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Genome Evolution in an Insect Cell: Distinct Features of an Ant-Bacterial Partnership

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Bacteria that live exclusively within eukaryotic host cells include not only well-known pathogens, but also obligate mutualists, many of which occur in diverse insect groups such as aphids, psyllids, tsetse flies, and the ant genus Camponotus *(Buchner, 1965; Douglas, 1998; Moran and Telang, 1998; Baumann* et al.*, 2000; Moran and Baumann, 2000). In contrast to intracellular pathogens, these primary (P) endosymbionts of insects are required for the survival and reproduction of the host, exist within specialized host cells called bacteriocytes, and undergo stable maternal transmission through host lineages (Buchner, 1965; McLean and Houk, 1973). Due to their long-term host associations and close phylogenetic relationship with well-characterized enterobacteria (Fig. 1), P-endosymbionts of insects are ideal model systems to examine changes in genome content and architecture that occur in the context of beneficial, intracellular associations. Since these bacteria have not been cultured outside of the host cell, they are difficult to study with traditional genetic or physiological approaches. However, in recent years, molecular and computational approaches have provided important insights into their genetic diversity and ecological significance. This review describes some recent insights into the evolutionary genetics of obligate insect-bacteria symbioses, with a particular focus on an intriguing association between the bacterial endosymbiont* Blochmannia *and its ant hosts.*

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The specific functions of most insect P-endosymbionts remain unknown. However, the typically unbalanced diets of their hosts suggest these bacteria play a nutritional role. In fact, nutritional functions are well documented in certain P-endosymbionts such as *Buchnera aphidicola,* which provides essential amino acids that are lacking in the plant sap diet of its aphid host (Sandstrom *et al.,* 2000), and *Wigglesworthia glossinidia,* which provisions its tsetse fly host with B-complex vitamins lacking in vertebrate blood (Nogge, 1981; Aksoy, 2000). The bacterium SOPE (*Sitophilus oryzae* primary endosymbiont) oxidizes excess methionine consumed by the weevil (Gasnier-Fauchet and Nardon, 1986) and increases mitochondrial enzymatic activity of the host by providing vitamins such as panthothenic acid and riboflavin (Wicker and Nardon, 1982; Heddi *et al.,* 1991, 1999). *Blattobacterium,* a symbiont that lives within fat bodies of cockroaches, may be involved in tyrosine biosynthesis (Goldberg and Pierre, 1969) and apparently recycles uric acid of the host, as evidenced by elevated uric acid levels in hosts from which the bacteria have been eliminated (Cochoran, 1985). The biosynthetic abilities of these bacteria allow hosts to exploit food sources and habitats that would otherwise be inadequate; therefore, symbiont acquisition can be viewed as a key innovation in the evolution of these insect lineages (Moran and Telang, 1998). Indeed, sister genera lacking P-endosymbionts typically utilize a different, more nutrient-rich dietary resource (Nardon and Grenier, 1991). These insect-bacteria relationships are reciprocally beneficial, as the highly specialized bacteria rely on the host cellular environment for their survival.

We are studying the evolutionary outcome of an obligate association between ants and a bacterial endosymbiont recently designated *Candidatus* Blochmannia gen. nov. (*Blochmannia*) (Sauer *et al.,* 2000). This bacterium lives

within host cells that are typically adjacent to or within the midgut epithelium, and forms an obligate association with at least three closely related genera in the ant subfamily Formicinae: *Polyrhachis, Colobopsis,* and *Camponotus* (Dasch *et al.,* 1984; Schroder *et al.,* 1996; Sameshima *et al.,* 1999). *Camponotus* is the second largest ant genus; it includes 931 species and is found in almost every biogeographic region (Bolton, 1995). Although *Blochmannia* is the best characterized ant endosymbiont, we know little about its gene content and nothing about its functional role. Building upon relatively rich molecular datasets currently available for *Buchnera* and *Wigglesworthia,* we are using comparative approaches to explore the impact of endosymbiosis on sequence and genome evolution in *Blochmannia,* including rates and patterns of DNA and protein sequence evolution and changes in genome size and content. In future work, genomic comparisons and experimental approaches promise to shed light on the physiological and ecological roles of this bacterium.

Forces Shaping Sequence Evolution in Endosymbionts

Phylogenetic analysis of insect mutualists

Analyses of 16S rDNA genes show that many insect endosymbionts group with the γ-3 subdivision of Proteobacteria, along with *Escherichia coli* and related enterobacteria (Fig. 1) (Munson *et al.,* 1991; Schroder *et al.,* 1996; Sameshima *et al.,* 1999). Furthermore, many previous studies suggest that *Buchnera, Wigglesworthia,* and *Blochmannia* share a very close phylogenetic relationship and may be monophyletic (*e.g.,* Schroder *et al.,* 1996; Spaulding and von Dohlen, 1998; Sauer *et al.,* 2000). However, these phylogenies were often estimated using maximum parsimony or distance approaches, which may be highly biased by the fast evolutionary rates and strong AT bias of endosymbiont sequences. In contrast, maximum likelihood analysis (Fig. 1) strongly suggests that *Buchnera* is phylogenetically distinct from *Wigglesworthia* and *Blochmannia,* but that the last two are closely related to each other. This aspect of the phylogeny agrees with that proposed by Charles *et al.* (2001). Multiple, independent origins of endosymbiotic lifestyles are not entirely surprising, since endosymbiotic bacteria have arisen in many classes of bacteria, including α-Proteobacteria, β-Proteobacteria, and flavobacteria (Douglas, 1989; Moran and Telang, 1998). Indeed, multiple origins of endosymbiosis within the γ-Proteobacteria makes this group an ideal model to explore (i) phylogenetically independent transitions to an endosymbiotic lifestyle (*e.g., Buchnera versus Wigglesworthia* and *Blochmannia*), and (ii) adaptation of closely related bacterial species to quite different host associations (*Wigglesworthia versus Blochmannia*).

