Evaluating the Core Microbiome of *Manduca sexta*

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Abstract: Microbiomes are complex communities of microorganisms that colonize many surfaces of an animal's body, especially those niches lined with carbohydrate-rich mucosal layers such as the eyes, male and female reproductive tracts, and the gastrointestinal tract. While a vast majority of data from microbiome studies has relied almost extensively on metagenomics-based approaches to identify individual species within these small complex communities, the contributions of these communities to host physiology remain poorly understood. We used a combination of culture- and non culture-based approaches to identify and begin functionally characterizing microbial inhabitants stably colonized in the midgut epithelium of the invertebrate model *Manduca sexta* (tobacco hornworm), an agriculture pest of *Nicotiana attenuata* (wild-tobacco) and many additional solanaceous plants.

Keywords: Microbiome, *Manduca sexta*, Intestine, *Nicotiana attenuata*, Metagenomics

Introduction

The animal intestinal microbiome comprises a diverse community of microorganisms, which influence host development, physiology, and response to pathogens. However, the mechanism underlying these complex interactions remain poorly understood. While we reference the microbiome as a single entity, disruptions of homeostatic conditions in the intestinal environment (i.e. temperature, diet, or antibiotics) can lead to dramatic shifts in the relative abundance of each species, affecting the overall community composition and perturbing the equilibrium amongst residents. The intestinal microbiota of holometabolous insects is unique as they mature through different stages of development (egg → larvae → pupae → moth) and experience changes in intestinal physiology at each stage (Casanova and Gaudº 1934). Increasing the current understanding of how the intestinal microbiota of lepidopteran species (caterpillars) contributes to host physiology may offer possibilities to improve pest management methods targeting insects that affect the agricultural economy (Mereghetti et al. 2017).

The reoccurrence of some bacterial species from one generation to the next and regardless of diet supports the hypothesis that a core microbiome persists in the intestinal tract of some Lepidopteran species. When the bacteria are transferred vertically, they are passed on generationally. When the bacteria are transferred horizontally, they are passed directly via contact with other individuals (Voirol et al. 2018). However, recent reports suggest that a combination of the extremely alkaline conditions of the midgut epithelium and the physiological changes associated with holometabolous development have resulted in the evolution of caterpillars independent from microbial symbionts (Chapman 2012, Hammer et al. 2017).

Using the agricultural pest and experimentally tractable model, *Manduca sexta* (tobacco hornworm), our aim is to develop effective strategies and experimental approaches that will advance our understanding of how these complex microbial ecosystems shape animal health and behavior. Relative to mammalian models, the *M. sexta* gastrointestinal tract is morphologically simple, comprising of a foregut (FG), midgut (MG), and hindgut (HG) regions (Figure 1). The MG epithelium is the primary site of absorption, secretion, and colonization. Because it is exposed to xenobiotics and entomopathogens that cause damage to apically

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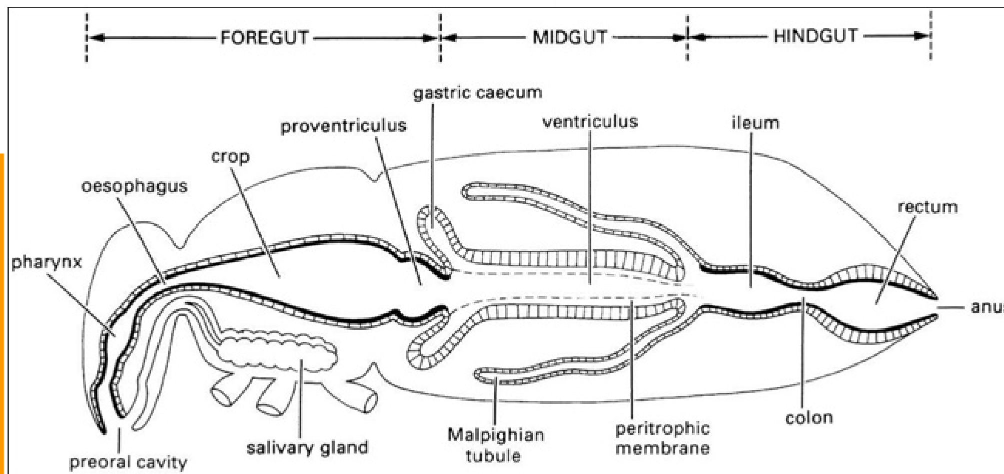

