Vertical Transmission of a Pentatomid Caeca-Associated Symbiont

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ABSTRACT We present molecular data for an endosymbiont of the insect family Pentatomidae, located in the gastric caeca of *Nezara viridula* (L.) (Hemiptera: Pentatomidae) stink bugs. Restriction fragment length polymorphism and polymerase chain reaction analysis suggest that this bacterium is consistently present in caeca of *N. viridula* from a variety of geographic locations. The bacterium is present in different midgut sections in nymphs versus adults. The bacterium also was detected on eggshells after nymphs had hatched but not in ovarioles, suggesting oral rather than transovarial transmission. Surface sterilization of egg masses generated aposymbiotic insects. Aposymbiotic individuals reached the adult stage, females laid viable eggs, and the offspring remained aposymbiotic in the following generation. No clear fitness decrease was observed in aposymbiotic individuals over two generations. Phylogenetic analysis of a partial 16S rRNA data set with 21 Gammaproteobacteria suggested the inadequacy of neighbor-joining and maximum parsimony models to account for homoplasy apparent in a molecular data set, including a range of insect endosymbionts. Maximum likelihood-based analysis suggests that the *N. viridula* endosymbiont is closely related to a caeca-associated symbiont found in another stink bug family (Plataspidae). The high AT content of the symbiont's 16S rRNA in relation to other insect endosymbionts, its location in the midgut of the host insect, oral transmission, and survival of aposymbiotic individuals suggest this symbiosis may be recently established.

KEY WORDS *Buchnera*, maximum likelihood, Heteroptera, stinkbug, symbiosis

The association between insects in the order Hemiptera and their bacterial symbionts has long been of interest to entomologists and microbiologists. Much work has focused on the suborders Sternorrhyncha and Auchenorrhyncha, most of which feed on nutrient-poor diets, such as phloem or xylem sap. For these insect hosts, bacterial symbionts provide nutrients lacking in their diet (Ishikawa 2003). Among the various insect–microbe symbiotic interactions studied with hemipteran hosts, the symbiosis between aphids and bacteria of the genus *Buchnera* (Munson) has attracted extensive attention (Tamas et al. 2002, Koga et al. 2003, Moran 2003). Transovarial transmission is a common characteristic of the biology of symbionts in both auchenorrhynchans (e.g., leafhoppers and planthoppers) and sternorrhynchans (e.g., aphids, whiteflies, psyllids, and mealybugs) (Buchner 1965). This vertical mode of transmission generates a high level of dependence between host and symbiont (Douglas 1996, Wernegreen 2002).

In contrast, oral transmission is the strategy used by many bacterial symbionts of the suborder Heteroptera (true bugs) (Buchner 1965). Mechanisms for oral transmission of microbial symbionts include coprophagy (Beard et al. 2002), ingestion of bacteria-filled capsules associated with egg masses (Fukatsu and Hosokawa 2002), and probing on the surface of eggs for symbionts smeared by females (Buchner 1965, Abe et al. 1995). This transmission strategy adds more challenges to the symbiotic association than the transovarial method because the bacterium must survive in an alternate environment outside the host (e.g., excrement or egg surface) and colonize an insect that might already have acquired other microbes from the environment. Sometimes, orally transmitted microbes can be cultured in vitro (Durvasula et al. 1997), suggesting the presence of more complete metabolic pathways than *Buchnera* or other endosymbionts that are vertically transmitted and to date cannot be cultured (Moran 2003).

Although advances have been made in recent years in our understanding of the biology of symbionts associated with various sternorrhynchans and blood-feeding heteropterans of medical importance, little is known about the caeca-associated symbionts of their plant-feeding heteropteran counterparts. Fukatsu and Hosokawa (2002) studied the microbes associated with the gastric caeca of *Megacopta punctatissima* Montandon, a stink bug in the family Plataspidae. *M. punctatissima* females lay eggs and symbiont-filled capsules, and hatching nymphs probe the capsules to acquire the symbiont. Physical removal and heat treatment of capsules impaired nymphal development

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(Fukatsu and Hosokawa 2002). The proposed transmission mode of symbionts for pentatomids (egg smearing) is different from plataspid bugs, but the general organization of the alimentary tract and caeca in which they are present is similar (Goodchild 1963, Buchner 1965). As with plataspids, elimination of caeca-associated symbionts (by surface sterilization of egg masses) was shown to negatively affect pentatomid fitness (Buchner 1965, Abe et al. 1995).

Although bacteria have been known to be associated with the gastric caeca of Pentatomidae since the late 1800s, these microbes have not been identified. The first comprehensive work on this topic was done by Glasgow (1914). He observed that although bacteria from caeca of different hosts were morphologically different, bacterial morphology was constant within each host species, and they were always present in an apparent monoculture. Those results led Glasgow to suggest that these bacteria were adventitious and that their major function was to exclude or inhibit other microbes from multiplying in the caeca. Kuskop (1924) and Rosenkranz (1939), cited by Buchner (1965), suggested that these bacteria had symbiotic roles related to insect nutrition. Rosenkranz (1939) introduced the hypothesis that transfer of symbionts occurred orally, suggesting that females coated their eggs with bacteria, which were acquired by nymphs after hatching.