The phylogenies of P-endosymbionts within a single host system are generally congruent with their hosts' species, indicating that the symbiont origin traces back to a single, often ancient, infection event within each host group. This pattern of host-symbiont cospeciation has been demonstrated for the aphid-*Buchnera* symbiosis (Munson *et al.,* 1991; Clark *et al.,* 2000; Funk *et al.,* 2000), the tsetse fly-*Wigglesworthia* symbiosis (Chen *et al.,* 1999), and the psyllid-*Carsonella* symbiosis (Thao *et al.,* 2000). Thus, in contrast to facultative symbionts or pathogens that can transfer horizontally to new hosts, P-endosymbionts have been vertically inherited over long evolutionary timescales. These ancient insect-bacterial associations date back as far as 150-200 MYA for the aphid-*Buchnera* association (Munson *et al.,* 1991) and about 50-100 MYA for tsetse fly-*Wigglesworthia* (Moran and Wernegreen, 2000). Phylogenetic congruence between *Camponotus* and *Blochmannia* also holds true across a large number of host species (Sameshima *et al.,* 1999; Sauer *et al.,* 2000; P. H. Degnan *et al.,* unpubl. data) as well as within a single *Camponotus* species (A. B. Lazarus *et al.,* unpubl. data), indicating the ant-bacterial association is evolutionarily stable and at least as old as the genus *Camponotus* (>20 MY; Wilson, 1985) if not even older.

Population bottlenecks and genetic drift

Maternal transmission of P-endosymbionts is thought to impose a population bottleneck that reduces the number of bacteria passed on from mother to offspring (Mira and Moran, 2002). Successive bottlenecks throughout the evolution of these ancient associations are expected to reduce the effective population size (N_e) of the bacterial partner. Consequently, endosymbiont population sizes may be determined by insect host population sizes, which are orders of magnitude smaller than the extremely large populations of free-living bacteria ($N_e \sim 10^9$ for species of enterobacteria; Selander *et al.,* 1987). Models of nearly neutral evolution predict that reduced N_e will lower the efficacy of natural selection and will elevate rates of fixation of deleterious mutations through random genetic drift (Ohta, 1973). Over time, the accumulation of deleterious mutations may negatively affect the fitness of the symbiont and host (Rispe and Moran, 2000; Wernegreen and Moran, 2000). Exacerbating the effect of genetic drift in P-endosymbionts is their apparent lack of recombination among genetically distinct lineages (Funk *et al.,* 2000; Wernegreen and Moran, 2001). This strict asexuality contrasts with recombination in free-living bacterial strains, and may produce an effect known as Muller's ratchet (Muller, 1964, Moran, 1996) in which genetic drift in small populations is increased because wild-type genotypes cannot be introduced through recombination.

Several studies show that the repair of slightly deleterious mutations is important in shaping sequence evolution of P-endosymbionts. Evidence for drift includes fast rates of

sequence evolution, changes that destabilize the 16S rRNA secondary structure, elevated rates of amino acid substitutions, and higher ratio of nonsynonymous to synonymous substitutions (dN/dS) compared to free-living bacteria (Moran, 1996; Brynnel *et al.,* 1998; Lambert and Moran, 1998; Wernegreen and Moran, 1999; Clark *et al.,* 1999). Similarly, protein-coding genes of *Blochmannia* show accelerated rates of evolution and elevated dN/dS, suggesting this ant symbiont may also experience strong genetic drift (unpubl. data). Pervasive acceleration of protein evolution across the genome is not easily explained by relaxed or positive selection, which is expected to act at individual genes. Nor can elevated mutation alone explain the observed rate increase, since mutation would affect dN and dS equally, with no expected change in dN/dS. Finally, population genetic analyses of *Buchnera* associated with two aphid species show low levels of sequence polymorphism consistent with population bottlenecks, and an excess of rare alleles and nonsynonymous polymorphisms that suggest strong effects of genetic drift (Funk *et al.,* 2001; Abbot and Moran, 2002). In total, the pervasive rate elevation across genes, elevated dN/dS, and intraspecific polymorphism data are most consistent with reduced efficacy of selection across the genome due to genetic drift in small populations. Intracellular pathogens also show elevated rates of protein evolution that suggest genome degradation through genetic drift (Andersson and Andersson, 1999).

