The taxophysiological paradox: changes in the intestinal microbiota of the xylophagous cockroach Cryptocercus punctulatus depending on the physiological state of the host

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Summary. The phylogenetic relationships of symbiotic bacteria from the xylophagous cockroach Cryptocercus (Cryptocercidae, Blattaria) were compared to those described in previous reports in lower termites. The 16S rDNA bacterial genes were PCR-amplified from DNA isolated from the entire hindgut using Bacteria-selective primers, and the 16S rDNA ampli-cons were cloned into Escherichia coli. The changes in the gut microbiota of Cryptocercus under three physiological conditions, “active,” “fasting,” and “dead,” were studied. Analysis of the active-clone library revealed 45 new phylotypes (clones sharing >97% sequence identity were grouped into the same phylotype) from 54 analyzed clones. The clones were affiliated with the phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, Synergistetes, Verrucomicrobia, and candidate phylum Termite Group 1 (TG1). Clones belonging to Spirochaetes, Bacteroidetes, and TG1 phyla clustered with previously reported sequences obtained from the guts of several termites, suggesting that these clones are common constituents of the intestinal microbiota of lower termites and Cryptocercus. In the fasting-clone library, 19 new phylotypes, from 49 clones studied, were distinguished. The new phylotypes were affiliated with the phyla Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Spirochaetes, Synergistetes, and the candidate phylum TM7. Finally, in the dead-clone library, 24 new phylotypes from 50 studied clones were found. The new phylotypes were affiliated with the phyla Firmicutes, Actinobacteria, and Proteobacteria. Thus, from active, to fasting, to dead physiological states, a decrease in the number of phyla present in the whole microbial gut was evident. However, in the dead physiological state, each phylum conserved contained more new phylotypes. This poses a taxophysiological paradox, because a stable, active physiological state of Cryptocercus—due to a continuous input of wood—supports a higher diversity of bacterial phyla, probably necessary to maintain a sharp O₂–H₂ gradient in the gut. By contrast, in the dead state, nutrient input is limited to the residual gut microbiota that is killed by the newly oxic environment, thus providing a food source for other, aerobic or facultative anaerobic bacteria. This results in an increase in the internal diversity of the few remaining phyla. [Int Microbiol 2009; 12(4):227-236]

Keywords: Cryptocercus punctulatus · Bacteroidetes · spirochetes · termite group TG1 · whole intestinal microbiota · host physiological state · coevolution

Introduction

Termites, cockroaches, and mantids form a well-established lineage of insects, the Dictyoptera. Six families of termites (order Isoptera) share with the xylophagous (wood-feeding) cockroaches (family Cryptocercidae, order Blattaria) the unusual ability to degrade lignocellulosic plant material.
Members of the genus Cryptocercus are subsocial, wood-feeding cockroaches that inhabit temperate forests of the northern hemisphere, living in extensive galleries excavated within decomposing logs. At present, nine species in the genus are recognized worldwide: two in eastern Eurasia, two in south-western China, and five in the USA. The distribution of C. punctulatus extends throughout western Virginia, and Pennsylvania. The ecological niche for the five Nearctic Cryptocercus species lies within a small range of the spectrum of annual mean temperatures and precipitation: annual mean temperatures from 6 to 17°C, and annual mean precipitation from 140 to 470 cm [20].

Termites evolved from omnivorous cockroach ancestors and are characterized by a reproductive system in which their oothecae are formed internally and by different degrees of intraspecific coprophagy (i.e., feces-eating) and gregariousness. These three characteristics have allowed specifically coevolved gut symbioses, because parental contact allows the transmission of a stable microbial assemblage from generation to generation by protodeal trophallaxis (i.e., nutrient transfer from the anus of one individual to the mouth of another). Since the intestinal contents pass between individuals, there are very few differences in the hindgut microbial populations of members of a single colony. The key evolutionary shift appears to have been the acquisition of mutualistic cellulolytic protists (flagellates) in both the termite ancestor and Cryptocercus, which allowed the cockroaches to become wood feeding (although this shift is found not only in the Cryptocercus clade, but also in other cockroaches, such as Parasphaeria boleiriana) [2,21].