Figure 1: Generalized physiology of the insect gastrointestinal tract (taken from Chapman 2012)

exposed enterocytes, the MG has the highest turnover rate of any fixed-cell population in the body. Consequently, the growth rate of microorganisms stably colonized in the mucosal layer of the MG must exceed the rate of developmental and damage-mediated tissue turnover. Our objective was to use a combination of metagenomics approaches to identify a core group of microorganisms colonized in the *M. sexta* MG mucosal layer of larvae reared on either artificial diet or its preferred host, *Nicotiana attenuata* (wild tobacco).

Methods

Insects:

M. sexta eggs were purchased from Carolina Biological Supply Company (Burlington, NC). After hatching, larvae were maintained on wheat germ-based artificial diet under natural light at 24°C. Larvae intended to be observed on the preferred host *N. attenuata* were placed on the base leaves of *N. attenuata* as eggs and kept under the same environmental conditions (lighting and temperature).

Plants:

N. attenuata sees were planted and grown in Miracle-Grow® potting mix. Plants were initially grown under 200W LED lights and watered as needed for roughly 4 to 6 weeks. They were then moved to 400W LED lights and fertilized weekly with a completely liquid fertilizer (Miracle-Grow®). Plants were transferred from the plant room to the lab in groups of 5 to 8 plants as needed.

Dissections:

Larvae from each instar were dissected by first anesthetizing on ice for 5 minutes. Midguts were removed by cutting behind the second set of true legs and prolegs, immersed in PBS in a micro-centrifuge tube, and gently vortexed for minutes. Midgut tissues were removed, and dislodged mucus and bacteria were pelleted by centrifugation followed by aspiration of the supernatant. All pellets were kept at 70°C.

DNA Extraction and Quantification:

Genomic DNA (gDNA) was extracted using the QIAamp PowerFecal® DNA Kit (Qiagen, Carlsbad, CA, USA) to obtain high-purity DNA template for more successful polymerase chain reaction (PCR) amplification. DNA obtained was quantified using a Biophotometer (Eppendorf, Hauppauge, NY, USA).

16s rRNA Gene Amplification:

The 16s rRNA gene was amplified by PCR using the following primers: 16S_rRNA_8Fwd (GAGTTTGATCCTGGCTCAG) and 16S_rRNA_1492R (GGTTACCTTGTTACGACTT). The 25-µl PCR reaction mixtures contained 0.4 µM of each forward and reverse primers (final concentration), 1 µl extracted template DNA, and 12.5 µl 2X GoTaq® Hot Start Green Master Mix (Promega). PCR reactions included an initial denaturation for 5 minutes at 95°C, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 45 seconds, and elongation at

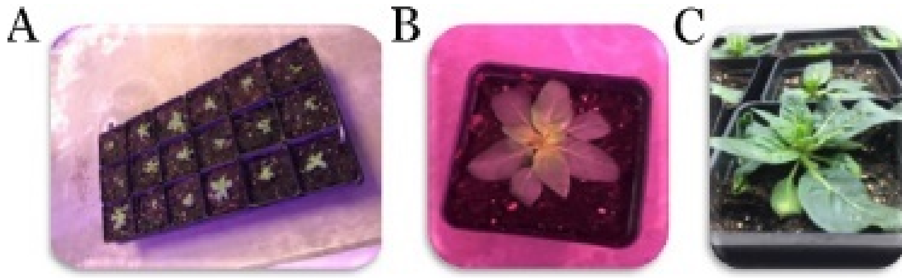

Figure 4: Overview of plant growth and maturation. N. attenuata *after (A) 3 weeks (B) 5 weeks (C) 8 weeks of growth*

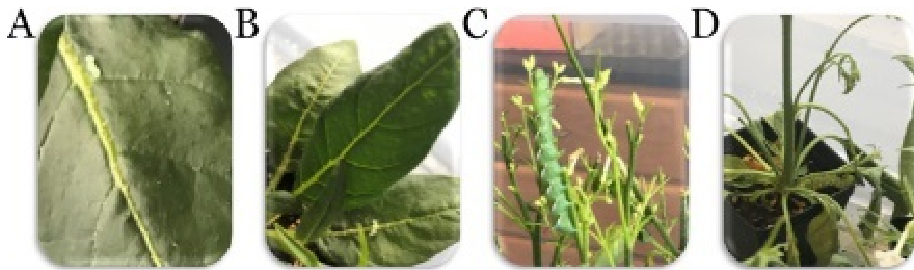

Figure 3: Stages of growth of M. sexta on N. attenuata. *(A)* M. sexta *eggs on base leaves of* N. attenuata *(B) 1st instar* M. sexta *larvae (C) 5th instar* M. sexta *larvae (D) Destroy plant as result of* M. sexta *feeding*

