth or agar after the media was autoclaved and cooled to below 42°C. This prevents degradation of the chloramphenicol. The resulting broth or plates were covered with tinfoil to prevent degradation of the chloramphenicol by light.

Chapter 3

Results

Which bacteria are present in *Manduca sexta*?

Manduca sexta (Tobacco Hornworms) have bacteria in their guts. The caterpillars obtain bacteria from their food, and reintroduce the bacteria to the environment with their frass and regurgitant. Eleven bacteria from the frass and regurgitant were isolated, cultured, and identified by 16S sequences. Diet-fed caterpillars contained *Bacillus amyloliquefaciens*; *Trichosporon faecale*; *Diutina catenulate* and a coccobacilli species. All of which were isolated from the regurgitant, although *B. amyloliquefaciens* was also obtained from the frass. When the caterpillars were fed *Nicotiana benthamiana* for a few days, *Pseudomonas putida*; a representative of the *Enterobacter cloacae complex*; *Enterococcus durans*; *Enterococcus casseliflavus*; *Candida duobus haemulonii*; *Bacillus megaterium* and a proteus species were isolated from the frass. *E. cloacae*, *Pseudomonas putida* and *Enterococcus durans* were also isolated from the regurgitant (Credit to Tracey Baumgartner). Subsequent experiments made use of *Pseudomonas putida, Bacillus megaterium* and *Enterobacter cloacae*.

How can we track the bacteria?

Several transformation techniques were attempted before successful methods were found. Heat shock was used to transform *E. coli* which served as a positive control to confirm that the

plasmid carried the correct genes, i.e. a gene conferring resistance to chloramphenicol and the GFP gene. *E. coli* also served as an "out group" in the feeding experiments since we did not isolate it from the caterpillars. *P. putida*, *E. cloacae* and *B*. *megaterium* were transformed by electroporation. Successful transformation was confirmed by PCR and plating onto selective chloramphenicol plates. Colonies that grew on the selective plates were imaged using fluorescence microscopy on the default GFP settings. A selection of those images (**Figures 1-4**) clearly show the bacteria fluorescing green. All of the selective plates with colonies growing showed fluorescence of the colonies. Based on this information, growth on a selective plate was equated with presence of the GFP plasmid. This provided three methods of tracking the bacteria: using PCR to verify presence of the plasmid, plating the bacteria onto Chloramphenicol selective plates, and inspecting the plates under a fluorescent microscope for GFP expression.

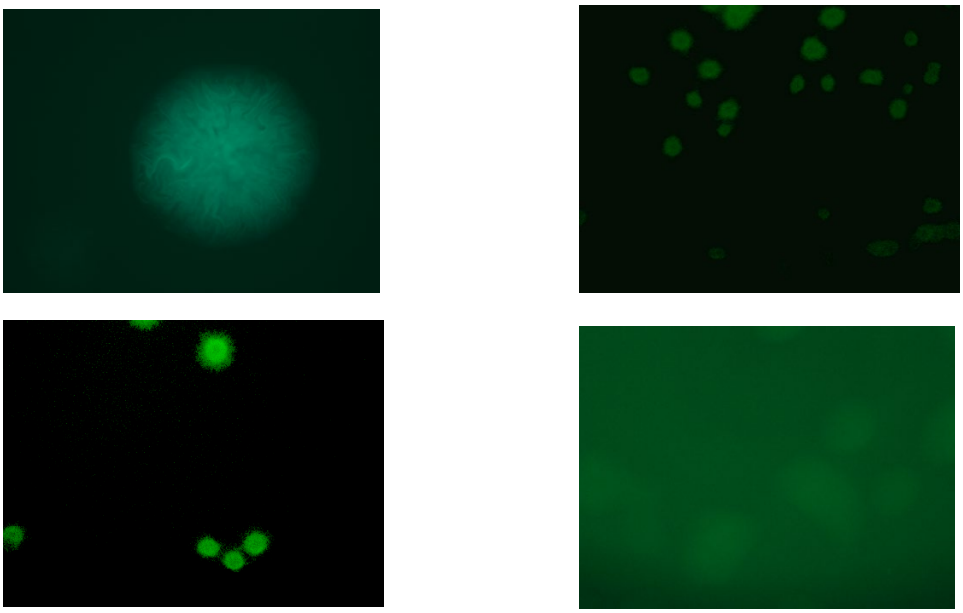


Figure 1 (Top Left): Single *E. coli* colony fluorescing green

Figure 2 (Top Right): *B. mega* colonies fluorescing green

Figure 3 (Bottom Left): More *B. mega* colonies fluorescing green

Figure 4 (Bottom Right): *E. cloacae* colonies fluorescing green

Do bacteria persist within the caterpillars?

Proof of concept pulse-chase feeding assays

Three proof of concept pulse-chase feeding assays (performed by a summer student – Bobbi Dunton) using the transgenic *E. coli* and *P. putida* strains showed that a GFP gene fragment could be amplified out of the DNA isolated from the frass of infected caterpillars (1-5a) for the three days they were fed diet inoculated with transgenic *E. coli* (**Figure 5**). The amount of GFP gene fragments amplified was highest on the first day and decreased daily before disappearing entirely after the caterpillars were switched to normal diet. To verify the identity of the PCR product (**Figure 6**), PCR products of samples 1a and 4a (fed on GFP-expressing *E. coli* inoculated diet), 8a (fed on normal diet immersed in LB media), and the bacterial inoculum were sequenced. 1a, 4a and *E. coli* aligned perfectly under the 200 base pair mark with the GFP gene of the plasmid (168-bp) (**Figure 7**) while the band amplified in 8a was not specific. These results show that transgenic bacteria can be amplified from the frass and that the bacteria infused into the diet can be tracked through the frass. With confirmation that the bacteria from the frass harbored the gene for GFP, it was decided that plating an aliquot of suspended frass onto a selective plate was an easier and cheaper method of tracking transgenic bacteria.

