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Phylogenetic Placement of Pentatomid Stink Bug Gut Symbionts

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Abstract Insect bacterial symbionts are ubiquitous, however, only a few groups of host families have been well studied in relation to their associations with microbes. The determination of the phylogenetic relationships among bacteria associated with different species within an insect family can provide insights into the biology and evolution of these interactions. We studied the phylogenetic placement of vertically transmitted bacterial symbionts associated with the posterior midgut (crypt-bearing) region of pentatomid stink bugs (Hemiptera, Pentatomidae). Our results demonstrate that different host species carried one major bacterium in their midgut. Phylogenetic analyses of the 16S rRNA gene sequences obtained from the midgut of stink bugs placed all symbionts in a clade with *Erwinia* and *Pantoea* species, both plant-associated bacteria. Results indicate that symbiont monophyly occurs among recently diverged taxa (e.g., within a genus) but does not occur in the Pentatomidae. Results suggest that these vertically transmitted symbionts are occasionally replaced by other taxonomically similar bacteria over evolutionary time. Our findings highlight how the evolutionary history of hemipteran symbionts in unexplored host families may have unpredictable levels of complexity.

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Introduction

Insects have a diversity of associations with bacterial symbionts [1]. Most well-characterized vertically transmitted insect symbioses are associations where one bacterial taxon resides within specialized cells of their respective hosts [2]. At the opposite extreme are gut-residing bacteria of various insect groups, such as termites, which have extremely complex microbial communities that assist with the digestion of nutrients. Although advances in molecular biology have allowed for in-depth studies of some of these systems, the vast majority of insect-microbe symbiotic associations are yet to be characterized.

The biology of heteropteran (Insecta, order Hemiptera, suborder Heteroptera) symbionts has been documented but remains poorly understood [1]. Pentatomorphan insects (e.g., stink bugs, shield bugs) have been reported to carry vertically transmitted symbionts in crypts in the midgut lumen. Buchner [1] summarized what was known about these systems before the advent of molecular tools, and recent research on the biology of pentatomorphan gut symbionts corroborates earlier work. It has been shown that gut symbionts in the family Plataspidae (shield bugs) are vertically transmitted via symbiont-filled capsules, resulting in strict host-symbiont cospeciation [3, 4]. Studies with another family of true bugs, Alydidae (broad-headed bugs), demonstrated that their gut symbionts belong to the genus *Burkholderia*, but those bacteria do not form a monophyletic group and are acquired every new generation from the environment [5, 6]. Therefore, despite the fact that the gut symbionts of both plataspids and alydids colonize a similar environment within hosts, the biology of bacterial transmission to new generations and evolutionary history are strikingly different. In the case of pentatomids (stink bugs, Pentatomidae), gut symbionts are smeared on the surface of

eggs by ovipositing females and are vertically transmitted from mother to offspring [1, 7]. However, the physiological role of these bacteria has not been determined and their putative mutualistic relationship with host insects remains controversial [1, 7–9]. The egg-smearing strategy may represent an intermediate state between the vertical and the environmental transmission of gut symbionts by plataspids and alydids, respectively. Thus, although vertical transmission occurs with the egg-smearing strategy, bacteria are acquired from an environment that may be prone to contamination by or competition with other microbes. If that is the case, one would expect that pentatomid gut symbionts have some degree of cospeciation with their host insects, but also find evidence of horizontal transmission. To determine the phylogenetic placement of pentatomid gut symbionts, we characterized the gut bacterial community of nine species of stink bugs.