The symbiotic protists inhabiting the gut of lower termites and wood-feeding cockroaches belong to the orders Trichomonadida, Cristamonadida, Hypermastigida, and Oxymonadida. Hypermastigids are unique in nature, as they are found only in lower termites and Cryptocercus. It is remarkable that Cryptocercus cockroaches retain more diverse flagellate species than any extant termite species [7]. Symbiotic flagellates were established in an ancestor common to Cryptocercus and lower termites, were vertically transmitted to their offspring, and subsequently became diversified to distinct levels, depending on the host and the symbiont lineages [32].

Hypotheses describing the relationships among termite and Cryptocercidae groups have provoked controversy. Recently, molecular phylogenetic analyses of the hosts have confirmed the sister-group relationship between Cryptocercus and termites. Termites can be considered as social cockroaches and may not constitute a separate order (Isoptera) from cockroaches (Blattodea). Instead, it has been proposed that termites should be treated as a family (Termitidae) of cockroaches [19].

Bacterial endosymbionts, found in many insect orders, provide a potential molecular clock for estimating divergence times among taxa [11]. Cockroaches (including Cryptocercus) and lower termites (e.g., Mastotermes darwiniensis) harbor endosymbiotic bacteria, e.g., Blattabacterium spp., within specialized cells—the bacteriocytes of the fat body—that are transferred vertically via the eggs. Based on the analysis of small subunit ribosomal genes (16S rDNA), Blattabacterium belong to the class Flavobacteria in the phylum Bacteroidetes [23]. There is a close relationship between endosymbionts from Mastotermes and Cryptocercus. The majority of the Blattabacterium spp. sequences appear to have undergone similar rates of evolution following divergence from a common ancestor, and an estimate of this rate was determined based on early Cretaceous fossils. Thus, modern cockroaches are thought to have radiated at some time between the late Jurassic and early Cretaceous, ca. 140 million years ago [22].

Molecular approaches for the detection and characterization of microbes have resulted in a dramatic change in our understanding of microbial diversity. For example, the analysis of 16S rRNA genes has allowed the microbial community of insects to be defined. The diversity of Bacteria from termite hindguts is extensive, with up to 15 Bacteria phyla described thus far. Among the phyla present in lower termite guts are the novel candidate phyla of termite groups 1 (TG1), TG2, and TG3, Spirochaetes, Bacteroidetes, low-GC gram-positive bacteria, and Proteobacteria [1,14,15]. In the present study, bacterial diversity obtained from the whole gut of the wood-feeding cockroach C. punctulatus was analyzed. Using 16S rRNA genes sequences, we compared the cockroach phylotypes with previously published sequences from termites and other C. punctulatus. We also analyzed the phylotypes in the gut microbiota of the cockroach under three physiological conditions, active, fasting, and dead. This approach allowed us to follow the changes in the number of phyla identified in each state, and also in the number of phylotypes belonging to each phylum.

Materials and methods

Cockroaches. Cryptocercus punctulatus was collected by Michael Dolan (University of Massachusetts at Amherst, MA, USA). Seven individuals were sent to our laboratory in Barcelona, Spain. During transport the cockroaches were maintained in tubes with wood at room temperature. Immediately after their arrival in the laboratory, three Cryptocercus individuals were used to determine the whole gut microbiota in the active physiological state. Two Cryptocercus were left in a tube without wood at 15°C for 15 days, to analyze their gut microbiota in the fasting physiological state. The remaining two Cryptocercus individuals were kept in a fasting state at 15°C until death.
**Isolation of bacterial DNA.** The entire hindgut of the insect was removed. The tissue was homogenized using a Mini-beadbeater (BioSpec Products, Bartlesville, OK, USA) with 0.1-mm glass beads, and bulk DNA was extracted by several washings with phenol-chloroform [1].

