Bacteria in the Gut of Southern Green Stink Bug (Heteroptera: Pentatomidae)

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ABSTRACT Laboratory studies with the southern green stink bug, *Nezara viridula* (L.), indicated the presence of bacteria *Klebsiella pneumoniae* (Schroeter) and *Enterococcus faecalis* (Andrewes & Horder) in the crop/stomach (ventriculus 1–3; V1–V3), and possibly *Pantoea* sp. in the gastric caeca (ventriculus 4; V4). Culturable bacteria were most abundant in V1–V3, and their abundance was drastically reduced in V4. The variable pH in the gut did not affect the presence of bacteria. Elimination of bacteria from the gut, by using the antibiotic kanamicin, did not affect nymphal developmental time or cause mortality, but it did cause reduced weight at adult emergence.

KEY WORDS *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Nezara viridula*, enteric bacteria

SOUTHERN GREEN STINK BUG, *Nezara viridula* (L.), is a highly polyphagous pentatomid with worldwide distribution and is of great economic importance (Panizzi et al. 2000). During development, newly hatched nymphs remain on the eggshells and only begin to disperse after the first molt (Kiritani 1964). The reasons for this behavior are speculated to be 1) humidity regulation, i.e., because of a high surface-to-volume ratio, first instars avoid desiccation by aggregating; 2) protection against predators because nymphs in groups suffer less predation than when isolated (Lockwood and Story 1986); and 3) acquisition of symbiotic microorganisms that are present on the egg surface (Abe et al. 1995).

Many phytophagous insects are associated with bacteria (Campbell 1990), which influence their development and fitness (Berenbaum 1988, Frederick and Caesar 2000). At least 10% of known insect species contain extracellular microorganisms (Douglas 1989). Extracellular bacteria that colonize the insect gut must compete with other bacteria to become established (Vries et al. 2001).

Stink bugs have appendices in the form of sacks in the alimentary tract. Those structures, called caeca or bacterial crypts, have different forms and sizes and always hold an enormous number of bacteria (Steinhaus 1967). However, the importance of these microorganisms to the biology of most bugs remains unclear. In the current study, the gut of *N. viridula* was examined and microbial diversity, localization, and effects on nymphal biology were investigated.

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Materials and Methods

***N. viridula* Rearing.** Adult southern green stink bugs were reared in transparent plastic pots (40 by 30 cm in diameter) that contained glass tubes of distilled water closed with cotton plugs. They were fed green bean pods and sunflower seeds. The insect colonies were kept in an environmental chamber at 25 ± 1°C, 60 ± 10% RH, and a photoperiod of 16:8 (L:D) h. The food was replaced every 2 d. The colony has been maintained without introduction of feral insects for >3 yr.

Dissection of *N. viridula*. Adults and nymphs of *N. viridula* were anesthetized with CO_2, and legs, wings, and lateral abdomen were excised. Immediately after excision, insects were surface sterilized by submerging in 2% sodium hypochlorite for 1 min, and each insect was transferred to petri dish (5 cm) with paraffin (the plate used in each dissection was sterilized in an oven at 130°C for 20 min). Insects were immobilized with pins, covered with sterile water, and dissected using forceps. The digestive system was divided in four sections: first (V1), second (V2), third (V3), and fourth (V4) ventriculus (Fig. 1).

Gut pH. For gut pH determination, 20 male and 20 female adults of *N. viridula* were dissected. The four gut sections, referred to above, were removed and the pH in each section (ventriculus) was measured. To avoid the disrupted gut tissue to change the pH, each section used was obtained from a different insect, and sections were put in the freezer (4°C). The sections were processed separately in microcentrifuge tubes (1.5 ml) with the addition of 50 μl of distilled water.