On a related note, a recent study attributed accelerated protein evolution in *Buchnera* to mutational bias alone (Itoh *et al.,* 2002). The authors argued against any role of genetic drift, on the basis that *Buchnera* presumably did not show an increase in dN/dS. However, the dN and dS values used to calculate this ratio were derived from different studies of different *Buchnera* loci. Thus, the apparent lack of elevated dN/dS must be weighed against the results of several studies that show a significant elevation of this ratio when dN and dS are calculated from the same dataset (Moran, 1996; Brynnel *et al.,* 1998; Wernegreen and Moran, 1999; Clark *et al.,* 1999). Itoh *et al.* (2002) also raise the intriguing suggestion that if slightly deleterious mutations are fixed in populations over time, these mutations will eventually render all genes functionless. However, mutations that severely impair or eliminate functions of necessary genes are not slightly deleterious; rather, these mutations would have high selective coefficients and would be eliminated even from small endosymbiont populations. For example, in the AT-rich *Buchnera* genome, high-expression genes (*e.g.,* chaperonins and ribosomal proteins) have distinct amino acid usage patterns compared to genes with putatively low expression levels (Palacios and Wernegreen, 2002). High-expression genes tend to use amino acids that are less aromatic and are encoded by relatively GC-rich codons, suggesting strong selection against aromatic amino acids and against amino acids with AT-rich codons. Thus, while AT mutational bias and genetic drift influence amino acid usage in *Buchnera,* selection at high-expression genes is sufficiently strong to attenuate the effects of mutational bias on amino acid content. We could generalize that, despite strong effects of genetic drift, selection still constrains deleterious amino acid changes in *Buchnera,* especially at high-expression loci.

Mutational bias in endosymbionts: random or adaptive?

Along with genetic drift, intracellular mutualists and pathogens also experience strong mutational pressure that, over time, can severely alter the base composition of their genomes. In contrast to the moderate base compositions of the enterics, sequences of intracellular bacteria are characterized by extremely low percentage content of GC (Fig. 2). A 37-kb fragment of the *Carsonella ruddii* genome was recently found to be just 19.9% GC, making this psyllid symbiont the most AT-rich bacterial genome yet characterized (Clark *et al.,* 2001). Analysis of six kilobases of *Blochmannia* sequences (unpubl. data) corroborates earlier evidence of low GC content for this bacterial genome (~30% GC; Dasch, 1975). This AT bias has a strong impact on the amino acid composition of *Buchnera, Carsonella,* and *Wigglesworthia* proteins, which are highly biased toward amino acids encoded by AT-rich codons (Clark *et al.,* 1999, 2001; Akman *et al.,* 2002; Palacios and Wernegreen, 2002).

Two main hypotheses have been proposed to explain base compositional biases observed in most obligately intracellular mutualists and pathogens. First, AT richness may reflect strong mutational bias resulting from the loss of DNA repair genes by random genetic drift (Eisen and Hanawalt, 1999; Moran and Wernegreen, 2000). According to this hypothesis, an underlying AT mutational bias is repaired less efficiently in small intracellular genomes that lack certain repair functions. Second, AT bias may be an adaptive feature of an intracellular lifestyle, explained by the high energetic cost and lower accessibility of GTP and CTP compared to ATP and UTP (Rocha and Danchin, 2002). ATP, for example, plays a significant role in cellular metabolism and is the most abundant nucleotide. Under this hypothesis, a nucleotide pool biased toward UTP and ATP and a corresponding AT mutational bias would be more efficient and thus would allow intracellular bacteria to exploit the host cell more effectively. Selection on each GC→AT mutation would be miniscule, but selection might favor larger changes (such as gene loss) that contribute to an overall mutation bias. Consistent with this adaptive hypothesis, the AT contents of other intracellular elements (*e.g.,* plasmids, phage, and insertion sequences) are also generally higher than those of their hosts, and base composition of phage corresponds to infection type, as virulent phages are more AT-rich than temperate ones (Rocha and Danchin, 2002).

Figure 1. Phylogenetic relationships among insect endosymbionts (boldface) and related γ-Proteobacteria, estimated from the 16S rDNA gene. Both maximum likelihood (ML) and Bayesian analyses give the tree topology presented. Values above nodes (in boldface) are bootstrap values for maximum likelihood analysis, and values below nodes are posterior probability values generated by the Bayesian analysis. Branch lengths reflect genetic distance under the maximum likelihood model used. This phylogeny strongly supports the following hypotheses: (i) a single origin of endosymbionts in the ancestor of the ant genera *Camponotus, Colobopsis,* and *Polyrhachis,* (ii) independent origins of symbiosis in the ants *Formica* and *Plageolepis,* and (iii), that *Buchnera* is a phylogenetically distinct lineage from *Wigglesworthia* and *Blochmannia,* which are closely related.

16S rDNA sequence data: Most 16S rDNA sequences were obtained from GenBank (with the exception new *Blochmannia,* obtained as described below). Nucleotide sequence accession numbers for other 16S rDNA sequences used in phylogenetic analysis are as follows: *Antonina crawii* S-endosymbiont AB030020; *Buchnera-Acyrthosiphon pisum* (P-endosymbiont) NC002528; *Arsenophonus-Triatoma infestans* (S-endosymbiont) U91786; *Colobopsis nipponicus* endosymbiont AB018676; *Dysmicoccus neobrevipes* S-endosymbiont AF476104; *Enterobacter asburiae* AB004744; *Escherichia coli* NC000913; *Erwinia herbicola* AB004757; *Formica fusca* endosymbiont AB018684; *Wigglesworthia-Glossinia austeni* (P-endosymbiont) AF022879; *Wigglesworthia-Glossinia brevipalpis* (P-endosymbiont) L37341; *Glossinia pallidipes* S-endosymbiont M99060; *Haemophilus influenzae* NC000907; *Klebsiella pneumoniae* AB004753; *Arsenophonus-Nasonia vitripennis* (S-endosymbiont) M90801; *Plagiolepis pigmaea* endosymbiont AB018683; *Pseudomonas aeruginosa* NC002516; *Polyrhachis lamellidens* P-endosymbiont AB018680; *Proteus vulgaris* J01874; *Buchnera -Schizaphis graminum* (P endosymbiont) L18927; *Sitophilus oryzae* P-endosymbiont AF005235; *Salmonella typhimurium* NC003197; *Uroluecon astronomus* S-endosymbiont (U type) AF293623; *Yersinia pestis* NC003143; *Yamatocallis tokyoensis* S-endosymbiont AB064515.

