BACTERIAL DIVERSITY IN THE INTESTINAL TRACTS OF THE FUNGUS-CULTIVATING TERMITES *Macrotermes michaelseni* (Sjöstedt) AND *Odontotermes somaliensis* (Sjöstedt)

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Declaration

The work described in this thesis was carried out at the International Center of Insect Physiology and Ecology during the period 2003-2004. The experimental work described is entirely my own. Information derived from published work is specifically acknowledged in the text and references appended. The contents of this thesis have not been submitted for any other award.

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Dedication

To my mother, Ruth and father, Charles. To my sisters Harriet, Tabitha, Christine, Gloria, and Catherine and my brother Nicholas. Thank you for all the support.

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Abstract

Termites are an important group of insects that harbor a complex community of gut microbes, which contribute to digestion, termite nutrition and gas (methane, carbon-dioxide and hydrogen) emission. The abundance of microbial communities in the intestinal tracts of two fungus-cultivating termites *Macrotermes michaelseni* and *Odontotermes somaliensis* were examined using, the Most Probable Number (MPN) and plate count techniques and diversity was assessed by analysis of 16S rDNA genes using PCR. The microorganisms were isolated using an enriched media using either glucose, gelatin or cellulose as substrates. The highest number of microbes was in *Macrotermes michaelseni* and was recorded in KMM1 medium giving a mean ± SD of 173.5 ± 28.9 x 10^3 CFU/gut and a mean ± SD of 156 ± 25.45 x 10^3 CFU/gut in *Odontotermes somaliensis* by plate count technique. Using the MPN technique, the highest number of microbes was recorded in a KMM1 medium giving approximately 43 x 10^4 cells/gut in *Macrotermes michaelseni* and in medium containing glucose an approximate of 480 cells/gut in *Odontotermes somaliensis*. Molecular studies were carried out by directly amplifying portions of the bacterial 16S ribosomal DNA (16S rDNA) genes from the mixed-population DNA of the microbial community in the termite gut by the PCR and these genes were clonally isolated. Analysis of the partial sequences of 16S rDNA showed the existence of bacteria species related to *Proteobacteria, Cytophaga-Flexibacter-Bacteroides,* low G+C gram-positive bacteria, *Anaerobaculum thermoterrenum* and Spirochetes in both termites. Unique sequences showing very low sequence similarity to known 16S rDNA were also found. Phylogenetic analysis revealed

that more the half of the clones isolated from *Odontotermes somaliensis* clustered with sequences from the *Cytophaga-Flexibacter-Bacteroides* group and few clones in *Proteobacteria,* low G+C gram-positive bacteria, *Anaerobaculum thermoterrenum* and Spirochetes groups. In *Macrotermes michaelseni*, the clones were well distributed in *Proteobacteria*, *Cytophaga-Flexibacter-Bacteroides,* and the low G+C gram-positive groups and very few clones related to *Anaerobaculum thermoterrenum* and Spirochetes groups. The results revealed an enormous diversity of bacteria in both termite species. Most of the clusters contained clones only from the respective termite species. The 16S rDNA gene sequence data show that the majority of the intestinal microflora of *Macrotermes michaelseni* and *Odontotermes somaliensis* consists of new uncultured species previously unknown to microbiologists.

Abbreviations

bp	Base pairs
BSS	Basal Salt Solution
CFB	*Cytophaga-Flexibacter-Bacteroides*
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetra-acetic acid
ICIPE	International Center of Insect Physiology and Ecology
JKUAT	Jomo Kenyatta University of Agriculture and Technology
LB	Luria Bertani
MPN	Most Probable Number
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
USA	United States of America
LGC	Low G+C content bacteria
KMM1	Kenya minimal media

CHAPTER ONE

1. GENERAL INTRODUCTION

Termites are insects that belong to the order *Isoptera*. *Isoptera* means equal winged, which, also reflects that the fore wings and hind wings of the reproductive are similar in size and venation (Nutting, 1990). Termites are among the most abundant and important soil micro-invertebrates (Nutting, 1990). There are approximately 2,753 species of termites in existence (Myles, 2000), whose biology and nutritional ecology are extremely diverse (Wood and Johnson, 1986).

Although frequently thought of as feeding on wood, the majority feed on other resources (Wood and Johnson, 1986). Half of the genera feed on soil (Noirot, 1992), while others cultivate gardens of fungi, which they ingest along with different components of plant litter, such as roots, leaves, and dead wood among others.

Termites are broadly divided into two groups, the lower termites and the higher termites (Donovan *et al.*, 2001). The lower termites comprise of six families, which are characterized by having symbiotic intestinal protozoa on which they depend for digestion of the complex polysaccharides e.g. cellulose and lignin in their food (Wood and Johnson, 1986). The diet of lower termites is restricted to sound wood (wood that is not decayed) or nearly sound wood (wood that is decayed) and grass (Noirot, 1992).

The higher termites belonging to the family Termitidae constitute approximately 75% of all termite species (Breznak, 1983). They harbor dense populations of gut bacteria but lack cellulolytic protozoa. Their digestive processes remain obscure, although they rely

partly on their own digestive enzymes, including cellulases and symbiotic gut microorganism (Breznak, 1983; Wood and Johnson, 1986). These termites colonize various biotypes due to their different dietary demands. Most of these thrive on relatively refractory nitrogen poor resources i.e. lignocellulosic plant material. These include the soil feeding, wood feeding; grass feeding, and fungus-cultivating termites (Noirot 1992).

The termites present in a colony consist of several castes, which are morphologically and functionally distinct (Noirot 1969). The castes may be divided into two broad groupings, reproductive and sterile. The most important of the sterile castes are the soldiers and the workers. The workers are the most numerous. They are also responsible for building the nest and for all foraging activity. The workers care for the eggs, feed the larvae, the soldiers and the queen, all of who are incapable of feeding themselves. Most biochemical and microbiological studies of termites have been carried out on the worker caste because of their importance in feeding the other castes (O'Brien and Slaytor, 1982).

All termites typically harbor a complex community of gut microbes, which may contribute to digestion, termite nutrition and gas (CH_4, CO_2 and H_2) emission (Breznak, 1982; Breznak and Brune 1994). These communities are substantial being found at an approximate concentration of 10^6-10^7 cells per µl gut volume (Anklin-Muhlemann *et al.*, 1995; Bignell *et al.*, 1980 and Schultz and Breznak 1978) and morphologically diverse. They are comprised of different combinations of representatives from life's three domains, and they include aerobic, facultative and anaerobic forms (Tholen *et al.*, 1997)

In a study of the microflora of *Reticulitermes flavipes* (Tholen *et al.*, 1997), almost one third of the bacteria cultivated on solid media consisted of strictly aerobic and

facultatively anaerobic strains and more than two thirds were aerotolerant lactic acid bacteria assigned to the group *Enterococcus*. This was also reported in earlier studies on the composition of the gut flora of wood-feeding termites by Schultz and Breznak (1978), who found that two thirds of the colonies isolated from dilutions of *Reticulitermes flavipes* gut homogenates were *Lactococcus lactis*. The other isolates were either facultatively or strictly anaerobic bacteria characterized as *Enterobacteriaceae* and as *Bacteroides* species. The prevalence of streptococci and enterobacteria has also been reported in the guts of various lower and higher termites (Eutict *et al.,* 1978). The presence of sulfate reducing bacteria has been reported in a number of termites (Kuhnigk *et al.,* 1996) and several *Methanobrevibacter* species associated with the hindgut epithelium of *Reticulitermes flavipes* have been successfully isolated (Leadbetter and Breznak, 1996).

Nevertheless, the bacterial numbers obtained by cultivation in earlier studies (Tholen *et al.,* 1997, Schultz and Breznak, 1978, Eutic *et al.,* 1978 and Schultz and Breznak, 1979) represented only a fraction of the total bacteria present. When direct microscopic counts of microorganisms in *Reticulitermes flavipes* hindgut, were compared with the sum of viable counts of Lactic acid bacteria, enterococci, strict aerobes, and methanogens, which predominated plate counts or liquid serial dilutions (Leadbetter and Breznak, 1996, Tholen *et al.,* 1997) it was apparent that about 90% of all prokaryotes in the hindgut escaped cultivation (Tholen *et al.,* 1997).

An indication of hindgut biodiversity can be obtained using molecular methods, which allow the detection and identification of microorganisms without the need for cultivation.

This is achieved by the analysis of 16S rDNA genes (Head *et al.*, 1998). The major groups that were detected in the hindgut of most termites comprise *Proteobacteria*, spirochetes, the *Bacteroides* group, and the low G+C (LGC) Gram-positive bacteria (Ohkuma *et al.*, 2002; Ohkuma *et al.*, 1999; Ohkuma and Kudo 1996 and Schmitt-Wagner *et al.*, 2003).

This study was designed to describe the diversity of bacterial community in the hindguts of two fungus-cultivating termites *Macrotermes michaelseni* and *Odontotermes somaliensis* in Kenya.

1.1 Justification

Termites digest high molecular weight food substances from their diet such as cellulose and hemicellulose (Brune, 1998), by using enzymatic action present in the termite and from the symbiotic microbiota that include bacteria, protozoa and fungi.

Where they are abundant, termites have a remarkable effect on the soil structure, nutrient cycling, growth of vegetation and wildlife through the movement of soil to build runways and nests and the remarkable conversion of large quantities of vegetable matter into feacal residues and termite tissues (Wood and Johnson 1986). Their role as important mediators of decomposition, humification, soil conditioning, aggregate binding and the formation of clay mineral complexes are widely recognized (Wood and Sands 1978; Nutting 1990).

Apart from their role in the degradation of lignocellulosic material and humus, termites have also been shown to emit methane, an important greenhouse gas (Breznak and

Switzer 1986, Brauman *et al.*, 1992). The soil-feeding and fungus-cultivating termites have the highest rates of methane emission compared to wood-feeding termites, and methanogenesis appears to be a major electron sink in their guts (Brauman *et al.*, 1992). Other possible processes in the termite gut attributed to the microbiota include, carbon and termite nutrition derived from lignocellulose digestion (Breznak and Brune, 1994), methanogenesis and acetogenesis from hydrogen and carbon dioxide (Brauman *et al.*, 1992), nitrogen fixation (Breznak *et al.*, 1973), recycling of uric acid and nitrogen (Potrikus and Breznak, 1980) and maintenance of low redox potential and prevention of entry of foreign bacteria (Veivers *et al.*, 1982).

The efficiency in degrading lignocellulosic compounds and the complex diversity of microbes resident in the termite guts may provide novel strains of microorganisms with equally new metabolic activities (Brune, 1998). A lot of scientific research has been done on wood-feeding and soil-feeding termites in which intestinal microbiota has been characterized (Schultz and Breznak 1978; Bignell *et al.*, 1980; Brune and Friedrich, 2000; Schmitt-Wagner *et al.*, 2003), but very little has been done to describe the microbial flora of the gut of fungus-cultivators. Fungus-growing termites are able to utilize lignocellulose (Darlington, 1994), and therefore play a dominant role in decomposition processes in many parts of the tropics (Buxton, 1981). Though the isolation of microbial cells may be successful, the cultivation conditions *in vitro* may not represent the natural state of the organisms. This is due to the complicated symbiotic relationships that the termite may have with other members of the communities involved.

Despite the isolation and cultivation of several bacteria and protists from within the termite guts (Eutick *et al.*, 1978; Odelson and Breznak, 1985; Schultz and Breznak 1978; Yamin, 1978 and Yamin 1981) our understanding of the biology and the physiology of intestinal microbiota remains limited as many of the predominant species within the community, have not yet been cultured and characterized (Ohkuma and Kudo 1996).

In this study the diversity of microbial communities in two fungus-cultivating termites was investigated using molecular phylogenetic analysis. In this approach, genes encoding small subunit ribosomal DNA (16S-like rDNA) derived from the extracted nucleic acids of mixed microbial populations are cloned and sequenced. These sequences are then compared with each other as well as with databases of 16S rDNA sequences from well-characterized microorganisms in order to determine the identity of organisms present in the termite guts. The application of molecular phylogenetic analysis to ecological studies has already been applied to investigate the diversity of the symbiotic microbial community in the gut of a lower termite *Reticulitermes speratus* and *Cryptotermes domesticus* (Ohkuma and Kudo, 1996; Ohkuma *et al.*, 1995; Ohkuma *et al.*, 1996; Ohkuma and Kudo 1998) and in soil feeding termite *Cubitermes* spp (Schmitt-Wagner *et al.*, 2003). These studies have shown that the termite symbiotic system includes many species yet uncultured in the laboratory (Ohkuma and Kudo 1998). A large discrepancy between the high diversity of microbial phenotypes and the few numerically and metabolically relevant species isolated underscores that new concepts for cultivation are necessary. As a prerequisite, the physiochemical environment within the termite gut need to be characterized in more detail, from which important clues for cultivation strategies

can be obtained. Knowing the phylogeny of the gut microorganisms could lead to the design of better cultivation methods.

Termites show a considerable variation in life-style, ecology and types of symbiosis. A comparison of the constituents of the microbial communities between termite species may help to understand the nature of the termite symbiotic systems (Ohkuma and Kudo 1998). A combination of culture dependent techniques and molecular techniques in this study is essential in establishing the diversity of microorganisms in the intestinal tracts of a number of termite species found in Kenya.

1.2 OBJECTIVES

1. To determine and compare the bacterial diversity in the guts of *Macrotermes michaelseni* and *Odontotermes somaliensis*
2. To isolate and characterize diverse microorganisms from both termites
3. To compare isolates got using the culture dependent techniques with microorganisms detected using molecular techniques

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Classification of Termites

Termites are classified into seven families three of which are divided into subfamilies. Members of the family Termitidae are often termed the 'higher' termites, as they possess more advanced features (Pearce, 1997). The other families are termed the 'lower' termites. A list of all families with authorities is given in Table 1 below.

Table 1.

A generic classification of termites with authorities (Pearce, 1997).

LOWER TERMITES
- Family Mastotermitidae (Desneux, 1904)
- Family Kalotermitidae (Banks, 1919)
- Family Hodotermitidae (Snyder, 1925)
- Family Rhinotermitidae (Light, 1921)
  - Subfamily *Coptotermitinae*
  - Subfamily *Heterotemitinae*
  - Subfamily *Psammoternitinae*
  - Subfamily *Termitogetoninae*
  - Subfamily *Stylotermitinae*
  - Subfamily *Rhinoternitinae*
  - Subfamily *Prorhinotermitinae*
- Family Termosidae (Grassé, 1949)
  - Subfamily *Termopsinae*
  - Subfamily *Porotermitinae*
  - Subfamily *Stolotermitinae*
- Family Serritermitidae (Emerson, 1965)

HIGHER TERMITES
- Family Termitidae (Light, 1921)
  - Subfamily *Termitinae*
  - Subfamily *Apicotermitinae*
  - Subfamily *Macrotermitinae*
  - Subfamily *Nasutitermitinae*

2.2 The Intestinal Tract of Termites

The digestive system of termites consists of the foregut, the midgut and the hindgut. The foregut consists of the oesophagus, the crop and the gizzard (Plate 1, 2, 3 and 4). The oesophagus is a simple, narrow tube, which extends as far as the middle of the thorax. The crop follows it and is not clearly separated, having practically the same structure (Plate 1, 2, 3 and 4). The gizzard is not generally separated from the crop, but its musculature is much more powerful. The foregut terminates with an esophageal valve which is always well developed and which penetrates deeply into the midgut (Plate 1, 2, 3 and 4). The midgut is a tube of uniform diameter and its histological structure is remarkably constant. The muscular connective envelope is only slightly developed. It includes a layer of almost continuous circular fibers and bundles of longitudinal fibers, some external and some internal to the circular fibers (Noirot and Noirot-Timothee, 1969; Bignell *et al.*, 1979).