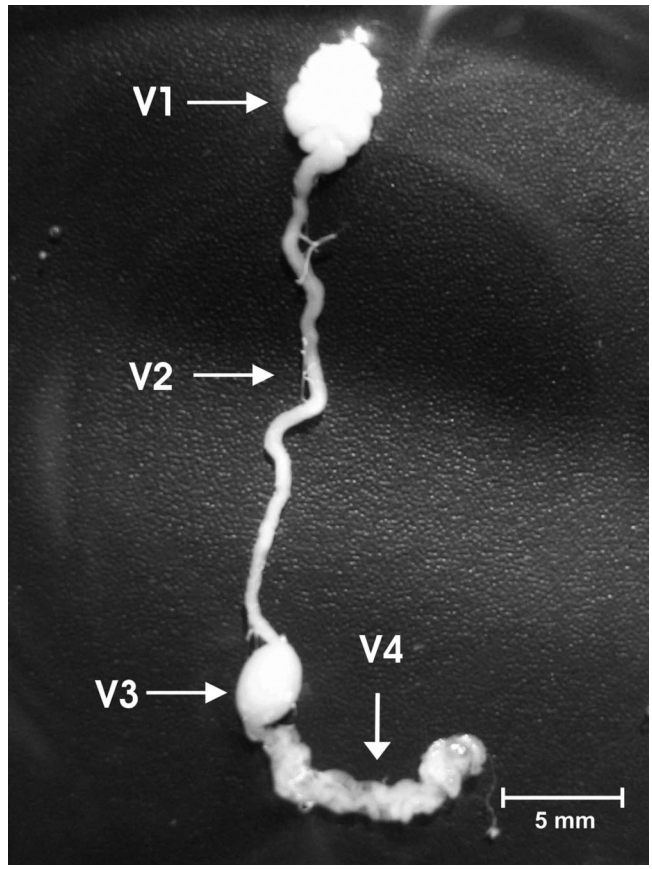

Fig. 1. Different gut sections (ventriculus 1–4; V1–V4) in *N. viridula.* V1 on the anterior end; V4 on the posterior end.

They were homogenized with a tissue grinder, and a 20-μl aliquot of the resulting suspension was placed on the pH meter probe Corning pH/ion meter 450 (Corning, New York, NY) to record the pH. The results are reported as mean ± SEM.

Bacterial Isolation. Another set of adult *N. viridula* (20 males and 20 females) was dissected. The four sections of the digestive system (V1–V4) were processed separately in microcentrifuge tubes (1.5 ml), as described above. They were carried through successive dilutions (three to seven) in 100 μl of water and distributed on Luria Bertani (LB) agar medium plates. After 48 h, the number of colony-forming units present was recorded. The results are reported as mean ± SEM.

Extraction of DNA. For culturable bacteria, the content of the gut tissue obtained from the microcentrifuge tubes (1.5 ml) was streaked on LB medium by using a sterile loop and maintained at 25°C. Bacteria from the gut of three specimens (one adult female, one adult male, and one fourth instar) were grown on LB medium at 25°C for 48 h. Ten bacterial colonies per insect were suspended in 100 μl of extraction buffer (0.05 M NaOH and 0.25% SDS) and boiled for 15 min. After lysis of the bacterial cells, the suspension was centrifuged at 10,000 rpm for 1 min., and 10 μl of supernatant was diluted 25× with sterile water. For the unculturable bacteria, insects were dissected, and the tissue of each section of the gut (V1–V4) was individualized in tubes, lyophilized, and then total DNA was extracted using standard protocols (Rehner and Buckley 2003).

16S rDNA Polymerase Chain Reaction (PCR) and Sequencing. To identify the culturable and unculturable bacteria, the 16S rDNA PCR was carried out in 50 μl of a mixture containing 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTDP, 20 pM each primer, 10 ng of DNA template matrix (bacterial DNA from heat-lysed bacterial suspension or total DNA from gut tissue), and 2.0 U of *Taq*DNA polymerase (Invitrogen, Carlsbad, CA). Primers 8F (5′-AGAGTTTGATCCTGGCTAG-3′) and 1429R (5′-ACGGCTACCDTTGTTAACGACTT-3′) were used to amplify the 16S rDNA (Esikova et al. 2002). The cycling program was conducted on a PTC-200 thermocycler (MJ Research, Watertown, MA) and had two phases: the first phase consisted of an initial denaturation at 94°C for 2 min, followed by 10 cycles of denaturation at 94°C for

1 min., primer annealing at 62°C for 1 min, decreased by 1°C each succeeding cycle, and elongation at 72°C for 2 min. The second phase consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 2 min, and a final extension of 72°C for 5 min. The amplified products were separated on a 1.5% low-melting point agarose gel, excised, and purified using standard methods (Sambrook and Russell 2001). The products obtained from the culturable bacteria were directly sequenced by using the *Taq*-mediated DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Warrington, United Kingdom) in an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA) as described previously (De Souza et al. 2003). Products obtained from the nonculturable bacteria from each gut section (V1–V4) were cloned into the T/A Cloning vector pCR2.1 (Invitrogen), following the manufacturer's instructions. Transformants were subjected to plasmid extraction by standard methods (Sambrook and Russell 2001). The inserts were amplified by using the M13 forward and M13 reverse primers (Invitrogen) with the following cycling program: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension of 72°C for 2 min. Products were separated and purified from low melting point agarose gels. Primers M13 forward, M13 reverse, 16SinF (5′-TGCCGCGTGAGTGAAGAA-3′), and 16SinR (5′-ATAAGGGCCATGATGACTTGAC-3′) were used for direct sequencing as described above. The LASERGENE (DNAStar, Madison, WI) sequence analysis software package and the BLAST software (Altschul et al. 1997) were used for DNA sequence analyses.