Despite the large number of studies on the identification and phylogenetic placement of hemipteran endosymbionts among other bacteria, the position of many taxa is inconclusive (Fukatsu and Nikoh 1998, Thao et al. 2002, Aksoy 2003). Variability in phylogenetic associations may be due to the taxa used in the analysis or to methods of tree construction, which can lead to inconsistent trees (Brocchieri 2001). Both distance-based and maximum parsimony (MP) methods have been shown to be vulnerable to homoplasy when the data sets cover relatively distantly related groups of organisms and deeper time divergences (Herbeck et al. 2005), probably because of substitution saturation of base pairs and possible nucleotide heterogeneity. Longer phylogenetic branches "attract" each other, leading to unification or proximity of taxa that are actually not closely related (Felsenstein 1978, Graybeal 1998, Hillis 1998). This homoplasy is exacerbated by nucleotide heterogeneity between taxa in analyses (Steel et al. 1995, Galtier and Gouy 1998, but see Conant and Lewis 2001, Rosenberg and Kumar 2003). Endosymbiont systematic studies frequently use distance and parsimony-based models, which are vulnerable to this phenomenon (Herbeck et al. 2005). Maximum likelihood (ML) is able to more effectively analyze data sets with different evolutionary rates, as is the case for data sets in the Gammaproteobacteria that include insect endosymbionts (Moran 1996). An additional confounding factor in sequence analysis may be that the alignments of 16S rRNA sequence differ in length, and most studies eliminate any sequence data that is not represented in all of the taxa in an analysis. Such a "no-gaps" data matrix makes all taxa of equal sequence length, but it eliminates large portions of data.

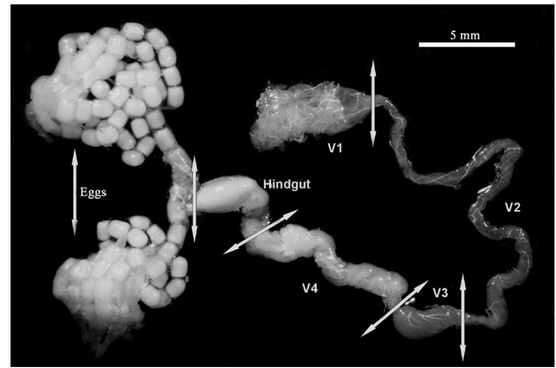

Fig. 1. *N. viridula* adult female gut sections relevant to this study. Picture shows different midgut sections (V1–V4), hindgut, and eggs and ovarioles. Lines illustrate regions of the midgut that were cut for PCR detection of symbiont.

We identified a bacterium molecularly that is consistently associated with the caeca of *Nezara viridula* (L.) (Hemiptera: Pentatomidae), an economically important and cosmopolitan pest of various crops. To phylogenetically place this bacterium and to assess the potential impact of homoplasy on endosymbiont phylogenetic reconstruction, we compared the results from distance and parsimony models with an optimized ML model designed to better account for homoplasy (Huelsenbeck 1997, Posada and Crandall 1998). We also showed that aposymbiotic insects can be generated after surface sterilization of egg masses.

Materials and Methods

Insects. We established an *N. viridula* laboratory colony in summer 2003 with adults collected from legumes growing adjacent to a macadamia farm in Hilo, HI. We occasionally added other individuals, collected at the same area, to the colony. Insects were reared in plastic containers in an insectary room with controlled temperature (25 ± 2°C). Green beans and peanuts were used as diet (Todd 1989). *N. viridula* adult females from California, South Carolina, and Florida were used to determine whether the bacterium was present in geographically distinct insect populations.

Transmission Electron Microscopy. The last section of the midgut (V4; Fig. 1) of second instars was dissected for transmission electron microscopy. We fixed the tissue in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.45. Samples were post fixed in 1% OsO_4 in 0.1 M cacodylate buffer, dehydrated through a graded series of alcohol, substituted with propylene oxide, and then infiltrated and embedded in LX-112 epoxy resin (Ladd Research Industries, Burlington, VT). Ultrathin sections were taken on a Reichert Ultracut E ultramicrotome and photographed on a LEO 912 EFTEM at 80 kV with a Proscan frame-transfer charge-coupled device.

