INVESTIGATING THE ACTINOMYCETE DIVERSITY INSIDE THE HINDGUT OF AN INDIGENOUS TERMITE, *Microhodotermes viator*

by

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ABSTRACT

Ever since the discovery of a cellulolytic actinomycete inside the gut of a termite, there has been considerable interest in the metabolic abilities and the diversity of these bacteria within this complex environment. However, until fairly recently, most investigations of termite hindguts involved the higher termites from the Termitidae family. Little attention was paid to the lower termites, and even less to the family Hodotermitidae. The main aim of this project was to explore the actinobacterial diversity of the paunch and colon hindgut regions of one particular member of the family Hodotermitidae, *Microhodotermes viator*. No previous work of this nature has ever been attempted on this particular termite species.

All termite specimens were collected from the Tygerberg Nature Reserve in Cape Town, South Africa and, after dissection of the termites, the contents of the paunch and colon regions of the hindgut were plated out on four different isolation media (M3, MIIC, MIIG and MIIX), each at four different pHs (pH 6.0, 6.5, 7.0 and 7.5). The results showed that the greatest number of actinomycetes was isolated from the paunch on MIIC pH 6.0 ($4.8x10^3$ CFU/ml) and MIIX pH 6.5 ($4.5x10^3$ CFU/ml), respectively. In terms of total diversity, the paunch proved to be the region from which higher numbers of all microorganisms were isolated.

A total of 80 actinomycete isolates was subcultured. Thirty nine of these strains were screened for antibacterial activity, with the majority of the isolates found to have activity against *Mycobacterium aurum* A+ (7 isolates) and *Staphylococcus aureus* ATCC 25923 (5 isolates), or against all three Gram positive test strains used (16 isolates).

Seventy six of the isolates were investigated for their carboxymethylcellulose (CMC)- and xylan-degrading ability. Four isolates showed no activity against either substrate, 14 were able to degrade both, and 75% were found to have specific CMC-degrading activity (after incubation for 21 days).

The presence of actinomycete filaments was observed during a morphological examination of the paunch and colon regions of both soldier and worker specimens, using both scanning and transmission electron microscopy. A great diversity of bacteria was found associated with the gut wall as well as within the lumen.

Of the 80 isolates cultured from the hindgut of *M. viator*, a molecular identification method found that 78 belonged to the *Streptomyces* genus, with the remaining two representing the genus *Nocardia*. Nineteen isolates were investigated as potential new species within their respective genera.

Of these isolates, two related *Streptomyces* groups were found to be noteworthy due to their rugose spore surface ornamentation. The Group 5 strains (encompassing isolates 5, 6, 26 and 39) were all found to be 99% similar by 16S-rRNA gene sequence comparison to *Streptomyces malaysiensis* ATCC

BAA-13^T. After BLAST analysis, the Group 33 isolates were found to be most closely related to six published *Streptomyces* species, also at 99% similarity.

Isolates 13, 16 and 37 were all found to have less than 96% sequence similarity to their top BLAST hits. Phenotypic differences were found between the three isolates and their top hit, *Streptomyces sodiiphilus* YIM 80305^T, indicating that they represent new *Streptomyces* species.

Isolates 18, 19 and 32 shared 98% 16S-rRNA gene sequence similarity with *Streptomyces avermitilis* NCIMB 12804^T, *Streptomyces cinnabarinus* NBRC 13028^T, and *Streptomyces kunmingensis* NBRC 14463^T. Morphological and physiological differences shown between all three isolates and their close phylogenetic relatives, supported the DNA-DNA hybridisation results which confirmed their identity as new species within the genus *Streptomyces*.

Isolate 14 was found to be more than 99% similar by 16S-rRNA gene sequence analysis to its four closest phylogenetic relatives. DNA-DNA hybridisation against the two closest relatives, *Streptomyces atratus* NRRL B-16927^T (35.45 ± 6.58% DNA-DNA similarity) and *Streptomyces sanglieri* DSM 41791^T (10.3 ± 0.42% DNA-DNA similarity) showed that isolate 14 represents a new species.

A total of 326 16S rRNA gene clones was obtained for the culture-independent analysis of the actinobacterial diversity in the colon and paunch (P3b region) hindgut regions of *M. viator* (both workers and soldiers) and the soil surrounding the mound from which the specimens were collected. PCR amplification using the actinobacterial-specific 16S rRNA gene primers S-C-Act-0235-a-S-20-F and S-C-Act-0878-a-A-19-R, and subsequent ARDRA analysis of the products using the restriction enzymes *Alu*I and *Rsa*I revealed 31 unique patterns from the paunch, 55 from the colon and 49 from the surrounding soil. A total of 222 of the 326 clones was sequenced, (58, 83 and 81 clones from the paunch, colon and surrounding soil samples, respectively).

The clone diversity within each sample (revealed from the sequencing and BLAST results) was used in the comparison of the samples. The paunch sample was dominated by clones with no nearest cultured relative, as well as three filamentous actinobacterial families – *Thermomonosporaceae* (15.52% of all clones from the paunch sample), *Streptomycetaceae* (10.34%) and *Micromonosporaceae* (8.62%). In contrast, the colon sample was dominated by members of the family *Propionibacteriaceae* (46.99%). The surrounding soil sample was dominated by three main groups – 23.46% uncultured clones, 12.35% unknown (clones with no specific taxonomic designation) and 13.56% *Nocardioidaceae*. In comparison to both the paunch and colon, the surrounding soil showed much greater filamentous actinobacterial diversity, with 13 families, including 21 genera (11 of which were filamentous groups), as opposed to the 10 families and 10 genera (3 filamentous) discovered for the paunch, and 14 families and 20 genera (5 filamentous) identified from the colon.

A phylogenetic analysis of these results served to emphasise the wide diversity of actinobacteria associated with the hindgut and surrounding soil of the *M. viator* termite.

A neighbour-joining phylogenetic tree was also generated to include the culture-independent streptomycete clones as well as the cultured *Streptomyces* representatives and their closest relatives. Here, a clear separation of colon and paunch clones from surrounding soil streptomycete clones lent support for the proposal of a termite hindgut-specific actinomycete population.

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1.1 INSECT SYMBIOSES

In nature, there are many interactions between insects and microorganisms, but the most interesting of these are those that would be considered symbiotic. The term symbiosis was first coined by a plant pathologist, Anton de Bary, and describes the permanent association between two or more distinct organisms during some part of their life cycle. Symbiosis therefore includes mutualisms (where both organisms benefit), commensalisms (where neither suffers), and parasitisms (where one partner benefits while the other suffers) (Hughes *et al.*, 2008). The term symbiosis is often used synonymously with mutualism, excluding the negative aspects, as this is the most well-understood and widely known form (Hughes *et al.*, 2008). Mutualisms are widespread in nature and range from very loose interactions, such as those between pollinating insects and flowering plants, to tightly co-evolved interactions between some insects and their endosymbionts, which live within specialised cells (Aanen *et al.*, 2009). Many symbioses involve microorganisms (symbionts) which are associated with unicellular or multicellular eukaryotic hosts, and these symbionts themselves can be either prokaryotes (Bacteria and Archaea) or eukaryotes. Often these associations are intracellular (where the endosymbiont resides within the host's cells – so-called mycetocytes or bacteriocytes), but are more frequently extracellular (where the two members are separate, but remain in close proximity). A good example of these is the many interactions that can be found in insect gut systems (Baumann & Moran, 1997; Kaltenpoth *et al.*, 2006).

1.1.1 Why symbiosis?

Insects are one of the most successful groups of animals in the world, both in terms of species richness and abundance. They play a vital ecological role in many terrestrial ecosystems, and have a vast capacity to adapt to entirely different habitats, not only in tropical areas, but also in extreme environments, such as Arctic or desert regions (Zientz *et al.*, 2005). One of the main reasons for their success and ability to adapt probably has to do with their ability to form such close associations with microorganisms. It has been estimated that at least 15-20% of all insects live in some form of symbiotic relationship with microorganisms. These interactions play roles in insect nutrition, defence and even reproduction and development (Boursaux-

Eude & Gross, 2000; Russell & Moran, 2005). In most cases, it is the versatility in the metabolism and biosynthetic capability of the microbes, which allows access to novel ecological niches and to new unbalanced food resources, such as plant sap, blood or woody materials (Boursaux-Eude & Gross, 2000; Zientz *et al.*, 2005). These symbionts can be housed in the lumen of the gut, in the insect hemocoel, or inside the specialised mycetocyte/bacteriocyte cells. The diets of these insects are nutrient poor, and they therefore rely on their endosymbiont's ability to utilise substrates for energy that would normally be inaccessible to the metabolism of the host itself (Baumann & Moran, 1997; Kikuchi *et al.*, 2005; Kaltenpoth *et al.*, 2006).

1.1.2 Examples of symbioses – a range of complexity

Soil-dwelling insects generally face a higher risk of bacterial and fungal infestation of their food stocks and their progeny from the surrounding soil. Contagious diseases are also a problem, especially for those insects that live in close proximity to each other, specifically social insects, whose close genetic relationship leaves them vulnerable to epidemics. It would therefore be expected that selection pressures would have resulted in the evolution of mechanisms to counteract pathogen attack (Veal *et al.*, 1992; Kaltenpoth *et al.*, 2005). Symbionts are often acquired because of their usefulness in the production of secondary metabolites e.g. antibiotics, and the cultivation of these antibiotic-producing bacteria would therefore be a valuable investment for the host in combating the threat of pathogen infestation (Kaltenpoth *et al.*, 2005). The high-G+C group of Gram-positive bacteria (class *Actinobacteria*, order *Actinomycetales*) are well known for their production of antibiotics and, as such, are the predominant bacteria utilised in these symbioses (Mueller *et al.*, 2008).

One such example involves the fungus-growing ants, from the tribe Attini. They are unique amongst insects in that they have a specialised metapleural gland that is able to secrete a substance over the ant's cuticle, which serves as a general defence mechanism (Beattie *et al.*, 1986; Bot *et al.*, 2002; Poulsen *et al.*, 2003). This secretion is linked to a larger, more complex quadripartite symbiosis, encompassing both mutualistic and parasitic associations and, in this instance, involving the more phylogenetically derived leaf-cutter ants, in the genera *Acromyrmex* and *Atta*. The attine ants are able to cultivate a crop of basidiomycete fungi. These fungi produce specialised structures called gongylidia (used by the ants for food), in exchange for the chewed pulp that the ants provide from their leaf-cutting activities (Currie, 2001; Cafaro & Currie, 2005).

It has been shown that the ants sometimes exchange their clonal crops with free-living fungi, and also share between colonies. This situation has probably evolved over time in response to a particularly virulent pathogen of their fungal gardens – a highly specialised parasite from the fungal genus *Escovopsis* (Shultz, 1999; Zhang *et al.*, 2007). To combat this pathogen, the ants have evolved a mutualistic association with an actinomycete that belongs to the genus *Pseudonocardia* (Cafaro & Currie, 2005). This bacterium grows on the cuticle of the ants as a whitish-grey, "waxy bloom", and produces antibiotics that have been found to have specific and potent inhibitory effects on the *Escovopsis* pathogen. The benefit for the actinomycete is that it is provided with food for growth, and more importantly, is dispersed by the virgin queen ants when they establish new colonies (Currie, 2001). This interaction between ant and actinomycete was initially thought to be a tightly controlled example of co-evolution, but more recent work has found this not to be the case (Mueller *et al.*, 2008).

Another example is found within the European beewolf wasp (*Philanthus triangulum*), where the females cultivate an actinomycete from the *Streptomyces* genus within specialised antennal glands. During egg-laying, the female applies secretions from these glands onto the ceiling of the nest site, which are taken up by the larvae and serve to protect both them and their food from fungal infestation (Kaltenpoth *et al.*, 2005; 2006).

1.1.3 Maintaining symbioses

Because of the importance and sometimes obligate nature of symbioses, it is beneficial for both the host and symbiont to maintain the association by transmission of the symbiont to the host's offspring. This is therefore closely linked to adaptations by the host, in order to provide suitable habitats for their symbionts, which include various structures that have evolved over time to serve this purpose.

1.1.3.1 Intracellular

In carpenter ants (*Camponotus ligniperdus*), their endosymbiotic *Blochmannia* bacteria are housed in specialised cells (bacteriocytes) that are found between normal epithelial cells of the midgut and in the ovaries of workers and queens. This location in the ovaries allows for strict maternal transmission, and their co-evolution has been supported by phylogenetic analysis (Boursaux-Eude & Gross, 2000; Zientz *et al*, 2005). This vertical transmission route brings together the interests of both symbiotic partners, as the spread of the symbiont depends on host reproduction and its prevalence is determined by its effects on host fitness (Russell & Moran, 2005).

A similar, ancient symbiosis has evolved between the aphid and the bacterium *Buchnera aphidicola*. This is an obligate symbiosis (as the microorganisms cannot be cultivated *in vitro*), and is essential for growth and reproduction of the host, as the symbiont has been found to provide essential nutrients, specifically the amino acids, leucine and tryptophan. The *Buchnera* symbiont is located intracellularly in 'symbiosomes' within the mycetocytes (specialised bacterial chambers) (Boursaux-Eude & Gross, 2000; Russell & Moran, 2005).

1.1.3.2 Extracellular

Within the Heteropteran insects, such as the Common Plataspid Stinkbug (*Megacopta punctatissima*) and Broad-headed Bug (*Riptortus clavatus*), the posterior end of the midgut is characterised by sac-like appendages or evaginations, called caeca or crypts. These insects are sap-feeding, and these specialised structures house specific groups of bacteria that aid in the digestion of this nutrient poor food source (Fukatsu & Hosokawa, 2002; Kikuchi *et al.*, 2005). These symbiotic bacteria are transmitted to the offspring via three possible mechanisms: superficial contamination of the eggs (egg smearing); probing of the mother's faeces (protophagy); or capsule transmission (where capsules containing the bacteria are deposited within the eggs) (Kikuchi *et al.*, 2005).

Special gut pouches filled with a dense bacterial mass have also been described in specific ant species and may have a similar function to the extracellular associations seen within the paunch (a special enlargement of the hindgut) of certain termites (Baumann & Moran, 1997; Zientz *et al*, 2005).

In some cases, the bacteria themselves are able to control their transmission. *Wolbachia* is an obligate intracellular *α-Proteobacterium* that is extremely widespread (it is estimated that around 15% of all insects are infected with these bacteria). It is transmitted maternally, via egg cytoplasm and, in order to enhance its transmission to the insect progeny, it is able to interfere with the reproductive systems of its hosts by causing phenomena such as: feminisation of males; male killing, induction of parthenogenesis; and differential killing of uninfected females via cytoplasmic incompatibility (Boursaux-Eude & Gross, 2000; Zientz *et al.*, 2005).

In general, once established, transmission of a symbiont is strictly vertical and the host and endosymbiont can co-evolve, often without there being any genetic exchange. These associations involve endosymbionts that are separated from the insect reproductive line. Even in those that are associated with the reproductive organs, the symbiont, such as those found in

aphids, seems to retain most of its genes (Baumann & Moran, 1997). There are some closely related bacteria that are general symbionts and are able to distribute themselves across distantly related insect hosts. These therefore reveal a history of horizontal transfer or host switching and suggest that they have maintained a general ability to survive, reproduce and be efficiently transmitted between novel hosts (Russell & Moran, 2005).

1.1.4 Insect gut systems

In some insects, the intestinal tract contains a wealth of microorganisms, many of which are thought to be directly involved in the processing of food. Since insects produce their own digestive enzymes, the specific role of the intestinal microbiota in some cases remains unclear. In order to gain insight into the significance of this community and its role in digestion, determining the numbers of bacteria in the different regions is an important first step (Cazemier *et al.*, 1997). In a study by Cazemier *et al.* (1997) involving a whole range of insects, they determined that the bacterial counts in the foregut were between 0.2 and 3.6 x 10^9 (ml gut)$^{-1}$, between 0.2 and 28 x 10^9 (ml gut)$^{-1}$ in the midgut, and between 0.1 and 190 x 10^9 (ml gut)$^{-1}$ in the hindgut. This supported the observation that substantial differences exist between the different gut regions both in distribution and numbers of bacteria. However, in all cases, the greatest number of bacteria was found in the hindgut – evidence of its important role in host digestion.

One of the most well understood gut systems is that of the termite. Much work has been done to understand the conditions found within this complex system and how the community of organisms that live there are involved in the termite's metabolic processes.

1.2 TERMITES AND THEIR ENVIRONMENT

Termites have an important ecological role to play in the environment. They play a role in the turnover and mineralisation of complex biopolymers to simpler compounds by degrading cellulose- and hemicellulose-containing materials, such as dead plant matter or wood (Varma *et al*, 1994). Termites are also notable for their role in nitrogen fixation and methane emission. In the tropics and sub-tropics and in areas with semi-arid soils, termites also have a significant impact on pedogenesis (the process of soil formation), soil properties, and soil functions by replenishing organics and increasing soil aeration and drainage (Varma *et al.*, 1994; Kurtböke & French, 2007). These physical and chemical changes are mainly caused by

their construction activities, specifically relating to the building of their mounds. Increases in the clay contents (5 times more), minerals (50 times more ammonium) and organic matter (5 to 7 times more carbon and nitrogen) compared with surrounding soils, have all been documented. These changes also have a corresponding impact on the bacterial and fungal communities in these areas (Keya *et al.*, 1982; Fall *et al.*, 2007).

This increase in the richness of the soil surrounding termite mounds also results in an increase in plant growth. Dense and vigorous growth of vegetation has been documented and certain grass species surrounding termite mounds have been shown to be different from the surrounding areas (Keya *et al.*, 1982).

The termite's gut community is vital to providing all of these functions – without their gut communities, termites would not be able to play the role that they do. In order to understand the biorecycling role that the termites have in these systems, the most important step is to understand the gut community and, by culturing these members, an understanding of their diversity, distribution and function can be gained (Kurtböke & French, 2007).

1.2.1 Termite taxonomy – higher vs lower

1.2.1.1 Taxonomy and evolution

Termites belong to the order Isoptera, with seven families currently recognised. The order as a whole is an ancient one and, for some families, there is even fossil evidence that dates back to the mid Cretaceous. Termites are divided into the more primitive, 'lower' termites and the highly evolved, 'higher' termites. The lower termites comprise the families: Hodotermitidae, Termopsidae, Kalotermitidae, Rhinotermitidae, Serritermitidae, and Mastotermitidae (which is considered the most primitive family and the closest relative to the cockroaches). The higher termites encompass only one family, the Termitidae, which alone accounts for 75% of the more than 2200 described termite species (Brauman *et al.*, 2001; Varma *et al.*, 1994). Their evolutionary radiation has coincided with three major events relating to diversification in their diets, as well as microbial symbioses, including the following: the flagellated protozoa (single-celled eukaryotes) found in the hindguts of all six lower termite families, have been lost in the higher Termitidae; an ectosymbiotic relationship was established with a fungus by the Termitidae subfamily, Macrotermitidae; and the independent evolution of soil-feeding behaviour among various members of three other higher termite subfamilies (Brauman *et al.*, 2001).

1.2.1.2 Higher vs Lower: the termite gut

The lower termites are typically xylophagous and feed primarily on wood or grass. They are characterised by a relatively simple gut structure and the presence of flagellated protists within the gut. The gut community also comprises prokaryotes (Bignell *et al.*, 1980a; Purdy, 2007). Many of the protozoa are found nowhere else in nature (except in members of the wood-feeding cockroach genus, *Cryptocercus*) and are affiliated to the orders Trichonymphida, Cristamonadida, Spirotrichonymphida, Trichonymphida (all phylum Parabasalia) and Oxymonadida (phylum Preaxostyla) (Varma *et al.*, 1994; Ohkuma *et al.*, 2007). These symbionts (housed in the paunch hindgut region) are the primary agents for the digestion of cellulose, converting it into various organic acids, some of which are absorbed by the host (Bignell *et al.*, 1980a; Bignell *et al.*, 1991; Brune, 2007).

Higher termites, on the other hand, have undergone extensive diversification, are typified by a lack of protists, and show various feeding habits. The gut community in these termites essentially consists of bacteria alone (Bignell *et al.*, 1980a; Varma *et al.*, 1994; Ohkuma, 2008). Higher termites feed on a wide range of plant material, ranging from wood to soil organic matter. The majority of these termites are soil-feeders and live on large amounts of mineral material, as well as highly humified and dispersed organic matter (humus) found in the soil. In fact, more than half of all termite genera are humivorous. As such, these termites can be quite diverse and abundant in terrestrial environments, especially in tropical forest soils (Bignell *et al.*, 1980a; Bignell *et al.*, 1991; Brune & Friedrich, 2000; Purdy, 2007).

1.3 THE TERMITE GUT SYSTEM

Like ants, termites also maintain symbiotic relationships with various microorganisms, which range from the cultivation of fungal gardens to housing bacteria intracellularly in bacteriocytes (Brune, 2006). However, their main symbiosis has to be that which has evolved inside the intestinal tract.

This symbiosis can be characterised as an obligate nutritional mutualism between the termite host and the various microorganisms that live within the gut and is comprised of members of all three domains: Archaea, Bacteria, and Eukarya. Within this environment, the microbiota can be found free-swimming, attached to the gut wall, or associated with its symbiotic protozoa – up to 85% of the total prokaryote population is thought to be involved in this association with protozoa (Stingl *et al.*, 2005; Brune, 2006; Kurtböke & French, 2007). This

community of microorganisms contributes to digestion, host nutrition, as well as gas emission (CH_4, CO_2 and H_2) and, as a result, is typically complex. Digestion in termites is closely related to gut structure, as well as the physical and chemical conditions and microbiota found in the different gut regions. As a result, the gut community is also substantial (10^6-10^7 cells per µl gut volume) and morphologically diverse (Brauman *et al.*, 2001; Purdy, 2007).

Because the termite gut is such a complex environment involving multiple diverse moving and flowing parts, a detailed understanding of all the individual parts is the only way that the system as a whole will ever be understood.

1.3.1 Termites – conditions within the gut

1.3.1.1 Gut anatomy

In all insects, the gut is generally divided into three main regions: a short foregut, the midgut (which is the main site of digestion) and a small hindgut. In termites, however, the hindgut has evolved over time, in both length and volume. As shown in Fig. 1.1a, the typical lower termite gut is still fairly simple, with the dilated hindgut 'paunch' now being the major site of digestion (Brune, 2006; Purdy, 2007). The hindgut can be divided into five successive segments: the proctodeal segment, the enteric valve (whose role is to prevent return of gut contents to the midgut), the previously mentioned paunch (generally composed of a dilated, thin-walled region, as well as a more tubular, thick-walled region), and lastly the colon and rectum (Varma *et al.*, 1994; Berchtold *et al.*, 1999). This simple design has been retained in the fungus-growing higher termites, but in all other higher termite lineages further elongation and compartmentalisation (and concomitant specialisation) have occurred (Fig. 1.1b). This change is most pronounced in the soil-feeding representatives (Brune, 2006). Following the foregut (crop and gizzard) and midgut, a region called the mixed segment is found. This unique mixture of both hindgut and midgut epithelia is associated with its own specific microbial community. Despite its relatively small volume of around 0.5-10 µl, the hindgut of these termites is morphologically complex. One other example is the modification of the posterior paunch region with numerous cuticular spines, which serve as attachment sites for the microbes found here. In the soil feeders, this whole system is kept full of soil, and the contents generally have a transit time of around 36-48 hours (Bignell *et al.*, 1980a; Berchtold *et al.*, 1999).

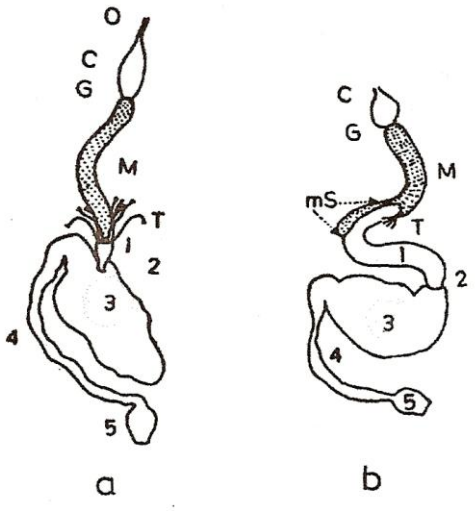

Figure 1.1: Gut diagrams for two termites, showing the different regions found in both a typical lower (a) and higher termite (b). C: crop; G: gizzard; M: midgut; T: malphigian tubules; mS: mixed segment; O: oesophagus; 1: proctodeal segment; 2: enteric valve; 3: paunch; 4: colon; 5: rectum (taken from Varma *et al*., 1994).

1.3.1.2 The termite rumen?

A combination of host and microbial activities within the gut is responsible for the creation of physicochemical gradients. These in turn result in specific conditions within the different gut compartments, relating to oxygen, hydrogen, redox potential and intestinal pH (Brune, 2006).

Termite guts, in particular the enlarged hindguts, were initially thought to be simple bioreactors or anaerobic digesters (Brune *et al.*, 2000; Brune, 2007), but are actually much more complex than that, not only in terms of digestion and the metabolic processes that take place there, but also in terms of the community of microorganisms that contributes towards this metabolic ability and the interactions that occur between the constituents and their termite host.

Much like in the rumen of sheep or cattle, the symbiotic gut microbiota is responsible for the depolymerisation of celluloses and hemicelluloses and the resulting carbohydrates are in turn fermented into acetate and short-chain fatty acids, which are absorbed by the host (Brune, 1998; Brune *et al.*, 2000). Termite guts therefore resemble the rumen in that they are characterised by high levels of fatty acids, as a result of the activities of fermentative bacteria and protozoa, and also the occurrence of typical anaerobic activities such as homoacetogenesis and methanogenesis. However, the same model as the rumen could never be applied to the termite gut, due to the following distinct and important reasons: (i) without oxygen, lignin and humic compound degradation (which is a recognised process within the termite gut), could not occur; (ii) most of the isolates that have been cultured from termite

guts include aerotolerant, facultatively anaerobic and obligately aerobic bacteria; and (iii) a system as small as the termite gut could only maintain its anaerobic status if the oxygen entering by diffusion were continuously removed (Brune, 1998).

1.3.1.3 Anaerobic or aerobic?

Support for the anoxic status of the termite gut was originally provided by sound scientific evidence, including the presence of oxygen-sensitive protozoa, as well as the occurrence of fermentative metabolism and oxygen-sensitive or strictly anaerobic processes, such as nitrogen fixation and methanogenesis. Coupled with the frequent isolation of strictly anaerobic bacteria, it is not difficult to understand why the termite gut was considered to be a simple anaerobic digester (Brune, 2006; Wertz & Breznak, 2007).

In contrast, several authors made use of the redox dye feeding technique to determine the redox state of the termite gut and found it to be aerobic. Other lines of evidence, such as lignin degradation and the isolation of oxygen-utilising bacteria lent support to the fact that some part of the gut must be aerobic (Varma *et al.*, 1994; Wertz & Breznak, 2007). The fact is that anoxia is established and maintained by the respiratory activity of the facultatively anaerobic and strictly aerobic members of the gut community (Brune, 1998).

This is no mean feat, as the continuous removal of oxygen is easier said than done – termite guts are surrounded by aerobic tissues that are aerated by the insect's tracheal system. Oxygen therefore moves via diffusion and, as such, easily makes its way into the gut system (Brune *et al.*, 2000). Another problem is the small size of the termite. There is an inversely-proportional relationship between surface area and volume in a spherical system, such as that of the gut. This means that for any surface related process, such as the diffusion of oxygen, there is an increase in its significance the smaller the volume of the system becomes. This is not a problem for the rumen of a cow, as this can be up to 100 litres in volume, but for the termite gut which, at 1µl, is 10^8 times smaller than the rumen, this has been estimated to result in around a 500 times larger influx of oxygen per unit volume (Brune, 1998; Brune *et al.*, 2000). It is simply a matter of scale which results in the fact that while the penetration of oxygen by less than one millimetre into a cow's rumen would have a negligible effect on the anaerobic status of the gut, for a termite, the penetration of oxygen by a fraction of this distance would have a significant impact on any anoxic environment. In *Mastotermes darwiniensis*, for example, the aerobic portion of its hindgut can reach up to 25% of the total gut volume (Brune, 1998; Wenzel *et al.*, 2002).

As a result of the facts described above, the oxic-anoxic interface at the gut wall has a very steep concentration gradient and, as such, there is a constant influx of oxygen into the gut through the gut wall. Microsensor measurements have shown that oxygen is able to penetrate between 50-200µm into the gut itself before it is totally removed by the respiratory activity of the gut microbiota. This implies that the microbiota at the gut periphery function as an important "oxygen sink" and are responsible for creating a microoxic zone around the gut wall, with the centre of the gut remaining an anoxic environment (Fig. 1.2) (Brune, 1998; Brune & Friedrich, 2000; Wertz & Breznak, 2007).

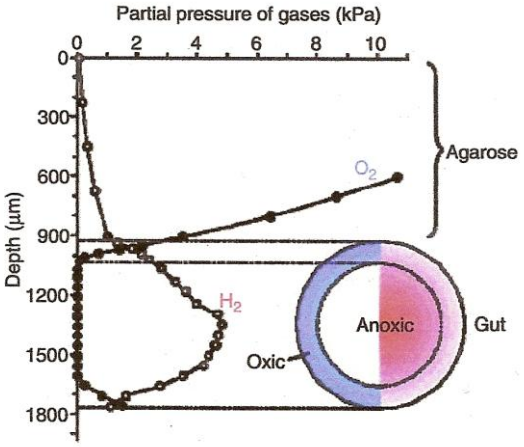

Figure 1.2: Radial gradients of both oxygen and hydrogen in an agarose-embedded paunch from a *Reticulitermes flavipes* worker larva. A pictorial cross-section through the paunch is shown to illustrate the relative size of the oxic and anoxic zones (Taken from Brune, 2006).

In a study testing exposure of termites to hyperbaric oxygen partial pressures, an extended exposure time resulted in irreversible damage to the oxygen-sensitive protozoa and subsequent defaunation of the hindgut. This demonstrated that there is a fragile balance within the termite hindgut between influx of oxygen into the gut and its removal by the respiratory activity of the gut microbiota (Brune, 1998).

1.3.1.4 The significance of hydrogen

As with oxygen in the gut, the accumulation of hydrogen in the anoxic centre of the hindgut, and its removal at the gut periphery, results in a steep gradient that runs in the opposite direction to the flow of oxygen, i.e. from the lumen of the gut outwards towards the gut epithelium (Brune & Friedrich, 2000). In lower termites, the high levels of hydrogen can be attributed to the activities of the intestinal protozoa during their carbohydrate fermentation processes. In higher termites, however, the lack of these symbionts makes the source of hydrogen in the gut unknown.

Despite the substantial accumulation of hydrogen in the gut, only small amounts actually escape into the environment. This can be attributed to the processes of methanogenesis and homoacetogenesis, which occur at the same time in termite hindguts and are considered the major hydrogen sink within the gut system (Brune, 1998; Brune *et al.*, 2000). Gut protozoa are often found associated with methanogens, hence the high levels of methane production within the lumen of the gut. However, a recent study in the termite *Reticulitermes flavipes* revealed, from the shape of the hydrogen gradient, that hydrogen consumption also occurs at the gut wall. In fact, the hindgut cuticle has been found to be densely colonised by members of the methanogenic archaea from the *Methanobrevibacter* genus. The basis for their oxygen tolerance at this location, as well as the significance of hydrogen-dependent oxygen reduction, is still to be determined (Brune, 1998; Brune *et al.*, 2000).

One problem with the dual activity of methanogenesis and homoacetogenesis in the termite gut, is that under the lower hydrogen partial pressures found in the posterior hindgut, methanogens should outcompete the homoacetogens as terminal hydrogen acceptors. Nevertheless, there is plenty of evidence for their co-existence in the same gut compartments and it has been suggested that the *in situ* proximity of the different gut regions would allow the diffusion of hydrogen from the anterior to the posterior of the gut. Also, homoacetogens consume most of their hydrogen within the lumen of the gut, leaving the protozoa-associated methanogens, as well as those attached to the gut wall, to utilise the remaining substrate. This situation suggests that it is the spatial and radial organisation of these two competing populations that allows for their co-existence within the termite gut system (Brune, 1998; Brune & Friedrich, 2000).

1.3.1.5 Termite gut pH

Recent studies have also made use of microelectrodes to show pronounced spatial differentiation of the termite gut in terms of its pH (Berchtold *et al.*, 1999). In the lower termites, the midgut pH has been found to be neutral, whereas the hindgut pH can range between 6 and 7.5. The most frequently measured value, such as that found in *Pterotermes occidentis*, is pH 6.8 (To *et al.*, 1980; Varma *et al.*, 1994). Except in the fungus-cultivating *Macrotermitinae*, early work documented the high alkalinity of the anterior hindgut compartments of the higher termites. Values up to pH 10.4 were observed in the gut of termites such as *Odontotermes obesus* – still considered tolerable for the growth of certain microorganisms. However, only recent more accurate work, using pH microsensors, has documented the highest pH value ever reported from a biological system. These values, ranging between pH 11-12.5, were found after investigating the anterior guts among the soil-feeding Termitinae. In all species tested, the pH increase starts at the midgut (which is around

neutral pH) and increases sharply, reaching its peak at the mixed segment region of the gut, and then decreases again towards the rectum (Varma *et al.*, 1994; Brune & Friedrich, 2000; Brune, 2006). As stated before, the diet of soil-feeding termites is low in carbohydrates and high in polyphenolics and humic compounds. It has therefore been suggested that the high pH in the anterior portion of the termite gut may be linked to the release of recalcitrant organic matter from the soil and its chemical decomposition, thereby allowing for subsequent microbial digestion of the solubilised substrates in the less alkaline gut compartments (Brune, 1998; Purdy, 2007).

1.3.2 Gut metabolism

As described previously, many termites, like other insects, survive on diets that are predominantly lignocellulose-based. They therefore have a large and dense gut community, housed in specially adapted gut compartments, which convert a substantial proportion of this material into useful products that the termites are able to utilise for their own metabolism. As such, it is assumed that this intestinal symbiosis provides for metabolic activities that would otherwise not be available to the termite host (Brune, 2006). There are three main metabolic activities that have been attributed to the gut microbiota: (1) hydrolysis of wood polysaccharides, such as cellulose and hemicellulose; (2) fermentation of the depolymerisation products to short-chain fatty acids; and (3) intestinal nitrogen cycling and nitrogen fixation (Brune & Friedrich, 2000).

1.3.2.1 Degradation of celluloses

The digestion of celluloses in the termite gut is a complex process that involves the activities of both the host and its gut microbiota. While the termite controls most of the processes in the foregut and midgut, events in the hindgut are largely dependent on the gut microbial community (Stingl *et al.*, 2005).

Plant material is one of the most complex dietary substrates, as plant cell walls are composed of three major components: cellulose, hemicellulose and lignin. As a result, the degradation of this food source requires the combined action of many different enzymes and, in the case of lignin, mechanisms to break up the lignocellulose complex. In nature, bacteria, protozoa and fungi are all efficient utilisers of these resources and, as such, termites and other animals have taken advantage of their specialised abilities in order to survive on plant material as a food-source (Schäfer *et al.*, 1996, Brune, 2006).

Little is known about the process of lignin degradation in the termite gut. The break-down of lignin is known to be an oxygen-dependent process, supported by the fact that its degradation increases in the well-oxygenated paunch region of the gut. However, the specific site where this occurs has not been determined (Varma *et al.*, 1994). Lignin depolymerisation by fungi is thought to be carried out by the activity of extracellular lignin peroxidases, which are also known to be produced by actinomycetes. Lignin-degrading actinomycetes (all from the *Streptomyces* genus) have even been isolated from the guts of a range of termites from the Termitidae, a result established from a study comparing their activity against the known lignin-degrader *Streptomyces viridosporus* strain T7A. However, no microorganism that is able to break down intact lignin has been found. It has been suggested that it is the combined efforts of cellulolytic and ligninolytic enzymes that allow for the break-down of lignocelluloses, as the activity of the cellulases may increase the accessibility of the lignin parts of the plant material to the ligninolytic enzymes (Pasti *et al.*, 1990; Varma *et al.*, 1994).

From very early on, it was a well-known fact (in the lower termites) that the symbiotic flagellates had a major role in the termite gut in terms of their cellulolytic and xylanolytic abilities. This is not surprising, as the bulk of the hindgut volume is taken up by these unicellular eukaryotes – as much as one third of the termite body weight can be contributed by these protozoa. It was originally thought that the gut microbiota were the primary contributors towards cellulose digestion but, more recently, the discovery of a cellulase gene of termite origin has provided strong evidence for the role of termites in their own digestive processes (Watanabe, *et al.*, 1998; Wenzel *et al.*, 2002, Stingl *et al.*, 2005). In its passage through the termite gut, the food is exposed to a variety of endogenous digestive enzymes (such as endoglucanases) that are secreted by the salivary glands and midgut. However, at this stage, the cellulose, particularly the amorphous regions, is only partially degraded and this product then passes to the hindgut where the protozoa endocytose and depolymerise the cellulose to completion. Therefore, it is the combined activity of the host enzymes and their gut protists that allows for the degradation of cellulose, despite these activities not being co-localised (Varma *et al.*, 1994; Ohkuma, 2008).

Investigations have shown that there are many more cellulolytic bacteria in termite guts than were previously surmised and it has therefore been suggested that, in the higher termites, these bacteria have taken over the role of cellulose degradation carried out by the flagellates in the guts of the lower termites (Wenzel *et al.*, 2002). An investigation of the paunch of a higher termite *Nasutitermes* species by Warnecke *et al.* (2007), showed the presence of a large and diverse set of bacterial genes for cellulose and xylan degradation, implicating spirochete and fibrobacter species in gut lignocellulose degradation. In soil-feeding termites,

their diets consist of fragments of plant material, fungal hyphae, large quantities of soil microbes and the undefined humic matter associated with the soil matrix. As discussed previously, the extremely alkaline anterior hindgut has been suggested to favour the extraction of organic matter from the soil, but it is the microbial biomass (which is assimilated more efficiently than cellulose), which is proposed as the more important dietary component for these humivorous termites (Brune, 2006).

Cellulose- and hemicellulose-degrading bacteria that have been isolated from termite guts include clostridia, staphylococci, micrococci, bacilli, as well as representatives of the actinomycetes, including members of the genera *Micromonospora* and *Streptomyces*. Gram negative representatives include isolates that belong to the genera *Pseudomonas* and *Acinetobacter* and to members of the *Enterobacteriaceae* family. It has also been suggested that certain yeasts and other fungi present in termite guts are also involved in hemi-cellulose break-down (Schäfer *et al.*, 1996, Wenzel *et al.*, 2002).

1.3.2.2 Fermentation

The process of fermentation of the soluble products released into the gut is where the prokaryote community within the termite gut has an important function. These products are produced by the activity of the host's digestive enzymes, or are due to the fermentative activity of the intestinal protozoa. In simple terms, the process involves the fermentation of the plant polysaccharides into acetate, carbon dioxide and hydrogen. The acetate is then absorbed by the termite and is used almost exclusively as their carbon and energy source. This model of fermentation in the termite gut fits the system quite well, as it allows for the acquisition of energy (ATP) by both the protozoa (via anaerobic fermentation of cellulose), and the termites (via aerobic oxidation of acetate) (Varma *et al.*, 1994; Brune & Friedrich, 2000; Brune, 2006; Ohkuma, 2008). In most termites, microbially produced acetate dominates the pool of fatty acids in the hindgut, making up almost 98 mol% of all volatile fatty acids and occurring at concentrations of up to 80mM. Although acetate is the major product of fermentation, other short chain fatty acids, such as propionate, butyrate and lactate, are also produced, although in smaller amounts (Brune, 2006; Wertz & Breznak, 2007).

In wood-feeding termites, it was initially assumed that the activities of the flagellates and acetogens would make the process of lignocellulose metabolism essentially homoacetogenic. However, a study using injected, radiolabeled metabolites into an immobilised hindgut of *R. flavipes* showed that reductive acetogenesis only represented 10% of the flow of carbon, with 30% proceeding via the production of lactate. The presence of lactic acid bacteria within the same gut system has been used to explain this discrepancy. It has been suggested that

these bacteria shift the fermentation balance from lactate towards acetate formation when oxygen (such as that found in the termite hindgut) is present (Brune & Friedrich, 2000; Bauer *et al.*, 2000).

1.3.2.3 Nitrogen cycling and fixation

There are almost no nitrogenous compounds found in wood (as little as 0.03 – 0.1% dry weight in some cases) and, as such, nitrogen fixing bacteria are probably a major source of nitrogen in the termite system. Nitrogen-fixing bacteria, such as *Enterobacter agglomerans*, have been isolated from the termite gut from as early as 1977. Many termites show a preference for wood that has been colonised by fungi, because the low C-to-N ratio created by the fungi aids the uptake of nitrogen. However, those termites that live off 'sound' wood, rely on the capacity of their gut bacteria to fix atmospheric nitrogen (To *et al.*, 1980; Varma *et al.*, 1994; Brune, 2006).