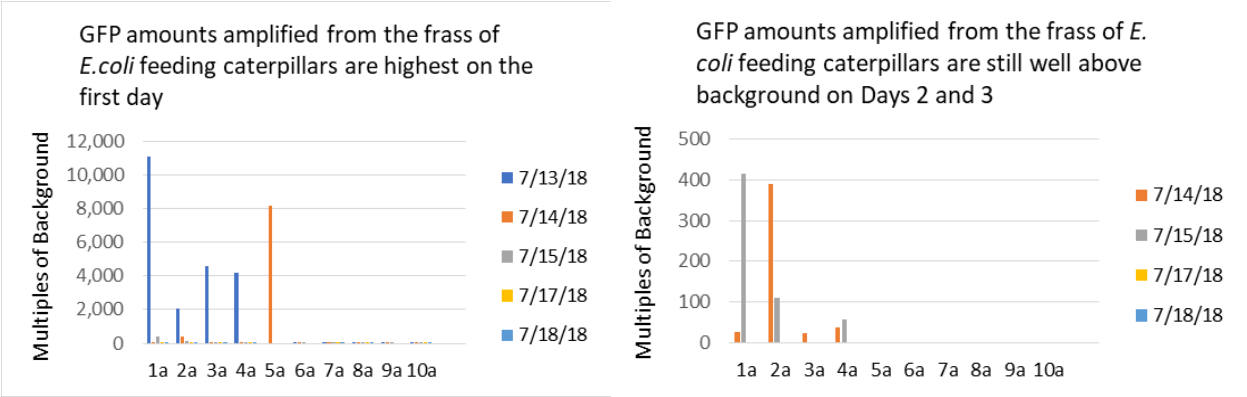


Figure 5: Quantitative PCR Results without normalization. Samples 1a-5a were fed on *E. coli* diet. Samples 6a-10a were fed on LB media diet. GFP could be amplified out of the frass produced by caterpillars fed on the *E. coli* diet. Caterpillar 5a died after Day 2 and caterpillar 3a died after Day 4. The highest amounts of GFP were amplified out of the frass on Day 1 (**left**) and decreased until Day 3 (**right**), before disappearing altogether when switched to normal diet.

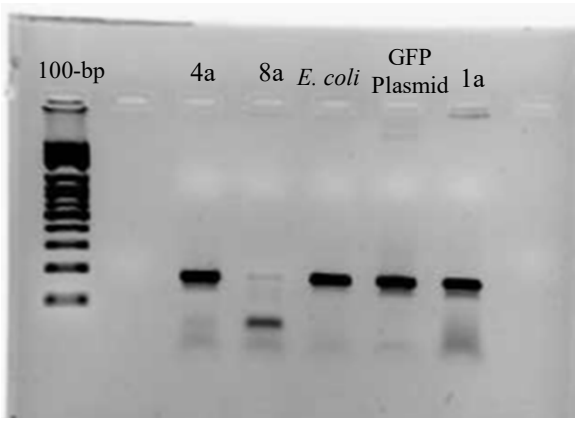


Figure 6: PCR gel showing bands for Green Fluorescent Protein plasmid, transformed *E. coli* and frass samples.

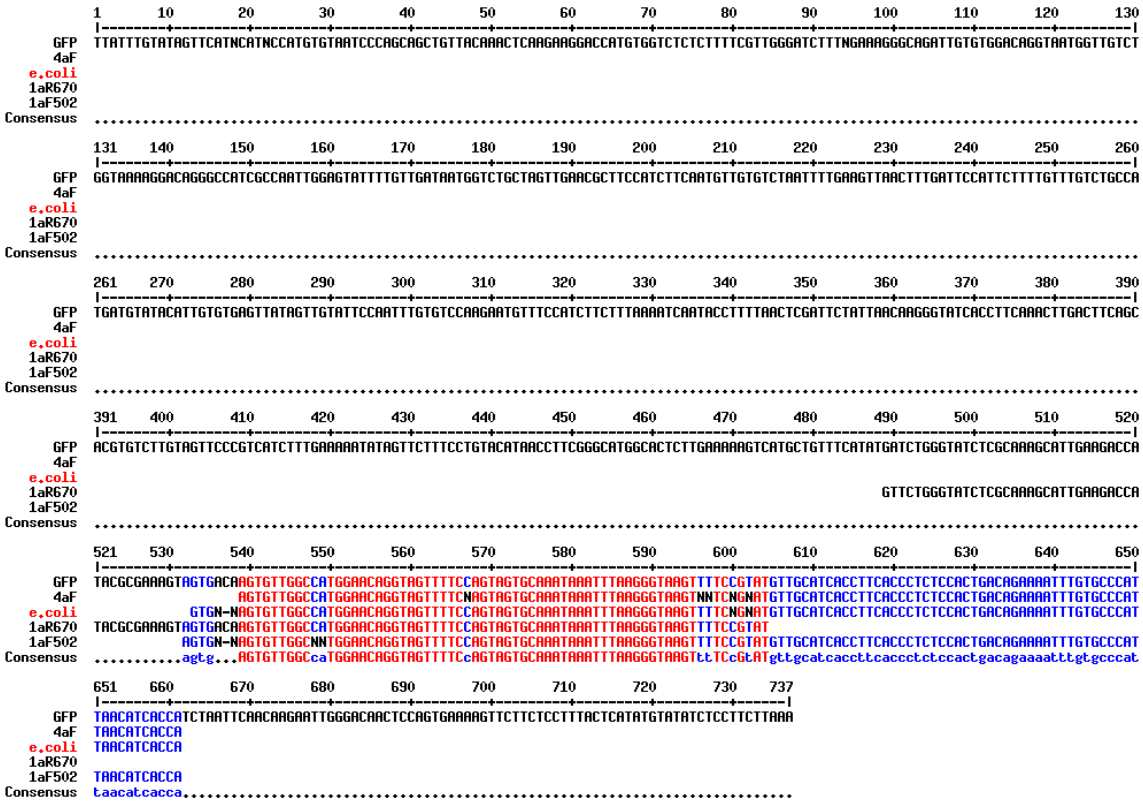


Figure 7: Gene sequencing of GFP, *E. coli* and the amplified PCR product of one frass sample. The targeted sequence of the GFP gene was 502 to 670 and the sequence from caterpillar 4a's frass falls within the two markers and aligns perfectly with the targeted GFP gene sequence of the plasmid (in blue).

