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Tetraponera ants have gut symbionts related to nitrogen-fixing root-nodule bacteria

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Some *Tetraponera* ants (Formicidae, Pseudomyrmecinae) subsist almost entirely on amino acid deficient honeydew secretions of pseudococcids and harbour a dense aggregation of bacterial symbionts in a unique pouch-shaped organ at the junction of the midgut and the intestine. The organ is surrounded by a network of intruding tracheae and Malpighian tubules, suggesting that these bacteria are involved in the oxidative recycling of nitrogen-rich metabolic waste. We have examined the ultrastructure of these bacteria and have amplified, cloned and sequenced ribosomal RNA-encoding genes, showing that the ant pouch contains a series of close relatives of Flavobacteria and *Rhizobium*, *Methylobacterium*, *Burkholderia* and *Pseudomonas* nitrogen-fixing root-nodule bacteria. We argue that pouch bacteria have been repeatedly 'domesticated' by the ants as nitrogen-recycling endosymbionts. This ant-associated community of mutualists is, to our knowledge, the first finding of symbionts related to root-nodule bacteria in animals.

Keywords: symbiosis; nitrogen recycling; eubacteria; *Tetraponera*; 16S rDNA; Formicidae

1. INTRODUCTION

Many insects depend on symbiotic microorganisms for essential nutrients (Douglas 1998; Moran & Telang 1998). Some symbionts deliver necessary enzymes for the digestion of plant cellulose (Martin 1992; Bacci *et al.* 1995), whereas others recycle nitrogen from metabolic waste (Potrikus & Breznak 1981; Cochran 1985; Sasaki *et al.* 1996; Douglas 1998), or produce essential amino acids for their hosts (Sasaki & Ishikawa 1995; Douglas 1998).

Colonies of the ant *Tetraponera binghami* (Formicidae, Pseudomyrmecinae) live exclusively in the hollow internodes of bamboo. These nest cavities contain numerous *Kermicus wroughtoni* pseudococcids, providing the ants with honeydew (Buschinger *et al.* 1994). Workers are rarely seen foraging outside their nests, so that *T. binghami* ants are believed to depend almost entirely on this amino acid deficient honeydew diet. The ants also harbour a dense aggregation of bacterial symbionts in a unique pouch-shaped organ at the junction of the midgut and the intestine (Billen & Buschinger 2000). The ant pouch is surrounded by a network of intruding tracheae and Malpighian tubules, transporting ample oxygen and nitrogen-rich metabolites to the tip of the pouch. The epithelium at the base of the pouch is specialized for absorption (Billen & Buschinger 2000), but the contorted shape of this section ensures that gut fragments cannot enter. The overall morphology (Billen & Buschinger 2000) of the pouch thus suggests that the bacterial symbionts are involved in the recycling of nitrogen-rich metabolic waste (Billen & Buschinger 2000), but there has, to our knowledge, been no direct evidence supporting this inference.

We sequenced the 16S rRNA gene from the bacteria in the ant pouch and compared this sequence with known bacterial sequences to identify the symbionts. We further show that the pouch organ is present in all castes of *T. binghami* and provide additional morphological and ultrastructural evidence for the taxonomic identity of the bacterial community in the pouch organ.

2. MATERIAL AND METHODS

(a) *Molecular analysis*

Bacterial pouches were dissected from five workers of *T. binghami* and externally sterilized by immersion in 70% ethanol, two rinses in double-distilled water and exposure for 20 min to UV radiation (250 nm). After immersion in liquid nitrogen, the pouches were ground and boiled for 20 min in 50 µl of a 10% Biorad Chelex 100 solution. The samples were centrifuged and stored at –20 °C until use. To prevent any contamination, the pre-extraction treatment and all DNA extraction procedures were performed under sterile conditions in a laminar flow hood.

