Phylogenetic Diversity and Physiology of Termite Gut Spirochetes

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SYNOPSIS. The hindgut microbiota of termites includes an abundant and morphologically diverse population of spirochetes. However, our understanding of these symbionts has remained meager since their first observation in termite guts by Leidy over a century ago, in part because none had ever been isolated in culture. Recently, this situation has changed dramatically with the application of cultivation-independent molecular methods to determine their phylogeny, and with the isolation of the first pure cultures. The emerging picture is that earth’s termites constitute an enormous reservoir of novel spirochetes, which possess metabolic properties (H₂/CO₂-acetogenesis and N₂ fixation) hitherto unrecognized in spirochetes and which contribute to the carbon, nitrogen and energy requirements of their termite host. These discoveries help to explain the enigmatic dominance of CO₂-reductive acetogenesis over methanogenesis in the hindgut of many termites, as well as the old observation that elimination of spirochetes from the gut results in decreased termite survival.

INTRODUCTION

Spirochetes are a monophyletic group of motile bacteria that possess a characteristic spiral or wavy shape (Paster et al., 1991). Cells typically consist of a coiled or undulate protoplasmic cylinder, which is bounded by the cell wall-cytoplasmic membrane complex and which contains the genomic DNA, ribosomes and other cytoplasmic constituents. Surrounding the protoplasmic cylinder is an outer membranous sheath; and interposed between the outer sheath and the protoplasmic cylinder are one or more periplasmic flagella (Canale-Parola, 1984) (Fig. 1). The latter are the organelles of motility (Charon et al., 1992). No other prokaryotes have quite this same body plan.

Spirochetes are widely distributed in nature, and as a group their biology is quite diverse. Some occur as free-living forms in freshwater, marine and hypersaline waters; whereas others associate with invertebrate and vertebrate hosts in relationships that range from benign commensalisms, to apparent mutualism, to parasitism. However, there are few habitats on earth in which spirochetes account for up to 50% of all prokaryotic microbes (Fig. 2). Hence, it is not surprising that termite vitality. Unfortunately, the nature of their contribution(s) remained obscure, as none had ever been isolated and studied in pure culture. Hence, much of the sporadic work on termite gut spirochetes over the past hundred years led to publications documenting their occurrence in various termite species and their morphological diversity and ultrastructure, and an assortment of new genus and species names were proposed for them based largely on morphological properties. Early work on termite gut spirochetes was reviewed by this writer and others (Breznak, 1973, 1984; Margulis and Hinkle, 1992).

Over the past seven years, our understanding of termite gut spirochetes has taken a giant step forward. In the mid-1990s, the first of what now are more than 300 16S rRNA sequences were determined for spirochetes from a variety of termite species by using a cultivation-independent, molecular approach that revealed their phylogenetic relationships to each other and to other spirochetes. Three years ago, the first pure cultures of these forms were obtained. Yet even in the relatively short time that such strains have been available, research on them has revealed metabolic properties heretofore unrecognized in spirochetes and, from that, some of the ways in which they contribute to termite survival.

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termite nutrition. This paper will summarize our recently-enlightened understanding of termite gut spirochetes.

PHYLGENY AND PHYLOGENETIC DIVERSITY OF TERMITE GUT SPIROCHETES

The first insight into the phylogeny of termite gut spirochetes came from analysis of cloned spirochetal 16S rRNA-encoding genes (16S rDNAs) obtained by PCR amplification of DNA purified from termite guts. The nucleotide sequence of clone MDS1 from the Australian termite *Mastotermes darwinensis* (family Mastotermitidae) grouped within the genus *Treponema*, but it was not closely related to any known treponeme (Berchtold *et al.*, 1994). This discovery was soon followed by analyses of additional spirochete 16S rDNA clones from *M. darwinensis* (Berchtold and König, 1996), as well as from *Nasutitermes lujiae* (family Termitidae) (Paster *et al.*, 1996), *Reticulitermes speratus* (family Rhinotermitidae) (Ohkuma and Kudo, 1996) and *Cryptotermes domesticus* (family Kalotermitidae) (Ohkuma and Kudo, 1998), and all of those clones grouped within the genus *Treponema* as well. As part of some of the investigations, fluorescent 16S rRNA-targeted oligonucleotide probes were designed and the probes were shown to react with hindgut spirochetes (Berchtold and König, 1996; Paster *et al.*, 1996).
al., 1996), thereby helping to confirm the spirochetal origin of the cloned rDNAs.

