Correlation of Biochemical and Biophysical Data from Microbes Grown from Honeybee (Apis mellifera) Gut Contents

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Abstract

The number of honeybee colonies has consistently been declining since the winter of 2006. The reason for this loss, which has been coined Colony Collapse Disorder (CCD), is not known. In this experiment, the culturable bacterial flora of the honeybee gut was examined to provide more information on the natural microbial populations of the honeybee. Since little data exists on the normal flora of the honeybee gut, being able to identify the normal flora could aid in identifying microbes associated with CCD. Honeybee gut content was extracted and cultured on standard microbiological media. Once bacteria were isolated, the microbe types were isolated for further analysis and characterization by biochemical and biophysical tests. Biochemical reactions were done on MacConkey and Triple Sugar Iron (TSI) agar and while spectral profiles were collected using Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF). All bacteria were found to have a rod morphology, with five being Gram-positive and two Gram-negative. One organism was found to be a yeast. MALDI-TOF displayed that four colony types had similar but unique mass spectrometry profiles. These data represent an effort to categorize microbe types found in honeybee gut by using a combination of biochemical techniques so that characterization of the

microbes can eventually be realted to the health of honeybee colonies.
Keywords: Apis mellifera, colony collapse disorder, bacteria, MALDI-TOF MS, biochemical reactions

Recent studies have identified a set of organisms typically found in the gut of healthy *Apis mellifera* (honeybees)[1] . Bacteria characterized in this way included *Lactobacillus* sp., uncultured *Firmicutes, Bifidobacterium* sp., *Bartonella* sp., *Gluconacetobacter* sp., *Simonsiella* sp., and two uncultured *Gammaproteobacteria*. There was an abundance of *Gammaproteobacteria* present, whereas organisms from the *Betaproteobacteria*, *Alphaproteobacteria*, *Firmicutes,* and *Actinobacteria* groups showed up less frequent. Since the bacterial community reported was similar to honeybees worldwide, it suggests these bacteria are part of the normal flora of the honeybee under normal conditions. A better understanding of the normal microflora of a healthy honeybee can aid in discovering microbes associated with the decline in honeybees, which has been reported in all regions of the world where apiculture contributes to agriculture.

In the United States, this problem in honeybees became evident as a significant decrease in the managed *Apis mellifera* (honeybee) populations in the winter of 2006/2007, and this situation is currently unresolved[2] . Exact reasons for these declines are not known, but factors including pathogens, parasites, and environmental toxins have been investigated. Northern California scientists have recently found a parasitic fly that hijacks the honeybees' body, causing them to abandon their hives. These flies could be a possible explanation for the honeybee die-off that has affected hives around the world .[3] This syndrome in honeybees is known as Colony Collapse Disorder (CCD) and threatens dire consequences for many essential food crops .[4]

Using mass spectrometry-based proteomics, Bromenshenk and co-workers compared thousands of proteins from healthy and collapsing bee colonies which led to discoveries of two new RNA viruses in the honeybees: Varroa destructor-1 virus and Kakugo virus[5] . They also identified an invertebrate iridescent virus (IIV) which is correlated with CCD colonies. Bees in failing colonies not only had IIV but also a microsporidia *Nosema*. Their findings

associate co-infection by IIV and *Nosema* with honeybee decline, giving reason to believe IIV, *Nosema*, and mites when found together are associated with losses of honeybees in the United States. While those studies link the presence of some potential honeybee pathogens to CCD, it has not been conclusively shown that these microbes are sufficient to cause the condition; and therefore, an additional microbe or other factor may be found in diseased bees.

At this time, there are few studies on the normal microflora of the honeybee intestine. Establishing a baseline is the first step to determining whether microbes are involved in CCD, and if so, which ones are involved. The goal of this study was to characterize the bacteria from the bee gut of honeybees from a healthy hive in Dunn County, Wisconsin. To do this, we correlated the biochemical reactions of the bacteria isolated from honeybees using different medium types to gather information obtained by Matrix-Assisted Laser Desorption/ Ionization Time of Flight mass spectrometry (MALDI-TOF MS).