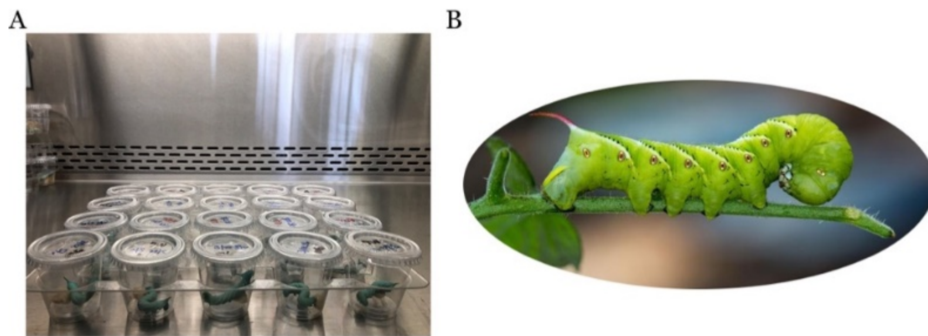

Figure 2: Hornworms. (A) maintained on artificial diet or (B) on N. attenuata

72°C for 1.5 minutes, with a final elongation step at 72°C for 5 minutes.

Electrophoresis:

Electrophoresis of PCR products was performed on 1% agarose (Fisher) using Tris-Acetate-EDTA (TAE) buffer at 100V.

Progress to Date

We have successfully grown *N. attenuata* and used plants to rear *M. sexta* larvae (Figure 2) Using *M. sexta* intestinal mucus to inoculate Brain Heart Infusion or Tryptic Soy Broth media, we obtained five Gram-positive isolates after incubation overnight at 30°C under aerobic conditions. Following growth *in vitro*, we were able to extract only small quantities of low-quality gDNA (A260:280 < 1.5) from these isolates that is insufficient to use PCR amplification and 16s rRNA sequencing. Similarly, we have obtained low-quality gDNA from mucus obtained from the MG epithelium of *M. sexta* larvae primarily during the third through fifth instars of development. First and second instar larvae were too small to consistently and reliably dissect the midgut and obtain mucus (Figure 3). Currently, we are optimizing an extraction protocol to efficiently lyse and isolate high-quality gDNA from these Gram-positive microorganisms in order to amplify the 16s rRNA gene and perform metagenomics analyses. No significant differences in growth rates were observed in *M. sexta* larvae maintained on artificial diet or *N. attenuata*. Relative to larvae fed artificial diet, larvae maintained on *N. attenuata* appeared bright green (Figure 4).

Discussion

If accurate descriptions of bacterial populations colonizing the gastrointestinal tracts of insect models are to be made, it will be necessary for scientists, regardless of primary discipline, to understand and apply the basic ecological principles of colonization as described by Rolf Freter. Using continuous flow culture models, Freter explained how multispecies communities are formed as a result of the

competition for nutrients (mucosal polysaccharides) in the gastrointestinal tract (Freter et al. 1983). Therefore, we have specifically targeted the mucosal layer in the *M. sexta* midgut as the primary site of colonization by bacterial residents. While the existence of a mucosal layer does not attenuate the extreme conditions of midgut, bacteria are routinely detected in the gastrointestinal tract of *M. sexta* and other species of Lepidoptera (Broderick et al. 2004, Brinkmann et al. 2008). In order to obtain a sufficient amount of gDNA for 16s sequencing in subsequent experiments, it may be necessary to pool mucus from technical replicates. With the proper controls in place (i.e. gDNA extracted from *N. attenuata* plant material and artificial diet), we expect the 16s sequencing results to be more reflective of the stably colonized bacterial population in the midgut epithelium, unlike many studies that have relied almost solely on sequencing 16s obtained from feces (Hammer et al. 2017).

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