The three pulse-chase experiments also showed that feeding *Manduca sexta* on *P. putida* or *E. coli* diets did not affect mortality rates of the experimental groups of caterpillars as compared to the control groups (**Figure 8**). Neither did feeding on food soaked in chloramphenicol. Each of these experiments also showed that the experimental group caterpillars' feeding and excretion patterns were not altered compared to the controls (**Figure 9**).

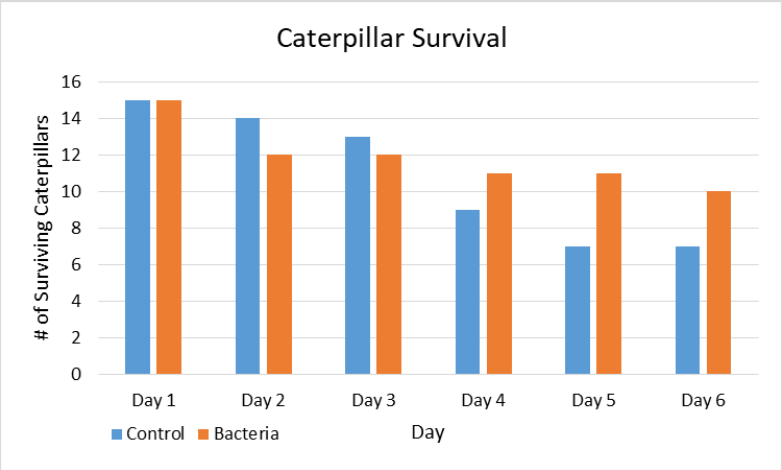


Figure 8: Control and bacterial inoculated (*P. putida* or *E. coli*) caterpillar survival combined from the three proof of concept pulse-chase assays.

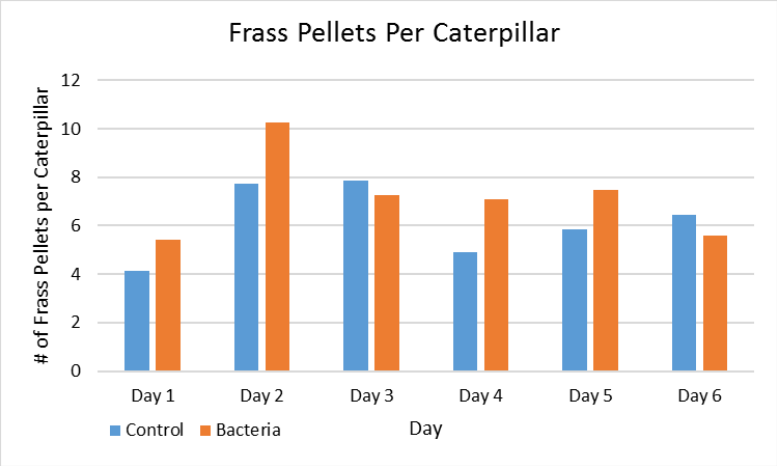


Figure 9: Number of frass pellets per caterpillar per day. (Data combined from the three proof of concept pulse-chase experiments).

Group pulse-chase feeding assay

A group pulse-chase assay was conducted over the course of five days using each of the four transgenic strains of bacteria (*E. coli, B. megaterium, P. putida* and *E. cloacae*). The experiment used 24 caterpillars sorted into four cohorts, each of which was assigned a bacterial strain. Each caterpillar was fed on a bacterial diet for two days before being switched back to normal diet on Day 2. Frass was collected and plated on Days 1, 2 and 5. Plating the frass on

selective medium provided a measure of viable bacteria in the frass (**Figure 10**). The *B. megaterium* persisted for at least 2 days after feeding and *P. putida* persisted for at least 5 days after feeding. *P. putida* persisted through at least one round of molting. Plates P2 and P3 from Day 5 were inspected under the fluorescent microscope and verified to be expressing GFP. This shows that the bacteria maintained the plasmid for at least 3-5 days. *E. coli* appeared on the plates only for the duration the caterpillars were fed on *E. coli* bacterial diet, but disappeared quickly afterwards. The *E. cloacae* did not appear on the selective plates from any of the days and were likely not transformed successfully. Minimal cross-contamination was seen on the selective plates. The caterpillars were returned to the colony after the experiment was concluded.

