Origin and Examination of a Leafhopper Facultative Endosymbiont

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Abstract Eukaryotes engage in intimate interactions with microbes that range in age and type of association. Although many conspicuous examples of ancient insect associates are studied (e.g., *Buchnera aphidicola*), fewer examples of younger associations are known. Here, we further characterize a recently evolved bacterial endosymbiont of the leafhopper *Euscelidius variegatus* (Hemiptera, Cicadellidae), called BEV. We found that BEV, continuously maintained in *E. variegatus* hosts at UC Berkeley since 1984, is vertically transmitted with high fidelity. Unlike many vertically transmitted, ancient endosymbioses, the BEV–*E. variegatus* association is not obligate for either partner, and BEV can be cultivated axenically. Sufficient BEV colonies were grown and harvested to estimate its genome size and provide a partial survey of the genome sequence. The BEV chromosome is about 3.8 Mbp, and there is evidence for an extrachromosomal element roughly 53 kb in size (e.g., prophage or plasmid). We sequenced 438 kb of unique short-insert clones, representing about 12% of the BEV genome. Nearly half of the gene fragments were similar to mobile DNA, including 15 distinct types of insertion sequences (IS). Analyses revealed that BEV not only shares virulence genes with plant pathogens, but also is closely related to the plant pathogenic genera *Dickeya*, *Pectobacterium*, and *Brenneria*. However, the slightly reduced genome size, abundance of mobile DNA, fastidious growth in culture, and efficient vertical transmission suggest that symbiosis with *E. variegatus* has had a significant impact on genome evolution in BEV.

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Introduction

Bacterial interactions with insect hosts have pronounced consequences for bacterial lifestyles and genome evolution. After millions of years of co-evolution, ancient insect endosymbionts have experienced irreversible changes in genome size and content, resulting in an inability to survive outside of hosts [24]. However, transitions from free-living to symbiotic lifestyles are likely ongoing in bacterial lineages [1, 12, 18]. Recently evolved facultative symbionts provide the opportunity to examine the initial processes that affected the genome evolution of long-term, obligate endosymbionts. Such a symbiont was identified and cultivated from the leafhopper *Euscelidius variegatus* (Hemiptera, Cicadellidae) [33], and its effects on hosts have been studied for over two decades.

The bacterium of *E. variegatus* (BEV) is transovarially transmitted [34], a factor that in some cases indicates long-term stability or mutualistic tendencies; however, BEV reduces the fitness of its host and is highly pathogenic when injected into other leafhopper species [6, 33, 34]. When tested in 1987, infected *E. variegatus* displayed reduced fecundity, longevity, and increased development

time [34]; since then, the degree of pathogenicity may have declined, under laboratory conditions. BEV can be horizontally acquired by naïve hosts when feeding on plants in the company of infected individuals, but it does not move, replicate, or cause disease within plants [35]. Microscopy of *E. variegatus* indicates that BEV cells are present in the hemolymph and invade the ovaries and midgut epithelium leading to tissue degeneration in the latter [5, 6]. BEV's ability to penetrate into different host tissues and avoid the insect immune system may be due in part to a tolerance for acidic conditions. Even at a pH of 5.2, BEV exhibited cell growth over a period of 24 h (Alexander Purcell, personal communication). Unlike obligate endosymbionts of insects, BEV is not essential for its hosts' survival or reproduction [23]. Additionally, BEV remains one of the few endosymbionts that can be cultivated axenically [32].

Here, we evaluated the efficiency of BEV vertical transmission in *E. variegatus* and calculated its growth rate in culture. Sequencing endosymbiont genomes generally requires complex isolation and enrichment techniques to acquire DNA for sequencing. However, we have taken advantage of the ability to grow BEV in culture to make a preliminary survey of its genome size, content, and phylogenetic origins.

Materials and Methods

History of the BEV Colony

BEV was discovered by Alexander Purcell in laboratory colonies of the leafhopper *E. variegatus* in Pont-de-la-Maye, France and has been maintained at UC Berkeley since 1984 [33]. Unfortunately, original material (insect or bacterial) from 1984 is not available. Infected leafhoppers are reared on a mix of barley, rye, and wheat grasses that are changed every 1 to 2 weeks. For the following experiments, BEV was axenically cultured from leafhoppers on Difco purple broth (PB) with 1.5–2.0% agar, acidified to pH 6.3 with 0.1 N HCl and incubated at 28°C in the dark [33].