Bacterial 16S rRNA-encoding sequences were amplified from a pooled sample using the universal primer pair 5′-TTGGGA TCCAGAGTTTGATCATCATGGCT-CAGAT-3′/5′-CACGA ATTCTACCTTGTTACGACTTCACCCC-3′ (Schröder *et al.* 1996), which is complementary to conserved regions characteristic of eubacterial ribosomal RNA genes (Amann *et al.* 1991, 1995). The resulting polymerase chain reaction products were cloned (Invitrogen Topo TA Cloning Kit), the DNA was purified (GFXTM, Amersham Pharmacia Biotec Inc.) and 10 positive transformants were sequenced using LI-COR chemistry and IRD-labelled M13 primers. A consensus sequence was calculated for sequences greater than 98% identical, so that minor Taq polymerase reading failures were corrected. This resulted in five consensus sequences. The 16S rRNA sequences of these five symbionts were submitted to GenBank (figure 2).

The partial 16S rRNA sequences were aligned using the CLUSTAL W program (Thompson *et al.* 1994) followed by manual

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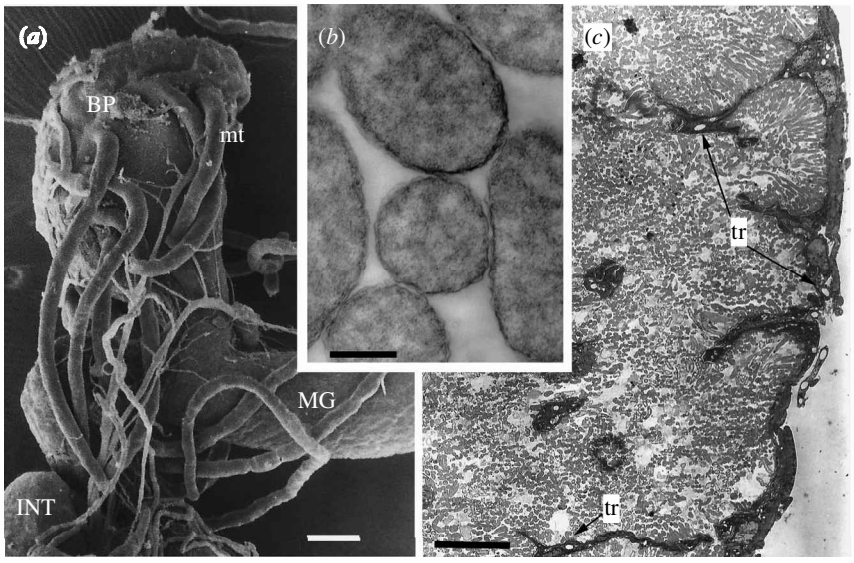

Figure 1. Electron micrographs of the bacterial symbionts and the gut pouch of a worker of the ant *Tetraponera binghami*. (*a*) Scanning electron micrograph showing the bacterial pouch (BP), Malpighian tubules (mt) and tracheal air supply. The intestine (INT) and midgut (MG) are also visible (scale bar, 100 μm). Reprinted from J. Billen & A. Buschinger 2001, with permission. (*b*) Detail (transmission electron micrograph (TEM)) of the bacterial contents of the pouch lumen (scale bar, 0.5 μm). (*c*) Overview (TEM) showing typical bacterial aggregations associated with the inner epithelium (right hand side of the figure) at the tip of the pouch. Cross-sections through tracheae (tr) are also indicated (scale bar, 10 μm).

refinement. The resulting dataset included 1517 nucleotide sites. The sequences were aligned to a subset of closely related reference sequences (29 sequences found in GenBank), including the two closest relatives of each pouch symbiont, a number of close relatives with sequence similarities greater than 95% (without discriminating between nitrogen-fixing and other bacteria) and a number of sequences representing taxa with known ecological features and sister genera of *Rhizobium*. The photosynthetic filamentous bacterium *Chloroflexus aggregans* (EMBL D32255) was included in the analysis as an outgroup sequence. The phylogenetic analysis was done using MEGA version 2.1 (Kumar *et al.* 2001). A neighbour-joining tree was calculated using a Kimura 2-parameter-based distance with pairwise deletion of insertions and deletions. Bootstrap analysis testing the reliability of the clades in the phylogeny included 1000 pseudoreplications. Additional maximum-parsimony and maximum-likelihood analyses were done using PHYLIP 3.5 (Felsenstein 1993), each including 500 pseudoreplications. There were no major changes in tree topology using parsimony, maximum-likelihood and neighbour-joining methods. We have chosen to present a neighbour-joining tree and to summarize small deviations obtained by alternative methods in the text.

