Random Genetic Drift and Selective Pressures Shaping the *Blattabacterium* Genome

Austin Alleman\(^1,2\), Kate L. Hertweck\(^1\) & Srini Kambhampati\(^1\)

Estimates suggest that at least half of all extant insect genera harbor obligate bacterial mutualists. Whereas an endosymbiotic relationship imparts many benefits upon host and symbiont alike, the intracellular lifestyle has profound effects on the bacterial genome. The obligate endosymbiont genome is a product of opposing forces: genes important to host survival are maintained through physiological constraint, contrasted by the fixation of deleterious mutations and genome erosion through random genetic drift. The obligate cockroach endosymbiont, *Blattabacterium* – providing nutritional augmentation to its host in the form of amino acid synthesis – displays radical genome alterations when compared to its most recent free-living relative *Flavobacterium*. To date, eight *Blattabacterium* genomes have been published, affording an unparalleled opportunity to examine the direction and magnitude of selective forces acting upon this group of symbionts. Here, we find that the *Blattabacterium* genome is experiencing a 10-fold increase in selection rate compared to *Flavobacteria*. Additionally, the proportion of selection events is largely negative in direction, with only a handful of loci exhibiting signatures of positive selection. These findings suggest that the *Blattabacterium* genome will continue to erode, potentially resulting in an endosymbiont with an even further reduced genome, as seen in other insect groups such as Hemiptera.

Comprised of over one million species, Class *Insecta* is the most speciose group among animals; at least half of extant genera are estimated to harbor obligate bacterial mutualists\(^1\)–\(^3\). While some intracellular bacteria can be harmful or even lethal to their insect host, many others play an important role in host survival and fecundity\(^3\)–\(^8\). These primary bacterial symbionts exist obligately within the cells of the insect, and are often required for the survival and reproduction of their host organism\(^1\)–\(^9\). An intercellular lifestyle affords endosymbiotic bacteria relative safety from competition and exploitation, in exchange for increased ecological flexibility imparted onto the host species. In many cases, these obligate bacterial mutualists function in the provisioning, recycling, or degradation of essential nutrients, and are vital to those insect species that subsist on nutritionally narrow diets, such as those composed primarily of woody material, plant sap, mammalian blood, or decaying organic material\(^8,10,11\). However, within some insect species primary bacterial endosymbionts also function in non-nutritional roles such as parasitoid defense\(^12\).

With the exception of a single cave-dwelling genus, *Noticola* (Blattodea, Nocticolidae), all cockroach species contain endosymbiotic bacteria within their fat bodies\(^13\)–\(^14\). These obligate endosymbionts belong to the genus *Blattabacterium* (Class Flavobacteria, Phylum Bacteriodetes)\(^15\)–\(^16\). Phylogenetic reconstruction suggests that cockroaches acquired these endosymbionts in a single infection event, dating between 300 million years ago - the approximate age of the first fossil roaches from the Carboniferous - and 140 million years ago, when currently extant families last shared a common ancestor\(^17\)–\(^18\). Initially, the function of these endosymbionts was subject to speculation, owing to their recalcitrance to culture outside their host. However, modern DNA-sequencing techniques have allowed for the study of a number of *Blattabacterium* genomes. From these genomes, it was discovered that the function of *Blattabacterium* is primarily the synthesis of amino acids and vitamins from the nitrogenous waste products of the cockroach host\(^16,19\). Cockroaches store excess nitrogen as uric acid within their fat body cells\(^20\). The decaying organic matter on which cockroaches typically feed is poor in nitrogen content. Thus, a mechanism for recycling nitrogenous waste would be beneficial to any organism whose diet is

---

\(^1\)Department of Biology, University of Texas at Tyler, 3900 University Blvd., Tyler, Texas, 75799, United States.

\(^2\)Present address: Institute of Organismic and Molecular Evolution, Johannes Gutenberg University Mainz, Johannes von Müller Weg 6, Mainz, 55128, Germany. Correspondence and requests for materials should be addressed to A.A. (email: aalleman@uni-mainz.de)
nitrogen-deficient. Unlike most insects, which excrete waste nitrogen as uric acid, cockroaches excrete ammonia. Blattabacterium are capable of utilizing both urea and ammonia because they contain an active urease as well as a functioning urea cycle that converts host urea to ammonia. In addition, increases in dietary nitrogen intake by host cockroaches correlates with increases in uric acid buildup within that host’s fat bodies.

Cockroaches represent an evolutionary lineage consisting of diverse and ancient taxa that have adapted to many habitats and exhibit broad nutritional ecology; their endosymbionts, therefore, represent an excellent system in which to assess relationships between these traits. To date, eight Blattabacterium genomes have been sequenced from the following cockroach host species: Periplaneta americana, Blatta germanica, Cryptococcus punctulatus, Blaberus giganteus, Blatta orientalis, Panesthia angustipennis, Nauphoeta cinerea and the termite, Mastotermes darwiniensis. While these genomes share similar gene composition and genome architecture, each also displays unique capacities for metabolic and physiological function. Thus, while the results of phylogenetic analysis support the hypothesis of co-cladogenesis between the endosymbionts and hosts, genome composition of Blattabacterium is not directly congruent with host phylogeny; rather it varies likely as a function of host nutrition, its relative importance in the mutualism, and the interaction between phenotypic constraint, environmental natural selection, and genetic drift.

An intracellular lifestyle strongly influences the selective pressures and evolutionary trajectories of bacterial endosymbionts. Evolution of the bacterial endosymbiont genome is characterized by elevated mutation rates and biases resulting from the combined effects of physiological constraint preserving symbiont-critical genes, random genetic drift – driven by frequent population bottlenecks, bacterial asexuality, and lack of genetic recombination – and environmental selection acting to reduce genome size. Endosymbionts have been shown to have higher substitution rates and values of non-synonymous to synonymous substitution rates a result attributed to small \( N_e \). Acting through Muller’s Ratchet, asexual reproduction can prevent the recovery of wild-type genotypes through recombination. Loss of recombination is a result of lost DNA repair, uptake, and recombination genes; which is a common pattern of all sequenced bacterial endosymbionts.

However, selection in the form of physiological constraint acts to maintain genes important to the bacterial-insect symbiosis, although its role in the continued erosion of non-essential genes in the bacterial genome is largely unknown. Described genomes from endosymbionts suggest that physiological constraint acts to maintain a gene set that retains its functionality for the host; though selection might also be driving the erosion of bacterial endosymbiont genomes. Certain metabolites ordinarily produced by the bacteria itself may now be obtained directly from the host; under such circumstances, these genes become superfluous and are necessary for neither bacterial survival nor continued host fecundity. As such, a smaller genome results in a cell that is faster and more efficient to reproduce. Particularly at the beginning of endosymbiosis, rapid loss of unnecessary genes may be advantageous.