The hindgut is always well developed, and it exhibits important variations depending on the different groups and their nutritive regime. According to Holmgren (1909), five successive segments may be distinguished. The first segment has an extremely variable length and volume. It may be very short, long, tubular, or it may even have a pronounced dilation. The second segment is formed by a differentiation, which is characteristic of the termites, the enteric valve (Grassé and Noirot, 1954). This valve generally assumes the form of a muscular funnel invaginated into the third segment. The third segment, whose entrance is controlled by the enteric valve, consists of a voluminous dilation, or paunch, containing an abundance of symbiotic microorganisms. This segment narrows

progressively in its posterior part and drains into the following segment without any clear limits. The fourth segment is, generally, a narrow and contorted tube, often called the colon, of variable, but always-considerable length (Grassé and Noirot, 1954). The rectum, which has a uniform structure and exhibits varying degrees of development, forms the fifth segment. It is an elongated ampul, muscular, larger than the preceding sections, and capable of dilation. As in many insects, the rectum of the termites has six more or less conspicuous longitudinal thickenings, forming the rectal papillae, or rectal glands. The papillae do not extend as far as the anus. A chamber with a thin pleated wall forms the terminal part of the rectum, which is capable of considerable dilation. The importance of this terminal chamber varies greatly among the species (Grassé and Noirot, 1954).

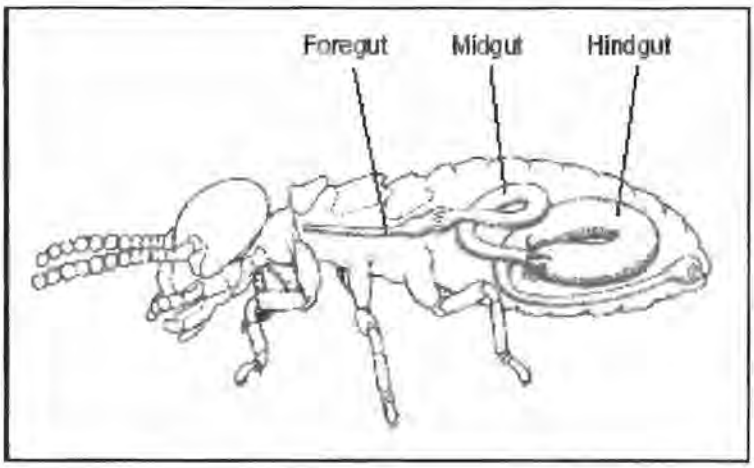


Plate 1: Morphology of the gut in worker castes of wood-feeding termite *Reticulitermes flavipes* belonging to the lower termites (Brune 1998).

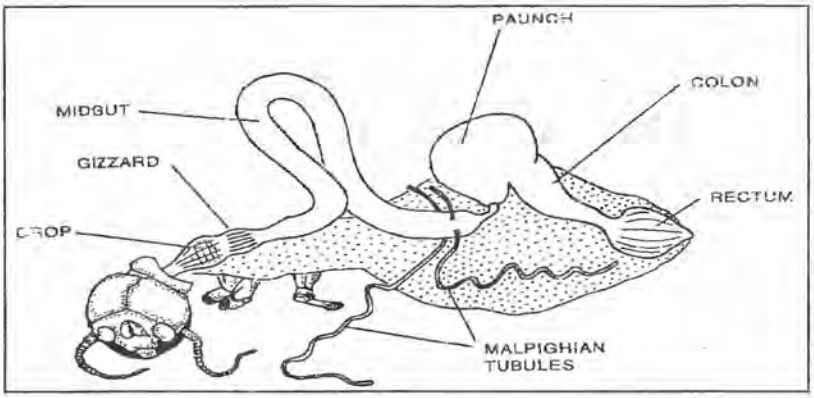


Plate 2: Morphology of the gut in worker castes of fungus-growing termite *Macrotermes subhyalinus* belonging to the higher termites. (Anklin-Muhlemann *et al.*, 1995).

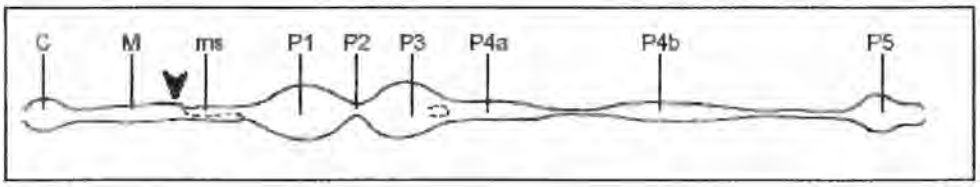


Plate 3: Gut morphology of the 'higher' soil-feeding termite *Thoracotermes macrothorax* (worker). Abbreviations: C, crop; M, midgut; ms, mixed segment; P1–5, proctodeal regions. Arrowhead marks the insertion point of the malpighian tubules. (Brune 1998).

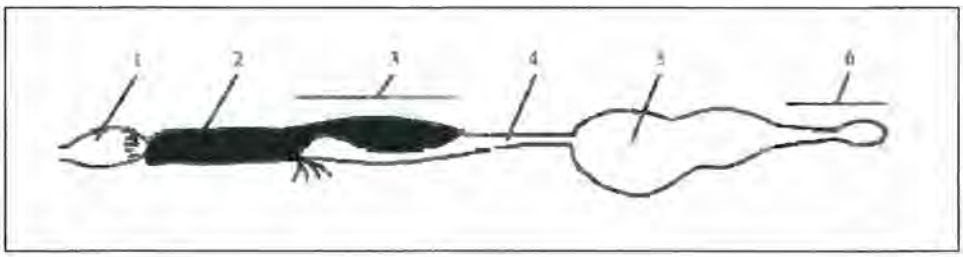


Plate 4. Gut morphology of the 'higher' wood-feeding termite, *Nasutitermes. takasagoensis.* A schematic drawing of the gut showing the following: 1, foregut; 2, midgut; 3, mixed segment; 4, first proctodeal segment; 5, enteric valve and paunch; and 6, colon and rectum (Tokuda *et al.*, 2000).

2.2.1 The intestinal tracts of lower termites

The arrangement of the digestive tube in the lower termites is not very variable, probably due to the constant presence of symbiotic flagellates in the paunch, which indicates a rather uniform physiology (Grassé and Noirot, 1954). The crop is essentially symmetrical. The gizzard possesses a typical chitinized armature. The midgut, which is relatively long, always terminates in a normal manner (Plate 1). The malpighian tubules are inserted at the limit of the mesenteron-proctodeum (Grassé and Noirot, 1954). There are eight malpighian tubules except in *Mastotermes*. The first segment of the hindgut forms a cone, more or less elongated and regular, whose tip corresponds to the enteric valve. This valve invaginates more or less deeply into the paunch but is never armed. The paunch, which is always very voluminous, is sometimes incompletely subdivided by a transverse constriction; the colon is always very short (Grassé and Noirot, 1954). The hindgut of lower termites has been considered an anoxic habitat where anaerobic microorganisms ferment wood polysaccharides to short-chain fatty acids, which in turn serve as the sole carbon and energy source for the host (Noirot and Noirot-Timothee, 1969; Bignell, 1994). Recent results emphasize that the termite gut is not a simple anoxic fermenter, but rather the microbial activities render it an axially and radially structured heterogeneous system characterized by steep gradients, which, in turn, govern the spatial arrangement of individual metabolic groups of the gut microbiota (Brune, 1998).

2.2.2 The intestinal tracts of higher termites

In Termitidae the arrangement is varied (Plate 2, 3 and 4). There are always four malpighian tubules. In many cases the mesenteron is considerably prolonged on one of

the faces of the intestinal tube, resulting in a mixed segment (Grassé and Noirot, 1954), so-called because the intestinal lumen is limited on one side by the mesenteron and on the other by the proctodeum. When such a mixed segment occurs, it always contains an abundance of bacteria, having the appearance of a pure culture. The mixed segment remains exterior to the peritrophic membrane and do not mix with the alimentary bolus (Grassé and Noirot, 1959). One may suppose that the constant association of the mixed segment with bacteria is of a symbiotic character.

In Macrotermitinae, the structure of the digestive tube is very uniform and very close to that of the lower termites, notably the Kalotermitidae and Rhinotermitidae which lack the mixed segment and the relatively un-extended nature of the hindgut (Plate 2) (Noirot and Noirot-Timothee, 1969; Bignell, 1994). By comparison the gut structure is very diverse in other Termitidae with extensive compartmentalization of the hindgut (Noirot and Noirot-Timothee, 1969; Bignell *et al.*, 1979). The gizzard possesses a well-developed typical armature. The midgut is relatively long and. there are four malpighian tubules, which are inserted in a symmetrical manner at the junction of the mesenteron-proctodeum (Grassé and Noirot, 1954). The hindgut begins with a very short, narrow segment and terminates in a muscular enteric valve, which commands the entrance to a voluminous paunch (Grassé and Noirot, 1954). It is unclear whether the relatively simple gut of Macrotermitinae lacking the elongated and highly differentiated hindgut typical of other higher termites, reflects the absence of a symbiotic relationship with intestinal bacteria (Anklin-Muhlemann *et al.*, 1995). Except in the fungus-cultivating Macrotermitinae, the anterior hindgut compartments of higher termites are generally quite alkaline (Bignell and Eggleton, 1995). The hindguts of soil feeding termites are more elongated and

compartmentalized than those of any other feeding guilds (Plate 3). Microscale pH measurements showed that the pH increases sharply in the anterior hindgut, and decreases again towards the rectum (Brune and Kühl 1996). Gut alkalinity in the soil-feeding Termitinae exceeds pH 12 and belongs to the highest pH values ever encountered in biological systems (Brune and Kühl, 1996).

2.3 Nutritional biology of termites

Of all the intriguing activities and properties of termites, none seems as widely recognized as the ability of termites to utilize wood as a food resource. Indeed many species of termites thrive on sound (decay free) wood, which contains very little nitrogen dry weight (Cowling and Metril, 1996). However it is also important to recognize that there are over 2,700 species of termites with varying biological and nutritional features (Wood and Johnson 1986). For example, many species of termites prefer wood that is particularly decayed by fungi (Lee and Wood, 1971), while others (subfamily *Macrotermitinae*) are known to cultivate fungi in elaborate gardens (fungus combs) for use as a nutrient source (Sand, 1969), yet other species feed on leaves, roots, grass, dung of herbivorous animals and humus (Donovan *et al.*, 2001).

The diet of termites is diverse but basically rich in cellulose, hemicellulose and lignin or lignin derivatives. This trait places termites in an important ecological position, particularly in the tropical region, where their activities can dominate the process of decomposition and nutrient cycling. Further the biomass density of termites can be so large (10-20 g m^{-2}) that its impact is similar to and may surpass that of grazing mammals (Wood and Sands, 1978).

2.3.1 Wood feeding termites

These are termites that feed on wood and woody litter, including dead branches still attached to trees. Most lower termites are wood-feeders and there are wood-feeding species in all subfamilies of the Termitidae except the Apicotermitinae (Bignell and Eggleton, 1995). The main part of the digestion takes place in the hindgut especially in the paunch, under the action of symbiotic microorganisms. It is well established that lower termites digest cellulose using synergetic actions of the cellulolytic enzymes that originate from the termites guts and symbiotic protozoa in the hindgut. The protozoan fauna accounts for most of the cellulolytic activities in the hindgut (Breznak, 1982; O'Brien and Slaytor, 1982). Despite the efforts to define the origin of cellulolytic enzymes in the digestive system, the precise involvement of each protozoa species still remains unsolved in cellulose metabolism (Hogan *et al.*, 1988; McEwen *et al.*, 1980; O'Brien *et al.*, 1979; Yamaoka and Nagatani, 1975; Veivers *et al.*, 1982). The role of bacteria in the digestion processes of lower termites is controversial. However, Potrikus and Breznak (1977) recorded evidence of significant nitrogen fixation in both microbial isolates and intact termites.

2.3.2. Soil feeding termites

The higher termites undergo trophic diversification and utilize not only majority of the plant resource types including wood, but also soil organic matter (Lee and Wood, 1971). Their symbiotic association has been examined in wood feeding termites *Macrotermitinae* (Martin and Martin, 1979; Rohmann and Rossman, 1980) and wood feeding *Nasutitermitinae* (Eutic *et al.*, 1978). Due to the complex organization of the gut

(Crousse and Noirot, 1958), it is assumed that bacteria play a primary role in digestive process. Protozoa are not thought to contribute significantly to the nutrition of higher termites (Honigberg, 1970). The humivorous mode of nutrition rendered the Termitidae independent of the necessity to harbor cellulolytic flagellates as symbionts, and thereby probably removed important evolutionary constraints, allowing further diversification of the gut (Noirot, 1992). Soil-feeding termites ingest large amounts of soil (Wood, 1978; Okwakol, 1980), and due to their high biomass densities, their feeding activity is important for the biomass turnover in tropical and subtropical ecosystems (Wood and Johnson, 1986; Wood, 1988; Collins, 1989; Martius, 1994; Bignell *et al.*, 1997). The food ingested by soil-feeding termites is quite heterogeneous. The gut contains predominantly soil minerals and humus, but also plant tissue fragments, plant roots, fungal mycelia and macerated organic material (Donovan *et al.*, 2001).

2.3.3 Fungus-cultivating Termites

Fungus-growing termites are abundant in the African and Asian tropics (Wood and Sands, 1978; Abe and Matsumoto, 1979). They play a significant role in the decomposition of plant litter, for example consuming more than 90% of dry wood in some arid tropical areas (Buxton, 1981) and directly mineralizing up to 20% of the net primary production in wetter savannas (Wood and Sands, 1978). They have evolved a unique mutualism with basidiomycete fungi of the genus, *Termitomyces*. The symbiotic fungi grow on a special culture within the nest maintained by the termites and called 'fungus comb' (Hyodo *et al.*, 2000).

The fungus comb is made from partly digested foraged plant litter, which passes rapidly through the termite's gut. The resulting faecal pellets are pressed together to make a comb-like matrix. As the comb matures, mycelium develops and produces conidial nodules, which together with older, senescent comb are consumed by workers. Because of the unique symbiotic relationship, many studies have been conducted on the termite-fungus association (reviews by Sands, 1969; Darlington, 1994). Several roles have been suggested for the fungal symbiont, for example, the provision of heat and moisture (Lusher, 1951), the provision of a concentrated nitrogen source as conidia (Matsumoto, 1976) and the enrichment of nitrogen in foraged foodstuffs by virtue of the fungal metabolism (Collins, 1983). Compared with other termites, for example those with protozoa and bacteria as mutualists or those with intestinal bacteria only, it is unclear whether the fungus-growing termites can utilize cellulose in plant material with greater overall efficiency, because white-rot fungi also consume cellulose for metabolism. From the ecosystem point of view, however, it has been noted that by associating with the lignin decomposer, the fungus-growing termite make it possible to utilize lignocellulose nearly completely as reflected in the small volume of their final faeces (Darlington, 1994) and therefore to play a dominant role in decomposition processes in many parts of the tropics (Abe, 1980; Buxton, 1981).

