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Small genome of *Candidatus* Blochmannia, the bacterial endosymbiont of *Camponotus*, implies irreversible specialization to an intracellular lifestyle

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***Blochmannia* (*Candidatus* Blochmannia gen. nov.) is the primary bacterial endosymbiont of the ant genus *Camponotus.* Like other obligate endosymbionts of insects, *Blochmannia* occurs exclusively within eukaryotic cells and has experienced long-term vertical transmission through host lineages. In this study, PFGE was used to estimate the genome size of *Blochmannia* as approximately 800 kb, which is significantly smaller than its free-living relatives in the enterobacteria. This small genome implies that *Blochmannia* has deleted most of the genetic machinery of related free-living bacteria. Due to restricted gene exchange in obligate endosymbionts, the substantial gene loss in *Blochmannia* and other insect mutualists may reflect irreversible specialization to a host cellular environment.**

Keywords: genome reduction, symbiosis, bacteriocytes, asexuality, genetic drift

INTRODUCTION

Obligate bacterial mutualists are critical to the reproduction and success of over 10% of insect species (Douglas, 1989). In contrast to some facultative bacterial associates, these primary endosymbionts are typically required for host growth and reproduction, occur within specialized host cells called bacteriocytes, and experience stable, maternal transmission through host lineages (Buchner, 1965; Hinde, 1971). Insect endosymbionts are widespread phylogenetically, but several lineages group with the γ-3 subdivision of the *Proteobacteria* and are closely related to *Escherichia coli* and other enterobacteria. Molecular evidence shows that γ-3-subdivision endosymbionts include *Buchnera aphidicola* associated with aphids, *Wigglesworthia glossinidia* associated with tsetse flies, *Carsonella ruddii* of psyllids, and *Blochmannia* of *Camponotus*, among other insect associates (Charles *et al*., 2001). Although their functional significance is often unknown, these symbionts are generally thought to play nutritional roles such as providing essential amino acids, vitamins or other nutrients that are lacking in the hosts' diet (Buchner, 1965; Baumann *et al*., 1995; Douglas, 1989). For example, *Buchnera*, the best-characterized insect mutualist, provides essential amino acids that are deficient in the aphid's diet of plant sap (Douglas, 1998; Baumann *et al*., 1998).

Like most intracellular pathogens, bacterial endosymbionts of insects have undergone severe genome reduction in the context of their obligate associations with hosts. The small genomes of *Buchnera* (630–650 kb; Charles & Ishikawa, 1999; Wernegreen *et al*., 2000) and *Wigglesworthia* (705–730 kb; Akman & Aksoy, 2001) approach the smallest known bacterial genome size (*Mycoplasma genitalium*, 580 kb; Fraser *et al*., 1995) and are very reduced compared to the 4·5–5·5 Mb genome size range for *E. coli* (Bergthorsson & Ochman, 1995, 1998). Since most bacterial genomes contain primarily coding DNA, genome reduction in endosymbionts must involve the loss of metabolic functions and physiological capacities, with important phenotypic implications (Andersson & Kurland, 1998; Moran & Wernegreen, 2000; Ochman & Moran, 2001). Indeed, full genome sequence data for *Buchnera* (Shigenobu *et al*., 2000) and microarray analysis of gene content in *Wigglesworthia* (Akman & Aksoy, 2001) demonstrate the deletion of several loci for metabolic functions that are not essential within the host cellular environment. The loss of these biochemical capabilities may account for the inability to culture insect endosymbionts without the host.

The GenBank accession number for the sequence reported in this paper is AF495758.

Blochmannia, first discovered in 1887 (Blochmann, 1887) and recently assigned to the new genus '*Candidatus* Blochmannia gen. nov.' (Sauer *et al*., 2000) is the primary endosymbiont of the ant genus *Camponotus*, the largest genus in the family Formicidae (ants) that includes 931 species in every biogeographical region (Bolton, 1995). Like most intracellular bacteria, *Blochmannia* shows accelerated evolutionary rates (unpublished data) and an extremely low G+C content (23 mol%; Dasch, 1975; Dasch *et al*., 1984). *Blochmannia* occurs exclusively within ant ovaries and within bacteriocytes, specialized host cells that are intercalated among enterocytes of the ant midgut. *Blochmannia* is widely distributed across most species of *Camponotus*, and congruence of host and symbiont phylogenies (Schroder *et al*., 1996; Sameshima *et al*., 1999; Sauer *et al*., 2000) suggests the association is evolutionarily stable and as old as the host genus (20 million years; Wilson, 1985), if not older. In this study, we used PFGE to estimate the genome size of *Blochmannia*. In the course of this analysis, we further developed methods for isolating genomic DNA of unculturable endosymbionts in sufficient quantity and at sufficient purity for PFGE.

