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Genomic Features of a Bumble Bee Symbiont Reflect Its Host Environment

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Here, we report the genome of one gammaproteobacterial member of the gut microbiota, for which we propose the name "*Candidatus* Schmidhempelia bombi," that was inadvertently sequenced alongside the genome of its host, the bumble bee, *Bombus impatiens*. This symbiont is a member of the recently described bacterial order *Orbales*, which has been collected from the guts of diverse insect species; however, "*Ca*. Schmidhempelia" has been identified exclusively with bumble bees. Metabolic reconstruction reveals that "*Ca*. Schmidhempelia" lacks many genes for a functioning NADH dehydrogenase I, all genes for the high-oxygen cytochrome *o*, and most genes in the tricarboxylic acid (TCA) cycle. "*Ca*. Schmidhempelia" has retained NADH dehydrogenase II, the low-oxygen specific cytochrome *bd*, anaerobic nitrate respiration, mixed-acid fermentation pathways, and citrate fermentation, which may be important for survival in low-oxygen or anaerobic environments found in the bee hindgut. Additionally, a type 6 secretion system, a Flp pilus, and many antibiotic/multidrug transporters suggest complex interactions with its host and other gut commensals or pathogens. This genome has signatures of reduction (2.0 megabase pairs) and rearrangement, as previously observed for genomes of host-associated bacteria. A survey of wild and laboratory *B. impatiens* revealed that "*Ca*. Schmidhempelia" is present in 90% of individuals and, therefore, may provide benefits to its host.

Autochthonous gut microorganisms greatly influence animal health by providing a range of nutritional, developmental, and protective benefits (e.g., energy, vitamins, immune priming, detoxification, and pathogen exclusion) to their hosts (1–3). Highly consistent gut-associated microbes are common among eusocial insect species (4–8). These microbes can be actively passed between generations through trophallaxis (mouth-to-mouth or anus-to-mouth food sharing) or passively transmitted via a fecal-oral route due to communal living (9, 10). Beneficial gut bacteria are often selectively transmitted between generations and form well-established interactions with their hosts (11); however, many of the eusocial-insect-associated microbes have unknown relationships with their hosts. Genome sequencing can provide insight into the metabolic capabilities and biological significance of these host-associated microbes.

Guts of bumble bee (*Bombus* sp.) are commonly inhabited by two bacterial species that are closely related to the honey bee (*Apis mellifera*)-associated lineages of *Gilliamella apicola* (previously called the "Gamma-1" phylotype, *Gammaproteobacteria*) and *Snodgrassella alvi* (previously called the "Beta" phylotype, *Betaproteobacteria*) (6, 12–14). Metagenomic sequencing of the *A. mellifera* gut bacteria revealed that *Gilliamella* and *Snodgrassella* contain genes that may contribute to pollen digestion, detoxification of mannose, and defense against pathogens (15). The closely related gut microbiota of *Bombus terrestris* was experimentally determined to provide an extended-immune phenotype against the trypanosomatid gut parasite *Crithidia bombi*, yet the mechanism of this defense was not identified (16).

The *Bombus impatiens* genome sequencing project recovered the genome sequence of a gammaproteobacterium related to the *Gilliamella apicola* clade. This genome sequence provides insights into the phylogenetic relationships and lifestyle of *Gilliamella*-related bumble bee symbionts, as well as clues about the role of this gammaproteobacterium in *B. impatiens* biology. Here, we describe the first genome sequence from the gammaproteobacterial order *Orbales* and compare it to the metagenome recovered from the *A. mellifera* microbiota to identify basic metabolic and ecological attributes and potential effects that this symbiont may have on its host.

MATERIALS AND METHODS

Source DNA, sequencing, identification and assembly of bacterial reads. A single adult male *Bombus impatiens* approximately at 24 h posteclosion was collected from a colony purchased from Biobest Biological Systems (Leamington, Ontario, Canada). DNA was extracted from the entire specimen using a standard phenol-chloroform preparation.

Three paired-end libraries were constructed with fragment sizes of 400, 4,000, and 8,000 bp. DNA was sequenced using Illumina GAIIx sequencing technology to generate eight lanes of 125-bp raw sequences. After error correction, the average read length was 105 bp, and the total number of reads used in assembled contigs was 150,442,748. Based on an estimated genome size of 250 megabases (Mb), this yields approximately 65-fold coverage of the *B. impatiens* genome. The reads were assembled using the CABOG assembler (17) modified to handle short Illumina reads. The draft assembly contained 69,944 contigs (including symbiont contigs) with a length greater than 100 bp, of which 6,658 contigs were longer than 10,000 bp. In addition, the assembly contained 1,086,650

Received 29 January 2014 **Accepted** 8 April 2014
Published ahead of print 18 April 2014
Editor: H. Goodrich-Blair
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00322-14.

doi:10.1128/AEM.00322-14

degenerate contigs, primarily small repetitive sequences or contigs with very low quality. Another 60,355,858 reads remained as unassembled singletons.

To scan the 69,944 contigs for sequences of possible bacterial symbionts, we initially aligned them to the genomes of four strains of *Wolbachia*, which we considered the first candidates because *Wolbachia* bacteria are the most ubiquitous endosymbionts of invertebrates. We aligned all assembled contigs against genomes of each of these four strains using the promer program from the MUMmer package (18), which translates both the reference and the query sequences to amino acids in all six reading frames. This allowed a more sensitive alignment than a DNA-based approach. We identified multiple contigs that had strong homology to at least one of the *Wolbachia* species, indicating that bacterial sequences might be present in the whole-genome data. We extracted these contigs and used the BLASTX program to map them against the complete nonredundant protein database at NCBI in order to find bacteria that were closer to the symbiont in *B. impatiens*. The best hits from this mapping were to three *Gammaproteobacteria* species: *Photorhabdus asymbiotica*, *Yersinia enterocolitica*, and *Proteus mirabilis*, indicating that the bacterial sequences were from *Enterobacteriales* (Gammaproteobacteria) and not *Wolbachia* (*Alphaproteobacteria*).

We then aligned all assembled contigs and all singleton reads to the complete genomes of each of these three bacteria. We ran DNA alignments and translated protein alignments using the nucmer and promer programs from the MUMmer package and mapped all contigs against both the DNA and the protein sequences of the three bacterial genomes. The more sensitive protein-based alignments were compared to the annotated coordinates of the proteins in each of the bacterial genomes. Each contig that contained at least one complete protein was considered possibly bacterial. In addition, contigs not longer than 500 bp that contained at least a partial match at greater than 60% identity to any bacterial protein were also considered bacterial. This analysis identified 367 regular contigs, 1,129 degenerate contigs, and 255,589 singleton reads as possibly bacterial in origin. Next, we used BLASTX to search each of these contigs and reads against the entire NCBI protein database, and we eliminated any contig with a better match to a eukaryotic species than to a bacterial genome. This left 343 regular contigs, 941 degenerate contigs, and 255,589 singleton reads as likely bacterial sequences.

We used the CABOG assembler's raw output files to locate all reads used to build these bacterial contigs and extracted these reads from the original sequence files with their paired-end mates. This resulted in 615,185 mate pairs from the 400-bp insert size library, 20,716 mate pairs from the 4-lb insert size library, 8,164 mate pairs from the 9-kb insert size library, and 121,568 unpaired reads. These reads were assembled *de novo* with the CABOG assembler. The final bacterial assembly contained 1,998,543 bp in just 79 contigs. The largest contig contained 110,984 bp, and the assembly had an N_{50} size of 39,885 bp. The 79 contigs were combined into 33 scaffolds spanning 2,004,741 bp, with a scaffold N_{50} size of 98,624 bp and a maximum scaffold of 204,248 bp. The approximate average coverage of the genome is 37-fold.

Annotation of the bacterial genes. Gene annotation was completed in the automated Integrated Microbial Genomes Expert Review (IMG/ER; Joint Genome Institute) pipeline (19). Protein-coding sequences and RNA-coding genes were predicted within its framework using Prodigal and tRNAS-can-1.23 (19). Functional annotations were assigned to genes based on protein domain characterization according to clusters of orthologous groups of proteins (COG) terms, Pfam, TIGRfam, InterPro domains, Gene Ontology (GO) terms, and KEGG Orthology (KO) terms with metabolic pathway maps. Additional manual assessment with KEGG (20), EcoCyc (21), and the MetaCyc Pathological program (22) was performed to check pathway completeness. Genes identified as missing from main pathways (e.g., tricarboxylic acid [TCA] cycle or NADH dehydrogenase I) were manually investigated using BLASTP searches against the *B. impatiens* symbiont genome with corresponding genes from *Escherichia coli*. A metabolic map was manually created for the *B. impatiens* gammaproteobacterium (*Bi*G). The annotated contigs for this genome are available at the IMG/ER website (https://img.jgi.doe.gov/cgi-bin/er/main.cgi) (proposed name, "*Candidatus* Schmidhempelia bombi Bimp").

