Appl Entomol Zool (2011) 46:483–488
DOI 10.1007/s13355-011-0066-6

ORIGINAL RESEARCH PAPER

Obligate association with gut bacterial symbiont in Japanese populations of the southern green stinkbug *Nezara viridula* (Heteroptera: Pentatomidae)

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Received: 10 June 2011 / Accepted: 27 July 2011 / Published online: 17 August 2011

Abstract The southern green stinkbug *Nezara viridula* (Linnaeus) has a number of sac-like outgrowths, called crypts, in a posterior section of the midgut, wherein a specific bacterial symbiont is harbored. In previous studies on *N. viridula* from Hawaiian populations, experimental elimination of the symbiont caused few fitness defects in the host insect. Here we report that *N. viridula* from Japanese populations consistently harbors the same gammaproteobacterial gut symbiont, but, in contrast with previous work, experimental sterilization of the symbiont resulted in severe nymphal mortality, indicating an obligate host–symbiont relationship. Considering worldwide host–symbiont association and these experimental data, we suggest that *N. viridula* is generally and obligatorily associated with the gut symbiont, but that the effect of the symbiont on host biology may be different among geographic populations. Possible environmental factors that may affect the host–symbiont relationship are discussed.

Keywords *Nezara viridula* · Symbiotic bacterium · Midgut crypts · *Gammaproteobacteria* · Obligate symbiosis

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Introduction

Many insects that feed exclusively on nutritionally limited diets, for example plant sap, have symbiotic microorganisms in their bodies (Buchner 1965; Kikuchi 2009). The insect suborder Heteroptera, so-called true bugs or stinkbugs, embraces over 40,000 described species with sucking mouthparts (Weirauch and Schuh 2011), and symbiotic associations with bacteria tend to occur in plant-sucking species. Such phytophagous stinkbugs have many sacs or tubular outgrowths, called crypts or caeca, in a posterior region of the midgut, which contain a dense population of symbiotic bacteria (Buchner 1965; Glasgow 1914; Kikuchi 2009). Experimental removal of the symbiotic bacteria from the host stinkbugs often results in retarded growth and high mortality (Abe et al. 1995; Fukatsu and Hosokawa 2002; Hosokawa et al. 2006; Kikuchi et al. 2007, 2009), indicating the biological importance of the symbionts for their hosts.

The southern green stinkbug *Nezara viridula* (Linnaeus) (Fig. 1a) is a cosmopolitan species distributed from tropical to temperate regions of North and South America,