Table 1. Information on taxa used for phylogenetic analysis

Bacterium (or insect host[a])	Accession no.	Biological information	Host	No. sites
Baumannia cicadellinicola (Moran et al.)	AF465793	Primary symbiont	Leafhopper, *Homalodisca coagulata* (Say)	1,432
Cand. *Blochmannia rufipes* (Sauer et al.)	X92552	Primary symbiont	Ant, *Camponotus rufipes* (Fabricius)	1,450
Buchnera aphidicola–Ap (Munson et al.)	M27039	Primary symbiont	Aphid, *Acyrthosiphon pisum* (Harris)	1,421
Buchnera aphidicola–Mr (Munson et al.)	M63255	Primary symbiont	Aphid, *Melaphis rhois* (Fitch)	1,436
Wigglesworthia glossinidia (Aksoy et al.)	AB063521	Primary symbiont	Tse-tse fly, *Glossina brevipalpis* (Newstead)	1,425
SOPE (Akman et al.)	AF005235	Principal symbiont	Weevil, *Sitophilus oryzae* (L.)	1,417
Cand. *Hamiltonella defensa* (Moran et al.)	AF293616	Secondary symbiont	Aphid, *A. pisum*	1,422
Sodalis glossinidius (Dale and Maudlin)	AF548136	Secondary symbiont	Tse-tse fly, *G. brevipalpis*	1,414
[1]*Paratrioza cockerelli* (Spaulding and von Dohlen)	AF286127	Secondary symbiont	Psyllid, *Paratrioza cockerelli* (Sulc)	1,417
Cand. *Serratia symbiotica* (Moran et al.)	M27040	Secondary symbiont	Aphid, *A. pisum*	1,416
[1]*Megacopta punctatissima*	AB067723	Caeca-associated symbiont	Stink bug, Plataspidae	1,420
[1]*Nezara viridula*	AY679762	Caeca-associated symbiont	Stink bug, Pentatomidae	1,416
Xenorhabdus poinarii (Akhurst)	X82253	Pathogen/symbiont	Insect/nematode	1,418
Erwinia herbicola (Lohnis)	AF290417	Pathogen	Plant	1,407
Pantoea agglomerans (Ewing and Fife)	AJ583011	Pathogen	Plant	1,377
Cand. *Phlomobacter betae* (Gatineau et al.)	AY057392	Pathogen	Plant/insect	1,418
Escherichia coli (Migula)	AE000452	Pathogen	Animal	1,415
Klebsiella pneumoniae (Schroeter)	AF453251	Pathogen	Animal	1,415
Salmonella typhi (Schroeter)	U88545	Pathogen	Animal	1,415
Serratia marcescens (Bizio)	AF124042	Pathogen	Animal	1,416
Yersinia pestis (Lehmann and Neumann)	AJ232236	Pathogen	Animal	1,417

[a] Insect host of bacterial symbiont.

Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism, and Sequencing. We used standard procedures for all experiments unless noted. We dissected 10 adult females under a microscope, extracted the gut, and transferred the last portion of the midgut with four rows of gastric caeca (segment V4; see below for description) to a tube with sterile phosphate-buffered saline (PBS). Samples were washed multiple times with PBS to eliminate surface contaminants and ground with a plastic pestle. DNA was extracted with a commercial DNA extraction kit (#A1120, Promega, Madison, WI). Primers 16SA1 and 16SB1 were used for amplification of a fragment of the 16S rRNA gene from bacteria present in samples as described previously (Fukatsu and Nikoh 1998). PCR products were ligated into vector pGEM-T Easy (#A1360, Promega). *Escherichia coli* DH5α was transformed with plasmids, and mutants were selected on solid media with ampicillin (70 μg/ml). Clones were grown overnight at 37°C on LB liquid medium with ampicillin, and plasmids were extracted (#27104, QIAGEN, Valencia, CA). We amplified the inserts using flanking sequences of the vector as primers (T7 and Sp6 promoters) and determined fragment size. Restriction enzymes RsaI, DdeI, SauIIIaI, and TaqI were used for restriction fragment length polymorphism analysis of the insert. Sequencing was done for eight clones (clones obtained from different individuals) with plasmids as template, by using 16SA1 and 16SB1 primers initially, then with internal primers designed based on the preliminary reads obtained. Sequencing was done at the Greenwood Molecular Biology Facility (Pacific Biomedical Research Center, University of Hawaii at Manoa, Honolulu, HI).

PCR Detection of Symbiont. The forward primer 16S-PNv (5′-GCCTAATATGCATGATC-3′) was designed for *N. viridula*'s caeca symbiont based on sequences obtained and sequence comparisons with other bacteria and used with the reverse primer 16SB1 (Fukatsu and Nikoh 1998) for PCR amplification of an ≈1-kb fragment of the 16S rRNA gene of the symbiont. The temperature profile was: 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min, 71°C 2 min, and one cycle at 71°C for 5 min. We compared detection of bacteria in caeca and *N. viridula*'s thorax muscles, leafhoppers [*Macrosteles* spp. (Hamilton)], and fruit flies (*Bactrocera* spp.). To determine the location of the bacterium in the midgut of individual insects, we used 10 other adult females from our colony and dissected them in sterile PBS. The midgut and ovarioles were transferred to a clean glass slide, and samples were washed multiple times during and after dissection to reduce superficial bacterial contamination. Tissue was cut and transferred to 100 μl of PBS; dissected samples included ovarioles and midgut sections V1, V2, V3, and V4, all individually tested (Fig. 1; Silva and Terra 1994). We also dissected 10 fourth instars of *N. viridula*, but midgut sections V1, V2, and V3 were combined into one sample, and V4 was tested separately. In this case, V1+V2+V3 were separated from V4 by cutting the constriction tissue separating these sections of the midgut. DNA from samples was extracted as described previously.