When compared to free-living bacteria, endosymbionts exhibit increased levels of mutation at synonymous and non-synonymous sites, as well as higher \( d_s/d_o \) ratios, indicating an increase in positive selective pressures and rapid protein evolution. Thus, we may conclude that the endosymbiotic genome is the result of interplay between random genetic drift and the reduction of genes through relaxed selection within large portions of the genome, and physiological constraint acting to preserve those genes vital to host survival and fecundity.

Genome evolution in insect endosymbionts has been the topic of a number of studies. Full genomes from several endosymbionts have been published, including Buchnera aphidicola from aphids, Wigglesworthia from the tssetse fly, Blochmannia from carpenter ants, and Blattabacterium from cockroaches. However, comparatively few of these genomes have been examined for signals of positive selection. Eight fully sequenced Blattabacterium genomes, in addition to the five fully-annotated free-living Flavobacterium genomes for comparison, offers a unique opportunity to investigate the patterns and processes that drive endosymbiotic genome evolution.

We estimated the positive and negative selection events in the genomes of all sequenced Blattabacterium strains, and compared them to those present within the closely-related but free-living Flavobacterium species (F. indicum, F. johnsoniae, and F. psychrophilum), to examine the similarities and differences between these two evolutionarily related, but divergent, groups. We hypothesized that patterns of selection acting upon the Blattabacterium genome will manifest as an elevation in both non-synonymous and synonymous mutation events, as well as a higher \( d_s/d_o \) ratio at sites under significant levels of selection than the free-living Flavobacterium - indicating increased positive selection pressures and an elevated rate of protein evolution. Additionally, we sought to determine whether patterns of selection observed in previous studies across limited numbers of genes are effective at predicting patterns of selection across an entire endosymbiotic genome.

Materials and Methods

Sequence data. Homologous genes for eight Blattabacterium and five Flavobacterium species were manually compiled from genomes available in GenBank (Table 1). With Blattabacterium sp. Cryptococcus punctulatus as the model genome (as it is the smallest Blattabacterium genome and thus frames the “core” gene set of Blattabacterium and Flavobacterium) we manually BLASTed each loci against all other Blattabacterium genomes, as well as against existing Flavobacterium genomes. Resulting BLAST hits were then manually compiled into single homologue files in nucleotide fasta format. We applied Clusters of Orthologous Groups (COGs) to categorize the function of genes in our dataset. Given that genome-wide COG composition is very similar among Blattabacterium, we assessed composition genome-wide as well as in the subset of homologous genes found in all taxa using the Bacterial Annotation System (BASys).
Trimming, Alignment of Homologs, and Phylogeny Building. All scripts developed for this analysis (pre-processing, alignment, phylogenetics, and tests for selection) can be found at https://github.com/k8hertweck/Blattabacteria. Phylogenetic reconstruction of the evolutionary relationships of the eight Blattabacterium and five Flavobacterium species (with eight Escherichia coli strains as outgroup) was carried out using PhyloPhlan under default parameters and whole genomes obtained from GenBank (Table 1). For each set of homologous genes, the last three base pairs (e.g., the stop codon) of each sequence were removed using Prinseq and each homolog group was then aligned using TranslatorX. Gaps present in more than 10% of an alignment were removed using trimAl and alignments summarized using readAl. The best fitting model of molecular evolution for gene alignments, as assessed by both AIC and BIC in jModelTest2, was GTR + G. A maximum likelihood tree for each homologous gene was calculated using this model in PhyML and assessed using 100 bootstrap replicates (alternative models of evolution did not significantly affect tree topology, data not shown). The maximum likelihood tree for each gene (except mia, which possessed two gene copies for some taxa) was used to create a reconciled species tree using ASTRAL v4.7.8.

Selection Analysis. Selection analysis was performed using the HyPhy v2.2.1 (github.com/veg/hyphy) suite of programs. For this analysis, three different selection tests were used: HyPhy's BUSTED, Quick Selection Detection (implementing MEME [Mixed Effects Model of Evolution]) and Branch Site REL. Each of these programs used the same sets of input data; namely the HyPhy alignment combined with the PhyML tree for each gene. Parameters used for these tests may be found here: github.com/k8hertweck/Blattabacteria/blob/master/blattabacteriaBUSTED.tar, github.com/k8hertweck/Blattabacteria/blob/master/blattabacteriaQSD.tar, and github.com/k8hertweck/Blattabacteria/blob/master/blattabacteriaBranchSiteREL.tar.

Summary statistics for all selection analyses performed were produced using an in-house script that may be found here: github.com/k8hertweck/Blattabacteria/blob/master/blattabacteriaSelectionSummary.sh. Statistics of particular import are those referencing branches under positive selection. This information was drawn from the BUSTED and Branch Site REL output. Additional statistics were obtained from the output of blattabacteriaGeneSummary.sh (see Methods section "Trimming, Alignment of Homologs, and Phylogeny Building").

Statistical Analyses. For analyzing the number of positive and negative sites of selection per gene length, a Linear Model was implemented using R version 3.4.4. Within these models, number of positive and negative selection sites were log transformed. A handful of outliers were noted but retained, as their inclusion had a non-significant impact upon the resulting models.

**Table 1.** GenBank accession numbers for bacterial genomes used within this study.
Number of positive and negative selection events vs. individual COG size were carried out using Generalized Linear Models with Poisson distribution with number of selection sites as the response variable and total number of nucleotides in the genome associated with a specific COG as the explanatory variable. As COG and gene length analyses used different datasets - number of selection events per total number of nucleotides of all genes associated with a given COG and number of selection events by gene length, respectively - we found that differing models better fit each type of analysis.

Results and Discussion

Selection by Gene Length and COG Groups. COG analysis indicates an uneven distribution of functional groups within the 304 genes selected for this analysis (Fig. 1). This figure illustrates the functional ‘core’ genes shared by all thirteen genomes analyzed. The majority of these genes are ribosomal in function. Perhaps unsurprisingly, the number of positive selection events (F-value: 40.872, Df: 1, p-value: 6.16e-10, adjusted R-squared: 0.12) as well as negative selection events (F-value: 189.15, Df: 1, p-value: 2.2e-16, adjusted R-squared: 0.38) both showed strong positive correlation with gene length (Fig. 2a,c, respectively). This finding is consistent with the conclusions of previous studies, where natural selection is also correlated with gene length.83 Building upon this on a functional level, however, we also noted that signatures of both positive and negative selection (response variable) correlated strongly with the total number of nucleotides assigned to a specific COG (explanatory variable) across the Blattabacterium genome (Positive selection events: Chi-square p-value: 2.2e-16; Negative selection events: Chi-square p-value: 2.2e-16; Fig. 2b,d, respectively).

