Habitat Visualization and Genomic Analysis of “Candidatus Pantoea carbekii,” the Primary Symbiont of the Brown Marmorated Stink Bug

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Accepted: January 7, 2015

Data deposition: This genome project has been deposited in the National Center for Biotechnology Information GenBank sequence database under the following accession numbers: CP010907, CP010908, CP010909, CP010910, CP010911.

Abstract

Phytophagous pentatomid insects can negatively impact agricultural productivity and the brown marmorated stink bug (Halyomorpha halys) is an emerging invasive pest responsible for damage to many fruit crops and ornamental plants in North America. Many phytophagous stink bugs, including H. halys, harbor gammaproteobacterial symbionts that likely contribute to host development, and characterization of symbiont transmission/acquisition and their contribution to host fitness may offer alternative strategies for managing pest species. “Candidatus Pantoea carbekii” is the primary occupant of gastric cecal lumina flanking the distal midgut of H. halys insects and it is acquired each generation when nymphs feed on maternal extrachorion secretions following hatching. Insects prevented from symbiont uptake exhibit developmental delays and aberrant behaviors. To infer contributions of Ca. P. carbekii to H. halys, the complete genome was sequenced and annotated from a North American H. halys population. Overall, the Ca. P. carbekii genome is nearly one-fourth (1.2 Mb) that of free-living congenerics, and retains genes encoding many functions that are potentially host-supportive. Gene content reflects patterns of gene loss/retention typical of intracellular mutualists of plant-feeding insects. Electron and fluorescence in situ microscopic imaging of H. halys egg surfaces revealed that maternal extrachorion secretions were populated with Ca. P. carbekii cells. The reported findings detail a transgenerational mode of symbiont transmission distinct from that observed for intracellular insect mutualists and illustrate the potential additive functions contributed by the bacterial symbiont to this important agricultural pest.

Key words: Halyomorpha halys, gammaproteobacteria, pentatomid, Hemiptera, invasive pest.

Introduction

Insect-bacterial mutualisms are widespread in insects and have had significant impacts on the evolution and diversification of insects, which remains the most speciose of macrofauna (i.e., Baumann 2005; Moran et al. 2008; McFall-Ngai et al. 2013; Douglas 2014). Many insects that feed exclusively on plant vascular fluids (e.g., xylem or phloem) or parenchymal cell contents can successfully exploit these abundant and nutritionally imbalanced diets with the assistance of bacterial symbionts that can provision essential amino acids and vitamins to their host (Moran et al. 2008; Douglas 2013). Complete genome sequencing and annotation of Buchnera aphidicola revealed a highly reduced genome lacking many genes typically present in free-living relatives of B. aphidicola, namely Escherichia coli, yet the presence of nutrient-yielding biosynthetic pathways. This pattern of gene loss/retention suggests that in spite of dramatic genome reduction due to gene loss, B. aphidicola maintains a genic repertoire that is comprised largely those encoding processes generative of host-supportive nutrients (Shigenobu et al. 2000), and the genomes of other intracellular bacterial mutualists of evolutionarily distant insects, including cockroaches, cicadas, and carpenter ants, exhibit similar patterns of genome streamlining and maintenance of host-supportive genic repertoires (reviewed in Moran et al. 2008). With a few notable exceptions, available genomes of bacterial mutualists of insects have...
been largely derived from transovarially transmitted, intracellularly incarcerated species.

Among heteropteran insects, alternative means of intergenerational transmission and domiciling of symbionts have been observed (reviewed in Hosokawa et al. 2013). Herbivorous females of the Pentatomidae and Plataspidae have been observed to deposit gammaproteobacterial symbiont-laden gut secretions that are either smeared on eggs or encapsulated and positioned proximally to eggs (Fukatsu and Hosokawa 2002; Hosokawa et al. 2005; Tada et al. 2011; Kikuchi et al. 2012). Unlike intracellular mutualists that are present within immature tissues prior to emergence, these symbionts persist in an unknown state of activity outside of host tissues prior to nymphal acquisition by oral consumption of maternal secretions and are presumed to travel to and fill the extracellular lumina of gastric ceca located on the distal midgut region. For example, *Megacopta punctatissima* (Plataspidae) nymphs acquire the gammaproteobacterial symbiont, *Candidatus Ishikawaella capsulata*, by consuming maternally generated capsules affixed to eggs, whereas *Plautia stali* (Pentatomidae) receive an inoculum of an unnamed gammaproteobacterial symbiont as nymphs from consuming maternal secretions smeared on eggs and both insects domicile their symbionts in the ceca of specialized crypts (Abe et al. 1995; Fukatsu and Hosokawa 2002; Hosokawa et al. 2006). Denial of either species from acquiring their symbionts resulted in delayed growth, retarded development, and reduced fecundity (Abe et al. 1995; Fukatsu and Hosokawa 2002; Hosokawa et al. 2008). Although the occurrence of gammaproteobacterial symbionts inhabiting specialized midgut ceca of stink bugs has been well-documented, relatively few complete genomes for these symbionts are currently available (e.g., Nikoh et al. 2011; Brown et al. 2014; unpublished *Pl. stali* symbiont genome GenBank: AP012551.1) to assist in inferring the specific contributions of these symbionts to their hosts (e.g., vitamins, essential amino acids, etc.) or possible genomic consequences (e.g., reduction, skewed genic profile, A+T-bias) of their host associations.

In this regard, the complete sequencing of the primary gammaproteobacterial symbiont, *Candidatus Pantoea carbekii*” (hereafter referred to as *P. carbekii*; Bansal et al. 2014), of the highly invasive (Hoebeke and Carter 2003; Wermelinger et al. 2008; Fogain and Graff 2011; Zhu et al. 2012; Leskey et al. 2012; Vetek et al. 2014; Xu et al. 2014) and polyphagous (Seetin 2011; Bergmann et al. 2014) pentatomid, *Halyomorpha halys*, commonly known as the brown marmorated stink bug, is reported. Although the *P. carbekii* genome is reduced relative to free-living gammaproteobacteria, it encodes enzymes that can generate essential nutrients potentially limited in the host’s diet and that may assist symbiont survival on the egg surfaces prior to nymphal consumption and infection of the distal midgut. As in the aforementioned stink bugs, *P. carbekii* is domiciled within the lumina of pigmented distal midgut gastric ceca and is obtained by nymphs when they consume maternal egg secretions following hatching (Taylor et al. 2014). Prevention of symbiont acquisition by nymphs through surface-sterilization of *H. halys* eggs results in aberrant nymph behavior and developmental delays (Taylor et al. 2014). To detail the transgenerational symbiont transmission strategy in pentatomids, in situ electron and fluorescence microscopy was used to obtain high-definition spatial and taxon-specific imagery of *P. carbekii*, yielding a more detailed description of the location and structural characteristics of the egg surface inhabited by *P. carbekii*. Collectively, these data suggest that genomic characteristics typically observed in insect mutualists are evident in *P. carbekii*, and the symbiont expresses a suite of enzymes that 1) may facilitate survival on egg surfaces prior to nymphal uptake and 2) play an essential role in *H. halys* development through provisioning of nutrients limited in the host’s diet.