For the biochemical reactions, MacConkey and Triple Sugar Iron (TSI) agars were used for testing. MacConkey agar distinguishes Gram-Positive from Gram-Negative bacteria and determines whether the bacteria can ferment lactose[6]. The TSI agar is able to test for a variety of biochemical characteristics. TSI agar tests bacteria to see whether the bacteria can ferment glucose, lactose, or sucrose. It also tests to see if the bacteria can produce hydrogen sulfide (H_2S)[7]. Information obtained from staining results and biochemical profiles can assist in organism characterization.

MALDI-TOF MS was used to provide further characterization of the microbes isolated from the healthy honeybees. MALDI-TOF MS is an analytical method used to characterize biological molecules according to their masses[8]. Using this technique, the mass of the compounds can be collected for a given microbe, which in turn creates a distinctive profile for that specific microbe.

Use of MALDI-TOF MS has proven invaluable to the profiling and identification of bacteria because it can generate fingerprints unique to a microbial species based on the presence of metabolites and peptides with high sensitivity, flexibility, and speed. For example, in the clinical lab, MALDI-TOF MS

can be used to generate profiles for microbes infecting human patients and enable a more rapid diagnosis and treatment[9].

By combining specific microbiological data with biophysical markers produced by microbes grown from the gut of the honeybees, we were able to create specific microbial profiles representative of the normal flora from honeybees in a thriving colony. This information could enable future studies that provide the information needed to correlate other specific microbes with diseased honeybees and the likelihood of colony failure due to CCD.

Methods and Materials

Cultivation and Preparation of Honeybee Intestinal Content

Bee guts were harvested in the Bio 370 Biotechnology course by undergraduate students at the University of Wisconsin Stout. The honeybees came from Gardner Apiaries (Spell Bee Company) of Baxley, Georgia. The hive was set up in May 2011 in Menomonie, Wisconsin. Honeybees were euthanized in September 2011 by freezing at -20° C. The bee mid-guts were extracted and placed in tubes and rinsed three times with 750 μl of 1X PBS buffer Solution. The PBS and mid-guts were transferred to the Dounce tissue homogenizer and macerated. The homogenized honeybee mid-guts were stored briefly at -20° C prior to culture analysis.

Figure 1

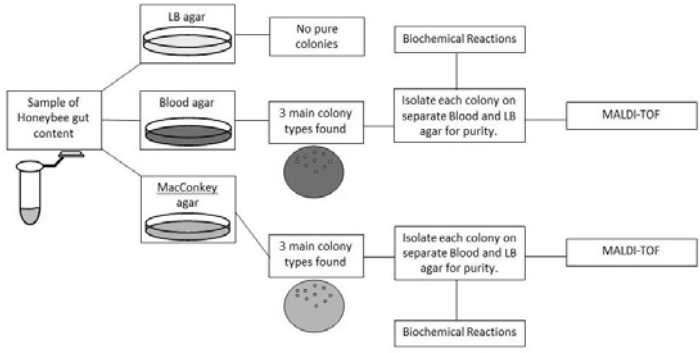

Figure 1 Honey bee gut content was characterized by culturing and isolating the bacteria on three different agars. Isolated colonies were tested for biochemical reactions on MacConkey and TSI. Isolated colonies were also evaluated by MALDI-TOF MS

Growing Bacteria from Honeybee Intestinal Content

All agar types were purchased from Difco Company. The honeybee gut content sample was streaked for isolation on Luria Broth (LB) agar, blood agar, and MacConkey agar plates. 10 μl of the sample was placed on the plates, followed by streaking for isolation. Colonies were grown on plates at room temperature in the dark. MacConkey agar was used to grow Gram-negative bacteria and to stain them for lactose fermentation. Once bacteria were successfully grown, sub-culturing of six individual colonies was done to isolate each type of colony observed. The six colony types chosen were then cultivated on the blood and MacConkey agar plates. The colonies were restreaked on blood agar for purity.

Biochemical Testing of Colonies

Biochemical testing was done on MacConkey and Triple Sugar Iron (TSI) agar. MacConkey agar is a differential and selective medium used to identify Gram-negative from Gram-positive and lactose fermenting from lactose nonfermenting organisms. MacConkey contains crystal violet and bile salts that inhibit the growth of Gram-positive bacteria and will identify lactose-fermenting bacteria[10]. TSI agar is a differential medium used to identify bacteria based on fermentation of glucose, lactose, and sucrose, and on hydrogen sulfide (H_2S) production[11]. The six colonies were streaked for isolation onto MacConkey agar plates and TSI agar tubes. MacConkey plates were placed at room temperature in the dark for 48 hours of growth, and the TSI tubes were placed in an incubator for 48 hours at 37°C.