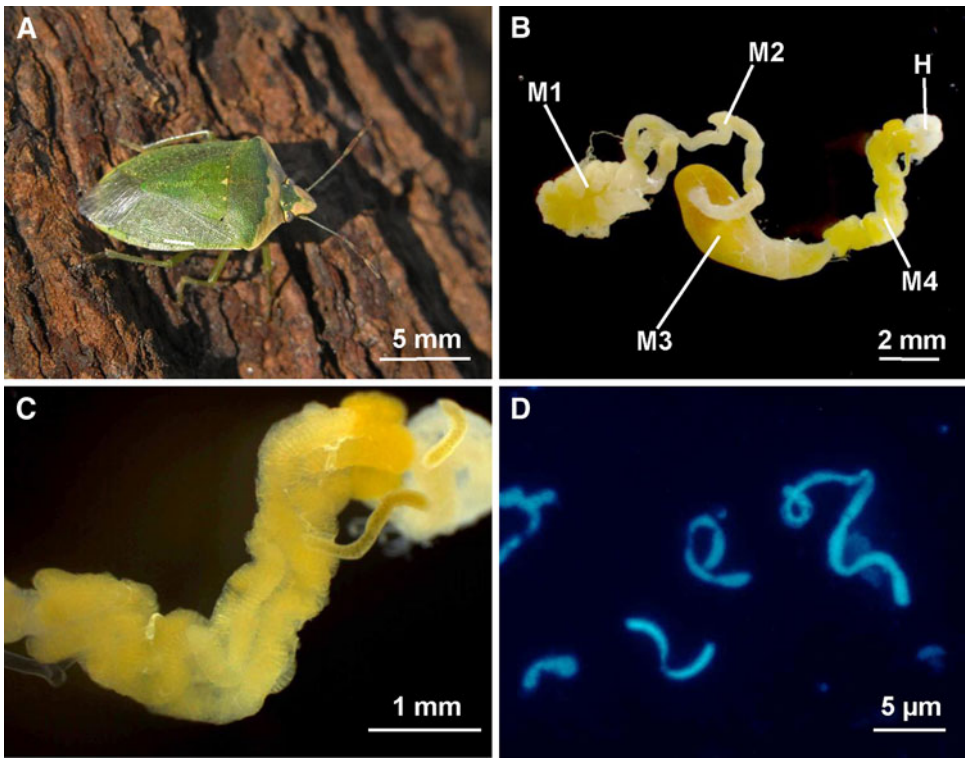

Fig. 1 The southern green stinkbug *Nezara viridula* and its gut symbiotic system. **a** An adult female of *N. viridula*. **b** A dissected alimentary tract of an adult female. *M1* midgut first section, *M2* midgut second section, *M3* midgut third section, *M4* midgut fourth section with crypts, *H* hindgut. **c** An enlarged image of the midgut fourth section with crypts. **d** Fluorescence image of the gut symbiont cells visualized by DNA staining with 4′,6-diamidino-2-phenylindole

Africa, Asia, Australia, and Europe (Todd 1989), and known as a notorious agricultural pest that damages diverse crop plants worldwide (Schaefer and Panizzi 2000). As in many phytophagous heteropteran species, *N. viridula* has midgut crypts that harbor a specific bacterial symbiont. Previous studies on Hawaiian, North American, and Brazilian populations of *N. viridula* consistently identified the same gammaproteobacterial symbiont in the midgut crypts (Hirose et al. 2006; Prado et al. 2006, 2009). Female insects vertically transmit the gut symbiont to their offspring via surface contamination of eggs with symbiont-containing excrement (Prado et al. 2006). With laboratory strains of *N. viridula* from the Hawaiian populations, Prado et al. (2006, 2009) showed that experimental elimination of the symbiont by egg-surface sterilization caused few fitness defects in the host insect: symbiont-free insects developed normally and reproduced as normal symbiotic insects did.

These previous studies suggest a facultative host–symbiont relationship in Hawaiian populations of *N. viridula*. Here, by contrast, we report that in Japan, *N. viridula* is associated with the gammaproteobacterial symbiont in the midgut crypts, but the effect of the symbiont on the host insect is strikingly different: without the gut symbiont, the host insect can neither grow nor reproduce normally, suggesting an obligate host–symbiont relationship in Japanese populations of *N. viridula*.

Materials and methods

Insect materials

Table 1 lists samples of *N. viridula* examined in this study. For rearing experiments, we used a laboratory stock of *N. viridula* collected from a field of tomato (*Solanum lycopersicum* Linnaeus) and okra (*Abelmoschus esculentus* Linnaeus) in Katano, Osaka, Japan. We maintained the insects on dry soybean seeds (*Glycine max* Linnaeus), raw peanuts (*Arachis hypogaea* Linnaeus) and distilled water containing 0.05% ascorbic acid at 25°C under a long-day regimen (16 h light, 8 h dark).

Microscopic observation

The whole midgut was isolated from adult insects using a pair of fine forceps under a dissection microscope in a plastic Petri dish filled with phosphate-buffered saline (PBS: 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4; pH 7.5), and photographed using a digital camera (EC3, Leica) connected to a dissection microscope (S8APO, Leica). From the whole midgut, the midgut fourth section with crypts (Fig. 1b, c) was isolated and homogenized in PBS. The suspension was stained with 4 μM 4′, 6-diamidino-2-phenylindole (Invitrogen) and observed under an epifluorescence microscope (Axiophoto, Carl Zeiss).