Obtaining Blochmannia 16S rDNA data: Genomic DNA was extracted from individual *Camponotus festinatus* and *C. pennsylvanicus* workers using the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. This DNA was used as template for PCR reaction using SL and SR universal eubacterial 16S rDNA primers (Schroder *et al.,* 1996). The single 1.6-kb band PCR product was then cleaned up using a column purification kit (Qiagen) and sequenced on an ABI 3700 automated sequencer. SL, SR, and two internal primers were used to sequence the PCR product. The resulting sequences were assembled and edited using PHRED,

Notable exceptions to the link between an intracellular lifestyle and AT bias include the relatively GC-rich genomes of the pathogen *Mycobacterium leprae* (57.8% GC; Cole *et al.,* 2001); the weevil endosymbiont, SOPE (54% GC; Heddi *et al.,* 1998); and the β-Proteobacterial subdivision endosymbiont of mealybugs, *Tremblaya* (57.1% GC across a 35-kb region; Baumann *et al.,* 2002). Interestingly, both SOPE and *M. leprae* also differ from most other obligately intracellular bacteria in having relatively large genome sizes (~3 Mb and 3.27 Mb, respectively) (Fig. 2). The genome size of *Tremblaya* is unknown, but its close relative *Burkholderia pseudomallei* has a very large genome (7.25 Mb; unpubl. data of the *B. pseudomallei* Sequencing Group at the Sanger Institute; http://www.sanger.ac.uk/Projects/B_pseudomallei/). These larger genomes may retain DNA repair functions that are missing from small, AT-rich genomes of most intracellular bacteria. Understanding the physiology of *M. leprae,* in particular, may help to distinguish whether the AT mutational bias of most intracellular bacteria is due to a loss of DNA repair functions by genetic drift or to selection for energetic efficiency. This pathogen experiences elevated mutation rates that drive genome deterioration, yet is relatively GC-rich. The hypothesis of adaptive mutational bias would predict that *M. leprae* has access to more energetic resources and competes less severely with its host for nutrients.

Genome Evolution in Endosymbionts: Size Matters

Full genome sequences of endosymbionts have provided new insights into the mechanisms and consequences of genome reduction. Recently published endosymbiont genomes include those of *Buchnera aphidicola* associated with the pea aphid *Acyrthosiphon pisum* (Ap) (Shigenobu *et al.,* 2000), the greenbug *Schizaphis graminum* (Sg) (Tamas *et al.,* 2002), and the gall-forming aphid *Baizongia pistacea* (van Ham *et al.,* 2003); and *Wigglesworthia glossinidia* of the tsetse fly *Glossina brevipalpis* (Akman *et al.,* 2002). Additional endosymbiont genomes, including that of *Blochmannia,* are being sequenced.

Comparative genome analyses illustrate striking parallels between P-endosymbionts of insects and intracellular pathogens. As described previously, pathogens and insect mutualists are characterized by reductive genome evolution, a syndrome that includes severely reduced genome size compared to free-living bacterial relatives, elevated rates of sequence evolution, and low genomic GC contents (Andersson and Kurland, 1998) (Fig. 1). The small chromosome sizes of *Buchnera* (450-650 kb; Charles and Ishikawa, 1999; Wernegreen *et al.,* 2000; Gil *et al.,* 2002) and *Wigglesworthia* (698 kb; Akman *et al.,* 2002) imply substantial gene loss since their divergence from the enteric bacteria (4.5-5.5 Mb genome size range for *E. coli;* Bergthorsson and Ochman, 1995). Because most bacterial genomes contain primarily coding DNA, genome reduction in endosymbionts

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PHRAP, and CONSED. These 16S rDNA genes of *Blochmannia-C. pennsylvanicus* and *Blochmannia-C festinatus* are assigned GenBank accession numbers AY196850 and AY196851, respectively.

