Papers & Publications: Interdisciplinary Journal of Undergraduate Research

Volume 8 Article 4

2021

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Acknowledgments

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Abstract: The gut microbiome plays an essential role in the health of many organisms, including insects. Here we report initial findings of bacteria present in the caterpillar midgut of the cabbage looper, *Trichoplusia ni*, grown in lab conditions on artificial diet and identified using biochemical tests. We identified one species previously reported as part of the gut microbiota of *Trichoplusia ni* and three species not previously reported. Our results support the need for multiple types of bacterial identification when looking at gut microbiomes, with the most confidence in identification being when multiple tests are in agreement.

Organisms are colonized by a large community of microbes early in life, and microbes that colonize the digestive tract are collectively called the gut microbiota (Cenit et al. 2014). The relationship between the gut microbiota and their host has been shaped by millions of years of coevolution, but the complexity and function of this relationship is still being determined (Engel and Moran 2013, Cenit et al. 2014). The gut microbiome plays an essential role in the health of many organisms, including insects (Tang et al. 2012, Engel and Moran 2013). Studies have shown that insect gut microbiota provide nutrients, detoxify harmful secondary metabolites, and contribute to the development and functioning of the immune system (Tang et al. 2012, Pennington et al. 2017). Studies have also shown, especially in lepidopterans, that large components of the gut microbiome are colonized from food (Hammer et al. 2017). So if we can understand the species in the gut and what function they play, we may be able to manipulate them to encourage population growth of beneficial insects and discourage pests. Therefore knowledge of the role of gut microbiota in insects has many potential future applications in fields such as medicine, agriculture, and ecology (Engel and Moran 2013). For example, secondary plant compounds and environmental contaminants have been shown to change the gut microbiome of insect larvae as well as increase larval mortality (Nunez-Mejia et al. 2016, Pennington et al. 2017). And while we are far from application of this knowledge, identifying the taxonomic composition of bacteria in the gut of lepidopteran larvae is a relevant first step in understanding the reciprocal relationship between the environment and life histories of both microbes and host.

There has been much debate about how gut microbes should be identified. Traditional culturing techniques are biased because many microbes cannot grow in standard lab conditions (Broderick et al. 2004). In response to this limitation, direct sequencing of genes from microbes has been employed. However, given the level of horizontal gene transfer

among bacterial species, this type of method may inaccurately identify species present in the gut (Riley and Lizotte-Waniewski 2009). Instead, some have proposed the use of the Core Genome Hypothesis (CGH) to identify bacterial species (Riley and Lizotte-Waniewski 2009). This hypothesis suggests that species should be identified based on a core set of metabolic housekeeping and informational processing genes which are shared by all members of the same species. Based on this species concept, it might be more accurate to identify species based on biochemical tests of their essential metabolic functions such as carbon utilization, amino acid synthesis, and salt tolerance (Biolog 2016).

Here we report initial findings of the bacteria identified in the caterpillar midgut of *Trichoplusia ni*, a common lepidopteran also known as the cabbage looper, grown in lab conditions on artificial diet and identified using biochemical tests that assess metabolic functions.

Materials and Methods

Insect culture

Trichoplusia ni was reared in the lab following standard practices (Strand 1989). Larval stages were fed a cornmeal based artificial diet (Southland Products Cabbage Looper Diet) ad lib and maintained in a Percival environmental chamber under constant conditions (27 °C, 16L:8D, 20% relative humidity).

Bacterial Isolation

Five caterpillars in the 5th instar, as determined by head capsule width (McEwen and Hervey 1962), were haphazardly selected for midgut dissection. All caterpillars were starved for 4 hours prior to dissection. After separation from both the foregut and hindgut, the midgut was surface sterilized with a cotton swab soaked in 70% ethanol. Then the mid-gut was smeared on a tryptic soy agar (TSA) plate. The plates were incubated for 48 hours at 25 degrees Celsius under aerobic conditions. After the 48 hour incubation period, the developing colonies were visually identified by general colony morphology and were isolated onto separate TSA plates. After two days, the TSA plates were visually inspected to verify that the colonies were correctly isolated and appeared to be pure cultures. If the colonies were not pure as evidenced by colonies of varying morphologies on the same plate, they were further isolated. To confirm the visual inspection of colony purity, all bacterial colonies were also Gram stained using standard techniques and visually assessed for shape. Purity was indicated as all bacteria having the same Gram staining results and bacterium shape.

