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Full Length Research Paper

Bacterial diversity in the intestinal tract of the fungus-cultivating termite *Macrotermes michaelseni* (Sjöstedt)

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Microorganisms in the intestinal tracts of termites play a crucial role in the nutritional physiology of termites. The bacterial diversity in the fungus-cultivating *Macrotermes michaelseni* was examined using both molecular and culture dependent methods. Total DNA was extracted from the gut of the termite and 16S rRNA genes were amplified using bacterial specific primers. Representatives from forty-one (41) RFLP patterns from a total of one hundred and two (102) clones were sequenced. Most of the clones were affiliated with 3 main groups of the domain Bacteria: Cytophaga-Flexibacter-Bacteriodes (73), Proteobacteria (13), and the low G+C content Gram-positive bacteria (9). Two RFLPs related to planctomycetes, but deeper branching than known members of the phylum, were detected. In addition, 1 and 2 RFLPs represented the spirochetes and TM7-OP11 groups, respectively. In studies using culture dependent techniques, most of the isolates obtained belonged to the Gram-positive bacteria with a high G+C content. However, only one of the clones represented Gram-positive bacteria with High G+C content. These results show a high bacterial diversity in the intestinal microbiota of *M. michaelseni*, which continues to escape cultivation. As is the case in other termites many of the clones represent previously uncultured bacteria. The fact that most of the clones clustered with clones from *Macrotermes gilvus* provides further support for the hypothesis that microorganisms in intestinal tracts of termites have co-evolved with their hosts.

Key words: Fungus-cultivating termites, bacterial diversity, intestinal tract, 16S rRNA gene, RFLP.

INTRODUCTION

Termites are terrestrial social insects, which belong to the order Isoptera (Nutting, 1990). The order Isoptera contains 5 families of lower termites and 1 family of higher termites (Noirot, 1992) and contains over 2600 described species (Ohkuma et al., 2001). Termites are important arthropods involved in the biodegradation of complex substances like cellulose and hemicellulose found in plant materials (Wood and Sands, 1978; Brune, 1998), and their biomass densities in tropical and sub-tropical > 50 gm^{-2} often surpass those of grazing mammalian herbivores (0.01-17.5 gm^{-2}) (Lee and Wood, 1971, Collins and Wood, 1984). Because of their apparent ecological importance in tropical environments, termites have been the subject of many studies looking at: their role as ecosystem engineers and influence on soil properties (Anderson et al., 1991; Dangerfield et al., 1998; Donovan et al. 2001), their contribution to trace gas emission (Khalil et al., 1990; Eggleton et al., 1999) and their role in the degradation of lignocellulose (Amund et al., 1988; Breznak et al., 1994; Hinze et al., 2002). Recently termites have also attracted interest through the architecture of the mounds of *M. michaelseni* (Turner, 2000), which are being viewed as a possible model for cheap and energy efficient buildings. With the current energy crisis, the ability of termites to degrade lignocellu-

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lose within 24 h is increasingly being investigated for production of cellulosic ethanol from biomass.

Diverse microorganisms inhabit the intestinal tracts of all termite feeding groups (Brune, 1998; Brauman et al., 2001) at a high density of 10^6–10^7 cells per µl of gut volume (Schultz and Breznak, 1978; Bignell et al., 1980; Brauman et al., 2001). The ability of these Isoptera to digest complex molecules is mostly attributed to the action of these intestinal microbes. The diversity of microbes in the guts of diverse termites includes bacteria, fungi and protozoa (Brune, 1998), which have been characterized and shown to include many uncultured species (Ohkuma and Kudo, 1996; Ohkuma and Kudo, 1998; Schmitt-Wagner et al., 2003; Hongoh et al., 2005; Shinzato et al., 2005; Hongoh et al., 2006). In the case of the fungus-cultivating termites, the symbiosis between the termite and fungi of the genus *Termitomyces* is crucial to the digestive process (Anklin-Mühlemann et al., 1995). Despite the symbiosis with the fungi, the fungus-cultivating termites are also reported to harbor dense populations of bacteria and archaea (Anklin-Mühlemann et al., 1995; Brauman et al., 2001). Fungus-cultivating termites, play an important role in degradation processes in the tropics (Buxton, 1981) but their symbiotic microbial community, which plays a role in this process has hardly been studied. Earlier studies involved enumeration of bacterial cells and quantification of metabolite pools in the gut compartments of *Macrotermes subhyalinus* (Anklin-Mühlemann et al., 1995). In another study using groups specific probes *M. subhyalinus* and *Macrotermes bellicosus* were also reported to harbor dense populations of bacteria and a smaller population of archaea (Brauman et al., 2001). Extensive studies have also tried to unravel the symbiosis between the fungus and the termite (Bignell et al., 1994; Crosland et al., 1996, Aenen et al., 2002, Hyodo et al., 2003). Recently, Hongoh et al. (2006) published a comprehensive study of bacterial diversity in *Macrotermes gilvus* from Thailand, which showed that most of the major bacterial phyla were represented. The microbial diversity in *M. michaelseni*, the dominant fungus-cultivating termite in tropical and sub-topical Africa has yet to be studied. Apart from their importance in the nutrition of the termites, the microorganisms in the termite may also include novel species with potential for exploitation in biotechnology. The current study reports on the diversity of the intestinal bacterial community of the higher termite *M. michaelseni* from Kenya studied using both cultivation and molecular techniques.