Matrix-Assisted Laser Desorption/Ionization

To prepare the bacterial samples for MALDI-TOF analysis, 1mL of LB broth was pipetted in eight different test tubes, one test tube per sample and one control. *Serratia marcescens* was used as a control for specificity in spectrum analysis. Next, each colony was picked from each sample with a sterile toothpick and placed in the test tube. The test tubes were placed on a shaker at room temperature for 24 hours for growth[12]. On the MALDI-TOF target plate, 0.5 μl of the bacterial suspension was mixed with a 0.5 μl matrix solution that was composed of 0.1% trifluro acetic acid (TFA), 70% acetonitrile, and

saturation with alpha-cyano-hydroxy-cinnamic acid (ACCA). The dried droplet method was used for spotting on the MALDI-TOF plate. Mass spectra were acquired in linear mode over an m/z range from 0 to 5,000 Da. Mass spectra were calibrated using a standard solution composed of bradykinin (1060.2 Da) and neurotensin (1672.9 Da).

Results

Several types of bacteria were isolated from the gut contents of the honeybee. The blood and MacConkey agars were used to obtain the six different types of colonies grown. These colony types were sub-cultured for further analyses. Three colonies were selected from the MacConkey agar and the other three from blood agar. On the MacConkey agar plate, there were a few dark and light pink colonies and one larger orange/tan colony. On the blood agar plate, there were numerous small white round colonies and a few yellow/tan and orange/tan colonies (Table 1). Six colony types were streaked for isolation on blood agar to verify each type was unique and once again on LB agar plates to ensure purity of colonies and stored in a refrigerator for stock.

Table 1

Sample	Morphology	Gram-Stain	Agar Types	TSI
1a	Gray small round colonies	Gram (+), rod	Blood, LB	No growth
1b	White/cream small round colonies, lactose fermenter	Gram (-), rod	Blood, LB, MacConkey	No growth
2	Gray spreading colony, central raised area (on mac tiny colony non lactose)	Gram(-), rod	Blood, LB, MacConkey	Sucrose fermentation
3	Small tanish colony (on mac small non lactose)	Gram(+), rod	Blood, LB, MacConkey (very small)	No growth
4	White average size colony looks pure on blood	Gram(+), cocci	Blood, LB	No reactions
5	Spreading gray colony on blood (pure), growing well on mac	Gram(+), short/fat rod	Blood, LB, MacConkey	Glucose fermentation
6	Tan colony average size pure on blood, on mac it is small non lactose	Gram(+), short/fat rod	Blood, LB, MacConkey	Glucose fermentation

Table 1 Characteristics of the main colony types found. Colonies were tested for Biochemical reactions using MacConkey and Triple Sugar Iron agar. Colonies were also gram stained to identify the shape and whether they were gram positive or negative.

The six different pure colony types were tested for biochemical reaction on MacConkey and TSI agar for characterization. When looking at sample 1, there were two types of colonies present on the blood agar and only one on the MacConkey agar. Since this happened, the two colonies (sample 1a and 1b) needed to be streaked for isolation on MacConkey to identify which one could grow on MacConkey agar. Sample 1b, 2, 3, 5, and 6 all grew on MacConkey agar. The MacConkey agar also showed that sample 1b is a lactose fermenter. The TSI was able to identify glucose, lactose, and sucrose fermentation in bacteria. None of the bacteria were capable of producing H2S gas or other gases which was also tested with the TSI. Sample 4 had no positive reaction on TSI agar. Samples 2, 5, and 6 all had a reaction indicating they ferment glucose. Sample 2 reacted, signifying it was a sucrose or lactose fermenter; however, since it did not show that is was a lactose fermenter on MacConkey, results suggest that it ferments sucrose and not lactose (Table 1).