### Materials and Methods

#### Genome Sequencing and Annotation

Complete genome sequencing of *P. carbekii* was performed using DNA extracted from adult *H. halys* specimens collected in Wooster, OH in 2013 and briefly maintained in a lab colony. DNA was extracted from the V4 region of the midgut using the DNEasy Blood and Tissue (QiAGEN) kit. Illumina MiSeq sequencing platform with the v2 reagent kit was used to generate 7.7 million 250-bp paired-end reads with an expected 250 bp insert size. Reads were quality trimmed (parameter: Base calls <Q30 Phred score were trimmed and reads <150 bp were excluded) and assembled within the CLC Genomics Workbench (version 6, CLC Bio; parameters: mapping mode = map reads back to contigs, automatic bubble size = yes, minimum contig length = 200, automatic word size = yes, scaffolding = yes, auto-detect paired distances = yes, mismatch cost = 2, deletion cost = 3, length fraction = 0.5, similarity fraction = 0.8, word size = 23, and bubble size = 241), generating 111,569 contigs. A BLASTx search (e value = 0.0001) versus the National Center for Biotechnology Information (NCBI) “nt” database was done for all 111,569 contigs. Only 67 contigs had significant hits and of these, 67 had hits to bacterial species (*supplementary material S1, Supplementary Material online*). The four largest of these (contig_3, contig_28, contig_43, and contig_139) were further evaluated. The four large contigs with the largest genomic size (>20 kb) were contigs_3, contig_28, contig_43, and contig_139, which were longer than 20 kb (range: 65–792 kb, average: 284 kb). Concurrent with our *P. carbekii* assembly efforts, an unpublished *H. halys* symbiont genome from *H. halys* specimens of unknown origin was released in GenBank in October 2013 (GenBank: NC_022547) by researchers in the Nihon University, School of Pharmacy in Japan (hereafter called *P. carbekii* JPN). Pairwise alignment of the de novo generated four large contigs with the *P. carbekii* JPN genome was performed using BLASTN (parameters: e value 0.001) to obtain a
putative genome scaffold. Evident in the contig arrangement with *P. carbekii* JPN and the sequence annotations at the ends of the four large contigs, ribosomal RNA (rRNA) operons were likely between each of these contigs and assembly of a single contiguous chromosome was failing due to the considerable sequence conservation between rRNA regions. Specifically, putative 5S and partial 23S sequences were annotated at both ends of contig_3, a partial 16S sequence was detected at one end of contig_43, a partial 16S sequence was detected at one end of contig_28 and a partial 23S was detected at one end of contig_139 whereas a partial 16S sequence was detected at the opposite end of this contig. To close gaps between these four contigs de novo, outward-oriented polymerase chain reaction (PCR) primers were designed to amplify sequence-spanning gaps between contigs using Primer3Plus (Untergasser et al. 2012) with the ends of the four large contigs as references. All primers used in this study are reported in supplementary material S1, Supplementary Material online.

Both combinatorial and guided (i.e., inferred contig ordering based on alignments to *P. carbekii* JPN) primer pairing in long-range PCR reactions performed with Platinum High Fidelity Taq polymerase (Invitrogen-Thermo, MA) according to manufacturer’s recommended thermocycler conditions. Successful PCR amplifications yielded amplicons that were approximately the expected size of rRNA operons based on the lengths of related *Pantoea* spp. rRNA operons (region that spans complete 23S, 5S, and 16S sequences; *Pantoea ananatis* LMG20103, NC_013956.2 average: 5.2 kb, range: 5.1–5.4 kb; *Pantoea vagans* C9-1, NC_014562.1 average: 5.2 kb, range: 5.0–5.3 kb). PCR products were purified using the Beckman Coulter Agencourt AMPure XP purification system and Sanger sequencing of all amplicons was performed at The Ohio State University Plant-Microbe Genomics Facility. Sequences were manually edited and quality-checked in Geneious (version 7.0.4, Biomatters Limited). A Sanger-based, primer-walking strategy was used to sequence gap-spanning amplicons and yielded a single, contiguous chromosome. The remaining seven contigs that were of putative bacterial origin were then mapped to the contiguous chromosome. Of these seven bacterial contigs, three mapped to the chromosome in the three rRNA operon regions. In silico translations of open reading frames (ORFs) detected using Prodigal (default parameters; Hyatt et al. 2010) on each of the remaining four contigs (contig_578, contig_74, contig_85, and contig_131) that did not assemble de novo with the *P. carbekii* chromosome were 77–91% identical (BLAST 2.2.30+, program: BLASTp, parameters: default, database: refseq_protein; Altschul et al. 1997) to gammaproteobacterial plasmid replication proteins (e.g., RepA and RepPFB) and were tested to determine whether they were plasmids. Inverse PCR (Life Technologies Platinum Taq DNA Polymerase High Fidelity; see supplementary material S1, Supplementary Material online, for primers) using pairs of outward-facing primers positioned at the ends of contig_578, contig_74, contig_85, and contig_131 resulted in positive amplification products that, when sequenced, closed the contigs upon themselves. The average read coverage for each of the contigs was as follows: contig_578 (pBMSBPS1): 2,960 x, contig_74 (pBMSBPS2): 7,487 x, contig_85 (pBMSBPS3): 1,412 x, and contig_131 (pBMSBPS4): 610 x. The lengths for the plasmids are pBMSBPS1: 14,562 bp, pBMSBPS2: 6,287 bp, pBMSBPS3: 17,880 bp, and pBMSBPS4: 7,593 bp.

MiSeq-generated reads were quality-filtered using FASTX-Toolkit v 0.0.13 (Gordon and Hannon 2010; “fastq_quality_trimmer” was used to trim nucleotides with quality <Q30 from the ends of the reads and reads less than 100 bp after trimming were discarded, “artifacts_remover” was implemented to remove reads with only three of the four possible bases, “fastq_quality_filter” was used to remove reads with less than 80% of bases with >Q28, and “fastq_to_fasta” was used to remove reads with any “N” ambiguous base calls). These high-quality reads were mapped to the *P. carbekii* chromosome to detect any polymorphisms and to detect any inaccurate base calls using BWA (default parameters; Li and Durbin 2010) with an average per base read coverage of 745 x as determined by the “genomeCoverageBed” program in BEDTools (Quinlan and Hall 2009). Mapping results were visually inspected using the Integrative Genomics Viewer (Thorvaldsdóttir et al. 2012) and base calls supported by less than 99% of mapped reads were manually edited in Artemis Genome Viewer (Rutherford et al. 2000), unless they were supported by the Sanger sequencing. Only ten polymorphic sites (out of 1,150,626 nt) were detected in intergenic spaces, where two different bases were supported nearly equally by the reads. In these ten cases, the base with majority (>50%) of the reads supporting it was called.