MATERIALS AND METHODS

Termites

The study was conducted on the fungus-cultivating termites *Macrotermes michaelseni* (Sjöstedt), which belong to the higher termites, family Termitidae, subfamily Macrotermitinae (Ruelle, 1975). These termites are widely distributed throughout the savanna habitats of sub-Saharan Africa (Turner, 2000). Like all Macrotermitinae, *M. michaelseni* maintains a symbiosis with a fungus, *Termitomyces* spp. that aids in digesting cellulose (Rouland et al., 1991; Turner, 2001) and lignin (Brune 2003).

Termites were collected once from one mound in the compound of Jomo Kenyatta University of Agriculture and Technology at Juja, in Thika District, Kenya. The sampling site, Juja is located in the transitional zone between the high rainfall area to the north and northwest and the low rainfall area to the east. The mean annual rainfall ranges from 782 -933 mm. The vegetation is grassland with scattered bushes, dominated by *Digitaria scalarum* (Schweinf.) Chiov, *Eragrostis atrovirens* (Desf.) Steud and a herb *Ocinum* sp.; *Acacia seyal* is the dominant bush. The soils are generally poorly drained and the texture of the topsoil is clay (Muchena et al., 1978). During sampling, the termites together with nest fragments were brought to the laboratory; adult major worker caste termites were used in experiments, within one day of collection. The Invertebrate Zoology section of the National Museums of Kenya identified the termites.

Molecular characterization of bacterial community

Termites were degutted using fine tipped forceps as previously described (Schmitt-Wagner et al., 2003) and 10 guts (70 mg) were pooled and total DNA was extracted using GENECLEAN® Bio101 soil DNA extraction kit according to manufactures instructions. DNA was also extracted from isolates obtained in cultivation experiments (see below) using the GENECLEAN® Bio101 soil DNA extraction kit. Purified total DNA from each sample was used as a template for amplification of 16S rRNA genes using HotStarTaq Master Mix kit (Qiagen®) according to the manufacturer's instructions.

Bacterial 16S rRNA genes were amplified using universal primers 27F 5'-TAG AGT TTG ATC CTG GCT CAG-3' forward primer and 1392R 5'-GAC GGG CGG TGT GTA CA-3' reverse primer according to the position in relation to *Escherichia coli* gene sequence (Lane, 1991). PCR reactions were started by an initial activation of the enzyme at 95ºC for 10 min followed by 25 cycles consisting of denaturation (30 sec at 94ºC), annealing (1 min 45 sec at 55ºC) and extension (2 min at 68ºC) and a final extension at 68ºC for 10 min. The PCR product was visualized on a 1% agarose gel stained with ethidium bromide.

For the construction of the clone library of the 16S rRNA genes, the PCR product was excised and eluted using the QIAquick® gel extraction kit protocol according to the manufacturer's instructions and used for cloning. Cloning was done using Invitrogene TOPO-TA® cloning kit according to the manufacturer's instructions, and introduced into *E. coli* cells for transformation. One hundred and four clones were picked and an EcoRI digestion and vector targeted PCR was conducted. Restriction fragment length polymorphism (RFLP) analysis of PCR products from clones was conducted using a cocktail of the enzymes AvaI, EcoRI, BamHI, HindIII, KpnI, and XbaI. A representative clone for each unique RFLP patterns was sequenced. The PCR products of isolates were also purified and sequenced.

Sequence data were analyzed with the ARB software package (http://www.arb-home.de) as previously described (Schmitt-Wagner et al., 2003). Phylogenetic trees were constructed by the Phylip distance method and the maximum-likelihood method executed in ARB. The CHECK-CHIMERA program of the Ribosomal Database Project (Maidak et al., 2001), was used to check sequences to detect the presence of possible chimeric artifacts. Diversity coverage by the clone libraries was analyzed with Analytic Rarefaction software (version 1.2; S. M. Holland, University of Georgia, Athens, GA; http://www.uga.edu/strata/Software.html).

Cultivation of microorganisms

Termites were dissected with sterile fine-tipped forceps, and guts were homogenized (10 guts ml^{-1}) were serially diluted and plated