Other ways in which termites obtain their metabolic nitrogen include extraction from their diet, digestion of symbionts (detailed below), ingestion of faecal matter, as well as nitrogen waste recycling. Some termites, such as *P. occidentis*, also feed on their dead siblings to provide another source of nitrogen (To *et al.*, 1980).

Nitrogen recycling is one of the most important ways in which termites are able to maintain high levels of ammonia in the hindgut. This is important, as it allows for the maintenance of an active gut community, ensuring high levels of carbon utilisation. Nitrogen recycling involves the assimilation of ammonium into microbial biomass, avoiding its loss in the faeces. This assimilated nitrogen is then recycled into other termites by a process called 'proctodeal trophallaxis', whereby worker larvae feed off droplets of hindgut fluid, solicited from adult workers. This behaviour is unique to the termites and has been found to increase with increasing levels of nitrogen limitation (Brune, 2006).

In the case of soil-feeding termites, such as *Cubitermes* spp., the nitrogen content of their diet increases with increasing humification of the organic matter in the soil and potential sources of dietary nitrogen include peptides, amino acids and microbial biomass. Studies have shown that in humivorous termites, it is dietary nitrogen and not nitrogen fixation that is the most important source of nitrogen. The high levels of ammonium in the mounds and faeces of soil-feeding termites suggest that this group is not nitrogen limited (Brune, 2006).

1.4 ADAPTATIONS FOR COLONISATION IN THE TERMITE GUT

Despite their relatively simple gut morphology, in the lower termites there are four very different environments or microhabitats that provide for the association of the termite and its gut community. The first is the midgut, which is characterised by host enzymes; second is the wall of the hindgut paunch (described previously as a highly oxygenated environment); third is the gut lumen, a space filled with digestive fluids and protozoa; and fourth is the protozoa themselves. All of these various habitats provide specific niches which allow the different communities that live there to interact and function in different ways (Yang *et al.*, 2005).

1.4.1 Midgut

The colonisation of the surfaces of other animals by microbes is common in nature, with attachment to gut epithelial surfaces being well-documented in a variety of insects and termites in particular (Breznak & Pankratz, 1977). In termites, the midgut epithelium, as with all other insects, is not protected by a cuticle. Instead, a peritrophic membrane separates the epithelial surface from the gut fluid. This membrane serves to protect the midgut epithelium from abrasion, facilitates the access of soluble digestion products for absorption, and acts as a physical barrier to infection. More importantly, this membrane also serves as an attachment site for the microorganisms that live here (Bignell *et al.*, 1980b; Brune 2006). It has also been reported that actinomycete bacteria colonise the space between the membrane and the epithelium – called the ectoperitrophic space – showing no pathogenic effects, and also form intimate associations with the epithelial microvilli. In soil-feeding termites, the unique mixed segment region of the gut also provides attachment sites for its own specific bacterial community (Bignell *et al.*, 1980b; Brune 2006).

1.4.2 Hindgut

As with the midgut, numerous associations with the epithelium have been demonstrated, except in this case they involve interactions with the cuticle (Brune, 2006). In soil-feeding termites, there has been considerable development and specialisation of the hindgut. One such example, which has been mentioned previously, is the development of numerous, greatly elongated spines from the cuticle lining of the gut wall in the posterior portion of the colon. The spines are around 200µm in length and form a matrix with mineral material, as well as the numerous microbial filaments found there. Because the spines are directed away from the rectum, this matrix across the gut lumen serves to filter the ingested soil moving through the gut by peristalsis (Bignell *et al.*, 1979; 1980a).

Within the paunches of two wood-feeding termites, *R. flavipes* and *Coptotermes formosanus*, cup-like indentations of the epithelial surface are found. An abundance of mitochondria and infoldings of the cytoplasmic membrane at these sites resemble transport epithelia and it has been suggested that these cups are the sites of active transport of ions. The bulk of cellulose fermentation occurs via the protozoa in the paunch and, because of the enteric valve, no acetate produced by this process is able to return to the midgut. Therefore, the hindgut must be able to absorb this energy source and the cup-like structures are thought to be the specific sites for this very function (Breznak & Pankratz, 1977).

1.4.3 Protozoa

The association of prokaryotes with the protozoa found in termites' guts has often been observed, but the physiological basis for these associations is not known. In these associations, the symbionts are either intracellular, found in the cytoplasm or in the nucleus (endosymbiotic), or found attached to the cell surface (ectosymbiotic). Associations with the surface-associated spirochetes, the ecto- and endosymbiotic *Bacteroidales* members, and the endosymbiotic methanogens, have been shown for multiple species of protozoa, based on molecular sequence analysis (Ohkuma *et al.*, 2007; Ohkuma, 2008).

In terms of the ectosymbionts, one functional adaptation exists between the protist, *Mixotricha paradoxa*, and its surface associated spirochete (identified as belonging to the *Treponema* cluster). *M. paradoza* only occurs in the gut of the termite, *M. darwiniensis*, and is propelled by the helical movements of the specific spirochetes that adhere to the host membrane via specialised cell junctions (Stingl *et al.*, 2004). It has also been suggested that the interactions between protozoa and their epibionts have a metabolic function. Bacteria known to be associated with the protozoa include polysaccharide-fermenting anaerobes, some of which are known to produce cellulases, and might therefore complement the activities of their hosts (Stingl *et al.*, 2004).

Cytoplasmic endosymbionts of the *Trichonympha* and *Pyrsonympha* protozoa species found in the gut of *Reticulitermes santonensis* include a group that belongs to the recently described *Elusimicrobia*, which seem to be exclusively associated with termite gut flagellates. Currently, there is no information on their function (Geissinger *et al.*, 2009; Brune, 2006).

1.4.4 Adherence mechanisms

Attachment of bacteria to the paunch or gut epithelia frequently appears to be mediated by epicellular holdfast elements. Two general types have been observed, one consisting of fuzzy, fibrous or thread-like material that coats the entire surface of the cells and is probably some form of adhesive capsular or slime substance. The other type consists of a cluster of thin fibres that originate from the poles of rod-shaped cells (Breznak & Pankratz, 1977). Attachment to the gut wall is also known for some protist species belonging to the order *Oxymonadida*. These species, present in the gut of *Reticulitermes speratus,* have attachment organelles or holdfasts on their anterior cell portion that allow them to adhere to the gut wall (Nakajima *et al.*, 2005).

1.4.5 Attachment advantages

Colonisation of and attachment to the intestinal epithelia have been suggested to provide a survival advantage to the adhering bacteria and to the termite in retaining its symbionts. The gut environment poses a constant threat of washout due to the movement of food by peristalsis. In the paunch, strong attachment is even more important, as the attached community must also contend with the disruptive and turbulent movements of the masses of fast-moving protozoa (Breznak & Pankratz, 1977; Nakajima *et al.*, 2005). The abundant bacterial groups associated with the gut wall include members of the *Actinobacteria*, *Bacteroidetes*, *Clostridiales*, and *Lactococcus* – all including non-motile species. In comparison, spirochetes and *Desulfovibrio* are associated with the gut lumen and are well-known for their high levels of motility, thereby allowing them to maintain their positions within the gut (Nakajima *et al.*, 2005).

Within the termite gut, the different gut regions provide specific habitats and conditions, such as the highly alkaline mixed segment of the higher termites. These distinct physicochemical conditions, as well as specific termite gut structural adaptations, provide unique environments that support a unique microbial diversity within the different parts of the termite gut (Hongoh *et al.*, 2005).

1.5 DIVERSITY WITHIN THE TERMITE GUT

1.5.1 Cultured vs Culture-independent

In view of the variety of conditions and habitats that exist in the termite gut, it is not surprising that there is an equally large variety of microorganisms that live there. Morphological and phylogenetic studies have been performed in many different termite guts and, although culture-based studies are important in order to understand their diversity, distribution and function, they can only reveal a small proportion of the gut community – those that are amenable to current cultivation methods (Brauman *et al.*, 2001; Brune, 2006; Kurtböke & French, 2007). By far the greatest amount of information and diversity has been revealed by cultivation-independent means. Studies involving the amplification and sequencing of 16S-rRNA genes extracted from the guts of *Reticulitermes* species (one of the most well studied termite genera), have shown that the majority of the recovered sequences represent novel and as yet-uncultured species. As such there is often very little overlap between the results obtained using the two different techniques (Brauman *et al.*, 2001; Brune, 2006; Kurtböke & French, 2007).

The microbial communities within the termite gut are substantial, making up 61% of the hindgut contents and ranging between 10^6 and 10^7 cells per µl of gut volume. They are also morphologically diverse and comprise members of all three domains – the Eukarya, Bacteria and Archaea and, because of the uniqueness of the termite gut environment, include aerobic, facultatively anaerobic and anaerobic forms (Varma *et al.*, 1994; Brauman *et al.*, 2001).

1.5.2 Archaea

There is a considerable diversity of archaea found in the termite gut. What is most noteworthy is that this diversity appears to be different between the lower and higher termites and is related not to termite phylogeny, but to differences in feeding group. In fact, in terms of relative abundance, results have shown that the ratio of archaea to bacteria is, on average, twice as high for soil-feeders as for wood-feeders. Most termites, regardless of their diet, appear to be able to host methanogens, but their relative abundance and/or activity is closely linked to their association with soil (Brauman *et al.*, 2001; Purdy, 2007).

Three methanogenic strains have been isolated from the wood-feeding lower termite *R. flavipes*, all belonging to the *Methanobrevibacter* genus. Other methanogens related to

Methanobrevibacter, as well as *Methanobacterium*, have also been reported from two *Nasutitermes* higher termites (Purdy, 2007).

In culture independent studies in *R. flavipes*, the gut wall was found to be the only site associated with methanogens, although this observation does not apply to all lower termites. The methanogens grow best with hydrogen and carbon dioxide and, to some degree, seem to be tolerant of oxygen due to the presence of catalase activity - which may explain their ability to colonise the microoxic gut periphery (Brune & Friedrich, 2000; Purdy, 2007). As discussed previously, methanogens are also associated with the hindgut protozoa and sequences related to *Methanocorpusculum parvum* have been cloned from the hindgut of *R. speratus*. In the higher termites, studies have shown much greater archaeal diversity, including members of the *Methanobacteriales*, *Methanosarcinales* and the *Methanomicrobiales*. In general, most of the studies performed on higher termites have found evidence of methanogens that belong either to the *Methanobacteriaceae* or to the *Methanosarcinaceae* families and, in the study by Brauman *et al.* (2001), these accounted for 60% of the archaeal signal (Brauman *et al.*, 2001; Purdy, 2007).

Thus, it seems that higher termites, whether they are soil- or wood-feeders, maintain a more diverse archaeal community than their lower termite relatives and the sequences found in termites differ quite considerably from known methanogens, indicating that unique lineages exist within this environment. The methanogens are not the only archaea that can be found in the termite gut – *Thermoplasmatales* and *Thermococcales* are two examples of non-methanogenic archaea (Brune, 2006; Purdy, 2007).

1.5.3 Bacteria

As with other members of the gut community, the bacterial component is highly structured in terms of its spatial distribution. Bacteria are often found attached to the gut wall, but can also be found suspended in the gut fluid/lumen, or located within or on the surface of the intestinal protozoa (Berchtold *et al.*, 1999; Nakajima *et al.*, 2005). Most bacteria have been found to be associated with the gut wall and its associated cuticular spines, such as those found in the termite *Procubitermes aburiensis*, whereas smaller numbers have been observed free within the lumen, or in the midgut region (Varma *et al.*, 1994).

1.5.3.1 Culture-based analyses

In terms of the culturable fraction that can be isolated from the termite gut, results have shown that between 0.3-1.3 × 10^6 colony-forming units per gut can be isolated from workers of *R. flavipes* (Varma *et al.*, 1994).

It has been shown that the majority of the carbohydrate-utilizing bacteria cultivated from the hindguts of this lower termite are aerotolerant lactic acid bacteria, along with facultatively anaerobic or even strictly anaerobic bacteria. The presence of these bacteria has been established for all of the wood-feeding termites that have been investigated so far, including three representatives from the lower termite families and one from the higher termites (Bauer *et al.*, 2000). In a study performed by Wertz & Breznak (2007), an attempt was made to cultivate oxygen-consuming bacteria in order to understand and identify those bacteria associated with the microoxic hindgut wall environment and what substrates they use for their respiratory activity. One particularly abundant bacterium was identified as being related to *Eikenella corrodens*, grouping within the family *Neisseriaceae* in the phylum β-*Proteobacteria*. Strains of this microbe were estimated to comprise between 2 and 7% of the total prokaryotic community within the gut of *R. flavipes* and, because of their phylogenetic novelty, as well as their apparent ability to oxidise acetate under hypoxic conditions (suggesting they might be true microaerophiles adapted to life at the hypoxic periphery of the gut), they were described as a novel species within their own novel genus, *Stenoxybacter acetivorans*. The bulk of the other isolates that were cultivated included members of the *Streptococcaceae*, *Enterococcaceae* and *Enterobacteriaceae* (Wertz & Breznak, 2007).

1.5.3.2 Culture-independent analyses

Methods used in this form of analysis include the sequencing of the 16S-rRNA gene and subsequent clonal analysis, or the hybridization of group-specific, fluorescently labelled, rRNA-targeted oligonucleotide probes. In combination with confocal laser microscopy, this latter method has been widely used in the identification and enumeration of non-cultivated microorganisms and allows their *in situ* localisation within complex environments, such as the termite gut (Berchtold *et al.*, 1999).

In one culture independent study alone, 700 gut bacterial phylotypes were discovered from the analysis of almost 2000 clones in the lower termite *R. speratus*. Of the more than 15 phyla detected, *Spirochaetes* (particularly from the genus *Treponema*) were found to be the most dominant and *Bacteroidetes*, *Firmicutes* and the candidate phylum Termite Group 1 (or the

Elusimicrobia) were the second most important groups. It was also shown that approximately 99% of the phylotypes that were discovered showed less than 97% sequence similarity to any other clones from other environments and to any cultured strains in the public databases, implying that they represent novel and as yet uncultured bacteria. In total, the three major groups discovered make up approximately 80% of the total bacterial component of the gut community, with even greater degrees of diversity found in other termite species (Hongoh *et al.*, 2005; Ohkuma, 2008).

Spirochetes are a constant and abundant component of the hindgut flora, particularly within the paunch, where they can be free-living or attached to the surface of protozoa in lower termites (Varma *et al.*, 1994). They are a morphologically diverse group that may account for up to 50% of all prokaryotes within some termites and molecular studies have indicated that they may represent a lineage that is distinct from other spirochetes (Brune & Friedrich, 2000; Brune 2006).

Bacteroidetes clones have been recovered from numerous termite species, either attached to the hindgut epithelium or to the surface of protozoa and form monophyletic clusters that are only distantly related to described taxa that have been found in other termite species (Brune, 2006).

Because of the unique physico-chemical conditions found at the gut wall, this habitat within the termite gut is associated with its own fairly large, diverse and very specific bacterial community, despite the abundance being lower than that of the gut lumen (Nakajima *et al.*, 2005). In a study by Nakajima *et al.* (2005), comparing the *R. speratus* communities associated with the gut lumen and wall, they found 38 novel phylotypes associated with the gut wall. One particularly prominent component of this gut wall fraction included a rod-shaped actinobacterial isolate from the family *Propionibacteriaceae*. Most members of this family are known to be non-motile and are either aerobes or facultative anaerobes. Other groups known to be associated with this unique habitat include *Bacteroidetes*, *Clostridiales* and *Lactococcus* (Nakajima *et al.*, 2005).

In comparison to the results found for *R. flavipes* and *R. speratus*, the situation in the gut of various soil-feeding *Cubitermes* spp. is quite different, because clone libraries have shown the presence of very few spirochetes and no evidence of members of the TG1 (‘Termite Group I’) phylum – as would be expected from a higher termite (Brune, 2006). Schmitt-Wagner *et al.* (2003b) demonstrated that the gut bacterial microbiota in these termites is quite specific and that changes in the structure and function of specific gut regions are reflected by changes in

the composition of the bacterial community. These differences in community structure relate not only to the high alkalinity that is found in the anterior gut regions, but are also due to the probable digestion of soil microorganisms in this region of the gut (Schmitt-Wagner *et al.*, 2003b). In fact, a steep increase in the pH between the midgut and the first portion of the hindgut coincides with a sharp drop in the density of bacteria. This is evidenced by the fact that the only clones that were obtained from this region of the gut were members of the low G+C Gram-positive Firmicutes and only one member of the Gram-negative *Bacteroidetes* (Schmitt-Wagner *et al.*, 2003a; Brune, 2006). In contrast, the posterior regions of the gut are represented by a larger variety of phyla, including different sub-groups of the *Proteobacteria*, as well as the *Bacteroidetes* and the spirochetes (Schmitt-Wagner *et al.*, 2003a).

The Firmicutes that were found in the *Cubitermes orthognathus* gut encompassed many clones that were affiliated with *Clostridium piliforme*, as well as other clostridial clones localised to the alkaline hindgut segments of the termite *Nasutitermes takasagoensis*. As such, many of these clones form phylogenetic clusters that are unique to termites, but are also related to other insects with alkaline intestinal tracts and may therefore represent unique lineages of alkaliphilic bacteria (Schmitt-Wagner *et al.*, 2003a; Brune 2006).

Representatives of the *Bacteroidetes* are also typical members of the gut microflora in mammals, including humans, and have been recovered from the gut of wood- and soil-feeding termites. Many are specialised degraders of plant fibres and proteins, which would explain their presence in all of these diverse environments (Schmitt-Wagner *et al.*, 2003a).

Comparisons of gut bacterial diversity indicate that these communities are conserved significantly within termite genera, forming lineages that are unique to termites from all bacterial groups. This conservation across gut communities, as well as their uniqueness, suggests that the diversity is endemic (autochthonous) and does not represent the diversity that is found in the surrounding environments (Ohkuma, 2008). These conclusions could never have been made without the use of cultivation-independent approaches that have resulted in the documentation of many new and previously uncultivated microbial phylotypes in the intestinal tracts of termites. However, the large difference between this high diversity and the relatively few metabolically relevant species that have actually been cultivated, highlights the need for new cultivation methods and strategies, the first of which would be to understand the physico-chemical environment found within the termite gut in more detail and to use this information in devising these new strategies (Brune & Friedrich, 2000; Brune 2006).

1.5.4 Actinomycetes

The first reported isolation of an actinomycete from a termite gut was by R.E. Hungate in 1946, when investigations into cellulose decomposition in the gut of an *Amitermes minimus* worker resulted in the isolation and identification of *Micromonospora propionici* (this species name was never validly published and the type strain no longer exists in any culture collections).

The cellulolytic and lignin-solubilising ability of the actinomycetes that have been isolated from termite guts is the main reason why they have been investigated in relation to this environment. However, despite earlier work performed in the 1980s, very little information is available about the diversity and role that these bacteria have in the complex termite gut system.

1.5.4.1 Early morphological evidence

Many of the studies done up to this time involved investigations of the lower termite families, due to their interesting association with gut protozoa. Bignell *et al.* (1979) were some of the first researchers to investigate the gut of soil-feeding termites from the higher termite family, Termitidae. In their examination of the guts of *P. aburiensis* and *Cubitermes severus*, scanning electron microscopy results showed the prominence of actinomycete-like bacteria attached to the elongated cuticular spines found in the posterior hindgut of these two termite species. Despite the fact that no sporing structures were seen, these filaments were assumed to be actinomycetes based on the fact that they were never more than 0.5 µm in diameter, were infrequently branched, not obviously septate and were clearly prokaryotic in ultrastructure. However, evidence of these filaments was found from the enteric valve all the way through to the posterior hindgut and also in the midgut as well – colonisation of the peritrophic membrane, the ectoperitrophic space, as well as intimate associations with the epithelial microvilli (Bignell *et al.*, 1980b). A detailed analysis was performed in the gut of *P. aburiensis*, involving a comparison of all of the regions. Standing crops of bacteria were determined by direct observation and showed a dramatic decrease in filaments in the anterior portion of the hindgut (similar to that found in the crop) when compared with the midgut, but subsequently increased until just past the colon. Dilution plates incubated under aerobic conditions supported this observation (Bignell *et al.*, 1980a). Later work by Bignell *et al.* (1991) in the same two soil-feeding termites also showed that at the transition from the midgut to the mixed segment, which coincides with the abrupt rise in pH of the intestinal lumen, there is a correspondingly steep decline in the number of actinomycetes.

Despite there being no conclusive evidence at the time that actinomycetes could degrade humus, Bignell *et al.* (1979) suggested that the ability of soil-dwelling strains to secrete phenoloxidases might imply that the actinomycetes found in soil-feeding termites have a role in the formation of humus from phenolic complexes derived from plant tissues. Whether the increases in the numbers of actinomycetes as well as other microorganisms in the ingested soil could be attributed to growth (inside the termite gut) by specific populations sourced from the indigenous soil flora, or represent a decrease in the diversity of the unique host symbionts as a result of leaving the gut system, was not clear. What was clear at the time was that their abundance in both the midgut and hindgut suggested that they may form an important component of the gut microflora (Bignell *et al.*, 1979; 1980a; 1980b).

1.5.4.2 Cultured isolates - Cellulolytic activity

Cellulose and hemi-cellulose are digested to a large extent during the passage of food through the termite digestive tract and in lower termites this activity has been assigned mainly to the symbiotic protozoa and host activities. However, many cellulolytic bacteria have also been isolated from the guts of lower and higher termites and their contribution to cellulose degradation in the termite gut is still a matter for debate (Schäfer *et al.*, 1996; Brune 2006). This question of whether the cellulolytic bacterial community has a functional role in the termite gut system is important and needs to be addressed, as the most abundant and ecologically important species (higher termites from the subfamilies *Termitinae*, *Apicotermitinae* and *Nasutitermitinae*) do not form the associations between protozoa and fungi seen in other termites and must therefore rely almost exclusively on their intestinal prokaryotes (Bignell *et al.*, 1991).

Actinomycetes from genera such as *Streptomyces* and *Thermomonospora* have been reported to produce active cellulases. As a result, Pasti & Belli (1985) attempted to isolate cellulolytic actinomycetes from the hindgut of four different termites – *Macrotermes*, *Armitermes*, *Odontotermes* and *Microcerotermes*. They found that mesophilic actinomycetes from these higher termite groups possessed very active cellulase complexes and some even seemed to be thermotolerant. Of the 20 strains that were isolated, 19 were found to belong to the genus *Streptomyces*, while the last was found to be a *Micromonospora*. They found that the optimum temperature for growth was 28^{o}C, with a pH optimum between 6.2 and 6.7. Three strains were even able to grow at 48^{o}C when cellulosic substrates were used (Pasti & Belli, 1985).

A more extensive analysis was performed much later by Watanabe *et al.* (2003), this time including isolations from a wide range of termite species, covering both the lower and higher

termites. The termite species sampled included: *Hodotermopsis japonica* (family Termopsidae), *Neotermes koshunensis* (Kalotermitidae), *R. speratus* and *C. formosanus* (Rhinotermitidae) and, from the higher termite Termitidae family – *Odontotermes formosanus* (subfamily *Macrotermitinae*). Despite the fact that they made extensive use of novel methods and media for isolation, including many different cellulosic substrates as well as lignin-based media, only members of the *Streptomyces* genus were cultured (Watanabe *et al.*, 2003). A comparison of the higher termite species with all other termites sampled, showed that the number of actinomycete colonies isolated per individual termite was small, but the diversity, based on cultural characteristics, was quite varied and the strains were nearly all different from the actinomycete types isolated from the lower order termites. Therefore, as with other studies looking at the whole bacterial gut community, they found that the actinomycete population depended largely on the area or habitat where the termites naturally occur and also that it seemed to be genus specific (Watanabe *et al.*, 2003).

As discussed before, the termite gut is not a simple environment and can actually be considered a well-aerated anaerobic gradient system. As such, it is important to investigate the aerobic, facultative, and microaerophilic components of the cellulolytic bacteria (Wenzel *et al.*, 2002). In this regard, the gut contents of the termite *Zootermopsis angusticollis* was investigated and was found to contain 119 cellulolytic bacterial strains belonging to 23 different groups of aerobic, facultatively anaerobic and microaerophilic cellulolytic bacteria. Cell counts of up to 10^7 cellulolytic bacteria per ml of gut debris indicated that these strains may play a significant role in cellulose digestion. The diversity included low G+C bacteria from the order *Bacillales*, Gram-negative isolates from the α-Proteobacteria, and more interestingly, members of the Gram-positive, high G+C subdivision (order *Actinomycetales*). The latter was made up of representatives from the genera *Microbacterium*, *Kocuria*, *Oerskovia* and *Cellulomonas* (Wenzel *et al.*, 2002).

The isolation of a member of the *Cellulomonas* genus is significant, as a related actinomycete was isolated from the hindgut of an Australian termite species, *M. darwiniensis*. *Cellulosimicrobium variabile* was discovered based on its cellulolytic and xylanolytic activity and was distinguished from the closely related genera *Promicromonospora* and *Cellulomonas*, because of phenotypic, chemotaxonomic and 16S-rRNA gene sequence differences (Bakalidou *et al.*, 2002). Interestingly, the closely related *Promicromonospora pachnodae* (subsequently renamed *Xylanimicrobium pachnodae*), was isolated from the gut of the rose-chafer beetle *Pachnoda marginata* which, like its termite counterparts, feeds on cellulose-rich plant material. Whether this indicates that these actinomycetes are specially

adapted to the gut systems of plant-feeding insects has yet to be determined (Bakalidou *et al.*, 2002; Cazemier *et al.*, 2003).

The importance of the microoxic nature of the termite gut in relation to the actinomycete population was also highlighted by Bignell *et al.* (1991), when they showed that under anaerobic culturing conditions, no streptomycete-type microbes could be observed by any microscopical method, nor could they be cultivated. However, they were able to isolate colonies of bacteria that occasionally consisted of filamentous organisms with sessile or short-stalked spores, which they said are characteristic of the genus *Micromonospora* (Bignell *et al.*, 1991).

1.5.4.3 Cultured isolates – Hemi-cellulolytic activity

Hemicelluloses are chemically complex compounds that can be made up of various different polysaccharides - the most common being xylan. The full set of enzymes required to degrade all of the constituents of xylans has yet to be found in any bacterial species. However, there are some filamentous fungi which are able to produce all the enzymes necessary to hydrolyse this compound. This fungal ability, in particular from a member of the *Termitomyces* genus, is utilised by the fungus-eating higher termite *Macrotermes mülleri* when it ingests the fungus with its feed (Schäfer *et al.*, 1996).

In comparison to the situation with cellulases, xylanases of host origin have yet to be found, although it has been shown that the gut protozoa of lower termites are able to fulfil this function. In other termites, hemicelluloses seem to be digested with the help of enzymes secreted by symbiotic bacteria (Schäfer *et al.*, 1996; Brune, 2006). Using xylan, arabinogalactan and carboxymethylcellulose as substrates, Schäfer *et al.* (1996), were able to isolate between 10^6 and 10^7 xylan-degrading bacteria per ml of gut contents from the hindgut of *M. darwiniensis*. A range of different bacteria from both Gram-positive and Gram-negative groups were isolated, with isolates belonging to the genus *Streptomyces*, as well as representatives of coryneform actinomycetes. When aromatic compounds were used, the *Streptomyces* and *Nocardia* genera became the predominant Gram-positive representatives (Schäfer *et al.*, 1996). While xylanolytic and arabinogalactan-hydrolysing abilities seemed to be a general trait for all the bacteria isolated, the ability to degrade crystalline cellulose was confined to a coryneform strain and to a strain from the *Streptomyces* genus. Their investigation was able to show that the paunch region of the termite gut, at least for this particular species, harbours a significant number of hemicellulose-degrading bacteria (and yeasts) and, although none of the isolates was able to completely digest the hemi-cellulose

alone, it was clear that these microbes have a significant role to play in the digestion of this complex food-source in the termite gut (Schäfer *et al.*, 1996).

1.5.4.4 Cultured isolates – Lignin-solubilising activity

As with hemicellulose, the abilities of lignolytic fungi (and their extracellular lignin peroxidases) have been harnessed by the fungus-cultivating termites in order to break down this recalcitrant component of the wood-feeder's diet. As discussed previously, this process is an aerobic one and, as such, various aerobic bacteria with the ability to degrade lignin-derived aromatic compounds have been isolated from the gut of wood-feeding termites (Brune, 2006). The work done by Pasti *et al.* (1990) is some of the only evidence of the lignin-solubilising ability of actinomycetes isolated from termite guts. In their study, they found 11 novel actinomycete strains, isolated from the gut of higher termites sourced from Kenya. All were found to be *Streptomyces* isolates, related either to *Streptomyces chromofuscus* or *Streptomyces rochei*.

1.5.4.5 Gut vs mound soil and the soil-feeding lifestyle

Except for the *Macrotermitinae*, a large proportion of the higher termites are soil-feeders that ingest great quantities of mineral material from the soil, including the dispersed organic matter generally termed humus. Soil-feeding termites from sub-tropical habitats can be very abundant but, like other such termites, little is known about the bacterial population that is housed in the termite hindgut and its role in the digestive process. What is known is that a substantial proportion of the putative symbionts appear to be actinomycetes (Bignell *et al.*, 1991).

Due to their function in the ecosystem, termites can have profound effects on their environments, mainly in the vicinity of their mounds. They generally have an effect on both the physical and chemical properties of the soil, which often leads to changes in the vegetation surrounding their mounds. This effect on their environment was the basis of a study by Keya *et al.* (1982) that was done in order to understand their effect not only on the soil, but also on the populations of fungi, protozoa, bacteria, and in particular the actinomycetes, associated with termites living in a semi-arid savannah ecosystem in Kenya. Their results were based on comparisons of mounds that had active termite colonies ('live') versus those without termites ('dead'), as well as comparisons during the wet and dry seasons. What they found was that the actinomycete population in these mounds was higher in the live mounds during the wet season compared with the dry season, and that the dead mounds only increased their actinomycete population during the dry season. The other components of the

mound's microbiota were found to be more moisture sensitive, with their populations declining to as few as 10^4 cells/g dry soil, compared with the highest counts of actinomycetes of 10^5 cells/g dry soil during the wet season (Keya *et al.*, 1982). This dominance of actinomycetes during the wet season was also found to be significantly correlated with the amount of carbon dioxide evolved and it was also shown that the numbers of cellulose decomposers was more numerous in the mound soil than in the soil adjacent to the mounds. All of these results suggested that in terms of the relationship between termites and their natural environment, their effect results in the microbial activity of the bacteria and actinomycetes being more significant during the wet season, thereby proving that termites and their symbionts have a profound role to play in the natural ecosystem (Keya *et al.*, 1982).

Similar work was done in order to understand and make comparisons between the internal and external actinomycete population in two African soil-feeding termites. An examination of the aerobic actinomycete population found in the gut of both *P. aburiensis* and *C. severus* was carried out by Bignell *et al.* (1991) in order to make comparisons between these populations isolated from the parent soil (surrounding soil), the mound material, and the gut contents. The mound is constructed largely from material that has passed through the termite gut, but despite this fact, their results showed that the mound population was not different from the parent soil, or to other mounds. Also, the passage of soil through the termite intestinal tract would eliminate many actinomycetes at the mixed segment and anterior portion of the hindgut. However, this apparently had no effect on the overall composition of the populations surviving in the mound. Because termite mounds are fairly permanent structures in their natural habitats, it was suggested that the mounds become recolonised from the surrounding soil through direct contact or via the activities of the termites (Bignell *et al.*, 1991).

A comparison of the internal (gut) population against the external population, based on cultural and phenotypic characteristics, showed that the gut actinomycete population had a more variable response to the carbohydrate sources offered, as well as differences in amino acid utilisation, when compared with the soil populations. Differences in the percentages of various morphological types were also shown between the two (Bignell *et al.*, 1991).

1.5.4.6 Novel isolation methods

As mentioned previously, culture-independent studies have shown on many occasions that the majority of the microbiota found in the termite gut has yet to be cultured. However, in order to be of benefit to applied microbiology and biotechnology and to understand the diversity, distribution, and function of this microbiota (including the actinomycetes) among termite species, it is necessary to cultivate these organisms. As such, it is important that efforts

continue to be made toward the design and utilisation of improved methodologies and techniques, in order to culture the vast diversity of bacteria that have been shown to be present (Kurtböke & French, 2007).

One such effort was made by Kurtböke & French (2007), as part of their aim to isolate, selectively, the actinomycete microflora from the gut of the Australian wood-feeding termite species, *Coptotermes lacteus*. Their method involved exploiting the phage susceptibility of different gut associated bacteria, which prevent the growth of actinomycetes on isolation plates. This non-actinomycete bacterial fraction was removed by exposing the gut contents to polyvalent bacteriophages that specifically target different non-actinomycete bacteria, allowing for the isolation of previously undetected and novel actinomycetes from the gut samples. As certain groups of actinomycetes are generally more abundant and easy to cultivate, actinophages against these major groups (including phages for streptomycetes, micromonosporae and *Nocardia*/*Rhodococcus*) were also used in conjunction with the non-actinomycete bacteriophages in order to determine whether some of the rarer actinomycete genera could also be isolated (Kurtböke & French, 2007).

This method proved to be highly successful, as a wide diversity of never-before-seen actinomycetes were isolated from the gut of *C. lacteus*. The spectrum of actinomycetes isolated included members of the following genera: *Microbispora*, *Geodermatophilus*, *Actinomadura*, *Saccharopolyspora* and *Pseudonocardia*. Also, despite the use of specific actinophages against these groups, isolates from the *Streptomyces*, *Micromonospora*, *Nocardia* and *Rhodococcus* genera were also cultivated. This finding was significant, because it implied that there are actinomycetes found in the gut that are different from the common soil isolates and, as such, may represent actinomycetes that have a functional role in the host termite gut, including the possible function of nestmate recognition by producing attractant compounds (Minkley *et al.*, 2006; Kurtböke & French, 2007).

1.5.4.7 Culture-independent analysis

A review of published work relating to the termite gut and its bacterial population, suggests that there has never been any culture-independent analysis exclusively relating to investigations of the association between actinomycetes and their termite hosts.

However, despite other studies postulating that the filamentous actinomycetes may have an important role in the posterior hindgut of the termite *C. severus* and actinomycetes with cellulolytic and lignin-solubilising activity having been isolated from soil-feeding termites, some culture-independent analyses have shown no evidence of the presence of actinomycetes.

This was the case in the work performed by Schmitt-Wagner *et al.* (2003a), where none of the clones in their clone libraries from the gut of *C. orthognathus* contained sequences belonging to any bacteria from the high G+C Gram-positive group. This may have been due to the fact that this group of organisms has been shown to be associated with the microoxic conditions found only associated with the gut wall. This was proven by Berchtold *et al.* (1999), when they found that a significant fraction of the posterior gut wall flora consisted of Gram-positive bacteria with a high G+C content. They even went as far as to suggest that rod-shaped and coccoid cellulolytic *Cellulomonas* species isolated from *M. darwiniensis* may represent some of the bacteria associated with this site.

The actinomycetes fall within the broader group of bacteria called the actinobacteria and, in the work performed by Nakajima *et al.* (2005), they found the gut wall of *R. speratus* to be associated with a greater degree of diversity than that found in the gut lumen. Their results showed that the actinobacteria make up a large part of this diversity – up to 16% of the clone library, with one phylotype in particular dominating all others. The presence of actinobacteria has also been found in the gut of *R. flavipes* by the use of 16S-rRNA gene sequencing and amplified rDNA restriction analysis (Fisher *et al.*, 2007).

A more informative analysis of the actinomycete composition found associated with the soil-feeding termite, *Cubitermes niokoloensis*, similar to that described in section 1.5.4.5, involved a culture-independent comparison of the termite mound soil and surrounding soil with different regions of the gut. Here, Fall *et al.* (2007) compared the bacterial community structure in these different sampling sites using PCR-denaturing gradient gel electrophoresis (DGGE) analysis, as well as cloning and sequencing of PCR-amplified 16S-rRNA gene fragments. What they found was that the mound material was dominated by bacteria from the *Actinobacteria* phylum, whereas the gut sections and surrounding soil were dominated by the Firmicutes and Proteobacteria, respectively. The details of the DGGE analysis proved more interesting in terms of the actinomycetes, because they found three clones in the posterior part of the gut that could be assigned to the actinomycete genera *Nocardia*, *Nocardioides* and *Cellulomonas* (Fall *et al.*, 2007). A single *Tsukamurella* clone was found associated with the anterior gut/ midgut sample. The mound soil showed a much greater abundance of actinobacteria, with 7 out of the 12 clones isolated from this sample affiliated with this group. In the 16S-rRNA gene clone library, of a total of 212 clones, approximately 50% of all assigned clones were made up of actinobacteria, with the surrounding soil being made up of approximately 30% from this same group (Fall *et al.*, 2007). The diversity of these clones consisted of representatives from the genera *Streptomyces*, *Rhodococcus*, a single *Propionibacterineae* representative, and a few uncultured bacteria. A phylogenetic analysis

based on the phylotypes obtained from the mound soil and the surrounding soil showed a clear separation of the clones from the different sample sites. Interestingly, although the termite gut and surrounding soil did not share any common actinobacterial phylotypes, nearly all of the phylotypes from the termite gut were also found in the mound soil. Overall, Fall *et al.* (2007) were able to demonstrate that the termite mound harbours its own specific bacterial community, in terms of structure and diversity, which is distinct from the surrounding soil and is characterised by the dominance of actinobacteria, which they suggested probably originates from the termite gut.

Despite all of the examples described above of actinomycetes that have been isolated from many different termite guts, their significance in the degradation of lignocellulose in the termite gut system remains a mystery, especially because it is still not clear whether these isolates are simply the result of spores ingested with the food (Brune, 2006). Microscopical evidence suggests otherwise, as these results imply that the actinomycetes in termite guts are not in fact accidental occupants, but are intimately associated with the gut epithelia (Kurtböke & French, 2007).

However, our understanding of the biology of the termite gut community in general remains poor – mainly due to the vast complexity of the system, but also due to the difficulty of cultivating the majority of its members. Recent culture-independent analyses have revealed the unexpected and impressive nature of this environment (Ohkuma, 2008) and findings suggest that the great majority of the bacteria found in the gut are members of uncultured lineages that are unique to termites. One such example is the TG1 phylum bacteria or *Elusimicrobia* (Ohkuma *et al.*, 2007; Geissinger *et al.*, 2009). Therefore, it is only through the synthesis of these two valuable techniques – the culture-based and the culture-independent – that a complete understanding of the functioning of the termite gut community will ever be achieved.

1.6 SYMBIOSIS AND CO-EVOLUTION IN THE TERMITE GUT

1.6.1 Symbiosis

Our understanding of the microbial populations found in termite guts is far from complete. The population has been shown to change with age and developmental stage, with the seasons and other environmental conditions, as well as with time when maintaining laboratory collections. The microbial components that are absolutely required for host viability are still not known, but the gut community is known to only be severely reduced in dying individuals or colonies. Although the gut system is complex, factors such as these, as well as the abundance and diversity of the microbes and their associations within the gut, suggest that their presence is indispensable to the health of the termites (To *et al.*, 1980). As such, a complex symbiosis does exist within the termite hindgut. The termite host creates an environment that is optimal for growth and reproduction, as well as transferring microbes to successive generations and, in return, the microorganisms serve a variety of important functions for the termite host (Brune, 2006; Fisher *et al.*, 2007).

In some cases this relationship could be termed an obligate mutualism, such as that which exists between lower termites and their fibre-digesting flagellates. However, in other instances, such as with most of the prokaryote gut symbionts, there appears to be little advantage for the host. If the benefit is only for the microbe, then the association must now be termed commensal, but due to the massive intake of microorganisms in their diet, it is impossible for the termites to maintain gut sterility. There is plenty of evidence for the existence of a gut specific microbiota, and this is important, as a normal flora within the gut prevents the proliferation of potentially pathogenic local or externally sourced microbes (as in almost all gut systems) (Brune, 2006; Fisher *et al.*, 2007). Another example of a critical component of the termite gut system, which could also be termed an obligate symbiosis, is between the archaea (acting as hydrogen sinks) and both lower and higher termites. Although there is a difference in the rates of methanogenesis and acetogenesis in these two groups, relating to wood- or soil-feeding lifestyles, it has been suggested that this association is an ancient one. Analysis of termite evolution suggests that the higher termites evolved as a result of the ‘externalisation’ of the gut for the fungus-feeding *Macrotermitinae*, followed by the evolution of soil feeding. It is therefore likely that the first step in this process would have been the acquisition of an archaeal strain, such as a member of the *Methanomicrobiales*, which had the potential to reduce hydrogen partial pressures to levels that would allow for the effective exploitation of soil organic matter (Purdy, 2007).