METHODS

Collection and maintenance of *Camponotus* host species. We selected *Blochmannia* associated with *Camponotus pennsylvanicus* because of the local abundance of this carpenter ant. *C. pennsylvanicus* colonies were collected from two sites in Falmouth, MA, USA, less than 1·5 miles (~2·4 km) apart. Colonies were maintained for 4–6 months in the laboratory on a modified Bhatkar diet and live or frozen insects (Hölldobler & Wilson, 1990).

Endosymbiont preparation. *Blochmannia* was isolated from ant hosts, using a Percoll density-gradient protocol previously described by Charles & Ishikawa (1999) for the isolation of *Buchnera* cells from aphid hosts. For *Blochmannia* isolations, we modified the protocol as follows: buffer A was supplemented with 100 mM and 250 mM EDTA, pH 8, and $MgCl_2$ was omitted, to reduce the activity of nucleases. The endosymbiont preparation was performed in buffer A with 250 mM EDTA, except for steps involving Percoll, for which we used buffer A with 100 mM EDTA. Filtration through 5 and 3 μM filters was omitted.

Endosymbiont cells were resuspended in buffer A with 100 mM EDTA and embedded in plugs of InCert agarose (BioWhittaker Molecular Applications) to preserve the structural integrity of chromosomal molecules. DNA preparations were performed in plugs as previously described (Charles & Ishikawa, 1999). DNA sequence data support a low genomic G+C content *Blochmannia* (e.g. Schroder *et al*., 1996, and our unpublished results). Therefore, we digested the genomic DNA within plugs using G+C-rich (rare-cutting) restriction enzymes, including *Apa*I, *Not*I, *Sma*I and *Asc*I. Before digestion, plugs were washed twice in the appropriate restriction buffer (30 min per wash). Digestions were performed with an excess of enzyme (40 U per 150 μl reaction) and incubated for 8 h at the appropriate temperature.

PFGE. PFGE was performed in a contour-clamped homogeneous electric field system (CHEF-XA Mapper; Bio-Rad) at 14 °C using 0·5× TBE, pH 8, and gels of 1% pulsed field gel-grade agarose (Bio-Rad). All gels were run at 6 V cm^{-1}, a 120° angle, and a constant ramp time. Switch times and total run times were programmed using the CHEF XA Mapper automated algorithm to span a wide range of fragment sizes (5–950 kb; see legend of Fig. 1; Ausubel *et al*., 1987). Standard size ladders included high-molecular-mass DNA markers (8·3–48·5 kb; Life Technologies), the Midrange II PFG ladder (24–267 kb; New England BioLabs), the Lambda Ladder PFG marker (48·5–1018 kb; NEB) and the Yeast Chromosome PFG marker (225–1900 kb; NEB). Digested fragments were sized by comparison with standard ladders, using the software package GelPro 2.0 (Image Processing Solutions). Sizes were determined based on the standard ladder closest to the lane of interest. In cases where experimental lanes were equidistant between two standard ladders, fragment sizes based on each ladder were averaged. Sizes of fragments generated by each enzyme were summed to estimate the genome size of *Blochmannia*.

DNA sequence analysis of *Blochmannia* 16S rRNA. We prepared DNA from a portion of an agarose plug used in PFGE by digesting the agarose with Beta-Agarase (New England BioLabs) and precipitating the DNA by following the manufacturer's instructions. This DNA was used as template in a PCR with universal eubacterial primers to the 16S rRNA gene (primers SL and SR; Schroder *et al*., 1996). The single resulting PCR product was the expected size of 1·6 kb. This PCR product was cleaned with a column purification kit (Qiagen) and sequenced directly on an ABI 3700 automated sequencer using SL, SR and two internal sequencing primers (primer sequences available upon request). The DNA sequence was edited and assembled using the PHRED/PHRAP/CONSED software package (version 10.0 for UNIX). The assembled sequence was assigned GenBank accession number AF495758.