Quantifying Vertical Transmission Efficiency of BEV

Ten cages were prepared each containing four rye grass plants and twenty BEV-infected *E. variegatus* adults. The adults were allowed to oviposit for 7 days then removed and frozen. After 5 days of maturation, 5–7 eggs per cage were collected and surface sterilized. DNA was extracted from the eggs and screened with BEV specific 16S ribosomal RNA (rRNA) PCR primers (see Supplemental Methods). Amplicons were run on a 1% agarose gel and

visualized with ethidium bromide (EtBr) and several were randomly sequenced for confirmation.

Estimation of BEV Growth Rate

A triple-cloned isolate (re-plated using a single colony, three times) was plated as a lawn on PB agar, collected and used to inoculate two replicate 1 ml liquid PB cultures. Two sets of ten culture tubes were then inoculated from each of the replicates, wrapped in foil, and incubated at 28°C and shaken at 180 rpm. Culture tubes from each replicate were removed serially starting at day zero, plated and colony-forming units per milliliter (CFU/ml) were counted.

Genome Size Determination

Briefly, BEV DNA was purified for pulsed-field gel electrophoresis (PFGE) by first scraping colonies from PB agar plates and spinning down the cells. Then, the pellet was resuspended in PBS, mixed with an equal volume of 1.5% w/v pulsed-field gel agarose (BioRad) in TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) and solidified in plastic plug molds. The plugs, containing intact cells, were then lysed, washed, and digested with the homing endonuclease I-CeuI (NEB). Plugs and appropriate size standards were separated on a PFGE rig (BioRad) using $0.5\times$ TBE buffer, 1% w/v pulsed-field agarose gels and run conditions listed in the Supplemental Methods. Gels were visualized with EtBr and fragment sizes were estimated manually by measuring fragment migration of the size standard and plotting it by size on a semi-log plot.

BEV Genome Library Construction and Annotation

Total genomic DNA was extracted from a single triple-cloned BEV isolate grown on PB agar using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). This required two extractions to obtain a 20 µg pooled DNA sample (as determined by UV spectrophotometery). This sample was used to generate a blunt-end, short-insert pUC19 library according to published protocols [36] (Supplemental Methods). Transformants bearing plasmids were purified using a boiling mini prep protocol [36], quantified, and Sanger sequenced with M13F primer on an ABI3730xl (Life Technologies). Individual BEV reads were trimmed, dereplicated, and annotated following standard protocols [11]. Reads containing identical transposase gene fragments were manually clustered then assembled into complete insertion sequences. Raw sequence reads have been submitted to the GenBank Trace Archive (2292004866–2292005569).

Reconstruction of BEV Phylogeny

Identified BEV CDSs were screened for orthologs of 203 single-copy, vertically inherited, gammaproteobacterial core genes identified by Lerat et al. [22]. Orthologs from BEV and enterobacterial genomes in GenBank were identified (as in [22]), aligned and manually edited to remove uncertain amino acids (Xs) and gap containing columns (Table S1). Single gene alignments were concatenated and analyzed using maximum likelihood methods with a gamma model of rate heterogeneity estimated from the data and WAG or JTT amino acid substitution model in RAxML [38] and PhyML [16], respectively. Sequences from BEV matching multilocus sequence type (MLST) loci from *Pectobacterium* spp. *Dickeya* spp. and *Brenneria* spp. (EF550652–EF550704, EF550758–EF550810) were aligned and analyzed as above.

Results

BEV Transmission and Growth

Transmission of BEV occurs both vertically and horizontally in laboratory-reared *E. variegatus* [35]. In this experiment, however, we explicitly tested the vertical transmission efficiency of BEV. We randomly selected eggs laid by 200 adults, and surface sterilized and screened them by PCR for BEV. All of the *E. variegatus* eggs were positive for BEV infection (64/64). Adult leafhoppers were not screened for BEV infections, as all of the eggs were positive for BEV. Although BEV does not propagate within plant tissues, it can be grown axenically under microaerophilic conditions (5–18% O_2) at room temperature (22–28°C) in the dark [33]. We measured its growth rate in PB media and estimated that BEV cells doubled every 7.0 h during exponential growth. The lag phase for BEV was about 24 h, the exponential growth phase lasted 36 h, and was followed by a very short stationary phase, after which the numbers of colony-forming units quickly declined (Fig. S1).