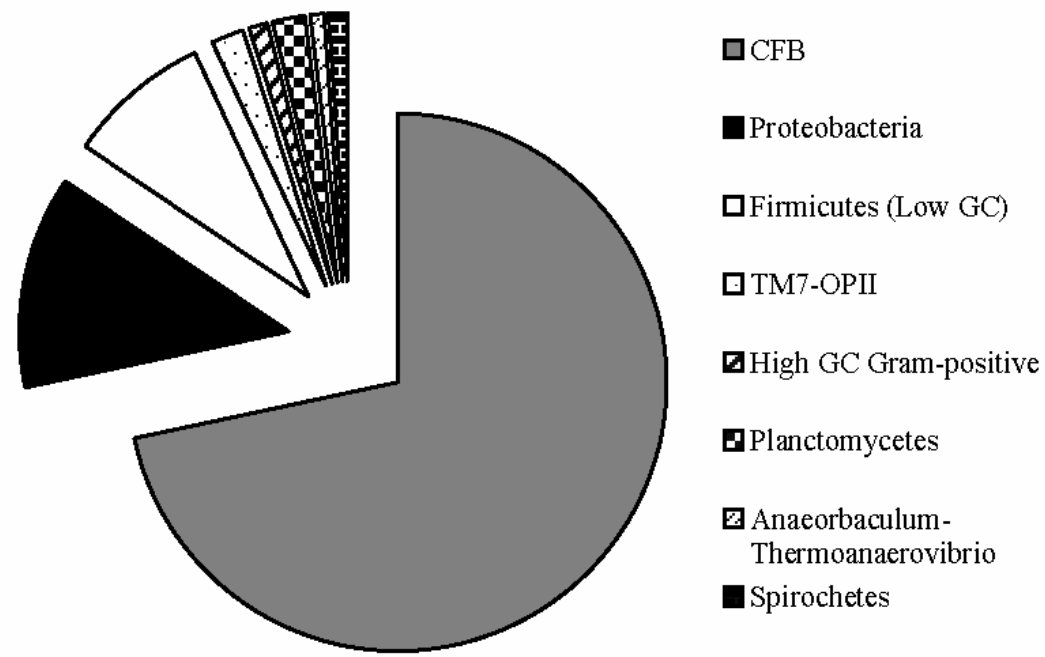

Figure 1. Relative clone frequencies in major phylogenetic groups of the *M. michaelseni* clone library. The number of clones in each phylum is indicated on the side.

on solid KMM1 (Ngugi et al., 2005) without additional substrate, and on KMM1 medium with either glucose (1%) or gelatin (1%). All solutions, cultures and media were prepared and maintained under oxic conditions and at pH 7.0. Cultures were incubated at 30°C in the dark. Colonies were counted after two weeks of incubation. Colonies were selected based on morphology, purified, and their 16S rRNA genes sequenced as described above. Sequences have been deposited with GenBank database and assigned accession numbers DQ307690- DQ307728 for clones and DQ312466-DQ312473 for isolates.

RESULTS

Molecular bacterial diversity

Phylogenetic analysis of 16S rRNA gene sequences obtained from representatives of the 41 RFLP revealed the following groups: *Cytophaga-Flexibacter-Bacteroides* (CFB) (73 clones), *Proteobacteria* (13 clones), *Firmicutes* (low G+C gram-positive bacteria) (LGC) (9 clones), a group distantly related to *Planctomycetes* (2 clones), *Anaerobaculum-Thermoanaerovibrio* (Synergestis group) (1 clone), TM7-OP11 (2 clones), High G+C content gram-positive bacteria (HGC)(1 clone) and spirochetes (1 clone) (Figure 1, 2 and 3). Two of the sequences were obvious chimeras.

The *C*FB group clones included MTG-1 (DQ307694), MTG-12 (DQ307702), MTG-19 (DQ307704), MTG-24 (DQ307706), MTG-25 (DQ307707), MTG-38 (DQ307711), MTG-47 (DQ307714), MTG-56 (DQ307715), MTG-58 (DQ307716), MTG-60 (DQ307718), MTG-85 (DQ307722), MTG-88 (DQ307723), MTG-89 (DQ307724), MTG-100 (DQ307695) and MTG-108 (DQ307700). The CFB clones were closely related to *Alistipes (*formerly *Bacteroides) putredenis* with a sequence similarity of 94.4-96.7%. All the clones likely belong to the genus *Alistipes* in the family Rikenellaceae. The RFLP pattern for clone MTG-104 (Accession DQ307696) was shared by 10 clones and was only distantly related to *Alistipes putredenis* and *Alistipes microfusus* with an 87.8 and 87% sequence similarity, respectively.

The second major phylum represented in the clone library comprises the *Proteobacteria* group with a total of 13 clones. Clone MTG-26, 27, 42, 45, 93 (3 clones), 102 and 109 assigned the accession numbers DQ307708, DQ307709, DQ307712, DQ307713, DQ307726, DQ307797 and DQ307701, respectively, clustered with *Desulfovibrio vulgaris* and *Desulfovibrio cuneatus*, of the delta subdivision of Proteobacteria with a sequence similarity of 90 and 92%. Two RFLPs belonging to clone MTG-6 (2 clones) (Accession DQ307717) and MTG-9 (1 clone) (Accession DQ307725) were closely related to *Escherichia hermannii* and *Escherichia senegalensis* with a 99.3% sequence similarity.