**PCR and clone libraries.** PCR was carried out with the Bacteria-specific primer pair 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1391R (5′-GACGGGCGGTGWTGTRCA-3′). To selectively amplify spirochetal 16S rDNA, a spirochete-selective reverse primer Spiro1483R (5′-GTTACGACTTCACCTCTCCT-3′) with a universal forward primer 8F was used. PCR amplification was carried out as described in [1]. Four clone libraries were constructed, three of them (corresponding to each physiological state, i.e., active, fasting, and death) using bacterial universal primers, and the fourth using primers specific for spirochetes.

**Sequencing and phylogenetic analysis.** Purified PCR products were sequenced and analyzed as described in [1], with some modifications. The primer used for Bacteria 16S rRNA sequencing was 515F (5′-GTGCCAGCMGCCGCCGCGGTAA-3′), whereas for spirochetes it was M13F (5′-GTAAAACGACGGCCAG-3′). PCR mixtures consisted of 2 μl PCR product, 1 μl primer at 5 μM, 1 μl BigDye, and 3 μl BigDye solution in a final volume of 20 μl. Cycle sequencing was done using an ABI 9700, with one cycle at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 s, and annealing 55°C for 5 s and extension at 60°C for 4 min. Sequencing reactions were run on an ABI 3100 DNA sequencer. Partial 16S rRNA sequences were compared to known sequences in GenBank with the advanced gapped BLAST (basic local alignment search tool) algorithm. Phylogenetic analyses were carried out with MEGA version 2.1. The dendrogram was constructed using the neighbor-joining algorithm and the Kimura 2-parameter distance estimation method. One thousand bootstrap trees were generated, and bootstrap confidence levels were determined using the MEGA 2.1 program. Chimeric sequences were identified according to the Chimera check program in the Ribosomal Database Project II.

**Nucleotide sequence accession numbers.** The partial 16S rRNA gene sequences of clones representing novel phylotypes defined in this study and published sequences are available for electronic retrieval from the GenBank nucleotide sequence databases (accession nos. GU434646–GU434661).

**Results**

**Bacterial community in the whole gut of Cryptocercus punctulatus.** Analysis of the 16S rRNA clone library provided a relative census of the bacterial community of the whole gut of Cryptocercus in the active physiological state. Based on the 16S rRNA from 54 analyzed clones, 45 new phylotypes were distinguished (clones sharing >97% sequence identity were grouped into the same phylotype). The clones were affiliated with the phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, Synergistetes, Verrucomicrobia, and candidate phylum Termite Group 1 (TG1) (Fig. 1). Firmicutes-Clostridia (14 new phylotypes), Bacteroidetes (11 new phylotypes), and Proteobacteria (8 new phylotypes, of which 6 corresponded...
to Deltaproteobacteria) were the most abundant bacteria in the active physiological state of Cryptocercus.