Table 1 Samples of *Nezara viridula* examined in this study

Sample information					16S rRNA gene accession number	Refs.
Country	Prefecture/state	City/island	Year	Collector		
Japan	Osaka[a]	Katano	2008	D.L.M.	AB636641	This study
	Kochi	Nankoku	2007	D.L.M.	AB636642	This study
	Fukuoka	Kitakyushu	2008	Mitsuo Baba	AB636643	This study
	Kumamoto	Koshi	2008	Y.K.	AB636644	This study
	Kagoshima	Amami-Oshima	2008	Yuki G. Baba	AB636645	This study
	Kagoshima	Yakushima	2008	T.F.	AB636646	This study
	Kagoshima	Tanegashima	2009	T.H	AB636647	This study
	Okinawa	Nago	2009	T.H.	AB636648	This study
	Okinawa	Ishgakijima	2009	T.H.	AB636649	This study
	Okinawa	Miyakojima	2010	T.H.	AB636650	This study
USA	Hawaii[b]	Hilo	2003	–	AY679762[c]	Prado et al. (2006)
	–	–	–	–	EU072503[c]	Prado and Almeida (2009)
Brazil	–	–	–	–	AY830409–AY830414[c]	Hirose et al. (2006)

–, Information not available

[a] Fitness effect of the gut symbiont was examined in this study

[b] Fitness effect of the gut symbiont was examined in Prado et al. (2006)

[c] These 16S rRNA gene sequences were retrieved from DNA databases

DNA extraction, cloning, and sequencing

A midgut fourth section was dissected from each adult insect as described above, and subjected to DNA extraction using a QIAamp DNA Mini Kit (Qiagen). A 1.5-kb region of the bacterial 16S rRNA gene was amplified with primers 16SA1 (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 16SB1 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Fukatsu and Nikoh 1998). Polymerase chain reaction (PCR) was conducted with Ampli*Taq* Gold DNA polymerase (Applied Biosystems) using a temperature profile of 95°C for 10 min followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min. Cloning and sequencing of the amplified products were performed as described elsewhere (Kikuchi et al. 2007).

Diagnostic PCR

A 0.83-kb region of the 16S rRNA gene of the gut symbiont of *N. viridula* was amplified with specific primers MMAOf1 (5′-GGG ATA ATG CCT AAT AYG CAT G-3′) and MMAOr1 (5′-GCT TGC TCT TGC GAG GTT-3′) using a temperature profile of 95°C for 10 min followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min. To check the quality of the DNA samples, a 0.65-kb region of insect mitochondrial cytochrome oxidase I (*COI*) gene was amplified with primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al. 1994).

Molecular phylogenetic analysis

Multiple alignments of nucleotide sequences were generated using the software Clustal W (Thompson et al. 1994) and the final alignments were corrected manually. Maximum likelihood, maximum parsimony, and neighbor-joining phylogenies were inferred by use of the software PhyML 3.0 (Guindon et al. 2010), PAUP 4.0b10 (Swofford 2001), and Clustal W (Thompson et al. 1994), respectively.

Egg-surface sterilization

Each mass of eggs of *N. viridula* was divided into two parts. One part was left untreated and the other part was treated with 70% ethanol for 10 min, then with 4% formaldehyde for 5 min, and finally rinsed thoroughly with 70% ethanol twice. Each of the experimental masses of eggs was kept in a Petri dish with a wet cotton ball at 25°C until hatching. To confirm successful elimination of the symbiont, hatchlings from the experimental egg masses were subjected to DNA extraction and diagnostic PCR 2 days after the molt to the second instar.

Fitness measurement

Time to hatching and hatching rate were recorded for both the control and sterilized masses of eggs. The hatchlings from the experimental mass of eggs were separately reared as described above, and their growth and survival were monitored until all the insects either became adult or died.

The data were statistically analyzed by use of Fisher's exact probability test and the Mann–Whitney *U* test by use of the software R v2.11.1 (R Development Core Team 2010).