Materials and Methods

Bacterial Community in the Gut of Stink Bugs

Insects were field-collected in California and Hawaii, and reared in the laboratory by our group and two research groups in California (K. Daane and J. Millar, University of California, Berkeley and Riverside, respectively). We kept insects at −80°C prior to dissection. The species *Euschistus heros* was collected in Brazil (Piracicaba, Sao Paulo State) and shipped to Hawaii in 70% alcohol. Taxa were selected to determine the relationship of gut symbionts present within pentatomid hosts in a genus (*Chlorochroa*), tribe (Pentatomini) and among tribes in the family (see Fig. 1). We dissected two adult females of each of the nine species studied (except *E. heros*, for which only one female was used) under a dissecting microscope, extracting the midgut and transferring it to a clean glass slide (see Table 1 for a list of species). The posterior section of the midgut (V4, crypt- or ceca-bearing region) was then cut, washed, and transferred to a lysis buffer for DNA extraction as previously described [7]. Only this section of the gut was used for the work described here. We sterilized dissecting tools after each insect by flaming them after ethanol rinses. To reduce the chance of surface contaminants, we rinsed the whole midgut at least three times with sterile phosphate-buffered saline (PBS) buffer and subsequently rinsed only the posterior midgut. We extracted total DNA from the posterior midgut of all individuals with a commercial DNA extraction kit (DNeasy; Qiagen, Valencia, CA). The bacterial 16S rRNA gene was partially amplified by PCR

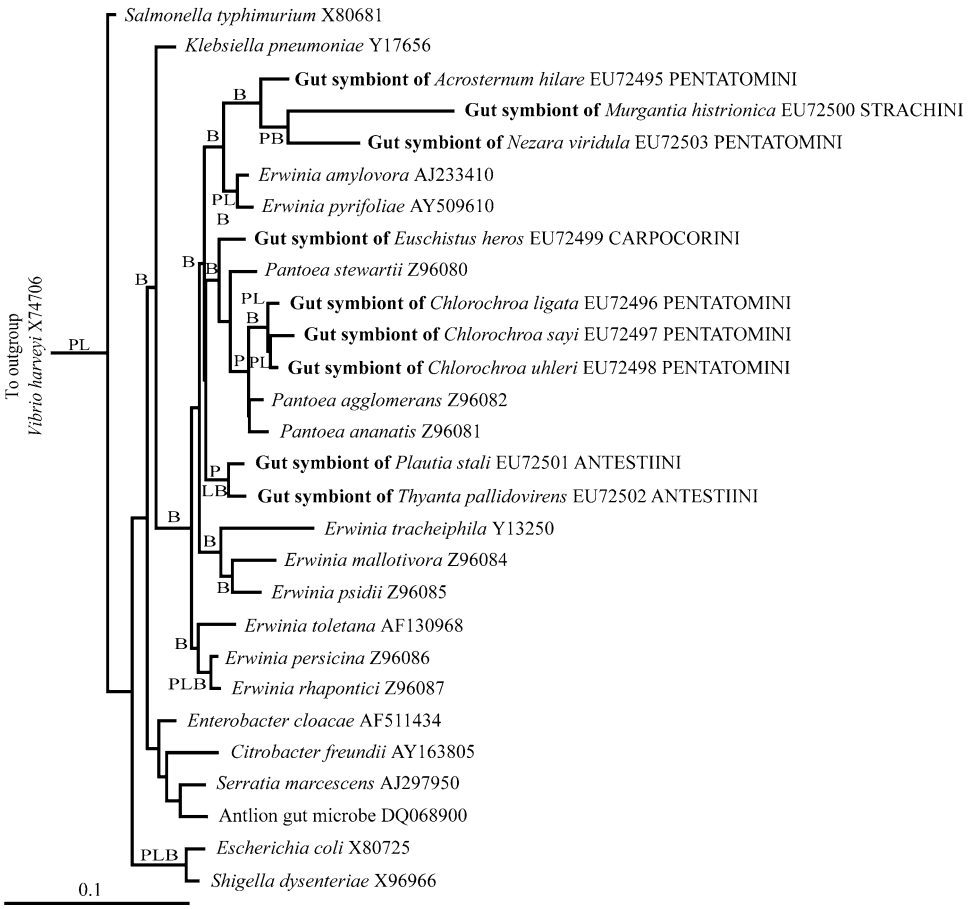

Fig. 1 Phylogenetic placement of pentatomid gut symbionts (in boldface) among closely related bacterial taxa. GS (pentatomid gut symbiont) precedes the species of insect host where the bacterium 16S rRNA gene sequence was obtained (in italics), which is followed by the accession number and pentatomid tribe to which the species belongs (in capital letters). The maximum likelihood tree is shown, but the tree topology was similar with maximum parsimony and Bayesian searches. Capital letters represent branch support with >70% bootstrap replicates using maximum parsimony (P; 1000 bootstraps) and maximum likelihood (L; 250 bootstraps) and with >90% support with Bayesian analysis (B)