2.4 Microbial Diversity in termite guts

The mutualistic relationship between termites and microorganisms inhabiting their hindgut is a well-known example of symbiosis, which aids termites to live exclusively on lignocellulose (Ohkuma, 2002). The isolation and cultivation of several members within the gut microbial community of termites have contributed to clarification of some

beneficial roles of the symbiotic microbiota to the host termites (reviewed by Breznak, 2000). However most of the predominant species of the community are difficult or even impossible to cultivate *in vitro,* rendering the understanding of the symbiosis limited. In the past decade molecular approaches mainly based on 16S rDNA sequences have improved the ability to assess naturally occurring biodiversity without cultivation of the resident microorganisms (Ohkuma, 2002).

The hindgut of *Reticulitermes flavipes*, which is probably one of the best investigated termite from the microbiological point of view, harbors at least six species of flagellates (Cook and Gold, 1998), and 20 - 30 different bacterial morphotypes (Breznak and Brune, 1994). Typically the diversity of bacterial 16S rDNA genes in termite guts is high. Analysis of 16S rDNA sequences in a wood-feeding termite, *Reticulitermes speratus*, revealed that of the 55 clones sequenced, most belonged to four of the major groups of the domain *Bacteria:* the *Proteobacteria*, the spirochete group, the *Bacteroides* group and the low G + C-content gram-positive bacteria. Of the 55 clones sequenced, 10 showed no close sequence similarity to any recognized bacterial phylum in the rDNA database (Ohkuma and Kudo, 1996). Clone libraries obtained from a soil-feeding termite, *Cubitermes orthognathus*, comprised a variety of phyla, including the gram-positive bacteria with low G + C content, the *Cytophaga-Flexibacter-Bacteroides* group various subgroups of *Proteobacteria* and the spirochetes (Schmitt-Wagner *et al.*, 2003). In another study, *in situ* morphology of the gut microbiota using electron microscopy, of the fungus-growing termite *Odontotermes formosanus* showed a diverse bacterial flora, consisting of at least seventeen morphotypes and among them were four morphotypes of spirochetes. The bacterial population was comprised mainly of long rods (Yara *et al.*,

1989). In a study on molecular profiling of prokaryotic communities in the guts of termites with different feeding habits (Brauman *et al.*, 2001), seven species of wood-feeding termites and two species of fungus-growing Macrotermitinae, showed abundance in bacterial and relatively low archaeal signals.

2.4.1 Spirochetes

Spirochetes are a morphologically diverse group and may account for as much as 50% of all prokaryotes in some termites. The 12 to 15 spirochete morphotypes in *R. flavipes* were paralleled by 21 different spirochete phylotypes, which were assigned to two major clusters of treponemes within the phylogenetic radiation of spirochetes (Lilburn *et al.*, 1999). Leadbetter *et al.*, (1999) were the first to isolate two gut spirochetes from *Zootermopsis angusticollis*. Both isolates were capable of hydrogen-dependent acetogenesis from carbon dioxide (Leadbetter *et al.*, 1999). This finding represents the first clue regarding the so far unknown metabolic function of the spirochetes colonizing the hindgut lumen and the surfaces of many protozoa in termite guts (Brune and Friedrich, 2000).

2.4.2 Lactic Acid Bacteria

Lactic acid bacteria are typical and numerically significant carbohydrate-utilizing microorganisms in the guts of many wood-feeding and soil-feeding termites (Tholen *et al.*, 1997; Bauer *et al.*, 2000). Their presence in *R. flavipes* was confirmed by 16S rRNA fingerprinting of the total hindgut community (Bauer *et al.*, 2000). Isolates obtained from the hindguts of *Reticulitermes flavipes* and *Thoracotermes macrothorax* show a

considerable genetic diversity, and comprise strains belonging to the genera *Enterococcus* and *Lactococcus* (Bauer *et al.*, 2000). All isolates proved to be aerotolerant and exhibit high potential rates of oxygen reduction in the presence of fermentable substrates, which may explain why they are regularly encountered in the intestinal tracts of termites and other insects (Bauer *et al.*, 2000).

2.4.3 Sulfate-reducing bacteria

Sulfate-reducing bacteria also seem to be common inhabitants of the intestinal tracts of many different termite species (Kuhnigk *et al.*, 1996; Fröhlich *et al.*, 1999; Ohkuma and Kudo, 1996). All isolates are related to free-living sulfate reducers of the genus *Desulfovibrio* and show high rates of O_2 reduction in the presence of H_2 (Kuhnigk *et al.*, 1996).

2.4.4 Methanogenic Archaea

Another abundant group of microorganisms in termite guts are the methanogenic Archaea. Only three species of methanogens from termite gut have been isolated in pure culture. These isolates from the hindgut of *R. flavipes* were identified as members of the genus *Methanobrevibacter* (Leadbetter and Breznak, 1996; Leadbetter *et al.*, 1998). Methanogens are among the few groups of organisms where one can infer metabolic information from the 16S rRNA gene sequence (Brune and Friedrich, 2000). Clonal 16S rRNA gene sequences of archaeal gut symbionts were retrieved from wood-feeding lower termites such as *Cryptotermes domesticus* (Ohkuma and Kudo, 1998), *Nasutitermes takasagonensis* (Ohkuma *et al.*, 1995), and *Reticulitermes speratus* (Shinzato *et al.*,

1999), the fungus cultivating *Odontotermes formosanus* (Ohkuma *et al.*, 1995) and the higher soil-feeding termite *Pericapritermes nitobei* (Ohkuma *et al.*, 1995).

2.4.5 Bacteroides

Isolation of *Bacteroides* species from termites has also been reported. In one reported case, cross feeding of lactate from a lactate-producer to a *Bacteroides* species was demonstrated (Schultz and Breznak, 1979), while in another case, a uric acid-degrading bacterium isolated from the termite gut was identified as *Bacteroides termitidis* (Potrikus and Breznak, 1980). Since most of the *Bacteroides* species are known to be fermentative and acidogenic, the organisms assigned to *Bacteroides* cluster may also have the same function (Ohkuma and Kudo 1996).

2.4.6 Actinomycetes

Bignell *et al.*, (1979) using electron microscope, observed actinomycete-like bacteria in the guts of termites. Actinomycetes were isolated from termite guts by Pasti and Belli (1985), and were found to have cellulolytic activity. Actinomycetes were also later observed to have lignin-solubilizing activity (Pasti and Belli, 1985; Pasti *et al.*, 1990). In another report, isolates obtained from *Coptotermes formosanus* and *Reticulitermes speratus* guts were identified using 16S rDNA analysis as actinomycetes belonging to the genus *Streptomyces* (Watanabe *et al.*, 2003).

2.4.7 Homoacetogenic Bacteria

Boga *et al.* (2003) isolated and characterized *Sporomusa aerivorans,* a homoacetogenic bacterium from the gut of the soil feeding *Thoracotermes macrothorax.* It was established that *Sporomusa aerivorans* could reduce oxygen with hydrogen and endogenous electron donors. Breznak *et al.,* (1988), Kane and Breznak, (1991), and Kane *et al.,* (1991), also previously isolated homoacetogenic bacteria from termite guts. The intestinal tracts of animals are generally characterized by the coexistence of homoacetogenic and methanogenic microorganisms (Breznak, 1994). In principle, homoacetogenesis is considered advantageous for the host organism, which is able to use acetate as a carbon and/or energy source (Breznak and Kane 1990). However the numbers of H_2-oxidizing methanogenic archaea cultivated from the rumen or cecum contents of different mammals are often orders of magnitude higher than those of homoacetogenic bacteria (Morvan *et al.,* 1996). The metabolic versatility of homoacetogens, which are generally capable of utilizing a wide variety of substrates (Breznak, 1994) including many of the fermentation products found in the hindgut fluid of soil-feeding species of Termitinae (Tholen and Brune, 1999), and methoxylated aromatic compounds derived from lignins or humic substances, might help them to maintain an active metabolism during phases of low H_2 pressure. Also their ability to simultaneous utilize of H_2 and organic substrates, as demonstrated for *Sporomusa termitida* isolated from wood-feeding *Nasutitermes nigriceps* (Breznak and Blum, 1991), would add to their competitiveness in an environment where H_2 is only temporarily available. It has been recently discovered that spirochetes isolated from the wood-feeding *Zootermopsis angusticollis* are homoacetogenic (Leadbetter *et al.,* 1999), but the

metabolic properties of the spirochetal morphotypes present in the P4b section of *Cubitermes umbratus* (Tholen and Brune, 1999), remain to be established.

3.2 Gut preparation for dilution series

Termites were dissected with sterile fine-tipped forceps, and guts were homogenized ten guts per milliliter, in sterile buffered salt solution (BSS) (Breznak and Switzer, 1986) by using a glass tissue homogenizer and serially diluted in ten-fold dilution steps up to dilution 10^{-10}. BSS contained K_2HPO_4 [2.0 g], KH_2PO_4 [1.0 g], KCL [1.5 g] and NaCl [1.5 g] per liter at pH 7.0.

3.3 Growth medium

Dilutions were inoculated in Kenya minimal medium for aerobes (KMM1), which was, based on MM5 medium (Boga *et al.*, 2003), except Napthoquinone, 4-hydroxyacetic acid and 3-indolyl acetic acid, aromatic fatty acids (AFAs) and menadion were excluded. KMM1 medium contained NaCl [1.7 g], KCl [6.5 g], $MgCl_2.6H_2O$ [0.50 g], $CaCl_2.2H_2O$ [0.10 g], NH_4Cl [5.6 g], $NaSO_4$ [1.0 g] and KH_2PO_4 [1.0 g] per liter. The medium was supplemented with yeast extract and casamino acids (each 0.1%; Difco, Detroit, MI, USA). After the medium cooled the following were added from sterile stock solutions; 1 M Na-phosphate buffer [40 ml; pH 7.0], SL 11 [2 ml], Se/W solution [2 ml], 7-vitamin solution [2 ml], Folic acid [2 ml; 50 mg/l], Riboflavin [2 ml; 50 mg/l], branched chain VFA's [2 ml; 25 mM] and Lipoic acid [2 ml; 1 mM]. Cultures were incubated at 30° C in the dark. All solutions, cultures and media were prepared and maintained under aerobic conditions and at pH 7.0. Substrates, glucose and gelatin were added to the medium at 0.1% w/v each and the medium was solidified with agar (1.5%w/v).

3.4 Culture-dependent methods

3.4.1 Plate counts

An aliquot of 0.1 ml from dilutions (10^3-10^6) in case of *M. michaelseni* and tubes with the lowest dilution (10^1-10^3) in case of *O. somaliensis* were plated in KMM1 plates containing solid KMM1 medium and also in plates containing KMM1 with the substrates (glucose and gelatin). All the experiments were prepared in duplicates with uninoculated controls which containing no inoculate for KMM1 medium only, KMM1 medium plus glucose and KMM1 medium plus gelatin and incubated at 30°C in the dark. The number of colonies was determined after two weeks of incubation. For each termite, seven colonies were selected according to different colony morphologies and purified by streaking them on to fresh KMM1 medium.

3.4.2 MPN Determination

Three-tube most-probable-number (MPN) determination was performed under aerobic conditions. Serial 10-fold dilutions of gut homogenates (10 guts ml^{-1}) were prepared in basal medium KMM1 (without agar) containing glucose or cellulose acetate membrane filter discs (as a source of carbon). Cellulose utilization was determined by addition of cellulose acetate filter paper discs in the liquid media for MPN analysis. The tubes were incubated for two weeks at 30°C and agitated at 200 rpm on a rotary shaker. Growth was evident by visible turbidity in culture media. Numerical data was computed using MPN probability tables (Alef and Nannipieri, 1995).

3.4.3 Molecular Characterization of Isolates

Eight bacterial isolates from *M. michaelseni* and seven bacterial isolates from *O. somaliensis* were picked and characterized using molecular techniques. Isolates were picked on the basis of different morphological characteristics. DNA was extracted and the 16S rDNA gene amplified and sequenced. 16S rDNA sequences were compared to sequences in public database with Blast

3.4.3.1 DNA Extraction

DNA was extracted from isolates obtained from the KMM1 plates using the GENECLEAN® Bio101 soil DNA extraction kit with a few modifications. The isolates were grown for three days in KMM1 liquid medium and centrifuged.

The pellet was transferred in a microcentrifuge tube with acid washed glass beads and 1 ml of bead beating buffer pH 8.0 (100 mM NaH_2PO_4, 100 mM EDTA, 100 mM Tris 1.5 M NaCl 0.5% Sarkosyl, 2% polyethylene Glycol) in the presence of 25 mg/ml lysozme. The microcentrifuge tubes were vortexed on high for 10 minutes and incubated at 37°C for two hours, to allow complete lysis of the cells. Incubation of the same isolates for 90 minutes had not yielded any DNA. After incubation, the tubes were centrifuged at 14,000 rpm for 30 seconds to pellet the sample. The supernatant was transferred to a clean 1.5 ml epitube and 20 µl of 3 M sodium acetate (pH 5.2) was added and mixed gently by inversion. Particulates including cell debris, beads and humic acids, form a pellet and DNA is left in the liquid supernatant at this stage. Tubes were centrifuged again at 14,000 rpm for five minutes and the supernatant was transferred equally into two 1.5 ml epitubes and 500 µ l of well-mixed binding matrix suspension was added to each tube. Tubes were

inverted for two minutes to allow DNA binding to take place and placed in a tube rack to settle for five minutes. The binding matrix is a DNA binding salt solution. DNA is selectively bound to the silica membrane in the presence of high salt concentrations. The supernatant was removed without disturbing the binding matrix and discarded. The matrix in both tubes was then resuspended and transferred into one spin filter. This was centrifuged at 14,000 rpm for one minute and the catch tube was emptied of flow through. A volume of 500 µl of 100% ethanol was added to the spin filter centrifuged at 14,000 rpm for one minute and the catch tube was emptied of flow through. This was carried out as a washing step to further clean the DNA. The dry filter was placed in a new catch tube and left standing for five minutes to dry. A volume of 50 µl of TE buffer (10 mM Tris-HCl [pH 7.6] 1 mM EDTA) was added and tubes vortexed to suspend the matrix. TE is an elution buffer, which passes through the silica membrane allowing the release of DNA. The tubes were then centrifuged at 14,000 rpm for one minute and an additional 50 µl of TE buffer was added and centrifuged again. Purified DNA was visualized by gel electrophoresis.

3.4.3.2 PCR amplification of bacterial 16S rDNA

Purified total DNA from each isolate was used as a template for amplification of 16S rDNA genes. This was done using HotStarTaq Master Mix kit (Qiagen®) according to the manufacturer's instructions. PCR amplification was performed with a model PTC-100 thermal cycler (MJ Research Inc., USA) 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). Each PCR reaction contained 25 µl of HotStarTaq Master mix, 0.5 µl of 27F forward primer, 0.5 µl of 1392R reverse primer, 1 µl of DNA template and 23 µl water in a final volume of 50 µl. A control containing all the above except the DNA template was also prepared. The PCR reactions were started by an initial activation of the enzyme at 95°C for 10 minutes followed by 25 cycles consisting of denaturation (30 seconds at 94°C), annealing (one minute 45 sec at 55°C) and extension (two minutes at 68°C) and a final extension at 68°C for 10 minutes. The amplification product was verified by electrophoresis in 1.0% agarose gel. The PCR products were purified and sequenced at Diversa Corporation (U.S.A) using an automatic sequence analyzer (Applied Biosystems model 3700).