Blattabacterium Selection Analysis. Initial analysis of Blattabacterium homolog sets was carried out across all eight of the fully sequenced strains, using a significance level of p ≤ 0.05 for homology. At this significance level, Blattabacterium displays a strong negative mutational bias, with a ratio of sites under negative selection to sites under positive selection of 11:1 across 304 genes. While most loci within Blattabacterium displayed a bias towards negative selection, a few did exhibit signatures of positive selection (Table 2a). That the vast majority of genes within the Blattabacterium genome are experiencing neutral (Table 2b) or negative (not shown in table) selection suggests conserved selective pressures and genome architectures within established endosymbiont lineages.28,69 Accordingly, only a small number of loci were found to show no signs of selection at all (Table 2c).

In recent years, a growing body of work seeks to place an increased emphasis on the role of selection in molecular evolution.84-86 While no predominant explanatory theory for molecular evolution has yet emerged to replace the largely disproven neutral theory, a re-evaluation of the classic, primarily neutral/drift-centric hypotheses for genome evolution in Blattabacterium is necessitated. With the data presented here - and in the light of previous studies into the genome evolution of Blattabacterium - we suggest that the Blattabacterium genome is shaped by a combination of random genetic drift, environmental selection, and physiological constraint on genetic variation. The Blattabacterium lifestyle is characterized by significant and repeated population bottlenecks with each host generation as bacterial cells are transmitted vertically from mother to offspring,17,18 a drastically reduced genome,25-30,39, and elevated rates of mutation. Previous studies into obligate bacterial endosymbiont evolution suggest that the reduction in effective population size through generational bottlenecks and lack of
genetic recombination resulting from Muller’s Ratchet elevates the rate of fixation of slightly deleterious mutations through random genetic drift. However, populations that experience a population bottleneck recover much of the lost genetic variation through rapid population growth. While it seems likely that this is the case for free-living and endosymbiotic bacteria as well, the strength of the bottleneck affects the loss of genetic variability much more so than subsequent rates of population growth. Within Blattabacterium and many other bacterial endosymbionts, these bottlenecks are not trivial, and are frequently recurring throughout the insect host’s lifespan, an environment that is completely atypical for most free-living populations. Thus, examined alone, population bottlenecks strongly reduce the genetic variation of Blattabacterium. Additionally, Blattabacterium – like other intracellular bacterial endosymbionts - reproduces asexually and lacks genetic recombination; two mechanisms otherwise crucial for the recovery of genetic variation. This combination of factors – lack of genetic recombination and repeated population bottlenecks – does seem to suggest that Blattabacterium and other obligate symbionts are less capable of recovering lost genetic variance after population bottlenecks than free-living bacteria.

However, bacterial endosymbionts also experience much higher mutation rates than their free-living relatives. Indeed, mutations are synonymous with increased genetic variation, and we show here that Blattabacterium experiences highly elevated rates of mutation compared to free-living bacterial populations. It is highly unlikely that the elevated mutation rates seen in Blattabacterium are adaptive or somehow function in recovering lost genetic variation, as mutations in Blattabacterium show a strong bias towards deletions rather than insertions; a pattern that is in agreement with previous studies as well as with Muller’s Ratchet. Additionally, it is suggested that reduced strength of selection on many genes in the endosymbiont genome increases the number of nucleotide sites that may be altered without consequences in fitness, strengthening the impact of deletion biases. Bacterial genomes are primarily functional DNA, and the drastic genome reduction observed within Blattabacterium and has come at the cost of physiological functionality. Intriguingly however, this drastic loss in functionality does not yet appear to have strong negative impacts on Blattabacterium survival or host fitness.

Figure 2. Plots outlining the relationships between number of selection events and gene length or COG size. (a) Relationship between gene length and number of nucleotides under positive selection within Blattabacterium (F-value: 40.872, Df: 1, p-value: 6.16e-10, adjusted R-squared: 0.12). (b) Relationship between total COG size and number of nucleotides under positive selection within Blattabacterium (Chi-square p-value: 2.2e-16). (c) Relationship between gene length and number of nucleotides under negative selection within Blattabacterium (F-value: 189.15, Df: 1, p-value: 2.2e-16, adjusted R-squared: 0.38). (d) Relationship between total COG size and number of nucleotides under negative selection within Blattabacterium (Chi-square p-value: 2.2e-16).
| Position | Locus   | Avg. Length (n) | COG | No. of Pos. sites | No. of Neg. sites |
|----------|---------|----------------|-----|------------------|------------------|
| a        | gyrB    | 2513           | L   | 18               | 11               |
| 96       | tarC    | 1045           | U   | 2                | 0                |
| 171      | purB    | 1843           | F   | 5                | 4                |
| 191      | marC    | 742            | U   | 2                | 1                |
| 239      | foeE    | 861            | H   | 2                | 1                |
| 265      | gmk     | 751            | F   | 1                | 0                |
| 266      | rpiB    | 606            | G   | 1                | 0                |
| 308      | rplX    | 324            | J   | 2                | 1                |
| 312      | rplP    | 541            | J   | 5                | 4                |
| 319      | rplC    | 821            | J   | 3                | 2                |
| 359      | entC    | 1374           | Q   | 3                | 1                |
| 365      | recC    | 2201           | LKJ | 3                | 0                |
| 387      | accA    | 1232           | I   | 5                | 3                |
| 388      | sdhB    | 985            | C   | 7                | 0                |
| 392      | phosA   | 1985           | R   | 5                | 3                |
| 405      | hinT    | 536            | FGR | 3                | 2                |
| 457      | psdRA   | 1362           | H   | 2                | 1                |
| b        | trmE    | 1821           | R   | 4                | 4                |
| 161      | accD    | 1098           | I   | 1                | 1                |
| 167      | purF    | 1997           | F   | 1                | 1                |
| 196      | accB    | 626            | I   | 1                | 1                |
| 208      | evoX    | 1009           | L   | 2                | 2                |
| 241      | m22     | 843            | O   | 1                | 1                |
| 304      | rplF    | 712            | J   | 1                | 1                |
| 318      | rplD    | 821            | J   | 2                | 2                |
| 430      | rpoD    | 1127           | K   | 7                | 7                |
| 438      | integral| 995            | P   | 1                | 1                |
| 478      | glyS    | 1910           | J   | 3                | 3                |
| 483      | pth     | 772            | J   | 1                | 1                |
| c        | truA    | 1001           | J   | 0                | 0                |
| 103      | rplT    | 317            | J   | 0                | 0                |
| 117      | aroK    | 674            | E   | 0                | 0                |
| 148      | rpmI    | 249            | N/A | 0                | 0                |
| 149      | rplT    | 456            | J   | 0                | 0                |
| 204      | rpmG    | 239            | J   | 0                | 0                |
| 240      | nadE    | 1028           | H   | 0                | 0                |
| 301      | rplO    | 601            | J   | 0                | 0                |
| 329      | rplM    | 587            | J   | 0                | 0                |
| 332      | cdsA    | 1035           | R   | 0                | 0                |
| 372      | rplL    | 487            | J   | 0                | 0                |
| 422      | sufE    | 567            | R   | 0                | 0                |
| 428      | rpsO    | 347            | J   | 0                | 0                |
| 491      | nfsA    | 442            | N/A | 0                | 0                |
| d        | dapF    | 1043           | E   |                  |                  |
| 112      | gcvH    | 523            | E   |                  |                  |
| 148      | rpmI    | 249            | N/A |                  |                  |
| 323      | rpsL    | 499            | J   |                  |                  |
| 398      | rpsU    | 267            | N/A |                  |                  |