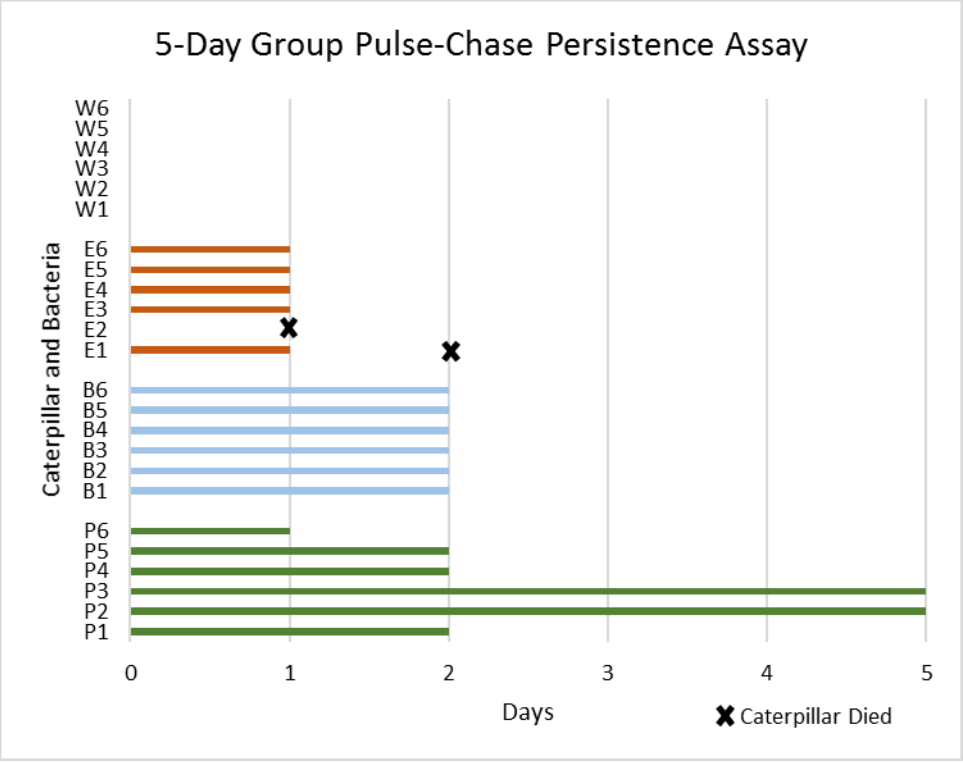


Figure 10: Five-Day Pulse-Chase Feeding Assay - W represents *E. cloacae*; E represents *E. coli*; B represents *B. megaterium*; P represents *P. putida*.

Individual pulse-chase feeding assays were conducted with *B. megaterium*, *E. coli* and *P. putida*. Each experiment involved a cohort of 10 caterpillars and one bacterial strain at a time. The caterpillars were fed on bacterial diet for the first day and then switched to normal diet in new cups.

B. megaterium **individual pulse-chase assay**

Frass was collected, suspended and plated on Days 2 through 10 with the exception of weekends. *B. megaterium* persisted in the caterpillars for the duration of the 10-day experiment, and over 2-3 rounds of molting as shown in **Figure 11**. Only presence or absence of *B. megaterium* was scored. **Figure 12** indicates that similar levels of bacterial growth were exhibited when plating one pellet suspended in 50µl of broth or 46 pellets suspended in 690µl. Cross contamination by *P. putida* was seen on many of the plates for the duration of the experiment. LB broth used for the resuspension of the frass pellets was plated and no bacterial growth was seen. Suspending the normal diet in LB broth and spreading it onto a selective plate resulted in scattered growth of both *P. putida* and *B. megaterium* colonies (**Figure 13**).

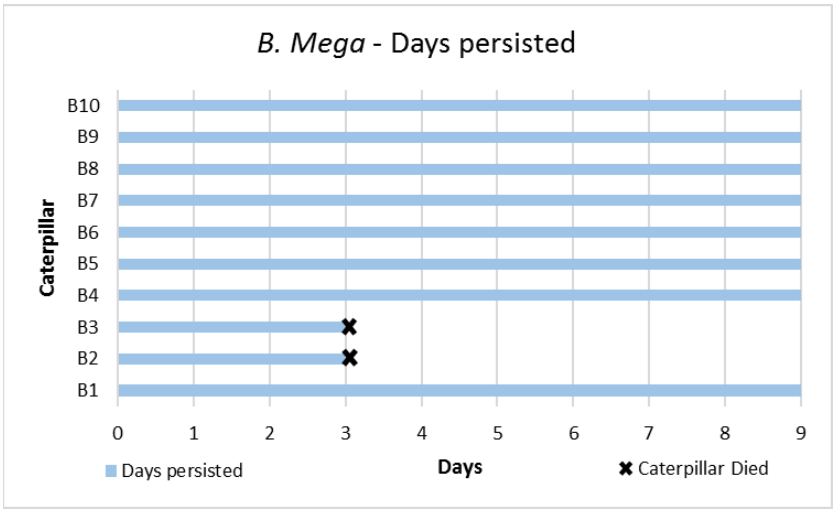


Figure 11 (Left): First Pulse-Chase Feeding Assay with *B. megaterium*

Figure 12 (Right Top): *B. megaterium* Day 1 - samples B5 (left half of plate) and B6 (right half of plate). B5 was sourced from one frass pellet, suspended in 50µl of LB broth, and 50µl was plated. B6 was sourced from 46 frass pellets, suspended in 690µl of LB broth (15µl per pellet) and 50µl were plated.

Figure 13 (Right Bottom): LB Broth (left half of plate) shows no bacterial colony growth. Normal Diet (right half of plate) shows some *P. putida* and *B. megaterium* growth.

E. coli individual pulse-chase assay

The next pulse-chase feeding assay tested the persistence of *E. coli*. Ten second instar caterpillars were chosen and fed on blue bacterial diet for the first day and then switched onto normal diets in new cups. The frass was suspended in LB broth and plated. Of the eight caterpillars that survived past the second day, all showed bacterial growth, primarily in the form of *B. megaterium* and *P. putida* throughout the 7 days that the experiment lasted. Only one caterpillar (E8) had *E. coli* show up on the plates, and only one or two *E. coli* colonies appeared on the plate, mixed in among the *B. megaterium* and *P. putida* (**Figure 14**). The *E. coli* persisted within the caterpillar until Day 4, after which it did not show up on any more plates. Both the bacterial diet (after contact with caterpillars) and the normal diet (before contact with the caterpillars) were plated and it was found that the normal diet contained *B. megaterium* and *P.*

putida, while the bacterial diet contained *E. coli* with a few scattered *P. putida* colonies (**Figure 15**).