Table 1 List of pentatomid species studied, including the number of taxa identified by RFLP analysis of the cloned 16S rRNA gene

Host species	RFLP of cloned 16S rDNA[a]	
	Dominant OTU[b]	Other OTU
Acrosternum hilare	44-44-44[c]	4-4-4
Chlorochroa ligata	44-44-42	0-0-2
Chlorochroa sayi	44-44-44	1-1-1
Chlorochroa uhleri	43-45-45	2-0-0
Euschistus heros	23-23-23	1-1-1
Murgantia histrionica	34-34-34	1-1-1
Nezara viridula	38-40-38	7-5-7
Plautia stali	43-43-43	2-2-2
Thyanta pallidovirens	49-48-25	0-1-0

[a] RFLP performed with three enzymes (*Dde*-*Hin*fI-*Rsa*I)

[b] Operational taxonomic unit

using 16SA1 and 16SB1 primers [10], and the amplicons were cloned into the pGEMT-easy vector following the manufacturer's instructions (Promega, Madison, WI). We determined the size of cloned fragments by amplifying inserts with flanking primers on the vector. Restriction fragment length polymorphism (RFLP) analysis of amplified products with three restriction enzymes (*Dde, Hinf*I, *Rsa*I) was conducted. Purified plasmids were submitted for sequencing of the 16S rRNA gene inserts of the dominant taxa determined by RFLP analysis at the Greenwood Molecular Biology Facility (Pacific Biomedical Research Center, University of Hawaii at Manoa, Honolulu).

Phylogenetic Analyses

The same sequence was obtained for both individuals from the same species sampled. The RDP Classifier [11] was used to infer the broader phylogenetic placement of these bacteria. After it was determined that the bacterial sequences obtained belonged to the Enterobacteriaceae, a preliminary phylogenetic analysis (maximum parsimony and likelihood searches) with 42 species of enterobacteria was conducted (data not shown). Based on these results, a second dataset was chosen for a more detailed analysis of the phylogenetic placement of pentatomid gut symbionts; *Vibrio harveyi* was used as an outgroup. Initial alignment of 16S rDNA sequences was done using the NAST alignment tool available at Greengenes (http://www.greengenes.lbl.gov) [12]. We used SeqMan II (Lasergene v5; DNASTAR, Madison, WI) to manually check the alignment. We searched all our sequences for the presence of chimeras using Bellerophon [13] and the Ribosomal Database II Chimera Check [11]. No chimeras were detected. Maximum parsimony analysis was conducted with PAUP* 4.0b10 [14] with 1000 bootstrap replicates. Modeltest 3.7 [15] was used to select a likelihood model (TIM + I + G model using the Akaike Information Criterion framework) for the maximum likelihood analysis, which we ran with PAUP*, with 250 bootstrap replicates for branch support. We also performed a Bayesian inference with MrBayes 3.1.2 [16], using MrModeltest 2.2 [17] for model selection. Posterior probability support for branches were based on 7500 trees (search parameters: 5 million generations, burnin = 2500, samplefreq = 500, 4 chains, GTR + I + G model selected by MrModeltest with Akaike Information Criterion). We used BioEdit 7.0.4 [18] to build a 16S rDNA sequence similarity matrix including several *Erwinia* and *Pantoea* taxa, in addition to sequences obtained here, to determine the degree of similarity among sequences; alignment was trimmed and insertions and deletions were deleted prior to analysis. The obtained sequences have been deposited in GenBank under accession numbers EU72495–EU72503.

Results and Discussion

Bacterial Community in the Gut of Stink Bugs

The posterior midgut of each stink bug species studied had one dominant bacterial taxon. The presence of other taxa in lower numbers, as determined by RFLP, may be the result of surface contaminants or the presence of other less abundant bacteria in that gut region (Table 1). The dominant operational taxonomic unit (OTU) recovered was considered to be the main bacterium associated with its respective host stink bug species, and these were the only ones used for phylogenetic analyses. Although all the OTUs from different species were most closely related to plant-dwelling bacteria in the genera *Erwinia* and *Pantoea*, there was moderate 16S rRNA gene sequence similarity to other species in those genera (Table 2). Following the general guideline that less than 98.7–99% 16S rDNA similarity is indicative of a different bacterial species [19], these gut bacteria would likely represent new undescribed species.