The phylotypes obtained in this study were compared with reported sequences from termites and other Cryptocercus. Spirochaetes, Bacteroidetes, and TG1 form special phylogenetic lineages that coevolved with their termite host [1,14,29,35]. Among the 20 clones (clone library with specific primers for spirochetes) analyzed from the whole gut of Cryptocercus, nine were attributable to the spirochetes. Clone Cp-LO43 was detected three times and Cp-LO11 twice. These spirochetal phylotypes fell into two previously defined clusters, designated Treponema-termite Clusters I and II (Fig. 2). Treponema-termite Cluster I comprises both ectosymbiotic spirochetes attached to protists and free-swimming gut spirochetes [17,26]. Based on the affiliation with reported sequences, phylotypes Cp-LO8, Cp-LO11, and Cp-LO43 were considered ectosymbiotic spirochetes attached to protists because they grouped with phylotypes NkS-Oxy70 (protist Oxymonas, from the lower termite Neotermes koshunensis) and NkS-Dev1 (protist Devescovina, also from N. koshunensis). Phylotypes Cp-LO10 and Cp-LO13 grouped with several sequences previously reported as belonging to Treponema-termite Cluster II (Fig. 2). Members of Cluster II are ectosymbiotic spirochetes of oxymonad protists; however, not all ectosymbiotic spirochetes are in Cluster II [1,17] as phylotype Cp-LO2 was apparently outside the Treponema-termite clusters. Treponema-termite Cluster III is related to the genus Spirochaeta [1,14]. The 16S rRNA gene sequence of strain SPN1 also belonged to the genus Spirochaeta but it was not closely related to sequences of Cluster III (Fig. 2). In contrast to all other known described spirochete species, strain SPN1 has a coccoid morphology and is immotile [8].
From the 54 analyzed clones comprising the active clone library, 11 new phylotypes related to the phylum Bacteroidetes were detected. Clones Cp-A37, Cp-A62, and Cp-A98 were detected twice. Among the 49 studied clones from the fasting clone library, 4 new phylotypes were detected. Phylotype Cp-F63 was detected twice. These results were compared to those obtained by Ohkuma et al. [30], who classified the Bacteroidetes in five clusters (I–V) (Fig. 3). Phylotypes Cp-A33, Cp-A37, Cp-A62, Cp-A82, Cp-A98, Cp-A209, Cp-F50, and Cp-F58 clustered with group V, made
up exclusively of sequences from uncultured strains obtained from termite guts. Phylotypes Cp-A12, Cp-A87, and Cp-F56 belonged to cluster IV, as was the case for those phylotypes previously described from different species of the protists Barbulanympha and Urinympa, both of which occur exclusively in the gut of Cryptocercus. Cluster IV also includes sequences identified from ectosymbionts on the protist Mixotricha paradoxa, an inhabitant of the lower termite Mastotermes darwiniensis [27]. Phylotype Cp-A62 was related to the Bacteroidetes phylotype Rs-N74, which was obtained from the gut of the lower termite Reticulitermes speratus. Thereafter, Rs-N74 was proposed to be a representative of a novel genus and species, ‘Candidatus Symbiothrix dinenymphae’ [16].

The candidate phylum TG1 [14] is highly diverse and widespread in the environment, with high numbers of TG1 bacteria also detected in the hindguts of lower termites and wood-feeding cockroaches. These bacteria are intracellular symbionts of flagellates. The name proposed for these symbionts is ‘Endomicrobia’ [13]. Phylotype Cp-A12 (from active state) was related to ‘Endomicrobia’ (Fig. 4).

**Physiological states of Cryptocercus punctulatus: active, fasting and dead.** Analysis of the clone library of 16S rRNA from the bacterial community of the whole gut of Cryptocercus in the fasting physiological state identified 19 new phylotypes among the 49 clones studied. These new clones were affiliated with the phyla Firmicutes (2 different clones from a total of 3), Bacteroidetes (4 different clones from 5 clones), Proteobacteria (7 different clones from 35 clones), Actinobacteria (2 different clones), Spirochaetes (1 different clone), Synergistetes (2 different clones), and the candidate phylum TM7 (1 different clone).

In the analysis of the clone library derived from dead Cryptocercus, 24 new phylotypes, from 50 clones studied, were distinguished. The new clones were affiliated with Firmicutes (7 different phylotypes from 12 clones), Proteobacteria (6 different clones from 16 clones), and Actinobacteria (11 different clones from 22 clones) (Fig. 5).

From the active to fasting to dead physiological state, there was a clear decrease in the number of phyla represented in the whole microbial gut. Firmicutes, Proteobacteria, and Actinobacteria were the only phyla present in all three physiological states. However, the internal constituents of each phylum changed. For example, among the Firmicutes, Clostridia was the major class found in the active state, whereas in the fasting and dead states its numbers decreased significantly. In the dead state, the predominant Firmicutes were the class Bacilli (orders Bacillales and Lactobacillales). Among the Proteobacteria, Deltaproteobacteria was the major class found in the active state, Betaproteobacteria in the fasting state, and Alphaproteobacteria in the dead state.
The relative abundance of the Bacteroidetes was maintained in the active and fasting states but in the dead state members of this phylum was undetectable. By contrast, the relative abundance of Actinobacteria increased significantly in the dead state. In general, the gut microbiota changed from strict anaerobes in the active state of the host to facultative or aerobic bacteria in its dead state (Fig. 5).