Genome Size Estimate

BEV cells were embedded in agarose, lysed, and enzymatically digested, permitting the estimation of its genome size through PFGE. The homing endonuclease I-CeuI recognizes and cuts the 23S rRNA gene. Therefore, the number of bands recovered from a circular chromosome is equivalent to the number of rRNA operons present. Multiple pulsed-field gels indicate that BEV possesses a 3.8 Mbp chromosome (fragment 1 = 1.73, 2 = 1.33, and 3 = 0.74 Mbp) and one ∼53 kb extrachromosomal element
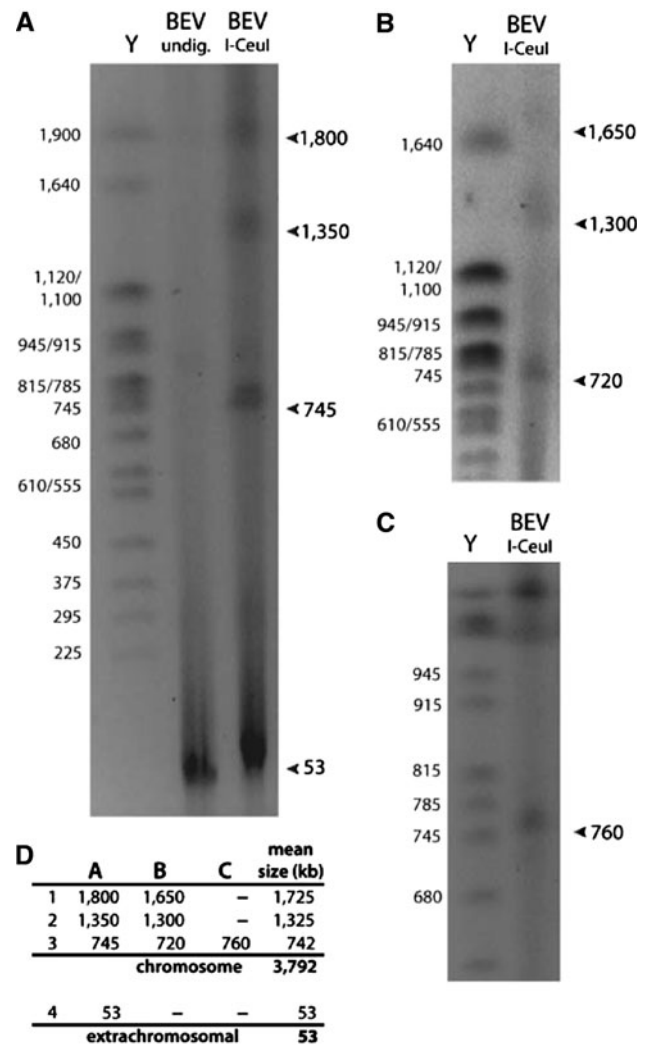

Fig. 1 Genome size estimation of BEV. The BEV genome size was estimated using pulsed-field gel electrophoresis (PFGE) and the *S. cerevisiae* chromosomal ladder (Y) was used as a size standard. **a** BEV DNA was run undigested and digested with I-CeuI to resolve a broad range of fragment sizes (50–1,500 kb). Three chromosomal fragments and one extrachromosomal fragment are apparent. Narrower range conditions were used to better resolve the upper **b** and middle bands **c**. **d** Size estimates for each BEV fragment were determined manually by plotting the migration of the size standard on a semi-log plot and mean fragment sizes are presented

(Fig. 1). We note that the BEV DNA seemed susceptible to degradation, which required the altering the pH of the recovery media (pH 6.5 vs. 7.4) and keeping the lysis and digestion times to a minimum.

BEV Genome Survey

To assess the genome content of BEV, we uni-directionally sequenced 854 clones from a random, short-insert library. Of these, 704 reads representing 626 unique clones and 438,406 bp were assigned to the BEV genome and

Table 1 Functional distribution of CDSs identified in BEV

	No. of unique partial CDSs	% of total CDSs
Core genome		
Cell processes	10	(1.4)
Cell structure	6	(0.8)
Information transfer	40	(5.6)
Metabolism	124	(17.2)
Regulation	4	(0.6)
Transport	40	(5.5)
Putative	83	(11.5)
Unknown	72	(10)
Mobile DNA		
Prophage	65	(9)
Transposase	276	(38.3)
Plasmid	1	(0.1)

annotated (Table 1, Table S2) the remaining reads were vector or poor sequence. The genome-wide G + C content of the reads is 52%, which is consistent with previous estimates from the 16S rRNA gene [33]. Despite size selecting for 1.5–2.0 kb inserts, many clones had inserts <400 bp. Therefore, most CDSs are partial gene fragments, which limits our ability to reconstruct the metabolism of BEV and to identify inactivated genes (pseudogenes). However, the reads still provided insight into the genome content and evolution of the BEV facultative endosymbiont.