Recently, studies on three insect families (Plataspidae, Alydidae, and Pentatomidae) within the hemipteran suborder Heteroptera (true bugs) have shown that the posterior midgut is colonized by one dominant bacterium [3, 6, 7]. A sac-like structure, or blister, appears to precede this gut region in nymphs of pentatomids and nymph and adult plataspids [3, 7]. This physical blockage may explain how these orally acquired (after hatching) symbionts occur in what seems to be a monoculture. However, it raises interesting questions about the initial stages of symbiont colonization of the gut; specifically, how do these bacteria reach this region of the gut, and how do host insects select

Table 2 Sequence similarity matrix of the 16S rRNA gene of pentatomid gut symbionts (GS; followed by host species) and several *Erwinia* and *Pantoea* species

	GS *A. hilare*	GS *C. ligata*	GS *C. sayi*	GS *C. uhleri*	GS *E. heros*	GS *M. histrionica*	GS *N. viridula*	GS *P. stali*	GS *T. pallidovirens*	*E. amylovora*
GS *A. hilare*	ID									
GS *C. ligata*	0.941	ID								
GS *C. sayi*	0.945	0.985	ID							
GS *C. uhleri*	0.943	0.992	0.987	ID						
GS *E. heros*	0.957	0.966	0.963	0.966	ID					
GS *M. histrionica*	0.930	0.909	0.912	0.909	0.914	ID				
GS *N. viridula*	0.954	0.942	0.947	0.945	0.945	0.927	ID			
GS *P. stali*	0.956	0.971	0.969	0.971	0.974	0.914	0.950	ID		
GS *T. pallidovirens*	0.956	0.969	0.964	0.969	0.973	0.915	0.948	0.987	ID	
E. amylovora	0.964	0.953	0.950	0.953	0.966	0.919	0.940	0.966	0.966	ID
E. mallotivora	0.951	0.942	0.938	0.943	0.953	0.916	0.934	0.954	0.953	0.962
E. pyrifoliae	0.969	0.954	0.953	0.956	0.969	0.925	0.948	0.970	0.969	0.986
E. rhapontici	0.957	0.953	0.949	0.953	0.968	0.922	0.938	0.967	0.968	0.974
E. persicina	0.958	0.953	0.950	0.953	0.970	0.922	0.938	0.969	0.969	0.974
E. tracheiphila	0.941	0.938	0.936	0.938	0.939	0.906	0.933	0.946	0.943	0.940
E. psidii	0.953	0.951	0.948	0.948	0.958	0.920	0.943	0.960	0.958	0.958
E. toletana	0.946	0.962	0.956	0.961	0.962	0.919	0.943	0.967	0.967	0.961
P. stewartii	0.957	0.969	0.963	0.969	0.971	0.921	0.945	0.965	0.961	0.963
P. agglomerans	0.946	0.979	0.972	0.979	0.970	0.909	0.944	0.976	0.974	0.956
P. ananatis	0.952	0.979	0.974	0.979	0.968	0.912	0.951	0.974	0.970	0.957

	E. mallotivora	*E. pyrifoliae*	*E. rhapontici*	*E. persicina*	*E. tracheiphila*	*E. psidii*	*E. toletana*	*P. stewartii*	*P. agglomerans*	*P. ananatis*
GS *A. hilare*										
GS *C. ligata*										
GS *C. sayi*										
GS *C. uhleri*										
GS *E. heros*										
GS *M. histrionica*										
GS *N. viridula*										
GS *P. stali*										
GS *T. pallidovirens*										
E. amylovora										
E. mallotivora	ID									
E. pyrifoliae	0.961	ID								
E. rhapontici	0.958	0.971	ID							