(b) *Morphology and ultrastructure*

A total of 15 workers, four males and four queens of *T. binghami* were dissected to investigate the consistent presence of the bacterial pouch in the sexes and female castes. Procedures for transmission electron microscopy and scanning electron microscopy were as previously described (Billen & Buschinger 2000). Transmission electron micrographs representing five pouches from workers were used to determine the morphology and size ranges of the bacteria present. The smallest and largest diameters were measured for at least 50 bacteria per pouch and cell shape and membrane structure were determined.

3. RESULTS

The pouch organ was present in all workers ($n = 15$), males ($n = 4$) and queens ($n = 4$) of *T. binghami* that were examined, and had the same overall morphology in all castes (figure 1*a*). All pouch organs were surrounded and partially penetrated by a dense network of tracheae. Moreover, the ant's Malpighian tubules typically open into the pouch. The highly specialized pouch organ described by Billen & Buschinger (2000) is thus not restricted to the workers, but also occurs in the males and females of *T. binghami*. The lumen of the pouch is filled with a dense aggregation of bacteria (figure 1*c*), which are rod-shaped, 1.4–6.0 μm long and 0.5–0.7 μm wide and typically have a double membrane structure and a granular appearance of their cellular content (figure 1*b*).

We have obtained and analysed the almost complete 16S rDNA sequences (1517 nucleotide sites) of the pouch symbionts of *T. binghami*. Comparison of these sequences with bacterial 16S rDNA sequences available in GenBank (figure 2) showed that five distinct taxa were present. Four were Proteobacteria and were statistically sufficiently supported (figure 2) to assign them unambiguously to the genera *Rhizobium* and *Methylobacterium* (α-Proteobacteria), *Burkholderia* (β-Proteobacteria) and *Pseudomonas* (γ-Proteobacteria). Nitrogen fixation has previously been demonstrated for each of these genera (Yadav *et al.* 1999; Ghiglione *et al.* 2000; Minerdi *et al.* 2001; Sy *et al.* 2001). This proteobacterial branch in the phylogeny was also obtained with maximum-likelihood and maximum-parsimony analysis, which is not surprising given the high bootstrap support values both for the external and internal branches in the neighbour-joining analysis (figure 2). The fifth sequence fell within the Flavobacteria, but was only 88% similar to the closest available *Flavobac-*