Table 2. (a) Loci within Blattabacterium displaying a positive selection bias. Positive selection is defined as those loci that display a greater number of sites under positive selection than under negative selection. (b) Loci within Blattabacterium displaying a neutral selection bias. Neutral selection is defined as those loci which display an equal number of sites under positive selection as negative selection. (c) Loci within Blattabacterium displaying no selection. These genes experience neither positive nor negative selection events. (d) Loci within Blattabacterium and Flavobacterium that display identical selection profiles. These genes display no selection.
events within either Blattabacterium or Flavobacterium genomes. ‘Position’ indicates that genes starting position within the Mastotermes darwineensis genome, the model Blattabacterium genome used here. Letters refer to COG functional categories as follows. C - Energy production and conversion; D - Cell division and chromosome partitioning; E - Amino acid transport and metabolism; F - Nucleotide transport and metabolism; G - Carbohydrate transport and metabolism; H - Coenzyme metabolism; I - Lipid metabolism; J - Translation, ribosomal structure and biogenesis; K - Transcription; L - DNA replication, recombination and repair; M - Cell envelope biogenesis, outer membrane; N - Cell motility; O - Posttranslational modification, protein turnover, chaperones; P - Inorganic ion transport and metabolism; Q - Secondary metabolites biosynthesis, transport, and catabolism; R - General function prediction only; S - COG of unknown function; T - Signal transduction mechanisms.

Indeed, many physiological tasks are now taken over by the cockroach host, rendering many Blattabacterium genes superfluous within the relatively safe and predictable symbiotic environment32–36,38. As in other obligate endosymbionts, many if not most of these genes come under relaxed selection, as their function is critical to neither Blattabacterium survival nor the symbiotic physiological requirements of its cockroach host32,35.

Whether or not elevated mutation rates in physiologically-important genes functions to actively reduce genome size and thus streamline bacterial reproduction, or are the result of random genetic drift is unknown; though that many genes lost by Blattabacterium since transitioning to an intracellular lifestyle coded for otherwise critical functionality - including the loss of many genes involved in DNA maintenance and repair34, reviewed in ref.37 – suggests that many losses are either only mildly non-adaptive or compensated for by the host and thus do not result in immediate impairment of symbiont or host. However, genome reduction is accompanied by a reduction and cell size and a substantial reduction in energy and nutrients requirements, providing an adaptive payoff for the active removal of non-essential genes. Indeed, a number of prokaryotic Prochlorococcus species display adaptive and rapid genome shrinkage, with genomic patterns similar to those observed in obligate symbionts including reduced G + C content, elevated rates of mutation, and the loss of DNA-repair genes87. However, despite these similarities, genome reduction in Prochlorococcus is characterized by largely neutral selection, as large population sizes impose low genetic drift and strong purifying selection87. Naturally, if genome reduction in Blattabacterium and other bacterial endosymbionts was being driven by adaptive forces and not random genetic drift, then we might expect patterns of selection similar to those in the free-living Prochlorococcus. Instead, we find here that the overwhelming majority of mutations in the Blattabacterium genome are negative in direction, strongly suggesting that genome reduction is not driven by selective processes, but rather by random genetic drift; as has been suggested for numerous other obligate bacterial endosymbionts32,35.

Specific genes within the endosymbiont genome are expected to vary among endosymbiont lineages as a function of the metabolic and physiological requirements of the host species. As such, these species-specific genes vital to bacterial survival and/or host fecundity experienced elevated selective pressures for their persistence within the Blattabacterium genome. We suspect that many genes in Blattabacterium involved in functions critical to this bacterial-host symbiosis display neutral or positive signatures of selection. Thus, while random genetic drift appears to play a strong role in shaping the Blattabacterium genome, physiological constraint acts to maintain Blattabacterium’s functionality as a primary nutritional endosymbiont across the cockroach lineage. Accordingly, the Blattabacterium genome architecture and composition is the result of the interplay between random genetic drift and the fixation of slightly deleterious mutations on one hand and physiological constraint promoting maintenance of cockroach-required metabolic functionality on the other.

When compared to the signatures of selection and patterns of evolution noted within other obligate bacterial symbionts, Blattabacterium shows striking similarity. While the ratio of negative to positive selection sites of 11:1 is specific to Blattabacterium-Flavobacterium comparisons, similar patterns of strong negative selection have been observed in other insect endosymbiont genomes69. Unsurprisingly then, our results conform to the findings of Brynnel et al.33, who also measured that the tuf gene of Buchnera is evolving more than 10 times as quickly than the same gene in the free living E. coli and S. typhimurium. Additionally, Blattabacterium - like Wigglesworthia and Buchnera – does show some evidence for maintaining those functions that are highly important to its insect host33,34,66,70. Indeed, the combined effects of Muller’s Ratchet appears to be ubiquitous within obligate insect bacterial symbionts: the Buchnera chaperonin groEL displays a 5-fold increase in non-synonymous mutations, and a 10-fold increase in synonymous mutations, when compared to E. coli72. Mutational pressure alone likely does not account for the magnitude of these dS/dN rate elevations. Within Buchnera, it has been suggested that this elevation of fixation occurs through random genetic drift resulting from the continual reduction of effective symbiont population size with each transmission from host parent to host offspring33,84. Given that this same elevation of polymorphisms is observed within Blattabacterium - and that Blattabacterium also undergoes similar population bottlenecks with each host generation - it is likely that similar mechanisms are shaping these two independent lineages. This also parallels the findings of Brynnel et al.33, whom suggested that the rate of synonymous codon substitution within Buchnera can be as much as 40 times higher than its free-living relatives.

Blattabacterium - Flavobacterium Selection Comparison. Blattabacterium displays elevated levels of both positive and negative selection events at a significance level of p ≤ 0.05 when compared to free-living Flavobacterium, indicating a genome-wide increase in mutation rates across the examined genes. In order to ensure that these patterns are not the result of sequences displaying radically different divergence times, we performed a phylogenetic analysis (Fig. 3) to elucidate the sequence similarity within each examined group. Phylogenetic analysis of both the Blattabacterium group (Table 3) and Flavobacterium group (Table 4) indicate similar levels of phylogenetic divergence between the individuals of each32,88,90.
In addition, each group displays comparable percentages of identical sites (Blattabacterium: 89.4%, Flavobacterium: 87.8%) as well as similar pairwise percent identities (Blattabacterium: 95.7%, Flavobacterium: 93.3%) when aligning the ribosomal 16S rRNA gene. Thus, extant Blattabacterium display signs of elevated rates of genome evolution in the form of increased levels of selection events. The increase in the number of sites experiencing negative or positive selection when compared to the free-living Flavobacterium suggests elevated levels of functional protein evolution in the endosymbionts. Only a limited number of loci display similar selection profiles between Blattabacterium and Flavobacterium (Table 2d).