Table 2 continued

	E. mallotivora	*E. pyrifoliae*	*E. rhapontici*	*E. persicina*	*E. tracheiphila*	*E. psidii*	*E. toletana*	*P. stewartii*	*P. agglomerans*	*P. ananatis*
E. persicina	0.959	0.974	0.992	ID						
E. tracheiphila	0.937	0.944	0.937	0.938	ID					
E. psidii	0.966	0.961	0.958	0.959	0.951	ID				
E. toletana	0.949	0.962	0.971	0.973	0.941	0.954	ID			
P. stewartii	0.956	0.966	0.959	0.961	0.945	0.966	0.961	ID		
P. agglomerans	0.949	0.958	0.958	0.958	0.943	0.955	0.961	0.971	ID	
P. ananatis	0.949	0.959	0.954	0.956	0.948	0.957	0.965	0.978	0.982	ID

Note: Accession numbers for taxa given in phylogenetic tree (Fig. 1)

for these organisms while eliminating others from this environment?

Phylogenetic Analyses

Because our sequences had moderate similarity to published sequences in databases, we first used the RDP Classifier [11] to infer the broader phylogenetic placement of these bacteria. All pentatomid gut bacteria were found to be closely related to *Erwinia* and *Pantoea*, both plant-associated genera. We conducted an analysis with pentatomid gut bacteria, various *Erwinia* and *Pantoea* species, and closely related taxa. Tree topology and branch support were similar with maximum parsimony (six most parsimonious trees were obtained), maximum likelihood, and Bayesian analysis (Fig. 1). All gut bacteria were placed in a strongly supported clade with *Erwinia* and *Pantoea* species. However, placement within that clade was variable. Symbionts were placed among *Erwinia* and *Pantoea* species, in three cases with strong branch support (except *E. heros*). The lack of monophyly is evident among taxa, as is the support for separate clades with symbionts. We observed monophyly for the genus tested, *Chlorochroa* spp., and for taxa in the tribe Antestiini—*P. stali* and *T. pallidovirens*. However, no monophyly was observed for individuals in the tribe Pentatomini. These results suggest that (i) this symbiotic relationship originated multiple times or (ii) symbiont replacement occurred in this insect family. Because other heteropteran bugs also have a crypt-bearing posterior midgut and harbor symbionts in a monoculture, we consider the symbiont replacement hypothesis more plausible and parsimonious than the multiple-origins one. However, studies with larger numbers of taxa that include phylogenetic analysis of host insects are necessary to better support this hypothesis. The fact that all symbiont clades identified here are phylogenetically related to *Erwinia* and *Pantoea* suggests that only closely related bacterial taxa are physiologically acceptable to hosts or capable of sustaining infections over generations. The lack of strict monophyly, coupled with the host association with bacteria of limited phylogenetic diversity (two genera in this case), resembles the association of alydids with *Burkholderia* [5].

The strategy for transmission of gut symbionts in plataspids allows for strict vertical transmission of symbionts, which contrasts with the environmental acquisition of *Burkholderia* in alydids. The vertical transmission of pentatomid symbionts by egg smearing may represent an intermediate state between the transmission strategies of these other two families. Our phylogenetic analysis supports that hypothesis. We found that pentatomid symbionts were polyphyletic, but monophyletic in more recently diverged taxa (*Chlorochroa* genus). It may be interesting for future studies to consider the evolutionary forces

responsible for the establishment and maintenance of these three distinct bug symbioses based on (i) strict vertical transmission (plataspids), (ii) vertical transmission with possible symbiont replacement (pentatomids), and (iii) acquisition of symbionts from the environment (alydids).

Our results, combined with those cited above on plataspid and alydid midgut symbionts, show that the posterior region of the midgut of pentatomorphans in different families is colonized by one dominant bacterial taxon, and that insects in these families are dependent on these symbionts for survival ([1, 3, 5, 8]; Prado and Almeida, unpublished data). However, a few studies with pentatomids suggest that gut symbionts do not provide clear fitness benefits to host insects (e.g., Refs. [7] and [9]). This variability in the Pentatomidae-symbiont studies may be hypothesized to occur due to the specific bacterium associated with different host species (potential for variable types of association) or the experimental diets used for fitness tests. In addition, the age of the association may be an indication of the degree of host-bacterium mutualism; the longer the association, the higher the degree of mutual reliance. Together, these studies suggest that heteropteran symbiotic associations are interesting systems not only for the study of mutualistic interactions, but also for comparative studies of gut bacterial infections.

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