Phylogenetic analysis methods: Alignments were created using the Ribosomal Database Project II sequence aligner (Maidak *et al.,* 2001), then manually edited in MacClade v. 4.05 (Maddison and Maddison, 2002). Maximum likelihood parameters were identified according to the Akaike information criterion (AIC) of Modeltest v. 3.06 (Posada and Crandall, 1998). The most likely model was a general time reversible (GTR) model in which invariant sites and the gamma distribution were estimated from the data. The optimized parameters (Rmat = [0.8676 4.6744 2.0447 1.0516 7.4521], shape of gamma distribution = 0.5500, and proportion of invariant sites = 0.5115) were used for all ML searches. The tree topology presented is the consensus of 100 separate heuristic ML searches, each starting from random trees, using PAUP v. 4.0b10; (Swofford, 2002). ML bootstrap values were determined from 100 bootstrap replicates, with each replicate starting from 10 random trees. Replicates were performed in parallel on a Beowulf cluster using the clusterpaup program (A.G. McArthur, http://jbpc.mbl.edu/mcarthur). Bayesian analysis was performed on the same data matrix (MrBayes ver. 2.01; Huelsenbeck and Ronquist, 2001) by running four simultaneous chains for 300,000 generations, sampling every 100 generations. Stationarity in likelihood scores was determined by plotting the -1nL against the generation. All trees below the observed stationarity level were discarded, resulting in a "burnin" of 5000 generations. The 50% majority-rule consensus tree was determined to calculate the posterior probabilities for each node. The selected model for Bayesian analysis was the GTR, using empirical base frequencies, and estimating the shape of the gamma distribution and proportion of invariant sites from the data. The Bayesian tree with the best likelihood score was identical to the ML tree presented, and the parameter values across this tree were virtually identical to those obtained in the ML analysis.

Limited sequence data (<570 bp of 16S rDNA gene) were available for four taxa included in the phylogeny (*Formica fusca, Plagiolepis pigmaea, Polyrhachis lamellidens* and *Colobopsis nipponicus),* compared to >1202 bp available for the rest of the taxa. Removal of these four taxa from ML or Bayesian analysis did not affect the overall tree topology; however, their removal greatly increased the statistical confidence in the node marked with the open circle. Comparisons of these analyses indicate that including *Plagiolepis pigmaea* drives down the confidence in that node, and suggest ambiguity in its position on the tree. However, given the incomplete sequence for that endosymbiont, this topology is the best estimate of its phylogenetic placement.