Chromosome and plasmid encoded ORFs were determined with Prodigal, transfer RNAs (tRNAs) were called using trNAscan and Aragorn, and transfer-messenger RNAs (tmRNAs) and rRNAs were detected using Rfam (Laslett and Canback 2004; Schattner et al. 2005; Hyatt et al. 2010; Burge et al. 2012). Annotation was completed using Rfam functional domain determination, COG assignments, KEGG Orthology Groups, TIGRFAM, and BLAST (Basic Local Alignment Search Tool) comparisons of ORFs to the “ECO,” “nr,” and “nt” databases (Altschul et al. 1997; Tatusov et al. 1997; Haft et al. 2003; Moriya et al. 2007; Punta et al. 2012; Zhou and Rudd 2013; BLASTP, e value = 0.001). Artemis Genome Viewer and DNAPlotter were used to assist with annotation and detecting G/C skew (Rutherford et al. 2000; Carver et al. 2009). Metabolic reconstruction was done using KEGG–KAAS with information from EcoCyc and MetaCyc (Moriya et al. 2007; Caspi et al. 2008; Keseler et al. 2009). *Pantoea carbekii* proteins that were fragmented due to internal stop codon(s) and/or frameshift mutations were annotated as pseudogenes. Average-per-base coverage in pseudogenes did not vary significantly from the average-per-base coverage across the
Comparative Analyses

Bioinsynthesis-related gene content and general genomic characteristics of P. carbeckii were compared with those of the following gammaproteobacteria inhabiting various ecological niches (NCBI GenBank accession numbers are parenthesized): E. coli str. K12 (NC_000913), P. ananatis (AP012032.1), Pl. stali symbiont (AP012551.1), Ca. I. capsulata symbiont of M. punctatissima (AP010872.1), and B. aphidicola str. APS (NC_002528) (hereby referred to by their generic names). General genome statistics were compared across these genomes as in Moran et al. (2008). Maximal protein length characteristics of P. carbekii were determined using OrthoMCL (Fischer et al. 2011). Orthologous protein groups from the proteomes of related gammaproteobacteria with complete and draft genomes were determined using OrthoMCL (Fischer et al. 2011). Orthologs and the sourced proteomes used for phylogenetic reconstruction are reported in supplementary material S1, Supplementary Material online. Eighty orthologs from 47 taxa were individually aligned in MAFFT using the L-INS-i algorithm (Kato et al. 2002) and gap-containing columns were removed using a custom Perl script, leaving a total of 18,678 characters per taxon. Aligned proteins were concatenated using a custom Perl script and maximum-likelihood trees were inferred using a web-based implementation of RAxML (parameters: rapid bootstrapping under the LG protein model and DAYHOFF substitution matrix, 100 bootstrap trees; CIPRES Science Gateway; Stamatakis et al. 2008; Miller et al. 2010) and the best-supported maximum-likelihood tree was reported. FigTree v1.3.1 and MEGAS.1 were used to prepare trees for publication (Rambaut and Drummond 2010; Tamura et al. 2011).

Molecular Phylogenetic Reconstruction

Orthologous protein groups from the proteomes of related gammaproteobacteria with complete and draft genomes were determined using OrthoMCL (Fischer et al. 2011). Orthologs and the sourced proteomes used for phylogenetic reconstruction are reported in supplementary material S1, Supplementary Material online. Eighty orthologs from 47 taxa were individually aligned in MAFFT using the L-INS-i algorithm (Kato et al. 2002) and gap-containing columns were removed using a custom Perl script, leaving a total of 18,678 characters per taxon. Aligned proteins were concatenated using a custom Perl script and maximum-likelihood trees were inferred using a web-based implementation of RAxML (parameters: rapid bootstrapping under the LG protein model and DAYHOFF substitution matrix, 100 bootstrap trees; CIPRES Science Gateway; Stamatakis et al. 2008; Miller et al. 2010) and the best-supported maximum-likelihood tree was reported. FigTree v1.3.1 and MEGAS.1 were used to prepare trees for publication (Rambaut and Drummond 2010; Tamura et al. 2011).

Single Nucleotide Polymorphism and Genomic Synteny Analysis

The P. carbeckii and P. carbeckii JPN chromosomes were compared to identify single nucleotide polymorphisms (SNPs) using BWA (Li and Durbin 2010) and the Genome Analysis Tool Kit (“GATK”; McKenna et al. 2010). Specifically, BWA-aligned genomes were subjected to an additional local alignment within GATK to improve the accuracy of SNP calling by locally realigning areas where there might be insertions or deletions (McKenna et al. 2010). GATK and Samtools (parameters: defaults; Li et al. 2009) were simultaneously used to call SNPs and only SNPs detected by both were considered. Furthermore, SNPs were only retained for analysis if greater than 99% of the P. carbeckii reads matched the called-SNP. Per base read depth for the SNPs averaged 857.5 reads (range: 51–1,784×), as determined by BEDTools genomeCoverage Bed program with the “-d” flag implemented for per base read depth calls (Quinlan and Hall 2009). SNPs were determined to be transitions or transversions within Microsoft Excel. For SNPs detected in coding regions, the “diffseq” program within EMBOSS was used to detect SNPs resulting in nonsynonymous or synonymous changes (Rice et al. 2000). SNPs resulting in nonsynonymous mutations in coding regions were categorized using the COG database (Tatusov et al. 1997). Structural rearrangements between the two genomes were detected using the “nucmer” program within the MUMmer package (Delsuc et al. 2002; default parameters except –maxmatch option) and visualized using the Genome Synteny Viewer (Revanna et al. 2011).

Halymorpha halys Gut Microbiome Analysis

Three adults from an Ohio population-derived, in-house H. halys colony were euthanized in 70% ethanol and immediately dissected in 1X phosphate-buffered saline (PBS). DNA was extracted separately from the V1–V2, V3, and V4 midgut tissues of the digestive tract (for details, see Bansal et al. 2014) using the QiAgen DNEasy Blood and Tissue Kit and submitted to the Institute for Genomics and Systems Biology Next Generation Sequencing Core (Argonne National Laboratory, Argonne, IL) for 16S rRNA amplicon library preparation and Illumina MiSeq 2×251 bp paired-end sequencing. Illumina-generated 16S rRNA sequence reads (hereafter referred to as “iTags” after Degnan and Ochman 2012) were assembled using Pandaseq (Masella et al. 2012), trimmed to remove base calls with less than Q30 Phred score and quality-filtered (parameters: reads with ≥1 errors in barcodes or primers and/or were less than 230 bp or greater than 260 bp in length were removed) using the CLC Genome Workbench. Quality-filtered reads were preprocessed using the mothur software package (version 1.29; Schloss et al. 2009) and operational taxonomic units (OTUs) were clustered at ≥95% identity using usearch (version 7; Edgar 2010). Sequences representing OTUs were taxonomically assigned by BLAST (program: BLASTn, parameters: default) searches of SILVA 16S small-subunit “SSU” (version 115; Pruesse et al. 2007) and NCBI GenBank nt (downloaded on March 18, 2014) databases. Best hits within these databases that aligned with greater than 99% of the OTU sequences informed OTU genus designations.