Clones MTG-23 and 96 (Accession DQ307705 and DQ307728, respectively) belonged to the TM7-OP11 cluster. MTG-23 was related to the oral clone CW040 (Accession AF308506) with an 89.7% sequence similarity. Clone MTG-96 was distantly related to an uncultured bacterium clone B02R018 (Accession AY197400)

Clones MTG-64 (DQ307719) and MTG-72 (DQ307721) were distantly related to planctomycetes with less than

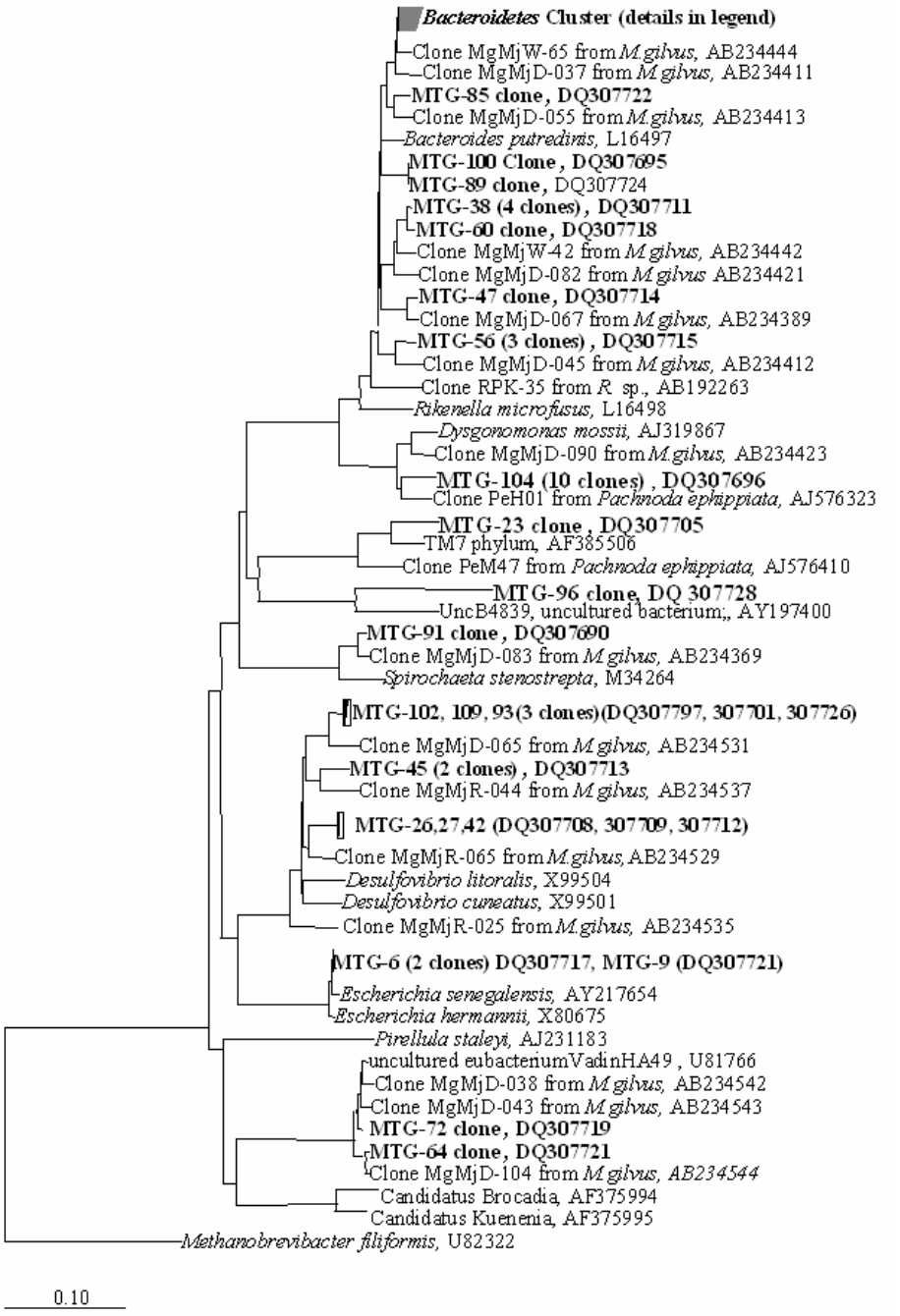

Figure 2. Phylogenetic tree showing positions of Bacteroides, Spirochetes, Proteobacteria and MTG-72 clones obtained from *M. michaelseni*. The scale bar indicates approximately 10% sequence difference. Clones from *M. michaelseni* gut are designated MTG with a number identifying each clone. Included are related sequences from *Macrotermes gilvus*, *Reticulitermes speratus* and *Cubitermes orthognatus.* The 16S rRNA gene sequence of *Methanobrevibacter filiformis* U82322 was used as the out-group. Accession numbers for clone in the Bacteroidetes cluster: MTG-1(DQ307694)(42 clones), MTG-12(DQ307702)(2 clones), MTG-19(DQ307704), MTG-24(DQ307706), MTG-25(DQ307707), MTG-47(DQ307714), MTG-58(DQ307716), MTG-60(DQ307718), MTG-88(DQ307723), and MTG-108(DQ307700)(2 clones).