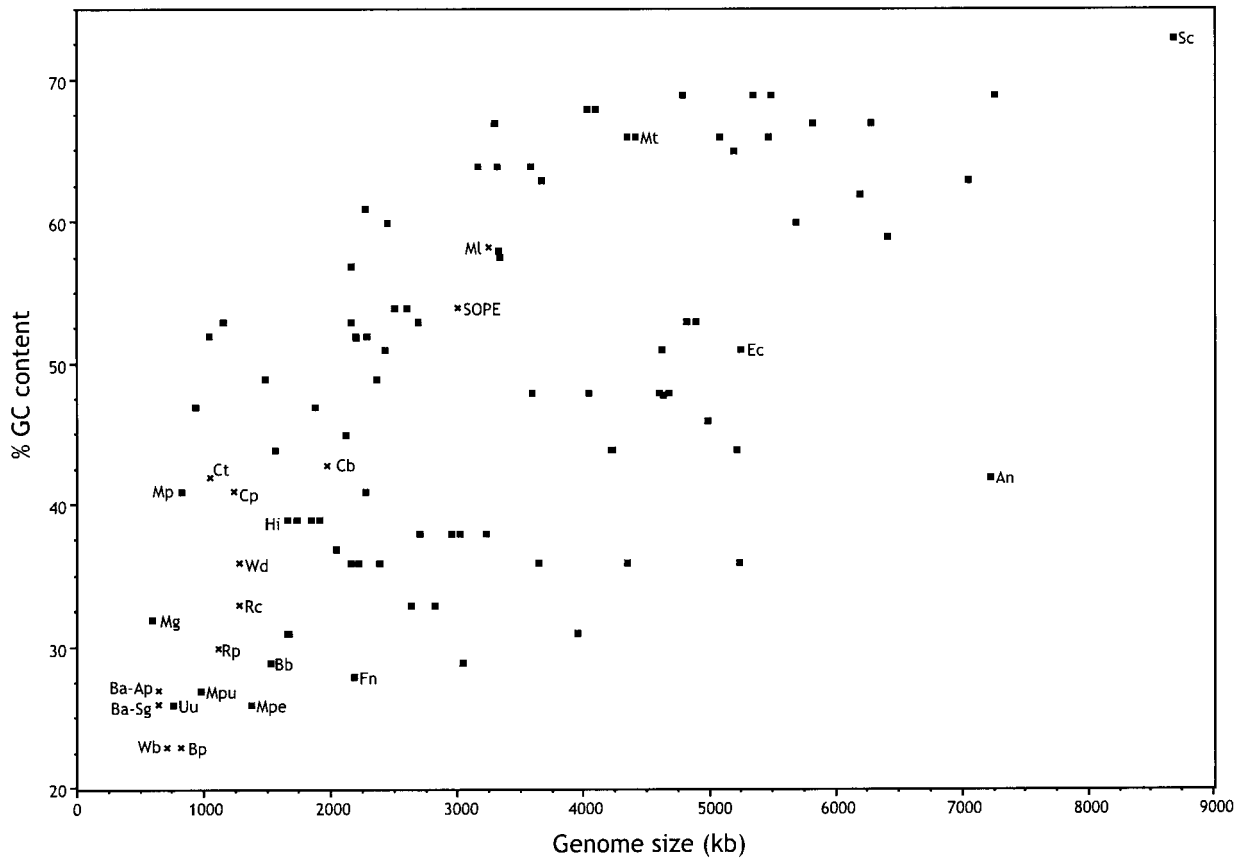

Figure 2. Comparison of genome sizes and %GC contents of select eubacterial genomes. Obligately intracellular bacteria, including pathogens and P-endosymbionts of insects, are marked with x, while facultatively intracellular or extracellular bacteria are marked with ■. Pathogens classified as obligately intracellular in this figure are classified as such on the IslandPath website (http://www.pathogenomics.sfu.ca/islandpath/current/islandpath.html). In certain cases, a close phylogenetic relationship of particular species may contribute to the observed positive relationship between genome size and genomic %GC content. However, since selected species span diverse subdivisions of Proteobacteria, the observed trend cannot be explained by shared phylogenetic history alone. Abbreviations for bacterial species of particular interest for this article are noted as follows: An *Anabaena* spp.; Ba-Sg *Buchnera aphidicola-S. graminum;* Ba-Ap *Buchnera aphidicola-A.pisum;* Bb *Borrelia burgdorferi;* Bp *Blochmannia-C. pennsylvanicus;* Ca *Clostridium acetobutylicum;* Cb *Coxiella burnetii;* Cp *Chlamydia pneumoniae;* Ct *Chlamydia trachomatis;* Ec *Escherichia coli;* Hi *Haemophilus influenzae;* Hp *Helicobacter pylori;* Mg *Mycoplasma genitalium;* Ml *Mycobacterium leprae;* Mp *Mycoplasma pneumoniae;* Mpe *Mycoplasma penetrans;* Mpu *Mycoplasma pulmonis;* Mt *Mycobacterium tuberculosis;* Rc *Rickettsia conorii;* Rp *Rickettsia prowazekii;* SOPE *Sitophylis oryzae* Primary Endosymbiont; Sc *Streptomyces coelicolor;* Uu *Ureaplasma urealyticum;* Wb *Wigglesworthia brevipalpis;* Wd *Wolbachia drosophila.* With the exception of SOPE and *Blochmannia,* all genome sizes and base composition data were obtained from full genome sequence data, summarized in the "DNA Structural Analysis of Sequenced Microbial Genomes" webpage (http://www.cbs.dtu.dk/services/GenomeAtlas/ created by Dr. David Ussery, Center for Biological Sequence Analysis, Technical University of Denmark). Genome size and base composition of SOPE were obtained from a previous study (Charles *et al.,* 1997). The genome size of *Blochmannia* has been estimated at ~809 kb by pulsed-field gel electrophoresis (Wernegreen *et al.,* 2002) and a GC content of 29.7%–31.6% was estimated by buoyant density and melting temperature (Dasch, 1975).

must involve the loss of metabolic functions and may have important, irreversible phenotypic implications (Moran and Wernegreen, 2000; Ochman and Moran, 2001). Strong genetic drift may contribute to this observed genome reduction by increasing the selective coefficient required to maintain a given gene in the genome (Lawrence and Roth, 1999).

Full genome sequences have taught us that obligate pathogens and mutualists retain a disproportionate number of essential genes (*e.g.,* those for cellular processes, translation, and protein fate and transcription), and have lost many genes for metabolic diversity that may be redundant in a nutrient-rich, relatively constant intracellular environment. As described above, the loss of DNA repair functions in these small genomes may contribute to elevated mutation

rates and AT mutational biases in pathogens and mutualists alike (Andersson and Andersson, 1999). The depletion of metabolic capabilities accounts for the inability to culture P-endosymbionts and obligate pathogens apart from a eukaryotic cell, and may constrain the ability of these bacteria to switch among lifestyles (Tamas *et al.,* 2002). For example, a lack of gene acquisition in *Buchnera* implies that this small genome cannot regain critical metabolic functions required for extracellular existence, nor can it acquire virulence loci or novel biosynthetic functions that could mediate new host associations.

In contrast to intracellular pathogens, the fitness of obligate mutualists depends not just upon their own replication and transmission but also on the success of their host. Not surprisingly, despite severe gene loss in most functional categories, endosymbiont genomes show conspicuous signatures of host-level selection and retain specific functions that are important for survival and reproduction of the insect (Zientz *et al.,* 2001). For example, *Wigglesworthia* retains genes encoding the biosynthesis of cofactors, prosthetic groups, and carriers, which is consistent with its known nutritional function for the host (Akman *et al.,* 2002). Likewise, *Buchnera* retains the genetic potential to synthesize practically all essential amino acids (Shigenobu *et al.,* 2000; Tamas *et al.,* 2002; van Ham *et al.,* 2003), as expected from its primary role to supplement the plant sap diet of the host (Sandstrom *et al.,* 2000). Nutrient exchange between *Buchnera* and its aphid host is complementary and mutually dependent (Shigenobu *et al.,* 2000): because it lacks the genes to synthesize them, the symbiont must import nonessential amino acids from the cytoplasm of its host. Another example of genome interdependence is that *Buchnera* can perform the pantothenate-to-pyruvate reaction, but only the host can convert pyruvate to CoA. Thus, while genomes of P-endosymbionts and pathogens show striking parallels, mutualists show reciprocally beneficial integration with host metabolism that is lacking in chronic pathogens.