Figure 2

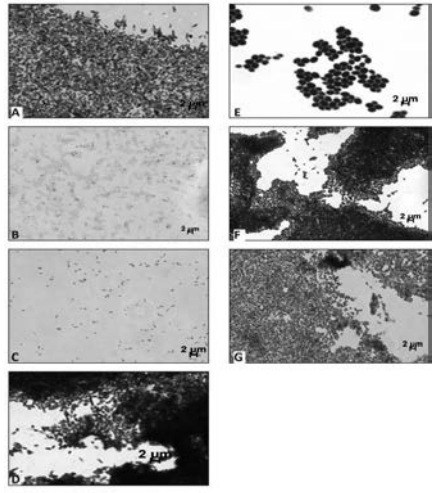

Figure 2 Gram staining results for sample 1a-6. (A) Sample 1a shows gram-positive rods. (B) Sample 1b shows gram-negative rods. (C) Sample 2 shows gram-negative rods. (D) Sample 3 shows gram-positive rods. (E) Sample 4 shows a yeast due to organism morphology. (F) Sample 5 shows gram-positive rods. These rods are different in that they are shorter and fatter in appearance compared to the others examined. (G) Sample 6 shows rods with gram variable reaction. Like sample 5 they are shorter and fatter in appearance.

Gram staining was done to identify the cell morphology and Gram reaction. All were rod- shaped except sample 4, which was rounded and of a typical yeast morphology. All bacteria were Gram-positive except samples 2 and 1b. Some of the samples appeared to be Gram-negative since they grew on MacConkey agar, though stained Gram-positive. Sample 6 appears to be Gram-variable (Figure 2).

Table 2

Mass of Molecules from Bee Gut Bacteria													
	~1007 m/z	**~1121 m/z**	**~1303 m/z**	**~1343 m/z**	**~1444 m/z**	**~1571 m/z**	**~1960 m/z**	**~2066 m/z**	**~2090 m/z**	**~2110 m/z**	**~2207 m/z**	**~2533 m/z**	**~2633 m/z**
Sample 1a		X	X					X		X	X	X	X
Sample 1b		X	X					X		X	X	X	X
Sample2							X		X		X	X	X
Sample 3		X	X					X		X	X	X	X
Control	X	X		X	X	X							

Table 2 Samples were examined by MALDI-TOF. Samples 1a, 1b, 2, 3, and the control (*Serratia marcescencs*) had acceptable mass spectra to examine. There were several similarities and differences in masses among the samples. The table shows which samples had similar mass peaks on their mass spectra.

Figure 3

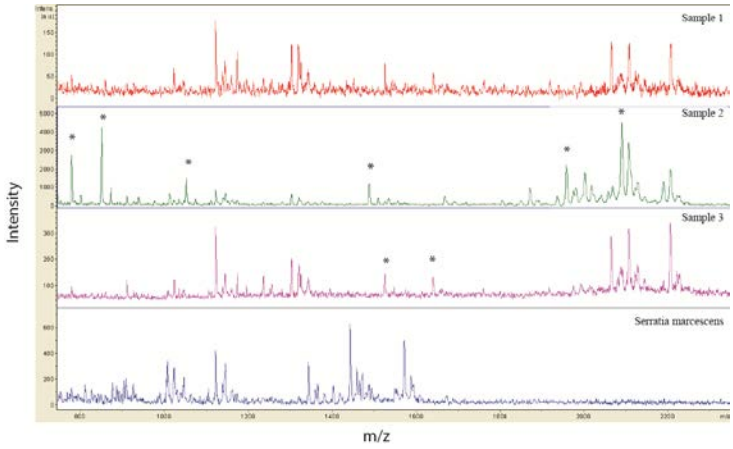

Figure 3 Matrix-Assisted Laser Desorption/Ionization Time of Flight mass spectra for samples 1-3 and the control (*Serratia marcescens*) using a matrix solution that was composed of 0.1% trifluro acidic acid (TFA), 70% acetonitrile, and saturation with alpha-cyano-hydroxy-cinnamic acid (ACCA). The peaks marked with asterisks correspond to unique peaks that are different from all the other samples.