Fluorescence In Situ Hybridization and Electron Microscopy

Fluorescence In Situ Hybridization

Crude homogenates were prepared from 1) freshly laid eggs with the extrachorion matrix removed (see below), 2) freshly laid eggs soaked in 10% bleach for 10 min and rinsed in
dH2O), 3) eggs extracted from gravid females, and 4) V4 midgut crypts for microscopic imaging. Additionally, lavages of the extrachorion matrix surrounding freshly laid eggs were also prepared for imaging by placing single eggs in 200 μl 1X PBS solution in a 1.5-ml centrifuge tube, vortexing for 10–15 s and the extrachorion matrix was further manually disrupted by pipetting approximately ten times, while being careful not to puncture the egg. Following the removal of the lavage, eggs were further rinsed with 1X PBS to remove any residual extrachorion matrix and homogenized in 1X PBS using a combination of vigorous vortexing, pipetting, and manual crushing with a sterile pestle until little or no intact egg fragments were visible. Eggs from gravid females and bleached eggs underwent a similar process (vigorous vortexing, pipetting, and manual crushing with a sterile pestle in 1X PBS) upon collection or after treatment (i.e., bleach treatment). V4 midgut sections were dissected from colony-maintained adults euthanized in 70% ethanol in 1X PBS and immediately transferred to a 1.5-ml microcentrifuge tube containing 200 μl 1X PBS. V4 tissues were homogenized until little or no intact pieces remained by vigorous vortexing, pipetting, and crushing with a sterile pestle. Three experimental replicates were performed for each of the treatments, except for the V4 midgut preparations, of which there were five replicates and the egg lavage treatments, of which there were nine replicates. Preparation for fluorescence in situ hybridization (FISH) followed Osborn and Smith (2005). Specifically, approximately 10–15 μl of each sample (egg lavage, crushed egg or V4 midgut homogenates) was placed on microscope slides, air-dried in a vacuum hood, and fixed over an open flame for 1–3 s. An amount of 40 μl 4% paraformaldehyde was immediately added to each sample, covered with a coverslip, and incubated for 24 h at 4 °C. The samples were then dehydrated in a series of ethanol washes for 3 min at each of the following concentrations of ethanol: 50%, 80%, and 95%. An amount of 1 μl (50 ng/μl) of each probe was warmed to 48 °C and mixed with 10 μl prewarmed hybridization buffer (180 μl 5 M NaCl; 20 μl Tris–HCl; 200 μl formamide; 599 μl ddH2O; 1 μl 10% sodium dodecyl sulfate, SDS) for each sample. A quantity of 10 μl of the hybridization buffer/probe solution (final probe concentration: 5 ng/μl) was added to each sample, covered with a coverslip, and incubated for 24 h at 46 °C. Cy3-labeled Enterobacteriaceae-specific (“ENT1251”: 5′-Cy3-TGCTTCGAGGTCGCTTCTCTT-3′, 50 ng/μl; Ootsubo et al. 2002) and TYE-563-labeled P. carbekii-specific (“CrbcK-TEX”: 5′-TYE-ATGCTGCCGTTCGACTTT-3′, 50 ng/μl; this study) FISH probes were used separately. Following incubation, samples were rinsed with washing buffer (1 ml Tris–HCl; 2.15 ml 5 M NaCl; 0.5 ml 0.5M ethylenediaminetetraacetic acid; 46.35 ml ddH2O) for 3 min with gentle agitation, then dipped in ice-cold ddH2O and air-dried in a vacuum hood. Slides were counterstained with 4,6-diamidino-2-phenylindole (1 μg/ml) prior to adding mounting buffer (10% glycerol in 1X PBS) and covered with a cover-slip to view with a Nikon Eclipse Ti inverted epifluorescence microscope.

**Transmission Electron Microscopy**

Midgut gastric ceca (V4) tissues were dissected from colony-maintained adult insects in ice-cold fixative (3% glutaraldehyde, 1% paraformaldehyde in 0.1 M potassium phosphate buffer, pH 7.2) in preparation for transmission electron microscopy (TEM), as described in Bansal et al. (2014). Briefly, samples were fixed for 3 h on a rocker at 23 °C, washed with 0.1 M potassium phosphate buffer (0.1 M KH2PO4, 0.1 M K2HPO4, pH 7.2), and treated with a postfixative solution (1% osmium tetroxide, 1% uranyl acetate in distilled water) at 23 °C for 1 h. Tissues were dehydrated through a graded ethanol series, resin infiltrated using a propylene oxide-resin series, and then embedded in EM Bed-812 resin (Electron Microscopy Sciences, Hartfield, PA). Ultrathin-sections were prepared using a Leica UC6 ultra-microtome. Three of the prepared V4 midgut thin-sections were used for FISH microscopy, using the protocol described above. After staining with 3% aqueous uranyl acetate for 20 min, followed by Reynolds’ lead citrate for 10 min (Reynolds 1963), sections were imaged using a Hitachi H-7500 transmission electron microscope and images recorded with a Optronics QuantiFire S99835 (SIA) digital camera.

**Scanning Electron Micrograph**

Freshly laid eggs (within 1 day of oviposition; 5–10 eggs per treatment) were subjected to one of the following treatments prior to scanning electron micrograph (SEM) imaging: 1) 10–15 s vortexing in 1X PBS; 2) soaking in 9% bleach for 2 min, followed by three washes with 1X PBS; 3) soaking in 10% SDS for 10 min, followed by three washes with 1X PBS; and 4) no treatment. Treated and control eggs were fixed in 3% glutaraldehyde, 2% paraformaldehyde, in 0.1 M potassium phosphate buffer pH 7.2 (PB) over night, and subsequently rinsed three times for 10 min with PB and postfixed in 1% OsO4 in PB for 1 h. After two PB washes, samples were dehydrated through washes in 50%, 75%, 95%, and 100% ethanol for 15 min each. Following dehydration, samples were dried in a critical-point dryer, spatter coated with platinum, and viewed on the Hitachi S-3500N scanning electron microscope.

**Results and Discussion**

*Pantoea carbekii* Dominates the *H. halys* Crypt-Bearing Midgut and Is Abundant on Egg Surfaces

iTags were generated from adult Ohio *H. halys* gut tissues to assess the diversity of bacteria comprising the microbiome of the V4 gut region and a location-specific, relatively high concentration of *P. carbekii* was detected in *H. halys* V4 midgut tissues. More than 98% of approximately 23,000 high-quality iTags for all of the V4-midgut region tissue samples were
unambiguously assigned to \( P. \) carbekii. In contrast, less than 1% of the iTags generated from all of the V1–V2 tissue samples and in most of the V3 tissue samples could be assigned to this symbiont. Additionally, \( P. \) carbekii has been detected in DNA preparations from \( H. \) halys egg clusters (Bansal et al. 2014; Taylor et al. 2014) and histological methods were used to localize egg-associated \( P. \) carbekii cells. \( Halyomorpha \) halys eggs are typically laid in clusters, affixed to one another with a maternally secreted extrachorion matrix (fig. 1E). Inspection of five eggs (41 SEM images) in which the extrachorion matrix was disrupted by brief vortexing in sterile, distilled 1X PBS (fig. 1A) revealed dense patches of \( P. \) carbekii on the egg surface (fig. 1B). Rod-shaped bacterial cells were observed beneath the matrix (fig. 1C) that were morphologically similar to those imaged by TEM of thin-sections of the V4 midgut gastric ceca (fig. 1D). Of the eggs that were treated with 10% bleach, three were observed (eight SEM images) and none had bacterial cells and or any of the extrachorion matrix present on untreated or vortexed eggs (fig. 1A). Four of the eggs that were treated with 10% SDS were observed (13 SEM images) and they maintained some of the extrachorion matrix but no bacterial cells were observed (fig. 1H). To confirm that the bacterial cells present on egg surfaces were \( P. \) carbekii, FISH microscopy was performed on lavages prepared from the egg extrachorion matrix (fig. 1G) and of V4 midgut tissues (supplementary material S2A and B, Supplementary Material online) using a \( P. \) carbekii-specific FISH probe. Rod-shaped cells that exhibited a strong fluorescence signal and were morphologically similar (fig. 1G and supplementary fig. S2A and B, Supplementary Material online) to those in the SEM (fig. 1C) and TEM (fig. 1D) were observed. Lavages prepared from eggs that were bleached or prevaxed multiple times in 1X PBS, or extracted from gravid females yielded no observable bacterial cells by FISH or light microscopic methods. Although pentatomomorphans exhibit a few types of symbiont acquisition and transmission mechanisms, varying from recruitment of environmental \( Burkholderia \) strains by each generation of alydids (Kikuchi et al. 2007) to vertical transmission of \( Ishikawaella \) through consumption of symbiont-filled capsules by plataspids (Nikoh et al. 2011), maternal egg smearing of gut fluids and subsequent nymphal feeding on these eggs has been documented in other pentatomomorphs examined, such as \( Nezara \) viridula (Prado et al. 2006), \( Eurydema \) spp. (Kikuchi et al. 2012b) and \( Sibaria \) englemani (Bistolas et al. 2014), and appears to be the mode of symbiont transfer in \( H. \) halys (Taylor et al. 2014).