In 1999, a comprehensive analysis was performed on nearly 300 spirochetal 16S rDNA clones obtained from termites representing five of the seven termite families (Lilburn et al., 1999). The emergent picture was that although all clones could be grouped in the genus Treponema, none were closely related (i.e., all bore ≦91% sequence similarity) to any known treponeme or to any 16S rDNA clone derived from not-yet-cultured treponemes. Moreover, the clones were remarkably diverse at what could be considered the species-level, a result consistent with the morphological diversity of spirochetes seen in guts of even individual termites. Conservative estimates implied that individual termite species contained as many as 21 different species of Treponema. However, rank-abundance plots indicated that the distribution of clone types in R. flavipes was quite equitable, suggesting that either the termite gut ecosystem is not in equilibrium or that there exist in the gut a relatively large number of niches capable of being filled by different species of treponemes. The data also revealed the existence of at least two major phylogenetic groups of treponemes: one consisting of all of the currently known isolates of Treponema from animals other than termites, as well as a large number spirochetal 16S rDNA clones from the human gingival crevice and dental dermatitis lesions of cattle, but containing a minority of termite gut clones; and another, termed the “termite cluster,” containing the vast majority of termite gut clones (Fig. 3), as well as Spirochaeta stenostrepta and the thermophile, S. caldaria. (The latter two spirochetes, as well as S. zuelzerae, were named before their 16S rRNA sequences were known, and as they were free-living anaerobes they were assigned to the genus Spirochaeta. However, they group with the genus Treponema on the basis of their 16S rRNA sequences ([Paster et al., 1991]).) Four nucleotide signatures were identified that almost perfectly distinguished members of the “termite cluster” from other treponemes, and the first hints of possible coevolution were seen, i.e., some subclusters of clones derived from a particular termite species were more closely related to each other than to clones derived from other termites (Lilburn et al., 1999). However, there were also numerous clones from some termite species that grouped among those derived from other termite species. This was also noted by Ohkuma and coworkers in a paper published later that same year (Ohkuma et al., 1999a). It had now become quite clear that the roughly 2,000 species of termites on earth themselves constituted an enormous reservoir of novel spirochetal diversity, but that patterns of evolution between spirochetes and termites were far from simple.

**Physiological Properties of Termite Gut Spirochetes**

By 1999, researchers in termite gut microbiology were literally drowning in spirochetal 16S rDNA sequences. A much better understanding of spirochete phylogeny and phylogenetic diversity had been obtained, and from that a basis for refined predictions about their probable physiological properties and symbiotic interactions with termites. As all of the 16S rDNA clones were affiliated with the genus Treponema, it seemed reasonable to assume that termite gut spirochetes possessed properties common to other members of this group (Miller et al., 1992). Hence, they were likely to be strict anaerobes, or possibly microaerophiles, with a capacity to degrade sugars and/or amino acids, forming acetate as one of the end products. This was mildly satisfying, as microbially-produced acetate was known to be a major carbon and energy source for termites (reviewed by Breznak, 1994). However, many microbes produce acetate, including termite gut protozoa and nonspirochetal bacteria that had been previously isolated from termite guts, but we still had no idea what factors contributed to the conspicuous abundance of spirochetes in guts or what other roles they might play. Fortunately, some of these questions were answered in that same year with the isolation and characterization of the first pure cultures, Treponema strains ZAS-1 (Fig. 1) and ZAS-2, from the dampwood termite Zootermopsis angusticollis (family Termopsidae) (Leadbetter et al., 1999).
H₂/CO₂-acetogenesis by termite gut Treponema strains ZAS-1 and ZAS-2