3.4.3.3 Phylogenetic analysis

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. Sequence data were analyzed with the ARB software package [Version 2.5b; O. Strunk and W. Ludwig, Technishe Universität München (http://www.arb-home.de)]. The new sequences were added to ARB database and aligned with the Fast Aligner tool (Version 1.03). Alignments were checked and corrected manually where necessary. Clonal 16S rDNA gene sequences were compared to sequences in public databases with Blast (Altschul *et al.*, 1990). 16S rDNA gene sequences with high similarities to those determined in this study were retrieved and added to the alignment. Highly variable regions of the 16S rDNA gene sequences and sequence positions with possible alignment errors were excluded by using only those positions of the alignment that were identical in at least 50% of all sequences. Phylogenetic trees were constructed by the neighbor-

joining method (Saitou and Nei, 1987) with the PHYLIP package (version 3.5c) (Felsenstein, 1989). Bootstrap analyses for 100 replicates were performed to attach confidence estimates for the tree topologies.

3.5 Culture-independent methods

3.5.1 DNA Extraction

A total of 10 guts from *M. michaelseni* termites weighing approximately 70 mg and a total of 50 guts from *O. somaliensis* termites weighing approximately 73 mg were removed and total DNA extraction was carried out using GENECLEAN® Bio101 soil DNA extraction kit as indicated below. DNA extraction from both termite species was carried out separately.

The guts from *M. michaelseni* were pooled together in a microcentrifuge tube and those from *O. somaliensis* pooled together in a separate microcentrifuge tube. Each tube contained acid washed glass beads and 1 ml of bead beating buffer pH 8.0 (100 mM NaH_2PO_4, 100 mM EDTA, 100 mM Tris 1.5 M NaCl 0.5% Sarkosyl, 2% polyethylene Glycol) in the presence of 25 mg/ml lysozme. The microcentrifuge tubes were vortexed on high for two minutes and incubated at 37°C for 90 minutes. This step aids in cell lysis. After incubation, the tubes were vortexed then centrifuged at 14,000 rpm for 30 seconds to pellet the sample. The supernatant was transferred to a clean 1.5 ml epitubes and 20 µl of 3 M sodium acetate (pH 5.2) was added and mixed gently by inversion. Particulates including cell debris, beads and humic acids, will form a pellet at this point. DNA is in the liquid supernatant at this stage. Tubes were centrifuged again at 14,000 rpm for five minutes and the supernatant was transferred equally into two 1.5 ml epitubes and 500 µl

of well-mixed binding matrix suspension was added to each tube. Tubes were inverted for two minutes to allow DNA binding to take place and placed in a tube rack to settle for five minutes. The binding matrix is a DNA binding salt solution. DNA is selectively bound to the silica membrane in the presence of high salt concentrations. Supernatant was removed without disturbing the binding matrix and discarded. The matrix in both tubes was then resuspended and transferred into one spin filter. This was centrifuged at 14,000 rpm for one minute and the catch tube was emptied of flow through. A volume of 500 µl of 100% ethanol was added to the spin filter centrifuged at 14,000 rpm for one minute and the catch tube was emptied of flow through. This was carried out as a washing step to further clean the DNA The dry filter was placed in a new catch tube and left standing for five minutes to dry. A volume of 50 µl of TE buffer (10 mM Tris-HCl [pH 7.6] 1 mM EDTA) was added and tubes vortexed to suspend the matrix. TE is an elution buffer, which passes through the silica membrane allowing the release of DNA. The tubes were then centrifuged at 14,000 rpm for one minute and an additional 50 µl of TE buffer was added and centrifuged again. Purified DNA was visualized by gel electrophoresis.

3.5.2 PCR amplification of bacterial 16S rDNA

Purified total DNA from each termite was used as a template for amplification of 16S rDNA genes. This was carried out as described in section 3.4.3.2 (page 28).

The amplified product was verified by electrophoresis in 1.0% agarose gel. The PCR product was excised and eluted using the QIAquick® gel extraction kit protocol according to the manufacturer's instructions and used for cloning into a sequencing vector. The DNA fragments were excised from the agarose gel with a clean scalpel and weighed in a

colorless tube. A volume of 221.7 µl Buffer QC was added. This was incubated at 50°C for 10 minutes till the slice dissolved completely. A volume of 73.9 µl of isopropanol was then added to the sample and mixed. To bind DNA, the sample was applied to a QIAquick® column and centrifuged for one minute. The flow-through was discarded and QIAquick® column placed back in the same collection tube. To wash the DNA, a volume of 0.75 of Buffer PE was added to the QIAquick® column and centrifuged for one minute. The flow-through was discarded and the column centrifuged again for an additional one minute at 13.000 rpm to remove residual ethanol from Buffer PE. The QIAquick® column was placed in a 1.5 ml microcentrifuge tube and 50 µl of Buffer EB (10mM Tris-Cl, pH 8.5) added to elute DNA. Tubes were then centrifuged for one minute.

3.5.3 Cloning PCR product into sequencing vector

Cloning of the PCR products was done using a kit from Invitrogene TOPO-TA® cloning kit for sequencing according to the manufacturer's instructions. The cloning reaction contained 3 µl of fresh PCR product, 1 µl of salt solution, 1 µl of sterile deionized water and 1 µl of TOPO® vector. These were mixed gently and incubated at room temperature for five minutes. For transformation of the cells, 3 µl of the cloning reaction was added into a vial of DHα5 chemically competent *E. coli* cells and mixed gently. Incubation was done for 30 minutes and the cells were heat shocked at 42°C for 30 seconds and then transferred immediately into ice for two minutes. A volume of 250 µl SOC medium was added and after shaking horizontally (200 rpm) at 37°C for one hour, 50 µl of the transformation was spread on a pre-warmed LB (Luria-Bertani) medium agar plates with

Kanamycin 50 µg/ml and incubated at 37°C overnight. Colonies were picked and inoculated in a tube with 3 ml of LB medium with Kanamycin 50 µg/ml and incubated in an incubator shaker (200 rpm) 37° C overnight. One hundred colonies were picked for each termite.

3.5.4 Plasmid extraction of individual clones

Volumes of 3 ml of the overnight cultures of *E. coli* grown in LB medium were centrifuged at 4000 rpm for 6 minutes to pellet down the cells. Plasmid extraction was carried out using QIAprep® Spin Miniprep kit protocol according to the manufacturer's instructions. Pelleted bacterial cells were resuspended in 250 µl of buffer P1 and transferred to microcentrifuge tubes. A volume of 250 µl of buffer P2 was then added and tubes were gently inverted to mix. Buffer N3 was added at a volume of 350 µl and mixed immediately. Tubes were centrifuged for 10 minutes at maximum speed in a tabletop microcentrifuge. Supernatants were transferred into QIAprep® column by decanting and then centrifuged for 60 seconds. The flow-through was discarded. The QIAprep® spin column was washed by adding 0.75 ml of buffer PE. Tubes were centrifuged for 60 seconds and the flow-through discarded. Residual wash buffer was removed by an additional centrifugation for one minute. QIAprep® columns were placed in clean microcentrifuge tubes and 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) was added to elute DNA. Tubes were left to stand for one minute and centrifuged for one minute. This was done to extract plasmid DNA containing the 16S rDNA inserts from the *E.coli* cells.

3.5.5 Restriction digest analysis

3.5.5.1 EcoRI digestion

To verify that the picked clones have the expected insert size, an EcoRI digestion was done on the extracted DNA. The digestion contained 1 µl EcoRI buffer, 1 µl EcoRI enzyme, 5 µl water and 3 µl of plasmid DNA per reaction to a final volume of 10 µl. Incubation was carried out at 37°C for 30 minutes and the digestion run on a 1.2% agarose gel stained with ethidium bromide and photographed (Sambrook *et al.*, 1989).

3.5.5.2 Restriction fragment length polymorphism (RFLP)

To compare restriction patterns of the selected clones, a digestion using a combination of six restriction enzymes was performed. The digestion contained 0.1 µl of the following restriction enzymes; AvaI, EcoRI, BamHI, HindIII, KpnI, and XbaI restriction enzymes, of each, 2 µl Buffer2, 0.2 µl BSA, 9.2 µl of water and 8 µl plasmid DNA per reaction. Incubation was done at 37°C overnight and the reaction run on a 2% agarose gel at 55 volts to visualize the different restriction patterns (Moyer *et al.*, 1994).

3.5.6 Phylogenetic analysis

Clones with unique restriction patterns were selected and sequenced using an automatic sequence analyzer (Applied Biosystems model 3700). 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. Sequence data were analyzed with the ARB software package [Version 2.5b; O. Strunk and W. Ludwig, Technishe Universität München (http://www.arb-home.de)]. The new sequences were added to ARB database

and aligned with the Fast Aligner tool (Version 1.03). Alignments were checked and corrected manually where necessary. Clonal 16S rDNA gene sequences were compared to sequences in public databases with Blast (Altschul *et al.,* 1990). 16S rDNA gene sequences with high similarities to those determined in this study were retrieved and added to the alignment. Highly variable regions of the 16S rDNA gene sequences and sequence positions with possible alignment errors were excluded by using only those positions of the alignment that were identical in at least 50% of all sequences. Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) with the PHYLIP package (version 3.5c) (Felsenstein, 1989). Bootstrap analyses for 100 replicates were performed to confidence estimates for the tree topologies.

CHAPTER FOUR

4.0 RESULTS

4.1 Enumeration of microorganisms

The gut of *M. michaelseni* showed the highest number of microbes in KMM1 medium giving a mean ± SD of 173.5 ± 8.9× 10^3 CFU/gut by plate count technique (Table 2). Using the MPN technique, the highest number of microbes was recorded in KMM1 medium without any substrate giving an approximate of 43 × 10^4 cells/gut. The highest number of microbes in *O. somaliensis* was recorded in KMM1 medium containing gelatin as a substrate giving a mean ± SD of 156 ± 25.45 × 10^3 CFU/gut (Table 2). Using the MPN technique, the highest number of microbes was recorded in medium containing glucose, which was approximately of 480 cells/gut. These may be due to the fact that *M. michaelseni*'s gut is much bigger than that of O. somaliensis. Cellulose was not utilized since the discs were still visible after two weeks.

Table 2. Results of plate counts and most probable numbers (MPN) of microorganisms obtained from gut dilutions of *M. michaelseni* and *O. somaliensis*

		KMM1	KMM1+ GELATIN	KMM1+ GLUCOSE	KMM1+ CELLULOSE
M. michaelseni	**Plate counts**	173.5 ± 28.9 × 10^3	139.5 ± 36.0 × 10^3	126.5 ± 23.3 × 10^3	n.d*
	MPN	43 × 10^3	n.d*	1.9 × 10^4	1.9 × 10^4
O. somaliensis	**Plate counts**	92.5 ± 12.02 × 10^3	156 ± 25.45 × 10^3	116.5 ± 4.94 × 10^3	n.d*
	MPN	48	n.d*	480	19

*n.d stands for not determined.

4.2 Characterization of Isolates

PCR amplification of bacterial 16S rDNA using bacterial universal primers was successful and yielded amplification products of approximately 1365bp as shown in Figure 1. This was visualized on a 1.0% agarose gel.

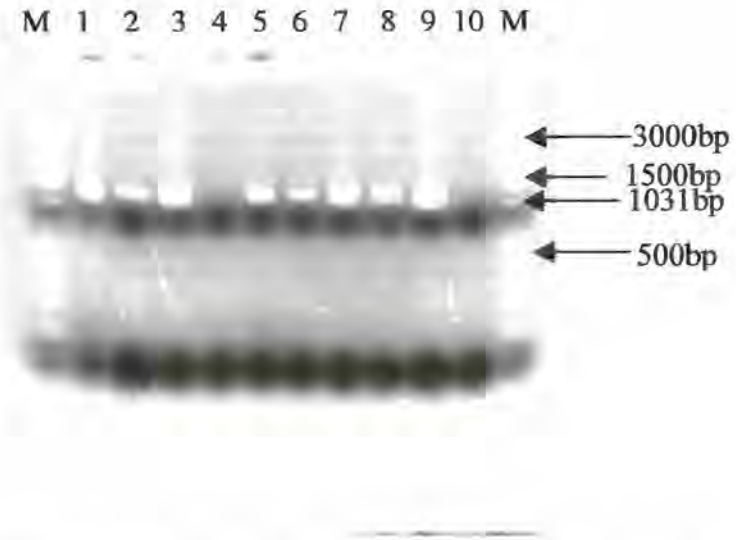


FIGURE 1. PCR amplification of 16S rDNA from isolates of *M. michaelseni* and *O. somaliensis* on a 1.0% agarose gel. Lanes 1-3 and 5-9 represent PCR products obtained from isolates. M is a 100bp DNA ladder (Biolabs) used as a molecular size marker.

The 16S rDNA sequences were compared to sequences in public database with Blast and sequences with high similarities to those of the isolates are shown in Table 3. Isolate IM 9A had a sequence similarity of 100% to unidentified *Streptomyces* species AY538691, while IM 14 had a 97% sequence similarity to *Streptomyces acidiscabies* AY207587. Isolate IM 15 had a sequence similarity of 99% to a *Streptomyces* species AF389343 isolated from China soil. Isolate IO 18 had a sequence similarity of 100% to *Streptomyces fungicidicus* AF429400 which was previously isolated from water damaged buildings, while IM 8A was related to a *Streptomyces* species AY529646 previously

obtained from marine samples with a 98% sequence similarity. Isolates IM 4, IO 11, IO 10A, IM 12, and IO 14C had a 99% sequence similarity to *Brevibacterium aureum* AY299093 previously isolated from a bioreactor. IM 5 had a sequence similarity of 99% to *Brachybacterium paraconglomeratum* AJ415377 previously isolated from deteriorated parts of a medieval wall painting. IO 14A was closely related to *Nocardia asteroides* AF430026 with a sequence similarity of 98%. Isolates IO 9 and IO 9B had a sequence similarity of 100% to *Bacillus licheniformis* AY291582 previously identified as natural cellulose degrading bacterium.

Table 3 Percentage sequence similarities of isolates with close relatives from the ribosomal database

ISOLATE	CLOSEST RELATIVE	ACCESSION NO.	% SEQUENCE SIMILARITY
IM 9A	*Streptomyces sp.*	AY538691	100
IM 14	*Streptomyces acidiscabies*	AY207587	97
IM 15	*Streptomyces sp.*	AF389343	99
IO 18	*Streptomyces fungicidicus*	AF429400	100
IM 8A	*Streptomyces sp*	AY529646	98
IM 4; IO 11; IO 10A	*Brevibacterium aureum*	AY299093	99
IM 12; IO 14C	*Brevibacterium aureum*	AY299093	99
IM 5	*Brachybacterium paraconglomeratum*	AJ415377	99
IO 14A	*Nocardia asteroides*	AF430026	98
IO 9; IO 19B	*Bacillus licheniformis*	AY291582	100

A total of 15 isolates were sequenced and shown to belong to the domain Bacteria. *M. michaelseni* isolates are represented by IM and IO represents *O. somaliensis* isolates. A phylogenetic tree containing each of the isolates is shown in Figure 2. The phylogenetic tree showed three main clusters. The first cluster comprised the genus *Streptomyces*. Isolates IM 9A, IM 15, IO 18 and IM 8A clustered with this genus and were supported by a bootstrap value of 100%. IM 9A clustered with *Streptomyces* sp. with accession number AY538691, IM 15 clustered with *Streptomyces* sp. AF389343 with a 100% bootstrap value, IO 18 formed a cluster with *Streptomyces fungicidicus* AF429400 with 100% bootstrap value and IM 8A clustered with *Streptomyces* sp. AY529646 with a bootstrap value of 100%. Isolate IM 14 also clustered with *Streptomyces* species with close relative IM 9A and *Streptomyces* species with accession number AY538691 with a bootstrap value of 100%. The second cluster comprised of *Brevibacterium, Arthrobacter, Brachybacterium* and *Nocardia* species. Isolates IM 4, IO 11, IO 10A, IM 12 and IO 14C clustered with the *Brevibacterium aureum* with a bootstrap value of 100%. IM 4 had the same nucleotide sequence as IO 11, IO 10A, IM 12 and IO 14C. IM 5 clustered with *Brachybacterium paraconglomeratum* with a bootstrap value of 100%. IO 14A clustered with *Nocardia asteroides* with a bootstrap value of 100%. The third cluster contained isolates IO 9 and IO 19B which clustered with *Bacillus licheniformis* with a bootstrap value of 100% and with *Bacillus subtilis* but with a low bootstrap value of 84%. Evaluation by CHECK-CHIMERA program of the Ribosomal Database Project (Maidak *et al.*, 2001) indicated one of the sequences of isolate IO 19A was chimeric hence it was removed from subsequent analysis.