Results

Characterization of gut bacterial symbiont associated with *N. viridula* collected in Japan

In dissected alimentary tracts of *N. viridula*, the midgut fourth section was consistently yellow in color and had a number of crypts arranged in four rows (Fig. 1b, c). Microscopic observations identified tubular bacterial cells packed in the lumen of the midgut crypts (Fig. 1d). A 1.5-kb region of the bacterial 16S rRNA gene was amplified, cloned, and sequenced from each of the midgut fourth sections dissected from ten adult females of *N. viridula* originating from different Japanese populations (Table 1). All the sequences were 1,464 bp in size and completely identical. BLAST searches with the sequence as query retrieved gammaproteobacterial 16S rRNA gene sequences; the top hit was the gut symbiotic bacterium of a Brazilian strain of *N. viridula* (accession number AY830411; 99.7% (1447/1452) sequence identity). Figure 2 shows the phylogenetic placement of the gut bacterial symbionts of the Japanese *N. viridula* samples on the basis of the 16S rRNA gene sequences. The symbiont sequences from Japanese *N. viridula* samples formed a well-defined clade, supported by 100% bootstrap values, together with the symbiont sequences from the Hawaiian, North American, and Brazilian *N. viridula* samples. Slight genetic divergence was observed between the Japanese symbiont sequences and the Hawaiian/American/Brazilian symbiont sequences.

Disruption of symbiont transmission by egg-surface sterilization

Egg masses of *N. viridula* were divided into two parts; one part was left untreated and the other was surface-sterilized. Nymphs from these experimental egg masses were reared until the second instar and subjected to diagnostic PCR detection of the symbiont. All the nymphs from the control egg masses were symbiont-positive (infected/total observed = 42/42, i.e. 100%), whereas all the nymphs from the sterilized egg masses were symbiont-negative (0/50, 0%).

Fitness effects of symbiont elimination on Japanese *N. viridula*

No significant differences between control egg masses and sterilized egg masses were detected for time to hatch (mean ± SD: control 5.66 ± 0.48 days, $n = 159$; sterilized 5.64 ± 0.48 days, $n = 168$; Mann–Whitney *U* test $P = 0.74$) and hatching rate (control 94.6% (hatched/total eggs = 159/168); sterilized 97.7% (168/172); Fisher's exact test $P = 0.17$). By contrast, drastic differences in post-hatch growth were recorded between the control and the sterilized group. Survival was significantly lower in the sterilized group than in the control group. In the sterilized group, survival decreased drastically during the 2nd and 3rd instars, the nymphs seeming to die without feeding. Only a few nymphs survived to adulthood in the sterilized group (Fig. 3).

Discussion

Our results indicate that:

1. *N. viridula* from Japanese populations is consistently associated with a gammaproteobacterial symbiont in the midgut crypts;
2. the gut symbiont is genetically very closely related to the gut symbionts of *N. viridula* from the Hawaiian, North American and Brazilian populations;
3. egg-surface sterilization disrupts nymphal infection with the symbiont, indicating vertical transmission of the gut symbiont via egg surface contamination; and
4. experimental elimination of the symbiont results in severe nymphal mortality and emergence of few adult insects, indicating the obligate nature of the host–symbiont association.

The results 1–3 are concordant with the previous studies on Hawaiian, North American, and Brazilian populations of *N. viridula* (Hirose et al. 2006; Prado et al. 2006, 2009). The prevalent and conserved host–symbiont association across Asia, North America, South America, and Oceania seems suggestive of important biological roles of the gut symbiont for *N. viridula*.

Result 4, on the other hand, contrasts sharply those from previous studies on Hawaiian populations of *N. viridula* (Prado et al. 2006, 2009), wherein experimental elimination of the gut symbiont caused few fitness defects in the host. It is currently unknown why the fitness effects of the gut symbiont differ so strikingly between the Japanese and Hawaiian *N. viridula* populations. Conceivably, environmental and/or genetic factors may be involved in the difference. In this study, the insects were fed with dry soybean seeds and raw peanuts under 16 h light and 8 h dark conditions, whereas in the experiment of Prado et al. (2006), the insects were reared on green beans, cabbage, and unsalted roasted peanuts under 14 h light and 10 h dark conditions. The different rearing conditions may affect the effects of the gut symbionts on their insect hosts.