Bacterial Associates of Ants: Distribution and Possible Functions

Independent origins of symbiosis in the Formicinae

Ants have evolved a wide range of interactions with other species, including plants, fungi, other insects, and as discovered more recently, associations with diverse bacteria (*e.g.,* Dasch *et al.,* 1984; Boursaux-Eude and Gross, 2000; Currie, 2001). These bacterial associates include intracellular endosymbionts, like *Blochmannia,* that live exclusively within ant cells. In contrast to the bacteriocytes of aphids and many other insects, ant cells that house beneficial bacteria are typically located beside or within the midgut epithelium (Dasch *et al.,* 1984). For example, *in situ* analysis shows that *Blochmannia* of *Camponotus* occurs exclusively in ant ovaries, consistent with its maternal transmission, and within bacteriocytes that are intercalated among enterocytes of the ant midgut (Sauer *et al.,* 2002). Phylogenetic analysis (Fig. 1) and a previous study (Sameshima *et al.,* 1999) suggest a single infection of *Blochmannia* prior to the divergence of *Camponotus, Colobopsis,* and *Polyrhachis.*

Bacteria related to *Blochmannia* infect the formicid genera *Formica,* in which the presence of symbionts varies among species, and *Plagiolepis,* which is poorly studied to date (reviewed in Dasch *et al.,* 1984). These bacteria can vary in their location within the ant host. In contrast to the endosymbionts of *Blochmannia,* those of *Formica* and *Plagiolepis* are not intercalated among midgut cells, but form symmetrical unicellular layers of bacteriocytes on either side of the midgut epithelium. Unlike the straight rods (1 μM by 5-15 μM) of *Blochmannia,* the *Formica* and *Plagiolepis* endosymbionts are shorter (3-4 μM) and crescent-shaped (Dasch *et al.,* 1984). Consistent with observed differences in bacterial morphologies and location in the host, the *Formica* and *Plagiolepis* endosymbionts are probably the results of independent infections (Fig. 1). The actual abundance, diversity, and origins of endosymbionts in ants remain unknown, since most ant taxa have not been tested for bacterial associates.

Possible functions of endosymbionts in ants: food and pheromones?

The function of even the best-studied ant endosymbiont, *Blochmannia,* remains a mystery. In a thorough study of the distribution of *Blochmannia* within host tissues, *Camponotus* workers were cured of their endosymbionts with no obvious harmful effects to laboratory reared animals (Sauer *et al.,* 2002). The ants' apparent ability to exist without *Blochmannia* raises the question of whether these endosymbionts are required for survival, or whether the presence of the bacteria is a relict from a past mutualism. However, the presence of *Blochmannia* in all *Camponotus* species sampled thus far and the long-term maintenance of this symbiosis suggest an important benefit to the host.

Whether *Blochmannia* plays a dietary role in ants is difficult to answer. It is clear that selection has favored mutualistic endosymbionts in specialized feeders, but *Camponotus* spp. are usually considered omnivorous and are thought to consume a wide assortment of arthropods and plant matter, in addition to homopteran secretions (Dasch *et al.,* 1984; Hölldobler and Wilson, 1990). Certain *Camponotus* species clearly have an unbalanced diet, particularly those species that live in the canopy of tropical rain forests and feed primarily on extrafloral nectar and insect exudates. Stable isotope technologies have determined the nitrogen (N) sources for several ant species in field populations (Davidson, 1997, 1998). Isotope profiles of many arboreal ants, including certain species of *Camponotus* and *Polyrhachis,* resemble those of herbivores (*i.e.,* low delta N) and

may even resemble those of sap-feeding insects (with still lower delta N values) (Davidson, 1997, 1998). The ancestral host of *Blochmannia* might have fed on a similarly restricted diet. However, given the current habitat diversity of its hosts (including live and dead trees and soil, across temperate, desert, and tropical regions), it seems unlikely that diets of all extant *Camponotus, Polyrhachis,* and *Colobopsis* are lacking any single nutrient that *Blochmannia* could provide.

Ant endosymbionts might also play a secondary nutritional role by augmenting an existing metabolic process in the host. For example, SOPE (the aforementioned weevil symbiont and a close relative of *Blochmannia*) has a positive effect on weevil mitochondrial enzymatic activity that results in increased female fertility, decreased larval development time, and longer flight distances (Heddi *et al.,* 1999). If *Blochmannia* had similar effects in ants, these traits would be especially advantageous to young ant queens starting new colonies. Any symbiont-induced increase in energetic efficiency would provide an important selective advantage during colony founding (Dasch, 1975). This stage of the colony life cycle imposes severe intra- and interspecific competition, when the queen must find an appropriate nesting site, raise a small worker force entirely from her own energy reserves, and then rely on this first generation of daughters to establish a viable colony (Hölldobler and Wilson, 1990). The gradual loss of bacteria from older queens (Sauer *et al.,* 2002) suggests that the significance of *Blochmannia* may lie in colony founding and growth rather than in adult maintenance. The fact that *Blochmannia* has been found only in formicine genera with relatively large body sizes (Dasch *et al.,* 1984; Sameshima *et al.,* 1999) deserves further investigation. The larger body size of these genera might reflect a longstanding relationship with a bacterium that increases metabolic efficiency.

In addition, pheromones and hydrocarbons mediate important behaviors ranging from kin recognition to suppression of non-queen egg-laying (Hölldobler and Wilson, 1990). It is possible that *Blochmannia* manufactures a component of these complex organic molecules. Unlike other P-endosymbionts that are sequestered within discrete bacteriocytes in the host body cavity, *Blochmannia* might be able to mediate precursors that it requires directly from the lumen of the gut. The proximity of *Blochmannia* to raw materials could augment pheromone production to any number of the ant's excretory glands. For example, it has been suggested that *Blochmannia* may play some role in producing the pheromones that *Camponotus* uses for food recruitment (Sauer, 2000).