Core gene phylogeny and metagenome gene phylogenies. A set of 89 single-copy orthologous (SiCO) genes was selected from an original set of 203 consistently present, single copy, non-horizontally transferred core genes (23); this set was used to reconstruct the phylogenetic placement of *Bi*G. SiCO genes were selected from 28 gammaproteobacterial genomes using SiCO gene lists in MaGE (24) or using a cutoff of a bit-score ratio of >0.30 in a BLASTP search with the 203 SiCO genes from *E. coli* (23). Inferred protein sequences of the 89 genes were individually aligned in MUSCLE (25) and concatenated together. The Gblocks (26) program was used to remove poorly aligned regions, and the resulting alignment consisted of 27,452 amino acid sites. Maximum-likelihood reconstruction was performed with RAxML (27) on 100 bootstrap replicates using the PROTGAMMA algorithm and the WAG substitution matrix, which were selected with ProtTest, version 2.4 (28). Individual SiCO gene trees were built with the same methods as the multiprotein data set and subsequently sorted with PhyloSort (29).

Gene content of the *Bi*G genome was compared to the gene content of the metagenome of the *A. mellifera* gut microbiota, which was determined in a previous study (15). The comparison was made to the taxonomic bins from that study including the all-bacteria bin and the gammaproteobacteria (Gamma) bin using the COG (clusters of orthologous genes) annotations from IMG/ER (Joint Genome Institute). Of the 195 SiCO genes present in *Bi*G, a set of 193 was identified as being shared between *Bi*G and the Gamma bin using BLASTP to identify pairwise protein sequence identities (SiCOs identified with a bit-score ratio of >0.30 to the set found in Lerat et al. [23]). The subset of 89 SiCO genes used to construct the multiprotein phylogeny was amended with corresponding SiCO genes from the Gamma bin, and individual gene trees were constructed using previously described methods. The phylogenetic relationships between the Gamma bin sequences, which contained genes from both *G. apicola* and *Frischella perrara*, and the *Bi*G were collected for each tree.

16S rRNA gene phylogeny. The 16S rRNA gene sequence was used to reconstruct and refine the phylogenetic relationships of the *Bi*G among a representative set of *G. apicola*, *F. perrara* (collected from the genera *Apis* and *Bombus*), and closely related sequences from the NCBI database. One of the four 16S rRNA gene copies in the *Bi*G genome was selected and aligned with the 16S rRNA data set using Infernal (30). A maximum-likelihood phylogeny was constructed with RAxML using the GTRCAT parameter and 100 bootstrap replicates (27).

Putative HGT. The IMG annotation pipeline identified putative horizontally transferred genes. Further analysis of potential horizontal gene transfer (HGT) was assayed with a phylogenetic pipeline modified from Moustafa and Bhattacharya (29). Briefly, the pipeline identifies closely related genes in the NCBI database, aligns the amino acid sequences, and constructs phylogenies for each. PhyloSort (29) was used to find trees which indicated horizontal transfer from *Firmicutes* to *Bi*G. The vast majority of genes from the *Bi*G genome have best hits to *Gammaproteobacteria*. The potential HGT genes are the outstanding examples having best hits to and clustering in a phylogeny with *Firmicutes*. These potential HGT genes come from many different contigs, most of which are fairly long and are otherwise composed of genes with top hits to *Gammaproteobacteria*. Because the phylogenetically near neighbors to *Bi*G have not been densely sampled, these computational findings may reflect sampling bias rather than true horizontal gene transfer, and further work will be needed to validate these findings.

PCR screen for *Bi*G among *B. impatiens* individuals. Specific primers BombusG2f (5′-CTGGTCGTCTGGAGTATTGT-3′) and BombusG2r1 (5′-AGGTCCGCTCACCATCGCTG-3′) were used to search for *Bi*G within *B. impatiens* individuals or gut organs from wild and laboratory individuals. Cycling was performed with an annealing temperature of 54°C for 35 cycles and a 1-min extension.

TABLE 1 General features of the *Bi*G genome

Parameter[a]	Value for the parameter
Chromosome length (bp)	>1,999,325
Extrachromosomal elements	Presence unknown
GC content (%)	36.6
Total no. of predicted CDSs	1,770
Coding density (%)	82
Average CDS length (bp)	954
No. of rRNA operons	
5S rRNA	7
16S rRNA	4
23S rRNA	5
No. of tRNAs	50

[a] CDS, coding sequence.

Nucleotide sequence accession number. The bacterial symbiont genome assembly was deposited in the GenBank database under accession number AWGA00000000.

RESULTS AND DISCUSSION

Retrieval of a nearly complete genome of a *B. impatiens* symbiont. Sequencing of the *B. impatiens* genome resulted in the byproduct sequencing of a gammaproteobacterial genome corresponding to an organism present in the bee from which DNA was extracted. From the ~250 Mb of assembled sequence from the project, 79 contigs representing 2 Mb were assigned to the bacterial genome (see Materials and Methods). Contigs that represent the gammaproteobacterial genome harbor at least one open reading frame (ORF) per contig and have an N_{50} length of 39.9 kb. To determine the completeness of the genome and the number of distinct bacterial genomes present, coverage of a preselected set of 203 single-copy, near-universal bacterial genes was assessed using BLASTP. We identified 195 of these 203 genes (96%), each with exactly one copy indicating the complete or nearly complete coverage of a single bacterial genome. Calculation of G+C content of the contigs produced a unimodal distribution with a mean of 36.6%, and the average depth of coverage (37-fold) was consistent across the contigs, providing further evidence for the retrieval of a single bacterial genome. Here, we refer to this organism as the *B. impatiens* gammaproteobacterium, or *Bi*G.

The *Bi*G genome is at least 1.99 Mb in size (Table 1). A total of 50 tRNA genes and 23 tRNA synthetase genes were identified, corresponding to all 20 amino acids. Altogether, 1,694 protein-coding genes were identified from the assembly, with 14% (236) of them having unknown functions. Roughly 72% had functions predicted as clusters of orthologous groups (COGs) (31). The largest COG categories represented were translation (9.7%), general function only (9.3%), amino acid transport and metabolism (8.6%), cell wall biogenesis (7.6%), replication (6.8%), coenzyme transport and metabolism (5.8%), and carbohydrate transport and metabolism (5.3%).

The small size and low G+C content resemble genomes of previously sequenced host-dependent commensals and pathogens (e.g., "*Candidatus* Hamiltonella defensa" [32] or *Histophilus somnus* [33]). Further, gut bacteria with strict host associations (e.g., *Helicobacter* sp., *Lactobacillus reuteri*, and *Pasteurellales* species) often have small genomes (34, 35), suggesting that *Bi*G may have a restricted host distribution. Overall, the genome lacks large regions of chromosomal synteny with other genomes, even for regions conserved among many species of *Enterobacteriales* and *Pasteurellales* (see Fig. S1 and S2 in the supplemental material for examples). However, contigs from the *A. mellifera* Gamma bin harbor regions with strikingly similar gene orders, even with interrupted operons for TCA cycle enzymes (*sucABCD*; only *sucCD* are retained) and the NADH dehydrogenase I complex (*nuoA-nuoN*; only *nuoJ-nuoN* are retained) (see Fig. S1 and S2). The conserved synteny between *Bi*G and sequences from the gut microbiota of *A. mellifera* confirms the presence of similar bacteria in both bee species and substantiates the robust assembly of the *Bi*G contigs.

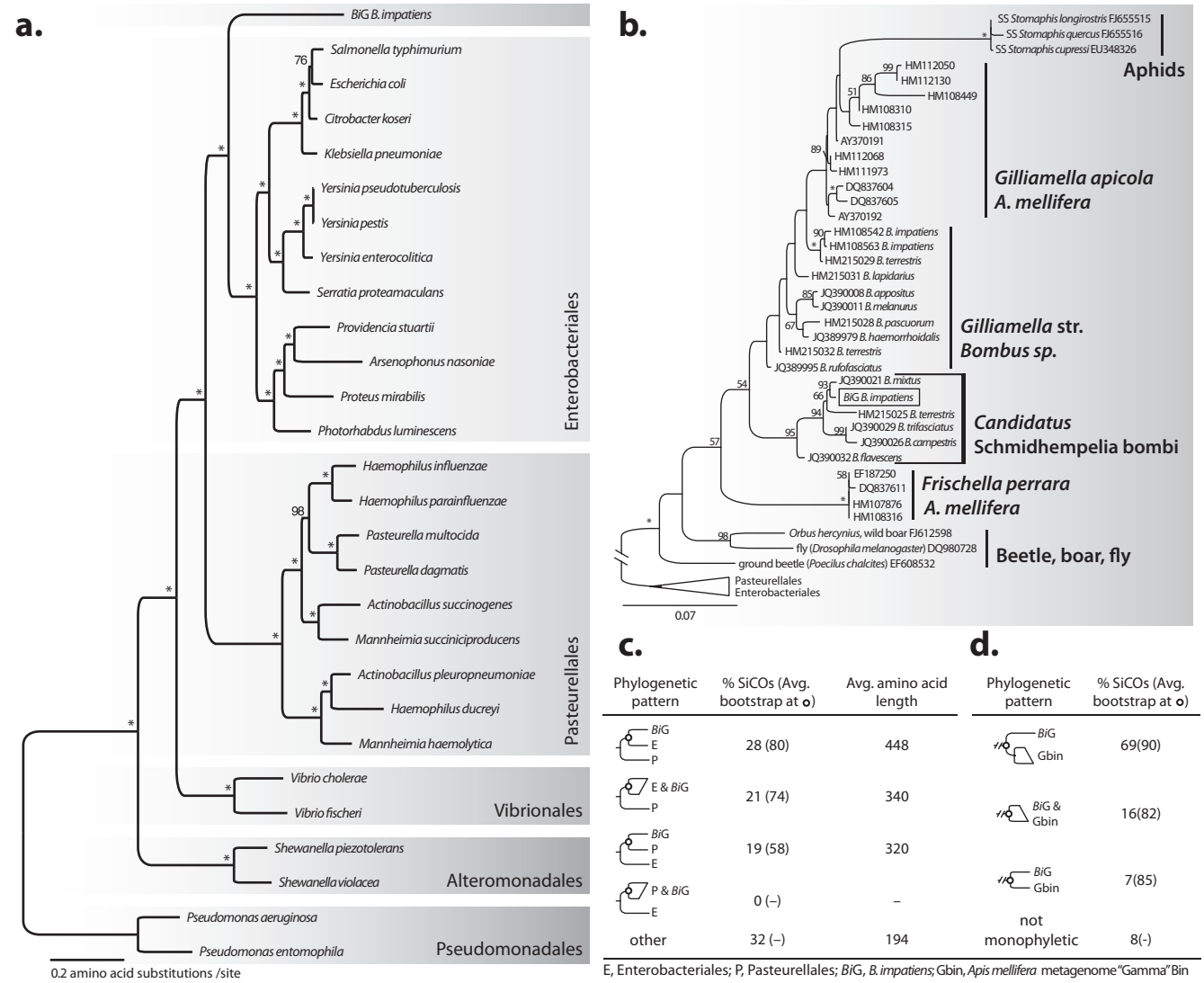
The majority of *Bi*G protein-coding genes (98%, 1,659/1,694) were shared with the Gamma bin of the *A. mellifera* gut microbiota metagenome. Notably, genes for the four subunits of the respiratory nitrate reductase A are present in *Bi*G but absent from the Gamma bin (see Table S1 in the supplemental material).