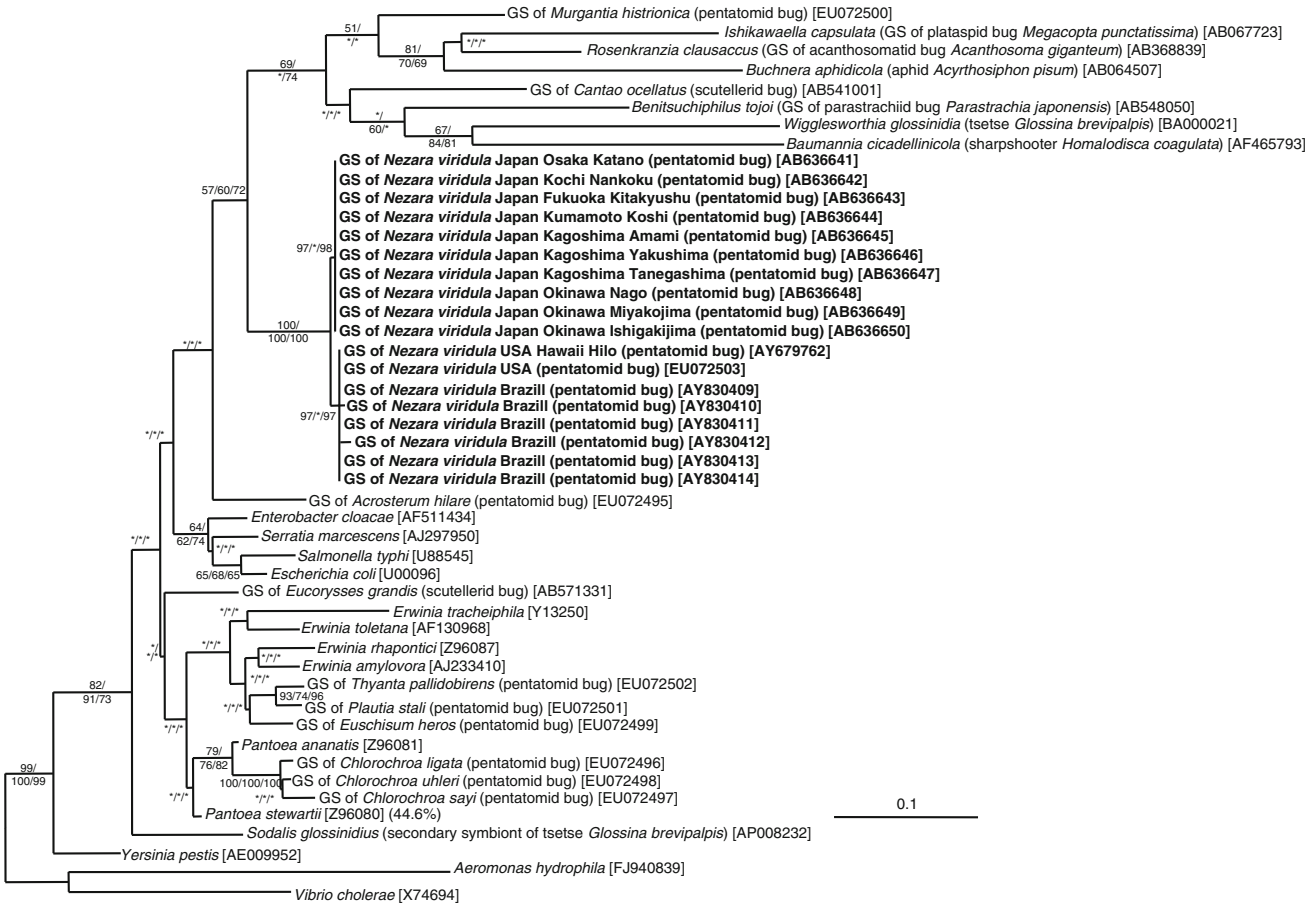

Fig. 2 Phylogenetic placement of the gut symbiotic bacteria of *Nezara viridula* on the basis of 16S rRNA gene sequences. A maximum likelihood phylogeny inferred from 1,221 unambiguously aligned nucleotide sites is shown. Maximum parsimony and neighbor-joining analyses gave substantially the same results (data not shown). Bootstrap values higher than 50% are indicated at the nodes in the order of maximum likelihood/maximum parsimony/neighbor-joining. *Asterisks* indicate support values lower than 50%. Sequence accession numbers are shown in *brackets*. For insect symbionts, the host common name, scientific name, and/or taxon name are also indicated in parentheses. *GS* gut symbiont

Alternatively, genetic differences of the gut symbionts and/or the host insects between the Japanese and Hawaiian populations could be responsible for the different fitness consequences. In this context, it may be notable that the gut symbionts of Japanese *N. viridula* insects are genetically slightly different from the gut symbionts of the Hawaiian, North American, and Brazilian insects (Fig. 2).

We demonstrated that the gut symbiont is required for normal nymphal growth of Japanese *N. viridula*, which accounts for the worldwide prevalence of the host–symbiont association. Normal growth and reproduction of symbiont-free Hawaiian *N. viridula* (Prado et al. 2006, 2009) looks, at least superficially, contradictory to the maintenance of the symbiotic association in natural host populations. Notably, Prado et al. (2009) reported few fitness defects for symbiont-free Hawaiian *N. viridula* insects reared at 25 and 30°C—delayed nymphal growth, extended life span, and ceased oviposition at 20°C were observed for the symbiont-free insects. Hence, although speculative, the