General Characteristics of the \( P. \) carbekii Genome

The \( H. \) halys symbiont, \( P. \) carbekii, forms a clade with the \( Pl. \) stali symbiont within the \( Pantoea \) group (fig. 2) and its genome is significantly reduced in size compared with congeneric and other gammaproteobacteria. Consisting of only 1,197,048 nucleotides, the \( P. \) carbekii genome is roughly one-fourth the size of related species (table 1). Although this represents a significant genome reduction, many intracellular insect symbionts, such as \( Buchnera or Blattabacterium \), have further reduced genomes, with many being less than 1Mb (Moran et al. 2008). The \( P. \) carbekii chromosome encodes 797 protein-coding genes, 2 tRNAs, 40 tRNAs, and two complete rRNA operons and one rRNA operon containing 165–235-encoding regions and lacking an identifiable 55-encoding region. Four plasmids were detected, all encoding RepA, and one encodes a similar gene content as the plasmid detected in \( Ishikawaella \). Twelve pseudogenes were predicted and annotated (table 1) and roughly 40 additional proteins appear truncated, but not pseudogenized, relative to orthologs in other \( Pantoea \) species. With the 40 tRNA species, \( P. \) carbekii is able to decode all 20 amino acids (table 1), including the translation initiating N-formyl-methionyl-tRNA (BMSBPS_0622), and a isoleucine-charged tRNA (BMSBPS_0773) that, following posttranscriptional lysiulation of a position 34 cytidine by Tis, changes the anticodon from CAU to AUA to prevent misreading of AUG as isoleucine (Soma et al. 2003). Multiple copies of genes encoding tRNAs that recognize the mRNA codons “AUG” and “GAA” (which correspond to methionine and glutamate, respectively) and “AUC” (isoleucine) are present. As with other primary symbionts of insects, the \( P. \) carbekii genome exhibits reduced G+C% (30.57%) compared with free-living relatives such as \( P. \) ananatis (53.76%), which is a hallmark feature associated with genome reduction that accompanies elevated fixation of mutations under relaxed selection in endosymbionts with stable host-restricted lifestyles (Moran et al. 2008). Although the genome is reduced, \( P. \) carbekii still encodes metabolic pathways for the production of peptidoglycan, generation of ATP by aerobic respiration, and other primary metabolic processes. The genes encoding enzymes for peptidoglycan biosynthesis are often missing in intracellular-residing insect mutualists with tiny genomes (e.g., \( Carsonella \) and \( Tremblaya \)) and their presence suggests that \( P. \) carbekii can produce a cell wall, which might be important for persistence in the extrachorion matrix. ATP synthesis by aerobic respiration is prevalent across members of the gammaproteobacteria and that \( P. \) carbekii retains this efficient energy-yielding pathway reflects the oxic state of the midgut crypts.

Another trait observed that is associated with a host-restricted lifestyle is the reduction in the maximal protein length in comparison to free-living relatives, such as \( P. \) ananatis and \( E. \) coli (table 1). Some proteins involved in toxin production, secondary metabolic processes, virulence, extracellular sensing, or are of unknown function were shown to be, on average, larger than those involved in primary metabolic processes such as DNA replication, transcription, translation and essential amino acid biosynthesis, and the former, being nonessential for long-standing host-restricted lifestyles, are largely missing from insect endosymbiont genomes and their absence likely contributes to the reduced
P. carbekii exhibits a somewhat similar genic profile in that the longest protein encoded by its genome is the 1,413 amino acids long \( \beta_0 \) subunit of RNA polymerase (RpoC), which is important for transcription, and it is one-third that of the longest protein encoded in the genome of a free-living relative, \( P. ananatis \) AJ13355 (putative secondary metabolite biosynthesis protein YP_005934773: 4,385 amino acids) that is of unknown function.

**P. carbekii Metabolism and Putative Role in \( H. halys \) Physiology**

Phytophagous diets are limited for essential amino acids and some vitamins and, based on the genome content, \( P. carbekii \)...
appears capable of supplementing the diet of *H. halys* with these nutrients. Although the genome shows evidence of reduction, *P. carbekii* encodes canonical pathways, like those typically observed in free-living gammaproteobacteria, namely *E. coli* and *P. ananatis*, for the biosynthesis of all essential and nonessential amino acids (fig. 3; supplementary material S1, Supplementary Material online) except for proline, isoleucine, leucine, and valine. The proline biosynthesis genes proA and proB, which encode glutamate-5-semialdehyde dehydrogenase and glutamate 5-kinase, respectively, are both absent from the *P. carbekii* genome and the ornithine-to-proline cyclodeaminase gene, *ocd*, is also missing. As no alternative pathways or functionally equivalent genes appear present, *P. carbekii* may not be able to synthesize proline de novo.

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**Fig. 2**.—Core gene phylogeny of *Enterobacteriaceae*. Maximum-likelihood-based phylogenetic reconstruction of *P. carbekii* and other gammaproteobacteria (*n* = 47 taxa) using 80 concatenated and aligned orthologous proteins, comprising a total of 18,678 characters per taxon, was performed in RAxML. Support values generated from 100 bootstrap trees are indicated at branch points and were greater than 95% (black dot) unless otherwise noted. Parenthesized values next to species names are genome sizes in megabases; asterisks indicate estimated sizes for draft genomes. Accession numbers for the genomes from which the proteomes were derived and the orthologs used are reported in supplementary material S1E, Supplementary Material online.
which is required for the final step in branched chain amino acid biosynthesis. Absence of this gene has been observed in both *Ishikawaella* and *Buchnera* and host participation in the production of these essential amino acids has been suggested (Shigenobu et al. 2000; Nikoh et al. 2011).