75% sequence similarity. However, MTG-64 and 72 were related to each other with 96.2% sequence similarity and to an uncultured eubacterium VadinHA49 (Accession U81766) with 95.8 and 96.2% sequence similarity, respectively. Both clones were also closely related to three clones from *M. gilvus* (Accession AB234542, AB234543 and AB234544) with sequence similarity of greater of 94-97%. MTG-64 and 72, VadinHA49 and two other clones,

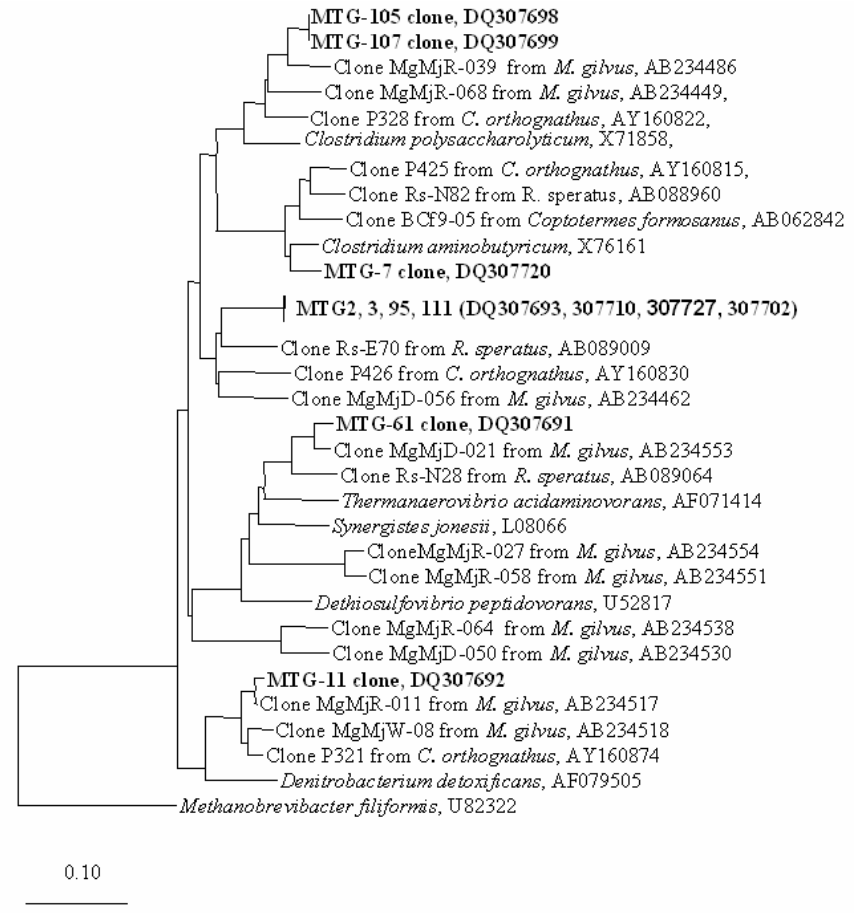

Figure 3. Phylogenetic tree showing positions of gram-positive bacteria clones obtained from *M. michaelseni*. Included are related sequences from *M. gilvus*, *R. speratus* and *C. orthognatus*. The scale bar indicates approximately 10% sequence difference. Clones from *M. michaelseni* gut are designated MTG with a number identifying each clone. The 16S rRNA gene sequence of *Methanobrevibacter filiformis* U82322) was used as the out-group.

uncultured bacterium "L" (Accession AF338766) and uncultured bacterium EO-IV (Accession AF149887), together with the three clones from *M. gilvus* formed a cluster distantly related to previously described members of the Planctomycetes.

The Spirochete group had only one clone MTG-91 (DQ307690), which was related to *Spirochaeta stenostrepa* with 89.3% sequence similarity and to MgMjD-083 treponeme clone (Accession AB234369) from *M. gilvus* with a sequence similarity of 89.3%.

The low G+C gram-positive bacteria were represented by MTG-2, 3, 7, 105, 107, 95 and 111 (Accession numbers DQ307693, DQ307710, DQ307720, DQ307698, DQ307699, DQ307727 and DQ307703, respectively). MTG-7 had a sequence similarity of 90.7% to *Clostridium aminobutyricum* (Accession, X76161). MTG-105 and 107, which were identical, had 86.7% and 90.1% sequence similarity with *Clostridium polysaccharoliticum* (Accession X71858) and clone MgMjR-039 (Accession AB234486) from *Macrotermes gilvus* (Accession), respectively. MTG-2, 3, 95 and 111 were closely related with a 99.8-100% similarity, but were distantly related to uncultured clone RS-E70 (Accession, AB089009) from *Reticulitermes speratus* and clone MgMjD-056 (Accession AB234462) from *M. gilvus*, with a sequence similarity of between 86.9 and 85.1%, respectively. Clone MTG-11 (1 clone) (Accession DQ307692) belonged to the gram-positive bacteria with a high G+C content. It was closely related to an uncultured Actinobacteriadae bacterium Rs-J59, Accession AY711125, obtained from *Reticulitermes speratus,* with a 97.1% sequence similarity. MTG-11 was also related to *Denitrobacterium detoxificans* (Accession AF079507) with a 90% sequence similarity.