Several factors contributed to the successful enrichment and isolation of strains ZAS-1 and ZAS-2. These were: (i) a medium containing pre-fermented, pH neutralized, clarified rumen fluid and a small amount of nutrient broth; (ii) inclusion of rifampin and phosphomycin (two drugs to which spirochetes are intrinsically resistant) in enrichment media; (iii) incubation under a headspace of 80% H₂/20% CO₂; (iv) addition of bromoethanesulfonate to media to inhibit H₂-utilizing methanogens; and (v) painstaking periodic microscopic examination of enrichment cultures to indicate which of a variety of media formulations supported a retention of spirochete motility and an increase in spirochete biomass. The latter was seen as an increase in the length of cells and the appearance of division stages. An effort was made to limit the amount of readily-fermentable carbohydrate in media (hence the use of pre-fermented rumen fluid) so as to discourage overgrowth of spirochetes by non-desired forms, a problem that plagued many previous isolation attempts. Successful enrichments took a long time to develop (10–12 wk), but eventually spirochetes outnumbered non-spirochetes by about 50:1, and from such enrichments pure cultures were isolated. It was later found that the same medium solidified with agar could be used to isolate spirochetes directly from dilutions of hindgut contents; and rumen fluid and nutrient broth could be replaced by yeast autolysate and a mixture of cofactors.

Of particular interest was the observation that growth of spirochetes in enrichment cultures was accompanied by acetate production and by the consumption of H₂ from the headspace of the sealed culture tubes, suggesting that the spirochetes might be capable of obtaining energy for growth by H₂/CO₂-acetogenesis, i.e., 4 H₂ + 2 CO₂ → CH₃COOH + 2 H₂O (ΔG°’ = –105 kJ per mole acetate). This suspicion proved to be correct when detailed experiments were performed on the two isolated strains (Leadbetter et al., 1999).

The performance of H₂/CO₂-acetogenesis by termite gut spirochetes, although offered as a speculation nearly 25 yr earlier (Breznak, 1973), was still quite surprising, because this mode of metabolism had never been described in spirochetes. Nevertheless, this metabolic property helped explain the enigmatic dominance of H₂/CO₂-acetogenesis over H₂/CO₂-methanogenesis in the hindgut of many wood-feeding termites (Breznak, 1994). Microelectrode-determined, radial profiles of H₂ gradients in hindguts of wood-feeding termites such as R. flavipes had revealed that the highest concentrations of H₂ (up to 50,000 ppmv) occurred in the luminal region (Ebert and Brune, 1997), being produced there largely by the protozoa in “lower” termites (such as R. flavipes) and by prokaryotes in “higher” termites. This is also the region of the hindgut in which spirochetes were most abundant. By contrast, H₂-consuming methanogens (and most other non-spirochetal prokaryotes) in R. flavipes were situated on or near the hindgut epithelium (Leadbetter and Breznak, 1996), where H₂ concentrations were lowest (ca. 3,000 ppmv). Thus, it appeared that the dominance of H₂/CO₂-acetogenesis reflected, in large part, the spatial separation of the H₂-consuming populations—with H₂/CO₂-acetogenic spirochetes consuming most of the H₂ at its source of production, and wall-associated methanogens using what H₂ was left over. That wall-associated methanogens were indeed limited for H₂ was shown by the fact that externally supplied H₂ stimulated methane emission (Ebert and Brune, 1997; Messer and Lee, 1989), but it had virtually no effect on in situ rates of acetogenesis from CO₂ (Tholen and Brune, 2000). The importance of microbial spatial relationships for the functioning of the termite gut microbiota have recently been discussed in detail (Brune and Friedrich, 2000).

The ability of spirochetes to grow by H₂/CO₂-acetogenesis also suggested that attachment of spirochetes to the surface of hindgut protozoa, an association that for some protozoa results in a spectacular “motility symbiosis” (Cleveland and Grimstone, 1964), reflects a strategy of some spirochetes to remain close to major sites of H₂ production. Recent elegant experiments with fluorescent, rRNA-targeted oligonucleotide probes imply that attachment to protozoa is limited to distinct phylogenetic types of spirochetes (Iida et al., 2000).