Consistent association of *Bi*G with laboratory-raised and wild *Bombus impatiens* individuals. The *Bi*G genome sequence was retrieved from a single male individual from a laboratory-raised *B. impatiens* colony. To understand the consistency of the association between *Bi*G and *B. impatiens*, we undertook a PCR survey of workers, males, and queens from natural and laboratory environments. Specific primers that differentiated between *Bi*G and the closely related *Gilliamella* revealed that *Bi*G is nearly ubiquitous (90%, 18/20) among laboratory adults and larvae and wild adults (see Fig. S3 in the supplemental material). The bacterium was detected in all gut organs (crop, midgut, ileum, and rectum), as well as from the leg DNA extraction. The leg sample may have been surface contaminated with feces because the source colonies lacked locations to remove excrement, possibly increasing fecal contamination within the hive relative to normal conditions.

The presence of this bacterium in both wild and laboratory-raised *B. impatiens* bees implies that *Bi*G is passed from one colony to the next, as documented for members of the closely related genus *Gilliamella* in honey bees and bumble bees (14, 16). Queens were shown to harbor this bacterium, thus affording a transmission link between the annual disintegration of *Bombus* colonies in the fall and the founding of new colonies in the spring. Overall, *Bi*G is a common associate of *B. impatiens* and potentially many other bumble bee species.

***Bi*G is a relative of *Gilliamella apicola* in the newly described order *Orbales*.** Our 16S rRNA gene tree placed *Bi*G as a member of *Orbales*, a bacterial order previously recovered from numerous honey bee and bumble bee species (13). *Bi*G clusters among sequences collected from native and commercially reared bumble bee species from around the world (Fig. 1b; see also Fig. S4 in the supplemental material). A survey of the bacterial associates of bumble bees (36) shows that *Bi*G clusters within a separate clade from the genus *Gilliamella* (found in honey bees and bumble bees [13]), *Frischella perrara* (Gamma-2 of honey bees [37]), and other *Orbales* species, with strong bootstrap support (95%) (Fig. 1b; see also Fig. S4). The *Bi*G sequences were identified in geographically and phylogenetically diverse bumble bees, but their 16S rRNA sequences are very similar (>98% identical). Therefore, *Bi*G is a member of a geographically widespread bacterial clade that is strictly associated with bumble bees, based on surveys conducted to date.

Our concatenated, multiprotein phylogeny retrieved high bootstrap support for previously established evolutionary relationships between the bacterial orders of *Gammaproteobacteria*



Phylogenetic pattern	% SiCOs (Avg. bootstrap at ○)	Avg. amino acid length
BiG, E, P	28 (80)	448
E & BiG, P	21 (74)	340
BiG, P, E	19 (58)	320
P & BiG, E	0 (–)	–
other	32 (–)	194

Phylogenetic pattern	% SiCOs (Avg. bootstrap at ○)
BiG, Gbin	69(90)
BiG & Gbin	16(82)
BiG, Gbin	7(85)
not monophyletic	8(-)

E, Enterobacteriales; P, Pasteurellales; *BiG*, *B. impatiens*; Gbin, *Apis mellifera* metagenome "Gamma" Bin

FIG 1 (a) Phylogenetic placement of *Bi*G as a singleton clade among five orders of *Gammaproteobacteria*. Maximum-likelihood reconstruction inferred from 89 concatenated SiCO genes (27,452 aligned amino acid sites). (b) Location of *Bi*G among members of the insect gut-associated *Orbales*. The tree is based on maximum likelihood with the 16S rRNA gene. (c) Proportions of the 89 individual SiCO gene trees that returned each phylogenetic pattern with their average (Avg) bootstrap support at the indicated node and average gene length. (d) Proportions of individual SiCO gene trees that united the *Bi*G and the *Apis mellifera* Gamma bin (Gbin) gene copies as a monophyletic clade and average bootstrap support. Asterisks represent bootstrap support values of 100, and values below 50 are not shown. Str, strain; SS, secondary symbiont.

included in the analysis (*Enterobacteriales*, *Pasteurellales*, *Vibrionales*, *Alteromonadales*, and *Pseudomonadales*) (23, 38, 39) (Fig. 1a). In this tree, *Bi*G is sister to *Enterobacteriales*, which supports the previous placement of *Orbales* (Fig. 1a) (13). Trees created with individual SiCO genes varied in their support, mainly due to their differing sequence complexities (46 to 1,407 amino acids in length) (Fig. 1c). The majority (61/89) of individual SiCO genes resulted in a tree topology uniting *Bi*G, *Enterobacteriales*, and *Pasteurellales*. Nearly three-fourths of those trees (44/61) placed *Bi*G sister to, or within, the *Enterobacteriales*, with an average bootstrap support of >80% (Fig. 1c).

To identify the relationship between *Bi*G and the sequences from the Gamma bin of the *A. mellifera* metagenome, which included sequences from the related genera *Gilliamella* and *Frischella*, the corresponding Gamma bin SiCO sequences were analyzed with each of the 89 SiCO genes. Many strains of *Gilliamella* and *Frischella* are present within the metagenomic data set for the *A. mellifera* microbiota, and the number of copies corresponding to the 89 *Bi*G SiCOs varies among the loci. The majority (92%, 81/89) of SiCOs retrieves the Gamma bin sequences and the *Bi*G sequence as a clade (Fig. 1d). The *Bi*G gene copy usually branches basal to the Gamma bin sequences (69%, 61/81), with a high average bootstrap support (90%) for this placement (Fig. 1d), which adds further support that *Bi*G represents a new genus of *Orbales* and not a member of *Gilliamella* or *Frischella*. This data set of protein-coding genes supports the sister relationship between the *Orbales* (i.e., *Bi*G and the Gamma bin clade) and *Enterobacteriales*, with high support (71% average bootstrap value; 36/57 trees) for this pattern, rather than *Pasteurellales* (50% average bootstrap value; 21/57 trees).

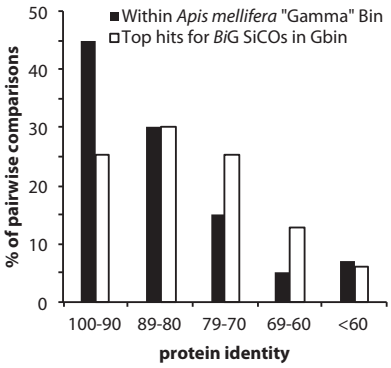


FIG 2 Pairwise comparisons between copies of SiCO genes separated by protein identity determined with BLASTP. Comparisons within the *Apis mellifera* Gamma bin show protein identities between 80 and 100% (15), whereas identities between the *Bi*G genome SiCOs and the Gamma bin (Gbin) are between 70 and 90%.

Pairwise protein identities of homologs in the *A. mellifera* metagenome reveal considerable strain variation within the Gamma bin. By collecting only the top hit in the Gamma bin of the *A. mellifera* metagenome for each of the 193 *Bi*G SiCO genes, we find that *Bi*G has a lower average protein identity to copies within the Gamma bin than these copies have to one another (Fig. 2) (15). Thus, the *Bi*G genome is considerably more divergent than the entire community of gammaproteobacterial strains within the *A. mellifera* microbiota, suggesting that *Bi*G be considered a separate genus of *Orbales*.

All phylogenies and the shared synteny of *Bi*G with contigs in the Gamma bin support placement of *Bi*G within the newly described order *Orbales*, close to *Gilliamella* (13). The 16S rRNA of the *Bi*G group sequences have an average distance of 5% or greater to *Gilliamella*, *Frischella*, and other *Orbales*, indicating sufficient divergence to be ranked as a separate genus.