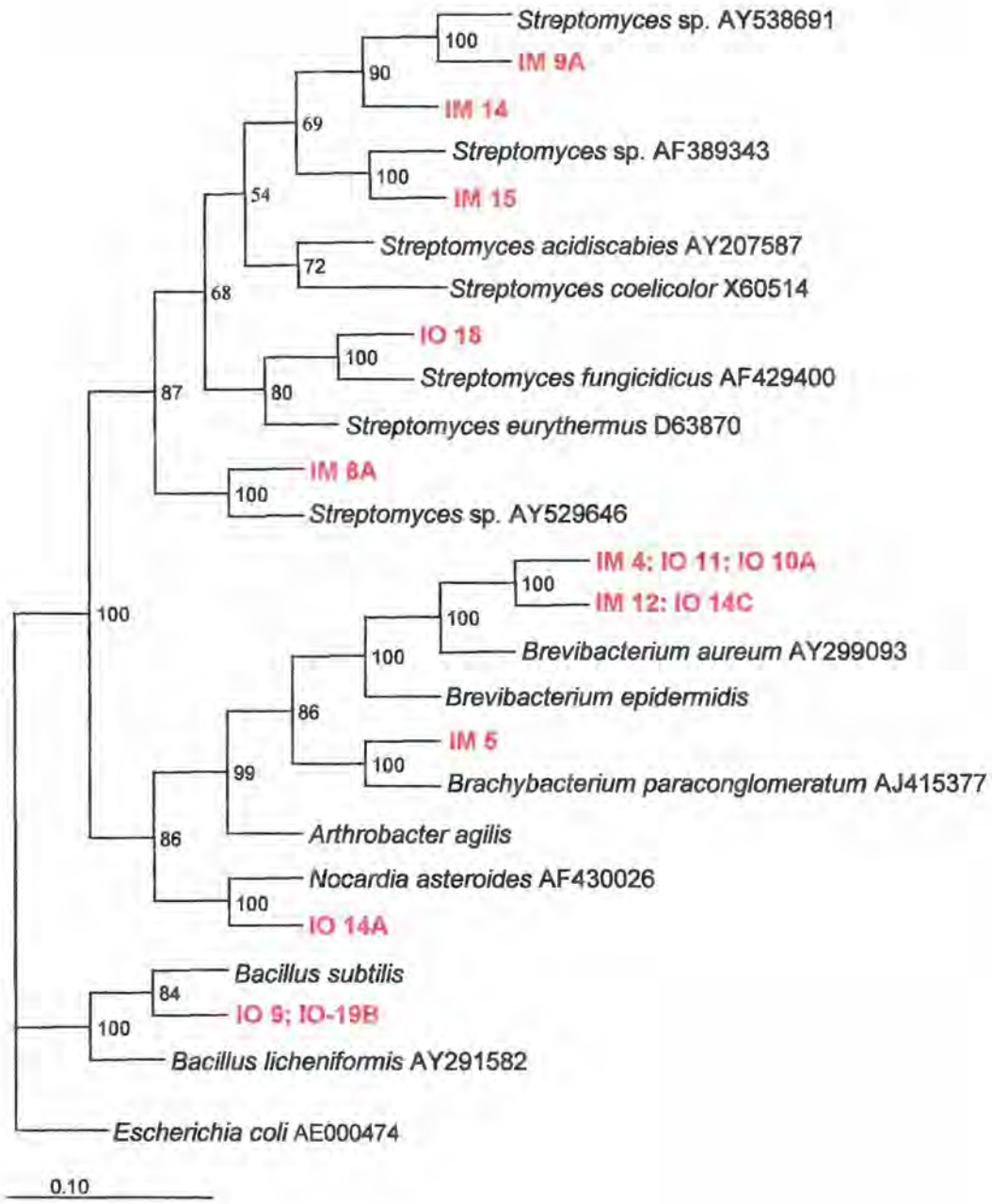


Figure 2. Phylogenetic tree showing positions of isolates obtained from *M. michaelseni* and *O. somaliensis*. The scale bar indicates approximately 10% sequence difference. Isolates obtained from *M. michaelseni* and *O. somaliensis* are shown in red. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resamplings. The 16S rDNA sequence of *Escherichia coli* AE000474 was used as the outgroup

4.3 PCR amplification of 16S rDNA gene

Polymerase chain reaction (PCR) amplification with bacterial universal primers for the ribosomal 16S rDNA gene portion was successful and yielded an amplification product of approximately 1365 bp from both termite samples as shown in Figure 3. This was visualized on a 1.0% agarose gel.

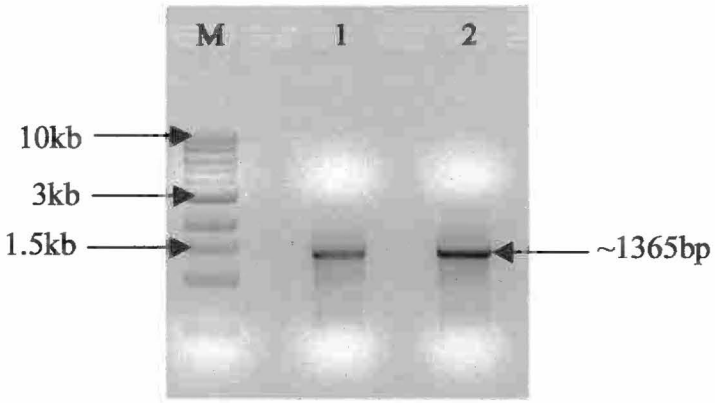
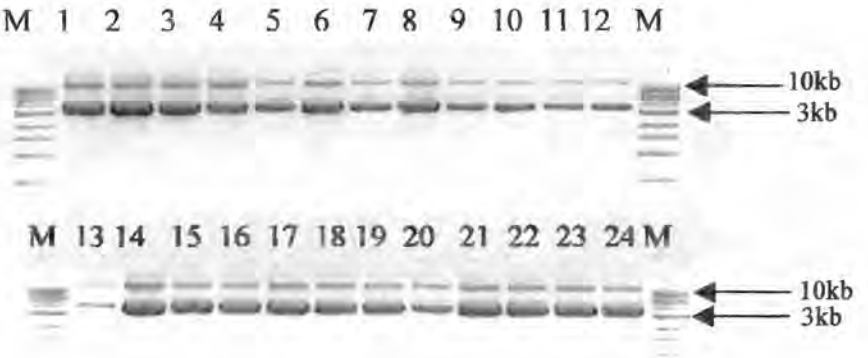


Figure 3. PCR amplification of 16S rDNA from the guts of *M. michaelseni* and *O. somaliensis* Amplified profile of 16S rDNA for *M. michaelseni* (Lane 1) and *O. somaliensis* (Lane 2) on a 1.0% agarose gel. M is a 1kb DNA ladder (Biolabs) used as a molecular size marker. (Size of bands of M 10,002 bp, 8,001 bp, 6,001 bp, 5,001 bp, 4,001 bp, 3,001 bp, 2,000 bp, 1,500 bp, 1,000 bp, 517 bp).

4.4 Plasmid purification

The 16S rDNA genes obtained from *M. michaelseni* and *O. somaliensis* were cloned in TOPO 10 vector separately. Transformation in DH5α cells produced both white and blue colonies. The presence of a large number of white colonies compared to blue colonies confirmed the transformation efficiency. A total of 100 white colonies were picked for each termite for plasmid purification. The expected size of the plasmid is approximately 4000 bp. The purified plasmids were visualized on 1% agarose gel (Figure 4 a, b) for *M. michaelseni* and *O. somaliensis* respectively.

a)



b)

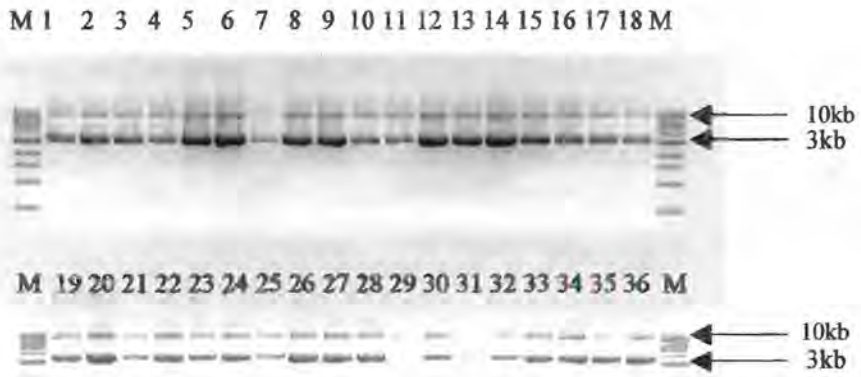


FIGURE 4. Plasmid purifications of clones **a)** Purified plasmid DNA carrying the 16S rDNA insert from *M. michaelseni*. Lanes 1-24 are representative plasmids from a total of 100 plasmids purified. M is a 1kb DNA ladder (Biolabs). (Size of bands of M 10,002 bp, 8,001 bp, 6,001 bp, 5,001 bp, 4,001 bp, 3,001 bp, 2,000 bp, 1,500 bp, 1,000 bp, 517 bp). **b)** Purified plasmid DNA carrying the 16S rDNA insert from *O. somaliensis*. Lanes 1-36 are representative plasmids from a total of 100 plasmids purified. M is a 1kb DNA ladder (Biolabs). (Size of bands of M 10,002 bp, 8,001 bp, 6,001 bp, 5,001 bp, 4,001 bp, 3,001 bp, 2,000 bp, 1,500 bp, 1,000 bp, 517 bp).

4.5 EcoRI Digestion

Digestion of purified plasmids with EcoRI restriction enzyme confirmed that all the plasmids had picked the 1365bp 16S rDNA gene insert. This was visualized on a 1% agarose gel (Figure 5 a, b). Some of the 16S rDNA genes had restriction sites for EcoRI enzyme hence displaying two bands on the gel with approximately 650 bp each for

example lanes 1 and 2 Figure 4 a, while others did not hence they had one band of approximately 1365 bp for example lanes 3 and 4 Figure 5 a.

a)

b)

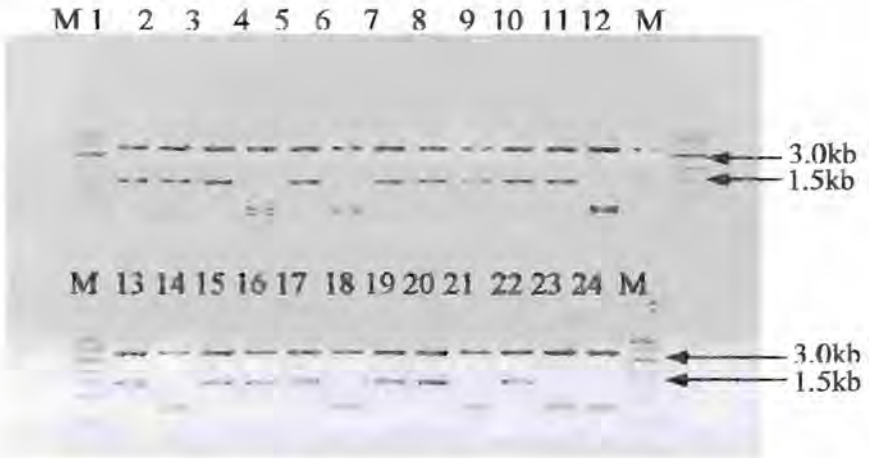


FIGURE 5. EcoRI digestion of purified plasmids Representative gels for: **a)** *M. michaelseni* Lanes 1-23 shows representative clones digested with EcoRI enzyme. M stands for the 1kb molecular size standard marker (Biolabs) (Size of bands of M 10,002 bp, 8,001 bp, 6,001 bp, 5,001 bp, 4,001 bp, 3,001 bp, 2,000 bp, 1,500 bp, 1,000 bp, 517 bp). **b)** *O. somaliensis* Lanes 1-12 shows representative clones digested with EcoRI enzyme. M stands for the 1kb molecular size standard marker (Biolabs) (Size of bands of M 10,002 bp, 8,001 bp, 6,001 bp, 5,001 bp, 4,001 bp, 3,001 bp, 2,000 bp, 1,500 bp, 1,000 bp, 517 bp).

4.6 Restriction fragment length polymorphism (RFLP)

Plasmids were analyzed by digestion with a cocktail of six enzymes. This digestion generated profiles that differed significantly (Figure 6 a, b). The banding profiles helped

distinguish the plasmids from each other. Plasmids with unique patterns were selected and sequenced for example lanes 1 and 2, Figure 6 a. Plasmids with the 16SrDNA insert obtained from *M. michaelseni* (Figure 6 a) formed patterns that were easy to distinguish from each other and hence 47 representative plasmids were picked for sequencing. Plasmids with the *O. somaliensis* 16S rDNA insert (Figure 6 b) formed patterns that were difficult to distinguish from each other for example lanes 8 and 12 (Figure 6 b) hence all the 100 plasmids purified were sequenced.