The BEV reads encode a number of CDSs involved in pathways related to BEV being a microaerophilic heterotroph (glycolysis, TCA cycle, NADH dehydrogenase I, and ATP synthase, etc.). BEV also contains several specific genes involved in anaerobic respiration including dimethyl sulfoxide (DMSO) reductase (*dmsABC*), nitrate reductase A (*narIJ*), and the biosynthesis of its cofactor molybdenum (*moaAB*). Partial CDSs were detected for the biosynthesis of nine of the amino acids essential for animals. Genes involved in various essential processes such as DNA replication, transcription, translation, and RNA, DNA and protein modification were also identified (Table S2). Unlike obligate mutualists, BEV has also retained a number of genes involved in lipopolysaccharide (LPS) biosynthesis, bacteriophage, as well as genes from type three secretion systems (T3SS).

Destroying Genomes one Transposition at a Time

Overall, 38% of the CDSs are homologous to transposases (276/721) (Table 1). All of the reads can be assembled into 15 distinct insertion sequence (IS) elements, nine of which have readily identifiable boundaries (inverted repeats) (Table 2). Several reads exhibited transposition of

IS elements within other elements (e.g., ISBEV08, ISBEV09). Other reads were identified with transposases adjacent to a bacterial CDS ($n = 44$), 18 of which clearly disrupt the CDS. If the library represents a random sampling of the BEV genome, then the read coverage of each IS element reflects its overall abundance and consequently its transpositional activity.

Origins of BEV

Previous phylogenetic analyses of BEV using 16S rRNA sequences found that it was a member of the Enterobacteriaceae and closely related to a bacterial endosymbiont in bedbugs (*Cimex lectularius* [Hemiptera, Cimicidae]) [4, 17]. Given that protein phylogenies provide a significantly better resolution of the enteric genera than those generated with 16S rRNA [26], we capitalized on the presence of numerous proteins to estimate a well-resolved phylogeny. Using 14 of 203 proteins conserved in most Gammaproteobacteria (Cca, DapA, GyrA, HslU, IspH, MiaA, MurG, PepA, Pth, PurH, RibA, RplQ, RpsC, SucB) we reconstructed a maximum likelihood phylogeny of the Enterobacteriaceae (Fig. 2). BEV was placed with high support within a clade containing the soft-rot plant pathogens *Dickeya* and *Pectobacterium*. A similar placement was found using BEV sequences for 2 MLST loci and a broader sample of taxa representing 49 strains of *Dickeya*, *Pectobacterium,* and *Brenneria* (Fig. S2). However, the delineation between BEV, *Pectobacterium* spp., *Dickeya* spp., and *Brenneria* spp. is unclear due to poor bootstrap support. Consistent with the findings of Naum et al. [26], we found that phylogenies and estimates of pairwise divergence using 16S rRNA were not useful in resolving the relationships of BEV and related enterics (unpublished results).

Discussion

Defining Endosymbiotic Origins

It is notoriously difficult to determine the evolutionary origins of long-term intracellular endosymbionts of insects due to their divergence from free-living bacteria and attendant changes in nucleotide composition [19]. However, recently formed insect endosymbioses provide the opportunity to compare the symbiont with closely related free-living bacteria and examine early changes that are involved in the establishment of the association (e.g., [7, 8]).

Previous work suggested that BEV is indeed a recently established endosymbiont [4]. Our current phylogenetic analyses clearly place BEV among the plant pathogenic genera *Dickeya*, *Pectobacterium*, and *Brenneria* (Fig. 2,

Table 2 Characteristics of consensus insertion sequence (IS) elements in BEV

Transposase	IS family	Total No. of reads[a]	Length	G + C%	Inverted repeats (IRs)	IR length (bp)
ISBEV01	IS1	19	768	51.0	–	–
ISBEV02	IS1650, IS4, IS427	18	920	49.8	+	15
ISBEV03	IS4	62	1,464	55.3	+	16
ISBEV04	IS2	52	1,336	54.1	+	11
ISBEV05	ISSod13 (integrase catalytic subunit)	50	1,201	48.9	+	8
ISBEV06	IS911	77	1,250	56.4	+	8
ISBEV07	–	13	2,269	55.1	+	5
ISBEV08	IS100, IS110, IS1328, IS902	14	1,194	46.8	–	–
ISBEV09	IS116, IS110, IS902	7	932	54.6	–	–
ISBEV10	IS4, IS903	20	1,046	49.8	+	15
ISBEV11	IS1414, IS285 (mutator type)	13	1,314	53.6	+	8
ISBEV12	IS630	20	1,242	51.0	+	8
ISBEV13	IS100, IS110, IS1328, IS902	4	1,063	48.1	–	–
ISBEV14	IS630	2	551	47.4	–	–
ISBEV15	IS204, IS1001, IS1096, IS1165	2	1,335	53.4	–	–