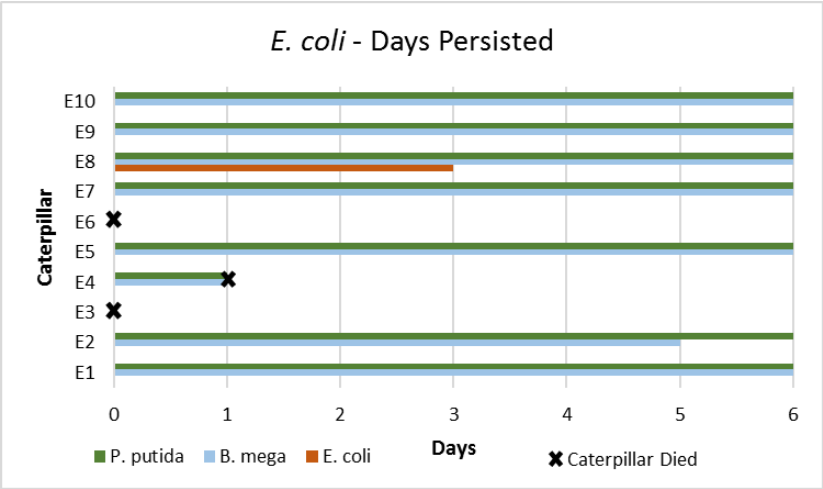


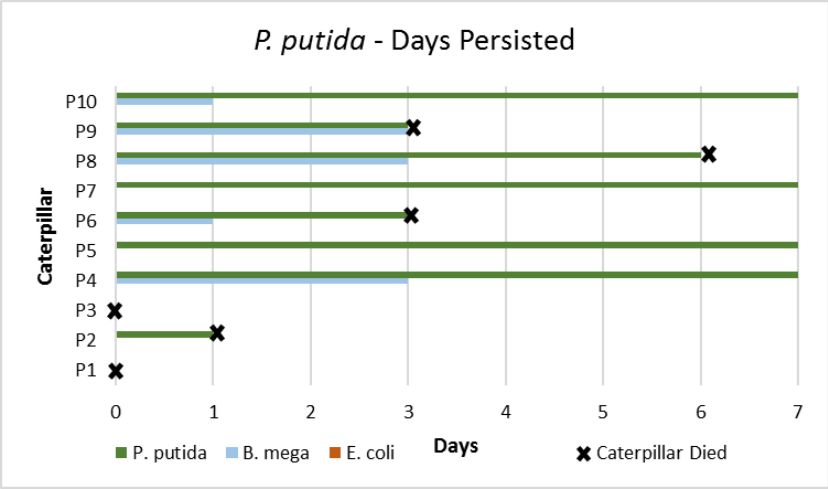
Figure 14 (Left): Pulse-Chase Feeding Assay with *E. coli* - (measuring *P. putida*, *B. megaterium*, and *E. coli*)

Figure 15: *E. coli* Day 1 - after 4 days of growth. Left side of plate: the normal diet before contact with caterpillars showed growth of B. megaterium (the larger colonies) and P. putida (the smaller colonies). Right side of plate: The bacterial diet (soaked in *E. coli)* showed growth of *E. coli* (the large green colonies) and a few *P. putida*.

***P. putida* individual pulse-chase assay**

This experiment took place after confirmation that the normal food source was contaminated with bacteria, and that the colony of *Manduca sexta* may have been exposed to the bacteria already. The blue food was autoclaved and new normal food was made and autoclaved. The *Manduca sexta* caterpillar cohort of 10 was moved to a different room in the lab (neonate Fall Army Worms in the insect room were getting into the *Manduca sexta* containers during the *E. coli* experiment). LB broth and normal autoclaved diet were plated and presented no bacterial colonies growing after 4 days of incubation, so the food source and suspension media were not sources of contamination in this experiment (**Figure 16**). The caterpillars were rolled over plates with selective medium to test what resistant microorganisms they were carrying on their skin.

Some of the caterpillars had *P. putida* or *B. megaterium* and one had mold from its skin. Frass was also collected and plated to determine what the caterpillars might be carrying inside their guts before the experiment started. Of the six caterpillars this was performed for, two had mostly *P. putida* with one *B. megaterium* colony, one had no colonies, one had heavy *B. megaterium* growth, one had only *P. putida*, and one had *P. putida* and mold. The blue autoclaved diet was soaked in *P. putida* overnight culture for one minute and fed the caterpillars for a day. On Day 2, the caterpillars were moved into their original containers with the original autoclaved food. On Day 3, they were moved into new containers, with new autoclaved pieces of diet in order to minimize transport of bacteria located on the bodies of the caterpillars. After being fed on the bacterial diet, all contained *P. putida* on Day 2; although five of them also had some *B. megaterium* and/or some mold (**Figure 17**). On Days 3 and 4, of the seven surviving caterpillars, all of them had *P. putida* and three of them showed contamination with one or two *B. megaterium* colonies. On Days 5 through 7, the five surviving caterpillars all showed *P. putida* with no contamination. On Day 8, the four remaining caterpillars still had *P. putida* with no other bacteria. The experiment was discontinued after Day 8. The number of frass pellets collected during this experiment was considerably lower (1-2 pellets/24 hrs) and there was no positive evidence that the caterpillars molted. From this experiment, it can be seen that the *P. putida* can persist in *Manduca sexta* for at least 7 days after exposure without being lost. On the final days of the experiment, the *P. putida* on the plates ranged from only four colonies from one caterpillar, to a completely covered section of plate, with most of the samples falling somewhere in the middle range (**Figure 18**).



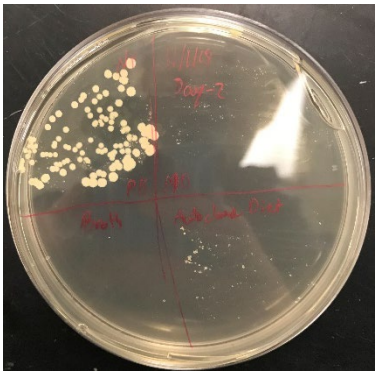


Figure 16: (Right) LB Broth (Bottom Left Quadrant) was plated and shows no bacterial growth. Autoclaved Diet (Bottom Right Quadrant) shows no bacterial growth, only frass remnants.

Figure 17 (Left): Pulse-Chase Feeding Assay with *P. putida* (measuring *P. putida*, *B. megaterium* and *E. coli*).

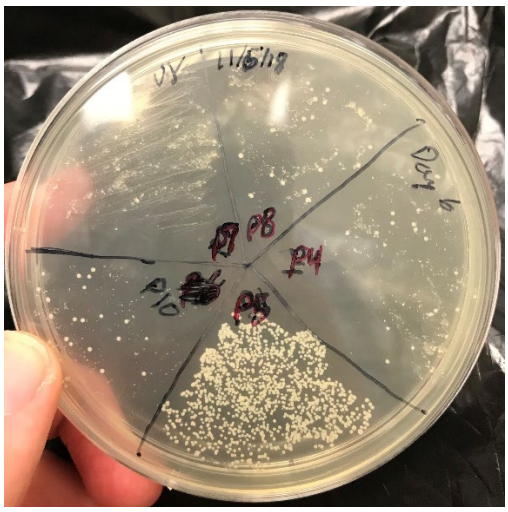


Figure 18: *P. putida* Day 6 plate containing (Clockwise from right) P4, P5, P10, P7, P8.