Phylogenetic Analysis. For the phylogeny, we selected bacteria that included primary and secondary endosymbionts from a range of families of sap-sucking Hemiptera and other insect orders as well as free-living bacteria within the Enterobacteriaceae (Table 1). One clone of *N. viridula* was used for our

phylogenetic analysis. All other bacterial nucleotide data were downloaded from GenBank (Table 1). Sequences were aligned with ClustalW version 1.83 (Chenna et al. 2003) and manually checked with MacClade version 4.05 (Maddison and Maddison 2002). Bacterial sequences used for analysis ranged from 1,377 to 1,450 bases (Table 1). After alignment, all sequences were cut at the first and last bases of *N. viridula.* All phylogenetic analyses were conducted using PAUP* 4.0b10 (Swofford 2002). Distance (neighbor-joining [NJ]) and MP searches were performed using all defaults. ModelTest version 3.5 (Posada and Crandall 1998) was used to optimize an ML model for the data set with the Akaike Information Criterion for both the "gaps" and "no gaps" data sets (see below). PAUP was used to implement the models recommended by ModelTest under an ML analysis. Bootstrap values for NJ, MP, and ML analyses (1,000 replicates) were calculated in PAUP*; decay index values were performed using TreeRot (Sorenson 1999).

The effects of retaining or eliminating gaps from the molecular data set also were investigated using identical search methods for a gaps and no gaps data set. Gaps, genomic regions in which insertions or deletions have occurred for some of the taxa in an analysis, are often removed during sequence alignment of bacterial 16S rRNA. To test the importance of gaps in our data set, we conducted identical phylogenetic analyses on data sets for which we removed nucleotides for all taxa in which one or more taxa had insertion/deletions (no gaps) and compared the results with data sets in which we left gaps as they occurred in the sequence (gaps). For the gaps data set, gaps were coded as missing data. All trees were rooted with *Pantoea agglomerans* (Ewing and Fife) and *Erwinia herbicola* (Lohnis), because they were hypothesized to represent likely outgroups for the other bacteria chosen for the analyses.

Surface Sterilization of Egg Masses. We surface sterilized eggs by dipping them for 5 min into 95% ethanol followed by 5 min in a 10% bleach solution. Eggs were allowed to air dry, and nymphs hatched normally. We sterilized eggs ≈4 to 5 d after oviposition; nymphs usually hatched within 6 d. Twenty egg masses were used for this experiment; each mass was divided into two halves, one half was nontreated (control group) and the other half was surface sterilized (treated group). All hatching nymphs were allowed to develop to adulthood and then maintained in individual containers. For each of the cohorts, we randomly selected insects and sampled the following (by PCR) for presence of the symbiont: 1) egg mass after nymphs hatched, 2) two fifth instars (V4), 3) one adult male (V4), and 5) one adult female (V4). In a few cohorts, we had high mortality or smaller number of insects available; thus, the total number of samples tested was smaller than the initial numbers might suggest. Adults from this first generation were combined into a cage (treatments separated) and allowed to mate and lay eggs. If any individual of a cohort in the first generation tested positive for the symbiont, the entire cohort

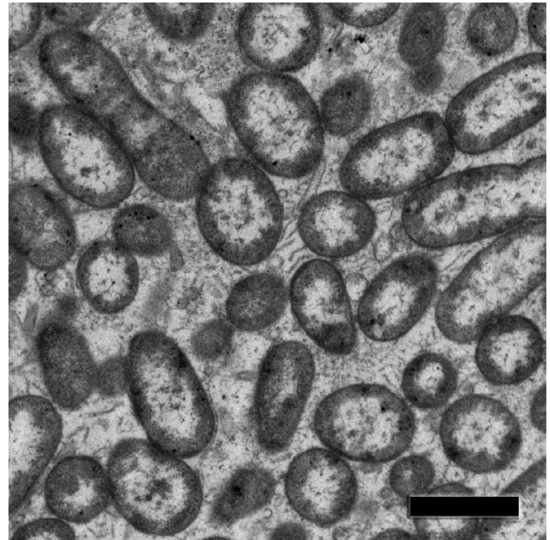

Fig. 2. Transmission electron microscopy cross section of the lumen of a crypt in the V4 section of a second instar *N. viridula* shows presence of bacteria. Bar = 1 μm.

was excluded from the mating cage. Offspring (second generation, from 12 symbiotic and 9 aposymbiotic egg masses) were reared to adulthood and sampled for presence of the symbiont as described for the first generation. We also counted, for both generations, the day the first nymph of each cohort molted into the next instar. We did not identify these individuals; thus, our observations may have been made on different individuals from the same cohort.