Metabolic reconstruction of *Bi*G and comparisons to the *A. mellifera* gut microbiota. The *Bi*G genome contains the majority of genes in predicted metabolic pathways for glycolysis, gluconeogenesis, the full pentose phosphate pathway, nucleotide metabolism, lipopolysaccharide production, peptidoglycan fabrication, heme and siroheme, and ubiquinone assembly (Fig. 3). Biosynthetic capabilities remain intact for the majority of the 20 protein amino acids and many cofactors. However, individual genes are missing for several pathways, including the final step in biosynthesis of the branched-chain amino acids (Ile, Val, and Leu), conversion of Ser to Gly, and synthesis of Pro (Fig. 3). Insects, including *B. impatiens*, encode enzymes for these reactions, and *Bi*G contains transporters for these amino acids, suggesting that these products may be imported from the host.

Numerous genes underlying the pathways in aerobic energy metabolism that are conserved in many *Enterobacteriales* and *Pasteurellales* are not encoded in the *Bi*G genome (Fig. 3). Incomplete pathways include NADH dehydrogenase I (missing *nuoABCDEFGHI*) and cytochrome *o* (missing *cyoABCDE*) (for synteny evidence, see Fig. S2 in the supplemental material). Of the 16 core genes that normally encode enzymes underlying the TCA cycle, *Bi*G is missing 14: *gltA*, *acnAB*, *icd*, *sucAB*, *sdhABCD*, *fumABC*, and *mdh*. *Bi*G has retained only the succinyl-coenzyme A (CoA) synthetase genes *sucC* and *sucD* (for synteny evidence, see Fig. S1).

In contrast, the *Bi*G genome possesses several low-oxygen and anaerobic mechanisms for energy production. It has kept the NADH dehydrogenase II (*ndh*) and cytochrome *bd* (*cydAB*), which have optimal conditions at low oxygen levels and have been shown to be critical for colonization of the hypoxic mouse gut by *Escherichia coli* (40, 41). In *E. coli* this cytochrome has its maximal level of expression and optimal conditions at low oxygen concentrations; additionally cytochrome *bd* reinforces the hypoxic environment by scavenging the remaining oxygen.

Additionally, *Bi*G encodes genes for anaerobic respiration. Together the periplasmic respiratory nitrate reductase A (*narGHI*) and the nitrate-induced formate dehydrogenase N (*fdnGHI*) can produce a respiratory chain, resulting in a proton motive force and cytoplasmic nitrite (42, 43). The NADH-dependent nitrite reductase (*nirBD*) detoxifies resulting nitrite to ammonia (44). Notably, the *narGHI* genes are not present in the *A. mellifera* gut microbiota metagenome Gamma bin, which further suggests that this *Gilliamella*-like bacterium is a divergent lineage among this group of common bee associates (see Table S1 in the supplemental material). However, the absence of the *narGHI* genes in the *A. mellifera* metagenome could reflect incomplete sampling of the gammaproteobacterial genomes.

*Bi*G has retained genes for citrate fermentation and for several branches of mixed acid fermentation. A Na/citrate symporter is adjacent to the complete citrate fermentation gene locus (*citABCDEFGX*), encoding capabilities for transporting citrate into the cell, sensing citrate in the environment, and degrading citrate to oxaloacetate and acetate via citrate lyase. Mixed-acid fermentation genes are present for lactate fermentation (*ldhA*), pyruvate cleavage to formate and acetyl-CoA (*pflB*), ATP generation via acetate formation (*pta* and *ackA*), and NAD^+ generation through ethanol production (*adhE* and *adhP*). Fermentation results in the excreted metabolites of ethanol, formate, and the short-chain fatty acids acetate and lactate. Short-chain fatty acids are the bulk of carbon and energy sources of ruminant animals (45). This raises the possibility that the bacterium is providing its host a nutritional benefit through biosynthesis of needed compounds (46). However, biosynthetic contributions (e.g., amino acids or vitamins) may not be very significant because the bee diet is composed of both easily accessible mono- and disaccharides and protein-rich pollen (47).

A primary constituent of the bee diet is pollen, which presents plant macromolecules that are difficult to degrade and that form barriers surrounding the pollen germ, the primary source of protein for bees. Some strains of *G. apicola* from *A. mellifera* encode pectate lyase and are able to degrade pectin, a function that may aid their host in nutrient acquisition by releasing pollen contents (15). This capability is not encoded within the *Bi*G genome; however, *Bi*G has transporters (*bglF*) and beta-glucosidases (*bglA*, glucoside hydrolase family 4) (48) that may confer the ability to import and metabolize some of the products of cellulase activity (e.g., cellobiose and cellotriose) potentially produced by other bacteria present in the gut or present in nectar or pollen. Metabolic scavenging of these compounds could provide the majority of energy for this organism since glucose and fructose (the major sugars in honey) are absorbed rapidly in the midgut and are not abundant in the hindgut (49, 50).

The pattern of missing genes implies that *Bi*G may inhabit a low-oxygen niche within the bumble bee gut, which is consistent with the cultivation conditions for related members of *Orbales*, both microaerophilic (*Gilliamella apicola*) and anaerobic (*Frisch-*

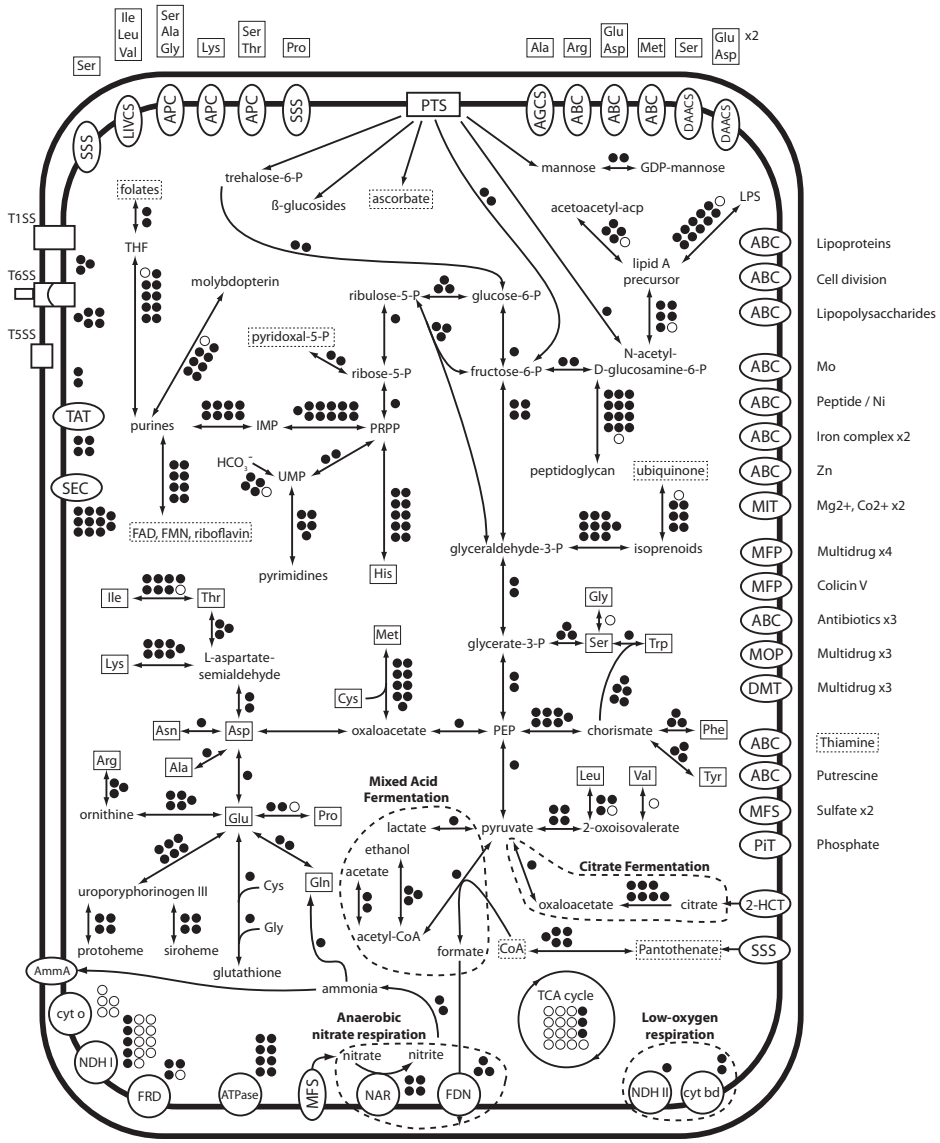


FIG 3 Metabolic reconstruction of *Bi*G. Dots next to each connecting line (arrows) represent genes involved with that pathway; filled dots represent intact genes that are present, and open dots represent missing genes. Amino acids are in solid boxes, and vitamins and cofactors are in dashed boxes; ovals represent membrane transporters, with their putative targets listed next to them outside the cell. T1SS, type 1 secretion system; T5SS, type 5 secretion system; T6SS, type 6 secretion system; TAT, twin-arginine translocation pathway; SEC, SEC system; NDHI, NADH dehydrogenase 1; ATPase, F-type ATPase; NDHII, NADH dehydrogenase 2; NAR, respiratory nitrate reductase A; FDN, formate dehydrogenase N; cyt *bd*, cytochrome *bd* complex; cyt *o*, cytochrome *o* complex; FRD, fumarate reductase.

ella perrara) (13, 37). Recent *in situ* analysis of the *A. mellifera* microbiota showed that the majority of the bacteria (including *G. apicola*) reside within the hindgut (14). Oxygen levels could govern colonization of the gut organs (i.e., anaerobic hindgut and aerobic foregut), restricting members of the *Orbales* to low-oxygen or anaerobic environments. Fluorescent *in situ* microscopy of the *Bombus* microbiota and microsensor surveys (i.e., O_2) of the *Apis* and *Bombus* gut are needed to test this hypothesis and determine the breadth of this pattern in corbiculate bees (51).