Results of MEME selection analysis indicate that all genes analyzed show at least some evidence of negative selection. Sites under negative selection comprise approximately 86 percent of examined loci. However, four

| BPLAN | BCpu | BBge | BGIGA | MADAR | BNCIN | BBor | BPane |
|-------|------|------|-------|-------|-------|------|-------|
| BPLAN | 0.048 | 0.037 | 0.044 | 0.056 | 0.04  | 0.015 | 0.04  |
| BCpu  | 0.048 | 0.038 | 0.043 | 0.048 | 0.043 | 0.043 | 0.045 |
| BBge  | 0.037 | 0.038 | 0.024 | 0.045 | 0.026 | 0.038 | 0.021 |
| BGIGA | 0.044 | 0.043 | 0.024 | 0.059 | 0.026 | 0.044 | 0.021 |
| MADAR | 0.056 | 0.048 | 0.045 | 0.059 | 0.049 | 0.051 | 0.056 |
| BNCIN | 0.04  | 0.043 | 0.026 | 0.049 | 0.039 | 0.021 |       |
| BBor  | 0.015 | 0.043 | 0.038 | 0.044 | 0.051 | 0.039 | 0.036 |
| BPane | 0.04  | 0.045 | 0.021 | 0.056 | 0.021 | 0.036 |       |

Table 3. Absolute sequence divergence in the 16S rRNA gene of Blattabacterium. A phylogenetic tree was created using the 16S rRNA gene from each sequenced Blattabacterium species. From this tree, phylogenetic distances were calculated in order to estimate sequence similarity and divergence. Host species abbreviations are as follows: BNCIN, N. cinerea; BGIGA, B. giganteus; BBge, B. germanica; BPLAN, P. americana; BCpu, C. punctulatus; MADAR, M. darwiniensis; BBor, B. orientalis; BPane, P. angustipennis spadica.
phylogenetic distances was estimated. Species abbreviations: Findic, Flavobacterium indicum; Fbranch, Flavobacterium branchiophilum; Fjohn, Flavobacterium johnsoniae; Fcolum, Flavobacterium columnare.

| Loci     | Protein Name                  | Putative Function                                      |
|----------|-------------------------------|--------------------------------------------------------|
| miaB     | 2-methylthioadenine synthetase B family tRNA modification enzyme | RNA modification                                       |
| Holliday Junction | Holliday junction resolvase-like protein | hydrolyase, nucleic acid binding, DNA recombination, transcription antitermination |
| rplT     | 50S ribosomal protein L25 | rRNA binding, negative regulation of translation, translation |
| atpG     | ATP synthase F1 subunit gamma | ATP binding, plasma membrane ATP synthesis coupled proton transport |

Table 5. Loci containing sites that display evidence for positive selection, according to MEME episodic selection analysis. First column denotes the locus of interest. Second column contains the names of the proteins coded by these loci; and the third column contains proposed functional information about these proteins, gathered from the UniProt gene database.

loci, at one site each, show evidence for positive selection (Table 5). Three of the four loci showing evidence for positive selection are involved in DNA or RNA modification: 2-methylthioadenine synthetase, Holliday Junction resolvase, and 50S ribosomal protein L25 subunit. Within E. coli and Salmonella typhimurium, variations of the protein 2-methylthioadenosine have been shown to stabilize codon-anticodon interactions through the restriction of first codon position wobble during tRNA aminoacylation\(^91,92\). This functionality prevents the misreading of the genetic code, thus reducing the likelihood of mutation. Additionally, Holliday Junction resolvase-like proteins have been shown to play key roles in DNA recombination and repair\(^93-95\). Finally, genes responsible for the production of ribosomes within a cell are crucial for the proper translation of proteins from mRNA\(^96,97\). Modifications to genes responsible for the production of ribosomal proteins will likely impact the efficiency and/or accuracy of protein translation and assembly. Given the broad reduction in functionality of the Blattabacterium genome, and the loss of many ancestral DNA and RNA maintenance and repair genes (Fig. 1)\(^8,98,99\), it is in some ways not surprising that all currently-described Blattabacterium strains display similar selection pressures on those remaining genes responsible for the maintenance of genetic material. However, of notable absence from our list of genes showing signatures of positive selection is the molecular chaperone and maintenance gene GroEL. These sequences are part of the large GroL locus in modern Blattabacterium genomes, regions of which were found previously in Blattabacterium to be under positive selection\(^100\). This inconsistency likely arises from the outgroups used in each study. We utilized Blattabacteriaceae’s closest free-living relative, Flavobacterium\(^101,102\), as an outgroup while Fares et al. utilized relatively distantly-related free-living Gammaproteobacteria\(^103\). Based on this methodological distinction, we can conclude that the selective pressure noted by Fares et al. was exerted prior to the split between Blattabacterium and Flavobacterium.

In contrast to the previous genes, however, which are involved in the maintenance of genetic material, the remaining locus found to show signatures of positive selection, atpG, codes for ATP synthase F1 subunit gamma. ATP synthase-family subunit proteins typically combine to form an ATP synthase complex, which is responsible for energy production in the form of ATP within the cell\(^101,102\). One of the primary functions of Blattabacterium within its host is amino acid synthesis. Amino acid production is a very endergonic process, requiring large amounts of energy in the form of ATP in order to effectively carry out biosynthesis\(^103\). Therefore, beneficial modifications to genes coding for an ATP synthase subunit that result in the more efficient functioning of ATP synthase as a complete complex are more likely to be favored within the Blattabacterium genome. In keeping with the previous findings that all Blattabacteriaceae strains examined to date are alike in their function to provide essential and nonessential amino acids to their cockroach hosts, here we demonstrate that Blattabacterium also share signatures of positive selection within genes responsible for the production of the ATP synthase F1 subunit.