Bacterial Identification

Bacteria were identified using GENIII MicroPlates (Biolog, Inc.) read automatically by a plate reader and compared to bacterial species

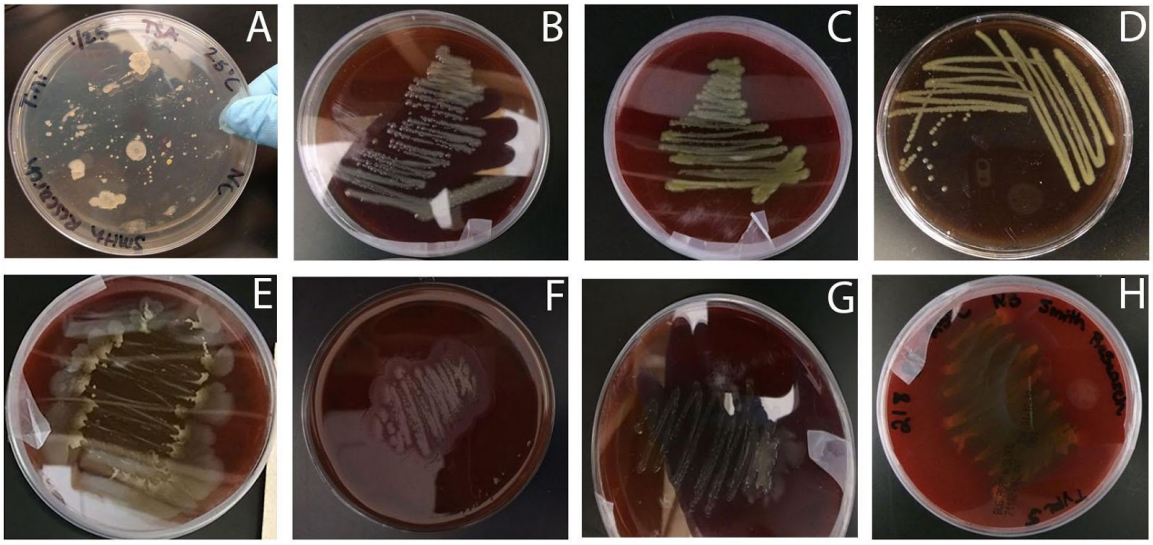

Figure 1. Representative images of bacterial colonies. (A) TSA plate with multiple colonies growing 48 hours after smearing of a single caterpillar cut. (B-H) Pure isolates on blood agar plates of each of the 7 different bacterial types initially isolated from *T. ni* midguts.

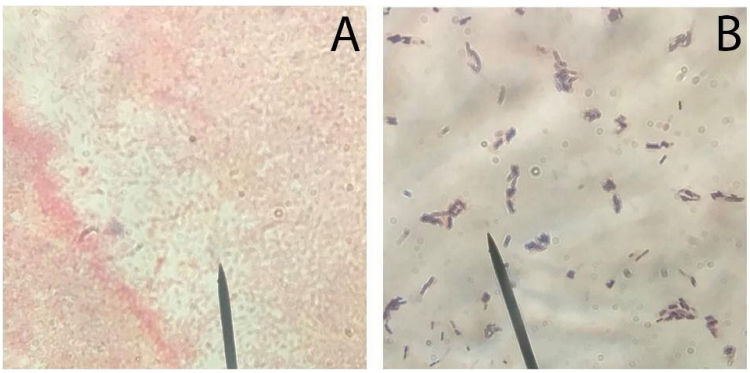

Figure 2. Representative images of Gram staining. (A) Gram negative (B) Gram positive

data in the Biolog database. Bacterial identification by this method requires that colonies grow in the presence of iron, so pure colonies were taken from TSA plates and grown on blood agar plates for 24 hours at 25 degrees Celsius prior to analysis. These colonies were then identified with the GENIII MicroPlates following manufacturer instructions (Biolog 2016). Biolog results were compared to the Gram-staining results to verify that they were consistent.