Candidate horizontal gene transfers from *Firmicutes* to *Bi*G. A total of 54 genes were identified by IMG as putatively horizontally transferred from *Firmicutes* to *Bi*G. Of these genes, PhyloSort supported horizontal transfer from *Firmicutes* for 39 genes (Table 2). Because *Bi*G is a novel genus, phylogenetic placement of its genes may not be as reliable as with a more thoroughly sequenced clade, and these 39 genes should be considered candidates worthy of further scrutiny rather than confirmed horizontal gene transfers. Closer analysis of potentially transferred genes identified several sugar uptake and degradation genes, including the previously mentioned *bglA* (Table 2). These genes may enable *Bi*G to utilize the numerous sugars found in nectar that cannot be metabolized by *B. impatiens* or that are abundant in the gut. An intact operon for the uptake of mannose (phosphotransferase system [PTS]) may have been transferred from a species related to *Bacillus* (Table 2). The mannose

PTS has been shown to have an extensive history of horizontal transfer in bacteria and is mostly found in bacteria associated with animal guts (52). Mannose is toxic to honey bees and bumble bees; therefore, microbial assimilation of this sugar could protect the host from small amounts of mannose, which is often present in nectar (53, 54). Alternatively, these transport systems can often act on a broad range of substrates (52). Evidence that mannose PTSs may be linked to the bee gut environment also comes from the finding that they are overrepresented in the *A. mellifera* gut metagenome relative to other gut metagenomes (15); however, this overrepresentation may merely reflect the taxonomic composition of the bacterial community.

Potential interactions with the host and other gut microorganisms. Several putative host interaction factors were present in the *Bi*G genome, including Sel1 repeat proteins, bacterial Ig-like domains, and bacterial α_2-macroglobulins; these could be critical for recognition of this bacterial strain by the host epithelium (55). A full Flp pilus gene set was present; this apparatus is known to be critical for adhesion and biofilm formation (56). Strains of *Gilliamella* associated with the honey bee have been shown to form thick biofilms within the ileum (14), and genes involved in biofilm formation/adhesion were overrepresented in the *A. mellifera* Gamma bin (15). More generally, adhesion to the gut wall may play a critical role for insect gut associates because, unlike mammals, insects do not secrete a mucus layer that facilitates microbial residence (57).

Similar to the *A. mellifera* Gamma bin of the metagenome, *Bi*G has a marked abundance of antibiotic/multidrug resistance transporters, including several ABC, drug metabolic transporter (DMT), multidrug/oligosaccharidyl-lipid/polysaccharide (MOP), membrane fusion protein (MFP), Eam/Emr, and arabinose efflux pumps. As mentioned in Engel et al. (15), the bee gut is exposed to plant defense compounds during pollen/nectar foraging and ingestion. These compounds could be a selective force for mechanisms enabling efficient elimination of these toxins in bee gut bacteria.

Complex interactions with the host epithelium are additionally supported by the presence of multiple secretion systems (types I, V, and VI) (Fig. 3). The recently described type VI secretion system (T6SS) evolved from viral tail fibers into a syringe-like effector delivery mechanism that is present in many bacteria and is becoming understood as pivotal to interactions among bacteria and between bacteria and eukaryotes (58–61). The cell-puncturing device (valine-glycine repeat protein G [VgrG] gene) of the T6SS, is critical for discriminating and attaching to target cells (62). The *Bi*G genome contains three intact VgrGs and 13 additional VgrG fragments (see Table S2 in the supplemental material), which may correspond to between 12 and 16 full VgrGs, a relatively large number for a genome of this size (63). These VgrG elements cannot be annotated with target specificity or function, but some have genes adjacent to the C terminus that may be T6SS effectors that interact with the host and other microbes present in the gut (64, 65) (see Table S2). For example, asymptomatic colonization of the mouse gut by *Helicobacter hepaticus* was disrupted when its T6SS was knocked out, leading to overcolonization by *H. hepaticus* and an inflammatory reaction by the host (66). This example suggests that the *H. hepaticus* T6SS facilitates signaling between the bacterium and the host or that the bacterium inoculates the gut epithelium with anti-inflammatory compounds. T6SS effectors also have lytic effects (67) that can be delivered to specific unicellular eukaryotic or bacterial targets, depending upon the VgrG utilized (62, 68). Thus, further investigation of the *Bi*G T6SS and its biological effects on the host bumble bee, gut pathogens, and members of the indigenous microbiota may demonstrate context-dependent interactions.

The bumble bee hive is a resource-rich environment that has a number of autochthonous bacterial species but also attracts a variety of pathogens, many of which are specific to the gut environment (69). Koch et al. (16) showed that protection from pathogens is provided by the resident microbiota in *Bombus terrestris*. The *Bi*G genome provides candidate mechanisms for protection against antagonistic microorganisms invading the gut, and future efforts to cultivate *Bi*G would facilitate direct tests of its effect on bumble bee biology.

Conclusions. As a member of the *Orbales*, the *Bi*G genome represents a recently described order of *Gammaproteobacteria* that is found in honey bees and bumble bees and that has been repeatedly collected from other insects (6, 12, 13, 36, 70). Our analyses show that the *Bi*G genome, sequenced concurrently with the genome of *B. impatiens*, is nearly complete, as indicated by the unimodal G+C content, the presence of a complete tRNA synthetase complement, exactly one copy of 96% of a defined set of single-copy genes, and a consistent coverage of 37-fold across all contigs. Previous genome sequencing projects have been shown to produce data sets corresponding to symbiotic microorganisms (71); however, most of these symbionts do not assemble as well as the *Bi*G genome retrieved in our study (79 contigs), indicating the presence of a clonal or near-clonal bacterial population in the source DNA sample. The loss of central metabolism components (i.e., TCA cycle) and the lack of synteny with other bacterial genomes suggest that this genome and genomes of closely related organisms (*Orbales*) underwent rearrangement, reduction, and specialization to the host environment similar to the processes observed in other symbiotic genomes (72, 73).

"*Candidatus* Schmidhempelia bombi." We propose the name "*Candidatus* Schmidhempelia bombi" for the bacterium identified within the bumble bee *Bombus impatiens* and several other bumble bee species (36). Phylogenetic reconstruction places this bacterium within the *Orbales*, a recently described family of *Gammaproteobacteria* that has been identified nearly exclusively within insect guts. "Ca. Schmidhempelia" is a distinct clade (95% bootstrap support) of *Orbales* that is separate from the named genera *Gilliamella* and *Frischella* that are symbiotic in honey bees and bumble bees, as well as from other non-bee-associated members of the *Orbales* (Fig. 1b; see also Fig. S4 in the supplemental material). This bacterium has an average of 5% 16S rRNA gene sequence divergence from *Gilliamella apicola* sequences and has been identified in several bumble bee species from around the world, yet it has not been found in the thoroughly surveyed honey bee microbiota (36). The proposed genus for "Ca. Schmidhempelia bombi" refers to the Swiss evolutionary parasitologist Paul Schmid-Hempel, who has studied the evolutionary ecology of bumble bee species and associated organisms, while the specific epithet reflects that this bacterium resides within bumble bees. Unique features of this organism include its apparent restriction to bumble bee hosts and the 16S rRNA gene sequence 5′-TTTAA AACTGGTCGTCTGGAGTATTGT-3′ (positions 636 to 662 of the 16S rRNA gene, with *E. coli* numbering).

TABLE 2 Genes putatively horizontally transferred into the *Bi*G genome from species of *Firmicutes*