B. mega **and** ***P. Putida*** **individual pulse-chase assays repeated**

We repeated the individual pulse-chase experiments for *B. mega* and *P. putida* following the updated procedure to avoid contamination. Each experiment had a cohort of twelve caterpillars. We attempted the *P. putida* assay two times. The first time, nothing grew on any of the plates, including the inoculated diet pellets. The lab was closed due to a snow day on the day

after feeding which may have contributed to the lack of growth. Starting the second time, the caterpillars were grown in an incubator set to 28°C with a light source, to keep them at a constant temperature and isolate them from flies in the lab that could be a source of contamination. The caterpillars grew quickly and appeared to molt every two days. When the inoculated food was plated, *P. putida* colonies grew, but no colonies were present in the frass. We dissected two of the caterpillars a week after discontinuing the second experiment. We plated the guts, hemolymph, skin and molted skin from the first caterpillar on a chloramphenicol selective plate (**Figure 19**) and found two colonies of *P. putida* in the guts, one colony of *B. mega* from the skin, and mold growing from the hemolymph, skin and molted skin. We plated the guts, hemolymph, skin and molted skin from the second caterpillar on a nonselective plate (**Figure 20**) and found what appeared to be *P. putida* growing from the hemolymph, an unknown white bacteria growing from the guts and mold growing from the guts, skin and molted skin. Replating the guts and hemolymph from both caterpillars on selective plates four days later resulted in the growth of one colony of *P. putida* and a different mold from the guts of the second caterpillar and no growth from the guts or hemolymph of the first caterpillar or the hemolymph of the second caterpillar.

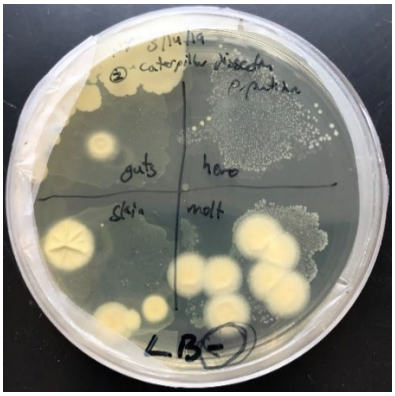


Figure 19 (Left): *P. putida* dissection 1 – chloramphenicol selective media

Figure 20 (Right): *P. putida* dissection 2 - nonselective media

During the repeat of the *B. mega* pulse-chase experiment, each of the caterpillars molted once within the first two days and again before the termination of the experiment. Eleven of the caterpillars retained *B. mega* for two days after feeding on inoculated diet and then cleared the bacteria from their frass by Day 3. *B. mega* persisted within one of the caterpillars (B3) for six days (**Figure 21**). None of the frass was contaminated with other transgenic bacteria, which can be seen in **Figure 22** and **Figure 23**. On Day 8 each of the caterpillars were dissected. The guts and hemolymph were spread onto chloramphenicol selective plates and no bacteria grew on any of the plates after four days of incubation.

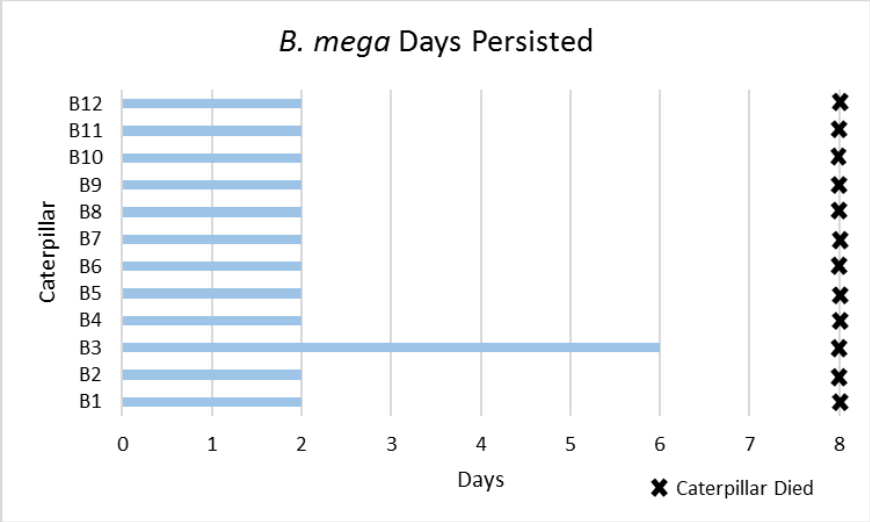


Figure 21: Second Pulse-Chase Feeding Assay with *B. megaterium*

Figure 22 (Left): *B. mega* Day 1 plate – after two days of growth

Figure 23 (Right): *B. mega* Day 2 plate – after two days of growth

Chapter 4

Discussion

Over the course of these experiments, bacteria were isolated from *Manduca sexta* and transformed with a plasmid containing genes for GFP and chloramphenicol resistance. Fluorescence microscopy and gene sequencing of the PCR product confirmed their transformation. In passaging experiments performed by Bobbi Dunton, GFP transgenic *E. coli* and *B. mega* grown in nonselective media and transferred to selective media retained the GFP plasmids over the course of the weeklong experiment, indicating that the bacteria do not lose their ability to be tracked in the timeframe of the pulse-chase experiments. In order to track the transgenic bacteria and determine whether they colonize the caterpillars gut, a pulse-chase assay was developed, tested and refined.