RESULTS AND DISCUSSION

One hallmark of bacterial genomes is their potential to undergo radical changes in size and organization through gene duplication, gene loss, and rearrangements of large portions of the chromosome, as well as to acquire genes via viruses, plasmids and transposable elements. Since most bacterial genomes contain over 85% coding DNA (Bergthorsson & Ochman, 1998) the acquisition or loss of a large portion of DNA may define the biochemical capabilities and range of environments available to particular bacterial lineages. Across bacteria, the most rapid and severe changes in genome sizes are coupled with transitions to an intracellular lifestyle (Moran & Wernegreen, 2000). Obligate endosymbionts that are sequestered within specialized host cells may lack opportunities for recombination with genetically distinct strains (e.g. Funk *et al*., 2000). Therefore, severe gene loss in endosymbionts may represent an irreversible evolutionary trajectory that constrains existence outside a eukaryotic cell, and limits transitions in symbiotic lifestyles (e.g. parasitic versus mutualistic associations with hosts) (Moran & Wernegreen, 2000; Tamas *et al*., 2001).

In this study, we demonstrate that the *Blochmannia* genome is severely reduced compared to closely related free-living bacteria such as *E. coli*. We estimated the size of the *Blochmannia* genome by summing the sizes of

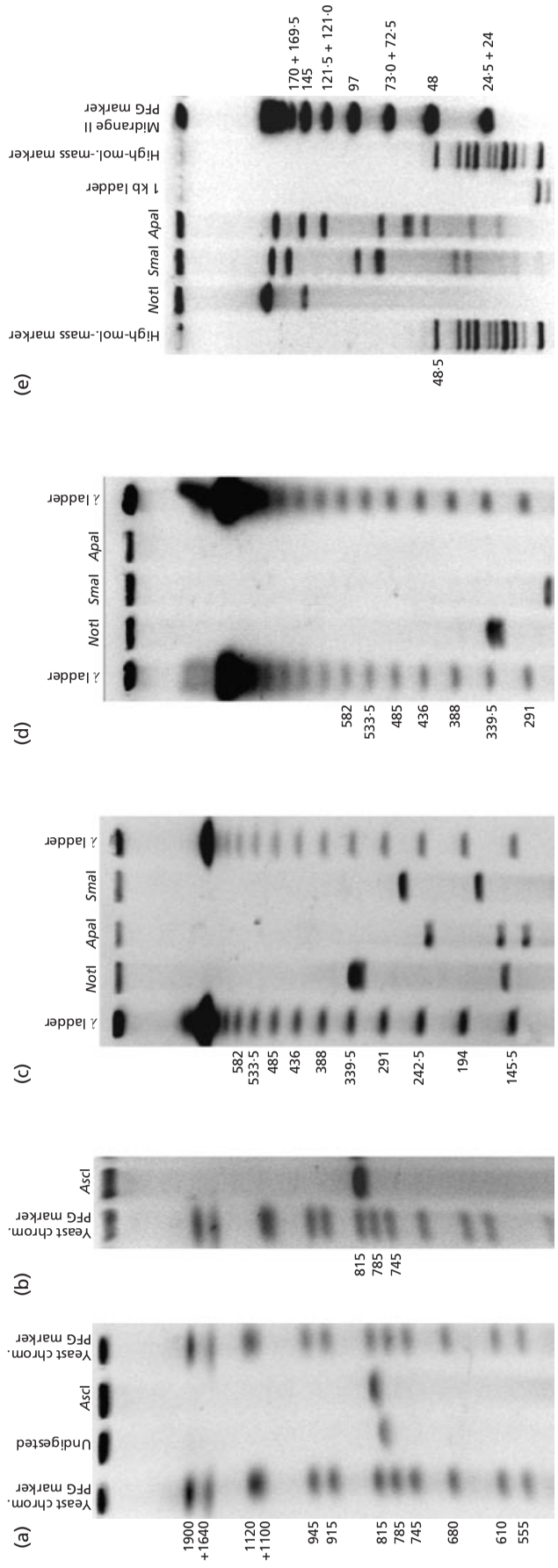

Fig. 1. PFGE of endonuclease-digested DNA of *Blochmannia* associated with *C. pennsylvanicus*. Sizes of standard ladders are marked in kb. *Blochmannia* DNA samples are labelled with the restriction enzyme used. Estimated sizes of *Blochmannia* DNA fragments were generally consistent across reactions and across gels (see Table 1). General running conditions are described in the text. Specific run conditions were programmed as follows: (a) 300–950 kb [initial switch time (IST) 35·43 s, final switch time (FST) 1 min 28·73 s, run time (RT) 34 h 8 min], 1 % gel; (b) 200–1000 kb (IST 24·03 s, FST 1 min 33·69 s, RT 29 h 57 min), 1 % gel; (c) 30–500 kb (IST 2·17 s, FST 44·76 s, RT 26 h 40 min), 1 % gel; (d) 60–650 kb (IST 7·93 s, FST 59·04 s, RT 27 h 47 min); (e) 5–100 kb (IST 0·23 s, FST 8·55 s, RT 15 h 16 min), 1 % gel.