IMG gene object ID[a]	IMG locus tag	Gene product name	Length (aa)	Scaffold IMG accession no.	Scaffold length (bp)	Contig GC%	COG	Genome source
2505924216	GBi_0012.00000200	Arabinose efflux permease	405	GBi_ctg820001289386	38219	0.39	COG2814	*Staphylococcus carnosus* subsp. *carnosus* TM300
2505924217	GBi_0012.00000210	Uridine phosphorylase (EC 2.4.2.3)	251	GBi_ctg820001289386	38219	0.39	COG2820	*Lactobacillus rhamnosus* Lc 705
2505924690	GBi_0029.00000900	Peptide methionine sulfoxide reductase (EC 1.8.4.11, EC 1.8.4.12, EC 1.8.4.11)	177	GBi_ctg820001289403	102,764	0.35	COG0225	*Listeria grayi* DSM 20601
2505924734	GBi_0036.00000050	Nitrous oxide-stimulated promoter.	112	GBi_ctg820001289410	14,474	0.35		*Bacillus coagulans* 2-6
2505924830	GBi_0041.00000130	Uncharacterized protein conserved in bacteria	124	GBi_ctg820001289416	38,071	0.35	COG4687	*Enterococcus faecium* E980
2505924831	GBi_0041.00000140	PTS, mannose/fructose/*N*-acetylgalactosamine-specific component IID	302	GBi_ctg820001289416	38,071	0.35	COG3716	*Enterococcus* sp. strain 7L76
2505924832	GBi_0041.00000150	PTS, mannose/fructose/*N*-acetylgalactosamine-specific component IIC	267	GBi_ctg820001289416	38,071	0.35	COG3715	*Bacillus thuringiensis* subsp. *israelensis* ATCC 35646
2505924833	GBi_0041.00000160	PTS, mannose/fructose/*N*-acetylgalactosamine-specific component IIB (EC 2.7.1.69)	323	GBi_ctg820001289416	38,071	0.35	COG3444	*Bacillus thuringiensis* IBL200
2505924834	GBi_0041.00000170	Uncharacterized protein conserved in bacteria	244	GBi_ctg820001289416	38,071	0.35	COG1636	*Enterococcus italicus* DSM 15952
2505924868	GBi_0043.00000060	Predicted hydrolases of the HAD superfamily	257	GBi_ctg820001289418	10,510	0.37	COG0561	*Listeria welshimeri* serovar 6b, SLCC5334
2505924928	GBi_0048.00000120	Adenine-specific DNA methylase Mod (EC 2.1.1.72)	628	GBi_ctg820001289423	59,508	0.38	COG2189	*Clostridium* cf. *saccharolyticum* K10
2505924929	GBi_0048.00000130	Restriction endonuclease (EC 3.1.21.5)	1,035	GBi_ctg820001289423	59,508	0.38	COG3587	*Clostridium* cf. *saccharolyticum* K10
2505924938	GBi_0048.00000220	Pyrimidine-nucleoside phosphorylase (EC 2.4.2.2)	433	GBi_ctg820001289423	59,508	0.38	COG0213	*Bacillus atrophaeus* 1942
2505924940	GBi_0048.00000240	Nucleoside permease	398	GBi_ctg820001289423	59,508	0.38	COG1972	*Bacillus pumilus* SAFR-032
2505925004	GBi_0051.00000020	Predicted membrane protein	173	GBi_ctg820001289427	39,885	0.37	COG4905	*Eubacterium cylindroides* T2–87
2505925044	GBi_0052.00000060	Putative glucose uptake permease	291	GBi_ctg820001289428	34,555	0.36	COG4975	*Listeria welshimeri* serovar 6b, SLCC5334
2505925059	GBi_0052.00000200	Type I site-specific restriction-modification system, R (restriction) subunit and related helicases (EC 3.1.21.3)	1,026	GBi_ctg820001289428	34,555	0.36	COG0610	*Bacillus coagulans* 36D1
2505925060	GBi_0052.00000210	Restriction endonuclease S subunits (EC 3.1.21.3)	417	GBi_ctg820001289428	34,555	0.36	COG0732	*Enterococcus italicus* DSM 15952
2505925061	GBi_0052.00000220	Type I restriction-modification system methyltransferase subunit (EC 2.1.1.72)	859	GBi_ctg820001289428	34,555	0.36	COG0286	*Bacillus coagulans* 36D1
2505925076	GBi_0054.00000020	Chorismate mutase (EC 5.4.99.5)	88	GBi_ctg820001289430	20,279	0.36	COG1605	*Anaerofustis stercorihominis* DSM 17244
2505925077	GBi_0054.00000030	Hypothetical protein	77	GBi_ctg820001289430	20,279	0.36		*Desulfitobacterium hafniense* DCB-2

2505925164	GBi_0059.00000040	Transposase	44	GBi_ctg820001289435	37,254	0.37		*Staphylococcus aureus* subsp. *aureus* ST398
2505925221	GBi_0060.00000290	Predicted branched-chain amino acid permease (azaleucine resistance)	234	GBi_ctg820001289436	77,226	0.37	COG1296	*Listeria welshimeri* serovar 6b, SLCC5334
2505925222	GBi_0060.00000300	Predicted membrane protein	107	GBi_ctg820001289436	77,226	0.37	COG4392	*Listeria welshimeri* serovar 6b, SLCC5334
2505925262	GBi_0061.00000100	PTS beta-glucoside-specific IIA component, Glc family (TC 4.A.1.2.5)	631	GBi_ctg820001289437	27,350	0.37	COG1263	*Bacillus subtilis* subsp. *spizizenii* W23
2505925263	GBi_0061.00000110	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (EC 3.2.1.86)	478	GBi_ctg820001289437	27,350	0.37	COG2723	*Clostridium bartlettii* DSM 16795
2505925412	GBi_0068.00000040	Transcriptional regulator	307	GBi_ctg820001289444	65,738	0.37	COG0583	*Syntrophomonas wolfei* subsp. *wolfei* strain Goettingen, DSM 2245B
2505925413	GBi_0068.00000050	Methionine synthase (B12-independent) (EC 2.1.1.14)	761	GBi_ctg820001289444	65,738	0.37	COG0620	*Paenibacillus curdlanolyticus* YK9
2505925414	GBi_0068.00000060	Tagatose-1,6-bisphosphate aldolase (EC 4.1.2.40)	345	GBi_ctg820001289444	65,738	0.37	COG3684	*Enterococcus gallinarum* EG2
2505925484	GBi_0069.00000240	Fructose-2,6-bisphosphatase (EC 5.4.2.1)	253	GBi_ctg820001289445	47,248	0.37	COG0406	*Listeriaceae* bacterium TTU M1-001
2505925526	GBi_0070.00000200	Amidases related to nicotinamidase	182	GBi_ctg820001289446	64,926	0.34	COG1335	*Geobacillus* sp. strain WCH70
2505925638	GBi_0072.00000210	Serine/threonine exchange transporter, LAT family (TC 2.A.3.8.12)	485	GBi_ctg820001289448	31,379	0.36	COG0531	*Bacillus cereus* subsp. *cytotoxis* NVH 391-98
2505925734	GBi_0076.00000160	Predicted membrane protein	221	GBi_ctg820001289453	45,947	0.35	COG2323	*Leptotrichia goodfellowii* F024
2505925735	GBi_0076.00000170	Acetyltransferases (EC 2.3.1.-)	150	GBi_ctg820001289453	45,947	0.35	COG0456	*Desulfitobacterium dehalogenans* JW/IU-DC1, ATCC 51507
2505925751	GBi_0076.00000330	Predicted hydrolases of the HAD superfamily	279	GBi_ctg820001289453	45,947	0.35	COG0561	*Listeria seeligeri* serovar 1/2b, SLCC3954
2505925796	GBi_0078.00000400	Predicted HD superfamily hydrolase	222	GBi_ctg820001289456	51,284	0.37	COG1418	*Pediococcus pentosaceus* ATCC 25745
2505925870	GBi_0082.00000030	Predicted glutamine amidotransferase involved in pyridoxine biosynthesis	197	GBi_ctg820001289460	6,516	0.39	COG0311	*Bacillus subtilis* subsp. *subtilis* strain 168
2505925871	GBi_0082.00000040	Pyridoxine biosynthesis enzyme	295	GBi_ctg820001289460	6,516	0.39	COG0214	*Bacillus subtilis* subsp. *subtilis* strain 168
2505925872	GBi_0082.00000050	Transcriptional regulator, GntR family	467	GBi_ctg820001289460	6,516	0.39	COG1167	*Listeria monocytogenes* 08-5923

[a] ID, identifier.

ACKNOWLEDGMENTS

We thank Ellen O. Martinson for her helpful discussion and comments on the manuscript and Jennifer H. Wisecaver for bioinformatics assistance.

V.G.M. was supported by the Center for Insect Science (University of Arizona) and a National Science Foundation award to N.A.M. (NSF 1046153). Sequencing and analysis were supported by NIH Director's Pioneer Award 1DP1OD006416-01 (G. E. Robinson) and by NIH grant R01-HG006677 (S.L.S.). H.K. was supported by Swiss National Science Foundation grants 140157 and 147881.