The proof of concept pulse-chase experiments illustrated that the pulse-chase assay was a viable method of tracking strains of transgenic bacteria. The experiments also showed that the assay did not increase the mortality rates or alter the feeding and excretion patterns of the caterpillars. The five-day pulse-chase experiment confirmed that plating suspended frass on chloramphenicol selective plates was a reproducible method to track transgenic bacteria. Using qPCR to track transgenic bacteria had the advantage of greater precision since it provided confirmation that the specific GFP plasmids were in the frass as well as the relative quantities of transgenic bacteria present. The disadvantages of qPCR are primarily the speed, cost and labor involved. Suspending and plating frass was a faster, less labor intensive and cheaper method of tracking transgenic bacteria. In subsequent pulse-chase assays, we plated resuspended frass pellets on chloramphenicol selective plates to track the presence and amount of viable transgenic bacteria.

The five-day pulse-chase assay provided initial evidence that it was possible for the transgenic bacteria to persist in the caterpillars beyond the duration of the inoculated food bolus passing through their digestive tracts. No cross contamination was seen on the plates at this stage of the experiment.

The first round of individual pulse-chase experiments served as further evidence that transgenic bacteria have the ability to persist within the caterpillars for at least a week and over the course of multiple molting events. In the individual pulse-chase experiments, *B. megaterium* persisted for the full ten days of the experiment, nine of which took place after switching to the normal diet. *P. putida* persisted for the full eight days of the experiment. *E. coli* persisted for three days in one of the ten caterpillars but did not appear in any of the other caterpillars' frass.

The diet inoculated with *E. coli* was suspended and plated on a selective plate and showed growth of *E. coli.* Therefore, the lack of *E. coli* persistence was not due to a problem with the inoculated food source. The lack of persistence was consistent with the proof of concept experiments where the *E. coli* disappeared quickly from the frass. It is possible that *E. coli* was unable to flourish in the *Manduca sexta* or was outcompeted by the *P. putida* and *B. megaterium* that appeared during the *E. coli* experiment. *E. coli* was not originally isolated from *Manduca sexta* while the *B. megaterium* and *P. putida* strains were isolated from *Manduca sexta* frass and regurgitant and it is possible that they are better equipped to persist within the caterpillars than *E. coli*.

During the first *B. megaterium* individual pulse-chase experiment, *P. putida* also appeared on the selective plates. During the *E. coli* pulse-chase experiment, both B. *megaterium* and *P. putida* appeared on the plates. Scattered colonies of both *B. megaterium* and *P. putida* grew after plating suspended normal diet, but not after plating the LB broth used as suspension

media, indicating that the normal diet was contaminated and acted as a continuous pulse. Plating suspended frass resulted in growth of *B. megaterium* and *P. putida* that covered the selective plates and was significantly greater than the amount of growth on the plate with normal diet. This suggests that the bacteria flourish and multiply within the digestive tract of *Manduca sexta* **Figures 12 & 13**.

The first *P. putida* experiment took place in a separate room away from the *Manduca sexta* and Fall Army Worm colonies using new diet sources that had been autoclaved to limit contamination. The caterpillars' were switched to new containers on two occasions to minimize the spread of bacteria hitchhiking on the outside of the caterpillars' bodies. The caterpillars' bodies were rolled onto selective plates and the frass was plated to determine if they carried any bacteria. Some of the plates showed growth of *P. putida* or *B. megaterium* before the start of the experiment. It is likely that the caterpillars from the five-day pulse-chase experiment retained the transgenic *B. megaterium* and *P. putida* and infected the other caterpillars when they were returned to the *Manduca sexta* colony. These caterpillars would have been infected before the start of the individual experiments, providing strong evidence for the persistence and colonization of the bacteria.

Repeating the individual pulse-chase assay with *P. putida* in uncontaminated caterpillars resulted in no persistence of *P. putida*. It did not appear on selective plates from the first day after feeding the caterpillars inoculated diet. The caterpillars used for this experiment appeared much healthier than the one used in the first *P. putida* individual experiment and they molted at a faster rate. This suggests that *P. putida* did not have had a chance to take hold and persist within the gut before cleared from the system during the course of a molting event. Alternatively, the caterpillars' immune system may have cleared the bacteria from the gut. The one colony of *B.*

mega that grew from the skin of the second dissected caterpillar was likely from the outside of skin and a carryover from the *B. mega* individual pulse-chase experiment occurring at the same time.

The repeated *B. mega* individual pulse-chase experiment showed that it is possible for *B. mega* to persist in the caterpillar for close to a week, but it also showed that the majority (eleven out twelve) of the caterpillars did not retain the bacteria for more than two days after inoculation. All of the caterpillars in this experiment molted within a day of feeding on the bacterial diet. This further supports the idea that molting wipes out a portion of the gut microbiome. If bacteria have not had a chance to establish a hold in the caterpillar, they may be extinguished from the gut microbiome. The dissections of the twelve caterpillars inoculated with *B. mega* showed that *B. mega* did not reside within the hemolymph or gut and was completely cleared from the caterpillars' system.

The two bacteria native to *Manduca sexta – B. megaterium* and *P. putida* – persisted within *Manduca sexta* for the duration of the first individual pulse-chase assays, through multiple molting events and even persisted in the colony across multiple experiments. They appeared to flourish within the caterpillar during the first round of experiments, as the number of colonies seen on the plates was significantly greater compared to similar amounts of plated bacterial diet. All of which provides evidence that there is a resident microbiome in the caterpillar.

In the repeated *P. putida* and *B. mega* experiments, the results were not as clear. The *P. putida* experiment indicates that the bacteria did not persist at all and may have been killed by the caterpillars' immune systems. The *B. mega* experiment indicated that the bacteria could persist for a short time before being cleared by a molting event unless the bacteria had taken a large enough hold and could persist through a molting event.

It appears that molting events act in a similar manner to evolutionary bottlenecks, in that they clear much of the microbiome away, leaving only a portion of the microbiome intact. Bacteria that have not managed to take hold have less chance of persisting than the bacteria that colonized in greater numbers. For this reason, caterpillars that molt more frequently would have fewer cases of persistent bacteria and would appear to have a transient microbiome. Bacteria would colonize caterpillars that grow at a slower rate more significantly and be more likely to persist through molting events. These bacteria would be more likely to be present in caterpillar regurgitant and would have a greater chance of reinoculating the caterpillars each time they feed.