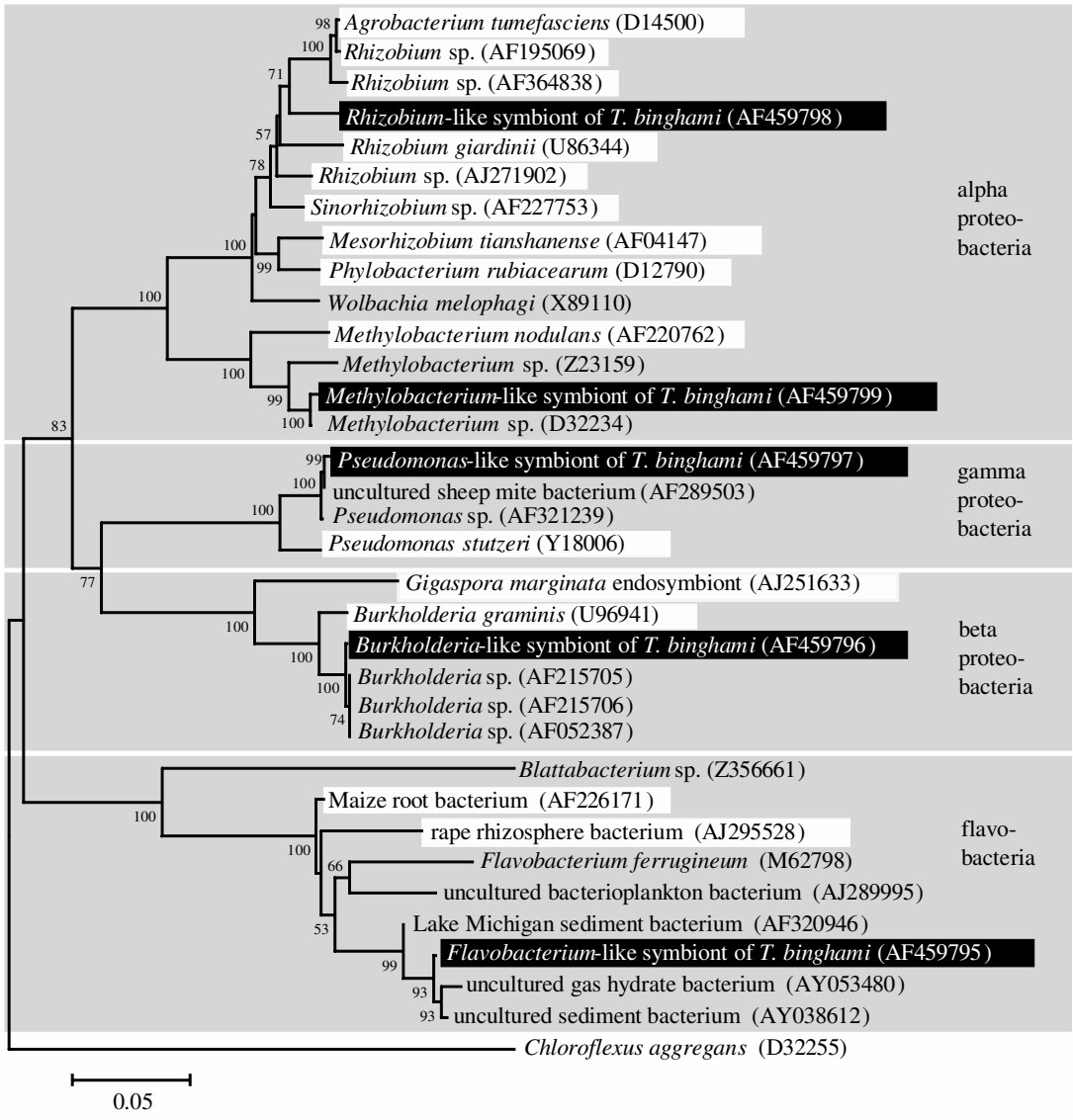

Figure 2. Rooted neighbour-joining tree showing the symbionts of *Tetraponera binghami*, their closest relatives and reference strains, based on aligned partial 16S rRNA-encoding sequences. Distances were calculated using the Kimura 2-parameter model in MEGA 2.0 (the scale bar represents a distance of 5%). Bootstrap support values (1000 pseudoreplicates) above 50% are given next to the branches. Sequences generated in this study are indicated in white on a black background. Names of strains are followed by their GenBank accession numbers. Plant-associated symbiotic bacteria are indicated by a white background. Note that *Wolbachia melophagi*, a gut symbiont of *Melophagus* sheep keds (wingless bloodsucking flies), is unrelated to intracellular *Wolbachia pipientis* symbionts of arthropods (Birtles 1994).

terium sequence and clustered mainly with bacteria of unclear taxonomical identity (figure 2). Using maximum-parsimony analysis, some of the internal branches were reversed, so that the *Flavobacterium*-like symbiont came out even closer to the plant-associated taxa 'rape rhizosphere bacterium' and 'maize root bacterium'. Using maximum-likelihood analysis, the topology of the 'Flavobacterial branch' was the same as in the neighbour-joining analysis. The ultrastructure of the bacteria (figure 1*b*) matched the known morphological ranges (Krieg & Holt 1984) for each of these five taxa. It is unknown whether Flavobacteria have nitrogen-fixing capacities, but two bacteria with GenBank sequences closely related to this ant-pouch symbiont are known to be associated with plant roots (Chelius & Triplett 2000; Macrae *et al.* 2000). Most if not all sequences obtained from the ant pouch bacteria thus have plant-associated nitrogen-fixing bacteria as their closest relatives (indicated by a white background in figure 2). In addition, representatives of three of the five known taxa (Moulin *et al.* 2001) of root-nodulating bacteria (the *Rhizobium*, *Methylobacterium* and *Burkholderia* branches in the phylogeny) coexist in this single specialized ant organ.