If this were the case, then it could be claimed that the evolution of one of the most important animal families on the planet was dependent on the acquisition of a microbe, in this case a methanogen and, in the case of lower termites, the acquisition of protozoa. This hypothesis totally changes our thinking on animal-microbe interactions and places microorganisms at the very centre of ecosystem evolution (Purdy, 2007).

1.6.2 Co-evolution

A study performed by Fisher *et al.* (2007) showed that over 90% of the 16S-rRNA gene sequences obtained from the gut of *R. flavipes*, that shared >90% sequence similarity with other sequences from a BLASTN search, only did so with uncultivated termite gut bacteria. As such, it appeared that there was a much higher similarity between sequences obtained from termite guts, compared with those that were found in other environments. This provides evidence for the possible co-evolution of termite hosts and their gut bacteria. In addition, these culture-independent methods of study provide an effective means for determining the phylogenetic species richness of the termite gut bacteria, a result that could not be achieved by the use of traditional culture-based analyses (Fisher *et al.*, 2007).

In another more extensive study, the bacterial gut microbiota from 32 colonies of wood-feeding termites, comprising four *Microcerotermes* species (Termitidae) and four *Reticulitermes* species (Rhinotermitidae) were analysed with the use of cloned 16S-rRNA gene sequences, as well as terminal restriction fragment length polymorphisms. Their results revealed that the bacterial community structure was very similar within each termite genus, irrespective of the individual, colony, location or host species. In contrast, there were considerable differences found between the two termite genera, with only one phylotype being shared between them. These results implied that the gut bacteria differentiated after acquisition by the ancestors of these termites and led them to the conclusion that the majority of the gut bacteria are not shared, but are in fact specific symbionts that have co-evolved with their termites and their community structures are consistent within termite genera (Hongoh *et al.*, 2005).

Co-evolution between gut microbiota and termite host has also been suggested for protistan symbionts. The majority of the protozoan gut community is unique to lower termites and wood-feeding cockroaches from the genus *Cryptocercus*. These symbiotic protozoa are basically species-specific to their termite hosts and are found and maintained in successive termite generations (Hongoh *et al.*, 2005).

1.6.3 Symbiosis and co-evolution combined

The hindguts of lower termites are packed with protozoa which are essential for the decomposition of lignocellulose in wood-feeding termites. These flagellates belong either to phylum *Parabasalia* or to the order *Oxymonadida* (phylum *Preaxostyla*) and consist of hundreds of different species that occur exclusively in the guts of lower termites and the wood-feeding *Cryptocercus* cockroaches. They are thought to represent the deepest branches of the eukaryotic phylogenetic tree and have been found to be specific to their host species. It has therefore been suggested that they may reflect their host's phylogeny (Noda *et al.,* 2007; Ohkuma, 2008; Ikeda-Ohtsubo & Brune, 2009). As described previously, the gut flagellates are regularly colonised by prokaryotes, which are either attached to the cell surface as ectosymbionts, or associate as endosymbionts when located inside the cytoplasm or nucleus. Despite the fact that an estimated 85% of the prokaryotes found in the termite gut are associated with the flagellates, only very few of the ectosymbionts have been properly identified. The same can be said for the endosymbionts of all larger flagellates, as the nature and identity of these microbes is almost completely unknown (Stingl *et al.*, 2005; Ikeda-Ohtsubo & Brune, 2009).

This dearth of knowledge has in part been rectified by the work of researchers such as Noda *et al.* (2007) and Ikeda-Ohtsubo & Brune (2009). In most cases, co-evolutionary studies focus on two interacting organisms. Relationships between more than two symbiotic members are rarely investigated, despite the prominence of these multiple symbioses in biological systems. This was not the case for the study performed by Noda *et al.* (2007), where they looked at the triplex symbiosis between the termites from the Rhinotermitidae family; their protists from the genus *Pseudotrichonympha* (phylum Parabasalia) and the intracellular endosymbiont of the protist, found to be affiliated with a unique phylogenetic lineage within the order *Bacteroidales*. It has been reported that this endosymbiont makes up a dense population of up to 10^5 cells per protist and accounts for more than two-thirds of the bacterial population in the gut. As such, it is likely that it makes a great contribution to the gut microbial metabolism, despite its exact role being unknown. In their analysis of the evolutionary history of this triplex symbiosis, they found that there is almost complete co-speciation of the *Pseudotrichonympha* with their host termites. Also, the overall topology of the phylogenetic tree used in their analysis showed significant congruence between the endosymbionts and both the protists and termite host. As such, they suggested that the three members of this symbiosis appear to have mostly co-speciated during their evolution (Noda *et al.*, 2007).

In contrast is the association that exists between the termite hosts of the *Trichonympha* flagellates and their associated endosymbionts from the candidate phylum TG1, or the

so-called ‘Endomicrobia’. This group represents a deep branch of bacteria that form a monophyletic lineage that occurs exclusively in the hindguts of termites and the wood-feeding cockroach – much like their protozoan hosts. However, there is no strict co-speciation between the Endomicrobia/*Trichonympha* partnership and their termite hosts, as the *Trichonympha* occur in four different termite families (Herlemann *et al.*, 2007; Ikeda-Ohtsubo & Brune; 2009). However, in the study done by Ikeda-Ohtsubo & Brune (2009), they did show that Endomicrobia found associated with a specific cluster of *Trichonympha* were not associated with any other genus of flagellates. This clear evidence of co-speciation resulted in their giving this group of Endomicrobia the name ‘*Endomicrobium trichonymphae’*, in order to distinguish it from those associated with other hosts. The TG1 phylum has since been renamed *Elusimicrobia*, with the isolation of the first cultured representative from this group, *Elusimicrobium minutum* (Geissinger *et al.*, 2009).

Despite the relative lack of information on the details of the subject, the relationship between termites and their hindgut protozoa remains one of the most well-documented examples of symbiosis within the complex termite gut environment.

1.7 TRANSMISSION OF THE GUT COMMUNITY

Termites shed most of their hindgut microbiota with every new moulting, and have abdomens that are typically translucent, containing none of the normal food material. As such, they depend exclusively on their nestmates to become newly inoculated with gut symbionts via a method of transfer termed proctodeal trophallaxis (or coprophagy), which essentially involves the solicitation of droplets of hindgut fluid from their nestmates (To *et al.*, 1980; Minkley *et al.*, 2006; Noda *et al.*, 2007). It has been speculated that this behaviour formed the basis for the evolution of sociality in ancestral termites – which is reinforced by the fact that termites such as *Hodotermes mossambicus* from the same colony harbour a nest-specific bacterial microbiota that is slightly, but significantly different from the gut microbiota of non-nestmates (Minkley *et al.*, 2006). Very young larvae also rely on this process, as they typically lack the conspicuous and morphologically distinct bacterial community characteristic of healthy older nymphs. At birth, their hindguts are presumably sterile, which requires that during their early development they gradually acquire their full complement of microorganisms via proctodeal feeding (To *et al.*, 1980).

A large part of the gut bacterial community is fairly oxygen tolerant. The expression of protective enzymes against oxygen radicals, such as catalase and superoxide dismutase, as

well as the ability to tolerate and adapt to high concentrations of oxygen may be important during trophallactic transfer and colonisation of newly hatched larvae. This adaptive tolerance would therefore ensure the survival of these gut bacteria until the correct aerobic/anaerobic balance was achieved inside the new termite gut (Wertz & Breznak, 2007).

This process of vertical transmission therefore allows for the propagation of the gut microflora into the next termite generation, ensuring the survival of both host and symbiont community. The transfer of the gut protozoa of lower termites is well described, but it is the gut community as a whole that is carried from the mother nest to newly founded termite colonies by the alates (king and queen). This mode of transmission explains why the gut protozoan composition is so well reflected by the termite phylogeny, as well as the significant consistency of the gut community as a whole within each termite genus (Noda *et al.*, 2007; Ohkuma, 2008; Ikeda-Ohtsubo & Brune, 2009).

Although the termite gut bacteria are also considered to be transmitted vertically from generation to generation, horizontal transfer has also been established as a mechanism for acquiring gut bacteria. This horizontal mode of transfer between congeneric termites is thought to be mediated via faeces that are excreted into the same environmental niches occupied by different termites (Hongoh *et al.*, 2005). However, despite all this evidence in the lower termites, the transfer of microbial symbionts via proctodeal trophallaxis has yet to be documented for the higher termite soil-feeders from the *Termitidae* family (Schmitt-Wagner *et al.*, 2003b).

1.8 THE ACTINOMYCETES

1.8.1 Antibiotic redundancy and actinomycetes from diverse environments

During screening programmes directed towards the discovery of novel compounds, it was found that some microbial metabolites were being discovered more frequently than others. As the number of these described compounds increased, so did the probability of their rediscovery. In fact, at one point it was estimated that the rediscovery rate of known compounds from terrestrial environments had even reached 95%. It was therefore clear that in order to increase the chances of discovering novel compounds, new strategies needed to be introduced into these screening programmes (Busti *et al.*, 2006; Zhang *et al.*, 2006).

The actinomycetes account for the greatest fraction of discovered microbial metabolites and, among them, *Streptomyces* is by far the most prolific genus. As a result, over the years the pharmaceutical industry must have screened millions of *Streptomyces* strains, with the consequence that the chances of discovering novel antibiotics from this genus are now severely diminished. However, it has been estimated that only a fraction of the antibiotics produced by streptomycetes have actually been discovered, which emphasises the need to look in other places for new sources of novelty (Watve *et al*. 2001; Busti *et al.*, 2006). One particular niche that has been overlooked as a source of novel streptomycetes is the various endophytes and symbionts that exist in numerous ecosystems around the world. This source would be a reasonable target as these streptomycetes are associated with eukaryotic organisms and, as such, may be less likely to produce compounds that are toxic to their host. Therefore, one of the major concerns in drug discovery, relating to the toxicity of drug candidates, may be reduced by dealing with these streptomycetes and their biologically active products (Bascom-Slack *et al.*, 2009).

The actinobacteria are widely distributed in terrestrial, freshwater and marine habitats where they are involved in the turnover of organic matter and xenobiotics. Some actinobacteria are pathogens of animals, humans and plants, whereas others form nitrogen-fixing associations with non-leguminous plants (Stach *et al.*, 2003b). The actinomycetes and other prokaryotes have also been found in various extreme environments that were previously considered to be hostile to life – in association with hydrothermal vents, in the deep subsurface of the oceans, inside Antarctic rocks, desert soils, and in symbiotic associations with invertebrates. Therefore, it is crucial that these unusual, unexplored or underexploited habitats be investigated for their potential to harbour new actinomycetes that can be pursued as sources of novel bioactive secondary metabolites (Maldonado *et al.*, 2005; Lam, 2006; Okoro *et al.*, 2009). It is the association with the invertebrates, in particular with termites, that is of interest for the work documented here.

1.8.2 The importance of drug discovery

Of all bacteria, the actinomycetes are the most economically and biotechnologically valuable and are possibly the single richest source of bioactive compounds. Some well-known examples of antibiotics include erythromycin (*Saccharopolyspora erythraea*), chlorotetracycline (*Streptomyces aureofaciens*) and vancomycin (*Amycolatopsis orientalis*). Other examples of some of the compounds that have been isolated from members of this group include: immunosuppressants (e.g. rapamycin), anticancer compounds (e.g. bleomycin), antifungal compounds (e.g. nystatin) and even antihelmintic agents (e.g.

avermectin). Their most important contribution to man, however, has to be the wealth of antimicrobials that specific members of this order produce (Weber *et al.*, 2003; Lam, 2006; Lefevre *et al.*, 2008).

Programmes that are aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources – the actinomycetes in particular – have yielded impressive numbers of compounds over the years, and these discoveries have found important applications in both human medicine and agriculture (Busti *et al.*, 2006). In fact, in September 2002, natural product sales (of which antibiotics are a part) accounted for 17% of the top best-selling drugs in the world, with an approximate value of US $28.9 billion (Knight *et al.*, 2003; van Wezel *et al.*, 2006).

Among infectious diseases, tuberculosis (TB) is still considered to be the leading cause of death and ranks as one of the most important burdens on human health (Bloom & Murray, 1992; Dye *et al.*, 2002). The causative agent of TB is *Mycobacterium tuberculosis* and, worldwide, it accounts for 6.7% of all deaths in developing countries, 18.5% of deaths in adults aged 15 to 59, and 26% of all avoidable deaths of adults. According to the World Health Organisation (WHO), in 2007 there were 9.27 million new cases of TB reported worldwide. South Africa is listed at number 5 on the list of the 22 highest-burden countries in the world, with 112 000 out of the 336 000 reported cases resulting in death (Bloom & Murray, 1992; Raviglione, 2003; WHO Global Tuberculosis Control Report, 2009).

Treatment of TB has been hampered in several ways, including problems with patient compliance with long drug regimens, which usually run for 6 months, as well as transmission of drug-resistant strains within the general population (Bloom & Murray, 1992; Telenti & Iseman, 2000). The development of TB and HIV co-infection, however, is the largest area of concern. The burden of TB in countries that are severely affected by HIV has increased rapidly as a result of this association. Of the 1.7 million people who died from TB in 2007, 456 000 were co-infected with HIV. TB is now documented as the most common cause of death in HIV-positive adults living in less-developed countries, with activation of latent TB thought to be the major mechanism leading to the development of TB in HIV-infected individuals. South Africa has the highest rate of TB-HIV co-infection in the world, reaching a phenomenal 73% (Johnson *et al.*, 2001; Corbett *et al.*, 2003; Andries *et al.*, 2004; WHO Global Tuberculosis Control Report, 2009).

Another cause for serious concern has been the development of multi-drug resistant (MDR) *M. tuberculosis*. Multi-drug resistance in *M. tuberculosis* is defined as resistance to at least

isoniazid and rifampicin (a derivative of rifamycin), two of the front-line drugs used in the chemotherapy of TB sufferers. In 2000, an estimated 3.2% of the 8.7 million new worldwide TB cases were multi-drug resistant, with fatality rates between 40 and 60%, and rates thought to be higher than 80% for individuals with HIV co-infection (Bloom & Murray, 1992; Dye *et al.*, 2002). Currently, of the 10.4 million episodes of TB recorded worldwide in 2007, an estimated 4.9% or 511 000 were found to be cases of MDR-TB. South Africa ranks fourth behind India, China and the Russian Federation in terms of the highest numbers of MDR-TB cases (WHO Global Tuberculosis Control Report, 2009). An even more serious situation has arisen with the development of extensively drug-resistant TB (XDR-TB), which is defined as TB caused by MDR strains that are also resistant to a fluoroquinolone antibiotic and at least one second-line injectable agent (amikacin, kanamycin and/or capreomycin). According to the WHO, by the end of 2008, 55 countries had reported at least one case of XDR-TB and, in combination with MDR-TB, makes the treatment of TB an ever-growing problem worldwide (WHO Global Tuberculosis Control Report, 2009).

Despite the fact that the incidence of TB has been increasing for the past ten years, very few drugs specifically targeting TB have been developed for the past four decades. A new diarylquinoline drug (TMC-207, formerly R207910), being developed by Johnson & Johnson, was discovered that targets the ATP synthase of *M. tuberculosis* and has, in combination with second line drugs, been found to be effective against MDR strains of TB tested in sputum cultures (Andries *et al.*, 2004; Lounis *et al.*, 2009). Recent efforts have resulted in an increase in the number of new drugs in the global TB drug pipeline. There a currently around 15 compounds and 28 projects in the global portfolio, including 9 compounds in different stages of clinical development, 6 compounds in preclinical development, and 28 projects at various stages of discovery. Among the 9 compounds in clinical development, 6 of them, including two fluoroquinolones, one rifamycin, the diarylquinoline mentioned above, and two nitroimidazoles, have passed proof-of-concept and are currently in Phase 2 and 3 clinical trials. The 3 remaining compounds, including one ethylenediamine and two oxazolidinones, are in Phase 1 trials (Ma & Lienhardt, 2009).

M. tuberculosis is not the only bacterium in which multiple-drug resistance has been found. Resistance to the glycopeptide antibiotics, vancomycin and teicoplanin, by the VanA forms of *Enterococcus faecium* and *Enterococcus faecalis* has also become a growing burden, ever since it was first discovered in 1988 (Nicas *et al*., 1997). Both antibiotics are used as last lines of defence against many serious infections caused by Gram-positive bacteria. These include various enterococci, methicillin-resistant *Staphylococcus aureus* and *Clostridium difficile*. The development of vancomycin-resistant enterococci is therefore a cause for great concern

for both doctors and their patients (Marshall *et al.*, 1998). The enterococci form a major component of the microflora that reside within the intestine and the fact that the mechanism for glycopeptide resistance is found on a plasmid, and is therefore highly transferable, makes the spread of vancomycin resistance to other human pathogens a veritable time bomb (French, 1998).

The information detailed above stresses the dire need for the discovery of new drugs. In their papers, Nicas *et al.* (1997), Telenti & Iseman (2000) and Dye *et al.* (2002) all emphasise that the support for drug discovery programmes should be a worldwide priority and that we cannot relax efforts in the development of new drugs against TB and other drug-resistant bacteria.

Despite the fact that actinomycetes were a known source of natural products, there was the perception that over 50 years of intensive research had exhausted the supply of compounds that could be discovered from this group. The advent of the bacterial genome sequencing age of the mid-1990s was supposed to revolutionise the identification of antibacterial targets and improve the efficiency of antibiotic discovery. As a result, there was a shift away from natural products as a source of small-molecule therapeutics. Many pharmaceutical companies began developing high-throughput target-based screening programmes and other drug discovery platforms, including methods such as combinatorial biosynthesis and rational drug design, which were to be used to augment these programmes. However, all these efforts have fallen short of their intended function of providing a 'panacea' for antibiotic discovery (Jensen *et al.*, 2005; Baltz, 2008). Antibiotics produced by actinomycetes (and other microbes) have been evolving for over one billion years and their efficacy has been tested by their ability to penetrate other microbes and inhibit target enzymes, macromolecules and other macromolecular structures. As such, one of the major obstacles of target-based approaches – converting *in vitro* discoveries into whole cell active leads - has already been solved by the evolution of natural product antibiotics. This fact, as well as the relative failure of high-throughput screening, is just one of the reasons why there has been a reversal of interest back to the discovery of natural secondary metabolites from environmental sources, combined with the new methods that have been discovered for their modification and use in drug discovery (Baltz, 2008).

1.8.3 Actinomycete taxonomy

1.8.3.1 Higher classification

The class *Actinobacteria* is one of the main phyla within the Bacteria, forming a distinct phyletic line in the 16S-rRNA gene tree. This important group encompasses bacteria that are diverse with respect to their morphology, biochemistry and their relationship to oxygen (Stach *et al.*, 2003b; Zhi *et al.*, 2009).

There are three main components that have contributed towards the classification of organisms belonging to the class *Actinobacteria*: (1) the establishment of chemotaxonomy that detects differences in the chemical composition of cell constituents; (2) the introduction of DNA-DNA re-association experiments that measure gross similarities between the single-stranded DNA of strains of closely related species; and (3) determination of 16S-rRNA gene sequence similarities, which reveal the extent of sequence variation among strains from all levels of relatedness (Stackebrandt *et al.*, 1997). The latter is one of the most important for all bacterial systematics, as it, more than any other taxonomic method, allowed the placement of organisms within a phylogenetic framework. Also, the revision of the higher levels of classification within the *Actinobacteria* did not change the prevailing descriptions of strains to the level of species or genus as, in most cases, these levels are based upon morphological, physiological and chemotaxonomic characteristics. All of the above methods have combined to provide a successful classification system that has been termed polyphasic taxonomy (Stackebrandt *et al.*, 1997).

The initial hierarchical classification system of the *Actinobacteria* was proposed by Stackebrandt *et al.* (1997), including 95 genera, belonging to 30 families and 10 suborders. However, their delineation of the taxa within this class, above the level of genus, was based solely on 16S-rRNA gene sequence-based phylogenetic clustering, and the presence of taxon specific rRNA signature nucleotides. The phenotypic characteristics, such as chemotaxonomic, morphological and physiological properties were not taken into account (Zhi *et al.*, 2009). However, at any rank, any significant increase in the numbers of species in any of the phylogenetic lineages (which has happened since 1997) results in a decrease in the number of signatures. As a result, a revised classification of the class *Actinobacteria* was proposed by Zhi *et al.* (2009), including an up-to-date set of signature nucleotides between the ranks of family and subclass. Currently, the class is composed of 219 genera, falling within 48 families and 13 suborders (Fig. 1.3).

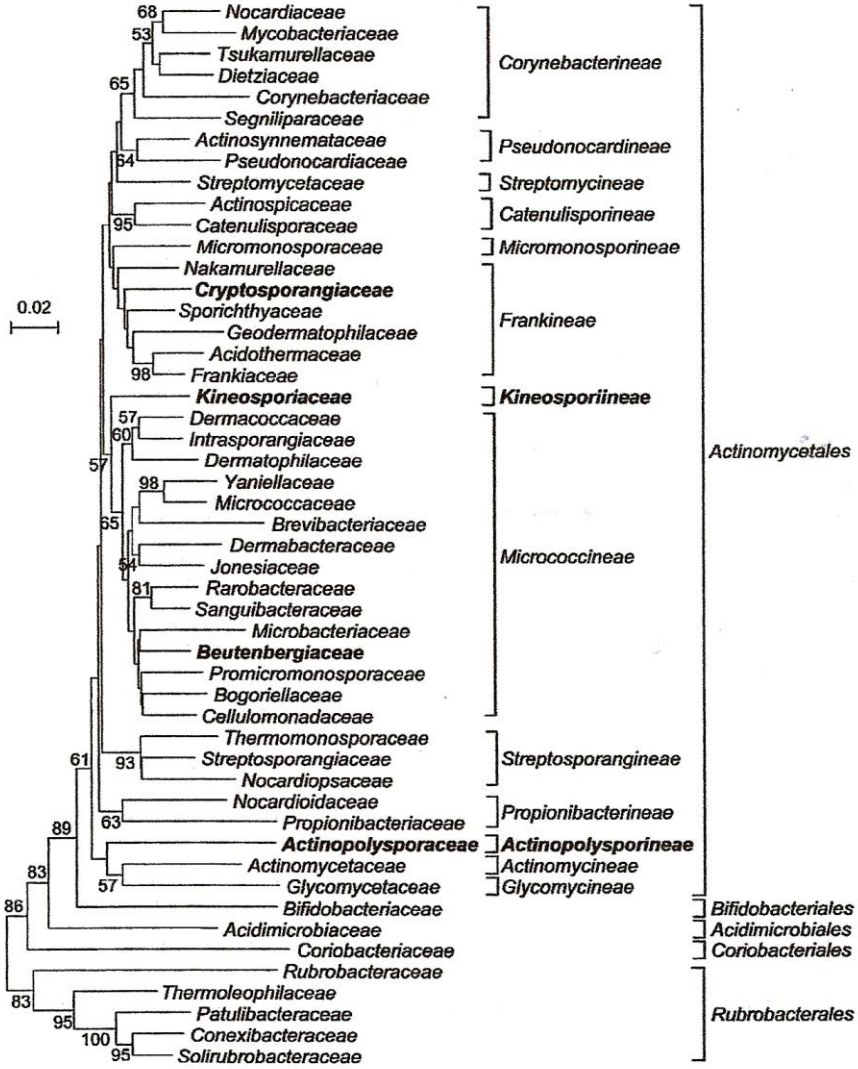

Figure 1.3: Phylogenetic tree of the relatedness found within the class *Actinobacteria*, encompassing the five orders, and based on 16S-rRNA gene sequence comparisons. All newly described taxa are highlighted in bold. Only bootstrap values over 50% are indicated at each branch point. Bar = 2 substitutions per 100 nucleotide positions (Zhi *et al.*, 2009).

1.8.3.2 The actinomycetes

Much early effort was directed towards establishing a better classification of the actinomycetes – established genera were revised and given tighter definitions, new genera were proposed to clarify the taxonomy and many nomenspecies were reduced to synonyms of well-described species (Goodfellow & Minnikin, 1977). However, actinomycete taxonomy, and bacterial taxonomy in general, is an ever-changing science that requires a constant effort in this regard, in order to provide the most accurate description of all published species and their relatedness to each other.

The actinomycetes fall within the class *Actinobacteria*, subclass *Actinobacteridae*, order *Actinomycetales* and are characterised by three main characteristics that differentiate them from other bacteria. They are Gram-positive, have (G+C)-rich genomes, and are mostly aerobic organisms (Lechevalier, 1989; Zhi *et al.*, 2009). The majority of the strains have been isolated from terrestrial soils, where they live primarily as saprophytes (Magarvey *et al.*, 2004).

Within the order *Actinomycetales*, there are currently 44 families with validly published names, containing 483 genera and over 3000 species. The genus *Streptomyces* alone contains a staggering 568 described species (Euzéby, 2009).

As with all modern bacterial classification systems, novel actinomycetes are described based on multiple phenotypic and genotypic characteristics, making their description a prime example of polyphasic taxonomy.

1.8.3.3 Polyphasic taxonomy – chemotaxonomy

The different cell constituents that are used to determine chemotaxonomic characters include cell wall peptidoglycan, polar lipids and fatty acids, isoprenoid quinones, cytochromes and the base composition of the DNA (Stackebrandt *et al.*, 1997).

Variations in the chemical structure of the peptidoglycan found in certain actinomycetes have enabled their differentiation based on variations in the qualitative amino acid and/or sugar composition. The majority of actinomycetes have a peptidoglycan that contains diaminopimelic acid (DAP) as a cell wall constituent. This amino acid has three stereoisomers (LL-, DD- and *meso*-DAP) and one analogue (3-OH-DAP). The relative proportions are distinctive for the genus or species, making the DAP configuration in the cell wall peptidoglycan one of the most important chemotaxonomic criteria for the classification of actinomycetes. For example, the genus *Nocardia* can be differentiated from the genus *Streptomyces* because streptomycetes contain LL-DAP in their cell walls, whereas the nocardiae contain *meso*-DAP. Also, the genus *Nocardia* contains galactose and arabinose as diagnostic sugars in whole-cell hydrolysates, whereas the genus *Streptomyces* is characterised by a lack of diagnostic sugars (Lechevalier & Lechevalier, 1970; Jones & Krieg, 1989).

Lipids occur in the cytoplasmic membranes of all bacteria and the fatty acid composition of these lipids has been shown to be characteristic for particular taxa. A particular category of fatty acids, the mycolic acids, for instance, have only been found in specific actinomycete genera, which include *Nocardia* and *Rhodococcus*. The polar lipids are also recognised for

their chemotaxonomic potential and include common types such as the phospholipids and glycolipids (Jones & Krieg, 1989). One in particular, called Lipid Characteristic of *Nocardia* (LCN-A), was just that, and was used by Mordarska *et al.* (1972) in a study differentiating this genus from other nocardioform actinomycetes. However, despite its uses, the fatty acid patterns obtained can often be influenced by a number of external factors, such as growth medium and temperature of incubation – making it essential that all strains in the study are grown under identical conditions in order for accurate comparisons to be made (Jones & Krieg, 1989).

Isoprenoid quinones are a class of terpenoid lipids located in the cytoplasmic membrane and encompass three main types – the ubiquinones, menaquinones and the demethylquinones. Their potential as an aid to classification was recognised as early as 1969 and, of the three, the menaquinones have been shown to have the most discriminatory power (Jones & Krieg, 1989).

The cytochromes are a specialised form of hemoprotein that are involved in various redox processes inside the cell. Their aid in identification and classification is based on either the 'pattern' or the 'structure' of this molecule (Jones & Krieg, 1989). As a taxonomic criterion, cytochromes are used very infrequently.

The DNA base composition, or specifically the mole percent guanine plus cytosine (mol% G+C) content, ranges in value from ~25 to 75%, but remains constant for a given organism and can therefore be used to compare two organisms, as those that are closely related have similar mol% G+C values (Johnson, 1989). For the class *Actinobacteria*, the DNA base composition has generally been found to be greater than 50 mol% G+C (Stackebrandt *et al.*, 1997).

1.8.3.4 Polyphasic taxonomy – morphology and physiological data

Historically, the classification of bacteria was based solely on similarities in phenotypic (morphological and physiological) characteristics. Phenotypic methods were not precise enough for distinguishing superficially similar organisms or for determining phylogenetic relationships. The results were also cumbersome, as they could not easily be compared by eye (Johnson, 1989; Sneath, 1989). However, at the level of genus and species, their importance cannot be denied.

In terms of morphology, the life cycle of actinomycetes offers three features that can be used for characterisation: (i) the vegetative or substrate mycelium (on solid and in liquid medium);

(ii) the aerial mycelium (on agar), if present, which bears the spores (in chains or in sporangia); and (iii) the spores themselves. The last two categories are the two most important, especially when it comes to the differentiation of streptomycete isolates from each other, as well as other spore-forming actinomycetes. As a result of the particular morphological characteristics of certain genera of actinomycetes, some of the tests mentioned above cannot be applied.

The spore chain morphology of *Streptomyces* species can be considered straight to flexuous (*Rectiflexibiles*), spiral (*Spirales*) or looped (*Retinaculiaperti*). The spore surface ornamentation includes the categories: smooth, spiny, hairy, warty, and the rarest of the five types, rugose (Locci, 1989).

Physiological data can be obtained from a wide range of different tests. Some of the tests that are routinely used in the classification and identification of *Streptomyces* species include the following: colour of the spore mass; pigmentation of the substrate mycelium, production of diffusible pigments, production of melanin, antimicrobial activity, enzyme activity, degradation activity on various substrates, resistance to antibiotics, growth at different temperatures and at specific pHs, growth in the presence of inhibitory compounds, the use of sole nitrogen sources, and the use of sole carbon sources (Locci, 1989).

1.8.3.5 Polyphasic taxonomy – DNA-DNA hybridisation and ANI

There is a general consensus among bacterial taxonomists that all the taxonomic information about a bacterium is incorporated into the sequence of its genome and, because whole genome sequencing did not become available until fairly recently, DNA-DNA hybridisation (DDH) experiments were used as the only way of determining whole-genome relatedness between bacteria (Goris *et al.*, 2007). A value of 70% DNA relatedness by DDH was proposed by Wayne *et al.* (1987) as the threshold for delineating species. As a result, the final word on the definition of species is based on the degree of DNA-DNA relatedness between two bacterial isolates and, as such, analysis of DNA-DNA hybridisation values has become the 'gold standard' for defining bacterial species. Despite this fact, there are drawbacks to this technique, as it can be quite expensive and time-consuming, and often requires specialised instrumentation. This technique also requires a high level of technical expertise and a database for comparison between strains cannot be established. Also, there are many parameters that can affect DNA-DNA reassociation, meaning that hybridisation values are often difficult to reproduce in different laboratories (Stach *et al.*, 2003a; Zeigler, 2003).

As a result of the limitations of DDH, investigations have been done to find alternative genome-based methods for the delineation of species. One such method is that of average nucleotide identity (ANI), which provides an average identity of all conserved genes between any two genomes and is based on pairwise alignment of genome stretches. Of all of the methods tested, ANI was found to be the one that best correlated with DDH values (Konstantinidis & Tiedje, 2007; Richter & Rosselló-Móra, 2009). Richter & Rosselló-Móra (2009) found that, for taxonomic purposes, it was not necessary to have complete genome sequences to compare strains. Randomly sequencing at least 20% of the genome of each of the query strains (producing an alignment equivalent to greater than 4% of their genome size) was sufficient, but a 50% genome coverage was recommended. Importantly, they also found that ANI could be used not only for cultured strains, but also for those uncultured strains that exhibited enough additional characters to allow for their identification. Ultimately it was determined that an ANI of 95-96% corresponded tightly to the frequently used 70% gold standard DDH value used for species delineation (Konstantinidis & Tiedje, 2007; Richter & Rosselló-Móra, 2009).

1.8.3.6 Polyphasic taxonomy – phylogenetics and 16S-rRNA gene sequencing

Phylogenetics essentially refers to the amount of change that has occurred in an evolutionary lineage since the time of its nearest common ancestor (Sneath, 1989). Phylogenetic analyses of the small subunit ribosomal RNA (16S-rRNA) gene have revolutionised the way that bacterial systematics studies have been done. The primary criterion for the designation of new taxonomic ranks is that the clustering in trees based on the 16S-rRNA gene should support this designation (Konstantinidis & Tiedje, 2007).

Also, importantly, a level of 16S-rRNA gene sequence similarity of 97% was widely accepted as the cut-off value for not requiring DDH in order to prove novel species status as, below this level, the DNA relatedness is expected to be below 70%. A value above this cut-off meant that DDH experiments had to be performed before species delineation would be accepted. However, at the beginning of the 1990s, there was a dramatic increase in the numbers of 16S-rRNA gene sequences available, with the consequence that it quickly became obvious that the relationship between 16S-rRNA gene sequence similarities and the corresponding DNA relatedness values is not linear (Stackebrandt & Ebers, 2006). Stackebrandt & Ebers (2006) used this information and, with a limited dataset, showed that below the threshold value of 98.5% 16S-rRNA gene sequence similarity, the corresponding DNA reassociation values were always below 70%. They therefore recommended that a 16S-rRNA gene sequence similarity range between 98.7-99% be the new threshold at which DDH experiments become mandatory in order to prove the uniqueness of an isolate.

One drawback of the reliance of bacterial systematists on the 16S-rRNA gene, is just that – it is only one gene from the entire genome and does not necessarily reflect the natural history of the organisms as a whole. More importantly, the 16S-rRNA gene offers limited resolution at the species level. The advent of whole-genome sequencing has resulted in an exciting alternative, as it provides a new way of investigating all DNA in the genome, thereby giving a truer reflection of the phylogenetic history of an organism (Konstantinidis & Tiedje, 2007). However, within the class *Actinobacteria* there are currently only 130 sequenced genomes out of the 12 460 known members within this group, making only 1% of these bacteria available for genomic comparisons with each other (Hugenholtz & Kyrpides, 2009). So far, this information has only been used in testing the robustness of the established 16S-rRNA phylogeny. However, until such time as there are more complete genome sequences available, researchers will have to rely on established methods in order to provide a deeper understanding of the genetic mechanisms that drive diversification of bacteria (Konstantinidis & Tiedje, 2007).

Multilocus sequence analysis (MLSA) is a method that was developed to overcome the reliance on 16S-rRNA single gene-based taxonomy, and is based on the concatenation of several housekeeping genes in order to determine genealogical relationships (Richter & Rosselló-Móra, 2009). Sequencing of six to eight genes/loci (as is typically done in MLSA) therefore provides bacterial taxonomists with a simple, sequence-based method that is a reasonable compromise between single gene-based and whole genome-based methods for species diversity studies (Konstantinidis & Tiedje, 2007). Other advantages of this technique include the fact that it is reproducible, allows for the generation of a cumulative database (unlike DDH), and it provides a level of resolution similar to that provided by DDH. There are drawbacks to the use of this method, which include the putative bias in gene selection as well as the availability of primers for PCR amplification of the target genes, but its use has already been proven in many different taxonomic studies. One such example is the work performed by Rong *et al*. (2009), where MLSA and DDH were used to clarify the relationship between members of the *Streptomyces albidoflavus* 16S-rRNA gene clade, which has been said to be overspeciated - as evidenced by the fact that most of the species within this clade have identical 16S-rRNA gene sequences. Based on the MLSA results, involving the concatenation of five separate genes (*aptD*, *gyrB*, *recA*, *rpoB* and *trpB*), as well as the DDH data and common phenotypic properties, it was recommended that all 13 strains that fall within this clade be merged into a single genomic species, *S. albidoflavus*.

Another taxonomic method that has proven to be useful not only at the family or genus level, but also at the species level for closely related strains, makes use of oligonucleotide frequency

distances. It has previously been shown that di-nucleotide frequencies within DNA sequences provide species-specific signals and this method also provides an alternative to whole genome comparisons (Takahashi *et al.*, 2009). Work by Takahashi *et al.* (2009) investigated phylogenetic analyses based on frequencies for oligonucleotides of greater than four nucleotides in length. A phylogenetic analysis based on Euclidian distances calculated from di- to deca-nucleotide frequencies was compared with the tree topologies generated for the 16S-rRNA gene as well as that from a concatenation of seven genes. It was found that when oligonucleotide frequency-based trees were constructed for bacterial species with a similar G+C content, the topologies were congruent with those based on homologous genes. At the family level, this was best seen for tetra- to octa-nucleotide trees, whereas at the genus level, penta- to hexa-nucleotides best estimated the phylogenetic relationships in homologous gene trees.

1.9 IMPORTANCE OF THIS WORK

The potential of renewable resources as a source of fuel is currently a very popular topic and is again receiving much worldwide attention. The world's production of waste cellulosic materials has been shown to exceed 4 billion tons per year (Pasti & Belli, 1985). The processing of these agricultural and forestry wastes is a highly attractive prospect, as it holds the potential for producing a renewable, clean energy source and at the same time solves the problem of cellulosic waste (Varma *et al.*, 1994).

The termite gut microbiota has been theorised to have the ability to convert a single sheet of A4 paper into two litres of hydrogen. Facts such as this have focused attention on termite guts as potential sources of microorganisms and enzyme systems that could be used in biofuel production. The largely unexplored diversity and biochemistry of the termite gut is therefore a prime target for the sourcing of these novel catalytic activities (Brune, 2007). In this regard, the benefit of an enzymatic process over acid hydrolysis is that the enzyme catalyst would be non-corrosive, environmentally non-hazardous, potentially renewable and offers an efficiency of more than 90% (Varma *et al.*, 1994). The use of suitable microorganisms is also to be preferred over other technologies (Pasti & Belli, 1985).

The termite soil and the termite gut bacteria both play a role in the depolymerisation of lignocellulosic matter, and the fact that the populations derived from the two different environments have been found to be distinct from each other, means that both are potential

sources of novelty, as well as bacteria that are able to degrade cellulosic and hemicellulosic materials more efficiently (Bignell *et al.*, 1991; Varma *et al.*, 1994).

In order to do all of this, it is imperative that we understand the physiology and biochemistry of termite metabolism, as well as characterising the microbiota that are involved in this process (Varma *et al.*, 1994). The work done so far has already gone a long way in providing us with this information, including the fact that the termite gut is composed of numerous different microenvironments, each with its own specific microbiota that is influenced by the aerobic/anaerobic balance, and specific conditions such as the extreme alkalinity of some of the gut compartments in many higher termites. The massive microbial species richness associated with these soil-feeding termites, as well as the extreme physicochemical conditions found in their guts, make them promising sources of new strains of bacteria (including actinomycetes), with potentially interesting metabolic activities. It is these microorganisms that can be used by researchers as a 'treasure trove' of useful molecules that can be applied to real-world problems and, in time, may be used for all manner of industrial applications (Brune, 1998; Brune, 2007). This is also relevant for economically important termite species, such as *R. flavipes*, as it might lead to the discovery of novel control mechanisms against termites (Fisher *et al.*, 2007).

It has been argued that, in order to ascertain the biotechnological applications of termite gut bacteria, more species richness information is needed (Fisher *et al.*, 2007). To date, few microorganisms have actually been isolated from termite guts, mainly due to the relatively small number of earlier microbiological studies relating to this work that have been done. However, this is rapidly changing as the immense potential of this group of insects is recognised for its great diversity, which is represented by more than 2000 species, as well as their broad spectrum of feeding habits and the diversification of their intestinal tracts (Brune, 1998).

The use of biochemical investigations into gene products, as well as other molecular and eco-physiological culture-independent work, is valuable, but only if it used in a synergistic way (in combination with what has already been discovered) that can, for example, direct the design of selective isolation methods. These methods can in turn be used to culture representatives of the microbial diversity found in the termite gut in order to gain a deeper understanding of this complex system and the symbiotic events that take place there (Kurtböke & French, 2007; Ohkuma, 2008).

Since there are so many associations happening in the termite gut, of which we know very little or nothing, the work done on trying to provide information on understanding these multiplex symbioses is therefore very important. There are also many questions about the underlying co-evolution between the termite and its gut community and its influences on sociality that remain to be answered (Noda *et al.*, 2007). The only way to do this is to continue the work already performed, in trying to put together all the pieces of the fascinating puzzle that is the termite gut system.

In an attempt to address some of the issues outlined above, the actinomycete diversity inside the hindgut of an indigenous South African termite species was investigated. The work done was a first step in trying to expand the current knowledge on the bacterial diversity that functions within this unique environment, by determining whether this diversity has the potential to be the source of new antibiotics as well as a potential biotechnological resource (in terms of its cellulose degradation abilities for biofuel production).

1.10 AIMS OF THIS PROJECT

The overall aim of this project was to conduct an investigation of the actinomycete diversity found within the hindgut of the lower termite *Microhodotermes viator*.