Although Treponema strains ZAS-1 and ZAS-2 are capable of H₂/CO₂-acetogenesis, they are not restricted to this substrate. Like many other so-called “homoacetogens” (i.e., anaerobic microbes that produce acetate as the sole or major fermentation product), they are also capable of fermenting various mono- and disaccharides, either alone or simultaneously with H₂ consumption. ZAS-2 is also capable of homoacetogenesis by using the methyl groups of methoxylated aromatic compounds (Graber and Breznak, 2000). Hence, spirochetes may contribute to demethoxylation of lignin (Esenther and Kirk, 1974) and other methoxylated aromatic components of termite food. However, not all termite gut spirochetes are homoacetogens. The more recently isolated strain, Treponema strain ZAS-9, which also groups within the “termite cluster” phylogenetically (Fig. 3), ferments sugars with the production of acetate and other products, including H₂, but it is incapable of H₂/CO₂-acetogenesis (Graber and Breznak, 2000). It is still too soon to appreciate the full range of metabolic capabilities of termite gut spirochetes given the few strains in culture at this time. Nevertheless, it seems safe to conclude that one important contribution of spirochetes to termite nutrition is via acetate production from a variety of substrates, including H₂ + CO₂.

Nitrogen fixation by termite gut spirochetes

With the availability of several strains of termite gut spirochetes in pure culture, their ability to fix N₂ was
also examined. N\textsubscript{2} fixation by termite gut microbes had been known for many years (reviewed by Breznak, 2000), and in light of the typically carbon-rich, but nitrogen-poor diet of termites, it was not surprising that N\textsubscript{2} fixation contributes as much as 60\% of the N in some termite colonies (Tayasu et al., 1994). However, N\textsubscript{2}-fixing microbes from termites were not well-represented in culture, and those that had been obtained—Citrobacter freundii (French et al., 1976), Enterobacter (now Pantoea) agglomerans (Potrikus and Breznak, 1977) and Desulfovibrio sp. (Kuhnigk et al., 1996)—were of uncertain quantitative significance to the process occurring in vivo. Indeed, surveys of \textit{nifH} (the gene encoding the Fe-protein of nitrogenase) present in termite guts indicated that the diversity N\textsubscript{2}-fixing organisms was far greater than that inferred by using cultivation-based methods, with most of such \textit{nifH} homologues not readily attributable to known microbial taxa (Ohkuma et al., 1999b, 1996).

Recent examination of ZAS-strains revealed that each possessed two homologues of \textit{nifH} and each exhibited nitrogenase activity, with ZAS-9 exhibiting the greatest specific activity, ca. 100-fold greater than that of ZAS-1 and ZAS-2 (Lilburn et al., 2001). Fixation of \textsuperscript{15}N\textsubscript{2} by ZAS-9 was also demonstrated. As \textit{nif} fixation was another property hitherto unknown in spirochetes, a survey was made for the presence of \textit{nifH} and nitrogenase activity in other members of this phylum. These traits were found to be restricted to certain species of \textit{Treponema} and \textit{Spirochaeta}. Of particular interest was the fact that the deduced NifH amino acid sequences of several spirochetes, including ZAS-strains, were identical or nearly identical to various NifHs previously observed in termite guts (above), including NifHs known to be expressed \textit{in vivo} (Noda et al., 1999), suggesting that the latter were likely to be of spirochete origin. Estimates of the potential contribution of spirochetes to the N\textsubscript{2}-fixing activity exhibited by live termites indicated that it could be significant (Lilburn et al., 2001).

**DISCUSSION**

Our understanding of termite gut spirochetes, and in particular their role in termite nutrition, has come a long way in a short time. It is now clear that spirochetes contribute to the carbon, nitrogen and energy requirements of termites via acetogenesis and N\textsubscript{2} fixation, and this is probably why their elimination from guts reduces the life span of termites. However, in light of the phylogenetic diversity of spirochetes in termites, it seems almost certain that the few strains now in culture offer but an introductory glimpse of the physiological diversity of the group as a whole, to be fully appreciated only as more representatives are coaxied into culture. Such efforts should be encouraged: they are an important companion to the powerful tools of molecular biology, and they are likely to be richly rewarded with even greater insight into the roles of spirochetes in termite nutrition. In the meantime, metabolic properties elucidated with the already available strains \textit{in vitro} must now be evaluated \textit{in vivo}. For example, what is the spirochete-specific contribution to H\textsubscript{2}/CO\textsubscript{2}-acetogenesis and N\textsubscript{2} fixation occurring \textit{in vivo}? Which phylogenetic groups of spirochetes are most important in these processes? What properties of spirochetes (or of the termite gut itself) enable them to become such a prominent component of the microbiota? Hopefully, creative and converging experimentation will soon provide answers to these intriguing questions.

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