Impact of Antibiotics on *N. viridula* Nymphal Development. Eggs masses of *N. viridula* were placed in a sterilized petri dish (9 cm in diameter) lined with filter paper and maintained in an environmental chamber at 25 ± 1°C, 60 ± 10% RH, and a photoperiod of 16:8 (L:D) h. When nymphs reached the second instar (first instars do not feed), they were placed singly in petri dishes ($n = 80$) with humidified cotton containing an antibiotic solution (60 μg/ml kanamicin). The control group ($n = 80$) was set up in a similar way, except that the wet cotton did not contain the antibiotic. Both groups were fed green bean pods. Nymphs were observed daily. The food and antibiotic solution were replaced every 2 d. Nymphal developmental time, survivorship, and weight at adult emergence were recorded.

Statistics. Data on pH of gut and size (millimeters) of different sections (V1–V4) of the gut and fresh body weight of *N. viridula* at emergence were analyzed with analysis of variance, and means were compared using the Tukey test ($P < 0.05$) (SAS Institute 1981).

Results and Discussion

The southern green stink bug midgut is a tubular organ and consists of four regions (Fig. 1). The first (V1) is a small globular structure. The second (V2) is the longest part of the midgut and is connected to V3 that is variously inflated depending upon its contents. The V2–V3 sections have the same histological features, whereas the last section (V4) is demarcated by the presence of four longitudinal rows of evaginations known as gastric caeca (Malouf 1933, Goodchild 1963, Silva and Terra 1994).

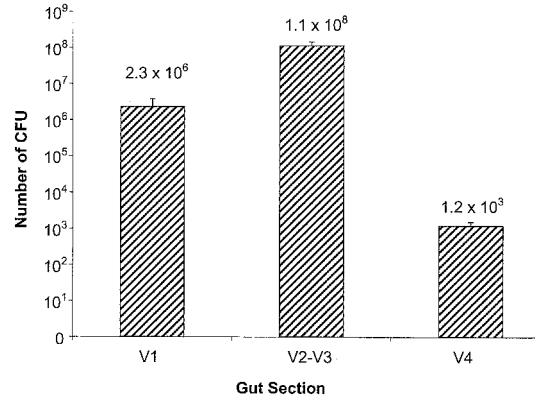

Fig. 2. Mean (±SEM) number of bacteria-colony units in LB agar medium obtained from different gut (ventriculus) sections (V1–V4) of *N. viridula.*

The number of culturable bacteria present in V1 and V2–V3 that grew in the LB medium was variable (Fig. 2). However, it was much greater in the V1 and V2–V3 sections than in the caecal region (V4), where the number was drastically reduced (six of 10 adults studied showed no culturable bacteria in V4).

The 16S rDNA segment sequenced from culturable bacteria was identified as belonging to *Klebsiella pneumoniae* (Schroeter) (Table 1). The segments 16S rDNA isolated directly from intestinal tissues indicated the presence of other two species of bacteria, *Enterococcus faecalis* (Andrewes & Horder), isolated from V1 to V3, and a bacterium (close to *Pantoea* sp.) present only in V4. *E. faecalis* is a fastidious bacterium that needs special nutritional requirements for growing not found in the media and growth conditions used in this study (Holt et al. 1994). The bacterium related to *Pantoea* sp. probably is not *Pantoea* because bacteria belonging to this genus can grow in the media and growth conditions used. The bacterium close to *Pantoea* sp. is identical to the one referenced from *N. viridula* in Hawaii (nucleotide sequence accession number GenBank AY679762) by R.P.P. Almeida and D. Rubinoff. *K. pneumoniae* was found in adults and in nymphs.

The pH measurements indicated that V3 has a more acidic environment than the adjacent structures (Table 2). In *Dysdercus peruvianus* Guèrin-Meneville (Heteroptera: Pyrrhocoridae), the pH of the midgut (V1–V3) is also variable, ranging from 5.6 (V3) to 6.4 (V1) (Silva and Terra 1994). However, when *K. pneumoniae* colonies isolated from the different parts of the gut (V1–V3) were placed in LB agar medium with adjusted pH of 5, 6, and 7, they multiplied equally well. Therefore, pH does not seem to be a factor that pre-

Table 1. Nucleotide sequences of bacteria found in the guts of *N. viridula* deposited in the GenBank with the respective accession numbers assigned

Species	Gut section	GenBank accession no.
K. pneumoniae	V1–V3	AY830394, AY830395, AY830396
E. faecalis	V1–V3	AY830397, AY830398, AY830399, AY830400, AY830401, AY830402, AY830403, AY830404, AY830405, AY830406, AY830407, AY830408
Bacterium close to *Pantoea* sp.	V4	AY830409, AY830410, AY830411, AY830412, AY830413, AY830414

vents the development of this bacterium in the caecal section, where its growth is definitely restricted. Apparently, the other bacteria present in the gastric caeca prevent or inhibit the development of *K. pneumoniae* in this area, but this needs further investigation. It is known that the diversity of the microbiota in an organ can be influenced by several factors, such as pH, presence of different digestive enzymes, and type of ingested food (Dillon and Dillon 2004).