**Conclusions**

Our findings indicate that the Blattabacterium genome is experiencing elevated rates of both positive and negative selection when compared to its free-living relative Flavobacterium, approaching a 10-fold increase in selection rate at the significance level \(p \leq 0.05\) across 304 individual genes. In combination with previous studies elucidating the evolutionary patterns in other insect endosymbionts, we conclude that the Blattabacterium genome is shaped by similar evolutionary mechanisms. Previous studies have outlined the current state of the Blattabacterium
genome, which is drastically reduced from its ancestral state and possesses a very strong bias towards A + T nucleotide base pairs. Analysis of these trends indicate that Blattabacterium are experiencing an accumulation of slightly deleterious mutations through the continued effects of random genetic drift resulting from consecutive population bottlenecks throughout Blattabacterium evolutionary history, with physiological constraint acting to maintain genes important to bacterial survival and host fecundity. Additionally, Blattabacterium has lost many of the genes involved in DNA repair, likely through similar mechanisms discussed here, thus exacerbating this evolutionary bias towards slightly deleterious mutations. That these mutations cannot be repaired increases functional protein evolution rates within this endosymbiont. The patterns discussed here are highly similar to those evolutionary and genomic trends observed in other intracellular insect endosymbionts.4,5,43,45,46,49,50. Additionally, our analyses also provide insight into the direction of selection of loci within the genome. A vast majority of loci in all Blattabacterium genomes analyzed here show signs of negative selection. Only a small fraction of loci (mitA, Holliday Junction, rplY, atpG) show signs of positive selection. These observations are in accordance with our previous understanding of the evolutionary history of Blattabacterium, as well as its function within its cockroach host as a nutritional endosymbiont aiding in the recycling of nitrogenous waste and the production of both essential and nonessential amino acids.

The analysis presented here could be augmented through a robust analysis of genome reduction within Blattabacterium. Using a parsimony approach, the ancestral genome of another primary insect endosymbiont, Buchnera-Ap, was reconstructed by Moran and Mira.11 The results of Moran and Mira’s analysis indicated that much of the ancestral Buchnera genome was lost during a relatively small number of large deletion events shortly after this bacteria’s transition to an intracellular lifestyle. While it is likely that that the Blattabacterium genome was reduced through similar mechanisms, a similar reconstruction within this group would offer us a more complete picture of the evolutionary origins of this unique cockroach endosymbiont.

**Data Accessibility**

All data used herein was procured from public NCBI databases; see Table 1.