These findings indicate that *Manduca sexta* have the potential for a resident gut microbiome, but that there is a large turnover rate, especially during periods of molting. When caterpillars grow quickly, they appear to have a transient microbiome that relies on ingesting new bacteria to maintain colonies. When caterpillars grow more slowly and have longer periods between molts, bacteria have the potential to establish a greater hold and persist for greater lengths of time. When bacteria persist across multiple molts, it seems likely that the caterpillars supplement the low remaining levels of bacteria by consuming their shed skin and reinoculating themselves with some of the bacteria that they lost.

In the future, results from experiments that varied the growth rates of the caterpillars with different feeding, temperature and light conditions would help to determine how easy it is for bacteria to persist within caterpillars under different conditions. Comparing these experiments to the growth rates of wild caterpillars would help to determine whether caterpillars primarily have a transient or resident microbiome. Experiments looking at metabolomics and operational taxonomic units within the guts of wild caterpillars would prove incredibly useful in determining the flux and flow of bacteria within the gut. These experiments would help to evaluate the

stability of the enzymatic composition of the gut microbiome. Results from these types of investigations would also be important biologically, helping to determine how much the bacteria within the gut microbiome actually aid caterpillars with digestion, supplementing missing nutrients and neutralizing plant defenses.

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efficacy, and toxicity. *Translational Research*. https://doi.org/10.1016/j.trsl.2016.08.002

ACADEMIC VITA

Noah Yoskowitz
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Education:

The Pennsylvania State University, University Park, Pennsylvania
Schreyer Honors College — Class of May 2019
B. S. in Toxicology, Minors in Biology and Psychology
Honors in Toxicology

Thesis Title: Do Manduca Sexta Have a Resident Gut Microbiome?
Thesis Supervisor: Dr. James H. Tumlinson

Work Experience:

Chemical Ecology Lab – The Pennsylvania State University, University Park, PA — Spring 2017-Present
Undergraduate Researcher, Chemical Ecology Lab, Dr. James H. Tumlinson
Examined green leaf volatiles and bacterial volatiles to understand the interactions between plants, insects and bacteria. Designed and conducted bacterial persistence experiments using caterpillars. Cared for insects and plants including caterpillars, coffin flies, corn and tobacco. Transformed bacteria using GFP plasmids.

FDA – National Center for Toxicological Research, Jefferson, AR — Summer 2018
Summer Student Researcher; Dr. Steven Foley & Dr. Bijay Khajanchi
Project Title: "Evaluation of Plasmid Encoded Factors in Virulence Potential of *Salmonella* Schwarzengrund Isolates from Different Food Sources"
Biosafety Level-2 Training; worked with virulent *Salmonella enterica* and human epithelial cells (Caco-2)
Presented findings at an internal conference capping off the program
Presented a poster at the Seventh Annual Central Arkansas Undergraduate Summer Research Symposium at UAMS

Sailing Coach — Summer 2013-2017, 2019
Seawanhaka Corinthian Yacht Club / SUNY Maritime / Centerport Yacht Club
Taught kids in groups of 5-20 (aged 6 to 16) to sail optimists, keelboats and 420s. My duties included keeping them safe, making sure they were having fun, getting them comfortable with being on the water, fostering the independence, skills and knowledge necessary to sail alone. Other duties included applying first aid or CPR if necessary, repairing boats and equipment, preparing lesson plans, and coaching from a powerboat. Learned how to stay calm and in control while defusing high tension or dangerous situations and emphasize safety at all times.

Publications:

Khajanchi, B. K., **Yoskowitz, N. C.,** Han, J., Wang, X., & Foley, S. L. (2019). Draft Genome Sequences of 27 *Salmonella enterica* Serovar Schwarzengrund Isolates from Clinical Sources. *Microbiology Resource Announcements*, *8*(12), e01687-18.

Abstracts Presented:

Bijay Khajanchi, **Noah Yoskowitz**, Jing Han, Christopher Grim, Shaohua Zhao and Steven Foley, 2019. Evaluation of Genetic Relatedness and Plasmid Mediated Virulence of *Salmonella* schwarzengrund Strains Isolated from Food and Clinical Sources. International Association for Food Protection, KICC Louisville, Kentucky July 22.

Yokowitz N., S. L. Foley and B. K. Khajanchi. 2018. Evaluation of plasmid encoded factors in virulence potential of *Salmonella* Schwarzengrund isolates from different food sources. 7th Annual Central Arkansas Undergraduate Summer Research Symposium, UAMS July 25th.

Grants, Awards and Professional Memberships:

Academic Excellence Scholarship 2015-2019
Schreyer Ambassador Travel Grant – Brazil and Colombia 2016
Dean's List All semesters, 2015-2019
Phi Kappa Phi Honor Society Membership 2018 – Present

Service Involvement:

- Penn State IFC/Panhellenic Dance Marathon (THON) through Club Sailing and Club Gymnastics – fundraising year round and coming together with over 15,000 Penn State students to provide emotional and financial support for children and families of Four Diamonds, collectively raising over $10 million each year (150+ hours)
- Various community service activities through Club Sailing
- RAM Medical Clinic in Ashtabula, Ohio (3 days long)

International Education:

Brazil and Colombia - Education and Culture Schreyer Signature Travel Program Spring 2016
Limited proficiency in French and Spanish

Skills and Activities:

Penn State Club Sailing Team 2015-2019

- Practiced 6-12 hours a week during season
- Taught new sailors how to sail during practice
- Competed in regattas every semester in MD, NY, NJ, PA, DE, and Ontario
- Safety Officer 2015-2019
- Thon Fundraising Chair 2016-2017
- Created designs for home regatta shirts 2015-2019

Penn State Club Gymnastics Team 2017-2019
Penn State Entrepreneurship Club – Innoblue 2015-2017
Penn State Remote Area Medical 2019

- Served at a free, three day long medical clinic in Ashtabula, Ohio

Adobe Photoshop and Illustrator, Advanced in Microsoft Excel, Word and PowerPoint; Google equivalents
Interests: Ceramics studio membership, painting, drawing, cooking, outdoor activities including hiking and stargazing