Although the genome of *P. carbekii* lacks argD (acyctylornithine aminotransferase), which catalyzes amination steps in lysine and arginine biosynthesis pathways (Ledwidge and Blanchard 1999), and argI (ornithine carbamoyltransferase), which catalyzes the sixth step of arginine biosynthesis (Glandsdorff et al. 1967), appears pseudogenized (frameshift around an adenosine 9-mer), the functional role of ArgD in arginine and lysine biosynthesis could be replaced by AstC (Kim and Copley 2007), which is encoded on the plasmid, pBMSBPS1, and similarly on the *Ishikawaella* plasmid (Nikoh et al. 2011). Although the gene encoding the asparagine synthetase A (asnA), which is missing from the asparagine biosynthesis pathway, asparagine synthetase B, asnB, which has homologous functions in *E. coli*, is present. However, asnB appears pseudogenized by a frameshift around an adenosine 8-mer but previous work has shown that a subset of transcripts from frameshift-based pseudogenes can encode intact enzymes due to transcriptional slippage at homopolymeric sites (Tamas et al. 2008). ArgI production may be also rescued in a similar manner if transcriptional slippage around the adenosine 9-mer corrects the internal frameshift. If these pseudogenized-by-frameshift genes are nonfunctional, then it is possible that host-encoded enzymes may complement the functions performed by these enzymes (Russell et al. 2013). *Pantoea carbekii* encodes complete or near-complete canonical pathways for the production of several vitamins and cofactors, including folate (vitamin B9), riboflavin (vitamin B2; although ribC is absent), pyridoxal-5’-phosphate (vitamin B6), glutathione, iron–sulfur clusters, and lipoate (fig. 3).

Unlike the *Ishikawaella* genome, *P. carbekii* is likely unable to synthesize biotin due to the complete absence of this pathway (supplementary material S1, Supplementary Material online). Like *Ishikawaella*, it is missing genes encoding enzymes involved in molybdopterin biosynthesis (*meaA, meaB, and meaC*), but the corresponding enzymes or metabolites may be supplied by other *P. carbekii* biosynthetic pathways, the host, or the host’s diet.

### Table 1

**Genome Characteristics Compared between *Pantoea carbekii* and Related Free-Living and Symbiotic Organisms with Varying Genome Sizes**

| Escherichia coli K-12 | Pantoea ananatis | Symbiont of *Plautia stali* | Pantoea carbekii | *Ishikawaella capsulata* | *Buchnera aphidicola APS* |
|----------------------|-----------------|---------------------------|-----------------|-------------------------|-------------------------|
| Genome size (bp)     | 4,641,652       | 4,877,280*                | 1,197,048*      | 754,729                 | 655,725                 |
| Plasmids             | —               | 1                         | 2               | 1                       | 2                       |
| Chromosomal CDS      | 4,140           | 4,038                     | 5,122           | 797                     | 623                     | 564                     |
| Plasmid CDS          | —               | 278                       | 26; 55          | 9; 5; 11; 6             | 8                       | 3; 7                     |
| rRNA coding genesb   | 22c             | 22c                       | 16d             | 8e                      | 9f                      | 3g                      |
| tRNAs                | 89              | 78                        | 59              | 40                      | 37                      | 32                      |
| Pseudogenes          | 184             | NA                        | NA              | 12                      | 35                      | 13                      |
| Maximum protein size (amino acids) | 2,358 | 4,385 | 1,843 | 1,413 | 1,415 | 1,407 |
| G+C content (%)      | 51              | 54 (52)                   | 57 (48; 49)     | 31 (30; 27; 26; 24)     | 30 (28)                 | 26 (27; 31)             |
| Habitat              | Free-living/enteric | Plant pathogen            | Insect symbiont | Insect symbiont         | Insect symbiont         | Insect symbiont         |
| Plautia stali (Brown-winged Green Bug) | Halyomorpha halys (Brown Marmorated Stink Bug) | Megacopta punctatissima (Japanese Common Plataspid Stink Bug) | *Acyrthosiphon pisum* (Pea Aphid) |
| NCBI accession       | NC_000913.3     | AP012032.1, AP012033.1    | AP012551.1, AP012552.1, AP012553.1 | AP010872.1, AP010873.1 | AP010872.1, AP010873.1 | NC_002528.1, NC_002253.1, NC_002252.1 |

*Genome size is the sum of the chromosome and plasmid(s).*

*Genes encoding the 5S, 16S, and 23S rRNAs were counted.*

*In addition to seven 55-235-16S rRNA operons, an additional 5S rRNA coding region has been annotated.*

*Two 23S rRNA-5S rRNA operons are present in addition to four 5S-23S-16S rRNA operons.*

*Two 5S-23S-16S rRNA operons are annotated and additional 16S-23S rRNA operon has been annotated.*

*Three complete rRNA operons are annotated.*

*3A 23S-5S rRNA operon and a separate 16S rRNA gene have been annotated in the genome.*
that include the entire ammonia-producing succinyl transferase pathway (astCADEB), glutamate dehydrogenase (gdhA), replication protein A (repFIB), small heat shock protein (ibpB), and 3-octaprenyl-4-hydrobenzoate decarboxylases (ubiD). The arginine succinyltransferase (AST) pathway allows *E. coli* to use arginine as a nitrogen source under nitrogen-starvation conditions and the total carbon requirement for *Klebsiella aerogenes* (reviewed in Cunin et al. 1986). Given that the *P. carbekii* genome encodes nearly all the enzymes for arginine biosynthesis (except for ArgF), it is possible that it employs the AST pathway as a means for recycling nitrogen from arginine under nitrogen demands. *Halyomorpha halys* is a generalist feeder and this dietary flexibility may be extended to diets that are particularly low in nitrogen as facilitated by *P. carbekii*. GdhA, glutamate dehydrogenase, is part of the ATP-dependent glutamate synthase pathway, catalyzing amination of alpha-ketoglutarate to glutamate (Veronese et al. 1975) and is one of the two pathways ammonium nitrogens are assimilated in bacteria to produce glutamate. Only the glutamine-yielding glutamine synthetase part of the GS-GOGAT ammonia assimilation pathway is present and, therefore, GdhA is likely the primary mechanism for glutamate production and was detected in the Mascot protein analysis of the egg lavage. Finally, AstC might replace the catalytic role of ArgD, which is missing from the *P. carbekii* genome but is necessary for arginine and lysine biosynthesis (reviewed in Cunin et al. 1986).
Among the 11 proteins encoded on pBMSBPS3, several appear to be involved in thiamine (vitamin B1) biosynthesis, which is not known to be produced de novo by insects (Sweetman and Palmer 1928; Craig and Hoskins 1940). Specifically, ThiSFGO are largely responsible for the production of the thiazole moiety, which, along with the pyrimidine moiety, are the two key precursors in prokaryotic thiamine biosynthesis (Du et al. 2011). In E. coli, the thiazole moiety is derived from condensation of tyrosine, cysteine, and 1-deoxy-d-xylulose-5-phosphate (Du et al. 2011), which involves thiazole synthase ThiH, which is not identifiable in the genome. ThiH stands, a bifunctional enzyme that adenylates tRNA and mobilizes sulfur in 4-thiouridine synthesis and has been suggested to participate as a persulfide carrier in thiazole synthesis (Taylor et al. 1998). Mutational analysis of Thi in Salmonella has shown that only a rhodanese domain in Thi, which can perform the sulfur transfer to thiazole, is necessary and thus an alternative sulfur transferase could, along with cysteine, perform this function (Martinez-Gomes et al. 2011). A conserved hypothetical protein in P. carbekii, BMSBPS_0563, has a rhodanese-like domain (PFAM: PF00581) and thus may perform this function. The remaining genes involved in thiamine biosynthesis are present on the chromosome. Specifically, ThiCD uses 5-aminomidazole ribotide from purine biosynthesis to generate the pyrimidine moiety, ThiE couples the thiazole and pyrimidine moieties to form thiamine monophosphate, and ThiL catalyzes the formation of the active form of thiamine.