MALDI-TOF mass spectrometry was able to provide a profile of cellular components, most likely peptides, based on masses over the region 800-2400 m/z. Mass spectrums were obtained for Sample 1a-3 and the control. Samples 1a, 1b, 3, and *S. marcescens* all have a peak around 1121 and 1303 m/z. Samples 1a, 1b, 2 and 3 have five peaks in common. These peaks are located at about 2066, 2110, 2207, 2534, and 2633 m/z. Mass specs for Samples 1a-3 are similar in appearance in some regions, except Sample 2 which is lacking peaks at 1122 and 1303 m/z. The control used, *S. marcescens,* does not have any peaks past 1575 m/z. The mass spec for *S. marcescens* show to be very different from the rest of the samples collected from the honeybee gut (Table 2, Figure 3).

Discussion

Biochemical reactions were done to help distinguish bacteria on the basis of carbohydrate fermentation. Fermentation is the process by which some bacteria derive energy from organic compounds in anaerobic conditions. During this process, energy is extracted from the oxidation of the organic compounds (sugars) with production of organic acids, alcohols, ketones and gases[13]. Some of the colonies had no reactions on MacConkey and TSI agar for fermentation. The MacConkey and TSI agar showed that colony samples 2, 5, and 6 produce positive reactions; therefore, they use at least one sugar for fermentation and get energy under anaerobic conditions. Several of the microbes have similar sugar fermentation characteristic patterns.

All microbes except for samples 1a and 4 grew on MacConkey agar. This is interesting, since after Gram staining only samples 1b and 2 appeared to be Gram-negative and only Gram- negative can grow on MacConkey agar. There are bile salts present in the MacConkey agar, which inhibit the growth of Gram-positive bacteria, but some have adapted and are able to grow and withstand the bile salts. Gram staining showed that sample 4 was a yeast and that all other bacteria have a rod morphology and are primarily Gram-positive. There are a few other types of Gram-positive bacteria that are capable of growing on MacConkey agar. Examples are *Enterococcus* and some species of *Staphylococcus.* These Gram-positive bacteria are able to tolerate bile salts, allowing them to grow on MacConkey agar. The

mechanism that allows these Gram-positive bacteria to withstand bile salts is unknown, but what is known is that this property is probably due to a combination of traits. The bacteria are not only overcoming damage to the membrane and DNA, but are also able to remove the bile salts with a pump. The removal of bile salts is important because it stops the damage that would lead to cell death[14].

Previous research was done on the gut flora of Malaysian honeybees. That study revealed several viable types of *Enterococcus* bacteria. *Enterococcus* is a genus of lactic acid Gram-positive bacteria which are facultative anaerobic organisms[15]. These organisms are capable of cellular respiration in both oxygen-rich and oxygen-poor environments. They are also tolerant of a wide range of environmental conditions. Bacteria grown in our study showed similar characteristics of the *Enterococci* in that they were able to withstand the bile salts in the MacConkey agar.

MALDI-TOF MS is important in profiling bacteria because it is fast, accurate, and reproducible. It is a soft ionization technique that allows the analysis of large organic biomolecules. MALDI-TOF MS can characterize the proteins that make up the bacteria, which helps identify microbes in the samples. As such, MALDI-TOF MS is able to characterize the chemical signatures of bacterial proteins for chemotaxonomic classification of bacteria[16]. It has been used to identify or differentiate bacteria by mass spectra of cells or cellular components. The MALDI-TOF mass spectral data obtained in our study were able to provide a few insightful findings. The spectral profiles of four of the samples were similar but unique. These spectra were compared to the profile obtained from the microbial control, *S. marcescens*, which proved to be different compared to the organisms we isolated from the honeybees, confiriming that these profiles were not an artifact produced by the growth medium. The data indicate metabolic relatedness among the microbes. These results emphasize the utility of this approach in further identification of bacterial associated with honeybees.

Conclusion

We observed seven different microbes grown from the gut of honeybees from a healthy colony. Biochemical testing of those microbes utilized different microbiological media while biophysical

profiling relied upon MALDI-TOF mass spectrometry. These data provided unique information on the bacteria isolated from the honeybee gut. We showed that four of the bacteria isolated can obtain energy from sugar fermentation. Through Gram staining, we showed one microbe isolated is a yeast. We were also able to observe the masses of the molecules that make up chemical fingerprints of bacteria through MALDI-TOF mass spectrometry. Now that the defining characteristics of these bacteria have been documented, future research can be shaped to characterize and compare microbes from healthy and diseased honeybee colonies using the techniques demonstrated in this research.

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