Table 1. Fragment sizes resulting from digestion of *Blochmannia* DNA with four endonucleases

Fragment sizes were estimated using GelPro 2.0, as described in the text. Restriction fragment sizes were summed for each enzyme to estimate genome size. Fragment sizes are labelled with the gel (Fig. 1, a–e) from which that estimate was derived. Sizes in bold are considered the most reliable, due to their close proximity to a standard size ladder and/or their location within a highly resolved region of the gel.

Restriction enzyme	Enzyme fragment sizes (kb)			Sum of fragments (kb)
AscI	**809** (a)	810 (b)		**809**
*Not*I	**332** (c)	334 (d)		**808**
	325 (c)	325 (d)		
	151 (c)	153 (d)	147·5 (e)	
*Sma*I	**267** (c)	264 (d)		**790***
	178 (c)	175 (d)		
	101 (c)		96 (e)	
	84 (c)		81 (e)	
	78 (c)		80 (e)	
	44 (c)		40 (e)	
	38 (c)		34 (e)	
*Apa*I	**234** (c)	227 (d)		**813**
	156 (c)	153 (d)	154 (e)	
	132 (c)		128 (e)	
	77 (c)		80 (e)	
	64		63 (e)	
	61 (c)		61 (e)	
	53 (c)		54 (e)	
	36 (c)		32 (e)	

* The slightly lower genome size estimate based on *Sma*I digestions may result from the presence of faint, low-molecular-mass restriction fragments that were not scored (see text).

linear DNA fragments resolved by PFGE (Fig. 1, Table 1). Based on digestions with four different endonucleases, we determined that the genome size of *Blochmannia* associated with *C. pennsylvanicus* ranges between approximately 790 and 813 kb, the strongest support being for a genome size of ~809 kb. This estimate is based on several independent digestion reactions and pulsed field gels, examples of which are shown in Fig. 1.

Furthermore, we confirmed that the agarose plugs used in PFGE are pure samples of *Blochmannia*–*C. pennsylvanicus* by amplifying and sequencing the 16S rRNA gene of the embedded bacterial DNA. Results of BLASTN (http://www.ncbi.nlm.nih.gov) show that the resulting sequence (assigned GenBank accession no. AF495758) is most similar to published *Blochmannia* 16S rRNA sequences. Phylogenetic analysis of this and other 16S rRNA genes (data not shown) demonstrated that our samples are most closely related to the published *C. pennsylvanicus* endosymbiont 16S rRNA gene (AJ245598; Schroder *et al.*, 1996). These sequence data confirm that the agarose plugs used for genome size analysis contained pure samples of *Blochmannia*–*C. pennsylvanicus* DNA.