Results

Midgut Organization. The midgut of a female adult *N. viridula* can be divided into four sections (ventricula): V1, V2, V3, and V4; gastric caeca are associated with V4 (Fig. 1). These four regions are similar to those observed in other Pentatomidae (Goodchild 1963, Silva and Terra 1994). However, when second, third, fourth, and fifth instars were dissected, all had a saclike section constricting the flow of ingested food from V3 to V4 (data not shown). This constriction was not observed in adults. Transmission electron microscopy sections of V4 showed the presence of bacterial rods in the V4 section of the midgut of nymphs (Fig. 2).

Restriction Fragment Length Polymorphism and Sequencing. Forty-two of 45 clones had the expected ≈1.5-kb insert (checked by PCR); all 10 insects were represented by at least one clone but no more than five clones. All 42 inserts had the same restriction fragment length polymorphism profile, with only one exception (an insert with sequence similarity to the genus *Brevibacterium*, which was eliminated from further analysis). Clones from different individuals were sequenced on both strands, and the partial 16S rRNA sequence (1,416 bp) was identical for all clones (with the exception of four nucleotides, assumed to be am-

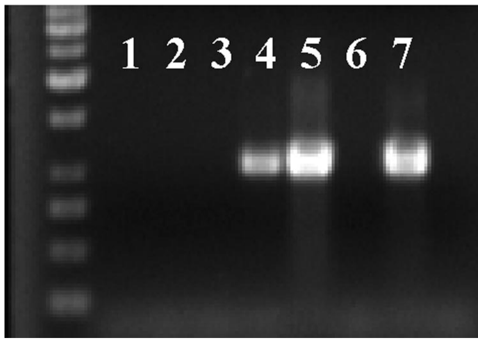

Fig. 3. PCR amplification of partial 16S rRNA gene of caeca-associated symbiont of a dissected adult female (lanes 1–5) and a fourth instar (lanes 6 and 7) of *N. viridula* by using caeca-symbiont-specific primers. Adult female: lane 1, ovariole; lane 2, V1; lane 3, V2; lane 4, V3; lane 5, V4. Fourth instar: lane 6, V1+V2+V3; and lane 7, V4. Fragments of ≈1 kb, expected with the primer pair used, indicate positive samples.

plification errors). One representative sequence was selected for phylogenetic analysis (AY679762).

PCR Detection of Symbiont. All DNA extracts from V4 yielded a single band of ≈1 kb after PCR, as expected (Fig. 3). DNA extracted from *N. viridula* thoracic muscle did not generate a PCR amplicon by using the same primers. The primer set did not generate a product when template DNA from leafhoppers or fruit flies was used. We also digested amplicons with restriction enzymes as described above. Digests matched the results expected when NEBcutter 2.0 (http://tools.neb.com/NEBcutter2/index.php) was used with the sequence from the 16S rRNA fragment expected to be amplified by the primers. Insects from California were tested for the presence of the symbiont by using these primers ($n = 20$ individuals); all these insects were positive. Some of the amplicons were subjected to restriction fragment length polymorphism, and these samples had the same expected profiles. We also sequenced the amplicon of individuals (V4 gut section) collected in California, South Carolina, Florida, and a macadamia farm in Hilo, HI. Amplicon sequences (≈1 kb) were identical to our initially obtained sequence. We also retrieved sequence AY830409 (16S rRNA) from GenBank, obtained from the midgut of *N. viridula* (location of insect collection not identified), which was also identical to that of our bacterium. These results suggest that this bacterium is consistently associated with the V4 section of *N. viridula*'s midgut regardless of geographic location.

We detected the symbiont in different sections of the midgut of *N. viridula* adults compared with nymphs (Table 2; Fig. 3). Symbionts occurred in both V3 and V4 sections of the adults, and in one adult we observed faint bands for V1 and V2 as well. The latter may have been surface contaminants or low titers of bacteria in these anterior sections of the midgut. Symbionts were only detected in V4 of nymphs, however, and none of the tested individuals was positive for any of the three other anterior sections of the midgut.

Table 2. Detection of *N. viridula* caeca-associated symbiont in different tissues of adult females and fourth instars

	Ovarioles	V1[a]	V2	V3	V4
Adult females[b]	0/10	0/10	0/10	9/10	10/10
			V1+V2+V3		V4
Fourth instars[b]			0/10		10/10

[a] Individual midgut sections sampled
[b] Number of PCR-positive samples/number of individuals tested.