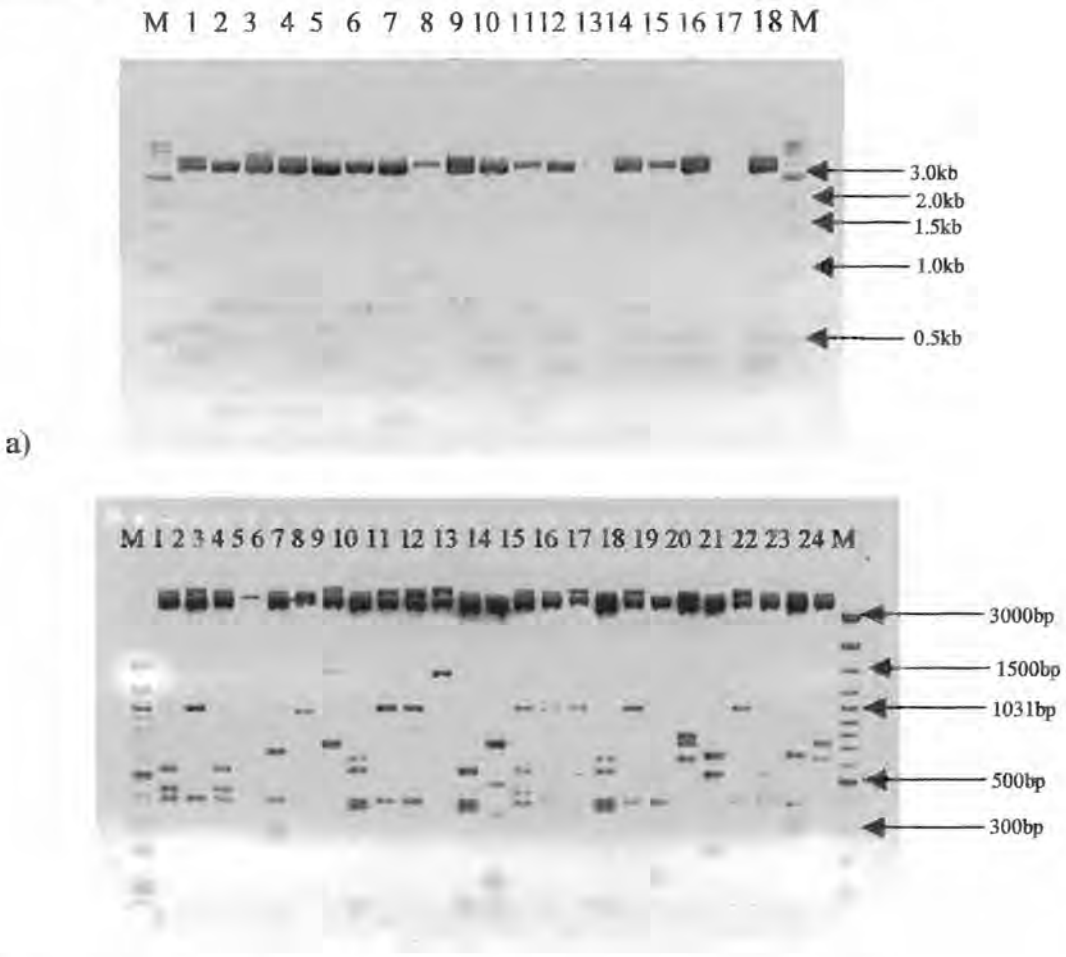


FIGURE 6. RFLP profiles of: *a) M. michaelseni* Lanes 1-18 represents clones digested with a cocktail of six enzymes. M stands for 1kb molecular size standard marker (Biolabs) (Size of bands of M 10,002 bp, 8,001 bp, 6,001 bp, 5,001 bp, 4,001 bp, 3,001 bp, 2,000 bp, 1,500 bp, 1,000 bp, 517 bp). b) *O. somaliensis* Lanes 1-24 represents clones digested with a cocktail of six restriction enzymes. M stands for 100bp molecular size standard marker (Biolabs).

4.7 Diversity of Bacterial community in *M. michaelseni*

The phylogenetic analysis of 16S rDNA gene sequences obtained from *M. michaelseni* revealed five main clusters, *Cytophaga-Flexibacter-Bacteroides* (CFB) cluster, *Proteobacteria* cluster, *Anaerobaculum thermoterrenum* cluster, low G+C content gram-positive bacteria cluster and Spirochetes cluster. A total of 47 clones were sequenced Four of the clones contained short sequences and were excluded from subsequent analysis. The CFB group was the largest with 15 clones followed by the *Proteobacteria* group with nine clones. Low G+C gram-positive bacteria group was third with seven clones then *A. thermoterrenum* and Spirochetes with two and one clone respectively. Three clones were unaffiliated (Figure 7).

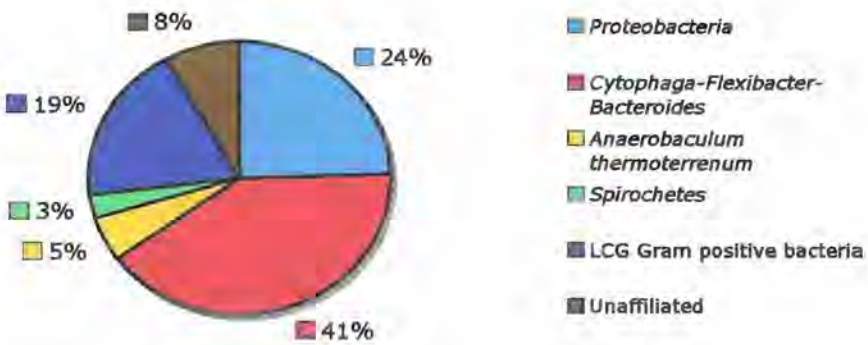
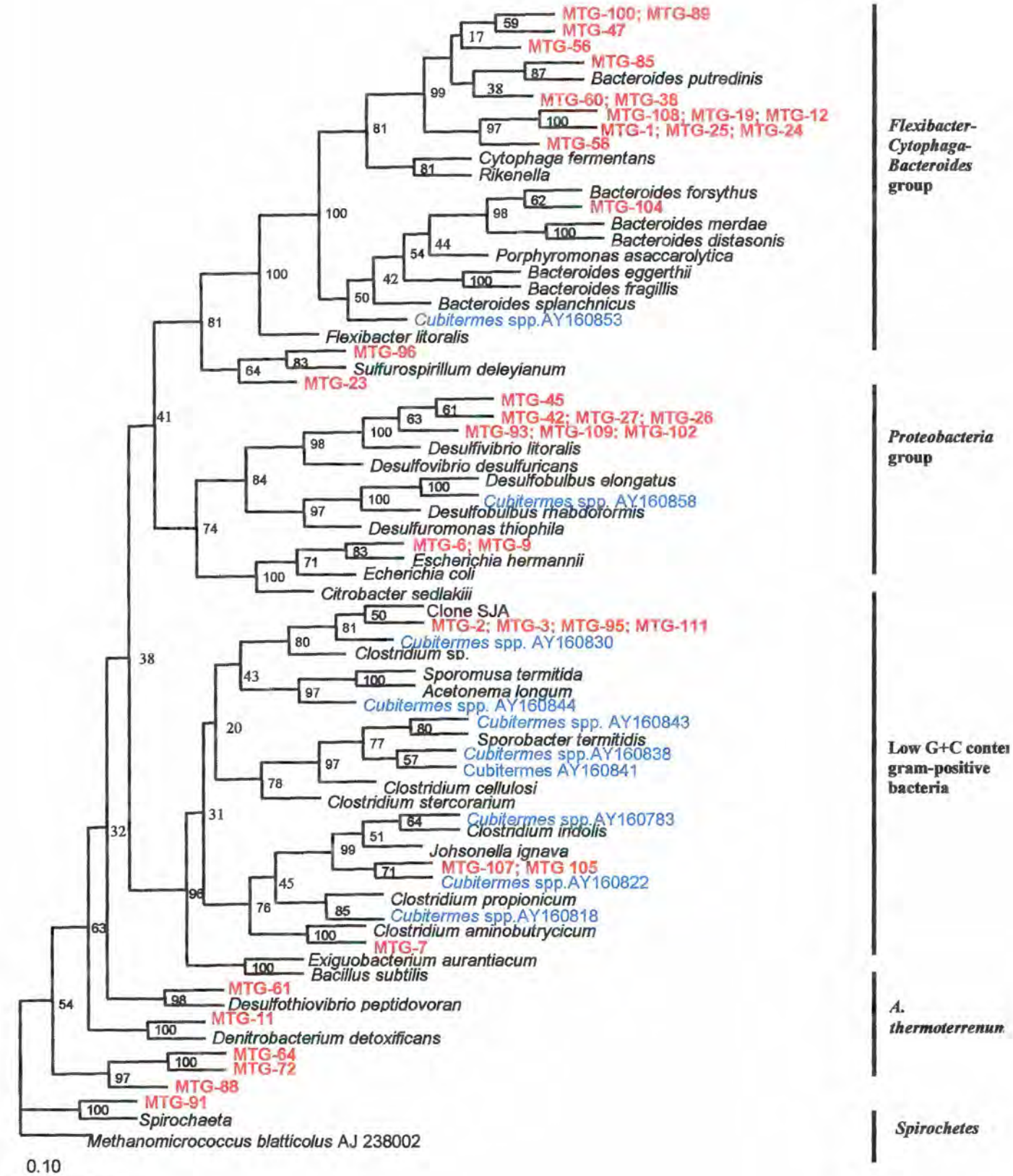


FIGURE 7. Percentage number of clones obtained from *Macrotermes michaelseni* in different bacteria groups

A large-scale phylogenetic tree containing members of each of the identified clusters is shown (Figure 8). The *Cytophaga-Flexibacter-Bacteroides* (CFB) group contained clones

MTG-100, MTG-89, MTG-47, and MTG-56, which are closely related but did not cluster with any known organisms in this group. Clone MTG-85 clustered together with *Bacteroides putredinis* with a bootstrap value of 87%. MTG-60 and MTG-38 joined this cluster with a low bootstrap value of 38%. Clones MTG-108, MTG-19, MTG 12, MTG-1, MTG-25, MTG-24 and MTG-58 did not show close relation to previously sequenced organisms but clustered together with high bootstrap values of 97 to 100%. The sequences of three clones, MTG-108, MTG-19 and MTG-12 were identical and so were MTG-1, MTG-25 and MTG-24. MTG-104 formed a cluster with *Bacteroides forsythus* with a low bootstrap value of 62%. Most of the clones affiliated with the CFB phylum showed a considerable phylogenetic distance of 89 to 92% from *Bacteroides* species. Clones MTG-96 and MTG-23 clustered together with low bootstrap values to *Sulfurospirillum deleyianum* with a sequence similarity of 91% and 86% respectively. The second cluster comprises the *Proteobacteria* group. Clones MTG-45, MTG-42, MTG-27, MTG-26, MTG-93, MTG-109, and MTG-102 clustered with *Desulfovibrio litoralis* of the delta subdivision of *Proteobacteria* with a high bootstrap value of 100% but had low bootstrap values within themselves. Clones MTG-42, MTG-27 and MTG-26 were identical and so were clone MTG-93, MTG-109, and MTG-102. Two clones MTG-6 and MTG-9 were closely related to *Escherichia hermannii.* Clones in this group had sequence similarities of 88 to 90% to *Proteobacteria* species. The third cluster belonging to the low G+C content gram-positive bacteria consisted of seven clones. Clones MTG-2, MTG-3, MTG-95 and MTG-111 shared the same nucleotide sequence and their closest relative was a clone of uncultured bacteria clone SJA with a phylogenetic distance of 89 to 90%. They also clustered with a clone obtained from a soil feeding termites

Cubitermes spp. (Schmitt-Wagner, 2003) but with very low bootstrap values. Clone MTG-107, which was identical to clone MTG-105, also clustered with a clone obtained from soil feeding termite *Cubitermes* spp. (Schmitt-Wagner, 2003) with a bootstrap value of 71%. MTG-7 clustered with *Clostridium aminobutrycicum* and was supported by a bootstrap value of 100% all the three clones had 95% sequence similarity to *clostridium* species. Clone MTG-61 had a sequence similarity of 89% to *Desulfothiovibrio peptidovoran* with 98% bootstrap value and clone MTG-11 was closely related to *Denitrobacterium detoxificans* with a sequence similarity of 88% and supported by 100% bootstrap value in the *A. thermoterrenum* group. Clones MTG-64, MTG-72 and MTG-88 did not fall in any of the clusters and could not be assigned to any of the known major groups of the *Bacteria*. The Spirochete group had only one clone, MTG-91 that is well supported by 100% bootstrap value and a sequence similarity of 90%. Chimeric rDNA clones composed of rDNA from different organisms, can arise during PCR amplification of mixed-population DNAs (Liesack *et al.,* 1991). Evaluation by CHECK-CHIMERA program of the Ribosomal Database Project (Maidak *et al.,* 2001) indicated that the sequences above showed no obvious evidence of chimeric artifacts.


MTG-100; MTG-89
MTG-47
MTG-56
MTG-85
Bacteroides putredinis
MTG-60; MTG-38
MTG-108; MTG-19; MTG-12
MTG-1; MTG-25; MTG-24
MTG-58
Cytophaga fermentans
Rikenella
Bacteroides forsythus
MTG-104
Bacteroides merdae
Bacteroides distasonis
Porphyromonas asaccarolytica
Bacteroides eggerthii
Bacteroides fragillis
Bacteroides splanchnicus
Cubitermes spp.AY160853
Flexibacter litoralis
MTG-96
Sulfurospirillum deleyianum
MTG-23
MTG-45
MTG-42; MTG-27; MTG-26
MTG-93; MTG-109; MTG-102
Desulfivibrio litoralis
Desulfovibrio desulfuricans
Desulfobulbus elongatus
Cubitermes spp. AY160858
Desulfobulbus rhabdoformis
Desulfuromonas thiophila
MTG-6; MTG-9
Escherichia hermannii
Echerichia coli
Citrobacter sedlakiii
Clone SJA
MTG-2; MTG-3; MTG-95; MTG-111
Cubitermes spp. AY160830
Clostridium sp.
Sporomusa termitida
Acetonema longum
Cubitermes spp. AY160844
Cubitermes spp. AY160843
Sporobacter termitidis
Cubitermes spp.AY160838
Cubitermes AY160841
Clostridium cellulosi
Clostridium stercorarium
Cubitermes spp.AY160783
Clostridium indolis
Johsonella ignava
MTG-107; MTG 105
Cubitermes spp.AY160822
Clostridium propionicum
Cubitermes spp.AY160818
Clostridium aminobutrycicum
MTG-7
Exiguobacterium aurantiacum
Bacillus subtilis
MTG-61
Desulfothiovibrio peptidovoran
MTG-11
Denitrobacterium detoxificans
MTG-64
MTG-72
MTG-88
MTG-91
Spirochaeta
Methanomicrococcus blatticolus AJ 238002
0.10
Flexibacter-Cytophaga-Bacteroides group
Proteobacteria group
Low G+C conte gram-positive bacteria
A. thermoterrenun
Spirochetes

4.8 Diversity of Bacterial community in *O. somaliensis*.

The phylogenetic analysis of 16S rDNA gene sequences obtained from *O. somaliensis* revealed five main clusters, CFB cluster, *Proteobacteria* cluster, *A. thermoterrenum* cluster, low G+C content gram-positive bacteria cluster and Spirochetes cluster. A total of 100 clones were sequenced. The CFB group was the largest with 64 clones followed by the *A. thermoterrenum* group with eleven clones. The Spirochetes was third with eight clones then *Proteobacteria* with five clones and the Low G+C content Gram-positive bacteria group had four clones. One clone was unaffiliated (Figure 9).

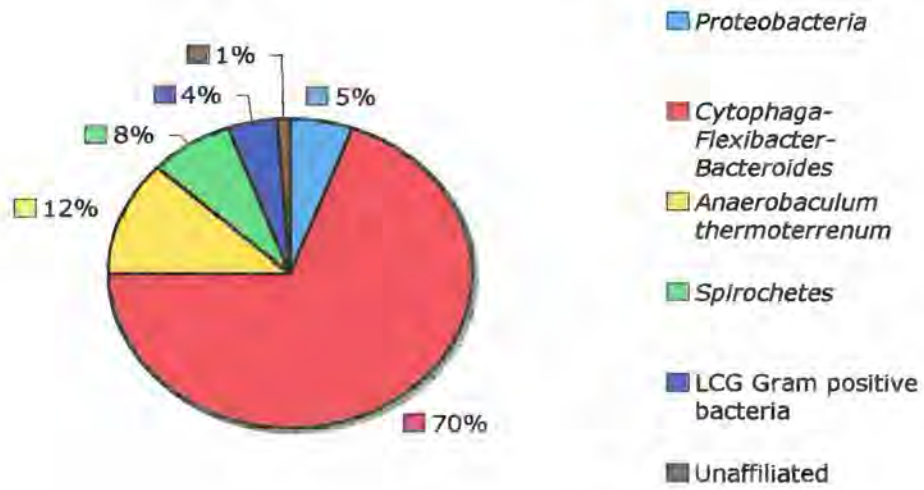


FIGURE 9. Percentage number of clones obtained from *Odontotermes somaliensis* in different bacteria groups.