REFERENCES

1. **Dillon RJ, Dillon VM.** 2004. The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. **49:**71–92. http://dx.doi.org/10.1146/annurev.ento.49.061802.123416.
2. **Shin SC, Kim SH, You H, Kim B, Kim AC, Lee KA, Yoon JH, Ryu JH, Lee WJ.** 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science **334:**670–674. http://dx.doi.org/10.1126/science.1212782.
3. **Stecher B, Hardt WD.** 2011. Mechanisms controlling pathogen colonization of the gut. Curr. Opin. Microbiol. **14:**82–91. http://dx.doi.org/10.1016/j.mib.2010.10.003.
4. **Dietrich C, Köhler T, Brune A.** 2014. The cockroach origin of the termite gut microbiota: patterns in bacterial community structure reflect major evolutionary events. Appl. Environ. Microbiol. **80:**2261–2269. http://dx.doi.org/10.1128/AEM.04206-13.
5. **Funaro CF, Kronauer DJC, Moreau CS, Goldman-Huertas B, Pierce NE, Russell JA.** 2011. Army ants harbor a host-specific clade of *Entomoplasmatales* bacteria. Appl. Environ. Microbiol. **77:**346–350. http://dx.doi.org/10.1128/AEM.01896-10.
6. **Martinson VG, Danforth BN, Minckley RL, Rueppell O, Tingek S, Moran NA.** 2011. A simple and distinctive microbiota associated with honey bees and bumble bees. Mol. Ecol. **20:**619–628. http://dx.doi.org/10.1111/j.1365-294X.2010.04959.x.
7. **Noda S, Kitade O, Inoue T, Kawai M, Kanuka M, Hiroshima K, Hongoh Y, Constantino R, Uys V, Zhong J, Kudo T, Ohkuma M.** 2007. Cospeciation in the triplex symbiosis of termite gut protists (*Pseudotrichonympha* spp.), their hosts, and their bacterial endosymbionts. Mol. Ecol. **16:**1257–1266. http://dx.doi.org/10.1111/j.1365-294X.2006.03219.x.
8. **Russell JA, Moreau CS, Goldman-Huertas B, Fujiwara M, Lohman DJ, Pierce NE.** 2009. Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. Proc. Natl. Acad. Sci. U. S. A. **106:**21236–21241. http://dx.doi.org/10.1073/pnas.0907926106.
9. **Lombardo MP.** 2008. Access to mutualistic endosymbiotic microbes: an underappreciated benefit of group living. Behav. Ecol. Sociobiol. **62:**479–497. http://dx.doi.org/10.1007/s00265-007-0428-9.
10. **Weiss MR.** 2006. Defecation behavior and ecology of insects. Annu. Rev. Entomol. **51:**635–661. http://dx.doi.org/10.1146/annurev.ento.49.061802.123212.
11. **Bright M, Bulgheresi S.** 2010. A complex journey: transmission of microbial symbionts. Nat. Rev. Microbiol. **8:**218–230. http://dx.doi.org/10.1038/nrmicro2262.
12. **Koch H, Schmid-Hempel P.** 2011. Bacterial communities in central European bumblebees: low diversity and high specificity. Microb. Ecol. **62:**121–133. http://dx.doi.org/10.1007/s00248-011-9854-3.
13. **Kwong WK, Moran NA.** 2012. Cultivation and characterization of the gut symbionts of honey bees and bumble bees: *Snodgrassella alvi* gen. nov., sp. nov., a member of the *Neisseriaceae* family of the *Betaproteobacteria*; and *Gilliamella apicola* gen. nov., sp. nov., a member of *Orbaceae* fam. nov., *Orbales* ord. nov., a sister taxon to the *Enterobacteriales* order of the *Gammaproteobacteria*. Int. J. Syst. Evol. Microbiol. **63:**2008–2018. http://dx.doi.org/10.1099/ijs.0.044875-0.
14. **Martinson VG, Moy J, Moran NA.** 2012. Establishment of characteristic gut bacteria during development of the honeybee worker. Appl. Environ. Microbiol. **78:**2830–2840. http://dx.doi.org/10.1128/AEM.07810-11.
15. **Engel P, Martinson VG, Moran NA.** 2012. Functional diversity within the simple gut microbiota of the honey bee. Proc. Natl. Acad. Sci. U. S. A. **109:**11002–11007. http://dx.doi.org/10.1073/pnas.1202970109.
16. **Koch H, Schmid-Hempel P.** 2011. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proc. Natl. Acad. Sci. U. S. A. **108:**19288–19292. http://dx.doi.org/10.1073/pnas.1110474108.
17. **Miller JR, Delcher AL, Koren S, Venter E, Walenz BP, Brownley A, Johnson J, Li K, Mobarry C, Sutton G.** 2008. Aggressive assembly of pyrosequencing reads with mates. Bioinformatics **24:**2818–2824. http://dx.doi.org/10.1093/bioinformatics/btn548.
18. **Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, Salzberg SL.** 2004. Versatile and open software for comparing large genomes. Genome Biol. **5:**R12. http://dx.doi.org/10.1186/gb-2004-5-2-r12.
19. **Markowitz VM, Chen IMA, Palaniappan K, Chu K, Szeto E, Grechkin Y, Ratner A, Jacob B, Huang J, Williams P, Huntemann M, Anderson I, Mavromatis K, Ivanova NN, Kyrpides NC.** 2012. IMG: the integrated microbial genomes database and comparative analysis system. Nucleic Acids Res. **40:**D115–D122. http://dx.doi.org/10.1093/nar/gkr1044.
20. **Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T, Yamanishi Y.** 2008. KEGG for linking genomes to life and the environment. Nucleic Acids Res. **36:**D480–D484. http://dx.doi.org/10.1093/nar/gkm882.
21. **Keseler IM, Bonavides-Martinez C, Collado-Vides J, Gama-Castro S, Gunsalus RP, Johnson DA, Krummenacker M, Nolan LM, Paley S, Paulsen IT, Peralta-Gil M, Santos-Zavaleta A, Shearer AG, Karp PD.** 2009. EcoCyc: a comprehensive view of *Escherichia coli* biology. Nucleic Acids Res. **37:**D464–D470. http://dx.doi.org/10.1093/nar/gkn751.
22. **Caspi R, Altman T, Dale JM, Dreher K, Fulcher CA, Gilham F, Kaipa P, Karthikeyan AS, Kothari A, Krummenacker M, Latendresse M, Mueller LA, Paley S, Popescu L, Pujar A, Shearer AG, Zhang P, Karp PD.** 2010. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res. **38:**D473–D479. http://dx.doi.org/10.1093/nar/gkp875.
23. **Lerat E, Daubin V, Moran NA.** 2003. From gene trees to organismal phylogeny in prokaryotes: the case of the gamma-proteobacteria. PLoS Biol. **7:**e19. http://dx.doi.org/10.1371/journal.pbio.1000019.
24. **Vallenet D, Labarre L, Rouy Z, Barbe V, Bocs S, Cruveiller S, Lajus A, Pascal G, Scarpelli C, Medigue C.** 2006. MaGe: a microbial genome annotation system supported by synteny results. Nucleic Acids Res. **34:**53–65. http://dx.doi.org/10.1093/nar/gkj406.
25. **Edgar RC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32:**1792–1797. http://dx.doi.org/10.1093/nar/gkh340.
26. **Talavera G, Castresana J.** 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst. Biol. **56:**564–577. http://dx.doi.org/10.1080/10635150701472164.
27. **Stamatakis A.** 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **22:**2688–2690. http://dx.doi.org/10.1093/bioinformatics/btl446.
28. **Abascal F, Zardoya R, Posada D.** 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics **21:**2104–2105. http://dx.doi.org/10.1093/bioinformatics/bti263.
29. **Moustafa A, Bhattacharya D.** 2008. PhyloSort: a user-friendly phylogenetic sorting tool and its application to estimating the cyanobacterial contribution to the nuclear genome of *Chlamydomonas*. BMC Evol. Biol. **8:**6. http://dx.doi.org/10.1186/1471-2148-8-6.
30. **Nawrocki EP, Kolbe DL, Eddy SR.** 2009. Infernal 1.0: inference of RNA alignments. Bioinformatics **25:**1335–1337. http://dx.doi.org/10.1093/bioinformatics/btp157.
31. **Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, Krylov DM, Mazumder R, Mekhedov SL, Nikolskaya AN, Rao BS, Smirnov S, Sverdlov AV, Vasudevan S, Wolf YI, Yin JJ, Natale DA.** 2003. The COG database: an updated version includes eukaryotes. BMC Bioinformatics **4:**41. http://dx.doi.org/10.1186/1471-2105-4-41.
32. **Degnan PH, Yu Y, Sisneros N, Wing RA, Moran NA.** 2009. *Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. Proc. Natl. Acad. Sci. U. S. A. **106:**9063–9068. http://dx.doi.org/10.1073/pnas.0900194106.
33. **Siddaramappa S, Challacombe JF, Duncan AJ, Gillaspy AF, Carson M, Gipson J, Orvis J, Zaitshick J, Barnes G, Bruce D, Chertkov O, Detter JC, Han CS, Tapia R, Thompson LS, Dyer DW, Inzama TJ.** 2011. Horizontal gene transfer in *Histophilus somni* and its role in the evolution of pathogenic strain 2336, as determined by comparative genomic analyses. BMC Genomics **12:**570. http://dx.doi.org/10.1186/1471-2164-12-570.
34. **Walter J, Britton RA, Roos S.** 2011. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm. Proc. Natl. Acad. Sci. U. S. A. **108:**4645–4652. http://dx.doi.org/10.1073/pnas.1000099107.
35. **Walter J, Ley RE.** 2011. The human gut microbiome: ecology and recent