PFGE analysis of obligate endosymbionts such as *Blochmannia* poses distinct experimental challenges. First, the inability to culture *Blochmannia* outside the host required that we isolate *Blochmannia* cells from *C. pennsylvanicus* workers prior to DNA preparation within agarose plugs. This direct isolation from hosts necessarily limited the sample size (e.g. the number of independent restriction enzyme digestions) that we could attain compared to PFGE analyses of culturable bacteria. Furthermore, difficulty in preparing highly concentrated *Blochmannia* DNA resulted in a low intensity of small chromosomal fragments (less than ~15 kb). The *Sma*I digestion may include faint, small fragments that are difficult to size accurately (Fig. 1e). If so, these small fragments would account for the slightly smaller genome size estimate based on this enzyme (790 kb). Despite these inherent limitations of PFGE analysis of obligately intracellular bacteria, independent restriction digestions gave remarkably similar estimates of *Blochmannia* genome size in this study. Our results

clearly demonstrate that the *Blochmannia* genome is severely reduced, and totals less than one-fifth the genome size of *E. coli*.

Genome reduction in *Blochmannia* and other obligately intracellular bacteria is consistent with a shift in the selection–mutation balance in these species. The persistence of a gene within a bacterial lineage depends on the selection coefficient for its maintenance, the efficacy of this selection, as determined by effective population size (N_e), and mutation rates (Lawrence, 1999). Each of these three factors may shift in obligate endosymbionts. First, selective coefficients for the maintenance of genes for metabolic diversity may be reduced in an intracellular environment, resulting in the loss of genes for the biosynthesis of compounds they can acquire from the host cytoplasm. For example, the full genome sequence of *Buchnera* sp. APS (Shigenobu *et al*., 2000) demonstrates that the exchange of gene products between the symbiont and host is complementary and mutually dependent. *Buchnera* provides the host with essential amino acids and, as expected, possesses a complete set of genes for the biosynthesis of essential amino acids. However, *Buchnera* lacks genes for the biosynthesis of non-essential amino acids that it must import from the host cytoplasm. In addition, the pantothenate→pyruvate→CoA pathway illustrates the mutual dependency between host and symbiont genomes. *Buchnera* performs the pantothenate→pyruvate reaction while the host converts pyruvate to CoA.

Second, endosymbionts may have small N_e due to severe bottlenecks upon transmission to host offspring, and few, if any, opportunities for recombination with genetically distinct strains. According to the nearly neutral theory (Ohta, 1973), reduced N_e is expected to increase the effect of genetic drift and to reduce the efficacy of selection. Previous studies show elevated rates of fixation of slightly deleterious mutations at particular genes of endosymbionts, consistent with a strong effect of genetic drift (Moran, 1996). On a genome-wide level, genetic drift may have a cumulative effect of reducing genome size if it increases the selective coefficient required to maintain a given gene (Lawrence, 1999; Lawrence & Roth, 1999).

Third, small genomes of intracellular bacteria may reflect stronger effects of mutational biases due to the loss of several DNA-repair genes (Moran & Wernegreen, 2000). Mutational bias toward deletions may account for shorter lengths of endosymbiont genes (Charles *et al*., 1999), and recent work suggests that deletion biases in bacteria may have greater effects on endosymbiont genomes, in which more sites are effectively neutral (Mira *et al*., 2001).

Further genomic studies of *Blochmannia* may inform our understanding of the functional significance of this endosymbiont, which is currently unknown. The proximity of *Blochmannia* to the ant midgut, and the ability to cure *Camponotus* of *Blochmannia* if the host is fed a very complex diet (Boursaux-Eude & Gross, 2000) both suggest that *Blochmannia* may provide the host with essential nutrients (Dasch *et al*., 1984). However, the insect and plant diet of *Camponotus* (Hölldobler & Wilson, 1990) is more complex than the unbalanced diets of other insects with bacteriocyte-associated symbionts (e.g. the phloem diet of aphids and other sap-sucking insects, and the blood diet of tsetse flies). Given the relative complexity of the *Camponotus* diet, it is quite possible that *Blochmannia* provides alternative, non-nutritional functions for the host. Notably, we found that the *Blochmannia* genome is slightly larger than those of the closely related insect endosymbionts *Buchnera* and *Wigglesworthia*. Genes that are present in *Blochmannia* but absent from related symbionts will provide promising candidates for loci with functional significance for this particular host association.

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