This was limited to two specific hindgut regions, namely the paunch and the colon, and encompassed the isolation of the culturable component as well as a culture-independent analysis.

Within the culturable component, a preliminary examination of all isolates for their antimycobacterial antibiotic and enzymatic potential was performed by antibiotic screening and analysis of their xylan- and CMC-degrading capabilities. A morphological examination of the hindgut was also undertaken, with the use of scanning electron microscopy and transmission electron microscopy, comparing the gut of both soldier and worker termites. The isolation component of the project also involved the description of potentially novel actinomycete isolates, with both detailed publishable descriptions, as well as more general indications of novelty.

The culture-independent component involved a metagenomic analysis of the actinomycete diversity using actinobacterial-specific primers and involved a comparison of the hindgut population with that found in the surrounding soil.

The work being presented here is groundbreaking, as no other work of this nature has ever before been attempted on this termite species, which is an endemic of the Cape Provinces of South Africa.

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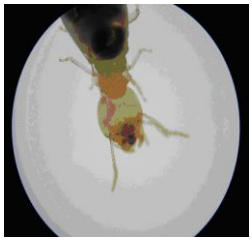
CHAPTER 2



ACTINOMYCETE ISOLATION, METABOLIC POTENTIAL AND A MORPHOLOGICAL EXAMINATION OF THE *M. VIATOR* HINDGUT

CHAPTER 2

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CONTENTS

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ACTINOMYCETE ISOLATION, METABOLIC POTENTIAL AND A MORPHOLOGICAL EXAMINATION OF THE *M. VIATOR* HINDGUT

2.1 ABSTRACT

Ever since the first discovery of a cellulolytic actinomycete inside the gut of a termite, there has been considerable interest in the metabolic abilities and the diversity of these bacteria within this complex environment. However, until fairly recently, most investigations of termite hindguts involved the higher termites from the Termitidae family. Little attention was paid to the lower termites, and even less so for the family Hodotermitidae. The aim of this part of the study was to explore the actinobacterial diversity of the paunch and colon hindgut regions of one particular member of this family, *Microhodotermes viator*.

All termite specimens were collected from the Tygerberg Nature Reserve in Cape Town, South Africa and, after dissection of the termite, the contents of the paunch and colon regions of the hindgut were plated out on four different isolation media (M3, MIIC, MIIG and MIIX), each at four different pHs (pH 6.0, 6.5, 7.0 and 7.5). Colony counts of the fungal, non-actinomycete and actinomycete components from both gut regions were recorded. The results showed that the greatest number of actinomycetes was isolated from the paunch on MIIC pH 6.0 ($4.8x10^3$ CFU/ml) and MIIX pH 6.5 ($4.5x10^3$ CFU/ml), and in terms of total diversity, the paunch proved to be the region from which higher numbers of all three groups of microorganisms were isolated.

A total of 80 actinomycete isolates was subcultured onto the same medium used for isolation and were separated into two groups, the 'Originals' (39 strains) and the 'Duplicates' (41 strains), based on various colony morphological and cultural characteristics.

The Original isolates were screened for antibacterial activity against: *Enterococcus faecium* Van A, *Mycobacterium aurum* A+ and *Staphylococcus aureus* ATCC 25923 (Gram positive) and *Escherichia coli* ATCC 25922 (Gram negative), tested on four different media. The majority of the isolates were found to have activity against *M. aurum* (7 isolates) and *S.*

aureus (5 isolates), or against all three Gram positive test strains (16 isolates). A list of the top sixteen antibiotic producers with activity against *M. aurum* was compiled and this information was used to determine which actinomycete isolates would be investigated further, as possible novel species.

All isolates were also investigated for their carboxymethylcellulose (CMC)- and xylan-degrading ability. Of the 76 isolates tested, four isolates showed no activity against either substrate, 14 were able to degrade both, and 75% were found to have specific CMC-degrading activity (after incubation for 21 days).

A morphological examination of the paunch and colon hindgut regions of both soldier and worker *M. viator* termites was also undertaken, using both scanning and transmission electron microscopy. The presence of actinomycete filaments was observed. As has been seen in other termites, a great diversity of bacteria was found associated with the gut wall, as well as within the gut lumen. Evidence of an actively reproducing and metabolising population of bacteria was provided in multiple transmission electron micrographs, along with suggestions of a tripartite symbiosis between the termite, some of its gut bacteria and their own endosymbiotic bacteria.

A molecular identification method was used to determine the genera to which the actinobacterial isolates belonged. Of the 80 isolates cultured from the hindgut of *M. viator*, 78 belonged to the *Streptomyces* genus, with the remaining two representing the genus *Nocardia*. Some of these genus assignments were confirmed by 16S-rRNA gene sequencing.

2.2 INTRODUCTION

The first report of an actinomycete being isolated from a termite gut was in 1946, with the discovery of *Micromonospora propionici* by R.E. Hungate (Hungate, 1946). Since then there has been a lot more interest in the organisms that inhabit the gut systems of termites, mainly because of the potential biotechnological applications relating to cellulose digestion by termites and their gut commensals (Varma *et al.*, 1994). However, the majority of the work to date involves a broad determination of bacterial species richness; mainly from the higher order termite species from the family Termitidae (Bignell *et al.*, 1991). Little attention has been paid to the actinomycete population found in termite guts, and even less so for the termite family Hodotermitidae.

Within South Africa, there are only two representatives of the lower termite family Hodotermitidae. These are *M. viator* (Southern Harvester Termite) and the related species *Hodotermes mossambicus* (Northern Harvester Termite) (Picker *et al.*, 2002). *H. mossambicus* is widely distributed throughout the drier savannah regions of the African continent, from Ethiopia to the southern karroid areas of the Cape Provinces of South Africa. *M. viator* is an endemic of the Cape Provinces (see Fig. 2.1 below), found nowhere else in the world, and its distribution overlaps with that of its relative. *M. viator* inhabits a range of vegetation types, preferring open veld and avoiding fynbos on sandstone, foraging and feeding on woody plants (twigs and sticks), especially *Pteronia* and 'vygie' species (Picker *et al.*, 2002; Scholtz & Holm, 1985).

Figure 2.1 Distribution of *M. viator* within South Africa (Picker *et al.*, 2002).

As with most other termite species, *M. viator* termites have a profound effect on their environment. They are a ubiquitous component of Karoo fauna, producing sharp conical mounds in soils with a high clay content. These mounds generally become covered in sand, forming very large, long-lived structures called 'heuweltjies' (up to 20m in diameter and 2m in height) that are evenly spaced across the region. Large amounts of frass (pelleted faeces) are deposited on the surface, and because of this, they are areas rich in nutrients, resulting in

colonisation by plants that favour disturbed sites, such as the colourful annuals, producing kaleidoscopic patterns across the landscape (Picker *et al.*, 2002).

The map shown in Fig. 2.2 provides details of the nature reserves that are found within the city of Cape Town, with the Tygerberg Nature Reserve circled (the site from which all *M. viator* specimens were obtained).

<u>Figure 2.2</u> Map of the Cape Peninsula of South Africa, providing details of all the nature reserves that fall within the boundaries of the city of Cape Town (http://www.capetown.gov.za/en/EnvironmentalResourceManagement/publications/Documents/Nature_Reserves_map_CCT_2008-06.JPG)

The work presented here is the first attempt at an actinomycete-specific isolation and investigation from the gut of a previously unexplored termite, *M. viator*, an endemic of the Cape Provinces of South Africa.

One of the primary aims was to provide initial isolation data, comparing the non-actinomycete, the actinomycete and the fungal populations that were isolated from both the paunch and colon regions of the hindgut of *M. viator,* on four different media, each at four different pHs. Another aim was to provide a detailed comparison of both the 'Original' and 'Duplicate' isolates (see explanation in Materials and Methods), in terms of the preference of the actinomycete isolates for different isolation media and pHs, and to determine the distribution of the isolates obtained from the paunch and colon.

Another important aim was to provide information on the metabolic abilities of the actinomycete isolates as an indication of their potential application in biotechnology. This involved an assessment of their antimicrobial spectra against a range of Gram positive and Gram negative bacteria, including *M. aurum*. *M. aurum* strain A+ was used because it has a similar antibiotic susceptibility profile to *M. tuberculosis* (Chung *et al.*, 1995), but has a much faster growth rate and is not pathogenic. All of the Original isolates were ranked according to the strength of their antibacterial activity against *M. aurum* A+. Antimycobacterial activity was one of the primary criteria used to determine which isolates to investigate further, as possible novel species.

All isolates, both Originals and Duplicates were also assessed for their ability to degrade carboxymethylcellulose (CMC) and xylan. This information indicated their ability to grow on alternative substrates and provides an indication of their potential uses for biotechnological applications.

The final aim of this section of the work was to provide a morphological examination of the hindgut of *M. viator* in both soldier and worker specimens, making use of both scanning and transmission electron microscopy to explore the community of microorganisms that reside within both the paunch and colon regions of this fascinating termite species. No detailed description of the bacterial morphology inside the hindgut of *M. viator* has been provided before.

2.3 MATERIALS AND METHODS

2.3.1 Gut morphology

It was decided that the hindgut paunch as well as the colon (P4 region) within the intestinal tract of *M. viator* would be sampled (labelled below in Fig. 2.3), based on previous evidence from the literature that suggested that these sites would be most likely to contain actinomycete populations. An initial review of the papers describing the gut morphology in lower termites suggested that the hindgut consisted of a single dilated hindgut region – the paunch. However, a much more detailed comparison of lower termite guts (Noirot, 1995) showed that the situation is more complex. This in-depth description of the histology and morphology found in these termites indicated that the paunch region (especially in the *Hodotermitidae* family) is divided into two distinct parts – the anterior paunch region (commonly designated P3a), which is thin-walled; and the posterior paunch region (P3b) which is thick-walled due to a stronger muscle sheath. The two regions, especially in representatives of *H. mossambicus* and *M. viator* are separated by a distinct narrowing of the gut. In the initial dissections, what was initially discarded as being an inflated midgut was actually the P3a region, and in fact only the posterior region (P3b) of the paunch was investigated during this project. In order to allow for accurate scientific comparisons, all subsequent investigations only involved this particular section of the paunch. As a result, all further references to the paunch region are in fact referring to the posterior paunch or P3b. The whole gut examinations shown here were made using a Wild M7A (Heerbrugg, Switzerland) dissecting microscope.

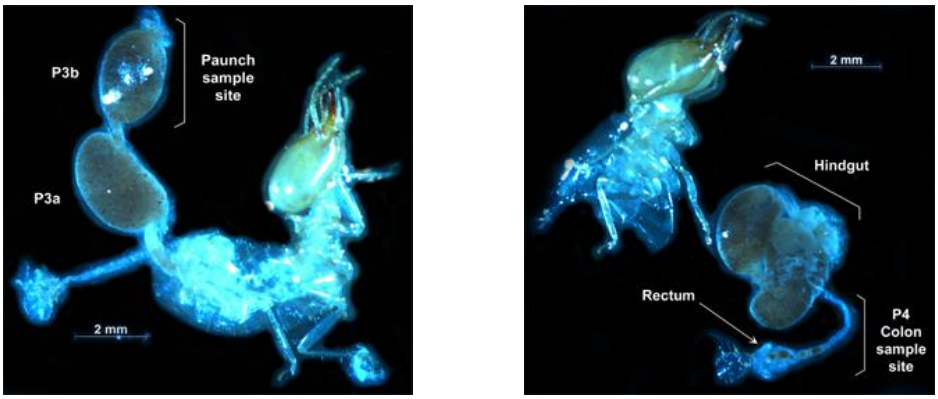


Figure 2.3: Dissection microscope photographs of two *M. viator* soldier termites showing the specific gut regions used during actinomycete isolation (6.3 X mag.)

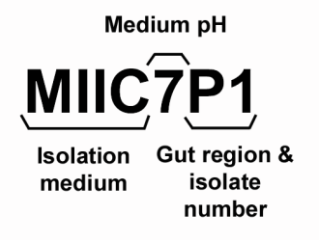
2.3.2 Dissection and Isolation

Twenty termites were obtained on 13 March 2006 from within a *M. viator* termite mound, located in the Tygerberg Nature Reserve, Cape Town, South Africa. Under sterile conditions, in a laminar flow hood, these termites were surface sterilized in 70% ethanol for 2 min before being washed three times in sterile distilled water (dH_20). The posterior paunch and colon regions were dissected from the termite abdomen and placed in separate microfuge tubes containing 2ml phosphate buffer (10 mM $Na_2HPO_4.2H_2O$, 1.8 mM KH_2PO_4; pH 7.0). The contents were vigorously vortexed for 5 min to disperse the contents of the gut region, before a standard dilution series was set up using sterile phosphate buffer.

One hundred microlitres (100µl) of each dilution was plated on four separate media (containing 50ug/ml cycloheximide and 10ug/ml nalidixic acid): Medium 3 (M3) [Wenzel *et al.*, 2002] and Medium II [Cazemier *et al.*, 2003] containing glucose (MIIG), CMC (MIIC) or xylan (MIIX) as carbon sources, with each medium at four different pHs (pH 6.0, 6.5, 7.0 and 7.5). This isolation strategy based on four different pH values is similar to that adopted by Pasti & Belli (1985), where their enrichment cultures were made at pH 6.0, 7.0 and 8.0, based on reports that the pH of the termite gut ranges between 6-6.5 and 7-7.5. All plates were incubated under aerobic conditions at 30^{o}C and were monitored for 28 days. Actinomycete isolates were selected based on colony morphology.

2.3.3 Bacterial maintenance and culture conditions

All actinomycete isolates were maintained either as spore stocks in dH_20 with 25% (v/v) glycerol at -20°C, or at -70°C as 40% (v/v) glycerol stocks made from liquid cultures. Initially, isolates were maintained on the same type of medium on which they were isolated, but later the predominant maintenance medium used was Yeast extract-Malt extract Agar (YEME) [International *Streptomyces* Project (ISP) Medium No.2, Shirling & Gottlieb (1966)]. Other media used for individual isolates were Difco Middlebrook 7H9 Agar (Becton Dickinson) amended with sterile glucose to a final concentration of 10mM (albumin-dextrose-catalase supplement omitted); Czapek solution agar (CZ) (Atlas, 1993); as well as Bennett's Medium agar (BM), and modified Bennett's agar (glycerol replaces glucose) (Atlas, 1993). All isolates were grown at 30^{o}C, generally for 5-7 days. A strain code was assigned to each isolate, and an example of this code is illustrated below. A 4 next to the gut region letter designates those isolates that were only identified and isolated after four weeks of incubation.



2.3.4 'Originals' versus 'Duplicates'

Very early in the project, all isolates were assessed for their ability to grow on media other than that of their original isolation medium. The media tested included three of the maintenance media described above: 7H9, CZ and YEME. Based on their ability to grow, their preferences for certain media, as well as the cultural characteristics (presence of aerial mycelium and colour of the aerial and substrate mycelia) on these media and their original isolation media, it was determined that some of the isolates were most probably repeat isolates or clones. As a result, these isolates were assigned to the 'Duplicate' isolate group, and were initially not characterised any further. The remaining isolates from the 'Original' isolate group were deemed unique, and were used for all further work (see Appendix A for a list of all Original isolates and their respective Duplicates).

The separation of the 'Original' isolates from the "Duplicates' was done to dereplicate the isolates and avoid investigating the same strain more than once. A similar strategy was adopted by Watanabe *et al.* (2003) in order to group their actinomycete isolates based on cultural characteristics observed after cultivation on ISP#2 medium after one week. They based their dereplication on the presence of aerial mycelium, colour of the aerial mycelium, colour of the substrate mycelium and whether a soluble pigment was produced.

2.3.5 Screening for useful metabolic abilities

2.3.5.1 Antibacterial testing

Antibacterial activity was assessed for the Original isolates only on 7H9, CZ and YEME media. Each isolate was stab-inoculated onto each of the three media using sterile toothpicks and incubated at 30°C for 14 days. The antibacterial activity was tested against a range of Gram-positive and Gram-negative bacteria: *M. aurum* A+, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *E. faecium* (a Van A strain). Each test bacterium was grown in 5ml Luria-Bertani Broth (LB) (Sambrook *et al.*, 1989) and incubated at 37°C with agitation for 24h (except for *M. aurum*, which required a longer incubation of 48h). Gram stains were

performed on each culture before measuring the optical density (OD) at 600nm using a Beckman DU®530 Spectrophotometer.

To ensure equivalent concentrations of each test bacterium were used from one experiment to the next, the equation $OD_{600} \times (x) = 4\ OD_{600}.\mu l$ (where x is the volume of culture required) was used for the *E. coli* culture, and for all other test bacteria, the volume required was determined from the equation $OD_{600} \times (x) = 160\ OD_{600}.\mu l$. These equations were determined empirically and ensure that an even lawn of the test bacterium is obtained in each overlay. Each test bacterium was inoculated into 6ml sloppy agar (0.7% agar; Sambrook *et al.*, 1989), overlaid onto each actinomycete-containing plate (being careful not to disturb any spores on the aerial mycelium) and the agar allowed to set before being incubated at 37°C for 24h. All *M. aurum* plates were incubated for 48h due to this test bacterium's slower growth rate. The presence of aerial mycelium, colony diameter and diameter of inhibition zone were recorded, and the antibacterial activity was calculated as a measure of the area of inhibition produced in mm^2.

2.3.5.2 Carboxymethylcellulose- and xylan-degrading activities

Degradation of xylan (0.4% w/v) and CMC (0.3% w/v) was detected in modified Bennett's agar, after the method described by Locci (1989). All plates were incubated at 30°C for 21 days. Xylan degradation was recorded by the presence of a zone of clearing. CMC degradation was visualised using the method described by Schäfer *et al.* (1996), which involves flooding the plates briefly with 0.1% Congo Red stain, and subsequent destaining using 1M NaCl (the 1M HCl step was omitted). A pale zone around the area of growth indicated that CMC degradation had occurred.

2.3.6 Termite hindgut morphological examination

A morphological examination was made of both the colon and paunch regions from the hindgut of individual worker and soldier termites, using both a scanning electron microscope (SEM) and a transmission electron microscope (TEM). Initially, the same preparation protocol was used for both SEM and TEM. However, this process proved to be too destructive for the preparation of TEM sections and a modified agarose-embedding protocol was devised.

The same washing protocol, described previously (section 2.3.2), was used before termite dissection. After dissection, the gut regions were immediately placed in a drop of 0.1M

$Na_3PO_4.12H_2O$ buffer (pH 7) containing 2.5% glutaraldehyde, on a strip of Parafilm. All gut segments were then transferred to eppendorf tubes containing the same solution for overnight fixation at 4°C. After fixation, this solution was discarded and the SEM samples were washed three times in glutaraldehyde-free 0.1M $Na_3PO_4.12H_2O$ (pH 7) buffer at room temperature (15min/wash). For TEM, only one wash was performed before gently placing the samples on a clean glass microscope slide. The samples were then covered with a drop of 2% low melt agarose and, after setting, the excess agarose was cut away. All further preparation for TEM used agarose-embedded samples. All samples (SEM and TEM) were then post-fixed in 0.1M $Na_3PO_4.12H_2O$ buffer (pH 7) containing 2% osmium tetroxide for two hours at room temperature in a fumehood. After discarding this solution, the samples were washed twice in 0.1M $Na_3PO_4.12H_2O$ buffer (pH 7) and twice in dH_2O. All segments were then dehydrated in a series of increasing ethanol (EtOH) concentrations, using solutions kept at 4°C (30, 50, 70, 80, 90 and 100% (v/v) EtOH, plus two more changes of 100% EtOH).

After this step, all SEM samples were critical point dried using carbon dioxide and sputter-coated with gold palladium, before they were visualised under the SEM. For TEM samples, the 100% EtOH was replaced with 100% acetone, and then with increasing concentrations of Spurr's Resin over the course of 3 days (25% Spurr's Resin/75% acetone, 50/50, 75/25 and 100%, and two more changes of 100% Spurr's Resin). After embedding and heating for another 24 hours, sections were made using a Reichert Ultracut S Ultramicrotome (Leica, Austria) and diamond knife, and placed on copper grids. The final step before visualisation under the TEM involved staining the grid sections with 2% aqueous uranyl acetate, followed by Reynolds' lead citrate (Reynolds, 1963) for approximately 5 min each.

For all SEM work, a Leica Stereoscan 440 scanning electron microscope was used, whereas a Leo 912 transmission electron microscope was used for all TEM-based examinations. TEM images were taken with a ProScan CCD camera and analysed using EsiVision Pro v.3.2 (Distributed by Soft Imaging Software (SIS) GmbH, Germany).

2.3.7 Molecular genus identification method

2.3.7.1 DNA extraction

In most cases, isolates were grown in 10ml YEME in 100-ml Erlenmeyer flasks for 2-7 days at 30°C, with agitation, and Gram stained before DNA extraction. The boiling method described by Cook & Meyers (2003) was used initially to extract the DNA necessary for 16S rRNA gene analysis. However, an alternative method was necessary for some isolates, in

order to obtain purer DNA (Wang *et al.*, 1996). Modifications were made to this protocol: the lysozyme concentration in the lysozyme buffer was increased from 8mg/ml to 25mg/ml, and proteinase K was added to a final concentration of 0.2 mg/ml. The RNase A treatment was performed for 40-45 min instead of the indicated 30 min. Depending on the concentration obtained, DNA was frequently diluted $^{1}/_{100}$ in Tris-EDTA buffer (pH 7.8) before use in PCR reactions.

2.3.7.2 PCR amplification and enzyme digestion

Amplification of the 16S rRNA gene of all isolates was carried out as described previously (Cook & Meyers, 2003), but 0.5 U *Taq* Super-Therm *Taq* polymerase (JMR Holdings, U.S.A.) was used instead of 2 U. PCR products were electrophoresed in 1% agarose gels containing ethidium bromide (0.8µg/ml) at 85V in tanks containing 1x TAE buffer for approximately 1 hour, in order to confirm that the correct product size had been obtained.

Standard restriction enzyme digestions were carried out on the 16S rRNA gene amplification products of all isolates, as described by Cook & Meyers (2003). Digests were performed overnight in a 37°C water bath, followed by electrophoresis in 1.5% agarose gels, as described above. The resultant restriction enzyme patterns of the isolates were visualised using a GelDoc (BioRad) and isolates were assigned to genera according to the dichotomous keys detailed in Cook & Meyers (2003). Isoschizomers *Mbo*I and *Vsp*I were used instead of *Sau*3AI and *Asn*I, respectively.

2.3.7.3 DNA sequencing

For some isolates, it was necessary to sequence the 16S rRNA gene PCR product in order to confirm the genus identification. For these isolates, the DNA was purified using a MSB Spin PCRapace® PCR purification kit (Invitek, Germany) as per the manufacturer's instructions. All sequencing was performed as a service by Macrogen, South Korea, using a ABI3730XL and ABI3700 DNA Capillary Sequencer (Applied Biosystems) with KB™Basecaller Software and a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing of the 16S rRNA gene made use of universal bacterial primers.

2.4 RESULTS AND DISCUSSION

2.4.1 Isolation of microorganisms

The dissected termite gut regions were dispersed in phosphate buffer before plating and as a result, the CFU/ml values described in this section are actually referring to CFUs per millilitre of termite gut extract (i.e. CFU/ml TGE). The term CFU/ml has been retained throughout to simplify discussion of the results.

2.4.1.1 Fungi

Looking at the M3 medium in Fig. 2.4, comparing the paunch and colon, the fungal isolates from the paunch were isolated at all four pH values. Whereas the average fungal colony count (across all four pH values, shown in Table 2.1) was $2.35x10^3 \pm 3.39x10^2$ CFU/ml for the paunch, the numbers in the colon were much lower ($< 3x10^2$ CFU/ml). This same trend was seen for the MIIC medium, except that in the colon, the fungal colony count for pH 6.0 was higher than the others at $7x10^2$ CFU/ml. MIIG paunch isolations showed the highest fungal colony count of all media, with an average of $3.29x10^3 \pm 1.2x10^3$ CFU/ml across all four test pHs. The counts for the MIIX medium were comparable with those recorded from M3, but noticeably greater than the numbers from medium MIIC in the paunch. Again, for both MIIG and MIIX, the fungal colon counts were much lower ($< 7.5x10^2$ CFU/ml), with the highest numbers recorded at pHs 6.0 and 6.5.

As the presence of actinomycetes in the termite hindgut has been suggested to be the result of spores ingested with the food (Brune, 2006), so too may be the fungal isolates that were cultured from both the colon and paunch regions of the *M. viator* hindgut. The fungal colonies that are recorded in Fig. 2.4 and Table 2.1 may therefore only represent the proportion of spores that were able to survive passage through the gut system and grow under the culturing conditions used in the initial isolation. This suggestion is supported by the SEM results, as no large fungal filaments were found inside either of the hindgut regions investigated. The lack of actively metabolising and lignin-degrading fungi in the hindgut would therefore mean that the putative actinomycetes are the only population inside the hindgut that has the potential to fulfil this important function.

However, the fact that the fungal numbers in the paunch far outweigh those recorded from the colon suggests that there is in fact a portion of the paunch that is occupied by an active population of fungi. Although Schäfer *et al.* (1996) suggested that fungi in the termite gut are

involved in hemicellulose breakdown, there is also the possibility that the *M. viator* fungal isolates may play an important role in lignin biodegradation, as this is a well-known ability associated with fungi (Kuhnick *et al.*, 1994).

One problem with the suggestion that the fungi and actinomycetes are important in the breakdown of lignin in the termite gut is that food is only retained in the gut for approximately 24 hours, a time frame that is too short for the degradation of such a complex polymer (Kuhnick & König, 1997). However, during the passage of food through the digestive tract, wood particles would be inoculated with microorganisms, which would start lignin breakdown near the aerobic paunch epithelium, and digestion should continue outside the gut, with the help of other microbes. Also, specific termite behavioural traits, such as proctodeal feeding and feeding on faecal matter by colony members, would repeatedly re-cycle the lignin through the gut, thereby increasing the efficiency of the digestion of lignin and other plant materials by the colony as a whole (Kuhnick & König, 1997).

2.4.1.2 Non-actinomycetes

In general, Fig. 2.4 indicates an approximately equal number of non-actinomycetes recorded across all media tested, as well as across all four pHs. Comparing the paunch numbers for medium M3, there were no significant differences between the tested pH values, with an average colony count of $2.88 \times 10^4 \pm 5.22 \times 10^3$ CFU/ml recorded across all four (see Table 2.1 for details). Although the numbers were also equivalent in the colon for pH 6.5, 7.0 and 7.5, they were lower and there appeared to be a spike in the colony count for pH 6.0, with a value of 2.63×10^3. For medium MIIC, the numbers of non-actinomycetes isolated from the paunch decreased steadily from pH 6.0 as the pH increased, a trend that was not shown for the colon sample. Again, the paunch average of $3.48 \times 10^4 \pm 8.57 \times 10^3$ CFU/ml was higher than that recorded from the colon ($1.69 \times 10^4 \pm 2.6 \times 10^3$ CFU/ml). Similar patterns as those seen in Fig. 2.4 for the paunch isolation on M3 and the MIIC colon sample were also seen for both gut region isolations on MIIG and MIIX, with the exception that the differences in colony counts did not appear to be as great between paunch and colon.

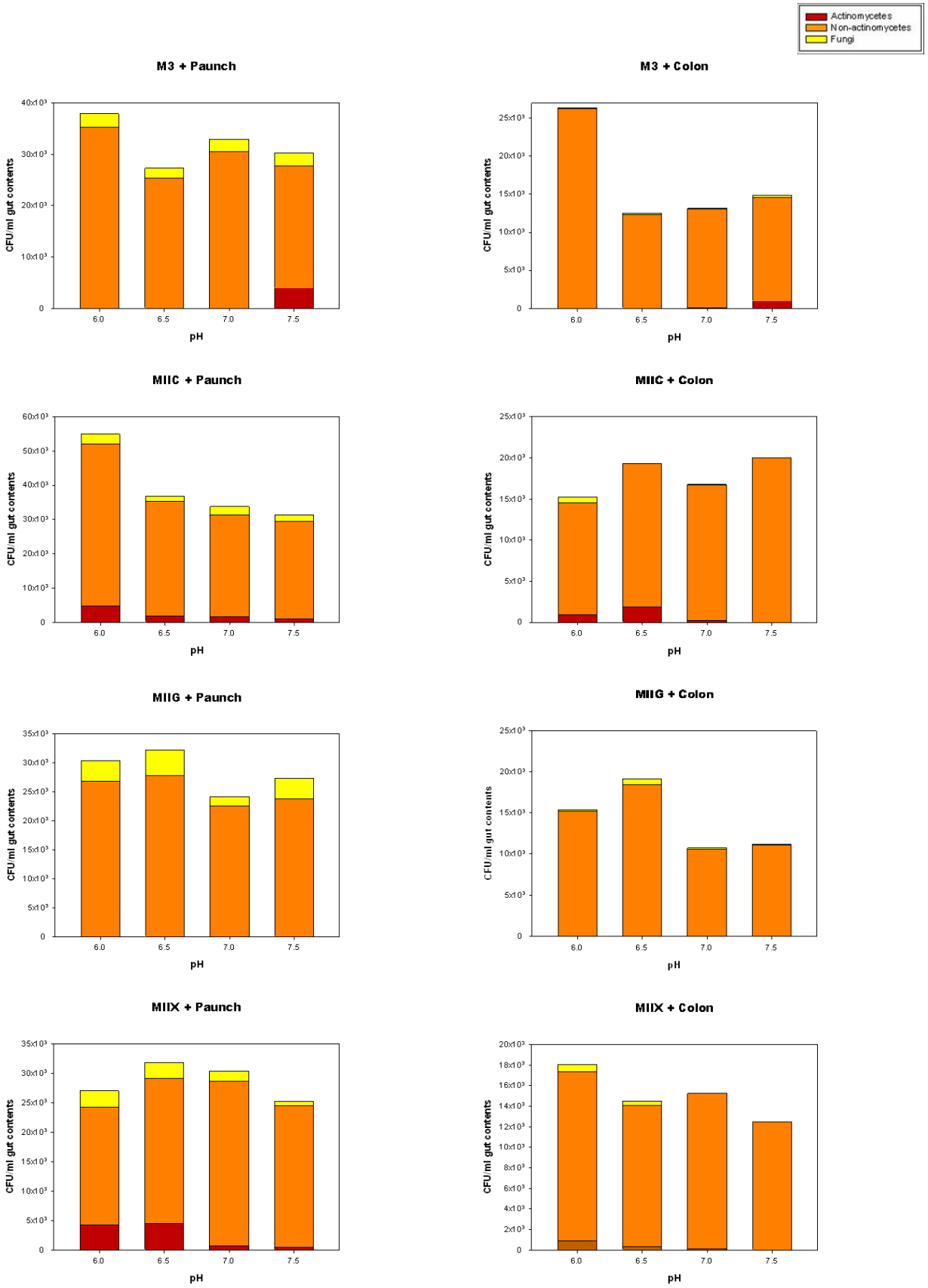


Figure 2.4: Graphs of total microbiological counts recorded from a standard dilution series of 20 termite hindgut regions, plated on four different media. Values are expressed as CFU/ml gut contents, plotted against the individual media and pHs.

2.4.1.3 Actinomycetes

M3 produced very few actinomycete isolates, except at pH 7.0 ($1x10^2$ CFU/ml from colon) and pH 7.5 ($4x10^3$ CFU/ml from paunch and $1x10^3$ CFU/ml from colon). Interestingly, no actinomycetes grew on MIIG from either of the gut samples, whereas a large number of actinomycete colonies were counted from the MIIC and MIIX media (Fig. 2.4). These results suggest that the actinomycetes in the termite gut are unable to utilise free glucose, or prefer to use non-glucose sugars as their carbon source. This is likely due to the food source that the termites are ingesting – woody plants from the surrounding fynbos environment (Picker *et al.*, 2002). For medium MIIC, there appeared to be a preference for pH 6.0 in the paunch, whereas the preference in the colon was pH 6.5, with no actinomycetes isolated from the colon at pH 7.5. However, the average colony counts from the paunch and colon samples were similar, with $2.28x10^3 \pm 1.73x10^3$ CFU/ml and $0.97x10^3 \pm 8.6x10^2$ CFU/ml recorded, respectively (see Table 2.1 for details). The pattern was quite different for medium MIIX, where greater actinomycete numbers were recorded for both sample sites at both pH 6.0 and 6.5, but much higher colony counts were recorded in the paunch than the colon. Again, no actinomycetes were isolated from the colon at pH 7.5, but values of $4.3x10^3$ CFU/ml and $4.5x10^3$ CFU/ml were recorded for the pH 6.0 and 6.5 paunch isolation, respectively, whereas for the colon the corresponding values were $9x10^2$ CFU/ml and $3x10^2$ CFU/ml.

Therefore, the greatest number of actinomycetes for both the paunch and colon were isolated at pH 6.0 and pH 6.5 when the media MIIC and MIIX were used, which may be a reflection of their natural conditions. This agrees with Pasti & Belli (1985), who found the optimum pH for growth of their actinomycete strains on cellulosic substrates to be between pH 6.2 and 6.7. The overall greatest number of actinomycetes in this study, $4.8x10^3$ CFU/ml, was isolated from the paunch on medium MIIC at pH 6.0. However, the greatest overall percentage actinomycete isolation (15.9% of the total isolates) was from the paunch at pH 6.0 on medium MIIX.

This reinforces the observation that the paunch has a greater diversity and abundance of microorganisms than the colon. This is to be expected, as the paunch is a major site for digestion in the termite gut (Yang et al., 2005).

Table 2.1: Detailed counts of the microfloral components of the M. viator hindgut, isolated on different media and at different pHs

Medium	pH	Paunch			Colon		
		Actinomycete	Non-actinomycete	Fungi	Actinomycete	Non-actinomycete	Fungi
M3	6	0	3.53×10^4	2.6×10^3	0	2.63×10^4	1×10^2
	6.5	0	2.54×10^4	1.85×10^3	0	1.23×10^4	2×10^2
	7	0	3.05×10^4	2.45×10^3	1×10^2	1.3×10^4	1×10^2
	7.5	4×10^3	2.38×10^4	2.5×10^3	1×10^3	1.35×10^4	3×10^2
MII + CMC	6	4.8×10^3	4.72×10^4	2.9×10^3	9×10^2	1.37×10^4	7×10^2
	6.5	1.8×10^3	3.35×10^4	1.5×10^3	1.9×10^3	1.74×10^4	0
	7	1.6×10^3	2.98×10^4	2.4×10^3	1.9×10^2	1.65×10^4	4×10^1
	7.5	9×10^2	2.85×10^4	1.9×10^3	0	2×10^4	0
MII + Glu	6	0	2.68×10^4	3.6×10^3	0	1.52×10^4	2×10^2
	6.5	0	2.78×10^4	4.45×10^3	0	1.85×10^4	6.5×10^2
	7	0	2.26×10^4	1.6×10^3	0	1.05×10^4	2×10^2
	7.5	0	2.38×10^4	3.5×10^3	0	1.11×10^4	1×10^2
MII + Xylan	6	4.3×10^3	1.99×10^4	2.8×10^3	9×10^2	1.64×10^4	7.5×10^2
	6.5	4.5×10^3	2.46×10^4	2.7×10^3	3×10^2	1.38×10^4	4×10^2
	7	7×10^2	2.8×10^4	1.7×10^3	1×10^2	1.52×10^4	0
	7.5	5×10^2	2.4×10^4	7×10^2	0	1.25×10^4	0

*Values are a measure of total microbiological counts recorded from a standard dilution series of 20 termite hindguts, expressed as CFU/ml gut contents.

2.4.1.4 Overall comparison and summary

In the paunch samples, there was an obvious preference by the actinomycetes for media MIIC and MIIX, whereas the non-actinomycetes and fungi grew on all four media at all four pHs. A similar result was obtained by Watanabe *et al.* (2003) for the hindgut of *C. formosanus*. They found the ratio of filamentous actinomycetes to other bacteria that appeared on cellulose-related Avicel and cellulose powder media to be 0.027 and 0.006, respectively. Also, when lignin-based media were used, there was a higher ratio of filamentous actinomycetes to other bacterial colonies. Interestingly, two of the isolated strains with the ability to grow on all of these media (including CMC) were identified by 16S rRNA gene analysis as belonging to the genus *Streptomyces* (Watanabe et al., 2003).

Although this preference for media MIIC and MIIX was also visible in the *M. viator* colon samples, the preference within the two media appeared to be related to the pH of the medium, rather than to the particular carbon source used. In the colon, actinomycete isolates were found on medium M3 at pH 7.0 and 7.5, but none were isolated from the pH 7.5 MIIC and MIIX media. In comparison, the paunch showed actinomycete isolations for both MIIC and MIIX media (pH 7.5), but not at pH 7.0 for medium M3. In terms of fungal colonies, as stated above, isolates were recorded for all media and pH values in the paunch, but for the colon, no fungi were recorded from MIIC at pH 6.5 and 7.5, nor were there any isolates for MIIX at pH 7.0 and 7.5. Comparing the actinomycetes with the fungi in terms of preference for particular media, it appears that the actinomycetes preferred media MIIC and MIIX (both complex substrates), whereas the fungi seemed to prefer media MIIG and M3, both of which contain glucose as the primary carbon source.

These facts imply that the majority of the actinomycetes that were isolated are unable to utilise free glucose, being much better adapted to utilising complex molecules such as CMC and xylan. This is not surprising when taking into account that in their natural environment (the termite hindgut), all of the easily accessible and digestible material would have been mobilised and absorbed by the time the gut contents reach the end of the midgut (Brune, 2006). This raises some interesting questions. Large molecules like CMC and xylan cannot be taken up, so they would have to be partially degraded before they can be transported into the cell. The question is what degradation products do these actinomycetes take up - di-, tri- and/or very short oligosaccharides, as well as xylose polymers of various lengths from the breakdown of xylan? Also, if they can take up glucose from the degradation of CMC, then why can they not take up free glucose in medium MIIG? This finding is supported by Pasti & Belli (1985), where they found that some of their actinomycete isolates from the hindgut of four different termite species exhibited high cellulase activities (which were constant for 5-7

days), but they also showed that inhibition by glucose was a common feature for almost all of their isolates.

In summary, the *M. viator* paunch has been shown to have much greater numbers of microorganisms, including actinomycetes, non-actinomycetes and fungi, than the colon. Across all media, there was a greater proportion of fungi identified (particularly in the paunch region) than actinomycetes. However, the non-actinomycete bacteria were by far the predominant microbes in the gut, in terms of numbers and in terms of the percentage of isolates recorded from all four media at all four pH values.

These results are in agreement with similar work, which has shown that the paunch region of the termite hindgut is an environment rich in microflora (Varma *et al.*, 1994; Brune, 2006). From the results presented in Fig. 2.4, it seems that the best combination for the specific isolation of actinomycetes involves the paunch region of the termite gut using medium MIIC at pH 6.0 or medium MIIX at pH 6.0-6.5.

2.4.1.5 Cultured actinomycete isolates

Looking at the information contained in the bacterial codes for all of the cultured actinomycete isolates shown in Appendix B and C, the following interpretations, described below, can be made.

In the Originals group of isolates totalling 39 strains, there were four actinomycete isolates that came from the M3 medium (pH 7.5). Only isolate 1 (M375C1) came from the colon and the other three were of paunch origin. For those isolated on medium MIIC, only three came from the paunch (one each for pH 7.0, 6.5 and 6.0), and the rest were colon isolates, with 8 out of the 10 isolates sourced from pH 7.5 and pH 7.0. A total of 22 Original isolates were cultured on MIIX, with approximately equivalent numbers coming from the paunch (12) and the colon (10). In both cases, compared with the MIIC isolates, the majority of the isolates were from the pH 7.0 and pH 6.5 plates.

The Duplicates group encompassed 41 strains, including the only actinomycete isolate to be found on medium MIIG – isolate 39D (MIIG65C1) sourced from the colon, at a pH of 6.5. The MIIC isolates from this group included 13 paunch and 7 colon isolates. All of the strains isolated from the colon on medium MIIC at pH 7.5 were clearly judged to be Original strains during the initial separation of Originals and Duplicates; the Duplicate colon actinomycetes only came from pH 7.0 (2), 6.5 (4) and 6.0 (1). There was a more even spread of paunch isolates across the different pHs for the MIIC medium, with only 2 isolates each from pH 7.0

and 6.0, and 5 and 4 isolates, respectively, identified from pH 7.5 and 6.5. The MIIX medium also provided 20 isolates, with the majority of the colon isolates obtained from pH 6.5 plates, with 2-3 isolates each for the remaining pH values. For the paunch isolates, as with the paunch sample for MIIC, most of the isolates came from pH 7.5 and pH 6.5.