[a] 68 reads have 2–3 separate transposase fragments

Fig. 2 Concatenated protein phylogeny of BEV and enterobacterial relatives. The BEV-symbiont lineage falls firmly among the plant pathogenic genera *Pectobacterium* and *Dickeya*, making BEV distinct from other facultative insect endosymbionts from tsetse flies and aphids (*in black text*). The BEV lineage is less divergent relative to most other facultative endosymbionts (shorter branch length). As for *Sodalis* the genome has not undergone as radical a reduction in size. Thick branches designate bacteria with large genomes (3.8–6.3 Mbp) and thin branches those bacteria with smaller genomes (1.8–2.5 Mbp). Support values for each node were estimated from 100 non-parametric bootstrap replicates in RAxML (*first*) and PhyML (*second*) and those less than 75 are shown in *gray*
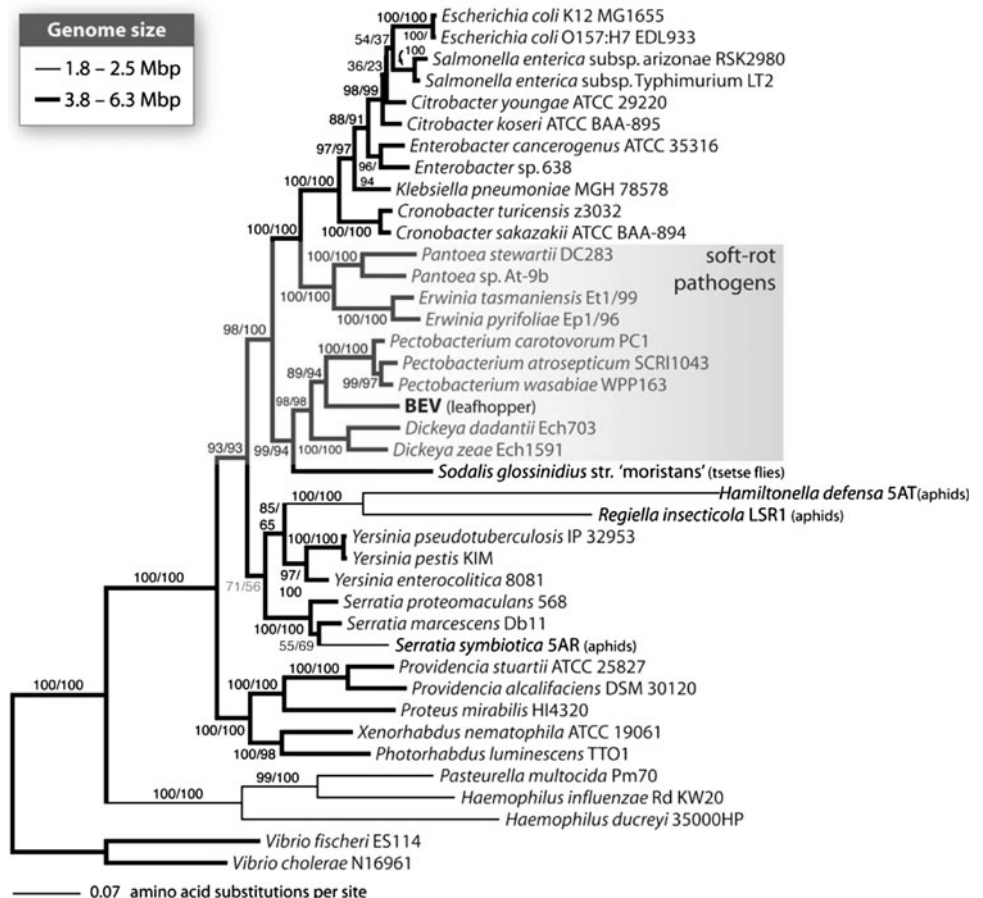
Fig. S2). This relationship is perhaps unsurprising as (i) its host *E. variegatus* feeds on the phloem sap of a variety of grasses and other plants, (ii) BEV is horizontally acquired during feeding, and (iii) plant and animal pathogens rely on common mechanisms to invade tissues, cause disease, and evade immune surveillance [35, 37]. These same molecular mechanisms are in fact crucial for some facultative and obligate endosymbioses [7, 8].