Phylogenetic Analyses. *No Gaps Data Set.* The NJ model under distance parameters gave a tree that was different from the parsimony consensus tree only in its level of resolution but not in any relationships between taxa (Fig. 4). Most interesting, perhaps, is the placement of the *N. viridula* symbionts branching basally or unresolved in relation to a clade containing other endosymbionts. Under the MP model, the data set for which gaps were eliminated yielded three most parsimonious trees of 1,106 steps, with 265 characters that were parsimony informative. The strict consensus of the three trees suggests a single, poorly resolved origin for hemipteran endosymbionts (Fig. 4). ModelTest chose a general time reversible model with a gamma distribution and a portion of invariable sites estimated from the data (best-fit model = GTR+I+G). The ML tree (Fig. 4), likelihood score $-\ln(L) = 7082.07704$, differed from the MP and NJ trees and suggests two independent origins for hemipteran primary endosymbionts, with the *N. viridula* endosymbiont in a clade containing *M. punctatissima* and *Buchnera.*

Gaps Data Set. For the data set in which all gaps were retained, there was a single best MP tree of 1,288 steps (Fig. 4), with 300 parsimony-informative characters. It differed from the no gaps tree in that the topology was better resolved (one best tree versus three for no gaps) for most terminal taxa, but the two trees did not conflict. The NJ analysis with gaps and no gaps have differences in the placement of the *N. viridula* symbiont, but they were slight and poorly supported by the bootstrap analysis (Fig. 4). ModelTest suggested the same GTR+I+G model as for the no gaps data set. The ML analysis gave a tree that was identical to the ML analysis under the no gaps data set, with a likelihood score of $-\ln(L) = 8013.34941$.

Surface Sterilization of Egg Masses. Nymphs hatching from surface-sterilized eggs were mostly free of the symbiont, as were adults that developed from these nymphs (G1) (Fig. 5). A high percentage of treated egg masses in this first generation, however, were positive for the bacterium. The symbiont was transferred to the second generation (G2) of the *N. viridula* control cohorts but not to G2 of the treated cohorts. The absence of the symbiont in a few individuals from control cohorts may be due to incomplete vertical transmission of the bacterium or detection limitations. We found no difference in the number of