When comparing the Originals and the Duplicates on their own, they both had almost equal numbers from the paunch and colon. However, for the Original isolates there initially appeared to be a preference for particular pHs, with 12 and 14 isolates each for pH 7.5 and 7.0 respectively, and only 9 and 4 for pHs 6.5 and 6.0. The Duplicates showed a preference for pH 7.5 and 6.5, with 11 and 16 isolates respectively, and 8 and 6 isolates sourced from pH 7.0 and 6.0.

From the combined data of the Original and Duplicate isolates, 39 actinomycetes were isolated from the paunch and 41 were isolated from the colon. This equality in the numbers of isolates from the paunch and colon is interesting, as it would be expected that, due to its larger size, more actinomycetes should be isolated from the paunch. However, as discussed in Chapter 1, only the periphery of the paunch is oxygenated and this is therefore the only region of the paunch that would be expected to harbour aerobic actinomycetes. The colon on the other hand is considered to be completely aerobic (Brune & Friedrich, 2000). Berchtold *et al.* (1999) compared the surface area of the paunch (P3a) and colon (P4) from the lower termite *M. darwiniensis*, and found them to be similar ($15.2 \pm 2.7 mm^2$ and $14.5 \pm 2.5 mm^2$, respectively). This could therefore explain why equal numbers of actinomycetes were isolated from the paunch and colon of *M. viator*, because the areas of the aerobic surfaces from which they were isolated may also be similar. Alternatively, the similar numbers could have been a consequence of the particular isolation conditions used, and the particular preferences that the paunch and colon isolates have for these conditions.

As detailed above, only one isolate was found on medium MIIG, 4 came from M3 medium, 33 came from MIIC and 42 were isolated on medium MIIX. In terms of the pH partitioning, 23 isolates came from pH 7.5, 22 from pH 7.0, 25 from pH 6.5 and only 10 were isolated at pH 6.0. This agrees with pH microsensor studies that have measured the pH within the different regions of the lower termite *R. flavipes* gut. Both the paunch and colon regions were found to have a pH around neutral, with the pH of the paunch confined to pH 6-8 (Brune, 2006).

2.4.2 Screening for antimicrobial and enzyme activities

2.4.2.1 Antibacterial spectrum

Fig. 2.5 provides a summary of the detailed antibacterial activities of the Original isolates shown in Appendix B, tested against the four bacterial test strains: *E. coli* ATCC 25922 (Gram-negative) and three Gram-positives, *E. faecium* (VanA), which was chosen because of its resistance to the important antibiotic vancomycin, *S. aureus* ATCC 25923 and *M. aurum* A+. The *E. coli* and *S. aureus* strains are standard antibiotic-susceptibility test strains.

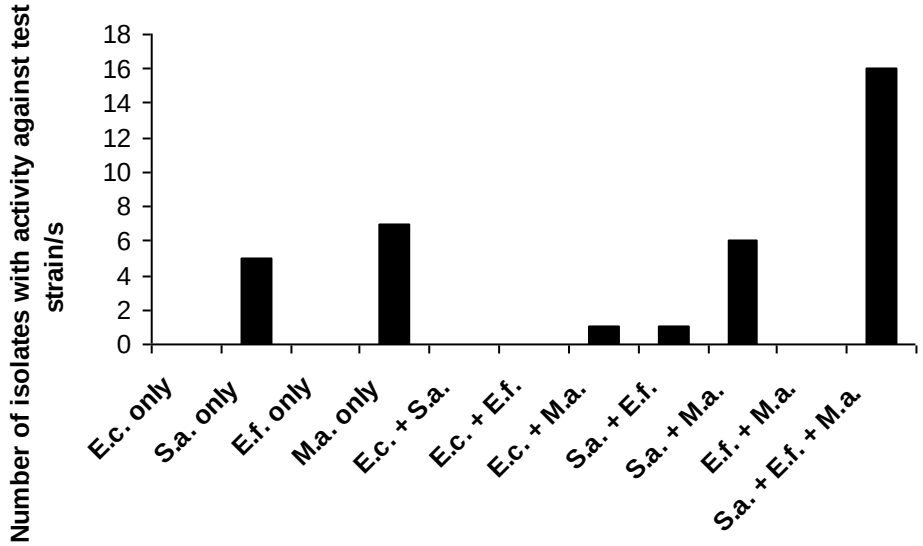


Figure 2.5: Summary of the number of Original isolates with activity against the various test bacteria

From Fig. 2.5 it appears that of the 39 isolates tested, there were none with any specifically anti-Gram negative activity. The majority of the isolates had activity against two or all three of the Gram positive bacteria. However, five isolates were found to have activity against *S. aureus* alone, whereas seven had only *M. aurum* activity. Those with dual activity included: isolate 2, which was the only one to have any activity against *E. coli*, combined with an anti-*M. aurum* result; isolate 15 which had combined activity against *S. aureus* and *E. faecium*; and six isolates with a positive result against *S. aureus* and *M. aurum* (strains 13, 17, 19, 31, 37 and 39). By far the greatest number of isolates had activity against all three Gram positive test bacteria – a total of 16 out of the 39 isolates (41% of the Originals). Although not shown in Fig. 2.5, three isolates were found to have no activity against any of the test bacteria. These were isolates 27, 12, and 20 – the last two being the only *Nocardia* strains that were isolated.

These results suggest that the antibiotics produced by the majority of the actinomycetes isolated from both the paunch and colon regions of the hindgut of *M. viator*, had general rather than selective antibacterial activities.

The top seventeen antibiotic-producing isolates are listed in Table 2.2, in order of decreasing activity against *M. aurum*, based on the areas of inhibition produced on the three different test media. Media 7H9 and CZ are defined media that have glucose and sucrose as carbon sources, respectively, whereas YEME is a rich medium containing glucose. Growth on CZ also requires that the bacteria use nitrate as sole nitrogen source.

Table 2.2: Isolates with the greatest anti-*M. aurum* activity, showing the areas of inhibition produced on three different test media

No.	Bacterium code	Area of inhibition (mm^2)			Order of highest activity
		7H9	CZ	YEME	
14	MIIC7P1	n/a	n/a	6229	1
36	MIIX65P4	3798	4508	493	2
33	MIIX7P41	2981	4044	75	3
6	MIIC75C2	3244	1144	1549	4
35	MIIX65P2	3167	2614	342	5
26	MIIX6C2	3063	251	398	6
5	MIIC75C1	2963	506	1208	7
39	MIIC75C21	n/a	n/a	2673	8
9	MIIC7C4	n/a	n/a	1809	9
30	MIIX7P2	1797	181	n/a	10
17	MIIX75C1	n/a	n/a	1508	11
23	MIIX65C3	n/a	n/a	1461	12
4	M375P3	n/a	n/a	1320	13
8	MIIC7C1	234	138	1320	14
22	MIIX65C2	n/a	n/a	1184	15
24	MIIX65C4	n/a	n/a	1131	16
38	MIIX6P1	1114	149	226	17

*n/a = no activity

The ability of the isolates to inhibit the growth of *M. aurum* varied widely according to the test medium. For instance, the greatest activity was produced by isolate 14 on YEME, whereas it showed no activity on the other two media. Seven other isolates also showed this pattern, with YEME being the medium on which the majority of the isolates produced activity against *M. aurum*. The two isolates with the next strongest activities produced this activity when grown on medium CZ, whereas six isolates produced their greatest activity on medium 7H9. Isolate 30 was the only one not able to show inhibition against *M. aurum* when grown on YEME, compared with all of the other CZ and 7H9 top producers, who were able to show activity on all three test media.

In almost all cases, there appeared to be a particular medium on which the isolates produced their greatest activity, but the relevance of the isolates' original isolation medium, pH or even gut region seems to have no importance as the whole spread of media, pH values and gut regions were represented among the top antibiotic producers.

Actinomycetes are well-known for their prolific antibiotic producing abilities, as discussed in Chapter 1 and, although there is currently no evidence for this, it could be that this portion of the termite gut community contributes towards the host's immune system by producing antibiotics in the gut. Examples of insects that make use of the antibiotics produced by actinomycetes have been discussed and include the *Pseudonocardia* used by the Attine ants (Cafaro & Currie, 2005), as well as the *Streptomyces* species used by the European beewolf wasps (Kaltenpoth *et al.*, 2005). There would therefore be an obvious benefit to the termite in cultivating these actinomycetes within its gut.

2.4.2.2 CMC and xylan degradation

The details of the results obtained for both the Originals and the Duplicates are shown in Appendix B and C, respectively.

Of the total of 38 Original isolates tested, 31 were found to degrade only CMC. None of the isolates, including both the Originals and Duplicates, showed degradation of xylan alone. However, three isolates (5, 6 and 26) were able to degrade both CMC and xylan. Isolates 12, 15 and 20 were unable to degrade either substrate, demonstrating a lack of antibacterial activity and an inability to utilise complex carbon sources for both *Nocardia* isolates (12 and 20). Isolates 18, 25 and 32 (which are included in the total CMC count) all showed weak CMC activity, whereas isolate 13 was unable to grow on either medium, despite it having been isolated on medium MIIC. This same lack of growth was also seen for some of the actinomycete isolates cultured by Pasti & Belli (1985), where they would all grow on rich media, but some could not be grown on minimal media supplemented with carbon or nitrogen sources. Further investigation proved that these strains had particular nutritional requirements for one or more growth factors.

The results presented in Appendix B and C therefore provide evidence of termite hindgut streptomycetes with the ability to hydrolyse CMC. In their investigation and identification of the cellulolytic actinomycete isolates from the hindgut of *Z. augusticollis*, Wenzel *et al.* (2002) also made use of Congo Red staining to visualise CMC degradation and found completely different CMC-degrading genera, including *Cellulomonas*, *Oerskovia*,

Microbacterium and *Kocuria*. In comparison, Pasti & Belli (1985) did find *Streptomyces* species, as well as strains of the genus *Micromonospora*, with CMC-degrading activity.

For the Duplicates, 26 out of the 38 isolates tested showed CMC-only degrading activity. Of these, isolates 48, 52 and 72 were the only ones that showed weak CMC activity, and isolate 47 the only Duplicate isolate that was unable to degrade either CMC or xylan. A total of eleven actinomycetes from this group were able to degrade both CMC and xylan (isolates 40, 41, 42, 44, 50, 60, 62, 66, 71, 74 and 76). These particular isolates are noteworthy because all of them were considered to be Duplicates (during the initial separation) of Original isolate number 5 (MIIC75C1) (Appendix A).

Another interesting fact is that all of the 8 isolates (except for isolate 18) that were initially identified as having the ability the degrade xylan, based on their isolation from MIIX plates (with xylan as sole carbon source) did not degrade xylan in the degradation test. This could be explained by different incubation times and media conditions between the isolation and degradation experiments.

Information about xylan-decomposing microbes is scarce, and few of these bacteria have actually been cultured from the termite gut. Schäfer *et al*. (1996), in an attempt to quantify the number of isolates with xylan-degrading activity from the hindgut of seven higher and lower termites, were only able to culture six strains obtained from xylan-containing isolation plates and two strains from plates containing CMC. This therefore agrees with the results provided here, which suggest that only a very small proportion of the culturable actinomycetes have the ability to degrade xylan. Of the actinomycetes isolated by Schäfer *et al*. (1996), all belonged to the *Streptomyces* genus.

In summary, of the 76 isolates that were tested for their ability to degrade CMC and xylan, four isolates showed an inability to degrade either substrate, whereas 14 isolates (making up 18.4% of those tested) had the ability to degrade both. An amazing 75% (57 isolates) of the total actinomycete isolates cultured from the hindgut of *M. viator* were found to have the ability to degrade CMC only.

The contribution of gut bacteria to cellulose degradation has always been a matter of debate, especially in the lower termites, where the role of the cellulolytic flagellates is well established. However, there have been many successful attempts at culturing cellulolytic bacteria, mainly using aerobic cultivation conditions (Schäfer *et al*., 1996; Brune, 2006). Numerous actinomycetes have been detected with cellulolytic abilities and, although their

significance in the degradation of lignocellulose in the lower termite gut remains to be established (Pasti & Belli, 1985; Wenzel *et al.*, 2002; Brune, 2006), what is clear from the evidence provided here, is that they do have both cellulolytic and xylanolytic abilities and they therefore have some role to play in this regard.

2.5 Molecular identification

Traditional methods used in the identification of aerobic filamentous actinomycetes are laborious and time-consuming, often requiring specialised tests. Chemotaxonomic criteria are used extensively, but often cannot identify an isolate to a single genus (Cook & Meyers, 2003). PCR-based techniques, in particular amplified rDNA restriction analysis (ARDRA), provide a more rapid and accurate way of identifying bacteria. In the search for novel antimicrobials, investigations of soil actinomycete populations frequently re-isolate known compounds due to the fact that 95% of the filamentous actinomycetes from soil represent streptomycetes, many of which have already been screened for their antibiotic-producing abilities. Isolation of the rarer, non-streptomycete genera therefore increases the chances that novel antibacterial molecules will be found (Cook & Meyers, 2003).

A rapid molecular method was devised by Cook & Meyers (2003) in order to distinguish streptomycetes from other actinomycetes and to identify actinomycetes isolated from environmental samples down to the genus level. This method was used to identify to which genera the Original and Duplicate isolates belong. Fig. 2.6 demonstrates how this method was used to identify a select group of Original isolates, making use of the dichotomous key provided by Cook & Meyers (2003).

Fig. 2.6A shows the results of the first restriction endonuclease digestion using *Mbo*I. All isolates (except for isolate 7) had the largest DNA fragment of the digest match the 'less than 750bp' designation, assigning them all to Group 1 and confining the rest of their identification to Table 1 of the key. Fig. 2.6B shows the result of the *Vsp*I digest, with isolates 7, 19, 28, 29, 30, 32 and 36 all undigested by this enzyme, whereas isolates 12 and 20 produced a banding pattern that according to the key required the next enzyme in the identification process to be *Sca*I. The resulting banding pattern of these isolates (Fig. 2.6C) resulted in their assignment to the *Gordonia*, *Nocardia* or *Skermania* genera. For isolates 19, 28, 29, 30, 32 and 36, the next step involved digestion with the enzyme *Kpn*I (Fig. 2.6C) and then *Sph*I (Fig. 2.6D), where the resulting banding patterns resulted in all of these isolates being assigned to the *Streptomyces* genus.

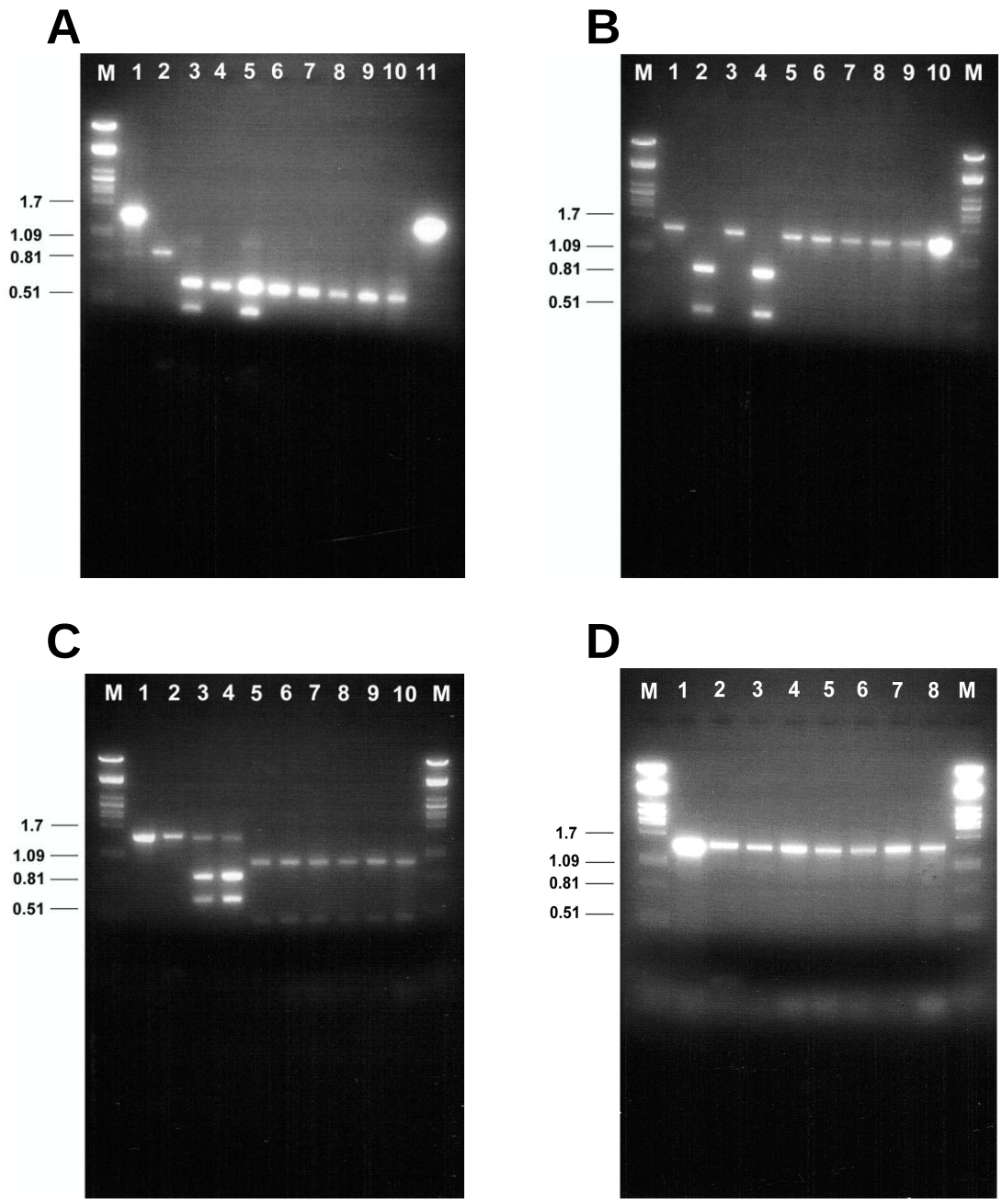


Figure 2.6: Representation of the molecular identification method, showing the enzyme digestion identification patterns obtained after digestion of the 16S rRNA gene amplification products of selected isolates. **A (*Mbo*I digests):** M: λ *Pst*I MW marker; 1 & 11: undigested 16S rRNA gene control; 2: isolate 7; 3: isolate 12; 4: isolate 19; 5: isolate 20; 6: isolate 28; 7: isolate 29; 8: isolate 30; 9: isolate 32; 10: isolate 36. **B (*Vsp*I digests):** M: λ *Pst*I MW marker; 1: isolate 7; 2: isolate 12; 3: isolate 19; 4: isolate 20; 5: isolate 28; 6: isolate 29; 7: isolate 30; 8: isolate 32; 9: isolate 36; 10: undigested 16S rRNA gene control. **C:** M: λ *Pst*I MW marker; 1: undigested 16S rRNA gene control; 2 (*Hind*III digest): isolate 7; 3 & 4 (*Sca*I digests): isolates 12 & 20; 5-10 (*Kpn*I digests): isolates 19, 28, 29, 30, 32 & 36. **D (*Sph*I digests):** M: λ *Pst*I MW marker; 1: undigested 16S rRNA gene control; 2-8: isolates 7, 19, 28, 29, 30, 32, 36. Molecular weight band sizes shown alongside all gels are in kb.

There are limitations to this method, as DNA that is not sufficiently pure, or unknown contaminants in the culture used for DNA extraction, can result in an isolate being identified incorrectly. In some cases, it is easier to sequence the 16S rRNA gene from the extracted DNA, as this immediately identifies any potential irregularities. This was found to be the case for isolate 7, where the molecular identification method was unable to correctly identify to which genus this strain belonged but, after sequencing of the 16S rRNA gene, identification to the *Streptomyces* genus was made.

The majority of the Original isolates were identified to the genus level using this method but, in some cases, as mentioned above, sequencing of the 16S rRNA gene was necessary in order to determine the genus to which some isolates belonged. This was especially true for isolates 12 (MIIC65C7) and 20 (MIIX7C4), because the molecular identification method did not have enough resolution for these particular actinomycetes. Sequencing results placed them in the *Nocardia* genus. The remaining 37 Original isolates were all identified as members of the *Streptomyces* genus. For the Duplicates, the molecular method identified all 41 isolates as streptomycetes. Therefore, of a total of 80 actinomycetes isolated from the paunch and colon regions of the hindgut of *M. viator*, 78 were found to be members of the *Streptomyces* genus, while only two isolates were identified as non-streptomycetes, both belonging to the *Nocardia* genus.

This is very interesting, as these particular genera are frequently represented in investigations into the actinomycetes found in various soil samples. This is not to say that there are no other actinomycete genera to be found in the *M. viator* hindgut, it just indicates that these genera are the ones that are best able to proliferate under the imposed isolation conditions. The presence of these particular genera has been found in other termite species. The *Streptomyces* genus is represented in both culture-independent and culture-based analyses from various different higher and lower termite species (Fall *et al.*, 2007; Pasti & Belli, 1985; Pasti *et al.*, 1990; Bignell *et al.*, 1991; Watanabe *et al.*, 2003; Kurtböke & French, 2007) and evidence of *Nocardia* has been found in the hindgut of the soil-feeding termite *C. niokoloensis* (Fall *et al.*, 2007).

Based on the results presented in this chapter, both *Nocardia* strains, as well as a selection of the *Streptomyces* isolates were identified as being worthy of characterising fully. The details of why they were chosen and their descriptions are presented in the next chapter.

2.6 Morphological examination - SEM and TEM

2.6.1 Actinomycetes in the hindgut of *M. viator*

The presence of actinomycetes in the gut of termites has been described previously. Bignell *et al.* (1980) described filaments from the midgut of *C. severus* (a higher termite), ranging from 0.15 – 0.8µm in diameter, that were not obviously segmented and were infrequently branched. On the basis of size, morphology and structure, they identified these filaments as actinomycetes. These results agree with those shown in Fig. 2.7, as the diameter of the filaments shown in Fig. 2.7B is approximately 200nm in diameter. These same authors in 1979 also found evidence of filaments attached to the cuticular spines associated with the P4b region of the hindgut of *P. aburiensis* and *C. severus*. Here, they measured the diameter of the filaments to be between 0.2 and 2.0µm. Under SEM, sporing structures appeared to be absent, but when small numbers of these filament-coated spines were inoculated onto starch-casein and chitin agar plates, a variety of actinomycetes were isolated. Later work by Bignell *et al.* (1991) in the same two termites again showed evidence of filamentous prokaryotes within the intestinal symbiont population. However, these filaments ranged between 0.2 and 0.5µm in diameter, were unbranched and lacked obvious sporing structures. Based on these morphological criteria alone, they suggested that an affiliation with the *Streptomyces* genus be ruled out, and queried whether the term 'actinomycete' was appropriate. By comparison, Fig. 2.8 shows clear evidence of branching (a lack of which can be attributed to the young growth stage of the actinomycetes found in the hindgut of *M. viator*), as well as evidence of spiral formation – a phenomenon known to be the precursor for spore formation in many *Streptomyces* species.

A

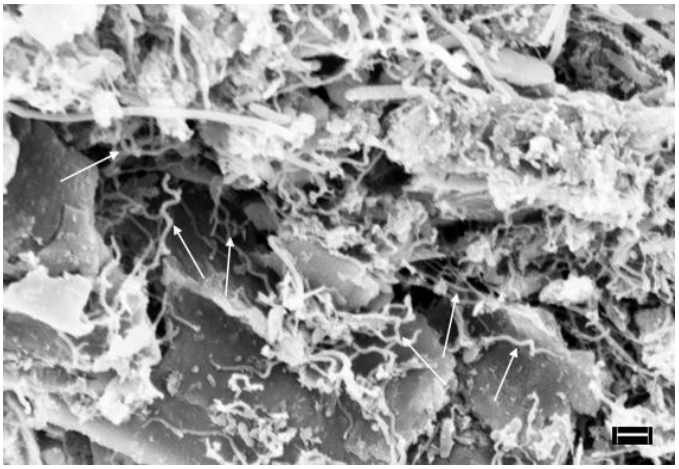


Figure 2.7: Scanning electron micrographs from the colon region of the hindgut of a soldier *M. viator* termite, showing the densely packed interior (A), and providing clear evidence of the presence of filamentous actinomycetes (indicated by arrows) (B). Bars = 1µm.

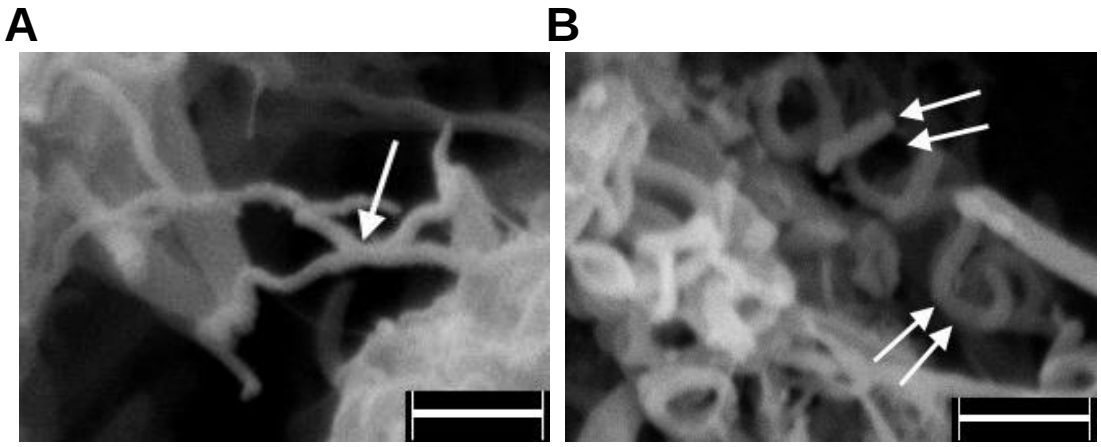


Figure 2.8: The majority of the scanning electron micrographs (from both the colon (B) and paunch (A) regions) show the actinomycetes to be fairly immature – evidenced by a general lack of branching and sporulation. The scanning electron micrographs shown here from a soldier *M. viator* termite, provide evidence that these filaments are actinomycetes, as there is evidence of branching (indicated by a single arrow in A) and there is also evidence of spiral formation (indicated by double arrows in B). Bars = 1µm.

2.6.2 Actinomycete or 'trichome'?

There is little work describing the presence of actinomycetes inside the hindgut of lower termites. Similar work to that being presented here was done by Breznak & Pankratz (1977), on the hindgut of two members of the Rhinotermitidae termite family – *R. flavipes* and *C. formosanus*. Unfortunately, there is no reference to actinomycetes, as all filaments are described as being bacterial 'trichomes', despite evidence in some of their scanning electron micrographs that suggests otherwise. These endospore-forming trichomes - a term used by

Breznak & Pankratz (1977) to describe chains of rods - (measuring between 1.4 and 2.1µm in diameter) all appeared to be covered by a layer of attached rod-shaped epibionts (Breznak & Pankratz, 1977). These same spore-forming filaments have apparently also been isolated from a cockroach, a millipede as well as two amphibians (To *et al.*, 1980).

In their investigation of the hindgut of *P. occidentis* (a member of the lower termite family, Kalotermitidae), To *et al.* (1980) found evidence of two distinguishable spore-formers inside the paunch. Both were filamentous and non-motile and corresponded to descriptions of the '*Arthromitus*' species described by Leidy in 1881. The larger filament was shown to be an endospore-former, with a diameter between 1.3 and 1.4µm, and a variable length that reached up to several hundred microns. The thinner filament had a diameter measuring approximately 0.8 - 1.0µm, and a length between 15 to several hundred microns. These filaments also had attached rod-shaped bacteria, their attachment mediated by a fibrous material thought to be produced by the epibionts.

It seems likely that the larger filament described by To *et al.* (1980) and the trichome-like filaments described by Breznak & Pankratz (1977) are one and the same. Their descriptions also match the filaments shown in Fig. 2.9, where the diameter is shown to be approximately 1.2 - 1.3µm. However, although the epibionts in Breznak & Pankratz (1977) were shown to align with their long axis parallel to the long axis of the trichome, the epibionts in Fig. 2.9 appear to encircle the filament, running cross-wise, as also appears to be the case for the epibionts shown in Fig. 2.10.

Various attempts have been made to identify these filamentous bacterial morphotypes associated with the hindgut wall, ever since the first description of '*Arthromitus*' by Leidy (1849). It has been proposed that they represent a different life stage of aerobic rod-shaped bacteria closely related to *Bacillus cereus*, however these conclusion were made after the isolation of these bacteria from the boiled intestines of ten species of soil arthropods containing '*Arthromitus*'-like filaments. Another suggestion is that they are part of a new lineage of Clostridiales (based on 16S rRNA gene sequence analysis), for which the generic name 'Candidatus Arthromitus' has been proposed for the segmented filamentous bacteria falling into this group. However, these filaments have yet to be cultivated and, as such, there can be no verification that they are in fact related to the filaments described by Leidy (Brune, 2006).

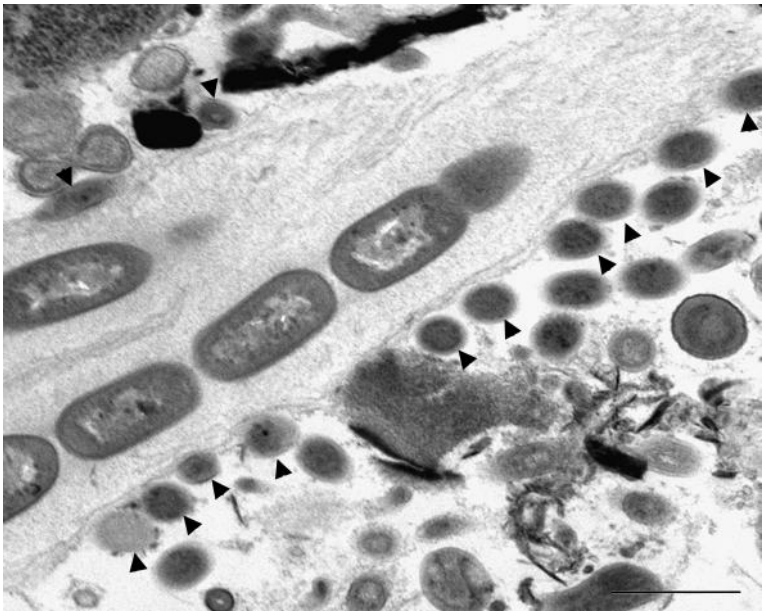


Figure 2.9: Transmission electron micrograph taken from within the paunch of a worker termite, showing a transverse section of 'trichome'-like filaments in the process of spore formation. Epibionts (indicated by arrowheads) are attached along the length of the filament. Bar = 1000nm.

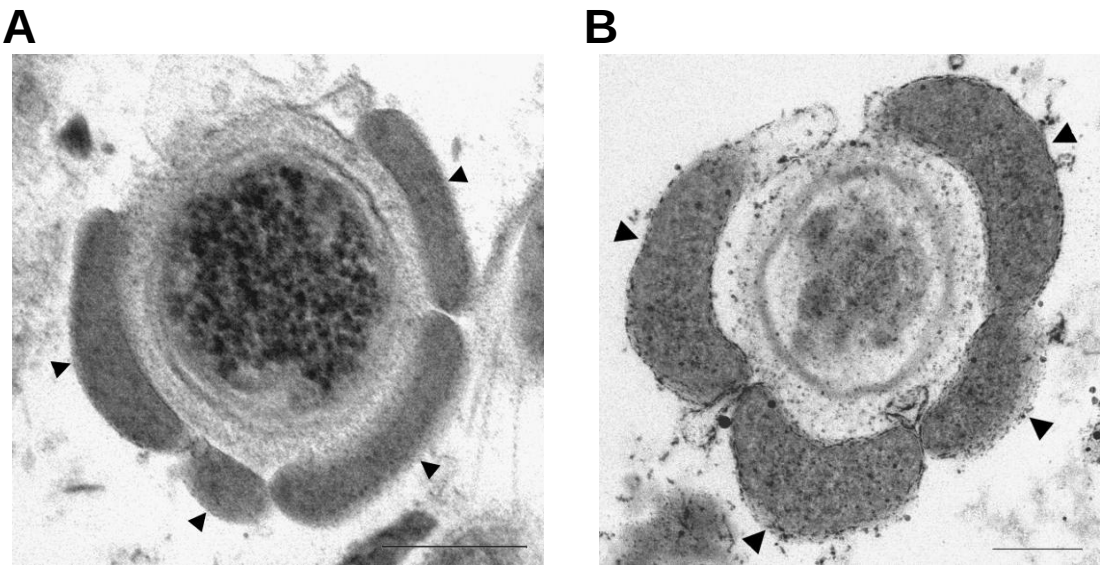


Figure 2.10: Transmission electron micrograph cross-sections from the paunch of a worker (A) and the colon region from a soldier termite (B), showing evidence of 'trichome'-like filaments, with attached epibionts indicated by arrowheads. Bar = 400nm (A) and 200nm (B).

Based on the evidence presented in the scanning electron micrographs from Figures 2.7, 2.8, 2.11 and 2.16 and the results described by Breznak & Pankratz (1977) and To *et al.* (1980), it is possible that if not the larger filament, then possibly the thinner filaments could be examples of actinomycetes. In fact, the scanning electron micrographs showing these 'trichomes', closely resemble the growth of the *Nocardia* isolates from the hindgut of *M.*

viator cultured on agar plates. The filaments in Fig. 2.10 measure between 600 – 800nm in diameter, which closely matches the description made by To *et al.* (1980). The presence of rod-shaped epibionts is also in agreement with their description. This therefore suggests that other lower termites also harbour actinomycetes in their hindgut, providing support for the suggestions that they do have a functional role within the termite hindgut community.

2.6.3 The *M. viator* hindgut community – diversity, structure and function

As shown in Fig. 2.11, the intertwined community of bacteria (including actinomycetes) found associated with the paunch wall appears to be similar for both worker and soldier termites. This result agrees with the observations made by Breznak & Pankratz (1977) that the morphologies of microbiota in the paunch of both workers and soldiers of *R. flavipes* are similar. This similarity can be explained by the previously described process of proctodeal trophallaxis. Evidence of this important behaviour is provided by the fact that termites from the same colonies harbour a nest-specific bacterial microbiota that is slightly, but significantly, different from the gut microbiota of non-nestmates (Minkley *et al.*, 2006).

It has been suggested that actinomycete isolates cultured from termite guts simply represent spores ingested with food or soil (Brune, 2006). In other words, the actinomycetes would enter the termite gut system as spores, and once they reached the paunch (in the case of lower termites), the abundance of food and oxygen would stimulate germination and the onset of mycelial growth. The SEM micrographs shown in this chapter provide evidence that actinomycetes are an abundant component of the gut community within the hindgut of *M. viator* (see Fig. 2.7 and 2.11). However, as pointed out in Fig. 2.8, the actinomycetes found in the hindgut all appear to be immature, as evidenced by a lack of branching and sporulation. This supports the suggestion that they are not a permanent part of the hindgut bacterial community, but are only transient residents.

Another interesting fact is that from all of the SEM figures shown here, as well as the TEM results, there appears to be no evidence of flagellates. In terms of the lower termites, this is unheard of. The scanning electron micrographs shown in Breznak & Pankratz (1977) show clear evidence of these organisms and one TEM micrograph even shows an attachment organelle produced by one of these paunch protozoa. In To *et al.* (1980), multiple examples of flagellates are discussed in detail, illustrated with both scanning and transmission electron micrographs. However, as discussed in section 2.3.1, the paunch region being investigated here is actually the P3b region. Noirot (1995) explained that the P3a and P3b paunch regions are separated by a large muscular ring or sphincter, and that the content of the two regions is very different. Whereas the P3a region was found to contain many symbiotic flagellates as in

all other lower termites, the P3b region (in all specimens investigated) showed a noticeable absence of these large organisms (Noirot, 1995). This explains why no evidence of flagellates was found in the 'paunch' of *M. viator*.

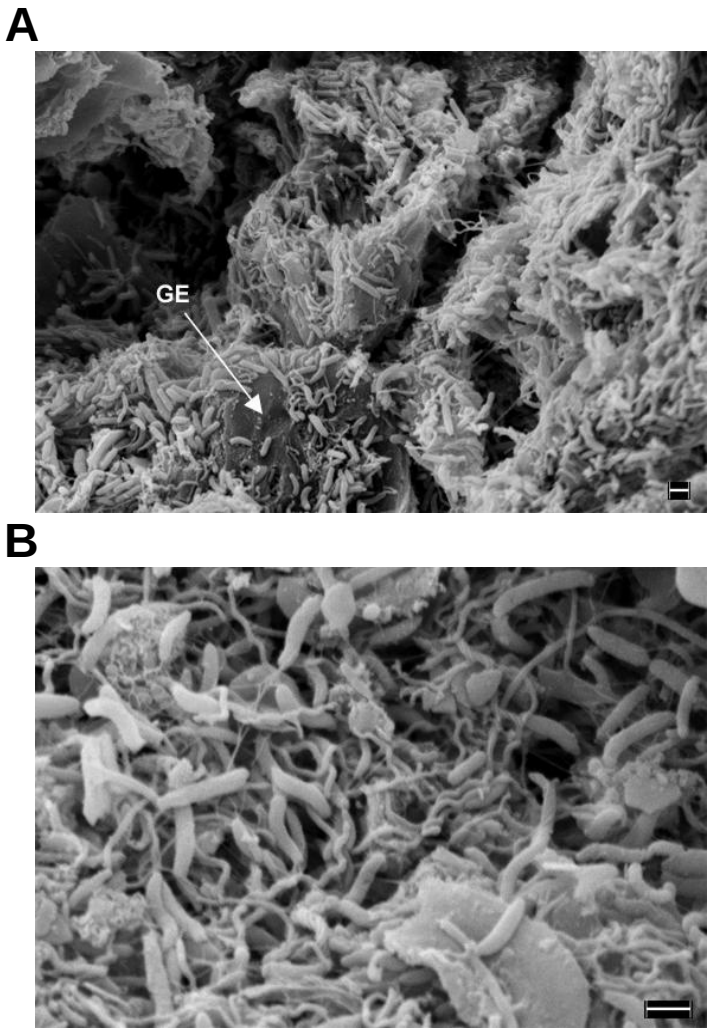


Figure 2.11: (A) Scanning electron micrograph from a soldier, showing the abundance of bacterial rods and filamentous actinomycetes in close association with the wall of the paunch, covering almost every available surface of the gut epithelium (GE). (B) This micrograph from the paunch of a worker termite shows the same densely packed community of filamentous actinomycetes and rod-shaped bacteria, showing no distinguishable differences in the bacterial morphologies of the hindgut community compared with the soldier sample. Bars = 1µm.

The lack of flagellates in the P3b region of the *M. viator* gut can be explained by higher oxygen fluxes into the P3b than those into the P3a. In their investigation of the hindgut of the lower termite *M. darwiniensis*, Berchtold *et al.* (1999) found that most of the P3a was occupied by large flagellates, whereas only low numbers of flagellates were present in the P3b/P4 regions. This situation is similar to that found in *M. viator*, except that for this termite

there is a complete lack of flagellates in the P3b region. Berchtold *et al.* (1999) suggested that this distribution may relate to oxygen concentrations in the respective hindgut regions and also the oxygen tolerance of the protists found there.

The association of the actinomycete population with the gut wall inside the paunch of *M. viator* is obvious from Fig. 2.11. Nakajima *et al.* (2005) have previously shown that the actinomycetes are primarily associated with the gut wall, which is to be expected as they are non-motile bacteria. The oxygenated layer surrounding the paunch wall (described in detail in section 1.3.1.3) also plays an important part in the position of the actinomycete population within the gut, as actinomycetes are mostly aerobic organisms. The close ties with the gut wall are not as important when actinomycetes inhabit the colon, such as those shown in Fig. 2.7. In these regions of the gut, where the gut diameter is much smaller than that of the paunch, the whole gut compartment is often completely aerobic (Brune & Friedrich, 2000).

As mentioned previously, attachment of bacteria to gut epithelial surfaces is a well documented phenomenon and confers a distinct survival advantage on those organisms that are able to take advantage of it. This is particularly relevant within the fast flowing and turbulent gut environment. Here, bacteria have to constantly resist washout due to the passage of food and they also have to contend with the disruptive movements of the community of organisms that inhabit the lumen of the gut (Breznak & Pankratz, 1977).