Clone MTG-61 (DQ307691) was closely related to MgMjD-021 (Accession AB234553) from *M. gilvus* with a sequence similarity of 91.2% and had a sequence similarity of 84.4% to *Thermoanaerovibrio acidamino-vorans* (Accession AF071414). The clone was assigned to the *Anaerobaculum-Thermoanaerovibrio* group also referred to as the "Synergestis" group. Rarefaction analysis of the

Table 1. Number of microorganisms (mean ± standard deviation) in the gut homogenates of *Macrotermes michaelseni* growing on selected media under oxic conditions.

Medium	Microbial population (cfu per ml × 10^8)
KMM1	1.74 ± 0.28
KMM1+ GELATIN	1.40 ± 0.36
KMM1+ GLUCOSE	1.27 ± 0.23

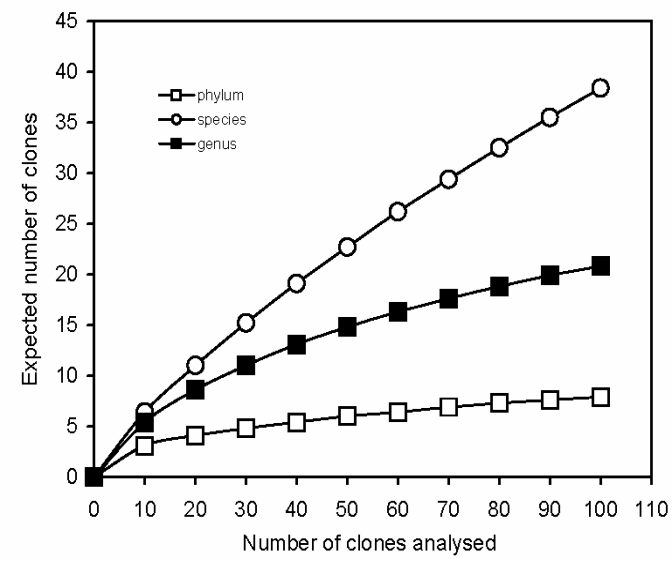

Figure 4. Rarefaction analysis of all bacterial 16S rRNA gene clones recovered from the hindgut of *M. michaelseni*. The expected number of clones was calculated from the number of clones analyzed at the species level with 97% sequence similarity (O), and at a sequence similarity of 94% (■) and ≤ 90% (□) arbitrarily defined as the genus and group (phylum) level, respectively. The slope of the curves indicates whether the diversity was covered (zero or gentle slope) or whether new Taxa can be expected if additional clones were to be analyzed (steep slope).

results of the clone library confirmed that the number of clones analyzed was not sufficient to describe the bacterial diversity at the species level (Figure 4). However, the slope of the rarefaction curve showed reasonable coverage at the genus and phylum group levels i.e. arbitrarily defined as 94 and 90% sequence similarity respectively

Cultivation of microorganisms

Enumeration of bacteria on media containing varied substrates revealed a mean population ± SD of 1.27–1.74 (× 10^5) per gut i.e. 1.27–1.74 (× 10^8) cells per ml (Table 1). All seven isolates sequenced belonged to the domain bacteria. A phylogenetic tree containing members of each of the isolates is shown below (Figure 5).

The phylogenetic tree contained three main clusters. The first cluster comprised the genus *Streptomyces*, which included isolates IM_9A & IM_9B (DQ312472 & DQ312473), IM_14 (DQ312466), IM_15 (DQ312467) and IM_8A (DQ312471). The isolates in this cluster shared a sequence similarity of between 95-99% with known *Streptomyces* species (Figure 5). The second group of isolates clustered with the *Brevibacterium-Brachybacterium* and comprised isolates IM_4 (DQ312468) and IM_5 (DQ312470). Isolate IM_4 had a 99.9% sequence similarity to *Brevibacterium sp.* (AY299093). IM_5 had a sequence similarity of 92.3% to *Brachybacterium tyrofermentans* (X916570) and most like represents a novel organism. Characterization of the isolates is in progress. Isolate IM_4B (DQ312469) clustered with *Bacillus licheniformis* (X68416). None of the isolates was represented in the clone library.

DISCUSSION

Prokaryotic microorganisms have been previously shown to inhabit the intestinal tracts of termites of all feeding groups. In this study we show that majority of the microorganisms from the gut of *M. michaelseni* belong to the CFB phylum (Figure 2) which has also been found to predominate in the fungus–cultivating termite *M. gilvus* (Hongoh et al., 2006), in wood-feeding termites (Kudo et al., 1998; Ohkuma et al., 2002) and in soil-feeding termites (Schmitt-Wagner et al., 2003). Representatives of the CFB group are typical components of the intestinal microflora of mammals including humans (Hold et al., 2002; Leser et al., 2002), and have also been cultured from wood-feeding termites (Schultz and Breznak, 1978). *Bacteroides* species are known to be fermentative and acidogenic, and organisms assigned to this cluster may have the same physiology. Many bacteria from the CFB phylum are also specialized in degradation of plant fibers and proteins (Shah, 1992) which are likely constituents of the termite diet. The exact role played by members of the CFB phylum in the nutritional physiology of *M. michaelseni* and in other termites will have to await the isolation and characterization of representatives of the CFB in pure culture. Techniques like stable-isotope pro-bing using appropriate substrates (Boschker et al., 1998) can also help to clarify the physiological importance of the different groups.