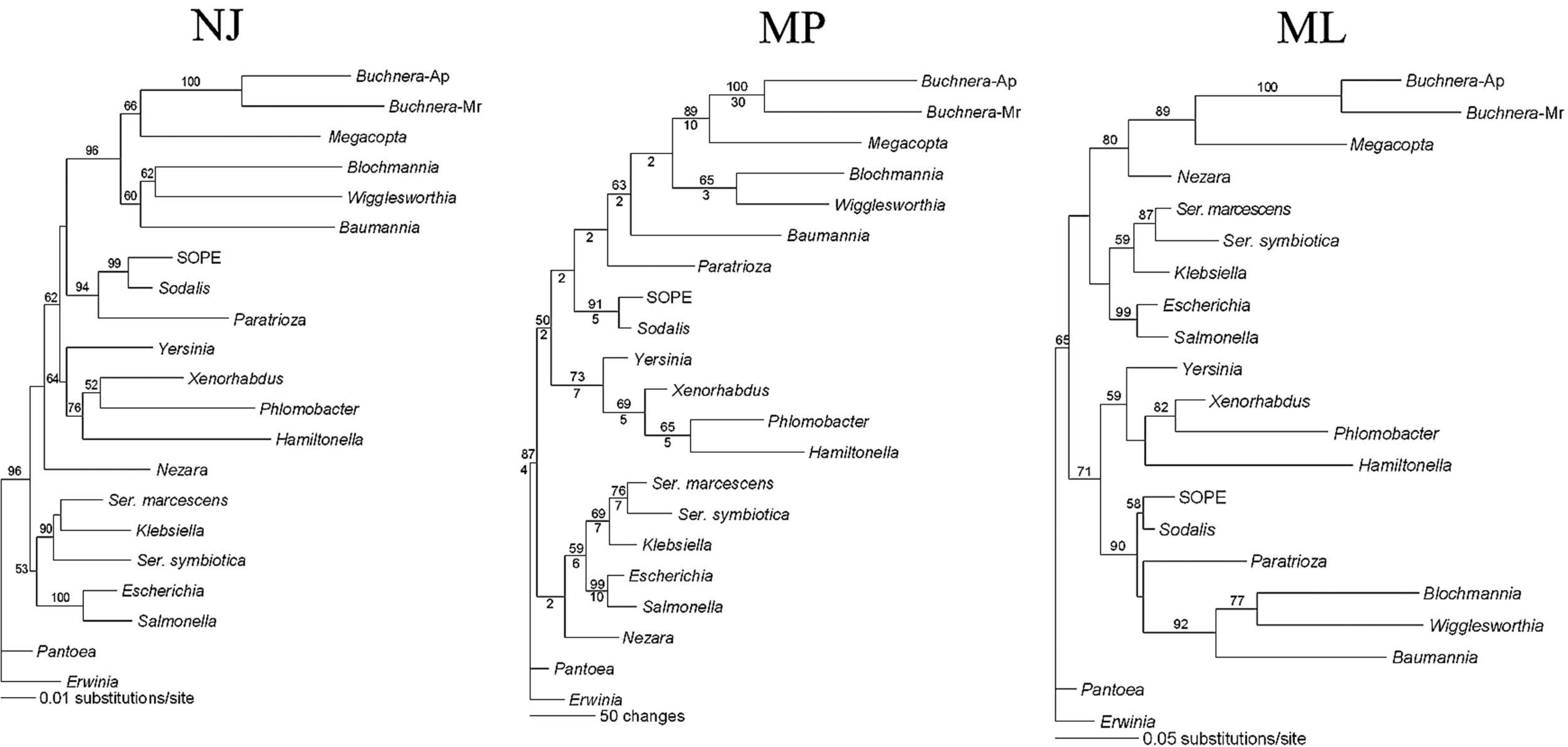

Fig. 4. For all three models, data set used is with gaps, clades retained by heuristic searches, but not represented in the 1,000 replicate bootstrap consensus, automatically have bootstrap values of <50%. Bootstrap values above nodes, and for MP tree, decay index values below nodes. See Table 1 for details about sequences used. Note the support for multiple origins for endosymbionts and placement of the *Nezara* symbiont. NJ no gaps data set phylogram was similar except that P-*Nezara* was placed as sister to all other endosymbionts, although this had poor branch support. MP no gaps phylogeny did not conflict but consensus tree was poorly resolved. ML no gaps topology was identical.

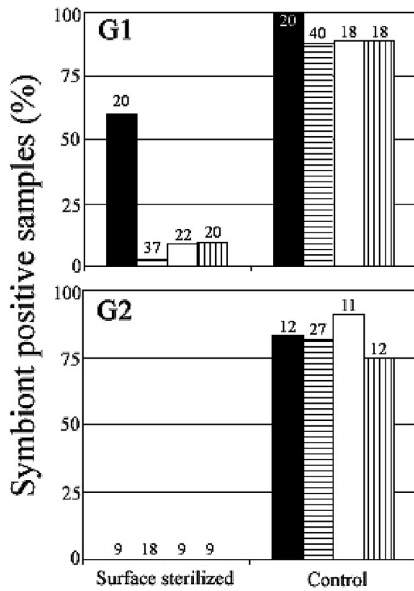

Fig. 5. Effect of egg surface sterilization on the presence of caeca-associated symbiont of *N. viridula* over two generations. Eggs were treated only in the first generation (G1). Adults from symbiont-free cohorts of G1 were combined and started a second generation (G2). Numbers on top of bars indicate total number of individuals tested by PCR for symbiont's presence. Black bar, egg mass; bar with horizontal lines, fifth instar V4; white bar, adult male V4; and bar with vertical lines, adult female V4.

days required for the first nymph in each cohort to molt to its subsequent instar in the different treatments studied (Fig. 6).

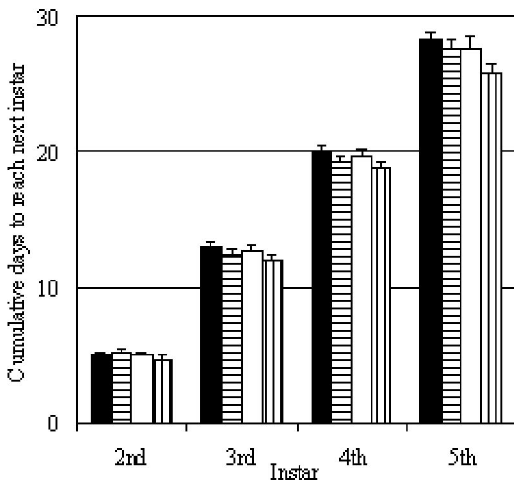

Fig. 6. Mean length of time (cumulative number of days) taken for the first nymph to molt from one stage to the next. Black bar, first generation of surface sterilized eggs; horizontal lines, first generation control; white bar, second generation of surface sterilized eggs; and vertical lines, second generation control. Standard error for each treatment shown on top of bars.

Discussion

We have molecularly identified a bacterial symbiont associated with the caeca of *N. viridula.* This bacterium is found in Hawaii, California, and South Carolina stink bug populations and may be the major bacterium found in the midgut section with caeca (V4) of this insect. The 16S rRNA partial sequence of the symbiont indicates that it is an Enterobacteriaceae. The sister clade in our ML analysis contains the symbiont of another hemipteran, in the family Plataspidae (Fukatsu and Hosokawa 2002). Our experiments strongly suggest that transmission occurs orally rather than transovarially. Surface sterilization of eggs generated aposymbiotic individuals, and aposymbiotic individuals survived well in our laboratory conditions.

The presence of the bacterium in the V3 and V4 sections of the midgut of adult females but in only V4 in fourth instars suggests a difference in gut morphology between adults and nymphs, as reported previously (Goodchild 1966). These results support previous reports that there is a blockage in the alimentary canal of pentatomids between V3 and V4 midgut sections in nymphs but not in adults (i.e., bacteria also present in V3 of adults). In this study, dissection of second to fifth instars of *N. viridula* showed the presence of a blister between these sections in all insects analyzed (data not shown). Detection of the bacterium in V3 in adults may have occurred after movement of cells during dissection rather than natural placement. It is unclear whether first instars have the restriction between V3 and V4, but our results raise the question of how bacterial cells reach the V4 region during initial stages of colonization.