Fig. 2.12 provides clear evidence of the fibrous holdfast material that seems to be the predominant material used by the wall-associated bacteria found in the paunch and colon of *M. viator* to attach themselves to the hindgut cuticle. A similar material is also used by the bacterial epibionts to attach themselves to the 'trichomes' shown in Figures 2.9 and 2.10. This material, used by both the wall-associated bacteria and the trichome epibionts, has been described previously by Breznak & Pankratz (1977). They also refer to another holdfast material used by certain rod-shaped bacteria, made up of a cluster of thin fibres that emanate from the poles of the bacterium – such as that shown in Fig. 2.19C. The undulate nature of the gut epithelium also provides attached bacteria with multiple attachment points – particularly relevant for the longer rod-shaped bacteria shown in Fig. 2.12C. It must be pointed out that, although the majority of the bacteria that are shown attached to the gut wall appear to be cocci, and may in fact be coccoid bacteria, the greater percentage of these are most probably cross-sections through attached rod-shaped bacteria. Evidence of this is provided by the fact that in all SEM micrographs, almost all of the bacteria found associated with the gut wall are rod-shaped bacteria.

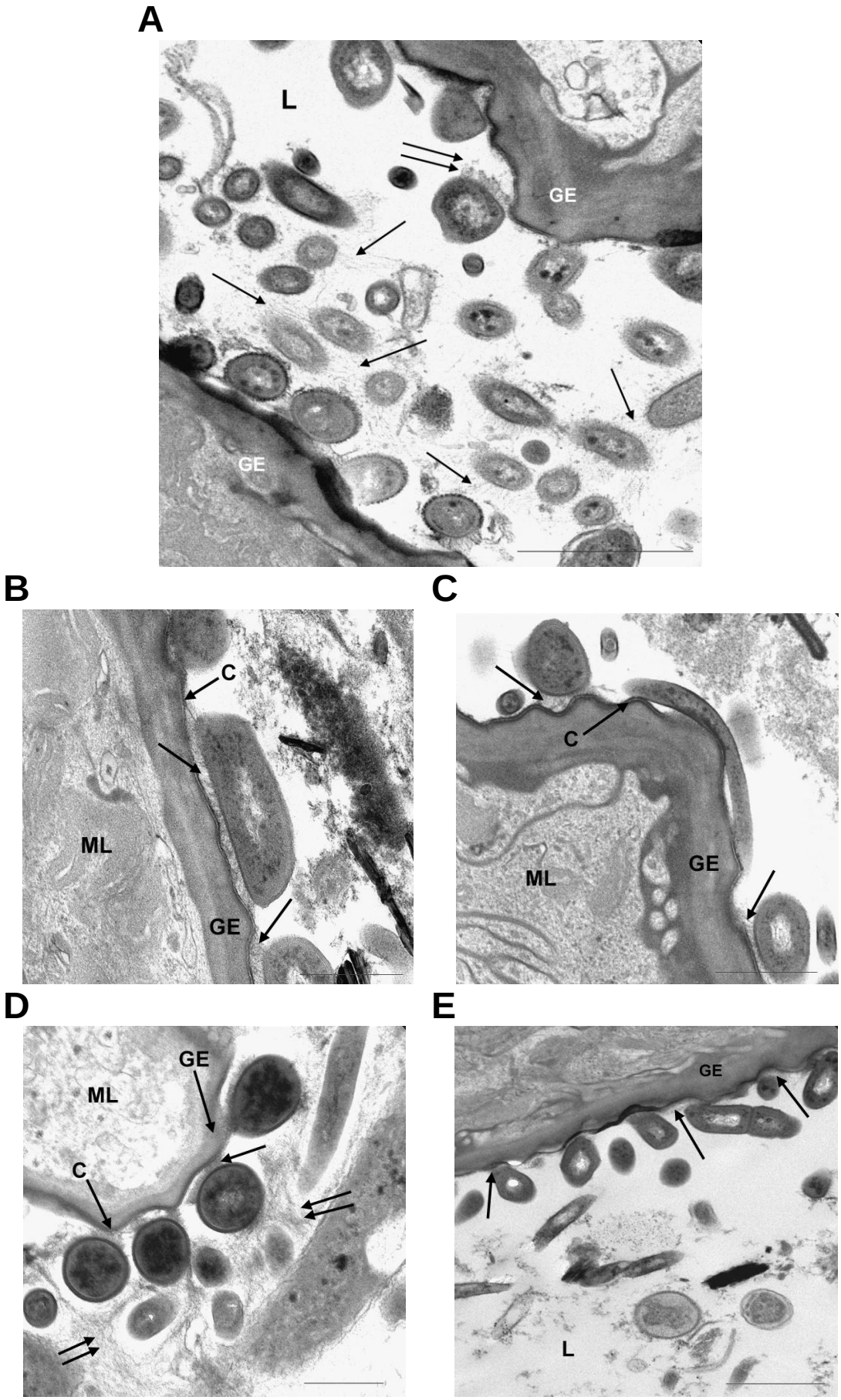

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Figure 2.12: Transmission electron micrographs of some of the bacteria associated with the hindgut epithelium (GE), showing the fibrous holdfast material (indicated by unlabeled single arrows in **B**, **C**, **D**, **E**) used by these symbionts to attach themselves to the cuticle (C) of this tissue layer. Bacteria from the lumen (L) interact and connect with the wall-associated bacteria via fibrils (indicated by single arrows in **A**) or a form of biofilm (shown in **D** by double arrows). The double arrows in **A** point to a bacterium that has been dislodged from the epithelium. Evidence of this association has been found in the paunch (**A**, **B**, **C**) and colon (**D**, **E**) regions of both worker (**A**, **B**, **C**, **D**) and soldier termites (**E**). ML = Muscle layer. Bar = 1000nm (**A** & **E**) and 500nm (**B**, **C**, **D**).

Bacteria within the gut lumen are also able to take advantage of the attachment benefits provided by the wall-associated community. For these bacteria, interaction between themselves, the attached bacteria and other bacteria found within the lumen, is mediated either by fibrous polar appendages or a fuzzy coating (see Fig. 2.12A), or via some form of epicellular matrix or biofilm (shown in Fig. 2.12D). This interaction has some very important advantages, as it allows these bacteria to stay within the oxygen-rich gut periphery, and could potentially allow for an exchange of ions or nutrients – creating an area of synergistic metabolism (especially relevant for the breakdown of recalcitrant plant matter).

The important muscle layers that lie below the gut epithelium are shown in many of the TEM micrographs, but of particular interest are the cellular structures that seem to congregate in the areas of bacterial attachment. Fig. 2.13 shows three of these important structures: the endoplasmic reticulum, Golgi apparatus and mitochondria. One of the many functions of the endoplasmic reticulum is to provide an attachment site for ribosomes, which produce proteins. The primary function of the Golgi apparatus is to process and package macromolecules, such as proteins and lipids, after their synthesis and before they make their way to their destination. They are particularly important in the processing of proteins for secretion. The mitochondria are important in their function of providing the energy necessary for all of these processes (Starr & Taggart, 1998). The presence of all of these structures near the gut epithelium therefore has particular significance, in conjunction with areas of bacterial attachment, as the whole system then functions to provide the digestive and absorptive enzymes necessary for the nutrition of the termite host.

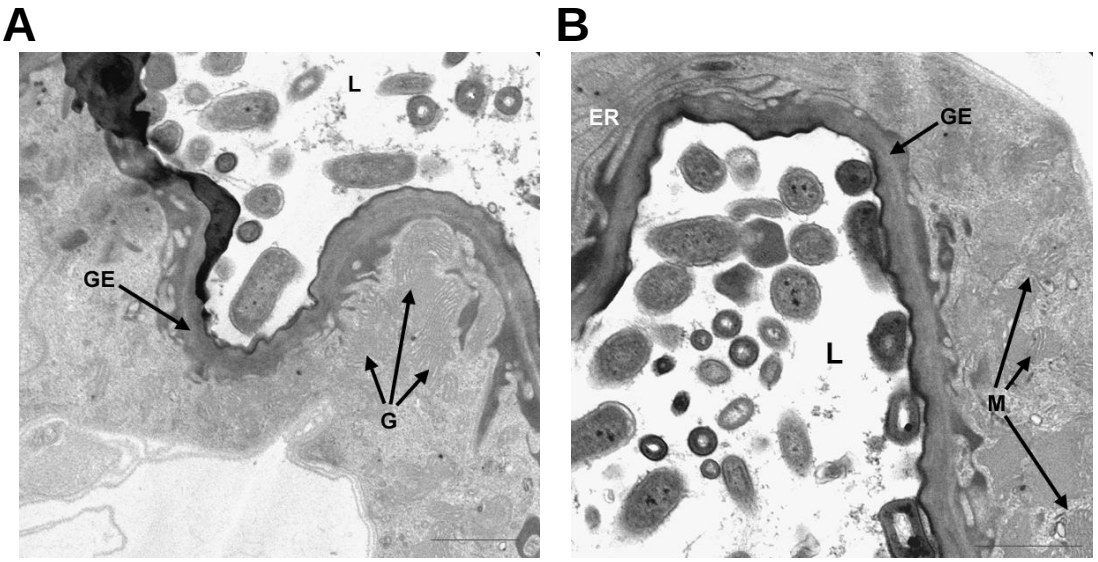

Figure 2.13: Thin sections through the paunch of a worker termite, showing an accumulation of cell structures, such as the endoplasmic reticulum (ER), Golgi apparatus (G) and mitochondria (M), in areas of attachment by gut bacteria – structures known to be associated with the production and secretion of proteins involved in host nutrition. GE = gut epithelium, L = lumen. Bar in A & B = 1000nm.

Besides the diversity that is found associated with the gut wall, there is an even greater degree of diversity found in the lumen of the hindgut. In Fig. 2.14A alone, more than 10 different bacterial morphotypes could be distinguished from the paunch of *M. viator*. Breznak & Pankratz (1977) and To *et al.* (1980) both produced comprehensive studies of the bacterial gut microbiota using transmission electron microscopy, providing detailed descriptions of the morphological diversity found in the hindgut of their respective termite species. In their morphological investigation of the hindgut of *P. occidentis*, To *et al.* (1980) were able to describe almost 30 morphologically distinct types of bacteria. Breznak & Pankratz (1977) were able to give a detailed description of 13 bacterial morphotypes from the paunch regions of *R. flavipes* and *C. formosanus*, seven only being observed in *R. flavipes*, three found only in *C. formosanus* and the remaining three types were common to both termite species. The considerable morphological heterogeneity was found to include bacteria that consisted of straight and slightly curved rods, undulate and endospore-forming cells, pleomorphic coccoid bacteria, and spirochetes. Spirochetes were also observed in the hindgut of *M. viator*, along with the termites' own diverse complement of rod-shaped and coccoid bacteria, as can be seen in Fig. 2.14B. Morphological features generally do not allow the affiliation of bacteria to specific taxa, but in some cases (such as with the spirochetes) the features seen under TEM are sufficient and distinct enough to allow for the proposal of a new species (Brune, 2006).

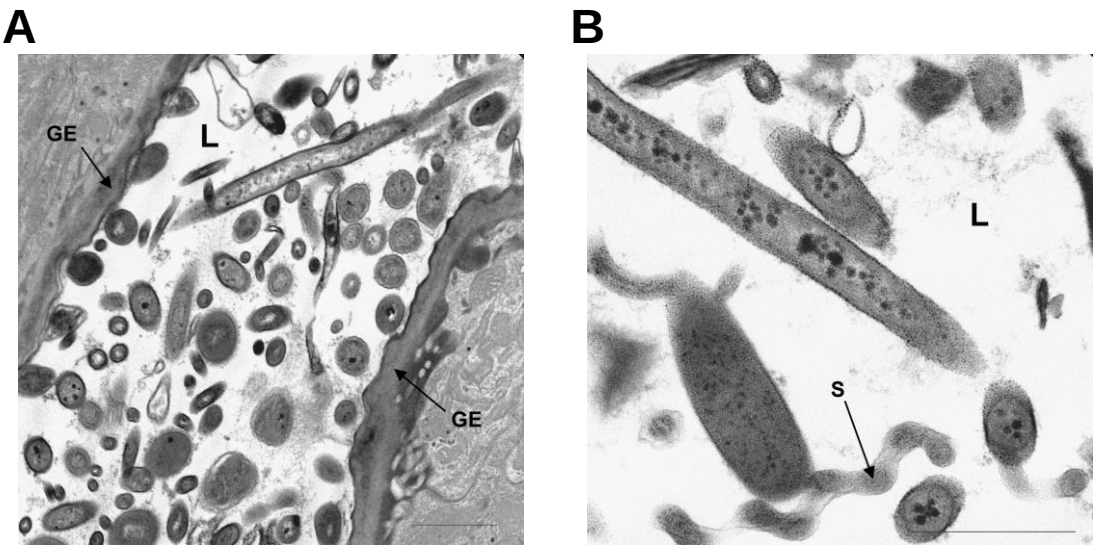

Figure 2.14: Thin sections through the paunch (A) and colon (B), demonstrating the diversity and abundance of different bacterial morphotypes within a single micrograph from both a worker (A) and soldier (B) termite - found attached to the gut epithelium (GE) as well as free in the lumen (L) of the gut. S = spirochete. Bars = 1000nm.

Despite the fact that the bacteria found in the lumen of the P3b paunch region no longer have to contend with the vigorous movements of the flagellates that are found in the P3a region, they still have to have a means of maintaining their position within the paunch (or colon). Fig. 2.15 shows some examples of the various different types and positions (both polar and peritrichous) of the flagella used by these bacteria to locate food within the lumen of the hindgut, and to counteract the movement of the gut contents in order to maintain their position within this nutrient-rich environment. An alternative explanation for the structures shown in Fig. 2.15A and 2.15B may be pili. Pili are short, filamentous proteins that project above the cell wall, and are used either in adherence to surfaces or for attachment to other bacteria as a prelude to conjugation (Starr & Taggart, 1998). However, they are not usually polar, such as those shown in Fig. 2.15A and B.

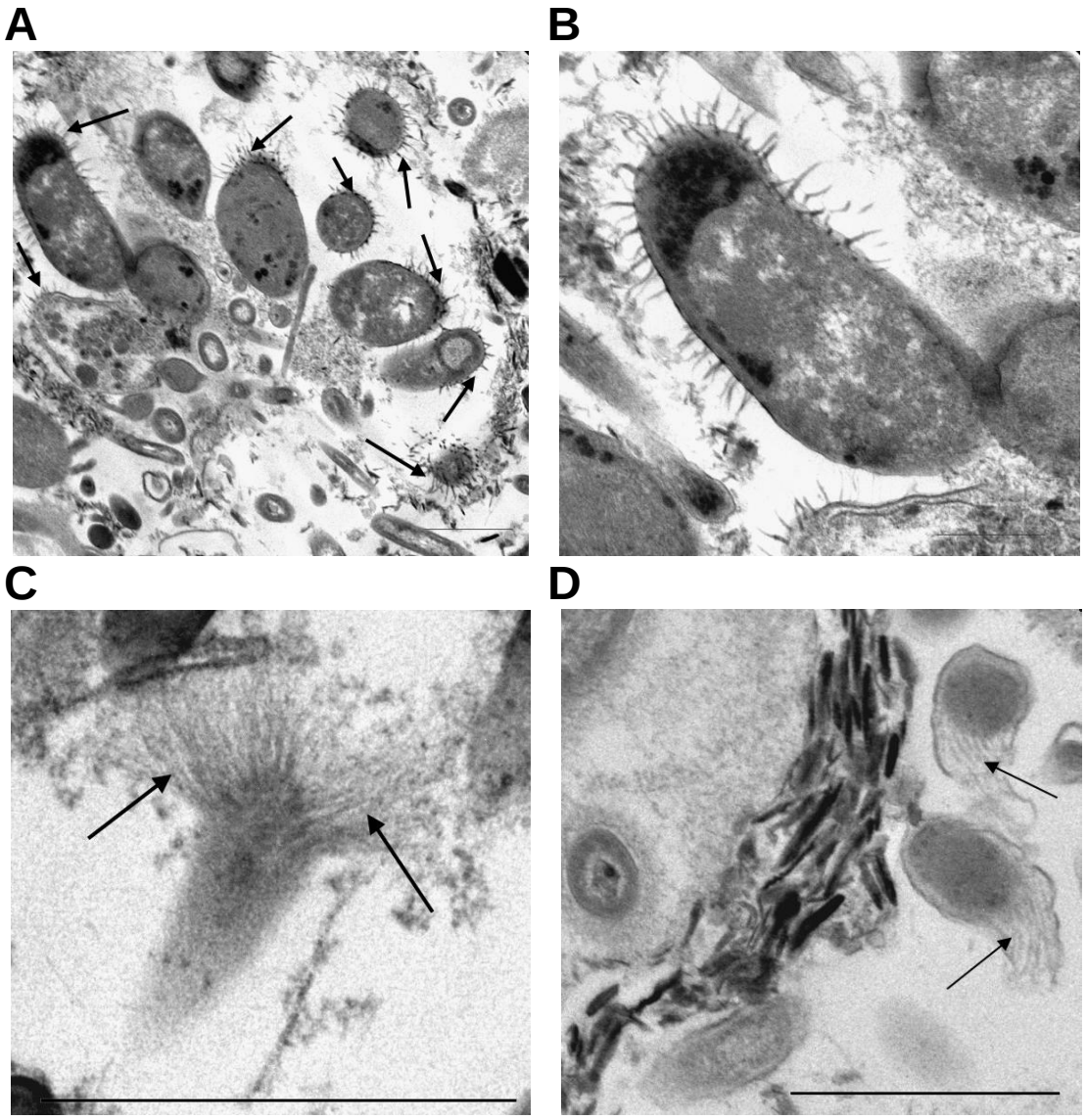

Figure 2.15: Bacteria within the lumen of the colon and the paunch of worker and soldier termites make use of flagella (indicated by arrows) to move around the hindgut in search of food. A, B, C and D all show the variation in the position and structure of these organs of motility. Alternatively, the structures indicated in A and B may represent pili. Bar A, C and D = 1000nm, B = 500nm.

2.6.4 Termite structural adaptations

Despite the fact that the microbiota of workers and soldiers is morphologically so similar, there are still some surprises when it comes to the hindgut of *M. viator*, such as the interesting non-actinomycete filamentous bacteria shown in Fig. 2.16B. Fig. 2.16A also shows evidence of some unusual cup-shaped structures. These structures resemble the cup-shaped depressions shown in Breznak & Pankratz (1977), which they discussed in detail. In both *R. flavipes* and *C. formosanus*, the cup-shaped depressions are distributed evenly across the surface of the gut epithelium, and serve as attachment sites for various wall-associated bacteria. These 'cupulae'

have also been described from other species within the Rhinotermitidae family (Noirot, 1995). However, these structures in *M. viator* are not associated with the gut epithelium and, as can be seen in Fig. 2.16A, do not appear to be specific attachment sites. The other SEM figures, as well as transmission electron micrographs show no evidence of the 'cupulae' anywhere on the gut epithelium of *M. viator*.

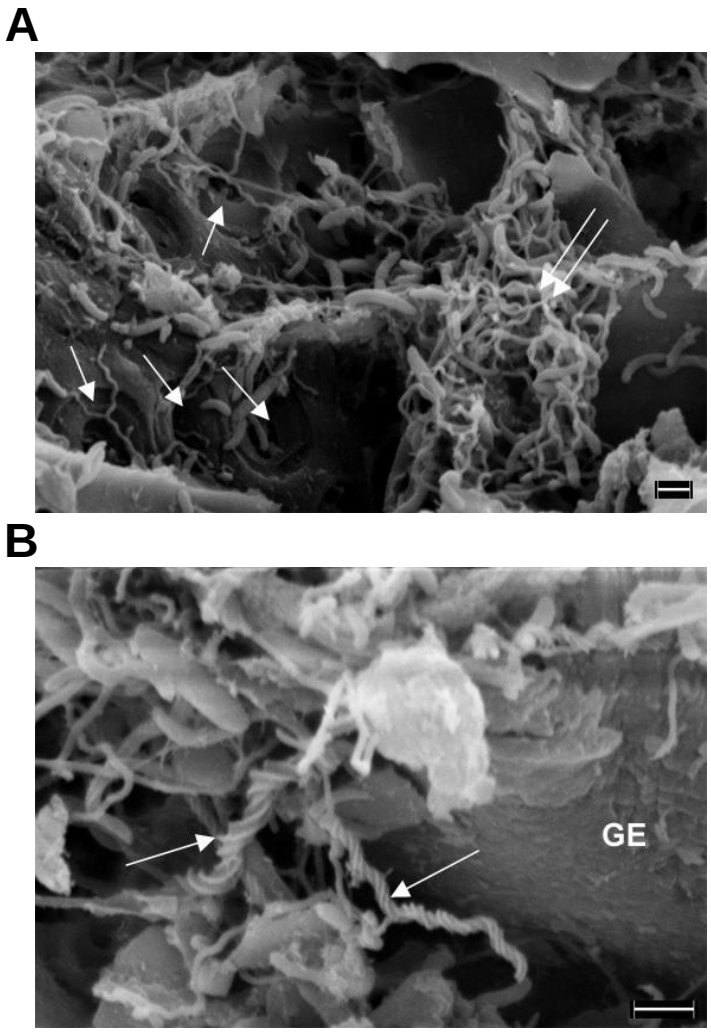

Figure 2.16: There is a close association between the actinomycetes and the non-actinomycetes within the paunch, the two populations intertwined at the gut wall - indicated by a double arrow in A. The scanning electron micrographs shown here also provide evidence of some unusual structures (indicated by single arrows in A), as well as some interesting non-actinomycete filamentous forms (shown by single arrows in B). GE = gut epithelium. Bars = 1µm.

M. viator also shows no evidence of cuticular spines (such as those seen in higher termite hindguts), which is to be expected from a lower termite, and is supported by observations made by Noirot (1995) that no lower termites showed signs of cuticular projections (spines, setae or scales). These facts suggest that spines and cupulae are specific attachment

modifications that have evolved in higher termites and some lower termites, respectively. There may also be a link to feeding habits. The cuticular spines serve as attachment sites for actinomycetes in the hindgut of soil-feeding higher termites, thereby filtering the soil that passes through the gut (Bignell *et al.*, 1979). The cupulae, on the other hand, may perform a similar function in wood-feeding lower termites by allowing for the attachment of important bacteria that may be involved in the breakdown of the ingested plant matter, preventing them from being removed from the gut during peristalsis.

2.6.5 An active, functional system – metabolism and cell division

It has already been established that protozoa have a vital role to play in the paunch of all lower termites, due to their cellulose-degrading abilities. This then begs the question as to why *M. viator* would need to have a second paunch region (P3b) that is devoid of flagellates. As discussed previously, only the lower termites harbour these organisms, as gut flagellates have been lost over evolutionary time in the higher termites from the Termitidae family (Brune & Friedrich, 2000; Brune, 2006). As a result, it was initially assumed that the prokaryotic symbionts took over this function in the hindguts of the higher termites. However, until fairly recently, there was still no conclusive evidence to suggest that bacteria play a major role in cellulose degradation, nor was there any evidence of large numbers of cellulolytic bacteria within these termite gut systems. This could possibly have been explained by evidence that has been accumulated about endogenous cellulases produced by the salivary glands and midgut epithelium of lower and higher termites, respectively. It has even been suggested that termites may be able to secrete a full complement of the enzymes necessary for the digestion of plant structural polysaccharides (Brune & Friedrich, 2000; Brune, 2006). Tokuda & Watanabe (2007) have since shown evidence for a major role of cellulolytic bacteria in higher termites. It appears from Fig. 2.17 that there is a population of bacteria inside the P3b paunch region of *M. viator* that is intimately involved in the breakdown of plant matter. Also shown here is a proportion of ingested plant material that has made its way past the host enzymes and the P3a region (containing all of the cellulose- and hemicellulose-degrading flagellates), without undergoing much degradation. The P3b paunch region may therefore be more like the hindgut of higher termites, colonised by prokaryotes that are able to degrade any plant material that reaches the posterior regions of the *M. viator* gut system. It also appears that this process involves the synergistic action of multiple bacteria from the hindgut community.

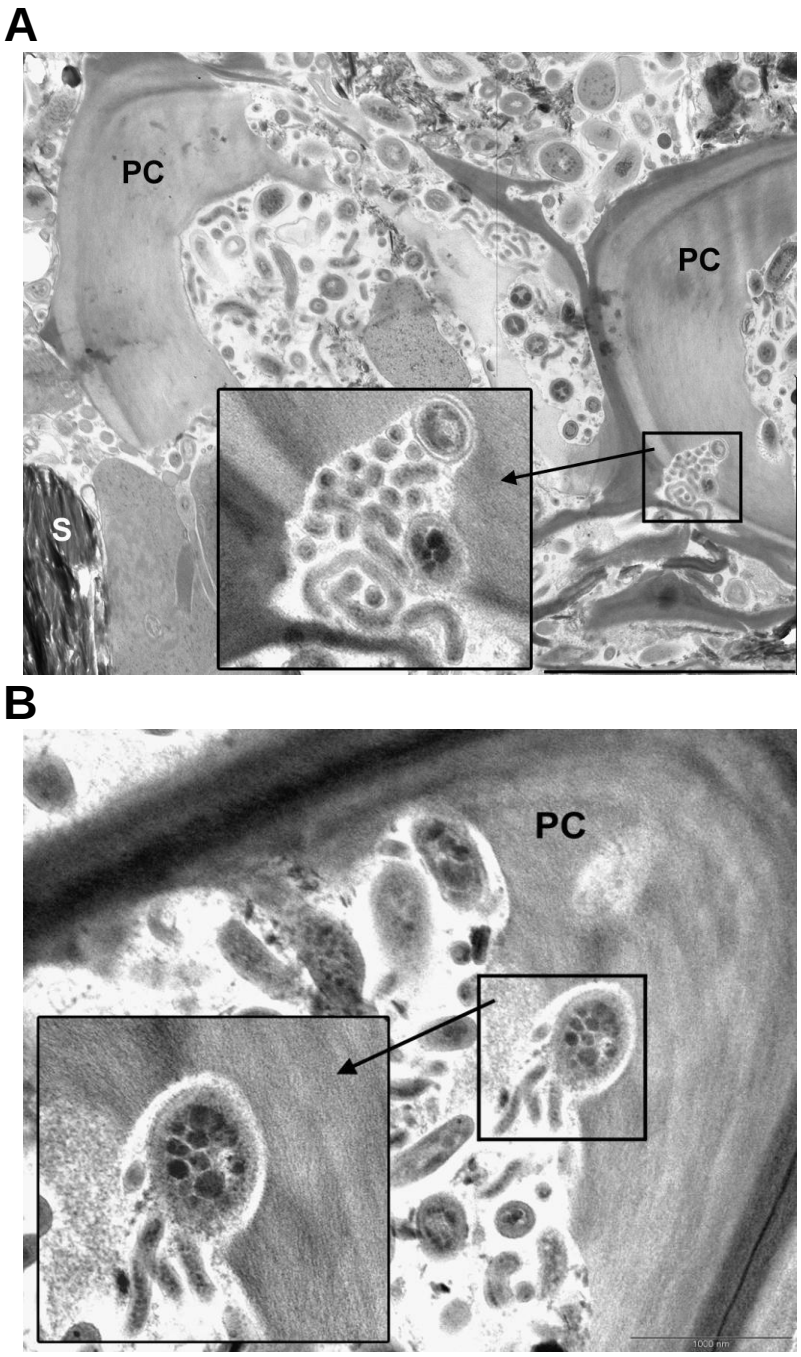

Figure 2.17: Transmission electron micrographs of the apparent synergistic breakdown of plant cells (PC) inside the paunch of a worker *M. viator* termite by its gut bacteria. S = silica crystals. Bar = 5000nm (A) and 1000nm (B).

It is clear that the bacteria found in the hindgut of *M. viator* are actively involved in the metabolic processes occurring there. Often bacteria are found to have storage bodies or cytoplasmic inclusions that contain either electron-translucent (as seen in Fig. 2.18A) or electron-dense (shown in Fig. 2.18B) materials. The electron-dense material may represent

stored glycogen (Stingl *et al.*, 2005), or possibly polyphosphate. A large number of bacteria with the 'electron translucent granules' were found to occur in the hindgut of *R. flavipes*, and the granules were likened to poly-β-hydroxybutyrate, which is known to be a carbon and energy reserve (Breznak & Pankratz, 1977; Wertz & Breznak, 2007). It is also possible that these storage bodies may contain plant cell breakdown products or substrates undergoing further digestion inside the bacterial cells.

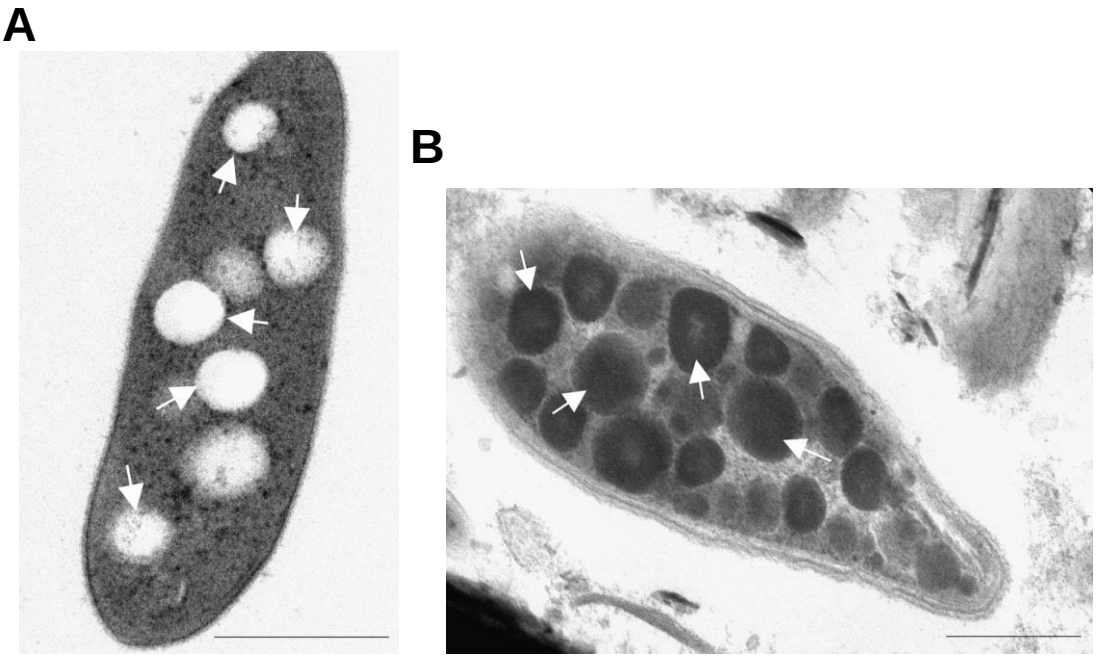

Figure 2.18: Transmission electron micrographs of two paunch bacteria, showing evidence of the electron translucent (A) and electron dense (B) storage bodies found inside some of the hindgut symbionts (indicated by arrows). Bars = 500nm.

The micrographs shown in Fig. 2.19 demonstrate that there is an actively reproducing bacterial community colonising both the colon and paunch regions of the *M. viator* hindgut. This is true not only for the community found inside the lumen of the gut, but also for those attached to the gut wall (as shown in Fig. 2.19B and 2.19C). This therefore suggests that at least some of the termite gut bacteria are symbionts that have co-evolved with their termite host (as has been found in other termite species - Hongoh *et al.*, 2005) and maintain their presence in the termite line through their own efforts of cell division and the efforts of the host through proctodeal trophallaxis.

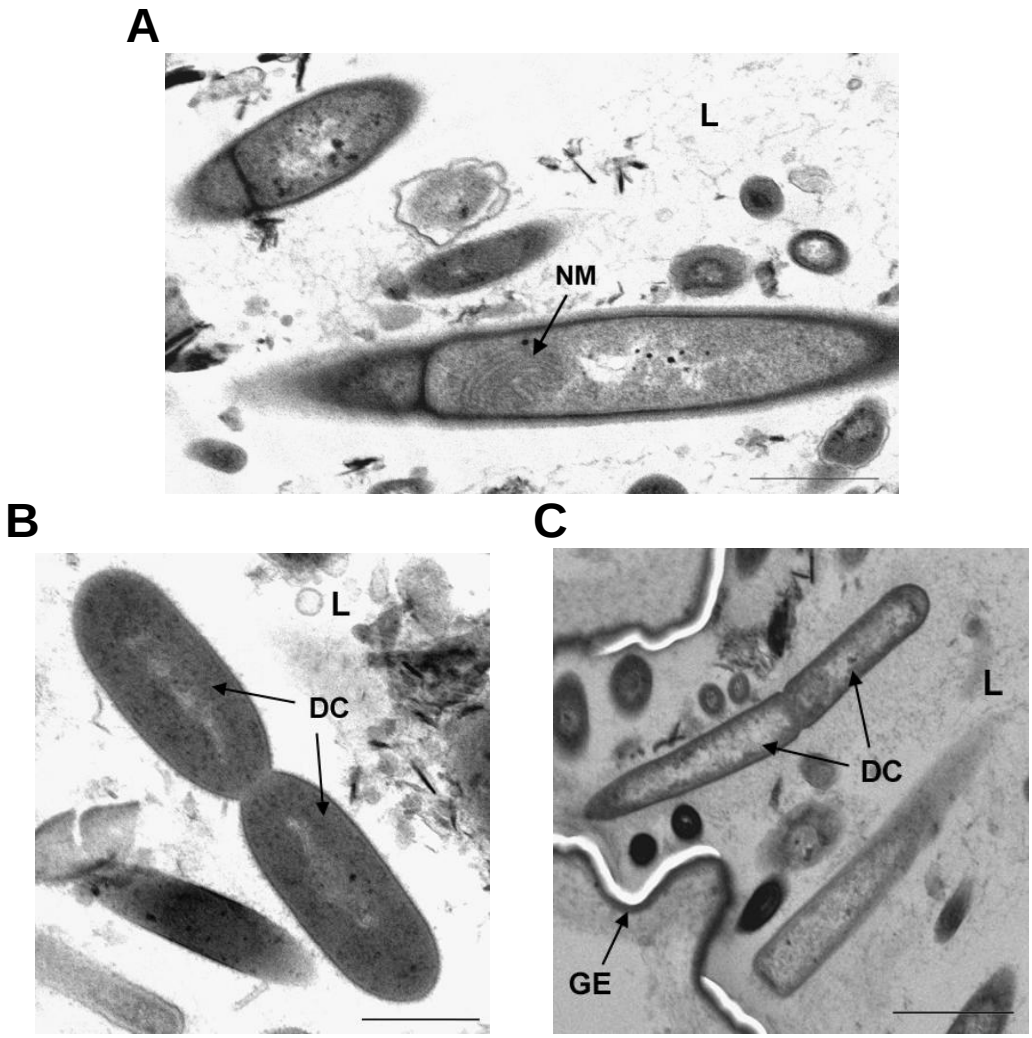

Figure 2.19: The bacterial population found inside the paunch (B and C) and colon (A) of the *M. viator* termite is able to continue colonisation of this environment through the active process of cell division, ensuring its progeny survive to colonise subsequent generations of termites. A, B and C are transmission electron micrographs showing examples of these cells during cell division. DC = daughter cells, GE = gut epithelium, L = lumen, NM = nuclear material. Bar A&C = 1000nm, B = 500nm.

Where there is an actively dividing population of bacteria, there will always be an actively infectious population of phages. A possible example of a paunch bacterium from *M. viator* that seems to have been infected by a phage is shown in Fig. 2.20. To *et al.* (1980) also found evidence of phage infection in their investigation of the bacteria in the hindgut of *P. occidentis*, finding structures resembling those that are shown in Fig. 2.20. It is this phage susceptibility of both the non-actinomycete and the actinomycete bacterial population within the termite gut, which enabled the isolation of rare actinomycetes from the hindgut of *C. lacteus* by Kurtböke & French (2007).

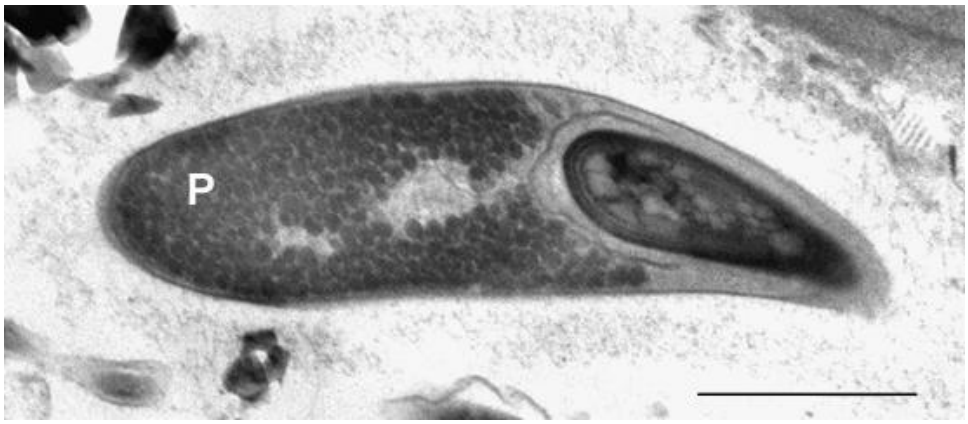

Figure 2.20: Transmission electron micrograph of a bacterial cell from the paunch of a worker termite that has been infected by a phage (P). Bar = 1000nm.

2.6.6 The 'undiscovered' tripartite symbiosis

The hindgut prokaryotes form part of a complex system that is made up of numerous different niches based on the different interactions and associations that occur within the spaces of the hindgut community. Evidence of many of these different niches has been provided by the scanning- and transmission electron micrographs shown in this chapter. These niches that the bacteria are able to occupy mean that they can be found: (1) free-floating in the hindgut lumen; (2) attached to the gut wall; (3) attached to the paunch flagellates; (4) inside the flagellates; (5) attached to other bacteria; and (6) inside other bacteria (To *et al.*, 1980).

The association between termite, flagellate and endosymbiont has been extensively discussed in Chapter 1 in relation to the symbiosis that exists between the three members of this tripartite alliance. However, just as the nature and identity of the endosymbionts associated with their flagellate hosts is almost completely unknown (Stingl *et al.*, 2005), so too is the relationship between hindgut bacteria and their own endosymbionts. Evidence of this relationship is shown in Fig. 2.21, where four large and very different bacteria (ranging in length from 3-5µm) from both the colon and paunch regions of the hindgut of *M. viator* contain a wide variety of intracellular bacteria. Each host bacterium seems to house its own specific and distinct complement of endobionts. However, whereas the bacteria inside the cytoplasm of those bacteria shown in Fig. 2.21A, C and D appear to occupy their own space within the host cell, it seems as if the bacteria in Fig. 2.21B are undergoing digestion. The bacterium in Fig. 2.21B is smaller than the other three, which may indicate that it is not in fact a host of other bacteria, but may be using the ingested bacteria as a food source. The endobionts of the other bacteria are presumably housed within the host's cytoplasm because they are able to provide some metabolic advantage for their host, either by aiding in the

digestion of nutrients or by providing metabolites that their host would not be able to access without their presence.