A total of 100 clones were sequenced and shown to belong to the domain *Bacteria.* A large-scale phylogenetic tree constructed on analysis of 16S rDNA sequences obtained from *O. somaliensis*, shows that the isolated clones correspond to many diverse groups of bacteria (Figure 10 a, b). Most of the clones were phylogenetically affiliated with five major subdivisions of the *Bacteria*: the group *Proteobacteria*, the CFB group, the Spirochetes, *Anaerobaculum thermoterrenum* and the Low G+C content gram-positive bacteria. The low G+C gram-positive bacteria group contained four clones. Clone OT-32 (Figure 10 a) clustered with a clone obtained from a soil–feeding termite *Cubitermes* spp. (Schmitt-Wagner, 2003) with a low bootstrap value of 85% and did not seem to be closely affiliated with any of the known bacterial sequences in the database. This clone had a 94% sequence similarity with uncultured *Clostridiaceae* bacterium. Clones OT-71, OT-86, OT-65 were identical and clustered with *Streptococcus sanguinis* and were supported by a bootstrap value of 100% and had a 90% sequence similarity to *Streptococcus Sanguinis* (Figure 10 a). The second cluster containing the *Proteobacteria* contained five clones. Clone OT-23 and OT-17 were identical and clustered with clone OT-92 with 100% bootstrap value and had a 91% sequence similarity to an uncultured bacterium. They however did not cluster with known sequences of the low G+C content gram-positive bacteria. OT-38 (Figure 10 a) clustered with *Propionibacter pelophillus* with a 100% bootstrap value, while OT-89 did not affiliate with any organisms in this group.

The *Anaerobaculum thermoterrenum* group contained eleven clones. Clones OT-20, OT-50, OT-31 and OT-11 were closely related and did not cluster with known sequences in this group. Clones OT-55 and OT-51 had the same nucleotide sequence and clustered

with clone OT-29 with a bootstrap value of 100%. These clones also did not affiliate with known sequences in this group. Clone OT-93, OT-70 and OT-78 with 100% bootstrap value and no close known relative formed another cluster. OT-13 was related with *Denitrobacterium detoxificans* and was supported by 100% bootstrap value. The clones in this cluster had a phylogenetic distance of 89 to 90% from the *Anaerobaculum thermoterrenum* species.

The Spirochete group consisted of eight clones, which are closely related and affiliated with *Spirochaeta.* Clones OT-87, OT-63, and OT-76 were identical and clustered with OT-1. These clones had a sequence similarity of 90 to 94% to uncultured *Treponema* species. This cluster was also closely related to clones OT-7, OT-57 and OT-45. OT-79 clustered with *Spirochaeta* with a bootstrap value of 100% and a sequence similarity of 94% (Figure 10 a).

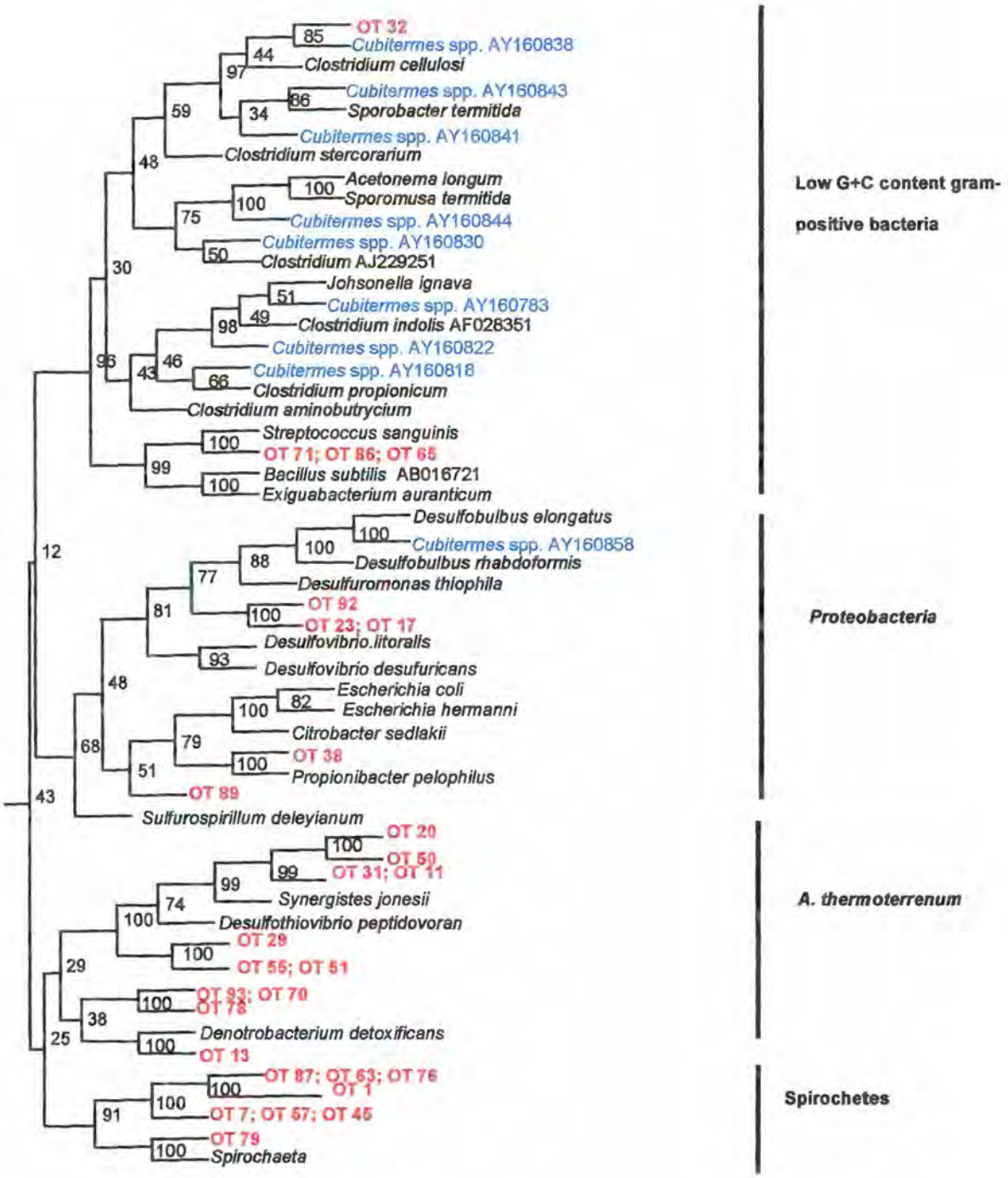


FIGURE 10. a). Phylogenetic tree showing positions of clones obtained from *O. somaliensis*. The scale bar indicates approximately 10% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resamplings Clones in red are those from *O. somaliensis* and those in blue were obtained from a soil feeding termite *Cubitermes* spp. (Schmitt-Wagner *et al.*, 2003). The 16S rDNA sequence of *Methanomicrococcus blatticolus* AJ238002 was used as the outgroup

The fifth cluster, which contained more than half the clones, obtained from *O. somaliensis* belonged to the group CFB (Figure 10 b). The CFB group formed two very distinct clusters indicating that the clones in this group were very divergent. Most of the clones in these two clusters did not seem to cluster with sequences of the known organisms in this group. The first cluster contained clones OT-59, OT-43, OT-34, and OT-21, which had the same nucleotide sequence and they clustered with OT-84 with a 100% bootstrap value. OT-60 and OT-47 joined this cluster with a 91% bootstrap value. These clones did not cluster with any known sequences of the *Bacteroides* group. OT-2, OT-10 and OT-35 formed a cluster with *Bacteroides forsythus* supported by a bootstrap value of 100%. Clones OT-85 OT-75 and OT-96 formed a cluster, which was supported by very, low bootstrap values. Clone OT-14, OT-24 and OT-30 clustered together with 100% bootstrap value and the same case applied to clones OT-72 and OT-5. Both clusters did not have a known close relative. Clone OT-91 and OT-54 each branched alone with clone OT-12 clustering with a clone obtained from *Cubitermes* species, a soil feeding termite (Schmitt-Wagner *et al.,* 2003). Clone OT-66, OT-73 and OT-44 did not form any clusters with other clones and had low bootstrap values indicating that the exact branching order may change when additional sequences become available. OT-69 formed a cluster with *Provotella enoeca* with a bootstrap value of 100%, while OT-3 branched alone. The second cluster had more clones most of which were identical. Clone OT-37, OT-42 and OT-33 were identical and clustered with OT-77 with 100% bootstrap value. Five clones OT-28, OT-26, OT-6, OT-8 and OT-39 were identical and did not cluster with other clones. OT-4 and OT-95 formed a cluster with a bootstrap value of 55%, while clone OT-41 did not seem to cluster with other clones. *Bacteroides putredinis* clustered

with clone OT-19 with a 96% bootstrap value. OT-83, OT-82 and OT-80 were identical and clustered with OT-68, OT-81 and OT-62. OT-61 and OT-74 did not fall within any clusters. Clones OT-98, OT-48, OT-40, OT-56 and OT-52 had the same nucleotide sequence and clustered with OT-25 and OT-18. Clones OT-97 and OT-94 formed a cluster and clone OT-88 and OT-64 did not cluster with any other sequences and neither did clone OT-67. Clone OT-15, OT-36, OT-53 and OT-22 were identical and did not form a cluster with known sequences and neither did clone OT-46. Clone OT-16 also branched alone. The clones in this cluster showed a considerable phylogenetic distance of 88 to 93% from different *Bacteroides* species. Evaluation by CHECK-CHIMERA program of the Ribosomal Database Project (Maidak *et al.,* 2001) indicated four sequences were chimera artifacts and these were removed from subsequent analysis.

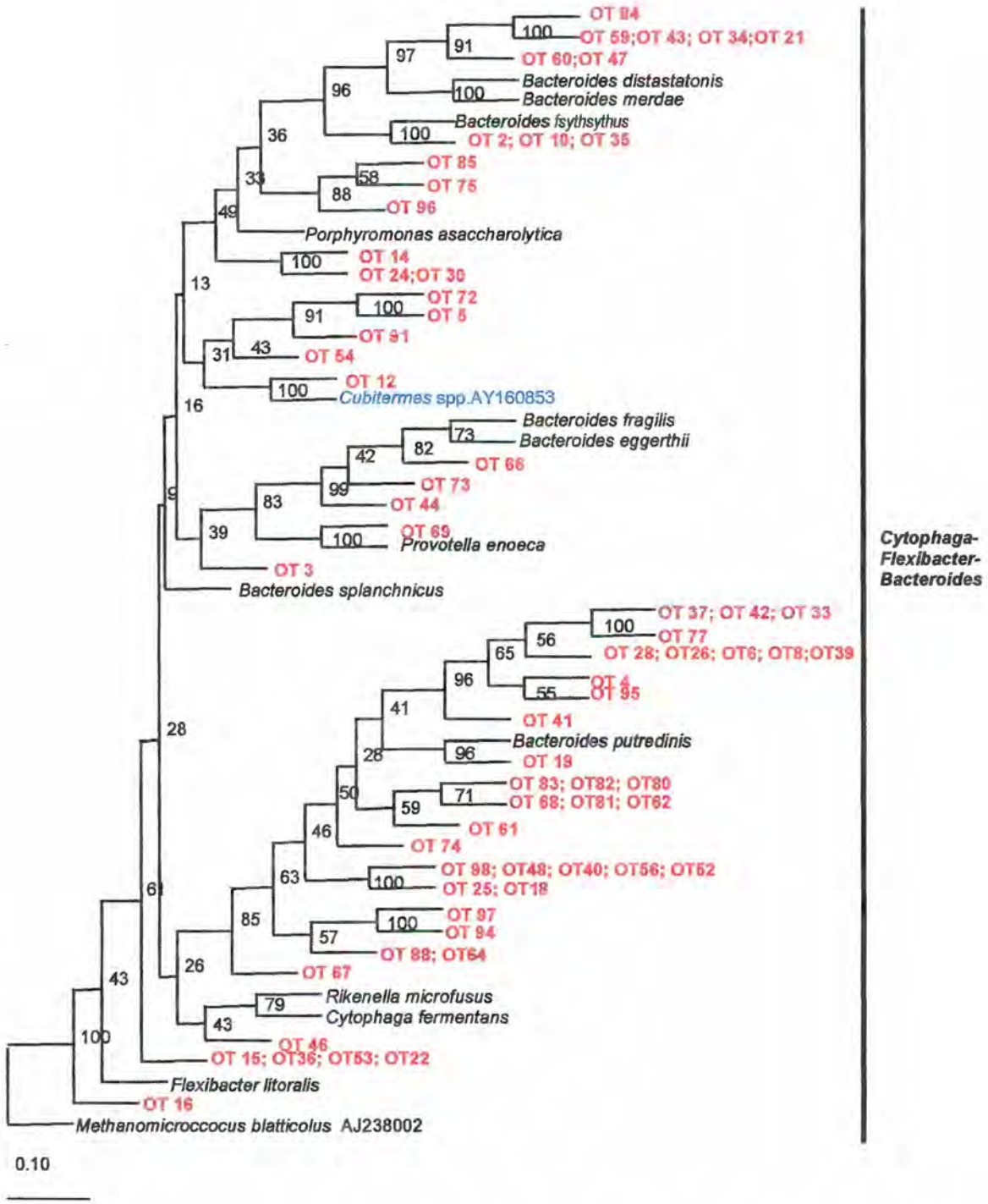


FIGURE 10. b). Phylogenetic tree showing positions of clones obtained from *O. somaliensis*. The scale bar indicates approximately 10% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resamplings. Clones in red indicate those obtained from *O. somaliensis* and clones in blue were obtained from a soil feeding termite *Cubitermes* spp. (Schmitt-Wagner *et al.*, 2003). The 16S rDNA sequence of *Methanomicrococcus blatticolus* AJ238002 was used as the outgroup.

CHAPTER FIVE

5.0 DISCUSSION

Using the plate count technique, the highest number of microbes in *M. michaelseni* was recorded in KMM1 medium giving a mean ± SD of 173.5 ± 28.9 × 10^3 CFU/gut (Table 2, page 36). In *O. somaliensis*, the highest number of microbes was recorded in medium containing gelatin as a substrate giving a mean ±SD of 156 ± 25.45 × 10^3 CFU/gut (Table 2, page 36). Results obtained from MPN also showed that *M. michaelseni* had a higher cell count than *O. somaliensis*. The highest cell counts in *M. michaelseni* were recorded in the KMM1 medium giving an approximate of 48 × 10^4 cells/gut, while in *O. somaliensis* it was recorded in KMM1+ glucose giving approximately 480 cells/gut (Table 2, page 36). These results however represent only the aerobic or facultative aerobic organisms only, since all experiments were carried out aerobically. The results are in line with the limited data available for other higher termites. Anklin-Mühlemann *et al.* (1995) recorded total densities of approximately 1.3 × 10^{11} organisms per ml in the paunch and colon of old minor workers of *Macrotermes subhylinus*, a fungus-cultivating termite by direct observation using the agar film method. Dilution plating on nutrient and MacConkeys agar media also showed large numbers of colony forming units throughout the alimentary canal (Anklin-Mühlemann *et al.,* 1995). Also a very diverse bacterial flora consisting of at least seventeen morphotypes was found in the paunch of *Odontotermes formosanus* a fungus- cultivating termite (Yara *et al.,* 1989). However, a previous investigation of higher termites by Bignell *et al.* (1980), revealed that total microbes isolable by dilution plating were between one and two orders of magnitude fewer than estimates of population size obtained by direct observation. This may be due to the fact

that most microorganisms elude cultivation hence new approaches are therefore needed to enumerate microbial counts in termite guts.