Glasgow (1914) demonstrated that the alimentary tracts of pentatomid embryos contained bacteria before hatching, suggesting transovarial transmission of the symbiont. However, Rosenkranz (1939) suggested that transmission does not occur transovarially but rather when nymphs probe on the surface of eggs after hatching. Lockwood and Story (1986) tested this hypothesis by surface sterilizing egg masses of *N. viridula* with 15% formalin for 20 min and determined that sterilization had no effect on nymphal mortality. Abe et al. (1995) showed that different surface sterilization treatments resulted in variable developmental rates for another pentatomid, *Plautia stali* (Scott). Thus, researchers studying the effect of surface sterilization of Pentatomidae eggs on insect fitness have produced conflicting results. We were able to eliminate a caeca-associated symbiont in the majority of *N. viridula* given access only to surface-sterilized egg masses after hatching. We observed no obvious fitness decrease in those insects over two generations, but we did not measure enough parameters for a conclusive answer regarding the importance of this association. Furthermore, we reared insects under controlled conditions with a constant supply of food. In the future, it would be worthwhile to study in detail the development and reproductive rate of aposymbi-

otic stink bugs under a range of conditions, including limited or unbalanced diets.

Because the partial 16S rRNA data set used here represents relatively large periods of evolutionary time during which unrelated endosymbionts were exposed to convergent genomic selection, including an AT bias, it is not surprising that there is a strong homoplastic signal pulling all of the endosymbionts into false monophyly. Extreme nucleotide heterogeneity, and the substitution saturation that comes with long divergence times is almost certainly the cause of the "long-branch" attraction we found with the distance (NJ) and MP models, and the phenomenon has been suggested by other studies (Brocchieri 2001, Huson and Steel 2004). Branch support for the NJ and MP trees is highly variable, and because the bootstrap represents a resampling of a subset of the original data (under the same specified model), it must be interpreted cautiously because it is not a measure of the accuracy of a particular model (Brocchieri 2001). Bootstrapping also assumes independence of nucleotides in the sequence and equal evolutionary rates among taxa, assumptions not valid for 16S rRNA data sets of bacteria that include insect endosymbionts (Moran 1996). Some minor topological differences were observed in analyses with and without gaps. However, those occurred primarily as branch swaps in nodes with low statistical support, or as a result of poor resolution within and between basal branching clades, and not taxa. This suggests that the elimination of gaps may not be affecting phylogenetic relationships in our data set. The placement of the endosymbiont we describe is ambiguous and poorly resolved with NJ and MP analyses. The MP analysis had better resolution than NJ with the data set that included gaps. This is likely because the no gaps data set eliminated 35 parsimony-informative characters. One of the three most parsimonious trees from the no gaps data set was congruent with the gaps data set MP tree. Although the MP and ML trees do not agree, this is probably due to homoplasy in the data set and is not a function of the elimination of gaps. Resolution and consistency were improved under a ML analysis, likely because the ML is better able to model data with varying evolutionary rates and nonconstant levels of AT richness. Concerns about AT bias and its effect on endosymbiont phylogeny have been raised previously (Fukatsu and Nikoh 1998, Charles et al. 2001). Small AT-rich genomes are probably a response to the similar, simplified, ecological conditions faced by endosymbionts (Herbeck et al. 2003) and therefore represent convergent evolution because of similar evolutionary pressures (Moran 2002).

Few recent studies have addressed the symbiotic flora of plant-feeding heteropterans. Fukatsu and Hosokawa (2002) identified the symbiont of *M. punctatissima* (Plataspidae), which was the bacterium phylogenetically closest to *N. viridula*'s symbiont in our ML analyses. Although these are the only stink bugs for which caeca symbionts have been molecularly identified, differences and similarities between these organisms are worth noting. Both symbionts are primarily restricted to the caeca of their host, but one symbiont is transferred to the offspring through capsules and the other symbiont likely through egg smearing by adult females during oviposition. Although ML phylogenetic analysis suggests that the stink bug symbionts are closely related, the AT content of the 16S rRNA gene of these bacteria is different (partial sequence analyzed had 45.6 [*N. viridula*] and 50.6% [*M. punctatissima*] AT content). It has been demonstrated that vertically transmitted symbionts have reduced genomes and AT bias (Wernegreen 2002). Similarly, the differences in AT content suggest that an organism transmitted within a capsule, without having to survive and compete on the surface of eggs, may undergo a higher rate of genome degeneration, as found in those bacteria that are transovarially transmitted.

The consistent association with *N. viridula* suggests that this symbiont may provide benefits to its host. However, this system is substantially different from intracellular symbiotic associations of other plant-sucking insects (e.g., aphids and *Buchnera*). The symbiont seems limited to a specific region of the midgut and to be orally transmitted to offspring. Additionally, under laboratory conditions, the host seems to survive and reproduce equally well without the bacterium, suggesting a lower level of dependence between the organisms.

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