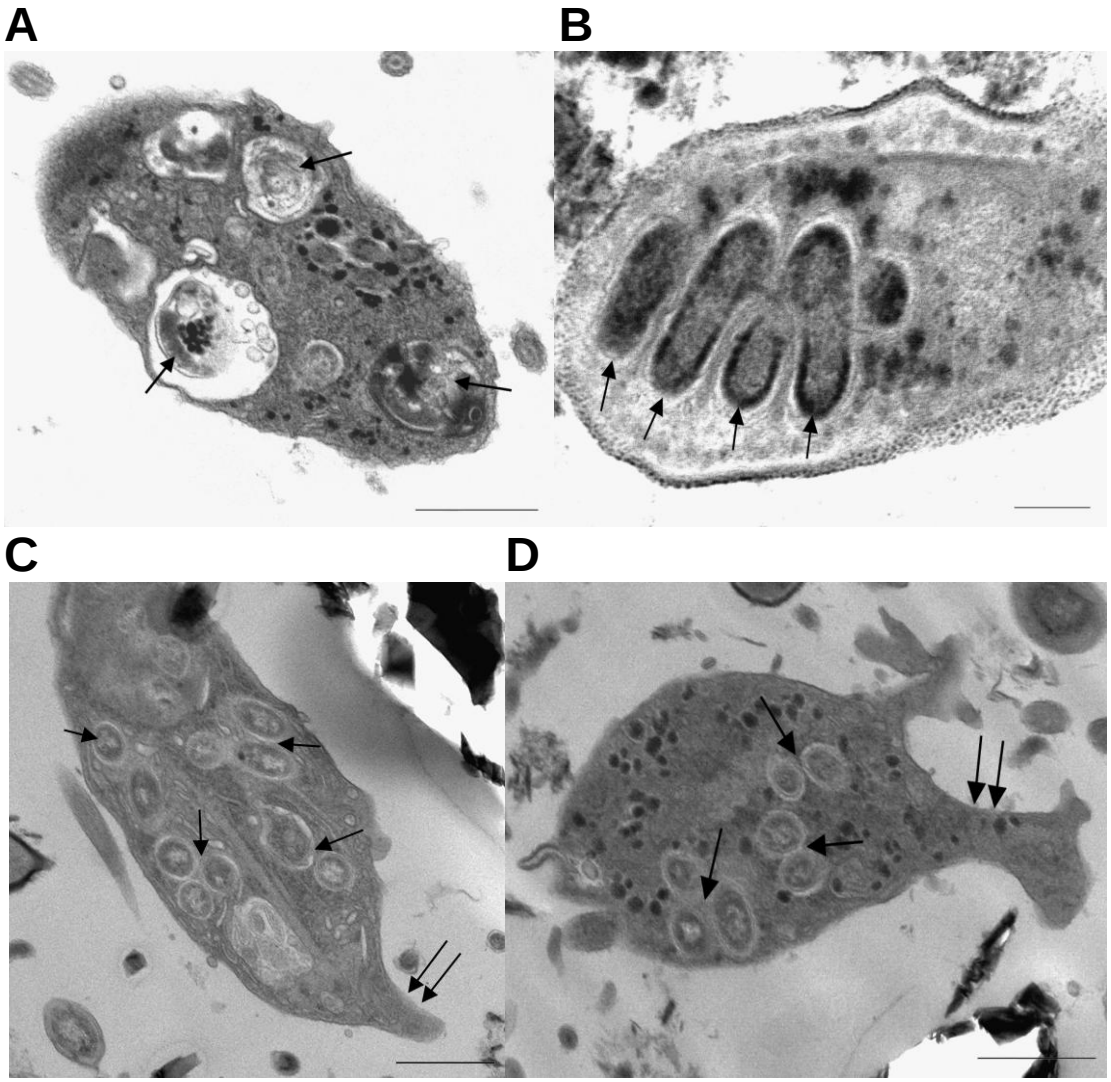

Figure 2.21: Large bacterial cells found in both the paunch (A, B and C) and colon (D) of the worker (C and D) and soldier (A and B) samples seem to have their own endosymbiotic populations. Arrows in the transmission electron micrographs of A, C and D indicate the presence of potential symbionts within the cytoplasm of their bacterial hosts. The rod-shaped cells inside the cytoplasm of the bacterium shown in B, seem to be undergoing digestion by the host cell, and may not actually be symbionts, but are in fact a food source. The cellular protrusions shown in C and D (indicated by double arrows) are reminiscent of the pseudopodia found in Amoeba, and may therefore have a possible role in sensing and engulfing food particles. Bars in A, C & D = 1000nm, B = 200nm.

The cellular protrusions shown in Fig. 2.21C and D, are noted in two of the bacterial hosts because similar 'tube-like elongations' have been shown from transmission electron micrographs of endosymbiotic bacteria found inside the cytoplasm of the flagellate *Trichonympha agilis*, located inside the hindgut of its termite host *R. santonensis* (Stingl *et*

al., 2005). The bacteria in this study were identified as belonging to the recently published *Elusimicrobium* genus (Geissinger *et al.*, 2009), previously known as TG1 bacteria or 'Endomicrobia'. This group represents bacteria that occur exclusively in the hindguts of all lower termites as well as the wood-feeding cockroach (Stingl *et al.*, 2005).

Evidence of this relatively unknown and 'undiscovered' tripartite symbiosis has been reported previously by To *et al.* (1980) in their investigation of the hindgut of *P. occidentis*, but this is the first evidence of this association in the hindgut of *M. viator*.

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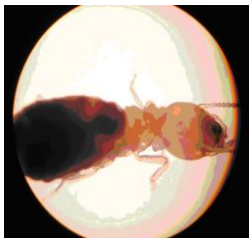
CHAPTER 3



IDENTIFICATION AND CHARACTERISATION OF NOVEL ISOLATES

CHAPTER 3

IDENTIFICATION AND CHARACTERISATION OF NOVEL ISOLATES

CONTENTS

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IDENTIFICATION AND CHARACTERISATION OF NOVEL ISOLATES

3.1 ABSTRACT

The discovery of novel secondary metabolites, such as antibiotics, has decreased over the years due mainly to the re-isolation of known producers, such as those from the genus *Streptomyces*. Therefore, the need to find new actinomycetes has seen scientists turning to under-explored or never before investigated habitats or environments in order to tap into the potentially novel biodiversity found there. This has been the case for this project, where the environment being investigated was the hindgut of the termite, *M. viator*.

Nineteen isolates were investigated as potential new species within their respective genera: two *Nocardia* and 17 *Streptomyces* strains.

For isolates 12 and 20, 16S-rRNA gene BLAST analysis against the GenBank database found them to be 99% similar to *Nocardia nova* ATCC 33726^{T}, with the presence of *meso*-DAP and the sugars galactose and arabinose in their whole cell hydrolysates supporting their placement within this genus. Isolate 2 was found to be 100% similar by 16S-rRNA gene sequence analysis to four *Streptomyces* species with validly published names, but was separated from them based on a number of phenotypic differences. Isolate 7 showed 98% sequence similarity to its three closest phylogenetic relatives. Despite sharing spiral spore chain morphology with these species, numerous physiological differences differentiated them from one another. However, like isolate 2, DNA-DNA hybridisation would be necessary in order to determine whether these isolates represent new species.

All members of Groups 5 and 33 were found to have rugose spore surface ornamentation. The Group 5 strains, encompassing isolates 5, 6, 26 and 39 were all found to be 99% similar by 16S-rRNA gene sequence comparison to *Streptomyces malaysiensis* ATCC BAA-13^{T}. A comparison of their morphological and physiological characteristics allowed for the separation of the isolated strains from their phylogenetic relatives. Isolates 5 and 39 were found to be strains of *S. malaysiensis* by DDH analysis (74 ± 3.68% and 81.1 ±1.41%, respectively). After BLAST analysis against the GenBank database, the Group 33 isolates

were found to be most closely related to six published *Streptomyces* species, also at 99% similarity.

Isolates 13, 16 and 37 were all found to have less than 96% sequence similarity to their top BLAST hits from the GenBank database. The absence of diagnostic sugars and the presence of LL-DAP in their cell walls supported their placement within the *Streptomyces* genus. Phenotypic differences were found between the three isolates and their top hit, *Streptomyces sodiiphilus* YIM 80305^T, indicating that they represent new *Streptomyces* species.

Isolates 18, 19 and 32 shared 98% 16S-rRNA gene sequence similarity with *Streptomyces avermitilis* NCIMB 12804^T, *Streptomyces cinnabarinus* NBRC 13028^T, and *Streptomyces kunmingensis* NBRC 14463^T. Isolate 19 was found to be different from all others due to spiny spore surface ornamentation, whereas isolate 32 was found to fragment when grown in liquid broth cultures. These differences, as well as the five, four and two physiological differences shown between all three isolates and the type strains of *S. avermitilis*, *S. cinnabarinus* and *S. kunmingensis*, respectively, supported the DNA-DNA hybridisation results which confirmed their identity as new species within the genus *Streptomyces*.

Isolate 14 was found to be more than 99% similar by 16S-rRNA gene sequence analysis to its four closest phylogenetic relatives. However, based on comparisons of the chemotaxonomy and physiological properties of isolate 14 against these published *Streptomyces* species, DNA-DNA hybridisation was performed against the two closest relatives, *Streptomyces atratus* NRRL B-16927^T (35.45 ± 6.58% DNA-DNA similarity) and *Streptomyces sanglieri* DSM 41791^T (10.3 ± 0.42% DNA-DNA similarity). All of these results supported the description of isolate 14 as a new species.

A de-replication protocol was devised, involving restriction enzyme digestion with the enzymes *Hpa*II, *Cfo*I and *Hinf*I in order to determine which of the Original isolates (that had not had their 16S-rRNA sequenced), as well as the Duplicates warranted further investigation into their species status. Based on unique digestion patterns, it was determined that 12 Originals and 10 Duplicates should be explored further as potentially novel species.

3.2 INTRODUCTION

The genus *Nocardia* (type genus of the family *Nocardiaceae*) represents a homogeneous group of actinomycetes that are characterised by the formation of extensively branched hyphae that fragment into rod- to coccoid-shaped non-motile elements and the presence of mycolic acids. The history of the recognition of novel *Nocardia* species has been problematic because of limitations in the primarily morphological and biochemical taxonomic tests used in the identification of these bacteria (Zhang *et al.*, 2003; Yassin & Brenner, 2005). However, with the implementation of molecular identification methods, particularly 16S-rRNA gene sequencing, as well as improvements in phenotypic approaches, the taxonomy of isolates that were previously collectively assigned to the *Nocardia asteroides* complex, has been resolved, facilitating the separation and discovery of novel species. The members of this genus form a distinct phyletic line within the actinobacterial 16S-rRNA gene tree and can be distinguished from each other and from related taxa, such as *Rhodococcus* and *Gordonia*, using a combination of chemotaxonomic and morphological features (Kim *et al.*, 2002; Zhang *et al.*, 2003; Yassin & Brenner, 2005).

The *Nocardia* genus has expanded considerably over the years, and currently stands at 90 species with validly published names (Euzéby, 2009). The vast majority of these species were isolated from human clinical samples (often from the sputa of patients with pulmonary infections), with a smaller percentage isolated from animal infections or soil sources. As such, much of the emphasis in the systematics of the *Nocardia* genus has focused on them as causal agents of actinomycetoma and nocardiosis (a potentially life-threatening infection caused by several *Nocardia* species); even though there is ample evidence of their presence in natural environments such as soil (Zhang *et al.*, 2003; Kageyama *et al.*, 2004; Yassin & Brenner, 2005). Despite the fact that the nocardiae are said to be underspeciated in both the clinical and non-clinical/environmental settings, it is important that their species richness in natural habitats also be determined in order to understand their role in these places, as well as for their potential usefulness as a source of bioactive compounds to be assessed (Zhang *et al.*, 2003; Cui *et al.*, 2005).

The genus *Streptomyces* (type genus of the family *Streptomycetaceae*) was proposed by Waksman & Henrici in 1943, for aerobic, spore-forming actinomycetes. Phenotypic characteristics are known to be shared within this genus, and they also constitute a distinct phyletic line in 16S-rRNA gene trees (Al-Tai *et al.*, 1999). Currently, the number of species with validly published names within this genus stands at 568 (Euzéby, 2009). The majority of the streptomycetes that have been discovered to date have originated from soil and, as such, this environment remains an intensively exploited source of novel actinomycetes. Some

species are pathogenic to plants while others have clinical importance (Rintala *et al.*, 2001; Taddei *et al.*, 2006).

Taxonomic relationships within the *Streptomyces* genus have been clarified in the past few decades due to the application of chemotaxonomic, sequence-based and phenotypic methods, and the genus is well defined (Saintpierre *et al.*, 2003; Goodfellow *et al.*, 2007). The *Streptomyces* genus has been said to be overspeciated based on polyphasic taxonomic studies on representatives from described species. However, studies on newly discovered streptomycetes show that the taxon as a whole is in fact rather underspeciated (Goodfellow *et al.*, 2007).

Streptomycetes are fairly difficult to classify below the genus level because of the complexity of the taxon, with various new techniques devised to aid in this process (Sembiring *et al.*, 2000). However, established standards and methods (such as chemotaxonomic characterisation) remain an entrenched part of the description of new members of this important genus. The morphological characteristics and physiological properties described by Shirling & Gottlieb (1966) and Williams *et al.* (1983) are still widely used. It is now standard practice to make use of both genotypic and phenotypic data in the delineation of new members of the *Streptomyces* genus (as is normal in the polyphasic taxonomic characterisation of all new bacteria). The phenotypic methods include chemotaxonomy (the isomer of diaminopimelic acid in the peptidoglycan, whole-cell sugars, fatty acid analysis, the major menaquinones and the phospholipid profile) and biochemical tests. The genotypic methods are all based on molecular data such as DDH, restriction digestion of total chromosomal DNA, randomly amplified polymorphic DNA, PCR assays and sequence comparisons. For both environmental samples and pure cultures, the detection and classification of *Streptomyces* isolates is now most commonly performed by molecular approaches that target the 16S-rRNA gene (Manfio *et al.*, 2003; Park & Kilbane, 2006).

In general, when the practice of using both the genotypic and phenotypic data is followed, streptomycete taxonomists can expect to yield well circumscribed species with comprehensive species descriptions and a stable nomenclature (Manfio *et al.*, 2003).

It was established that many streptomycete type strains could be assigned to multi-membered groups that contain various other strains and their subjective synonyms. Some of these groups include those classified under *Streptomyces albidoflavus*, *Streptomyces griseus* and *Streptomyces violaceusniger* (Goodfellow *et al.*, 2007). Members of the *S. violaceusniger* 16S-rRNA gene clade are characterised by the formation of grey aerial spore mass, with aerial

mycelia that differentiate into spiral chains of rugose ornamented spores. Putative members of this group are considered fairly easy to recognise because of the transformation of this aerial spore mass from grey to black and mucilaginous, when mature. The group currently contains 13 validly described species that include *Streptomyces malaysiensis*, *S. violaceusniger*, *Streptomyces javensis* and *Streptomyces yogyakartensis* (Goodfellow *et al.*, 2007). The *S. violaceusniger* clade is an important part of the *Streptomyces* genus, because its members are known producers of antibacterial and antifungal metabolites (in particular to several classes of plant pathogenic fungi), biological control agents, enantioselective biocatalysts, as well as immunosuppressants, such as rapamycin (Sembiring *et al.*, 2000; Goodfellow *et al.*, 2007). The vast majority of natural antibiotics in clinical use today are made by streptomycetes and fungi (Park & Kilbane, 2006) and, as such, the discovery of new members of this genus, including those from the *S. violaceusniger* group, is especially important not only for taxonomists, but also for medical science.

Despite their exploitation, streptomycetes remain a rich source of new secondary metabolites and attract continued interest for novel bioactive compounds. However, because of the increasing difficulties in discovering commercially useful secondary metabolites from already discovered members of the *Streptomyces* genus (and subsequent rediscovery of known compounds), it has become even more important to isolate and characterise novel members of this important genus. Streptomycetes from under- or un-explored environments (such as the gut of the *M. viator* termite) are proving to be a rich source of new species, and thereby also a rich source of new bioactive compounds, including antibiotics (Antony-Babu & Goodfellow, 2008).

3.3 MATERALS AND METHODS

3.3.1 Strain descriptions

A total of 17 *Streptomyces* isolates as well as both of the *Nocardia* strains (isolates 12 and 20) was chosen for investigation into their species status. The 17 streptomycetes strains were 2, 7, 5, 6, 26, 39, 33, 35, 36, 38, 13, 16, 37, 18, 19, 32 and 14. Isolates 12 and 20 were investigated because they were the only non-streptomycetes discovered. Two groups (isolates 5, 6, 26 and 39 and isolates 33, 35, 36 and 38), were chosen because of their rare spore surface ornamentation and, in the case of isolates 33, 35, 36 and 38, also because of their strong antibacterial activities. Isolate 19 was investigated further because of its novel spore surface

ornamentation, and isolates 2 and 14 were chosen because of their antibacterial activity. Isolates 7 and 18 were chosen due to interesting results obtained during their rapid identification to the genus level by a restriction endonuclease method. Isolate 32 was investigated because it was found to be an unusual, fragmenting streptomycete. Isolates 13, 16 and 37 were all chosen because of low sequence similarity to published *Streptomyces* species.

3.3.1.1 Morphological and physiological characterisation

In general, morphological characteristics were determined after growing all strains on ISP#4 (Shirling & Gottlieb, 1966) for 14 days at 30°C. Spore chain morphology and spore surface ornamentation were determined using scanning electron microscopy. A similar preparation protocol for scanning electron microscopy to that described in section 2.3.6 was used. Modifications included the use of an approximately $1cm^2$ stub of agar, containing a sporulating streak of each of the strains investigated for SEM, cut from their respective agar plates using a sterile scalpel blade. These stubs were then placed separately into sections of a standard 24-well tissue culture plate for all fixing and washing steps.

A determination of the aerial and substrate mycelium colours was also performed on medium ISP#4. Production of diffusible pigments was determined on ISP#5, while melanin production was determined on ISP#6 and ISP#7, as described by Shirling & Gottlieb (1966). Methods for growth determination at 37°C and 45°C, and in the presence NaCl (3%, 5% and 7% w/v) were as described by Locci (1989). Testing for the reduction of nitrate and production of H_2S, as well as the decomposition of allantoin, adenine, arbutin, casein, cellulose, aesculin, gelatin, guanine, hypoxanthine, starch, L-tyrosine, urea, xanthine and xylan, were performed as described previously (Locci, 1989; Gordon *et al.*, 1974). Determination of sole carbon source utilisation was performed as described by Shirling & Gottlieb (1966). All incubations were carried out at 30°C, unless indicated otherwise.

3.3.1.2 Chemotaxonomy

The isomer of diaminopimelic acid (DAP) and the whole cell sugar pattern were determined as described by Hasegawa *et al.* (1983), using freeze-dried cells grown in 100ml ISP#2 broth for 4 days at 30°C, with shaking. In some cases, it was necessary to concentrate the extract obtained from the sugar protocol. This was done by freeze-drying the 100µl extract and re-dissolving it in a smaller volume of water (25µl dH_20). Modifications to the DAP protocol involved filtering off the black residue into 2ml microfuge tubes using size 1A coffee filters, and subsequent freezing at -70^oC for 5-6 hours. These frozen samples were then freeze-dried

before being re-dissolved in 100µl dH_20. In almost all cases, for both DAP and sugar analyses, 2µl of each extract was run on a cellulose TLC plate and, in some cases, this concentrate needed to be diluted further in order to resolve the components. Isolates 12 and 20, as well as 14, 18, 19, 32, 13, 16 and 37 were the only isolates that had their DAP isomer and sugars analysed.

3.3.1.3 Phylogenetic analyses

The methods for 16S-rRNA gene PCR amplification, genus identification and 16S-rRNA gene sequencing have all been described previously in section 2.3.7. In this instance, however, the sequences from six primers (general bacterial primers F1, R1, F3, R3, F5 and R5) were used to generate a consensus sequence for the nearly complete 16S-rRNA gene sequence for all isolates. The resulting sequences obtained for all of the isolates described above were submitted to the GenBank database for comparison, using the BlastN search tool. The 16S-rRNA gene sequences of the top hits resulting from these analyses, as well as from submission to the EzTaxon database (http://www.eztaxon.org; Chun *et al.*, 2007) for isolates 13, 16, 37, 18, 19, 32 and 14 only, were aligned with those of their phylogenetic relatives using the MEGA version 4 software program (Tamura *et al.*, 2007). A neighbour-joining phylogenetic tree (Saitou & Nei, 1987) was then generated, based on common regions of the 16S-rRNA gene. All pairwise 16S-rRNA gene sequence comparisons were made using DNAMAN version 5.2.9 (Lynnon BioSoft, 2001).

3.3.1.4 DNA-DNA hybridisation

To date, isolates 5 and 39, as well as isolates 18, 19, 32 and 14 are the only strains to have undergone DDH with their closest phylogenetic relatives. DDH analysis was performed by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh) identification service. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DDH was carried out as described by De Ley *et al.* (1970) [incorporating the modifications described by Huss *et al.* (1983)] using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in situ* temperature probe (Varian).

3.3.2 Strain de-replication

A simple restriction endonuclease-based protocol was developed in order to determine whether any additional isolates (i.e. other than those that had already been investigated),

warranted further investigation into their potential novel species status. This method was also used to determine the validity of the separation of the Originals and the Duplicates isolates.

Initially, *in silico* digestions using restriction endonucleases *Hae*I, *Hae*II, *Hae*III, *Hha*I (*Cfo*I), *Hha*II (*Hinf*I), *Hpa*I and *Hpa*II were performed on the almost complete 16S-rRNA gene sequences obtained for 14 of the isolates already under investigation. A combination of three enzymes was chosen based on their combined ability to distinguish the sequenced isolates from each other. These were *Hpa*II, *Cfo*I and *Hinf*I. *Hpa*II was found to have good resolution for major groupings, whereas *Cfo*I and *Hinf*I were able to distinguish closely related isolates from each other. A comparison of the *in silico* predicted band sizes against the observed banding patterns on a gel showed that it was not possible to use the actual band sizes for comparison as many bands were too close together or too small, and a visual comparison of the banding patterns was sufficient for discrimination purposes.

The 16S-rRNA gene PCR amplification products of all 77 isolates investigated were subjected to restriction endonuclease digestion for 24 hours at 37^{o}C using *Hpa*II, *Cfo*I and *Hinf*I. All digestions were electrophoresed in 1.5% agarose gels, containing ethidium bromide at a concentration of 0.8µg/ml, for 1-2 hours at 85V. Banding pattern designations for all three enzymes were assigned to the sequenced isolates and all other patterns were then compared to these patterns. Isolates were assigned to established groups or to their own group based on the patterns obtained. The results were tabulated to facilitate analysis.

3.4 RESULTS AND DISCUSSION

3.4.1 The *Nocardia* isolates 12 and 20

The results of the BLAST analysis against the GenBank database showed that both strains 12 and 20 were most closely related to *Nocardia nova* ATCC 33726^{T}. Two other species that form a sister group to these three (*Nocardia jiangxiensis* and *Nocardia miyunensis*), as demonstrated in Fig. 3.1, also showed 99% sequence similarity to both of the isolated strains. All other *Nocardia* type strains had 16S-rRNA gene sequence similarities of <98.7 % to strains 12 and 20. However, the bootstrap support for the association of strains 12 and 20 with the type strain of *N. nova* was low, as was the bootstrap support for the association of these three strains with the type strains of *N. jiangxiensis* and *N. miyunensis*. Results of the

chemotaxonomic analysis confirmed placement of isolates 12 and 20 within the *Nocardia* genus. Both were found to have *meso*-DAP in their cell walls, as well as the sugars galactose and arabinose (cell wall Type IV and whole cell sugar pattern Type A; Lechevalier & Lechevalier, 1970).

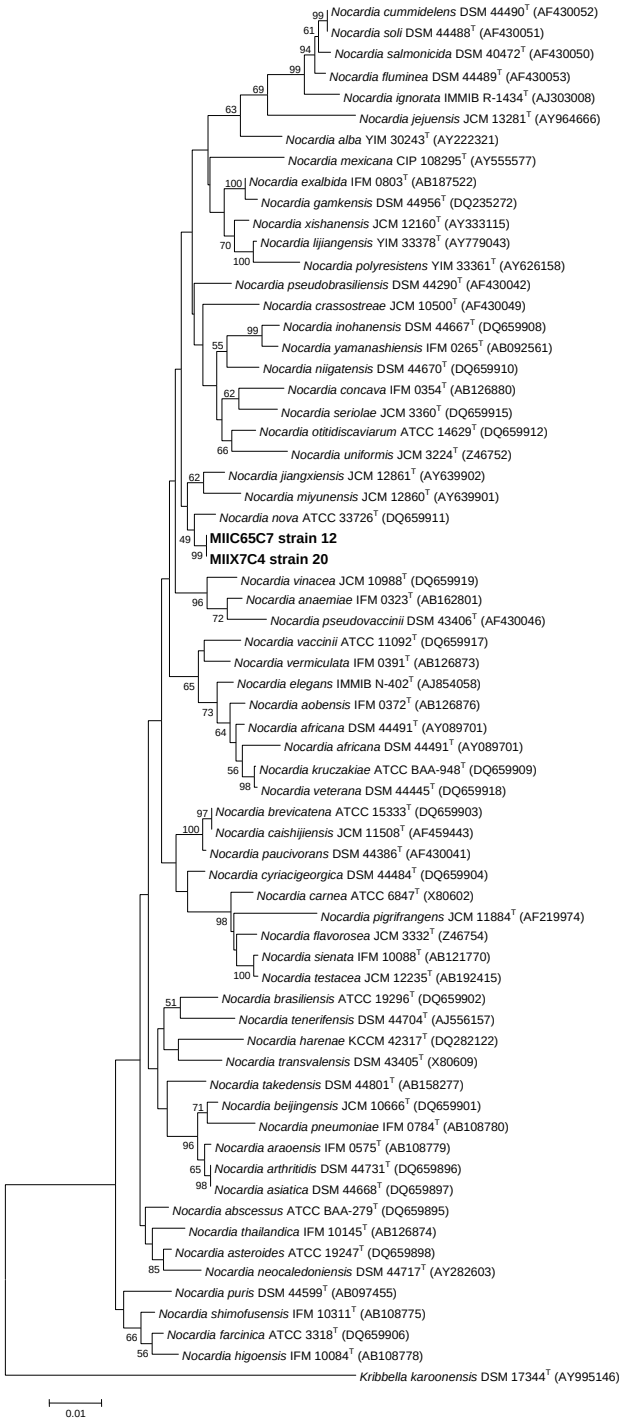

Figure 3.1: Neighbour-joining phylogenetic tree based on 1304 bp of 16S-rRNA gene sequence, showing the phylogenetic position of strains 12 and 20 amongst 63 members of the *Nocardia* genus. GenBank sequence accession numbers are shown in parentheses. Bootstrap support for each node is indicated as a percentage, calculated from 1000 randomly re-sampled datasets (only values above 48% are shown). Scale bar indicates 1 nucleotide substitution per 100 nucleotides. *Kribbella karoonensis* DSM 17344^T was used as an outgroup.

Based on the results shown in Table 3.1, it seems highly likely that strains 12 and 20 are the same species (see Appendix D for the complete set of results). The majority of the differences shown between the two isolates relate to differences in degree, such as those shown for production of H_2S and growth on D-(+)-galactose. The only clear difference was in the weak ability of 20 to degrade arbutin, compared with 12's negative result. Comparing the results for 12 and 20 against those published for *N. nova* was made very difficult due to contradictory results in the published literature. This situation is one of the reasons why a paper was compiled outlining a standard set of criteria for the publication of new species (Tindall *et al.*, 2009). One of these criteria involves having to perform phenotypic comparisons on the proposed new species and their closest relatives at the same time under the same conditions. This could not be done in this case, as *N. nova* is classified as a Risk Group 2 bacterium, and therefore requires specific facilities for its use. However, if it is assumed that those results for which more than one reference show the same result are correct, then a number of differences can be seen between *N. nova* and strains 12 and 20 (Table 3.1). These include the ability of *N. nova* to degrade arbutin compared with isolate 12. Also, a positive result for decomposition of urea for *N. nova*, as well as its ability to grow on fructose, D-(+)-galactose and D-(–)-mannitol, compared with both 12 and 20 provide some measure of support for separation of these two isolates as strains of a new species. However, due to the high level of sequence similarity between all of these strains, DDH will be necessary in order to determine the species status of strains 12 and 20.

Table 3.1: Physiological differences between *Nocardia* isolates 12 (1), 20 (2) and *Nocardia nova* ATCC 33726^T (3).

	1	2	3
Aerial mycelium colour (ISP#4)	bald?	white	N/D
Production of:			
H_2S	+(w)	+/–	N/D
Decomposition of:			
Adenine	–	NG	–[b,c,e]
Arbutin	–	+(w)	+[b,e]
Esculin	+	+	–[a]/+[b,e]
Urea	–	–	+[a,b,e,f]/–[c]
Growth on sole carbon source:			
Fructose	–	–	+[f]
D-(+)-Galactose	–	+/–	+[a,c,d]/–[f]
myo-Inositol	–	–	–[a,c,f]/+[d]
D-(–)-Mannitol	–	–	–[a,f]/+[b,e]
L-(+)-Rhamnose	–	–	–[a,b,c,e,f]/+[d]
Sucrose	–	–	–[a,f]/+[d]

NG = No growth; N/D = Not determined; +(w) = weakly positive; a: Yassin & Brenner (2005); b: Zhang *et al*. (2003); c: Kageyama *et al*. (2004); d: Kim *et al*. (2002); e: Cui *et al*. (2005); f: Tsukamura (1982). All other results were determined during this project.

3.4.2 The streptomycetes:

3.4.2.1 Isolates 2 and 7

Isolates 2 and 7 were isolated from the paunch and colon, respectively, of *M. viator*. Isolate 2 showed 100% sequence similarity to all four of the top BLAST hits (Fig. 3.2). The 100% bootstrap support also provided strong support for their phylogenetic association. Isolate 2 also showed phenotypic similarities with *Streptomyces costaricanus* NBRC 100773^T, *Streptomyces griseofuscus* NBRC 12870^T and *Streptomyces murinus* 12799^T, as evidenced by the spiral spore chain morphology shown in Fig. 3.3, and detailed in Appendix E. *Streptomyces graminearus* NBRC 15420^T could not be included in the physiological comparisons, as the only reference to this species is in Russian.

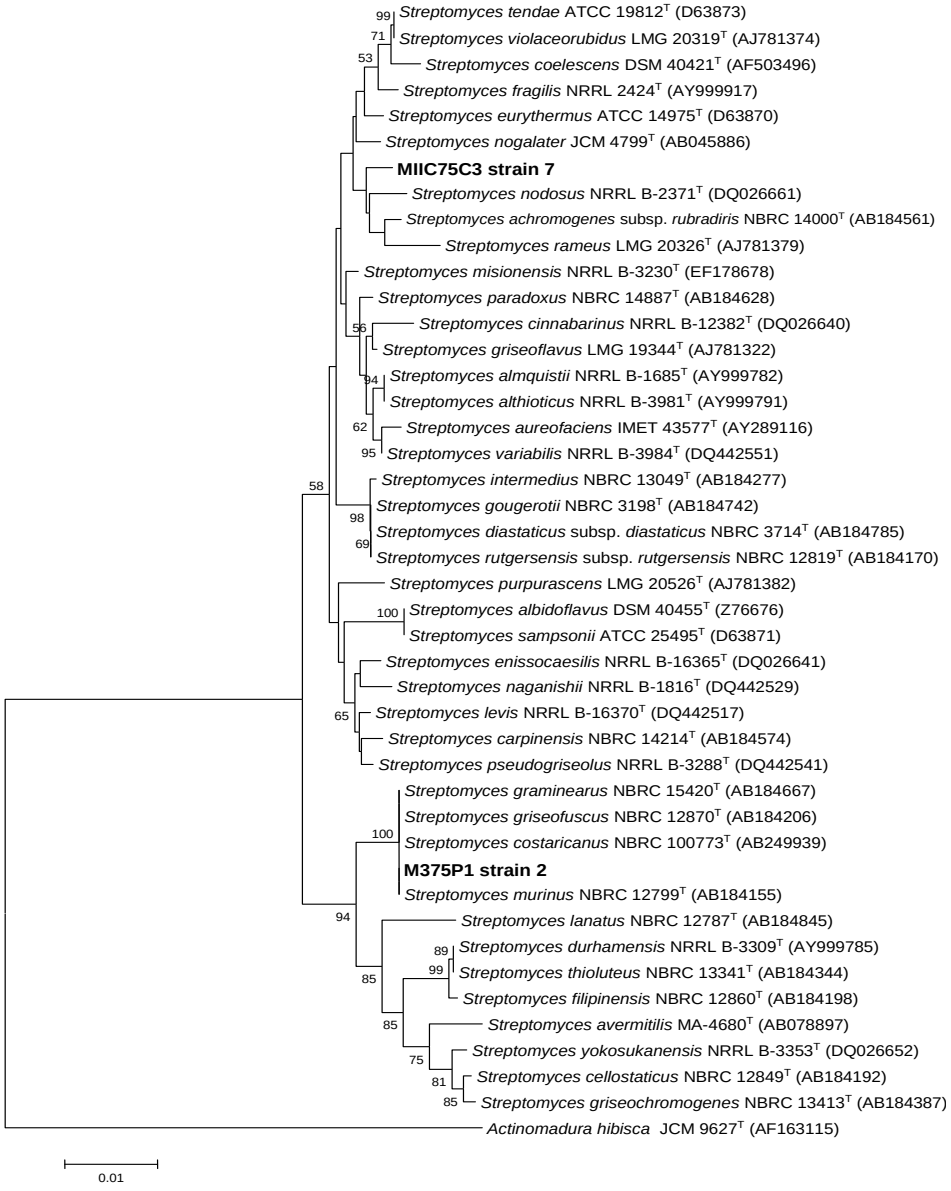

Figure 3.2: 16S-rRNA gene phylogenetic tree showing the position of isolates 2 and 7 in relation to their closest phylogenetic relatives. The tree was constructed using the neighbour-joining method based on 1460 bp of sequence. Accession numbers are shown in parentheses. Bootstrap support for each node is indicated as a percentage, calculated from 1000 randomly re-sampled datasets (only values above 50% are shown). *Actinomadura hibisca* JCM 9627^T was used as an outgroup. The scale bar indicates 1 nucleotide substitution per 100 nucleotides.

As shown in Table 3.2, differences were found in both the aerial mycelium and substrate mycelium colour of strain 2, compared with the three phylogenetic relatives. Excluding this result, there were a total of 4 differences between strain 2 and *S. costaricanus*, 6 differences for *S. griseofuscus* and 4 differences when compared with *S. murinus*. Whereas strain 2 and *S. griseofuscus* both produced no diffusible pigments on ISP#5, *S. costaricanus* and *S. murinus* both produce yellow pigments on this medium. In comparison, isolate 2 and *S. murinus* share the ability to grow in the presence of 5 and 7% (w/v) NaCl, whereas the other two cannot. In terms of their ability to grow on sole carbon sources, isolate 2 seems to be most different from *S. griseofuscus*.

Table 3.2: Physiological comparison between *Streptomyces* isolate 2 (1) and its closest phylogenetic relatives, *S. costaricanus* NBRC 100773^T (2), *S. griseofuscus* NBRC 12870^T (3) and *S. murinus* NBRC 12799^T (4).

	1	2	3	4
Aerial mycelium colour (ISP#4)	white	grey-brown[a]	pale greyish red-brown[a]	grey[a]
Substrate mycelium colour (ISP#4)	pale yellow-brown	yellow[a]	pale greyish brown[a]	greyish yellow[a]
Production of diffusible pigment on:				
ISP#5	–	yellow[a]	–[a]	yellow[a]
Growth in presence of:				
5% NaCl	+	–[a]	–[a]	+[a]
7% NaCl	+	–[a]	–[a]	+[a]
Growth on sole carbon source:				
L-(+)-Arabinose	–	–[a]	+[a]	+(w)[a]
D-(+)-Raffinose	+(w)	–[a]	+(w)[a]	+(w)[a]
L-(+)-Rhamnose	–	–[a]	+(w)[a]	+(w)[a]
Salicin	+(w)	+[a]	–[a]	+[a]
Sucrose	–	–[a]	+(w)[a]	+(w)[a]

+(w) = weakly positive; a = Esnard *et al.* (1995).

As shown in Fig. 3.2, isolate 7 has three close phylogenetic relatives, all of which were found to be 98% similar to this strain by BLAST analysis against the GenBank database. These were *Streptomyces achromogenes* subsp. *rubradiris* NBRC 14000^T, *Streptomyces nodosus*

NRRL B-2371^T and *Streptomyces rameus* LMG 20326^T. As with isolate 2, strain 7 shares its spiral spore chain morphology with all three of its relatives (see Fig. 3.3 and Appendix F for details). Of those physiological results shown in Table 3.3, isolate 7 shows seven differences when compared to *S. achromogenes* subsp. *rubradiris*, including differences in aerial and substrate mycelium colour, very weak growth of strain 7 on 7% (w/v) NaCl, the ability to reduce nitrate, a negative result for the decomposition of xanthine, and an inability to grow on *myo*-inositol and sucrose. Six differences were found between isolate 7 and *S. nodosus*. These also include aerial and substrate mycelium colour differences, but encompass the ability of isolate 7 to produce H_2S as well as 3 differences in their growth on carbon source plates. Of the limited number of tests that could be compared between isolate 7 and *S. rameus*, six differences were found, which included a different aerial mycelium colour on ISP#4, melanin production by *S. rameus* on ISP#6 and ISP#7 and the lack of growth of *S. rameus* on L-(+)-rhamnose and its growth on salicin and sucrose.

Table 3.3: Physiological comparison between *Streptomyces* isolate 7 (1) and its closest phylogenetic relatives, *S. achromogenes* subsp. *rubradiris* NBRC 14000^T (2), *S. nodosus* NRRL B-2371^T (3) and *S. rameus* LMG 20326^T (4).

	1	2	3	4
Aerial mycelium colour (ISP#4)	rose/pink	grey[a]	grey[b]	grey[a]
Substrate mycelium colour (ISP#4)	reddish-brown	yellow-brown[a]	green[b]	N/D
Production of melanin on:				
ISP#6	–	–[a]	–[b]	+[a]
ISP#7	–	–[a]	–[b]	+[a]
Growth in presence of:				
7% NaCl	+(vw)	–[a]	N/D	N/D
Reduction of:				
Nitrate	+	–[a]	+[b]	N/D
Production of:				
H_2S	+	+[a]	–[b]	N/D
Decomposition of:				
Xanthine	–	+[a]	N/D	N/D
Growth on sole carbon source:				
L-(+)-Arabinose	+	N/D	–[b]	+[a]
myo-Inositol	–	+[a]	+[b]	–[a]
D-(–)-Mannitol	+(vw)	+[a]	+[b]	+[a]
D-(+)-Raffinose	+(w)	+[a]	–[b]	+[a]
L-(+)-Rhamnose	+(vw)	+[a]	+[b]	–[a]
Salicin	–	N/D	N/D	+[a]
Sucrose	–	+[a]	–[b]	+[a]

N/D = Not determined; +(w) = weakly positive; +(vw) = very weakly positive; a = Locci (1989); b = Trejo & Bennett (1962). All other results were determined during this project.

Despite the fact that a number of differences were found between isolate 2 and isolate 7 versus their closest phylogenetic relatives, both share more than 98% sequence similarity to already published species. In order to determine whether they are new species within the *Streptomyces* genus, both would require DDH analysis against their closest phylogenetic relatives.

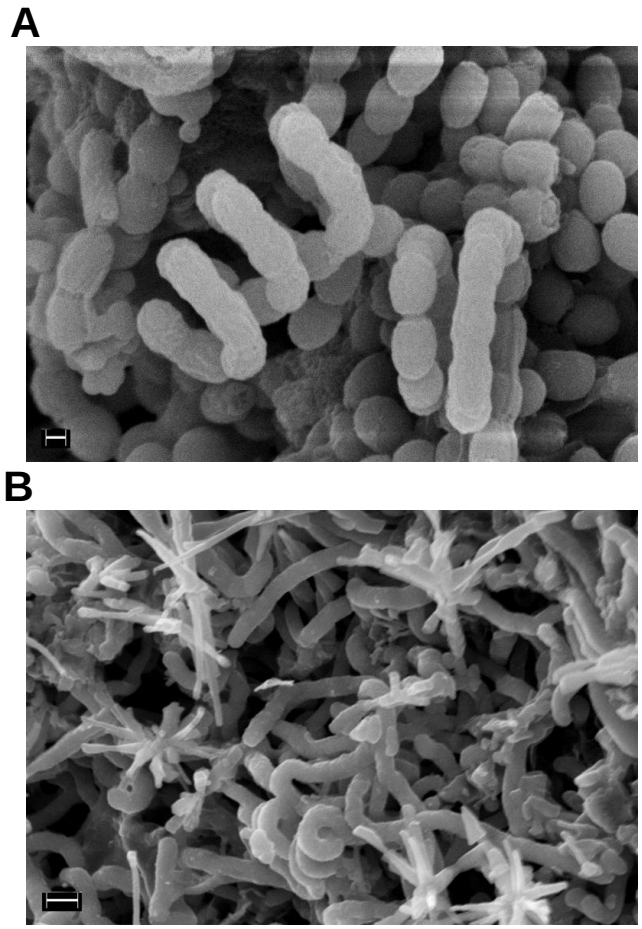

Figure 3.3: Scanning electron micrographs of the *Spirales* spore chain morphology and smooth spore surface ornamentation exhibited by isolates 2 (A) and 7 (B) when grown on medium ISP#4 for 14 days at 30°C. Bars = 300nm (A) and 1µm (B).

3.4.2.2 'Group 33' isolates – 33, 35, 36 and 38

Within the 16S-rRNA gene phylogenetic tree shown in Fig. 3.4, the Group 33 isolates form a sister cluster to six *Streptomyces* species with validly published names. Within the Group 33 cluster, isolate 38 forms an outgroup which is supported by a very high bootstrap value. The six closest phylogenetic relatives were *Streptomyces endus* NRRL 2339^T, *Streptomyces hygroscopicus* subsp. *hygroscopicus* NBRC 13472^T, *Streptomyces javensis* NBRC 100777^T, *Streptomyces sporocinereus* NBRC 100766^T, *Streptomyces violaceusniger* NBRC 13459^T and *Streptomyces yogyakartensis* NBRC 100779^T, and all have a sequence similarity of 99% with

all members of Group 33, as indicated by the GenBank BLAST results. The separation of these top BLAST hits from the four *M. viator* isolates in Fig. 3.4 is supported by a very high bootstrap value. The 16S-rRNA gene sequence similarity between all possible pairwise combinations of strains 33, 35, 36 and 38 ranged between 99.71-100%, based on 1396 bp of common sequence.