Phylogenetic analysis of bacterial 16S rDNA genes of the isolates obtained from the plating techniques showed that they all belonged to the high G+C content gram-positive bacteria (Figure 2 page 40). Isolates were selected according to different morphologies and seven isolates were picked from each termite species. The phylogenetic tree contained three different clusters. The *Streptomyces* cluster contained mainly of isolates obtained from *M. michaelseni* and only one isolate IO-18 from *O. somaliensis*. Most isolates from *M. michaelseni* could not be identified to the species level since they clustered with organisms whose species have not yet been identified. IO-18 however clustered with *Streptomyces fungicidicus* and was strongly supported by a bootstrap value of 100%. Both termite species were represented in the second cluster belonging to *Brevibacterium*. Isolates IM-4, IO-10A and IO-11, which had the same nucleotide sequences, had a bootstrap value of 100% with the IM-12 and IO-14C cluster. This cluster had a 100% bootstrap value with *Brevibacterium aureum*, indicating that all the five isolates are closely related to this organism. IM-5 clustered with *Brachybacterium paraconglomeratum* and IO-14C with *Norcadia asteroides*. Both were well supported by 100% bootstrap values (Figure 2, page 40). The third cluster had only two isolates from *O. somaliensis*, IO-9 and IO-19B that clustered with *Bacillus licheniformis* members of this genus have been shown to degrade cellulose (Brune, 2003). These results are in line with previous studies in which actinomycetes belonging to the genus *Streptomyces* were isolated (Watanabe *et al.*, 2003). Bignell *et al.* (1979), observed actinomycete-like bacteria in the guts of termites using an electron microscope. Actinomycetes were also

isolated from termite guts by Pasti and Belli in (1985), and were found to have cellulolytic and lignin solubilizing activities. Based on previous results the isolates described in this study may also have cellulolytic and lignin solubilizing activities but biochemical tests should be carried out to determine this hence giving an insight in to their roles in the guts of *M. michaelseni* and *O. somaliensis*.

The highest number of clones in both termites belonged to the CFB group (Figures 7, page 46; Figure 8, page 50; Figure 9, page 51; Figure 10 a, page 54 and Figure 10 b, page 57). The CFB group had 15 clones from *M. michaelseni* and 64 clones from *O. somaliensis*. However most of the clones from both termites did not seem to cluster with known *Bacteroides* species. The *Bacteroides forsythus* cluster contained some clones from both *M. michaelseni* and *O. somaliensis* species and none from *Cubitermes* spp. The *Bacteroides splanchnicus* cluster contained clones from *O. somaliensis* and *Cubitermes* and none from *M. michaelseni* species. Other clones from *O. somaliensis* and *M. michaelseni* clustered with *Bacteroides putredinis* and none from *Cubitermes* spp. The last cluster in the CFB phylum contained clones from *O. somaliensis* only. It is important to note that the clones obtained from *O. somaliensis* in this group were highly divergent forming two distinct clusters within the CFB group. Representatives of these phyla are typical components of the intestinal microflora of mammals including humans (Hold *et al.*, 2002; Leser *et al.*, 2002) and have also been recovered from wood and soil feeding termites by cultivation (Breznak and Brune 1994) or as 16S rDNA gene fragments (Kudo *et al.*, 1998; Ohkuma *et al.*, 2002). This also confirms results of previous studies in which CFB forms the largest number of clones in the clone library of a soil-feeding termite *Cubitermes orthognathus* (Schmitt-Wagner *et al.*, 2003). Ohkuma *et al.* (2001) also

reported the *Bacteroides* subgroup as one of the major populations within the gut community of *Reticulitermes flavipes* termite. *Bacteroides* species are known to be fermentative and acidogenic; organisms assigned to this cluster may have the same function. Many bacteria from the CFB phylum are also specialized in degradation of plant fibers and proteins (Shah, 1992).

Other clones clustered with the *Proteobacteria* group. *M. michaelseni* had nine clones while *O. somaliensis* had five clones (Figures 7, page 46; Figure 8, page 50; Figure 9, page 51 and Figure 10 a, page 54). Some clustered with *Desulfovibrio* species and others with *Citrobacter* species. *M. michaelseni* clones and *O. somaliensis* clones were well represented in this cluster, with only a few from *Cubitermes* spp. Since several *Desulfovibrio* species are know to be nitrogen fixing bacteria, the organisms in our clone libraries which cluster with them also represent possible N_2 fixing candidates in the termite gut. *Citrobacter freundii* isolated from several kinds of termites was also identified as a nitrogen-fixing bacterium. Nitrogen fixation mediated by gut bacteria is one of the crucial aspects of termite symbiosis since termites feed on nitrogen poor food.

The gram-positive bacteria with a low G+C content were not well represented by clones from *O. somaliensis*, which had four clones. *M. michaelseni* on the other hand had seven clones in this group (Figures 7, page 46; Figure 8, page 50; Figure 9, page 51 and Figure 10 a, page 54). Most of the clones from soil-feeding termite *Cubitermes* spp. (Schmitt-Wagner *et al.,* 2003) were found in this group. However none of the clones from *M. michaelseni* and *O. somaliensis* clustered with those from the soil-feeding termite. Moreover, none of the clones in our library showed high similarity to *Sporomusa* but a

few *Clostridium*-related clones were identified. A phylogenetic study of the symbiotic bacteria in the mixed segment of the termite *Nasutitermes takasagoensis* (Shiraki) (Tokuda *et al.*, 2000) revealed that they were closely related to the *Clostridium* group, which is one of the largest bacterial genera. It is commonly known that *Clostridium* species are usually obligate anaerobes, and some are facultative anaerobes (Rieu-Lesme *et al.*, 1996). It was suggested that probably these bacteria contribute to the dramatic decrease of oxygen concentration observed in the mixed segment (Brune *et al.*, 1995). Clostridia are usually known as soil bacteria and are also found in the rumens and intestines of animals and humans (Cummings and Macfarlane, 1997) Some predicted roles of the intestinal bacteria in termites (Breznak and Brune, 1994), are similar to those in humans, such as producing short-chain fatty acids from carbohydrates or synthesizing amino acids (Cummings and Macfarlane, 1997). Since many clostridia degrade polysaccharides to produce acetone, alcohol acetate, lactate, CO_2 and hydrogen (Chen, 1995) and others can ferment nitrogenous or lipidic compounds, it is expected that the clones in this group play a role in the nutritional physiology of both termites. A few CO_2–reducing acetogens have been isolated from termite guts in pure culture and identified as species of *Sporomusa termitida*, *Acetonema longum* and *Clostridium mayombei* (Breznak *et al.*, 1988; Kane and Breznak 1991).

Other clones from *M. michaelseni* and *O. somaliensis* were related to *A. thermoterrenum* group. *A. thermoterrenum* is a thermophilic anaerobic bacterium that was isolated from the production water of a petroleum reservoir (Rees *et al.*, 1997) This strain could not be placed in any previously described taxon based on its phylogenetic and physiological traits and hence it was named *A. thermoterrenum* gen. nov., sp. nov (Rees *et al.*, 1997).

This group contains organisms with interesting characteristics. *Denitrobacterium detoxificans* gen. nov., sp. nov., which clusters with this group, was isolated from the bovine rumen and respires on nitrocompounds (Anderson *et al.*, 2000). It is non-motile and none spore forming and is described as a phenotypically dissimilar anaerobe within the class Actinobacteria (Anderson et al., 2000). *Desulfothiovibrio peptidovoran* also belongs to the *A. thermoterrenum* group. *Synergistes jonesii*, a ruminal bacterium that is able to degrade the pyridinediol toxin in the plant *Leucaena leucocephala* (Hammond, 1995) also belongs to this group. *O. somaliensis* had eleven clones clustering in this group and *M. michaelseni* had two clones. Further analysis of clones in this group could help determine whether they have the same characteristics as those described or could lead to the discovery of bacteria with new interesting characteristics. *Desulfothiovibrio,* a sulfate-reducing bacterium, has been reported to be isolated from the termite gut by enrichment culture (Trinkeri, *et al.*, 1990).

The Spirochetes group had the least number of clones with *M. michaelseni* having one clone and *O. somaliensis* having eight clones. These clones were not related to any obtained from other termites. Spirochetes are believed to play an important role in the termite gut, as termites harboring them appear to be both vigorous and healthy (Ohkuma and Kudo, 1996). Recently pure cultures of spirochetes have been obtained from *Zootermopsis angusticollis* (Leadbetter *et al.*, 1999) and were found to be capable of CO_2-reducing acetogenesis, thereby implying a significant role for spirochetes in termite nutrition and helping explain why their presence in guts is important to termite vitality.

The clone libraries of both *M. michaelseni* and *O. somaliensis* show that even though both termites are fungus-cultivators, their microbial community was different. This is especially represented in the CFB group. Both termites had abundant clones but those in *O. somaliensis* formed two distinct clusters showing that the clones in this group were highly divergent while those in *M. michaelseni* formed only one cluster. However both termites had similarities in the *A. thermoterrenum* group in which both termites had clones clustering with *D. detoxificans*. The low G+C content group had no similarities between the two termites. *M. michaelseni* had more clones in this group than *O. somaliensis* and the organisms that clustered with the *M. michaelseni* clones were different from those that clustered with the *O. somaliensis* clones the same occurred in the *Proteobacteria* group. The difference in microbial diversity between the two termites could be species specific since both termite mounds were located in the same area with a distance of about 100 meters between them.

Our clone libraries did not represent any of the isolates obtained from the culture-dependent techniques in this study. Most of the isolates from both *M. michaelseni* and *O. somaliensis* were related to the High G+C content bacteria mainly *Streptomyces* species. This could be due to a methodological bias since the DNA extraction method employed for the extraction of DNA from the termite guts was not sufficient for the isolates. Most High G+C content bacteria form spores hence making their cells difficult to lyse. However increased vortexing time as well as increase in the incubation time during cell lysis, lysed the cells completely hence yielding sufficient DNA.

Similarly, some of these isolates may not be part of the normal flora of the gut and since conditions were not favorable in the gut they may have formed spores hence being difficult to extract DNA from as compared to other organisms represented by the clone libraries. Conditions in the laboratory may have been favorable for their cultivation.

The abundance of clones of high similarity within the 16S rDNA gene clone libraries constructed here may reflect their numerical abundance in the termite gut, especially the *Bacteroides* and *Proteobacteria* clusters. However since both clone libraries and analysis of the 16S rDNA of the isolates have the limitations inherent to all PCR-based approaches, including efficiency of DNA extraction, primer selectivity, possibility of PCR bias and different copy numbers of 16S rDNA genes, a quantitative analysis of abundance (Amann *et al.*, 1995) is necessary.

To fully describe the diversity of microorganisms in the two fungus-cultivating termites, it is important to analyze the Archaea. Methanogenic archaea are an abundant group of microorganism in termite guts (Brune and Friedrich 2000). Termites emit methane, and they are one of the few terrestrial arthropods to do so (Odelson and Breznak, 1983). The methane emitted arises from members of the methanogenic Archaea, which reside in the gut and appear to be one of the terminal "H_2 sink" organisms of the hindgut fermentation (Breznak and Switzer, 1986). Owing to this property and to their high biomass densities, particularly in tropical habitats, termites have been cited as a potentially significant source of atmospheric methane (Leadbetter and Breznak, 1996). However, their precise contribution to global methane emissions has been hotly debated (Leadbetter and Breznak 1996) Among the uncertainties in global estimates of methane emission by

termites are knowledge of the exact number of termites on Earth and of the extent of intra- and interspecific variation in emissiom rates among the 2,000 or so known species (Leadbetter and Breznak 1996). In soil-feeding and fungus-cultivating termites, methanogenesis dominates acetogenesis as an H_2 sink; however the reverse is true for wood and grass-feeding termites (Brauman *et al.*, 1992). Aside from the visualization of methanogens by F_{420} epifluorescence microscopy of termite gut contents (Lee *et al.*, 1999) and brief descriptions of the enrichment and isolation of such forms, our understanding of the physiology of termite gut methanogens is virtually nonexistent (Leadbetter and Breznak 1996). Clonal 16S rDNA gene sequences of archaeal gut symbionts have been retrieved from wood feeding fungus-cultivating and soil-feeding termites in previous studies (Ohkuma and Kudo, 1998; Ohkuma *et al.*, 1999; and Shinzato *et al.*, 1999).

Since molecular techniques allow detection and identification of microorganisms without cultivation, this gives an indication of the diversity of the many microorganisms we are unable to cultivate or isolate. Knowing the phylogeny of the microorganisms might lead to the design of better cultivation strategies. However 16S rDNA sequences alone cannot be used to assign function to individual microbial populations in the gut. Genes whose products are directly involved in metabolism can be used as molecular markers to identify potential metabolic groups using *in situ* hybridization techniques. The gene encoding dinitrogenase reductase (*nif*H) was used to detect a remarkable diversity of potentially nitrogen fixing microorganisms in the guts of various termites (Ohkuma *et al.*, 1996). In order to assign functions to the major populations within a microbial

community, information on diversity and physiological characteristics have to be combined with localization and characterization of their respective microhabitats.

To further advance our understanding of termite gut micro ecology, it is essential to proceed beyond a mere description of the microbial communities by their phylogenetic diversity. An integrative analysis of community structure and function will require the description of the environmental conditions, the localization of individual populations, and the characterization of their major metabolic activities, all *in situ* and at high resolution (Brune and Friedrich, 2000).

In conclusion, the guts of *Macrotermes michaelseni* and *Odontotermes somaliensis* show a high diversity in bacterial species in this study. More studies should be carried out to establish the biodiversity of the molecules they produce as a result of primary and secondary metabolism. This may lead to the discovery of microorganisms with significant economic value that may be used different application such as food production and preservation, production of antibiotics, manufacture of vaccines, management of pests and pathogens, increasing soil fertility, cleaning up of oil spills and waste water treatment.

Hence this study, as well as similar studies that help in understanding the functioning of ecosystems, play an important role in the process if changing the perception about the need to conserve biodiversity including microbial diversity.

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Appendix

1 **LB Agar (per liter)**

10 g of NaCl

10 g of tryptone

5 g of yeast extract

15 g of agar

Add deionized H_2O to a final volume of 1 liter

Adjust pH to 7.5 with NaOH and Autoclave

2. **LB-Kanamycin Agar (per liter) for growing colonies**

1 liter of LB agar, autoclaved

Cool to 55 °C

Add 10ml of 10 mg/ml filter-sterilized Kanamycin.

Pour into petri dishes (~25 ml/100-mm plate)

3. **SOC medium (per liter)**

20 g of tryptone

5 g of Yeast extract

0.5 g of NaCl

Add deionized H2O to a final volume of 1 liter and autoclave.

Add 10 ml of filter-sterilized 1 M Mgcl2 and 10 ml of filter-sterilized 1M of MgSO4 prior to use. In 100 ml of this medium, add; (Note. This medium was prepared immediately before use). 1 ml of filter-sterilized 2M glucose.

4. **1x TAE Buffer (50x)**

242 g Tris base

57.1 ml glacial acetic acid

100ml 0.5 M EDTA (pH 8.0)

Dilute to 1x TAE working solution with dH2O

5. **T E Buffer**

10mM Tris-HCl

1mM EDTA, pH adjusted to 8.0