Apart from members of the CFB phylum, members of the Proteobacteria were the second most dominant in the clone library. Ten of the clones clustered with *Desulfovibrio* species, while the other three clustered with *Escherichia hermannii* and *Escherichia senegalensis*. This group was also reported in a fungus-cultivating termite *M. gilvus* (Hongoh et al., 2006). *Desulfovibrio* species are known to be strict anaerobes capable of reducing sulfate and fixing nitrogen. The activity of sulfate reducing bacteria has been detected in the wood-feeding *Mastotermes darwiniensis* (Dröge et al., 2005) and sulfate-reducing

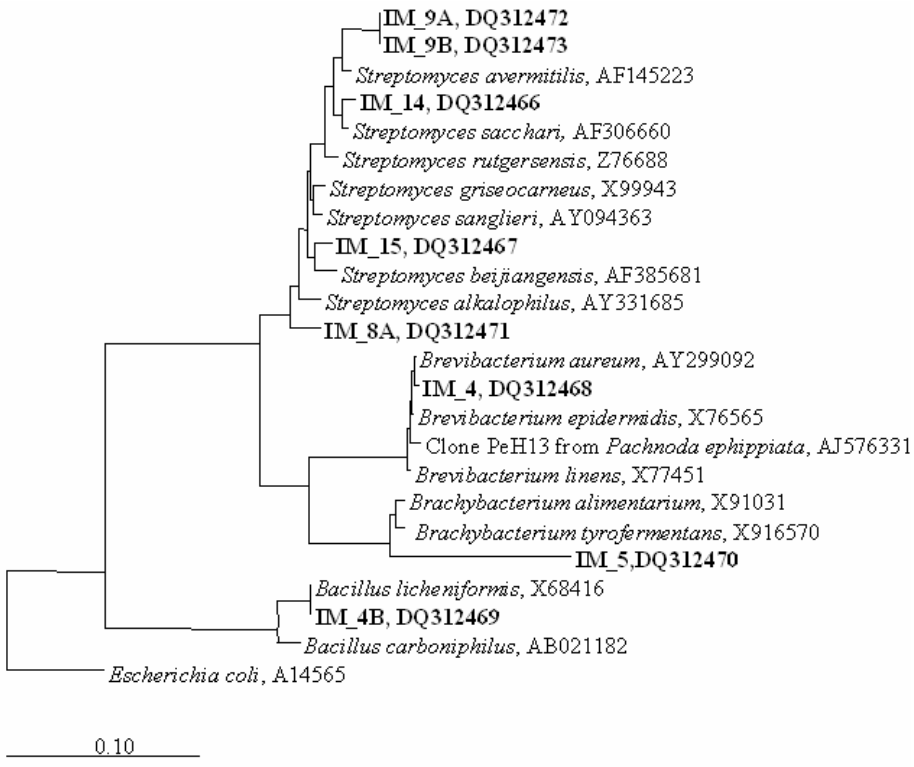

Figure 5. Phylogenetic tree showing positions of isolates from *M. michaelseni* relative to existing sequences. The scale bar indicates approximately 10% sequence difference.

bacteria have previously been isolated in pure culture (Kuhnigk et al., 1996, Fröhlich et al., 1999). Although, sulfate reducing bacteria were isolated from both lower and higher termites, sulfate reduction *in situ* is yet to be detected, and sulfate is not likely to be a significant electron acceptor in termites. Another likely role of the *Desulfovibrio* related organisms, is nitrogen fixation to supplement the N-poor diet taken by termites (Noirot, 1992). Nitrogen fixation was not detected in *M. michaelseni* although it was reported in *Macrotermes nataliensis* (Breznak, 2000). Studies using labeled $^{35}SO_4^{2-}$ and $^{15}N_2$ may held clarify the significance of the two processes in termite guts.

In contrast to the soil-feeding *Cubitermes orthognatus*, only nine clones in *M. michaelseni* belonged to the gram-positive bacteria with a low G+C content. In the soil-feeding *C. orthognatus* many clones belonged to this group (Schmitt-Wagner et al., 2003). Clostridia have also been detected in the mixed segment of *Nasutitermes takasagoensis* (Shiraki) (Tokuda et al., 2000) and also in *M. gilvus* (Hongoh et al., 2006). Many *Clostridium* species are obligate anaerobes, but some are facultative anaerobes (Rieu-Lesme et al., 1996). Clostridia are mostly soil bacteria, but can also be found in the rumens and intestines of animals and humans (Cummings and Macfarlane, 1997). Some predicted roles of the intestinal bacteria in termites, are similar to those in humans, such as producing short-chain fatty acids from carbohydrates or synthesizing amino acids (Cummings and Macfarlane, 1997). Many Clostridia degrade polysaccharides to produce acetone, alcohol, acetate, lactate, CO_2 and hydrogen (Chen, 1995). Representatives of this group may play a role of transformation of such compounds in termites. A CO_2–reducing acetogenic *Clostridium mayombei* was isolated from *Cubitermes speciosus* (Breznak et al., 1988, Kane et al., 1991). More studies targeting members of this group in diverse termites have to be carried out as a step towards explaining their role.