**References**

1. Buchner, P. Endosymbiosis of Animals with Plant Microorganisms. (New York: Interscience Publishers, 1965).
2. Ishikawa, H. Insect Symbiosis: an Introduction. P. 1–21. In Bourtitzis, K. & Miller, T. A. Insect Symbiosis. (Boca Raton: CRC Press, 2003).
3. Ruby, E., Henderson, B. & McFalls-Nagi, M. We get by with a little help from our (little) friends. Science. 303, 1305–1307 (2004).
4. Daesch, G., Weiss, E., Chang, K. Genus VIII. Rickettsiella Philip 1956. Bergy’s Manual of Systematic Bacteriology. 4:811–833 (New York: Springer, 2010).
5. Douglas, A. E. Mycetocyte symbiosis in insects. Biol. Rev. Cambr. Phil. Soc. 64, 409–434 (1989).
6. Margulis, L. Fester R. Symbiosis as a Source of Evolutionary Innovation. (Cambridge, MA, USA: MIT Press, 1991).
7. Moran, N. A. & Telang, A. Bacteriocyte-associated symbionts of insects. Bioscience. 48, 295–304 (1998).
8. Moran, N. A. & Baumann, P. Bacterial endosymbions in animals. Curr. Opin. Microbiol. 3, 270–275 (2000).
9. Zientz, E., Silfa, E. J. & Gross, R. Genome interdependence in insect-bacterium symbioses. Genome Biol. 2, reviews1032, https://doi.org/10.1186/gb-2001-2-12-reviews1032 (2001).
10. Douglas, A. E. Nutritional Interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria Buchnera. Annu. Rev. Entomol. 43, 17–37 (1998).
11. Bourtitzis, K., Miller, T. A. Insect Symbiosis. (Boca Raton: CRC Press, 2003).
12. Vorbacher, C., Gehret, L. & Rodriguez, P. A strain of bacterial symbiont Regiella insecticola protects aphids against parasites. Biol. Lett. 6, 109–111 (2010).
13. Blochmann, F. Über das regelmäßige Vorkommen von bakterienähnlichen Gebilden in den Geweben und Eiern verschiedener Insekten. Z. Bact. 24, 1 (1887).
14. Brooks, M. A. Comments on the classification of intracellular symbiotes of cockroaches and a description of the species. Journal of Invertebrate Pathology. 16, 249–258 (1970).
15. Bandi, C., Damiani, G., Magni, A. & Fani, R. Flavobacteria as intracellular symbionts in cockroaches. Proc. Biol. Sci. 257, 43–48 (1994).
16. Kambhampati, S. Genus Blattabacterium. Bergy’s Manual of Systematic Bacteriology. 4, 315–321. (New York: Springer, 2010).
17. Clark, J. W., Hossain, S., Burnside, C. A. & Kambhampati, S. Coevolution between a cockroach and its bacterial endosymbiont: A biogeographical perspective. Proc. R Soc. Lond. B. 268, 393–398 (2001).
18. Lo, N., Bandi, C., Watanabe, H., Nalena, C. & Beninati, T. Evidence for cladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. Mol Biol Evol. 20, 907–913 (2003).
19. Sabree, Z. L., Kambhampati, S. & Moran, N. A. Nitrogen recycling and nutritional provisioning by Blattabacterium, the cockroach endosymbiont. Proc. Natl Acad Sci USA 106, 19521–19526 (2009).
20. Mullins, D. E. & Cochran, D. G. Nitrogen excretion in cockroaches: uric acid is not a major product. Science. 177, 699–701 (1972).
21. Cochran, D. G. Nitrogen excretion in cockroaches. Annu. Rev. Entomol. 30, 29–49 (1985).
22. Bandi, C. et al. The establishment of intracellular symbiosis in an ancestor of cockroaches and termites. Proc. R Soc. Lond. B. 259, 293–299 (1995).
23. Mullins, D. E. & Cochran, D. G. A comparative study of nitrogen excretion in twenty-three cockroach species. Comparative Biochemistry and Physiology. 53, 393–399 (1976).
24. O’Donnell, M. Insect Excretory Mechanisms. Advances in insect physiology. 55, 1–22 (New York; Academic Press, 2008).
25. Lopez-Sanchez, M. J. et al. Evolutionary convergence and nitrogen metabolism in Blattabacterium strain Bge, primary endosymbiont of the cockroach Blatta germanica. PLoS Genet. 5, e1000721 (2009).
26. Neef, A. et al. Genome ecumenization in the endosymbiont of the wood roach Cryptocercus punctulatus due to drastic loss of amino acid synthesis capabilities. Genome Biol. Evol. 3, 1437 (2011).
27. Huang, C. Y., Sabree, Z. L. & Moran, N. A. Genome sequence of Blattabacterium sp. strain BGGI, endosymbiont of the Blaberus giganteus cockroach. J. Bacteriol. 194, 4450 (2012).
28. Patino-Navarette, R., Moyá, A., Latorre, A. & Pereto, J. Comparative genomics of Blattabacterium cuenoti: the frozen legacy of an ancient endosymbiont genome. Genome Biol. Evol. 5, 351–361 (2013).
29. Tokuda, G. et al. Maintenance of Essential Amino Acid Synthesis Pathways in the Blattabacterium cuenoti Symbiont of a Wood-Feeding Cockroach. Biol. Lett. 9, https://doi.org/10.1098/rsbl.2012.1153 (2013).
30. Kambhampati, S., Alleman, A. & Park, Y. Complete Genome Sequence of the endosymbiont Blattabacterium from the Cockroach Nauphoeta cinerea (Blattodea: Blaberidae). Genomics. 102, 479–483 (2013).
31. Sabree, Z. L. et al. Genome shrinkage and loss of nutrient-providing potential in the obligate symbiont of the primitive termite Mastotermes darwiniensis. Appl Environ Microbiol. 78, 204–210 (2012).
32. Moran, N. A. & Mira, A. The process of genome shrinkage in the obligate symbiont Buchnera aphidicola. Genome Biol. 2, research0054.1. https://doi.org/10.1186/gb-2001-2-12-research0054 (2001).
33. Brynnel, E. U., Kurland, C. G., Moran, N. A. & Andersson, S. G. Evolutionary rates for tuf genes in endosymbionts of aphids. Mol. Biol. Evol. 15, 574–592 (1998).
34. Clark, M. A., Moran, N. A. & Baumann, P. Sequence evolution in bacterial endosymbionts having extreme base compositions. Mol. Biol. Evol. 16, 1588–1598 (1999).
35. Wernegreen, J. J. & Moran, N. A. Evidence for genetic drift in endosymbionts (Buchnera): analyses of protein-coding genes. Mol. Biol. Evol. 16, 83–97 (1999).
36. Chen, X., Li, S. & Aksoy, S. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus Glossina and its bacteriome-associated endosymbiont, Wigglesworthia glossinidia. J. Mol. Evol. 48, 49–58 (1999).
37. Clark, M. A., Moran, N. A., Baumann, P. W. & Wernegreen, J. J. Cospeciation between bacterial endosymbionts (Buchnera) and a recent radiation of aphids (Uroleucon) and pitfalls of testing for phylogenetic congruence. Evolution. 54, 517–525 (2000).
38. Funk, D. L., Heibling, L., Wernegreen, J. J. & Moran, N. A. Intraspecific phylogenetic congruence among multiple symbiont genomes. Proc. R. Soc. Lond. B. Biol. Sci. 267, 2517–2521 (2000).
39. Clark, J. W. & Kambhampati, S. Phylogenetic analysis of Blattabacterium, endosymbiotic bacteria from the wood roach, Cryptocercus (Blattodea: Cryptocercidae), including a description of three new species. Molecular Phylogenetics and Evolution. 26, 82–88 (2003).
40. Barea, N. et al. Developmental Origin and Evolution of Bacteriocytes in the Aphid-Buchnera Symbiosis. PLoS Genet. 1, 70–76 (2003).
41. Mira, A. & Moran, N. A. Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. Microb. Ecol. 44, 137–143 (2002).
42. Moxon, R. & Russell, E. The impact of bottlenecks on microbial survival, adaptation, and phenotypic switching in host-pathogen interactions. Evolution. 71, 2801–2816, https://doi.org/10.1111/j.1558-5646.2017.01771.x (2017).
43. Mira, A., Ochman, H. & Moran, N. A. Deletional bias and the evolution of bacterial genomes. Mol. Biol. Evol. 17, 589–596 (2001).
44. Rispe, C. & Moran, N. A. Accumulation of deleterious mutations in endosymbionts: Muller’s ratchet with two levels of selection. Am. Nat. 156, 424–441 (2000).
45. Nei, M., Maruyama, T. & Chakraborty, R. The Bottleneck Effect and Genetic Variability in Populations. Science. 200, 589–596 (1978).
46. Tamas, I. et al. 50 million years of genomic stasis in endosymbiotic bacteria. Science. 296, 2376–2379 (2002).
47. Woolfit, M. & Bromham, L. Increased rates of sequence evolution in endosymbiotic bacteria and fungi with small effective population sized. Molecular Biology and Evolution. 20, 1545–1555 (2003).
48. Woolfit, M. Effective population size and the rate and pattern of nucleotide substitution. Biology Letters. 5, 417–420 (2009).
49. Muller, H. J. The relation of recombination to mutational advance. Mutat. Res. 2, 1–9 (1964).
50. Moya, A., Pereto, J., Gil, R. & Latorre, A. Learning how to live together: genomic insights into prokaryote-animal symbioses. Nat. Rev. Genet. 9, 218–229 (2008).
51. Moran, N. A., McCutcheon, J. P. & Nakabachi, A. Genomics and evolution of heritable bacterial symbionts. Annu. Rev. Genet. 42, 165–190 (2008).