gut symbiont could be involved in some aspects of cold tolerance in Hawaiian populations of *N. viridula*. In Japan, recent northward expansion of the distribution range of *N. viridula*, which may be related to global warming, has been reported and monitored (Kiritani 2011; Musolin 2007; Tougou et al. 2009; Yukawa et al. 2009). How the gut symbiont is involved in the ecological and evolutionary processes ongoing in the Japanese populations of *N. viridula* is of great interest, and currently under investigation.

In conclusion, we suggest that *N. viridula* is generally and obligatorily associated with the gut symbiont, and that the symbiont effect on the host biology may be different among different geographic populations. We also point out the possibility that the gut symbiont could be a target for controlling this cosmopolitan insect pest, at least in Japan and possibly in neighboring Asian countries where the host–symbiont association is obligate.

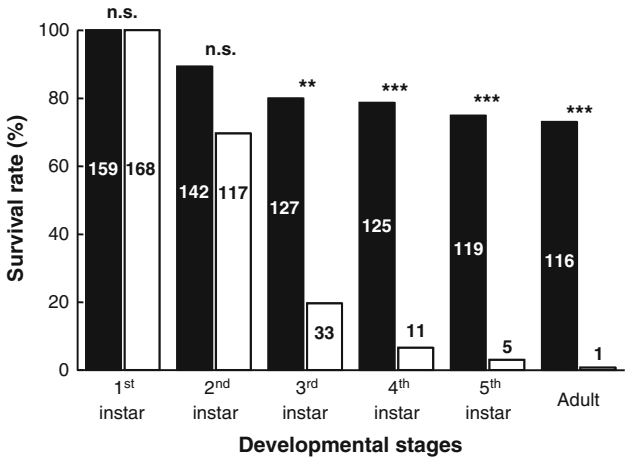

Fig. 3 Effects of symbiont elimination on survival of *Nezara viridula*. *Black bars*, untreated control group; *white bars*, experimental egg-surface sterilization group. The *number on each column* is the total number surviving. *Asterisks* indicate *P* values after Bonferroni correction (Fisher's exact probability test; $^{**}P < 0.001$; $^{***}P < 0.0001$)

Acknowledgments We thank M. Baba and Y. G. Baba for insect samples. This study was supported by the Program for Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry (BRAIN), the Japan Society for the Promotion of Science (JSPS), and The Council for Grants of the President of the Russian Federation and State Support of the Leading Scientific Schools (project # 3332.2010.4).

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