In addition to harbouring intestinal symbionts, some animal species also possess intracellular symbiotic microbes. The relative contributions of gut-resident and intracellular symbionts to host metabolism, and how they coevolve are not well understood. Cockroaches and the termite *Mastotermes darwiniensis* present a unique opportunity to examine the evolution of spatially separated symbionts, as they harbour gut symbionts and the intracellular symbiont *Blattabacterium cuenoti*. The genomes of *B. cuenoti* from *M. darwiniensis* and the social wood-feeding cockroach *Cryptocercus punctulatus* are each missing most of the pathways for the synthesis of essential amino acids found in the genomes of relatives from non-wood-feeding hosts. Hypotheses to explain this pathway degradation include: (i) feeding on microbes present in rotting wood by ancestral hosts; (ii) the evolution of high-fidelity transfer of gut microbes via social behaviour. To test these hypotheses, we sequenced the *B. cuenoti* genome of a third wood-feeding species, the phylogenetically distant and non-social *Panesthia angustipennis*. We show that host wood-feeding does not necessarily lead to degradation of essential amino acid synthesis pathways in *B. cuenoti*, and argue that ancestral high-fidelity transfer of gut microbes best explains their loss in strains from *M. darwiniensis* and *C. punctulatus*.

1. Introduction

Animals that feed on plant-based substrates often form symbiotic relationships with microbes. For example, termites harbour gut microbes that perform functions such as nitrogen fixation [1], whereas aphids harbour intracellular *Buchnera* that synthesize essential amino acids (EAA; [2]). Cockroaches and the termite *Mastotermes darwiniensis* are unique in harbouring specialized microbes in their guts as well as the intracellular bacterium (*Blattabacterium cuenoti*) in their fat bodies [3]. These insects provide an opportunity to investigate the evolution and relative contribution of spatially separated symbionts in animal nutrition, a topic that remains poorly understood.
Figure 1. Presence or absence (blue and white boxes, respectively) of genes associated with amino acid synthesis, urea recycling and sulfur assimilation in the genomes of six *B. cuenoti* strains. Green, pink and blue vertical bars indicate wood-feeding, omnivorous and litter-feeding hosts, respectively. Phylogenetic relationships among the six strains were inferred based on an alignment of 13 protein coding genes, and are congruent with host relationships (see the electronic supplementary material). Essential amino acid synthesis pathways are indicated by red horizontal bars, non-essential amino acid synthesis pathways are indicated by black bars. *cysD* in BGE is a pseudogene. See text for host species of each strain.

**Blattabacterium cuenoti** is passed from mother to offspring and is apparently essential for host growth and reproduction (reviewed in [4]). Congruence between phylogenetic trees of host and symbiont indicates that *B. cuenoti* was present in the last common ancestor of cockroaches at least 130 Ma, and has been transmitted in a strictly vertical fashion [5]. The role of *B. cuenoti* has long been thought to involve nitrogen recycling, via metabolism of uric acid [4], which is stored in specialized urocytes adjacent to bacteriocytes harbouring *B. cuenoti*. Surprisingly, the genomes of five *B. cuenoti* strains examined thus far [6–10] do not contain genes encoding known uricolytic enzymes. However, pathways for the conversion of the nitrogenous waste products ammonia and urea into glutamine are a common feature of all genomes, as are other pathways for amino acid and vitamin synthesis.

Of the sequenced *B. cuenoti* strains, two (CPU and MADAR) are from wood-feeding hosts—*Cryptocercus punctulatus*—a close relative of termites [11], and *M. darwiniensis*—the earliest branching lineage of termites [12]. The other three sequenced strains (BGE, BPLAN and BGIGA) are, respectively, from the household pests *Blatella germanica* and *Periplaneta americana*, and the litter-feeder *Blaberus giganteus*—the earliest branching lineage of termites [12]. The other three sequenced strains, two (CPU and MADAR) are from the decaying wood-feeder strain *M. darwiniensis* and BGIGA as sister groups, distant from strains CPU and BGE [12]. Gene loss owing to an intracellular lifestyle is similar to other *B. cuenoti* genomes (590–641 kb), and is a result of gene loss associated with an intracellular lifestyle [15] (genome sizes of free-living relatives are 3–6 Mb [9]). Gene loss owing to an intracellular lifestyle. Gene content in strain BPA’s genome is highly similar to that in other *B. cuenoti* genomes (590–641 kb), and is a result of gene loss associated with an intracellular lifestyle [15] (genome sizes of free-living relatives are 3–6 Mb [9]). Gene loss owing to an intracellular lifestyle. Gene content in strain BPA’s genome is highly similar to that in other *B. cuenoti* genomes (see the electronic supplementary material), suggesting their last common ancestor had a highly reduced genome [6–10]. Phylogenetic analysis placed strains BPA and BGIGA as sister groups, distant from strains CPU and MADAR (see figure 1 and electronic supplementary material).