The size (length and width) of the gut regions was variable (Table 2). V2 was much longer than the other parts of the midgut, followed but the caecal section (V4), V1, and V3. Considering the width of the gut regions, the size decreased in the following order: V1, V3, V4, and V2.

The association between microorganisms and insects may be casual and transitory, particularly when the former are derived from the diet fed upon by the latter (Douglas 1989). It is possible that the *K. pneumoniae* we found were acquired by the bugs through the food, became adapted to the laboratory-rearing conditions, did not cause significant damage to the insect colony, and helped to prevent the establishment of noxious microorganisms. For example, the colonization of germ-free locusts by *Pantoea agglomerans* (Ewing & Fife) was favored in the presence of two other indigenous species, *Klebsiella pneumoniae* subsp. *pneumoniae* (Schroeter) and *Enterococcus casseliflavus* Collins et al. A single inoculation with these three germs was sufficient to establish the colonies that persisted several weeks while feeding on the sterile diet (Dillon and Dillon 2004). In humans and domesticated animals, the indigenous intestinal microbiota shows a beneficial function by withstanding the colonization of the gut by pathogens and therefore prevents enteric infections (Berg 1996). Despite the numerous microbial species that *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae) consume through their feeding and foraging activities, only two species are routinely isolated from the alimentary canal, *Enterobacter* spp. and *Klebsiella* spp. These two bacterial genera form an extensive biofilm within the esophageal bulb, crop, and intestines. This specialized complex assemblage further supports the hypothesis that *Enterobacter* spp. and *Klebsiella* spp. jointly participate in the cycling of nitrogen (Lauzon 2003).

N. viridula nymphs were not affected in their developmental time (23.9 ± 0.47 d) ($n = 58$) compared with 23.6 ± 0.36 d ($n = 56$) for untreated control nymphs, nor in their survivorship (27.5% mortality compared with 30.0% mortality for the controls), when they were exposed to the antibiotic kanamicin, which eliminated *K. pneumoniae* from the gut. However, both females and males of *N. viridula* showed reduced fresh body weight at adult emergence when as nymphs, they were supplied water containing the antibiotic, compared with the control bugs that were exposed to water only (Fig. 3). The latter result suggests that the elimination of *K. pneumoniae* from the gut of nymphs results in smaller, probably less fit, adults of *N. viridula.*

Table 2. Values of pH and size (millimeters) of different sections (ventriculus 1–4; V1–V4) of the gut of *N. viridula* reared in the laboratory ($n = 5$)

Sex	Gut section			
	V1	V2	V3	V4
pH				
Female[a]	6.6 ± 0.18a	6.1 ± 0.08a	5.2 ± 0.46b	6.6 ± 0.06a
Male[a]	6.7 ± 0.22a	6.0 ± 0.35a	5.1 ± 0.56b	6.7 ± 0.03a
Size				
Length				
Female	5.3 ± 0.20c	14.9 ± 0.60a	4.3 ± 0.37d*	9.4 ± 0.61b
Male	5.1 ± 0.14c	14.5 ± 1.89a	3.3 ± 0.21d	7.7 ± 0.53b
Width				
Female	3.1 ± 0.23a	0.8 ± 0.01d	1.8 ± 0.24b	1.3 ± 0.09c
Male	3.1 ± 0.22a	0.7 ± 0.04d	1.9 ± 0.15b	1.2 ± 0.11c

Asterisk (*) indicates significant difference between genders.
[a] Means followed by the same letter in each line do not differ significantly ($P < 0.05$) using Tukey's test.

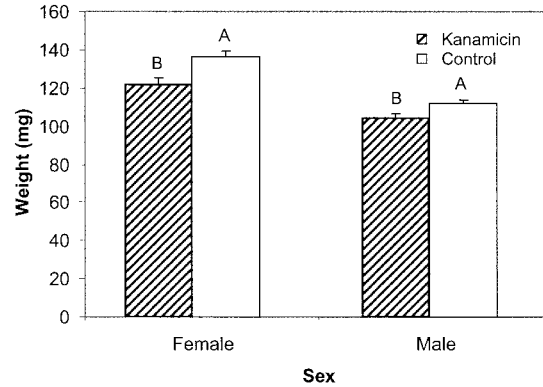

Fig. 3. Mean (±SEM) fresh body weight (milligrams) of *N. viridula* adults at day of emergence in controls and those treated with the antibiotic kanamicin (60 μg/ml), feeding on green bean pods in the laboratory. Means between treatments followed by the same letter do not differ significantly ($P < 0.05$) using Tukey's test.

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