Of the 11 protein-coding regions on the remaining two plasmids, pBMSBPS2 and pBMSBPS4, seven encode proteins with conserved domains of unknown function or are conserved hypothetical proteins.

pBMSBPS2 encodes five proteins, two of which could potentially be involved in stress-tolerance, namely the DNA mismatch repair protein MutT and inositol monophosphatase SuhB. A mutT deletion in E. coli significantly increases the spontaneous occurrence of A:T to C:G transversions 1,000-fold over the wild-type and increases transcriptional errors (Dukari et al. 2000; Shimokawa et al. 2000). Although the physiological role of SuhB is not well understood, a suhB mutation is sensitive to cold, suggesting a potential role in cold-tolerance (Matsuhisa et al. 1995; Chen and Roberts 2000).

Degradation of DNA Replication and Repair Mechanisms and Abundant SNPs between P. carbekii Strains

A few genes encoding products involved in DNA repair, transcription, homologous recombination, and metabolite conversions exhibit abnormal gene morphologies and/or harbor indel mutations, resulting in premature stop codons, frameshifts or truncations. Notably, DNA polymerase I, which is involved in DNA repair (Glickman 1975; Sharon et al. 1975; Smith et al. 1975), is present in the Buchnera, Ishikawaella, and P. ananatis genomes as single loci encoding approximately 900 amino acid enzymes, but it is split into two protein-coding regions (e.g., polA1 and polA2) in different reading frames separated by stop codons in P. carbekii. Although the frameshifts and stop codons may interfere with production of a classical DNA polymerase I, the presence of intact functional domains within polA1 and polA2 suggests that, together, the products of these two genes may be capable of performing the functions of PolA. Additionally, the NAD-dependent DNA ligase, LigA, which is active during DNA replication, recombination, and repair and joins DNA fragments, closely resembles orthologs in Ishikawaella, Buchnera, and other Panthoea species except for lacking approximately 90 C-terminal residues that comprise a BRCT domain (Pfam: PF00533) that binds to DNA but is not essential for DNA-joining activity in E. coli (Wilkinson et al. 2005).

Several genes involved in DNA repair are missing from the P. carbekii genome. Absent are phr, which encodes a deoxyribodipyrimidine photolyase that acts in a light-dependent reaction to split pyrimidine dimers after UV radiation exposure (Keseler et al. 2009), and xth (Exonuclease III) and rep (Rep helicase) whose products are also involved in DNA repair. Xth is an endonuclease that repairs DNA where damaged bases have been removed or lost in E. coli (Gossard and Verly 1978). The loss of the regulation of xth results in the hypersensitivity of E. coli mutants to UV radiation (Sak et al. 1989). Rep helicase is required for replication in E. coli, preventing double-stranded breaks and acting in a replication fork restart pathway (Michel et al. 1997; Sandler 2000). The absence of rep in E. coli results in severe growth problems, namely the accumulation of DNA within a single cell (Trun 2003). We detected the 8-oxo-dGTP diphosphatase (mutT) gene on pBMSBPS2 and it encodes half of the base excision repair (BER) system whereas the formamidopyrimidine DNA glycosylase, encoded by mutM was absent; mutM deletions in E. coli have been shown to result in an increase in GC→AT transversions (Cox 1976; Cabrera et al. 1988). Finally, recG (RecG DNA helicase) is not detected in the P. carbekii genome, but it is also missing in Ishikawaella and Buchnera (supplementary material S1, Supplementary Material online).

Pairwise comparison of the reported P. carbekii genome and P. carbekii JPN highlights areas of the genome that may have accumulated recent mutations, assuming that the former is recently (i.e., in the last ~30 years) derived from a related native regional Asian H. halys population. An amount of 99.8% nucleotide identity between the genomes of the two P. carbekii strains was determined by megaBLAST
alignment and the overall gene order between the genomes of the two P. carbekii strains was identical, with only two 76- and 66-bp inversions and a 100-bp deletion in intergenic regions being detected in the P. carbekii US genome. An SNP analysis was performed on the two strains to identify genes that may be experiencing rapid mutations and are either 1) the target of strong positive selection or 2) no longer being selected for since they are not required for maintenance of the organism (i.e., Brown et al. 2014). An average of 1 SNP per kb was observed and of 1,144 total SNPs observed in P. carbekii, 511 of these were in genic regions (supplementary material S1, Supplementary Material online). Most of the SNPs represented transitions (724) versus transversions (420). Over half of the SNPs in genic regions (293/511) coded for nonsynonymous protein mutations and these SNPs were distributed over 170 unique genes, with some genes having up to seven nonsynonymous mutations, but these did not result in any observable loss-of-function due to new stop codons (supplementary material S1, Supplementary Material online). Of the few SNPs that resulted in nonsynonymous changes in protein-coding regions, determining the impact of these changes on enzyme function is part of ongoing investigations. Furthermore, additional sampling in H. halys originating from both extant and archived populations in the United States and the Asian-Pacific regions would be useful in determining whether the observed SNPs are fixed and/or distributed by habitat.

Genes with more than one nonsynonymous SNP included ytfN (or tamB), uvrA, surA, and polA. ytfN encodes the integral inner membrane protein portion of the translocation and assembly module “TAM,” which was recently found to promote efficient secretion of autotransporters in proteobacterial species (Selkrig et al. 2012). Along with TamA (also encoded within P. carbekii genome), the integral outer membrane protein in this complex, TAM allows for the efficient translocation of Antigen 43 and EhaA, two autotransporter proteins that are involved in biofilm formation and pathogenesis in E. coli (Danese et al. 2000; Wells et al. 2008; Selkrig et al. 2012). Interestingly, Antigen 43 and EhaA are not encoded by the P. carbekii genome. This suggests that TAM might translocate other proteins in P. carbekii, not yet described, that may share similar features with these biofilm-formation proteins. uvrA, which encodes a subunit of the UvrABC nucleotide excision repair generalized DNA repair process also appears to have acquired new mutations (Kenyon and Walker 1981). This is notable as it is involved in SOS response in E. coli and therefore might play a similar role in P. carbekii, which may experience environmental stress during its time on the H. halys egg surface. surA is yet another gene involved in DNA repair in which four substitution mutations are observed but it is unclear as to how these may affect its ability to fold outer membrane proteins or respond to high or low pH (Dartigalongue et al. 2001).