Curiously, when comparing 16S rRNA genes, the most closely related bacterium to BEV is a symbiont of the unrelated blood-feeding bedbug (*C. lectularius,* ∼99% identical) [17]. This same pattern of close relationship between symbionts of blood-feeding and plant-feeding insects has been observed in three other facultative endosymbiont lineages, *Sodalis, Arsenophonus,* and *Rickettsia* [28–30]. These represent independent horizontal transmission events between unrelated plant-feeding and blood-feeding insects. BEV, *Sodalis,* and *Arsenophonus* are also among the few insect endosymbionts that can be cultivated in sterile media [32]. They are all microaerophilic, slow growing, and require rich media and moderate temperatures (25–30°C) [32]. The ability of these facultative endosymbionts to persist outside of a host possibly facilitates successful horizontal transmission.

Vertical Transmission

BEV is efficiently transmitted by transovarial transmission at a level sufficient for retaining it within a host population. Perfect vertical transmission, a hallmark of obligate endosymbionts, is also common among insect reproductive parasites (e.g., *Wolbachia, Cardinium*) [20, 31]. We did not test the possibility that *E. variegatus* nymphs lose the infection as they mature. If this occurs, horizontal transmission may play a role in BEV reinfection or exchange within an *E. variegatus* colony, potentially allowing the bacterium to maintain its mildly pathogenic qualities [6, 34].

Other leafhopper species are known for vertically transmitting bacterial plant pathogens, supporting the possibility of BEV transitioning from a plant pathogen to an insect endosymbiont. For example, rickettsia-like organisms vertically transmitted by leafhoppers are implicated in clover club leaf, rugose leafcurl, and Papaya bunchy top diseases [2, 10, 15]. Transitions may occur in the opposite direction as well, with insect-bacterial symbionts becoming plant pathogens, as may have occurred in the genus *Arsenophonus* [3].

Genomic Insights

Given the genome size estimate of 3.8 Mbp, we have recovered only a fraction of the entire genome (∼12%).

However, the partial genome survey reveals a number of clues regarding the metabolic potential of BEV. For example, we identified several partial gene sequences involved in plant pathogenicity consistent with its phylogenetic affinity with the soft-rot plant pathogens: pectinesterase A (b510), polygalacturonase (b159), polysaccharide deacetylase (b303), exopolysaccharide biosynthesis (b704), and CDSs for two T3SS (b48, b445, b674, b709) [14]. The pectinesterase A locus appears to have been disrupted by an IS element. Inactivation of this and possibly other plant pathogenicity loci due to changes in population dynamics and/or selective regimes likely underlies the inability of BEV to persist or cause damage to host plants [35].

Overall, the BEV genome data share several hallmarks common among young facultative endosymbionts including larger genome sizes (3.5–4.1 Mbp), evidence of diverse metabolic capabilities tempered by gene inactivations and the persistence of virulence-associated loci and mobile DNA [9, 39]. These features contrast sharply with the extremely streamlined genomes of obligate mutualists [24]. The large fraction of CDSs associated with mobile DNA in BEV (276/721) suggests that these elements are active, creating new pseudogenes and possibly leading to repeat-induced genome rearrangements and large-scale deletions [21, 27].

Genomes of bacteria are seldom inundated with such large numbers of mobile DNA elements; however, these elements accumulate in facultative endosymbionts and some pathogens in which small population sizes and changes in selective pressures result in an inability to purge such selfish DNA [11, 21, 25]. Coexistence of distinct endosymbionts within individual hosts may facilitate horizontal transfer of novel mobile elements.

Concluding Remarks

BEV represents the longest-studied, cultivatable insect endosymbiont [33], and studies continue to explore its interactions and impact on its host *E. variegatus* (e.g., [13]). Therefore, BEV provides important insights into the evolutionary transition from free-living bacteria to host-associated symbiont and raises pertinent questions. Considering that BEV can penetrate multiple host organs and is not maintained within a bacteriome, how does it interact with *E. variegatus* obligate endosymbionts? How does it escape the host immune system? Does it provide a benefit for the host under certain conditions? In light of our added genome data, BEV will continue to be an important model for the exploration of insect-bacterial symbiosis.

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