One clone (MTG-61) clustered with four clones obtained from young and old workers of *M. gilvus* (Hongoh et al., 2006) and a clone from *Reticulitermes speratus* (Hongoh et al., 2003), indicating an association of this group with termites. Among isolates clustering with MTG-61 are, *T. acidaminovorans* (Baena et al., 1999b), *D. peptidovorans*, isolated from the termite gut by enrichment culture (Trinkeri et al., 1990) and *Synergestis jonesii* isolated from a goat rumen (Allison et al., 1992). The rRNA approach tends to describe only the numerically dominant 16S rRNA genes within each ecosystem. The *Anaerobaculum-Thermoanarovibrio* in some cases referred to as the “Synergestis group” represents mostly microbes occurring infrequently in a given ecosystem (Godon et al., 2005). It is apparent that this group has also co-evolved with termites, since “Synergestis” clones from termites were found to cluster together (Godon et al., 2005). The role of members of the ‘*Synergistes* group’

in termite guts remains unknown. However, "*Synergistes*" seem to be anaerobic amino-acid degraders, suggesting that they may be involved in the turnover of amino acids in natural anaerobic ecosystems. All cultivated "*Synergistes*" strains such as *Aminomonas paucivorans* (Baena et al., 1999a), Th*ermoanaerovibrio acidaminovorans* (Baena et al., 1999b), *Dethiosulfovibrio* sp. (Magot et al., 1997; Surkov et al., 2001), *Aminobacterium mobile* (Baena et al., 2000) and *Aminobacterium colombiense* (Baena et al., 1998) degrade amino acids. Only a few of them, such as *Thermanaerovibrio velox* (Zavarzina et al., 2000) and *Anaerobaculum* sp. (Rees et al., 1997), are able to use carbohydrates as well.

Consistent with reports on other fungus-cultivating termites *M. gilvus* and *Odontotermes sp*, which have low abundance of spirochete-like cells in their gut contents (Hongoh et al., 2006), only one Spirochete clone was recovered from *M. michaelseni* . Spirochetes are believed to play an important role in wood-feeding termites, as termites harboring them appear to be both vigorous and healthy (Ohkuma and Kudo, 1996). Pure cultures of spirochetes have been obtained from *Zootermopsis angusticollis* and were found to be capable of CO_2-reducing acetogenesis, thereby implying a significant role in termite nutrition (Leadbetter et al., 1999). In a recently published study, analysis of the diversity and expression profiles of the bacterial *fhs* gene, a marker gene encoding a key enzyme of reductive acetogenesis, formyl tetrahydrofolate synthetase (FTHFS) in *Reticulitermes santonensis* (Rhinotermitidae) and *Cryptotermes secundus* (Kalotermitidae) revealed that most of the clones clustered among the 'Termite Treponemes', which comprise also the *fhs* genes of the two strains of the homoacetogenic spirochaete *Treponema primitia.* The results strongly support the hypothesis that spirochaetes are responsible for reductive acetogenesis in the hindgut of lower, wood-feeding termites (Pester and Brune 2006). The significance of reductive acetogenesis in fungus-cultivating termites is yet to be investigated.

The phylogenetic groups CFB, Proteobacteria, Firmicutes, High G+C gram positive, and Spirochetes reported in this study, are largely similar to those reported from the guts of other fungus–cultivating termites *Macrotermes gilvus* (Hongoh et al., 2006) and in many cases the clones from the two termites cluster together. Interestingly some of the clones also associate with those from wood-feeding and soil-feeding termites (Ohkuma and Kudo, 1998; Ohkuma et al., 2002; Schmitt-Wagner et al., 2003; Hongoh et al., 2005). This is consistent with understanding that termite gut microbes are unique to termites having co-evolved with their hosts (Ohkuma et al., 2001)

It is not surprising that low numbers of bacteria were also recovered from *M. michaelseni* using the culture dependent technique (Table 1), since cultivation is known to capture only a small insignificant fraction of the microbial diversity in any given sample. Aerobic cultivation yielded largely isolates belonging to the Gram-positive bacteria with high G+C content, which were hardly represented in the clone library. MTG-11 was the only clone of the high G+C Gram positive bacteria recovered in this study. Interestingly, some of the isolates obtained in this study are novel species. Despite their insignificance numerically, their potential use in biotechnology needs to be further investigated. Future cultivation attempts have to be directed at isolating groups detected in the clone library, with the aim of understanding the physiology of the different groups, their possible role in the termite gut and potential application in biotechnology.

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