evolutionary changes. Annu. Rev. Microbiol. **65:**411–429. http://dx.doi.org/10.1146/annurev-micro-090110-102830.
36. **Koch H, Abrol DP, Li J, Schmid-Hempel P.** 2013. Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees. Mol. Ecol. **22:**2028–2044. http://dx.doi.org/10.1111/mec.12209.
37. **Engel P, Kwong WK, Moran NA.** 2013. *Frischella perrara* gen. nov., sp. nov., a gammaproteobacterium isolated from the gut of the honey bee, *Apis mellifera*. Int. J. Syst. Evol. Microbiol. **63:**3646–3651. http://dx.doi.org/10.1099/ijs.0.049569-0.
38. **Gao B, Mohan R, Gupta RS.** 2009. Phylogenomics and protein signatures elucidating the evolutionary relationships among the *Gammaproteobacteria*. Int. J. Syst. Evol. Microbiol. **59:**234–247. http://dx.doi.org/10.1099/ijs.0.002741-0.
39. **Williams KP, Gillespie JJ, Sobral BWS, Nordberg EK, Snyder EE, Shallom JM, Dickerman AW.** 2010. Phylogeny of *Gammaproteobacteria*. J. Bacteriol. **192:**2305–2314. http://dx.doi.org/10.1128/JB.01480-09.
40. **Jones SA, Chowdhury FZ, Fabich AJ, Anderson A, Schreiner DM, House AL, Autieri SM, Leatham MP, Lins JJ, Jorgensen M, Cohen PS, Conway T.** 2007. Respiration of *Escherichia coli* in the mouse intestine. Infect. Immun. **75:**4891–4899. http://dx.doi.org/10.1128/IAI.00484-07.
41. **Jones SA, Gibson T, Maltby RC, Chowdhury FZ, Stewart V, Cohen PS, Conway T.** 2011. Anaerobic respiration of *Escherichia coli* in the mouse intestine. Infect. Immun. **79:**4218–4226. http://dx.doi.org/10.1128/IAI.05395-11.
42. **Jones RW.** 1980. Proton translocation by the membrane-bound fomate dehydrogenase of *Escherichia coli*. FEMS Microbiol. Lett. **8:**167–171. http://dx.doi.org/10.1111/j.1574-6968.1980.tb05072.x.
43. **Jormakka M, Tornroth S, Byrne B, Iwata S.** 2002. Molecular basis of proton motive force generation: structure of formate dehydrogenase-N. Science **295:**1863–1868. http://dx.doi.org/10.1126/science.1068186.
44. **Cole JA, Brown CM.** 1980. Nitrite reduction to ammonia by fermentative bacteria: short circuit in the biological nitrogen cycle. FEMS Microbiol. Lett. **7:**65–72. http://dx.doi.org/10.1111/j.1574-6941.1980.tb01578.x.
45. **Dehority BA.** 1997. Foregut fermentation, p 39–83. *In* Mackie RI, White BA, Isaacson RE (ed), Gastrointestinal microbiology, vol 1. Chapman & Hall, New York, NY.
46. **Breznak JA, Kane MD.** 1990. Microbial H_2/CO_2 acetogenesis in animal guts—nature and nutritional significance. FEMS Microbiol. Rev. **7:**309–313. http://dx.doi.org/10.1111/j.1574-6941.1990.tb01698.x.
47. **Kane MD.** 1997. Microbial fermentation in insect guts, p 231–268. *In* Mackie RI, White BA, Isaacson RE (ed), Gastrointestinal microbiology, vol 2. Chapman & Hall, New York, NY.
48. **Grabnitz F, Seiss M, Rucknagel KP, Staudenbauer WL.** 1991. Structure of the beta-glucosidase gene *bglA* of *Clostridium thermocellum*. Eur. J. Biochem. **200:**301–309. http://dx.doi.org/10.1111/j.1432-1033.1991.tb16186.x.
49. **Crailsheim K.** 1988. Intestinal transport of sugars in the honeybee (*Apis mellifera*). J. Insect Physiol. **34:**839–845. http://dx.doi.org/10.1016/0022-1910(88)90117-5.
50. **Crailsheim K.** 1988. Regulation of food passage in the intestine of the honeybee (*Apis mellifera*). J. Insect Physiol. **34:**85–90. http://dx.doi.org/10.1016/0022-1910(88)90158-8.
51. **Schramm A.** 2006. Microsensors for the study of microenvironments and processes in the intestine of invertebrates, p 463–473. *In* Konig H, Varma A (ed), Intestinal microorganisms of soil invertebrates, vol 6. Springer-Verlag, Berlin, Germany.
52. **Zúñiga M, Comas I, Linaje R, Monedero V, Yebra MJ, Esteban CD, Deutscher J, Pérez-Martinez G, González-Candelas F.** 2005. Horizontal gene transfer in the molecular evolution of mannose PTS transporters. Mol. Biol. Evol. **22:**1673–1685. http://dx.doi.org/10.1093/molbev/msi163.
53. **Barker RJ, Lehner Y.** 1974. Influence of diet on sugars found by thin-layer chromatography in thoraces of honey bees, *Apis mellifera* L. J. Exp. Zool. **188:**157–164. http://dx.doi.org/10.1002/jez.1401880204.
54. **Pawlikowski T.** 2010. Pollination activity of bees (Apoidea: Apiformes) visiting the flowers of *Tilia cordata* Mill. and *Tilia tomentosa* Moench in an urban environment. J. Apic. Sci. **54:**73–79.
55. **Budd A, Blandin S, Levashina EA, Gibson TJ.** 2004. Bacterial α_2-macroglobulins: colonization factors acquired by horizontal gene transfer from the metazoan genome? Genome Biol. **5:**R38. http://dx.doi.org/10.1186/gb-2004-5-6-r38.
56. **Tomich M, Planet PJ, Figurski DH.** 2007. The tad locus: postcards from the widespread colonization island. Nat. Rev. Microbiol. **5:**363–375. http://dx.doi.org/10.1038/nrmicro1636.
57. **Bignell DE, Oskarsson H, Anderson JM.** 1980. Specialization of the hindgut wall for the attachment of symbiotic microorganisms in a termite *Procubitermes aburiensis* (Isoptera, Termitidae, Termitinae). Zoomorphology **96:**103–112. http://dx.doi.org/10.1007/BF00310080.
58. **Leiman PG, Basler M, Ramagopal UA, Bonanno JB, Sauder JM, Pukatzki S, Burley SK, Almo SC, Mekalanos JJ.** 2009. Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. Proc. Natl. Acad. Sci. U. S. A. **106:**4154–4159. http://dx.doi.org/10.1073/pnas.0813360106.
59. **Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, Goodman AL, Joachimiak G, Ordonez CL, Lory S, Walz T, Joachimiak A, Mekalanos JJ.** 2006. A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. Science **312:**1526–1530. http://dx.doi.org/10.1126/science.1128393.
60. **Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, Heidelberg JF, Mekalanos JJ.** 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc. Natl. Acad. Sci. U. S. A. **103:**1528–1533. http://dx.doi.org/10.1073/pnas.0510322103.
61. **Schwarz S, Hood RD, Mougous JD.** 2010. What is type VI secretion doing in all those bugs? Trends Microbiol. **18:**531-537. http://dx.doi.org/10.1016/j.tim.2010.09.001.
62. **Zheng J, Ho B, Mekalanos JJ.** 2011. Genetic analysis of anti-amoebae and anti-bacterial activities of the type VI secretion system in *Vibrio cholerae*. PLoS One **6:**e23876. http://dx.doi.org/10.1371/journal.pone.0023876.
63. **Pukatzki S, McAuley SB, Miyata ST.** 2009. The type VI secretion system: translocation of effectors and effector-domains. Curr. Opin. Microbiol. **12:**11–17. http://dx.doi.org/10.1016/j.mib.2008.11.010.
64. **Jani AJ, Cotter PA.** 2010. Type VI secretion: not just for pathogenesis anymore. Cell Host Microbe **8:**2–6. http://dx.doi.org/10.1016/j.chom.2010.06.012.
65. **Murdoch SL, Trunk K, English G, Fritsch MJ, Pourkarimi E, Coulthurst SJ.** 2011. The opportunistic pathogen *Serratia marcescens* utilizes type VI secretion to target bacterial competitors. J. Bacteriol. **193:**6057–6069. http://dx.doi.org/10.1128/JB.05671-11.
66. **Chow J, Lee SM, Shen Y, Khosravi A, Mazmanian SK.** 2010. Host-bacterial symbiosis in health and disease. Adv. Immunol. **107:**243–274. http://dx.doi.org/10.1016/B978-0-12-381300-8.00008-3.
67. **Russell AB, Hood RD, Bui NK, LeRoux M, Vollmer W, Mougous JD.** 2011. Type VI secretion delivers bacteriolytic effectors to target cells. Nature **475:**343–347. http://dx.doi.org/10.1038/nature10244.
68. **Hood RD, Singh P, Hsu FS, Guvener T, Carl MA, Trinidad RRS, Silverman JM, Ohlson BB, Hicks KG, Plemel RL, Li M, Schwarz S, Wang WY, Merz AJ, Goodlett DR, Mougous JD.** 2010. A type VI secretion system of *Pseudomonas aeruginosa* targets, a toxin to bacteria. Cell Host Microbe **7:**25–37. http://dx.doi.org/10.1016/j.chom.2009.12.007.
69. **Schmid-Hempel P.** 1998. Parasites in social insects. Princeton University Press, Princeton, NJ.
70. **Chandler JA, Lang JM, Bhatnagar S, Eisen JA, Kopp A.** 2011. Bacterial communities of diverse *Drosophila* species: ecological context of a host-microbe model system. PLoS Genet. **7:**e1002272. http://dx.doi.org/10.1371/journal.pgen.1002272.
71. **Salzberg SL, Hotopp JCD, Delcher AL, Pop M, Smith DR, Eisen MB, Nelson WC.** 2005. Serendipitous discovery of *Wolbachia* genomes in multiple *Drosophila* species. Genome Biol. **6:**402. http://dx.doi.org/10.1186/gb-2005-6-7-402.
72. **Burke GR, Moran NA.** 2011. Massive genomic decay in *Serratia symbiotica*, a recently evolved symbiont of aphids. Genome Biol. Evol. **3:**195–208. http://dx.doi.org/10.1093/gbe/evr002.
73. **Nikoh N, Hosokawa T, Oshima K, Hattori M, Fukatsu T.** 2011. Reductive evolution of bacterial genome in insect gut environment. Genome Biol. Evol. **3:**702–714. http://dx.doi.org/10.1093/gbe/evr064.