In addition, a potential role of *Blochmannia* in ant reproduction is intriguing, and may involve genetic conflicts in the colony. Research on other maternally transmitted bacteria of insects (*e.g., Wolbachia*) has also illustrated that some endosymbionts can dramatically affect host reproduction, through feminization, male-killing, parthenogenesis, and cytoplasmic incompatibility (O'Neill *et al.,* 1998). All these effects impart a fitness advantage to the bacteria by increasing the number of transmitting hosts (*e.g.,* females) in a population. If *Blochmannia* is similarly involved in manipulating ant reproduction and sex ratios toward females, it would in part share this "preference" with colony workers, who are also favored to manipulate sex ratios owing to relatedness asymmetries under haplodiploid genetics. In a haplodiploid genetic system (diploid females produced from fertilized eggs and haploid males produced from unfertilized eggs), workers are more closely related to their sisters (3/4) than to either their brothers (1/4) or mothers (1/2) (Hölldobler and Wilson, 1990). Workers can therefore increase their inclusive fitness by preferentially caring for sisters or killing male larvae, resulting in an increase in the number of females produced (Sundstrom *et al.,* 1996; Chapuisat *et al.,* 1997; Passera *et al.,* 2001; Hammond *et al.,* 2002). With similar interests in increased female production, *Blochmannia* and workers could be considered allies in their respective genetic conflicts. However, this is speculative, and further research is needed to explore potential links among *Blochmannia,* pheromone production, and sex ratios of the host.

Genome evolution in Blochmannia

To further explore the effects of symbiosis on bacterial genome size and architecture, the genome size of *Blochmannia* was estimated using pulsed-field gel electrophoresis (Wernegreen *et al.,* 2002). Like other P-endosymbionts, *Blochmannia* has a very small genome (~809 kb) that contrasts with the much larger genomes of related enterobacteria. Clearly, *Blochmannia* has deleted most of the genetic machinery of free-living and commensal bacterial species, and consequently depends entirely upon its eukaryotic host. Since *Blochmannia* and other obligate endosymbionts experience restricted gene exchange, this severe gene loss may reflect irreversible specialization to the host cellular environment.

It is difficult to predict the gene content of *Blochmannia* when its functional role in the ant symbiosis is so unclear. However, the gene complements of fully sequenced insect mutualists such as *Wigglesworthia* might provide some hints (Akman *et al.,* 2002). Like *Wigglesworthia, Blochmannia* is probably a relatively young symbiont (~30–40 MY old), has a slightly larger genome size than *Buchnera,* and lives directly within cytoplasm rather than host-derived membranes (Sauer *et al.,* 2000). Therefore, *Blochmannia* may retain certain characteristics of free-living bacteria that have been found in *Wigglesworthia,* such as a robust cell membrane and perhaps a flagellar apparatus (Akman *et al.,* 2002). The strong AT bias of this symbiont might reflect the depletion of DNA repair pathways (Andersson and Anders-

son, 1999). If the *Blochmannia* genome parallels those of other "resident genomes," then one might expect the loss of many genes involved in central cellular pathways such as transcription and protein synthesis, but a more severe depletion of genes for metabolic diversity (Andersson and Kurland, 1998). One key to unraveling the functional role of this bacterium will be to identify host-selected functions. Good candidates for those functions include genes that are preserved in *Blochmannia* but are typically lost in other small bacterial genomes.

Conclusions and Prospects

Among obligate endosymbionts of insects, independent transitions to an intracellular lifestyle have been coupled with elevated rates of evolution, strong AT base compositional bias, and extreme genome reduction. This syndrome of reductive genome evolution parallels the patterns observed in chronic bacterial pathogens, and suggests that similar evolutionary forces operate across intracellular bacteria generally. Full genome sequences of *Buchnera* and *Wigglesworthia* have provided important insights into the evolutionary forces shaping mutualist genomes, including strong effects of mutational bias and genetic drift, but also host-level selection for beneficial traits. Determining the gene inventories of additional endosymbionts will allow comparisons among the diverse strategies by which mutualists specialize to their hosts, and the extent to which mutational pressure and genetic drift may limit their evolutionary potential.

Blochmannia, an obligate bacterial symbiont of ants, shows striking parallels with P-endosymbionts characterized to date, including close specialization with its insect host, reduced genome size compared to free-living relatives, and evidence for mutational bias and genetic drift. However, *Blochmannia* also shows important differences, including its distinct location among midgut-associated cells, and its slightly larger genome size compared to many other P-endosymbionts. The proximity of *Blochmannia* to the ant midgut suggests that the bacteria may provide the host with essential nutrients. However, the relatively complex and diverse diet of *Camponotus* hosts (Hölldobler and Wilson, 1990) contrasts with the strict, unbalanced diets of other insects with bacteriocyte-associated symbionts (*e.g.,* the phloem diet of aphids and other sap-sucking insects, the blood diet of tsetse flies, the grain diet of certain weevils) and suggests that this bacteria has an alternative role. Future genomic studies of *Blochmannia* may inform our understanding of its physiological and ecological significance for the host, which is currently unknown. Genes that are specifically retained in *Blochmannia* will provide promising candidates for host-specific, functionally important loci in this bacterial-ant association and will be the subject of further study.

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