Results

Seven visually distinct colonies were isolated from the midgut of *T. ni* (Figure 1). Of those seven distinct colonies (Figure 1B-F), four were identified using the Biolog system: *Achromobacter spanius, Cellulomonas fimi, Paenibacillus pasadenensis*, and *Bacillus subtilis* (Table 1). All species identifications via Biolog had a probability of > 50% and were also confirmed with Gram stains (Figure 2). The four identified genera were a mixture of aerobes and facultative anaerobes (Table 1).

Discussion

Four genera of bacteria in the gut of Trichoplusia ni were identified, including *Achromobacter, Paenibacillus, Cellulomonas,* and *Bacillus* (Table 1), and 3 isolates could not be identified by these methods. This small number of major bacterial genera is consistent within findings from bacterial communities in the midgut of other leaf-feeding lepidopteran caterpillars (Engel and Moran 2014, Xia et al. 2017, van der Hoeven et al. 2008).

We recovered a genus, Achromobacter that belongs to a family Alcaligenaceae, which was previously reported as part of the gut microbial community in organisms reared on artificial diet with bacteria identified through sequencing (Pennington et al. 2017). In contrast, the genera of *Bacillus, Paenibacillus* and *Cellulomonas* species have not previously been reported to be present in the gut of *T. ni* as far as the authors know. However, all three of these species have been recovered from other lepidopterans, such as *Helicoverpa amiga* (Priya et al. 2012) and *Hofmannophila pseudospretella* (Shannon et al. 2001).

We failed to recover two genera that are common residents of insect midguts, *Clostridium* and *Enterococcus* (Tang et al. 2012, Pennington et al. 2017). However, we believe this may be, in part, due to the limitations of

Table 1: Identification and descriptive features of the four microbial isolates from *T. ni* midguts

	Probability	Colony Description	Picture	Gram Staining	Shape
Achromobacter spanius	75.7%	Grey gel	Fig. 1B	Negative	Rod
Cellulomonas fimi	50.9%	Green-yellow gel	Fig. 1C	Positive	Rod
Paenibacillus pasadenensis	53.5%	Yellow gel	Fig. 1C	Positive	Cocci
Bacillus subtillus	54.2%	Brown-beige border growth	Fig. 1E	Positive	Rod

the Gen III microplates. These plates can only recover aerobic or facultative anaerobic species, those bacteria that can grow in the presence of oxygen. *Clostridium* sp. are obligate anaerobes and would not have grown in our lab conditions if present. Enterococcus species tend to be facultative anaerobes that potentially we could have cultured. We may not have recovered Enterococcus at all, or a species among the three bacteria that we were unsuccessful in identifying.

It is not completely unexpected that our results do not perfectly match previous studies, even with the limitations of which species we can culture. *T. ni* gut microbiome composition likely varies among populations given the evidence that insect gut microbiomes are generally thought to be colonized from the environment (Hammer et al. 2017, Engel and Moran 2013, Priya et al. 2012). Populations feeding on different food may have different bacterial gut residents, a pattern seen in other species (Priya et al. 2012). Collectively, these results highlight the dynamic reciprocal relationship between the host gut and the microbiome dwelling therein. The host food sources and microenvironment of the gut affects which bacteria can live there, and likely the bacteria affect the host life history in return. Understanding how the specific bacterial species we identified affect the host life history in *T. ni* is a future direction for research in this system.

Additionally, results may vary between studies because the methods used for bacterial identification, sequencing or biochemical, vary among studies. Our results support the need for multiple types of bacterial identification when looking at gut microbiomes, with the most confidence in identification being when multiple tests are in agreement. With confidence in bacterial species identification, future research could then include functional studies to understand how members of the gut microbiome impact the life history of the host.

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Contributor Bios

Naomi Childs received her undergraduate degree in Biology from the University of North Georgia in 2018. With a primary interest in animal physiology, she hopes to further her education at the University of Georgia to eventually practice as a Doctor of Veterinary Medicine. Bethany Gray graduated in Spring 2019 with a BS in Secondary Education and a focus in Biology. She is eager to teach young students about the joys of science. Robbie Scoggins is also a recent graduate of the Biology program at the University of North Georgia. He is currently a chemistry teacher at Pepperell High School in Lindale Georgia and applying to masters programs in the southeast. Drs. Barding and Smith are both Associate Professors of Biology at the University of North Georgia–Dahlonega campus.

Acknowledgements

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