**Discussion**

**Coevolution of Cryptocercus punctulatus and its gut bacterial community.** To understand the biology of insects it is necessary to consider their symbiotic microorganisms. The gut microbiota has evolved as a valuable metabolic resource for some insects feeding on suboptimal diets, such as wood-eating lower termites and the xylophagous cockroach *Cryptocercus*. The diversity of such microbiota depends, in part, on several factors: the variety of specialized structures present in the gut, the effect of pH, redox conditions, and the type of food ingested [6,24]. An indigenous biota is one that is present in all individuals, colonizes the gut habitat, and is maintained in “stable” climax communities. Clearly, the microbiota detected in the guts of lower termites and *Cryptocercus* greatly differs from that in the environment (e.g., soil) of these insects [6].

While PCR amplification together with the sequencing of small-subunit rRNA genes to investigate gut microbiota has several hindrances, such as PCR errors, PCR bias, and different copy numbers of the gene, this approach is widely accepted as a powerful and essential tool for the investigation of as yet-unculturable microorganisms. Previous studies of the bacterial community in the gut of wood-feeding insects have focused mostly on lower termites. The intestines of wood-feeding lower termites harbor a diverse population of prokaryotes and flagellates that degrade lignin, cellulose, and hemicelluloses to fermentable carbohydrates and are thus indispensable to their termite hosts. The diversity of *Bacteria* from termite hindguts as detected by 16S rRNA analysis is extensive [10,14,15,25]. The relative frequencies of the
clones identified in the present study in Cryptocercus at the phylum level are detailed in Fig. 1. Phylogenetic analysis showed that the clones corresponded to a diverse range of members of the domain Bacteria. All sequences grouped into one of eight bacterial phyla: Proteobacteria, Spirochaetes, Bacteroidetes, Firmicutes, Actinobacteria, candidate phylum TG1 (‘Endomicrobia’), Verrucomicrobia, and Synergistetes. The results imply that these phyla are common constituents of the gut microbiota in lower termites and Cryptocercus. Phylogenetic analysis of the clones (Spirochaetes, Bacteroidetes and TG1) detected in our study clustered sequences previously reported from several termite guts (Figs. 2–4).

While lower termites and Cryptocercus support a characteristic community of gut protists, many protist species are not necessarily restricted to one termite species [7]. Furthermore, many protist species are simultaneously associated with different bacterial ectosymbionts, and protists also contain endosymbionts [31]. The coevolutionary relationships of ectosymbiotic spirochetes that attach to the cell surfaces of protists in the termite gut are complex. A single protist cell usually harbors multiple spirochete species, and different protist genera share the same spirochete species [17,26]. Spirochetes detected in Cryptocercus grouped with Treponema-termite Clusters I and II (Phylotypes Cp-Lo8, Cp-Lo10, Cp-Lo11 and Cp-Lo43), which constitute separate phylogenetic lineages of spirochetes found in the gut of termites (Fig. 2).

Bacteroidetes are involved in associations with a wide variety of gut protist species as either intracellular endosymbionts or surface-attached ectosymbionts [16,27]. The close relationships of the ectosymbionts between the related protist species suggest that the symbionts were acquired before the diversification of these protist species (clone Cp-A87 related to Barbulanympha protist, and Cp-F50 to Devescovina protist) [5]. The Bacteroidetes ectosymbiont ‘Candidatus Symbiothrix’ genus may be distributed among various termites that harbor the protist Dinenympha. Phylotype Cp-A62 was closely related to phylotype Rs-N74 ‘Candidatus Symbiothrix’ (Fig. 3). Another example of cospeciation is the endosymbionts (Bacteroidetes) from the protist Pseudotrichonympha [28].

‘Endomicrobia’ form a separate line of descent in the bacterial tree. They are part of the TG1 phylum, but are host-specific intracellular symbionts of termite and Cryptocercus gut flagellates [13]. All flagellates studied by different researchers carry a unique phylotype of ‘Endomicrobia,’ which supports the hypothesis that the diversity of ‘Endomicrobia’ in each termite and Cryptocercus guts reflects the diversity of their flagellate hosts. Phylotype Cp-A12 was closely related to published phylotypes from Crypto-
cercus (Fig. 4), which could share similar protist populations. ‘Endomicrobia’ phylotypes associated with Trichonympha species constitute a monophyletic group that is phylogenetically distinct from the phylotypes recovered from all other flagellates. Trichonympha flagellates harbor a specific lineage of ‘Endomicrobia’ inherited by vertical transmission from their common ancestor [18].