3. Results and discussion

(a) Genome features and phylogenetic position of str. BPAA

Str. BPAA’s genome (GenBank accession no. AP012548) consists of a single circular genome of 632 490 bp. This small size is similar to other *B. cuenoti* genomes (590–641 kb), and is a result of gene loss associated with an intracellular lifestyle [15] (genome sizes of free-living relatives are 3–6 Mb [9]). Gene loss owing to an intracellular lifestyle. Gene content in strain BPA’s genome is highly similar to that in other *B. cuenoti* genomes (see the electronic supplementary material), suggesting their last common ancestor had a highly reduced genome [6–10]. Phylogenetic analysis placed strains BPA and BGIGA as sister groups, distant from strains CPU and MADAR (see figure 1 and electronic supplementary material).

(b) Nitrogen metabolism and maintenance of amino acid pathways in str. BPAA

Similar to other *B. cuenoti* strains, str. BPAA lacks known uricolytic pathway enzymes such as uricase and xanthine

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dehydrogenase. Such enzymes are common in the genomes of other Bacteroidetes, but appear to have been lost in the ancestor of B. cuenoti [9]. Two alternatives have been proposed for uric acid breakdown in the absence of these two enzymes: host enzymes, and metabolism by gut microbiota [1]. Sabree et al. [9] have argued for the latter, based on the fact that antibodies are known to reduce uricolytic activity, and the consistent isolation of uricolytic gut bacteria from cockroach and termite guts (see [9]). They propose that B. cuenoti’s role may be to use uric acid breakdown products (urea and ammonia) for amino acid production. The genomes of all examined strains encode enzymes for recycling urea and ammonia into glutamate.

Gene complements associated with nitrogen metabolism in str. BPAA are most similar to those of related strains from non-wood-feeding hosts, rather than other wood-feeding hosts (figure 1). In strains CPU and MADAR, genes encoding pathways for the production of six of ten EAAs are absent, with one additional pathway (lysine) being interrupted [6,10]. Str. BPAA’s genome encodes intact pathways for all EAAs, with the exception of methionine, which is interrupted in all known B. cuenoti strains. With regard to non-EAA pathways, str. BPAA is similar to all other B. cuenoti strains in missing glutamine and asparagine pathways (figure 1), and missing steps in serine, proline and methionine, and cysteine pathways. It may import these amino acids from the host, or use alternative mechanisms for producing them.

Str. BPAA’s genome content indicates that host wood-feeding does not necessarily lead to the loss of EAA pathways and is inconsistent with the hypothesis that EAA pathway loss occurred owing to the presence in rotting wood of microbes that provided an adequate supply of EAAs to an ancestral host [6]. The lineage leading to P. angustipennis has been feeding on rotting wood for at least 15 Myr, as evidenced by this habit in essentially all members of the Panesthiinae, and the age of this subfamily inferred from biogeographic evidence [13]. The presence of EAA pathways in str. BPAA is, therefore, not due to a recent shift to wood-feeding from a non-wood-feeding ancestor.

An alternative explanation for the absence of EAA pathways in strains CPU and MADAR is that ancestral gut symbionts, and the fidelity of symbiont transfer between parents and offspring, may have been responsible for the relaxation of selection upon their maintenance. Cryptocercus punctatus and M. darwiniensis both harbour unique cellulo-lytic parabasalid and oxymonad protists found nowhere else in nature [16]. It is possible that bacterial symbionts present in ancestral gut flagellates (or other ancestral members of the gut microbiota) synthesized EAA and vitamins [17], supplanting the need for their production by B. cuenoti.

All cockroaches examined contain gut microbiota that potentially provide their hosts with EAAs [18]. Why is it that only in strains CPU and MADAR we see loss of essential pathways? One possibility is that a consistent source of EAAs may depend upon reliable transfer of specific hindgut microbes, such that coevolution can occur. The social behaviour of C. punctatus and M. darwiniensis means that hindgut microbiota are reliably transferred between parent and offspring (e.g. via proctodeal trophallaxis), and indeed such transfer has been occurring with high fidelity for over 130 Myr in some cases [19,20]. In non-social taxa, gut microbes are obtained in a more random fashion from environmental sources [10], though particular bacterial groups are shared between distantly related taxa [18]. This may reduce the potential for coevolution between gut microbiota and host. Although P. angustipennis is a gregarious species, there is no evidence for direct transfer of symbionts between parents and offspring via trophallaxis, as is known in the social species C. punctatus and M. darwiniensis. Further studies of B. cuenoti from cockroach hosts that have high-fidelity transfer of symbionts are required to examine whether genome degradation is associated with this trait.

We thank Tatsuya Kitazume for Illumina sequencing, Joji M. Otaki and Nancy Moran for critical comments, and KAKENHI 18380045, 17405025, and NIBB Cooperative Research Program no. 11-723 for financial support. N.L. supported by ARC.

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