Degraded Cell Division Genes May Contribute to Nonuniform Cell Morphology

The cells of the gammaproteobacterial symbiont of Murgantia histrionica have been reported to exhibit nonuniform lengths (Prado et al. 2010) and in SEM, TEM, and FISH imaging of P. carbekii revealed cells with similar morphologies (fig. 1C, D, and G). The observed cell morphology may reflect cells undergoing cell division but the loss of the Rep helicase (rep) and presence of a truncated FtsK and pseudogenized FtsN gene may impair cytokinesis. Mutations in rep in E. coli resulted in severe growth defects and a near-doubling of the amount of chromosomal DNA present within each cell (Trun 2003). FtsK directs DNA translocation and chromosome segregation during cytokinesis in E. coli and the P. carbekii ortholog (932 amino acids) is approximately 80% of the length of FtsK in P. ananatis (1,112 amino acids) and the P. stali symbiont (1,143 amino acids). Although it lacks the DNA-binding C-terminal gamma domain (Pfam: PF01580), it retains the putative transmembrane FtsK-SpoIIE domain that is implicated in DNA translocation in E. coli (Begg et al. 1995) and Ba. subtilis (Fleming et al. 2010). Escherichia coli ∆ftsK-encoded proteins with substitutions in the C-terminal domain show impaired DNA binding but remain capable of functioning in DNA translocation and chromosome segregation (Sivanathan et al. 2006). Additionally, E. coli with FtsK lacking the C-terminal domain exhibits a higher proportion of filamentous cells compared with the wild-type (Sivanathan et al. 2006). Sequence analysis alone is not conclusive on whether or not P. carbekii FtsK performs similarly to orthologs in P. ananatis or E. coli, or if its lack of a C-terminal domain contributes to the observed P. carbekii cell morphology. Although 387 nt coding for an intact N-terminal portion of the protein is detected, ftsN has been annotated as a pseudogene because an in silico translation of an adjacent 382 nt reveals a region coding for the peptidoglycan-binding SPOR domain found within the C-terminal portion of FtsN that is peppered with stop codons and frameshifts. FtsN is important in cell division as it interacts with another key late-stage cytokinesis protein, FtsA, to trigger septation, yet FtsA has been shown to function independently of FtsN (Bernard et al. 2007). Although ftsN is absent in the Buchnera genome, truncated and near-full length orthologs were annotated in the Ishikawaella (170 amino acids) and the Pl. stali symbiont (279 amino acids) genomes. Consequences of these mutations could be nonlethal impairment of cytokinesis within P. carbekii, leading to the observed cell morphologies, but further experimental work to confirm the expression and functionality of P. carbekii FtsK and FtsN is needed.

Extrachorion Persistence and Stress Tolerance in P. carbekii

During the period between oviposition and consumption by emerged nymphs (~1 week; Nielsen and Hamilton 2009),
P. carbekii lives outside of host gastric tissues within a maternally secreted matrix on the egg surface. Although beneath and intercalated within the extrachorion matrix of H. halys eggs, P. carbekii is exposed to fluctuations in environmental conditions (e.g., temperature fluctuations, UV radiation, desiccation, etc.) and biotic interactions (i.e., competition with other microbes or predation) that may result in stress in P. carbekii cells. A number of putative stress–response genes were detected in the P. carbekii genome and are discussed briefly. Protein denaturation, misfolding, and aggregation can be detrimental to cells following heat stress and sigma-32, which is encoded by rpoH, is at the hub of rapid responses to heat stress through the expression of enzymes involved in refolding and stabilizing denatured proteins (chaperones: GroESL, ClpB, IbpAB, and DnaKJ) or degrading irrecoverably misfolded proteins (proteases: ClpAP, ClpXP, HslUV, and FtsH) (reviewed in Rosen and Ron 2002; Gunesekere et al. 2006).

Pantoea carbekii encodes all of the aforementioned enzymes, with ibpB present on pBMSBPS1. Ibpb binds aggregated or denatured proteins and has been shown to increase in expression in response to temperature up-shifts and during biofilm formation in E. coli (Lasokowska et al. 1996; Ren et al. 2004). Some of its functions are dependent on Ibpb (Kuczyńska-Wisnik et al. 2002), which is encoded on the P. carbekii chromosome and is present in both Ishikawaella and Buchnera. In Buchnera, Ibpb significantly impacts heat-tolerance in pea aphids as shown by a single nucleotide deletion in ibpb that resulted in sharp fitness declines in pea aphids harboring Buchnera. Ibpb were maintained at elevated temperatures (25–30°C) (Dunbar et al. 2007). The presence of ibpb and the pBMSBPS1-encoded ibpb in P. carbekii may indicate that it also confers elevated temperature tolerance to H. halys.

Additionally, host factor one (hfq) is also encoded on the P. carbekii chromosome and it has both RNA chaperone activity and regulates the expression of, among other stress response genes, the stationary phase and environmental stress response regulator, RpoS (Muffler et al. 1996; Moll et al. 2003; Battesti et al. 2011).

Many of the aforementioned genes are not present in Ishikawaella, which has a similar mode of inheritance to P. carbekii, but it is packaged in symbiont capsules, which are deposited next to the eggs (Fukatsu and Hosokawa 2002), rather than in a surface smearing. Ishikawaella might not have the same exposure to abiotic and biotic pressures as P. carbekii does within the extrachorion matrix. It has been suggested that the Ishikawaella capsule conditions mimic that of the host midgut, protecting it from environmental fluctuations (Fukatsu and Hosokawa 2002; Hosokawa et al. 2005). As a result, the environmental conditions for Ishikawaella may not necessitate retention of the same repertoire of stress response genes as P. carbekii.

Conclusions

We report the first ultrastructural characterization of P. carbekii within the extrachorion matrix of brown marmorated stink bug eggs by SEM, and provide the complete genome sequence of this agricultural pest primary symbiont. Detection of the symbiont within this extrachorion matrix confirms that H. halys shares its primary symbiont transmission modality with other phytophagous stink bugs that exhibit egg-smearing behavior and harbor gammaproteobacterial symbionts within the gastric ceca of the distal midgut. Elucidating the biochemical composition of the extrachorion matrix may reveal chemical attractants that stimulate nymphal feeding behavior immediately following emergence (i.e., presence of specific attractants) as well as compounds involved in improving the survivability of P. carbekii outside of host tissues following oviposition and prior to nymph consumption.

Detailed genomic analysis of P. carbekii indicates that it has the potential to provision a wide range of dietary supplements, namely essential amino acids and vitamins, to its herbivorous host, and that the genome displays hallmarks of long-term host association, including a low G+C% and a reduced genic repertoire and genome size. If P. carbekii is provisioning nutrients to its host insect, then it would support the ability of H. halys to exploit a wide range of host plants and would explain breadth of greater than 150 host plants H. halys is known to feed upon (Bergmann et al. 2014). The P. carbekii genome is reduced in size relative to known nonhost-restricted Pantoea spp., but many gammaproteobacterial intracellular symbionts have genomes less than 1 Mb in size and the retention of genes involved in peptidoglycan and cell wall biosynthesis, and stress response, which are absent in many gammaproteobacterial intracellular mutualist genomes, are among those contributing to the relatively modest size reduction of the P. carbekii genome. The multiphasic lifestyle (e.g., within the extrachorion matrix, insect gut during migration to the gastric ceca, and intraluminal crypt-dwelling) of the P. carbekii may necessitate a broader genic repertoire than bacterial symbionts that exist solely within host tissues.

Supplementary Material

Supplementary materials S1 and S2 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

Acknowledgments

The authors thank Andrew Michel, of the Department of Entomology and the Ohio Agricultural Research and Development Center, for Halyomorpha halys specimens and thoughtful comments. They also thank the anonymous reviewers for their many helpful comments.
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Associate editor: Daniel Sloan