4. DISCUSSION

Insects generally excrete between 55 and 80% of their nitrogen as uric acid or its primary degradation products allantoin and allantoic acid (Bursell 1970), or as ammonia (in aphids; Douglas 1998). These nitrogen losses are often a major constraint on growth and reproduction (Joern & Behmer 1997), so that symbioses for recycling nitrogenous waste products have evolved in a number of lineages, e.g. yeasts in planthoppers (Sasaki *et al.* 1996), intracellular flavobacteria (*Blattabacterium* sp.) in cockroaches

(Cochran 1985) and actinomycete gut bacteria in termites (Potrikus & Breznak 1981). However, compared with these previously known cases, the ant–bacterial symbiosis discovered in the present study is unique because (i) the ants have evolved a highly specialized organ for maintaining their bacterial symbionts. The fact that the pouch also occurs in males is remarkable, as ant males are generally short-lived and often emerge with all the body reserves that they need to disperse and mate (Boomsma & Isaaks 1985). This suggests that young adult *T. binghami* males are fed by trophallaxis of nitrogen-poor honeydew in the same way as workers and queens. (ii) The bacteria that they culture in their gut pouch are not related to any of these symbionts of other insects; the eukaryotic planthopper yeasts belong to a different kingdom (Woese *et al.* 1990) and the termite actinomycetes belong to a eubacterial lineage (Gram-positive bacteria with high guanine and cytosine content; Woese (1987)) that is unrelated to the bacteria in the *Tetraponera* pouch. *Blattabacterium* associates of cockroaches do not cluster together with the pouch Flavobacteria (figure 2). (iii) The pouch bacteria are close relatives of root-nodule bacteria that have never before, to our knowledge, been shown to enter associations with animals.

The exact mechanisms by which the pouch bacteria are involved in the ant's nitrogen metabolism remain to be studied, but the structural connection with Malpighian tubules and tracheae and the phylogenetic proximity to nitrogen-fixing bacteria suggest that they must be involved in such a symbiotic function. We suggest that the *Tetraponera* gut bacteria may reintegrate metabolic nitrogen waste of the ants into their own metabolic pathways and release amino acid precursors in the bacterial pouch, where they can be absorbed by the ant's proximal cylindrical epithelium. Alternatively, and not mutually exclusively, the pouch bacteria might combine dinitrogen gas from the tracheal air supply with metabolites provided by the ant to synthesize amino acid precursors.

The fact that *Tetraponera* ants use multiple close relatives of root-associated nitrogen-fixing bacteria suggests that these symbionts have convergently evolved enzymatic pathways to transform nitrogenous metabolites into amino acid precursors, i.e. a similar enzymatic machinery known to occur in unrelated gut bacteria of cockroaches and termites, but derived from different enzymatic pathways known to be operational in root-nodule bacteria. The most striking result is that the gut pouch symbiosis between *Tetraponera* ants and nitrogen-fixing bacteria has evolved 4–5 times by independent host-jumping between biological kingdoms. Alternatively, the ant and plant hosts may have acquired their symbionts independently from free-living nitrogen-fixing bacteria. The latter scenario, however, seems implausible, since no known free-living nitrogen-fixing bacteria seem to be related to the ant symbionts that we characterized. An entire community of relatives of root-nodule bacteria is apparently able to coexist in the gut pouch of these *Tetraponera* ants, which suggests that they either have segregated functional niches within the pouch, or that their densities are actively and differentially regulated by the ants.

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