52. Moran, N. A. & Bennett, G. M. The tiniest tiny genomes. Annu. Rev. Microbiol. 68, 195–215 (2014).
53. Wernegreen, J. J. Endosymbiont evolution: predictions from theory and surprises from genomes. Annu. N.Y. Acad. Sci. 1360, 16–35 (2015).
54. Moran, N. A. & Wernegreen, J. J. Lifestyle evolution in symbiotic bacteria: insights from evolution. Trends Ecol. Evol. 15, 321–326 (2000).
55. Silva, F. J., Latorre, A. & Moya, A. Why are the genomes of endosymbiotic bacteria so stable? Trends Genet. 19, 176–180 (2003).
56. Latorre, A. & Manzano-Marín, A. Dissecting genome reduction and trait loss in insect endosymbionts. Annu. N.Y. Acad. Sci. 1389, 52–75 (2017).
57. Andersson, S. G. & Kurland, C. G. Reductive evolution of resident genomes. Trends Microbiol. 6, 263–268 (1998).
58. Ochman, H. & Moran, N. A. Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. Science 292, 1096–1099 (2001).
59. McCutcheon, J. P. & Moran, N. A. Extreme genome reduction in symbiotic bacteria. Nature Reviews. 10, 13–26 (2012).
60. McDonald, I. H. & Kreitman, M. Adaptive protein evolution at the Afl locus in Drosophila. Nature. 351, 652–654 (1991).
61. Yang, Z. & Bielaszka, B. Statistical methods for detecting molecular adaptation. Trends in Ecology and Evolution. 15, 496–503 (2000).
62. Herbeck, T. H., Funk, D. J., Degnan, P. H. & Wernegreen, J. J. A conservative test of Genetic Drift in the Endosymbiotic Bacterium Buchnera: Slightly Deleterious Mutations in the Chaperonin groEL Genes. 165, 1651–1660 (2003).
63. Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. & Ishikawa, H. Genome Sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. Nature. 407, 81–86 (2000).
64. Akman, L. et al. Genome sequence of the endocellular obligate symbiont of tsetse flies, Wigglesworthia glossinidia. Nat. Genet. 32, 402–407 (2002).
65. Rio, V. M. R., Symula, R. E. & Wang, J. Insight into the Transmission Biology and Species-Specific Functional Capabilities of Tsetse (Diptera: Glossinidae) Obligate Symbiont. Wigglesworthia. mBio. 3(No.1), e00240–11 (2012).
66. Gil, R. et al. The genome sequence of Blochmannia floridanus: comparative analysis of reduced genomes. Proc. Natl. Acad. Sci. 100, 9388–9393 (2003).
67. Bernardet, J. F. & Bowman, J. P. Genus Flavobacterium. Bergey’s Manual of Systematic Bacteriology. 4, 112–154, (New York: Springer, 2010).
68. Wernegreen, J. J. Genome Evolution in Bacterial Endosymbionts of Insects. Nat. Rev. Genet. 3, 850–861 (2002).
69. Herbeck, J. T., Wall, D. P. & Wernegreen, J. J. Gene expression level influences amino acid usage, but not codon usage, in the tsetse fly endosymbiont, Wigglesworthia. Microbiol. 149, 2585–2596 (2003).
70. Van Domselaar, G. H. et al. BaSyc: a web server for automated bacterial genome annotation. Nucleic Acids Research. 33, 455–459 (2005).
71. Segata, N., Börmügen, D., Morgan, X. C. & Hüttenhower, C. PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. Nature Communications. 4 (2013).
72. Schmidt, R. & Edwards, R. Quality control and preprocessing of metagenomic datasets. Bioinformatics. 27, 863–866 (2011).
73. Capella-Gutierrez, S., Silla-Martinez, J. M. & Gabaldón, T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics. 25, 1972–1973 (2009).
74. Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. JModelTest 2: more models, new heuristics and parallel computing. Nature methods. 9, 772 (2012).
76. Guindon, S. et al. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Systematic Biology. 59, 307–321 (2010).
77. Mirarab, S. & Warnow, T. ASTRAL-II: Coalescent-Based Species Tree Estimation with Many Hundreds of Taxa and Thousands of Genes. Bioinformatics. (ISMB special issue). 31, 144–152 (2015).
78. Kosakovsky Pond, S. L., Frost, S. D. W. & Muse, S. V. HyPhy: hypothesis testing using phylogenies. Bioinformatics. 21, 676–679 (2005).
79. Murrell, B. et al. Gene-Wide Identification of Episodic Selection. Mol. Biol. Evol. 32, 1365–1371 (2015).
80. Murrell, B. et al. Detecting Individual Sites Subject to Episodic Selection. PLoS Genetics. 8(No. 7), e1002764 (2012).
81. Smith, M. D. et al. Less is More: An Adaptive Branch-Site Random Effects Model for Efficient Detection of Episodic Diversifying Selection. Mol. Biol. Evol. 32, 1342–1353 (2015).
82. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. http://www.R-project.org/; (2013).
83. Comeron, J. M., Krettmann, M. & Aguade, M. Natural Selection on Synonymous Sites Is Correlated with Gene Length and Recombination in Drosophila. Genetics. 151, 239–249 (1999).
84. Krettmann, M. The neutral theory is dead. Long live the neutral theory. BioEssays. 18, 678–683 (1996).
85. Hahn, M. W. Toward a selection theory of molecular evolution. Evolution. 62, 255–265 (2008).
86. Kern, A. D. & Hahn, M. W. The Neutral Theory in Light of Natural Selection. Mol. Biol. Evol. 35, 1366–1371 (2018).
87. Dufresne, A., Garczarek, L. & Partensky, F. Accelerated evolution associated with genome reduction in a free-living prokaryote. Genome Biology. 6, R14 (2005).
88. Funk, D. J., Wernegreen, J. I. & Moran, N. A. Intraspecific variation in symbiont genomes: bottlenecks and the aphid-Buchnera association. Genetics. 157, 477–489 (2001).
89. Ochman, H. & Wilson, A. C. Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. J. Mol. Evol. 26, 74–86 (1987).
90. Moran, N. A., Munson, M. A., Baumann, P. & Ishikawa. A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proc. R. Soc. Lond. B 253, 167–171 (1993).
91. Winson, K. & Roe, B. Presence of the hypermodified nucleotide N6-(A2-isopentynyl)-2-methylthioadenosine prevents codon misreading by Escherichia coli phenylalanyl-transferRNA. Proc. Natl. Acad. Sci. USA 86, 409–413 (1989).
92. Esberg, B. & Bjork, G. The Methylthio Group (ms2) of N6-(4-hydroxyisopentenyl)-2-Methylthioadenosine (ms2io6A) Present Next to the Anticodon Contributes to the Decoding Efficiency of the tRNA. Journal of Bacteriology. 177, 1967–1975 (1995).
93. Mandal, T., Mahdi, A., Sharples, G. & Lloyd, R. Resolution of Holliday Intermediates in Recombination and DNA Repair: Indirect Suppression of ruvA, ruvB, and ruvC mutations. Journal of Bacteriology. 175, 4325–4334 (1993).
94. Aravind, L., Walker, D. & Koonin, E. Conserved Domains in DNA repair proteins and evolution of repair systems. Nucleic Acids Research. 27, 1223–1242 (1999).
95. Avarind, L., Makarova, K. & Koonin, E. Holliday junction resolvases and related nucleases: identification of new families, phyletic distribution and evolutionary trajectories. Nucleic Acids Research. 28, 3417–3432 (2000).
96. Nakada, D. & Kaji, A. Function and Properties of the “Native” 30S and 50S Ribosomal Subunits of Escherichia coli. Proc. Natl. Acad. Sci. USA 57, 128–135 (1967).
97. Herold, M. & Nierhaus, K. Incorporation of Six Additional Proteins to Complete the Assembly of the 50 S Subunit from Escherichia coli Ribosomes. The Journal of Biological Chemistry. 262, 8826–8833 (1987).
98. Lawrence, J. & Roth, J. In Organization of the Prokaryotic Genome (ed. Charlesbois, R.). 263–289 (Washington DC: ASM Press, 1999).
99. Moran, N. A. Microbial minimalism: genome reduction in bacterial pathogens. Cell 108, 583–586 (2002).
100. Nakada, D. & Kaji, A. Function and Properties of the “Native” 30S and 50S Ribosomal Subunits of Escherichia coli. Proc. Natl. Acad. Sci. USA 57, 128–135 (1967).
101. Weber, J. & Senior, A. Catalytic mechanism of F1-ATPase. Biochimica et Biophysica Acta. 1319, 19–58 (1977).
102. Capaldi, R., Schulenberg, B., Murray, J. & Agger, R. Cross-Linking and Electron Microscopy Studies of the Structure and Functioning of the Escherichia coli ATP Synthase. Journal of Experimental Biology. 203, 23–33 (2000).
103. Umbarger, H. Amino Acid Biosynthesis and its Regulation. Annual Review of Biochemistry. 47, 533–606 (1978).

Author Contributions
S. Kambhampati responsible for concept and design of work. Genomic data collected from public repositories and prepared by A. Alleman. Selection analyses performed by K. Hertweck and A. Alleman. Manuscript drafted by A. Alleman, and was critically revised by all three authors.

Additional Information

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018