**Gut bacterial community structure in different physiological states of Cryptocercus punctulatus.**

Many herbivorous insects have a tubular hindgut with several dilated compartments that allow a dense gut microbiota. In these dilated compartments or “fermentation chambers,” the prolonged residence time of food allows its degradation by microbial symbionts, a situation analogous to that in the rumen or colon of mammals. Microorganisms supplement the digestive capacities of the host, especially with their ability to hydrolyze the major structural polymers of plant cell walls (cellulose and hemicelluloses). Fermentation products, typically acetate and other short-chain fatty acids, are reabsorbed by the host and contribute substantially to its nutrition [4].

Termite guts are axially and radially structured habitats containing numerous microniches created by a combination of host and microbial activities [4]. Insect guts are surrounded by aerobic tissues aerated by the insect’s tracheal system. Oxygen penetrates the peripheral hindgut contents to a depth of up to 150–200 μm below the epithelium; its removal by the respiratory activity of the gut microbiota creates a microoxic periphery around an anoxic center. This is consistent with the presence of strictly anaerobic bacteria and protists in termite guts, and also explains the presence of strictly aerobic and facultative anaerobic bacteria as numerically significant members of the gut microbiota (Figs. 1, 5) [3].

Physiologically active Cryptocercus requires high microbial diversity to maintain an O2–H2 gradient in the gut. In lower termites, H2 formation is mainly attributed to the dense populations of cellulolytic flagellates. Clostridia may produce hydrogen during fermentation and thus possibly contribute to the accumulation of hydrogen in the termite gut. Acetogenesis and methanogenesis are the major hydrogen–sink reaction in the guts of many wood-feeding termites [33]. Ectosymbiotic and free-swimming spirochetes appear to specialize in metabolic interactions with the host or with other co-occurring microorganisms. The main compounds produced by spirochetes are acetate, H2, and CO2, which are normally consumed by sulfate-reducing bacteria and methanogens (with both groups represented in termites) [8,12]. The acetate produced by gut microbiota supports up to 100% of the respiratory requirement of termites [4]. Oxygen is consumed by aerobic and facultative anaerobic bacteria and by the high respiratory
rates of the epithelial cells. The mitochondria of epithelial cells can metabolize the acetate produced by the gut microbiota.

When lower termites are either starch-fed or starved, the luminal H₂ partial pressures in the hindgut strongly decrease, as does the acetogenesis rate [9]. When the influx of oxygen surpasses the oxidative capacities of the gut microbiota, the gut becomes completely oxic and the luminal production of hydrogen ceases. Prolonged exposure of the hindgut microbiota to large oxygen partial pressures appears to be deleterious to the protists and probably also to the H₂-consuming anaerobic bacteria [4]. Those results corroborate our observations regarding changes in the bacterial community of Cryptocercus in fasting and dead physiological states (Fig. 5).

Cryptocercus offers an example of the taxophysiological paradox in the composition of its gut microbiota under different physiological conditions. A stable, active physiological state supports high microbial diversity, probably to maintain a sharp O₂–H₂ gradient in the gut. In the fasting state, the host may have difficulties to maintain this gradient as well as the highly structured microniches in the gut. The fasting physiological state, therefore, marks a transition between two very different environmental situations in the gut: active (sharp O₂–H₂ gradients, with continuous input of nutrients, e.g., wood), and dead (oxic environment with no external input of nutrients). While the fasting physiological state implies a perturbed environment, in the dead physiological state there is a new “stable” condition, in which the functional redundancy of survivors favors an increase in intra-phyla diversity. In the dead state, nutrient input is limited to the members of the dead insect’s own gut microbiota, which cannot survive in the newly oxic environment (e.g., strict anaerobic bacteria and protists). Instead, these microorganisms may serve as food for other bacteria, thus favoring an increase in the diversity of aerobic or facultative anaerobic bacteria.

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