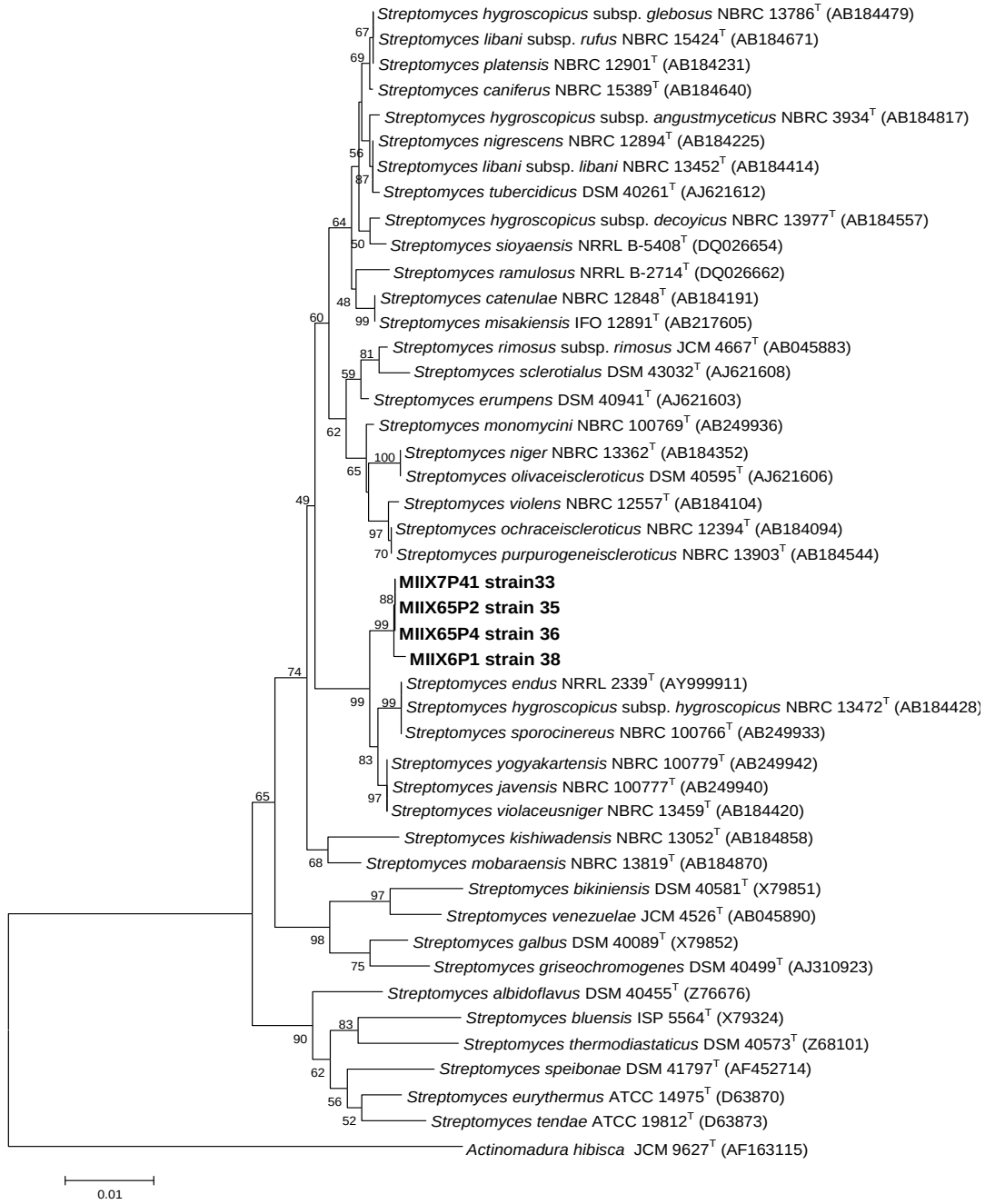

Figure 3.4: Neighbour-joining phylogenetic tree based on 1401 bp of 16S-rRNA gene sequence, showing the phylogenetic position of the 'Group 33' isolates (strains 33, 35, 36 and 38) amongst their closest phylogenetic neighbours from the *Streptomyces* genus. GenBank sequence accession numbers are shown in parentheses. Bootstrap support, calculated from 1000 randomly re-sampled datasets is indicated as a percentage at the nodes (only values above 47% are shown). The scale bar indicates 1 nucleotide substitution per 100 nucleotides. *A. hibisca* JCM 9627^T was used as an outgroup.

The most interesting discovery about the Group 33 isolates, as well as those from Group 5, was their rugose spore surface ornamentation (as evidenced in Fig. 3.5). This unusual ornamentation of the spore sheath is fairly rare in the *Streptomyces* genus, but is a well-recognised feature of all the members of the *S. violaceusniger* clade (Goodfellow *et al.*, 2007). *S. endus* and *S. hygroscopicus* subsp. *hygroscopicus* were not included in the phenotypic comparison as both are considered to be subjective synonyms of *S. violaceusniger* (Locci, 1989). *S. sporocinereus* was also not included because its description is only available in Russian. Another common feature that can be seen across all isolates and their relatives is the grey aerial mycelium and cream/ yellow substrate mycelium (see Table 3.4 and Appendix G for details). However, there were many differences between the Group 33 isolates and their phylogenetic relatives, as can be seen in Table 3.4.

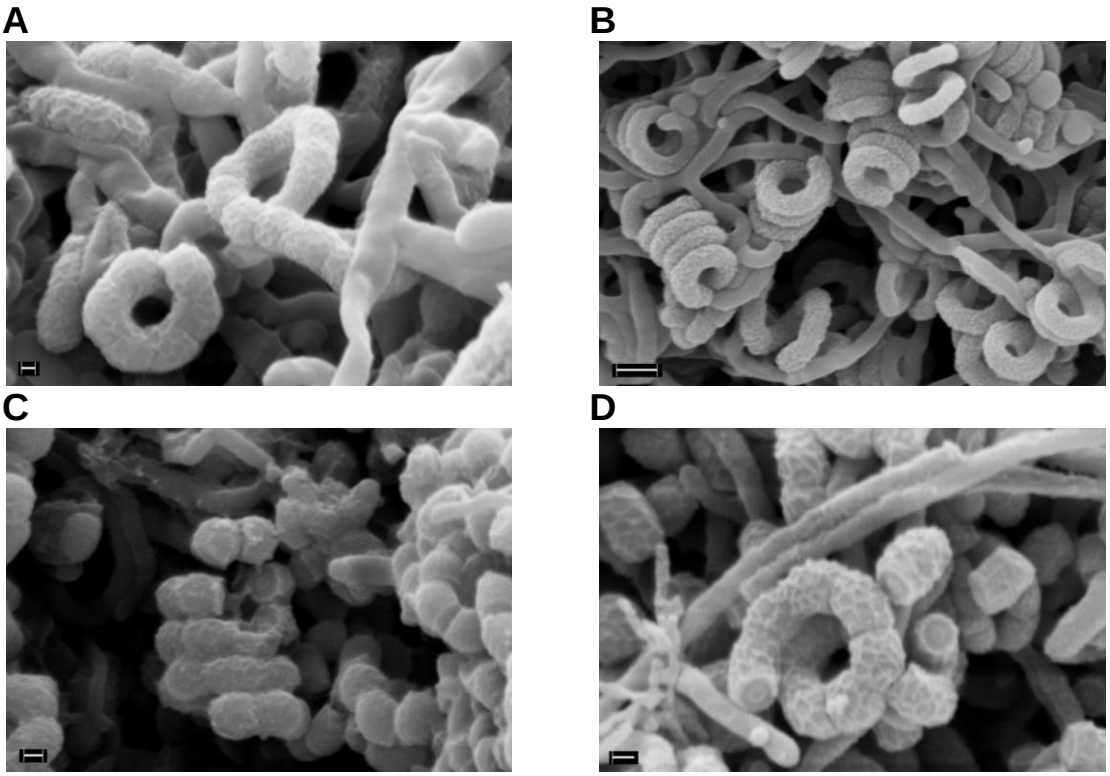

Figure 3.5: Scanning electron micrographs of the *Spirales* spore chain morphology and rugose spore surface ornamentation of isolates 33 (A), 35 (B), 36 (C) and 38 (D). Except for isolate 33 (which was grown on medium MIIX at pH 7), all strains were grown on ISP#4 medium for 14 days at 30°C. Bars = 200nm (A), 300nm (C) and 1µm (B & D).

Within the Group itself, strain 38 stands out because of a number of differences from the other three strains. Strain 38 was unable to grow on ISP#5 or ISP#7, compared with the negative results that were shown by 33, 35 and 36 for these tests. Strain 38 was also unable to grow at 37^{o}C and was the only isolate able to reduce nitrate – an ability it shares with *S. violaceusniger*. It was also the only strain of the four isolates that was unable to grow on L-

(+)-arabinose and sucrose. Strains 33 and 36 were found to be almost identical, but isolate 35 stood out from the others as the only isolate able to grow weakly in the presence of 5% (w/v) NaCl and as the only strain with the ability to produce H_2S (including the published species). All four Group 33 isolates share with *S. javensis* an inability to grow at 45°C, unlike *S. violaceusniger* and *S. yogyakartensis*. In their esculin decomposition result, they only differ from *S. yogyakartensis*. All four isolates show differences against all three published strains in their inability to degrade xylan, as well as their inability to grow at all on the allantoin and urea plates. Table 3.4 also shows that in terms of carbon source utilisation, strains 33, 35 and 36 only differ from *S. violaceusniger*, whereas strain 38 differs from *S. javensis* and *S. yogyakartensis* in its L-(+)-arabinose and sucrose results.

Table 3.4: Physiological comparison between *Streptomyces* isolates 33 (1), 35 (2), 36 (3) and 38 (4) and three close phylogenetic relatives, *S. javensis* NBRC 100777^T (5), *S. violaceusniger* NBRC 13459^T (6) and *S. yogyakartensis* NBRC 100779^T (7).

	1	2	3	4	5	6	7
Aerial mycelium colour (ISP#4)	dark grey/black	dark grey/black	dark grey/black	dark grey	grey (ISP#3)[c]	white-grey[a]	grey (ISP#3)[c]
Substrate mycelium colour (ISP#4)	cream	cream	yellowish-cream	cream	greyish yellow (ISP#3)[c]	greyish yellow[a]	greyish yellow (ISP#3)[c]
Production of diffusible pigment on:							
ISP#5	–	–	–	NG	yellow (ISP#3)[c]	–[a]	yellow (ISP#3)[c]
Production of melanin on:							
ISP#7	–	–	–	NG	–[c]	N/D	–[c]
Growth at:							
37°C	+	+	+	–	N/D	N/D	N/D
45°C	–	–	–	–	–[c]	+[b]	+[c]
Growth in presence of:							
3% NaCl	–	+	+	–	N/D	N/D	N/D
5% NaCl	–	+(w)	–	–	N/D	N/D	N/D
Reduction of:							
Nitrate	–	–	–	+	–[b]	+[b]	–[c]
Production of:							
H_2S	–	+	–	–	–[b]	–[b]	–[b]
Decomposition of:							
Allantoin	NG	NG	NG	NG	+[c]	–[b]	+[c]
Esculin	+	+	+	+	+[c]	+[b]	–[c]
Urea	NG	NG	NG	NG	+[b]	+[b]	+[b]
Xylan	–	–	–	–	+[c]	+[b]	+[c]
Growth on sole carbon source:							
L-(+)-Arabinose	+	+	+	–	+[b]	–[b]	+[b]
Sucrose	+	+	+	–	+[c]	–[b]	+[c]
D-(+)-Xylose	+	+	+	+	+[b]	–[b]	+[b]

NG = No growth; N/D = Not determined; +(w) = weakly positive; a = Goodfellow *et al.* (2007); b = Saintpierre *et al.* (2003); c = Sembiring *et al.* (2000).

In terms of the number of differences within the Group and compared against their phylogenetic relatives, it would seem that isolates 35 and 38 would be the best choices for DDH analysis, as these were found to be the most unique of the four Group 33 strains. The Group 33 isolates were all isolated from the paunch of *M. viator*.

3.4.2.3 'Group 5' isolates – 5, 6, 26 and 39

In comparison to the Group 33 isolates, the members of Group 5 only had one close phylogenetic relative, *S. malaysiensis* ATCC BAA-13^T, with a sequence similarity of 99% to all four members. Sequence similarities to all other *Streptomyces* species were <98%. Based on 1354 bp of common sequence, all possible pairwise combinations of strains 5, 6, 26 and 39 produced a 16S-rRNA gene sequence similarity of 100%. The BLAST analyses also contained hits from other genera closely related to the *Streptomyces* genus, namely, *Streptacidiphilus* and *Kitasatospora*. However, clustering of the Group 5 isolates amongst the other *Streptomyces* species shown in Fig. 3.6 supports their placement within this genus and the very high bootstrap support (100%) supports their close association with the type strain of *S. malaysiensis*.

As for *S. malaysiensis*, isolates 5, 6, 26 and 39 all had spiral spore chain morphology and rugose spore surface ornamentation, bringing the total number of rugose-spored streptomycetes isolated from the hindgut of *M. viator* up to eight. This spore surface ornamentation is shown clearly for the Group 5 isolates in Fig. 3.7. As with Group 33, the Group 5 isolates also have similar aerial and substrate mycelium colours to their closest relative (see details in Appendix H). Another commonality with *S. malaysiensis* is in their growth characteristics, where the aerial mycelium of the isolates on various media starts off grey, but rapidly becomes a smoky black and then slimy at maturity.

A number of differences was found when a physiological comparison was made between isolates 5, 6, 26 and 39 and *S. malaysiensis*, as shown in Table 3.5. Within the Group, there were clear differences between 5 and 6 compared with 26 and 39, as the former isolates were able to grow in the presence of 5% (w/v) NaCl and were able to produce H_2S – two differences that were also found against *S. malaysiensis*. Isolate 5 was the only strain not able to degrade esculin, while isolate 39 was unable to degrade L-tyrosine. Isolate 39 was also unable to grow on D-(+)-raffinose and salicin as sole carbon sources. There were also four results for which all four isolates differed from *S. malaysiensis*. These included the production of diffusible pigment on ISP#5 by *S. malaysiensis*, as well as a negative result for decomposition of arbutin. All of the Group 5 isolates were also unable to grow on the allantoin and urea plates, whereas *S. malaysiensis* showed negative and positive results for

these tests, respectively. Other differences in growth on sole carbon sources were also found, but these were only differences of degree.

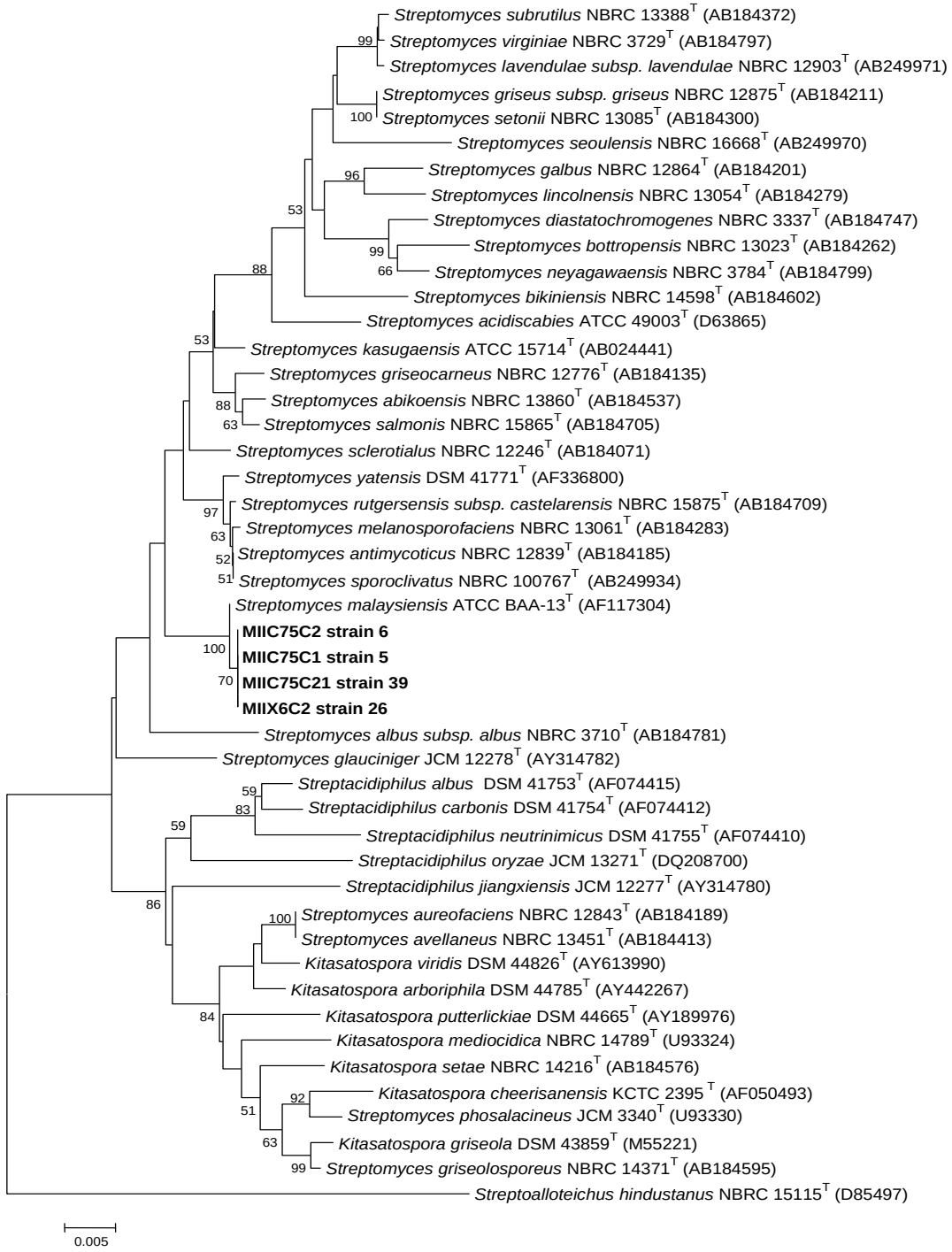

Figure 3.6: 16S-rRNA gene phylogenetic tree showing the 'Group 5' isolates (strains 5, 6, 26 and 39), in relation to 30 of their closest phylogenetic relatives from the *Streptomyces* genus, as well as 5 and 7 members of the related genera *Streptacidiphilus* and *Kitasatospora*, respectively. The tree was constructed using the neighbour-joining method based on 1371 bp of sequence. GenBank accession numbers are shown in parentheses. The percentage bootstrap values of 1000 replications are shown at each node (only values above 50% are shown). *Streptoalloteichus hindustanus* NBRC 15115^T was used as an outgroup. The scale bar indicates 5 nucleotide substitutions per 1000 nucleotides.

Table 3.5: Physiological differences between *Streptomyces* isolates 5 (1), 6 (2), 26 (3) and 39 (4) and *S. malaysiensis* DSM 41687^T (5).

	1	2	3	4	5
Production of diffusible pigment on:					
ISP#5	–	–	–	–	+[a]
Production of melanin on:					
ISP#7	–	–	–	–	–/+[a]
Growth in presence of:					
3% NaCl	+	+	+	+(w)	+[a]
5% NaCl	+	+	–	–	–[a]
Production of:					
H_2S	+	+	–	–	–[a]
Decomposition of:					
Allantoin	NG	NG	NG	NG	–[c]
Arbutin	+	+	+	+	–[c]
Esculin	–	+	+	+	+[b]
Starch	+	+	+(w)	+(w)	+[a]
L-Tyrosine	+	+	+	–	+
Urea	NG	NG	NG	NG	+[c]
Growth on sole carbon source:					
myo-Inositol	+(w)	+(w)	+(w)	+	+
D-(+)-Raffinose	+(w)	+(w)	+(w)	–	+(w)
L-(+)-Rhamnose	+	+	+	+(w)	+
Salicin	+(w)	+(w)	+(w)	–	+(w)

NG = No growth; +(w) = weakly positive; a = Al-Tai *et al.* (1999); b = Goodfellow *et al.* (2007); c = Saintpierre *et al.* (2003). All other results were determined during this project.

Based on the phenotypic differences that were found within the Group and to the type strain of *S. malaysiensis*, DDH analysis was performed between strains 5 and 39 and *S. malaysiensis* DSM 41697^T. Strains 5 and 39 were found to belong to the same species as their DNA-DNA similarity was 97.1 ± 3.68%, which is well above the threshold value of 70% recommended for the delineation of bacterial genomic species (Wayne *et al.*, 1987). Strain 39 was found to have a DNA-DNA similarity of 81.1 ± 1.41% to *S. malaysiensis*, again well above the cut-off value. When strain 5 was compared with *S. malaysiensis*, a value of 74 ± 3.68% was recorded. As this was also above the guideline threshold level of 70%, it was suggested that strain 5 might belong to the same species as *S. malaysiensis* (the 74% DNA-relatedness result was regarded as close enough to 70%)

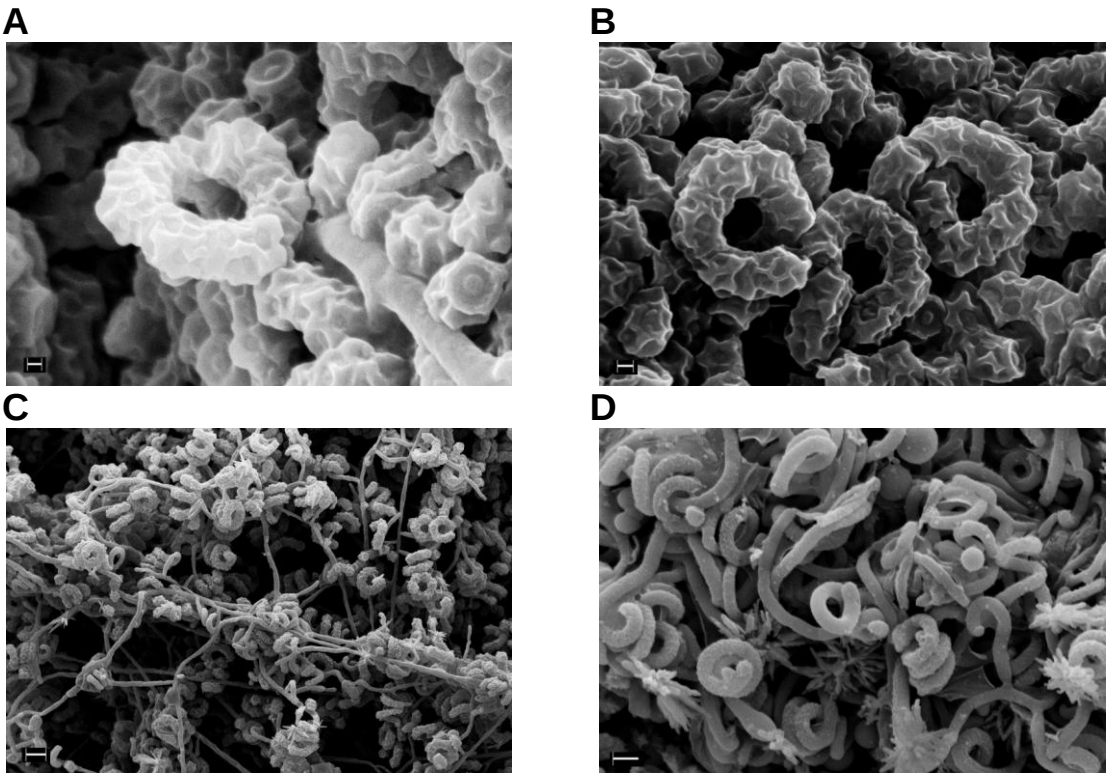

Figure 3.7: Scanning electron micrographs, all showing the rugose and *Spirales* nature of the spore surface ornamentation and spore chain morphology, respectively, of isolates 5 (A), 6 (B), 26 (C) and 39 (D). All isolates were grown on ISP#4 for 14 days at 30°C. Bars represent 200nm (A), 300nm (B), 2µm (C) and 1µm (D).

Interestingly, whereas the Group 33 isolates all originated from the *M. viator* paunch, the Group 5 strains were all isolated from the colon region. Another interesting observation relates to the antibacterial activities of these two groups. The separation of the Group 5 isolates from those in Group 33 is noteworthy, as it can be seen in Table 2.2 of Chapter 2 that except for isolate 38, all of the Group 5 and Group 33 isolates fall within the top eight antibiotic producers, with zones of inhibition well above 2000 mm^2. In comparison, the rest of the isolates that are shown in Table 2.2 only produced values below 2000 mm^2. This was one of the reasons why these isolates, along with isolate 14, were chosen to be investigated further. The Group 33 isolates belong to the *S. violaceusniger* clade, the members of which are known to produce antibacterial antibiotics. *S. malaysiensis*, to which the Group 5 isolates belong, is also known to produce antibacterial antibiotics.

3.4.2.4 Isolates 13, 16 and 37

As seen in Fig. 3.8, the separation of isolate 13 from isolates 16 and 37 is supported by a 100% bootstrap value. All three form a deep branch within the phylogenetic tree, with their closest phylogenetic relative being *Streptomyces sodiiphilus* YIM 80305^T. However, the

association with *S. sodiiphilus* YIM 80305^{T} was supported by a very low bootstrap value (<50%). The most significant feature of these three isolates is their low sequence similarity to all other 16S-rRNA gene sequences in the GenBank database. BLAST analysis revealed that strain 13 only shared 96% sequence similarity with its closest hit, whereas strains 16 and 37 were only 95% similar to their top hits. These values are significant, as they are all different enough from all published sequences to warrant immediate recognition as new species within the *Streptomyces* genus (using the 16S-rRNA gene sequence similarity threshold of 97%; Stackebrandt & Goebel, 1994). Their very low sequence similarity to all other *Streptomyces* species suggested that they may not belong to the *Streptomyces* genus at all. However, as can be seen in Fig. 3.8, the three strains cluster amongst other *Streptomyces* species within the tree, providing support for their placement within this genus. The 16S-rRNA gene sequence similarity between all possible pairwise combinations of strains 13, 16 and 37, based on 1476 bp of common sequence, ranged between 98.58-100%.

Isolates 13, 16 and 37 were all analysed for their cell wall DAP isomer and whole-cell sugar patterns to check whether these chemotaxonomic characteristics were consistent with their assignment to *Streptomyces*. All were found to have the LL-DAP isomer, and only contained the sugars ribose and glucose in their whole cell hydrolysates (cell wall Type I; Lechevalier & Lechevalier, 1970) – both of which are non-diagnostic, and therefore in combination with the LL-DAP result support the placement of isolates 13, 16 and 37 within the genus *Streptomyces.* By comparison, the genus *Kitasatospora* is characterised by the presence of LL-DAP and *meso*-DAP as well as the sugar galactose in its whole cell hydrolysates, whereas *Streptacidiphilus* is characterised by the presence of LL-DAP and the sugars galactose and rhamnose (Kim *et al.*, 2003).

All three isolates were quite a challenge to maintain in the laboratory. Initial growth from freezer stocks on a range of different media was always sparse, and very seldom showed any signs of sporulation, as seen by the aerial mycelium colour result in Table 3.6. However, once established on agar plates or in broth cultures, they were usually fairly easy to sub-culture. The phylogenetic separation of isolate 13 from 16 and 37 shown in Fig. 3.8 was supported by the phenotypic comparison (see Appendix I and Table 3.6 for details). This confirmation by both phylogenetic analysis and physiology is important, as the minimal standards for the delineation of *Streptomyces* species need to be based on a complimentary set of genotypic and phenotypic data (Saintpierre *et al.*, 2003). The morphological differences are obvious when looking at Fig. 3.9. Whereas, strains 16 and 37 both have straight spore chains with smooth spore ornamentation, strain 13 showed spiral spore chain morphology and hairy spore surface

ornamentation. Isolate 13 also differed in its ability to grow weakly in the presence of 3% NaCl, as well as having a negative result for the decomposition of allantoin and urea tests, compared with a total lack of growth by strains 16 and 37 on these plates (see Table 3.6). The only difference that was found between isolate 16 and 37 was the ability of 16 to weakly degrade hypoxanthine. Unfortunately, due to problems in culturing isolate 37 on the carbon source media, all results were found to be negative, including the glucose control, and in comparison to almost all of the results being positive for both strains 13 and 16, suggested that these were false negatives. A pilot study was done to investigate whether including yeast extract in the carbon source medium would allow strain 37 to grow. The addition of 0.05% (w/v) yeast extract to both the no carbon source and glucose control plates allowed for a clear distinction to be made between no growth and growth. In order to publish these isolates as new species, the carbon source results will need to be repeated using plates containing 0.05% (w/v) yeast extract in addition to the test carbon sources.

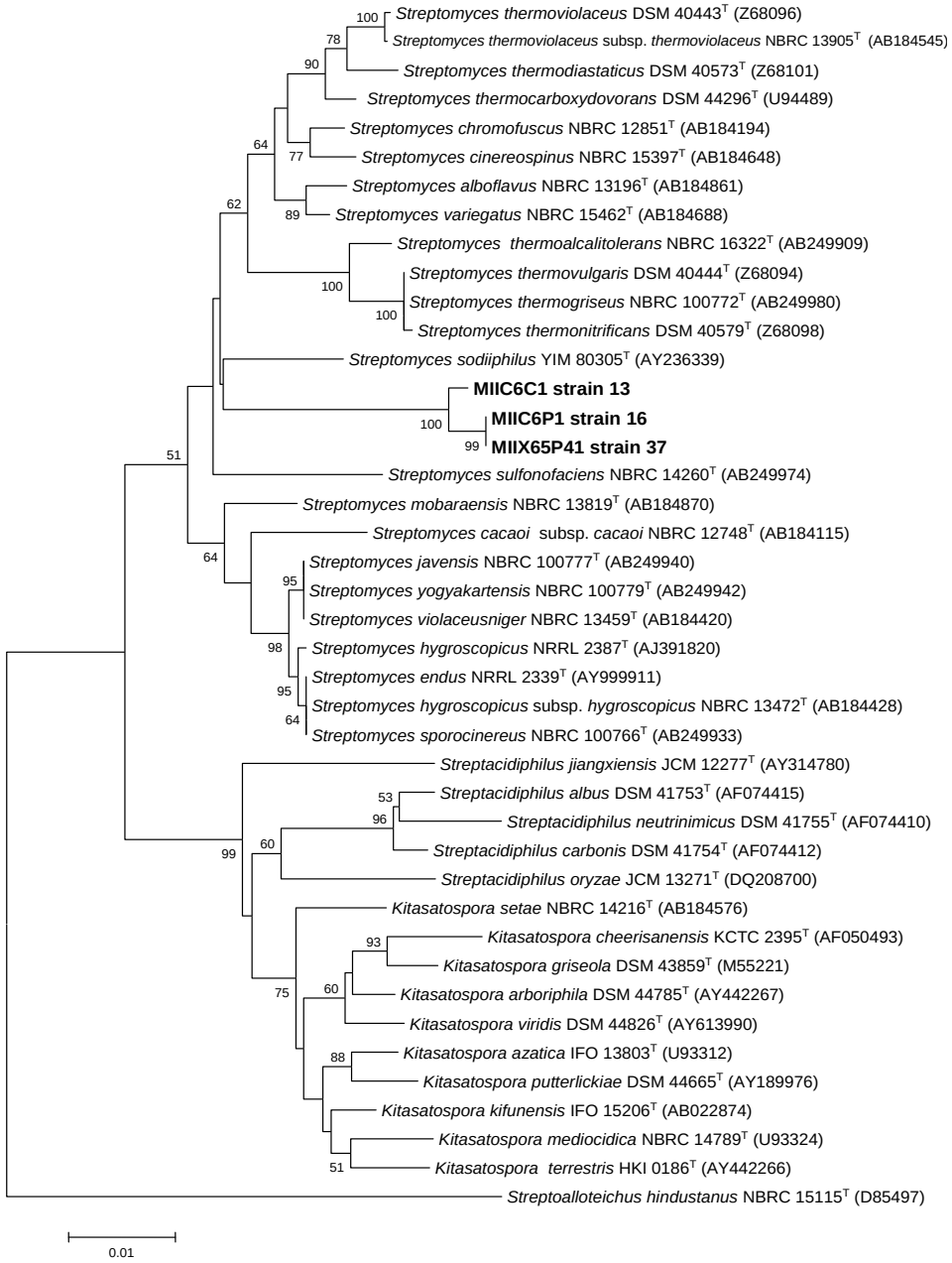

Figure 3.8: Neighbour-joining phylogenetic tree based on 1424 bp of 16S-rRNA gene sequence showing the phylogenetic position of strains 13, 16 and 37 amongst 23 members of the genus *Streptomyces*, as well as 5 and 10 members of the related genera *Streptacidiphilus* and *Kitasatospora*, respectively. GenBank accession numbers are shown in parentheses. The percentage bootstrap values of 1000 replications are shown at each node (only values above 50% are shown). The scale bar indicates 1 nucleotide substitution per 100 nucleotides. *S. hindustanus* NBRC 15115^T was used as an outgroup.

Table 3.6: Differences in morphological and physiological characteristics between *Streptomyces* isolates 13 (1), 16 (2) and 37 (3) and *S. sodiiphilus* YIM 80305^T (4).

	1	2	3	4
Spore surface ornamentation	hairy	smooth	smooth	smooth
Spore chain morphology	spiral	straight?	straight	straight to flexuous
Aerial mycelium colour (ISP#4)	bald	bald	bald	grey-white (ISP#2, pH 9)
Growth at:				
37^{o}C	+(w)	+(w)	+	N/D
Growth in presence of:				
3% NaCl	+(w)	–	–	N/D
Reduction of:				
Nitrate	–	–	–	+
Decomposition of:				
Allantoin	–	NG	NG	N/D
Hypoxanthine	+(w)?	+(w)	–	N/D
Starch	+	+	+	–
Urea	–	NG	NG	–
Growth on sole carbon source:				
Glucose	+	+	–?	–
Fructose	+(w)	+	–?	–
D-(+)-Galactose	+	+	–?	–
myo-Inositol	+	+(w)	–?	–
D-(–)-Mannitol	+	+	–?	–
D-(+)-Raffinose	+	+	–?	–
Salicin	?	+	–?	N/D
Sucrose	+	+	–?	–
D-(+)-Xylose	+	+	–?	–

All *S. sodiiphilus* results were taken from Li *et al.* (2005). N/D = Not determined; NG = No growth; ? = Result uncertain; +(w) = weakly positive.

One of the differences between isolates 13, 16 and 37 and *S. sodiiphilus* is in the fact that *S. sodiiphilus* is alkaliphilic and, when grown on ISP#2 (pH 9.0), has well developed aerial and substrate mycelia (Li *et al.*, 2005). It is also able to reduce nitrate and is unable to degrade starch (see Table 3.6). Excluding the carbon source data for strain 37, strains 13 and 16 differed from *S. sodiiphilus* in all carbon source results, as *S. sodiiphilus* was unable to grow on any of the carbon sources. The only similarity found was between strain 13 and *S. sodiiphilus* in their negative urea decomposition result.

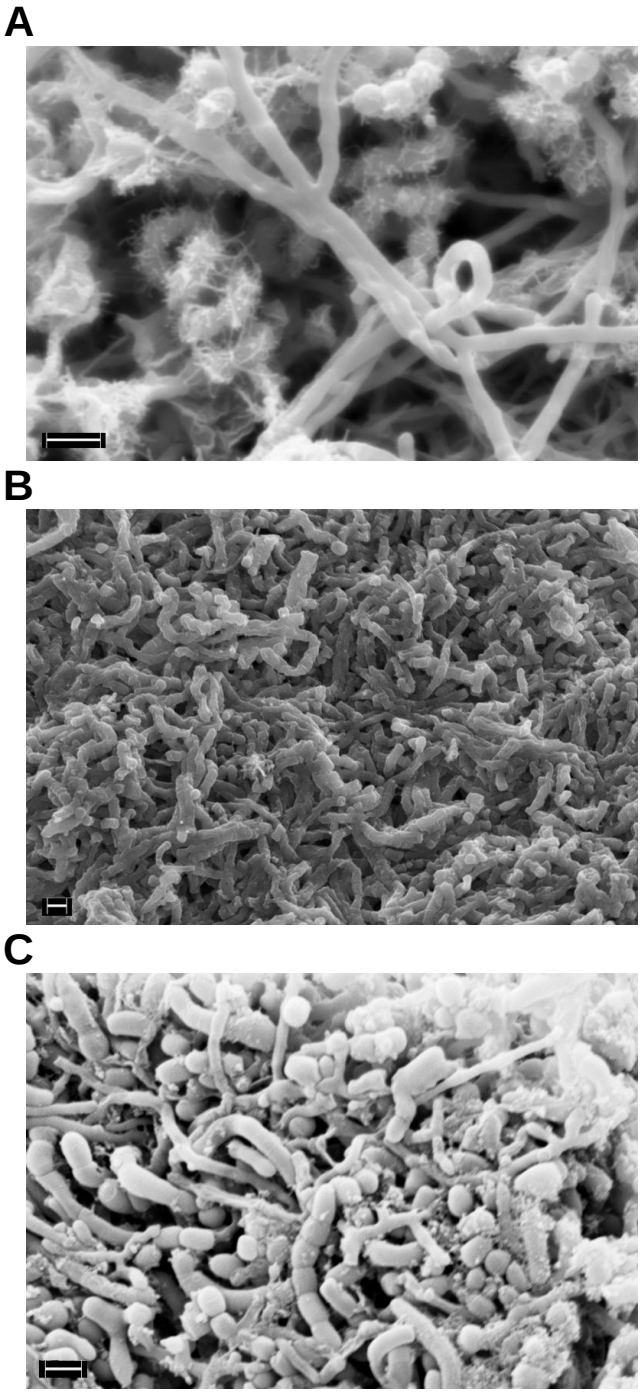

Figure 3.9: Scanning electron micrographs showing the hairy spore surface ornamentation and *Spirales* spore chain morphology of isolate 13 (A) in comparison to the smooth spore surfaces and *Rectiflexibiles* spore chains of isolates 16 (B) and 37 (C). Isolate 13 was grown on MIIC medium at pH 6, 16 on ISP#4 and 37 on modified Bennett's medium; all for 14 days ate 30°C. Bars = 1µm.

It is clear from both the low sequence similarity of all three isolates to other *Streptomyces* species, as well as the differences that have been shown between strains 13, 16 and 37 and *S. sodiiphilus*, that strains 13 and 16/37 warrant description as new species within the genus *Streptomyces*. Isolates 16 and 37 are most probably strains of the same species.

3.4.2.5 Isolates 18, 19 and 32

In Fig. 3.10, there appear to be four major clusters within the phylogenetic tree of the selected *Streptomyces* species. Strains 18, 19 and 32 occupied one of these with their close phylogenetic relatives *Streptomyces avermitilis* NCIMB 12804^T, *Streptomyces cinnabarinus* NBRC 13028^T, and *Streptomyces kunmingensis* NBRC 14463^T. There was very low bootstrap support for the clustering of these six strains, except in the case of the affiliation of isolate 32 with *S. kunmingensis*. However, BLAST analysis against the GenBank database revealed that isolates 18, 19 and 32 all share 98% sequence similarity with their top hits. The 16S-rRNA gene sequence similarities between all possible pairwise combinations of strains 18, 19 and 32 are shown in Table 3.8.

A chemotaxonomic investigation of the DAP and sugar content of their cell walls confirmed that all belong to the *Streptomyces* genus. The presence of LL-DAP and ribose and glucose as whole cell sugars (i.e. no diagnostic sugars) placed them firmly within cell wall Type I (Lechevalier & Lechevalier, 1970).

There was a clear morphological distinction between isolate 19 and isolates 18 and 32. Whereas strains 18 and 32 had straight spore chain morphologies with smooth spores, 19 had spiny spores in tight spiral chains, as shown in Fig. 3.11.

Strain 19 differed from the other two in its lack of nitrate reduction and an inability to degrade either allantoin or urea (see Appendix J and Table 3.7 for details). Isolate 32 differed from 18 only by degree for the allantoin, starch and urea decomposition tests, and was the only isolate not able to grow on *myo*-inositol. One distinctive difference exhibited by strain 32 was the unusual characteristic of fragmentation in all broth cultures. This characteristic is fairly rare in the *Streptomyces* genus and was the origin of the proposed species name for this strain.

It was found that *S. avermitilis* shared its spiral spore chain morphology with 19, but shared its smooth spore surface ornamentation with 18 and 32 (and *S. cinnabarinus*). *S. kunmingensis* differed remarkably from all three isolates due to the formation of sclerotia. There were 5 differences shown between *S. avermitilis* and all three strains, including production of diffusible pigments on ISP#5 and melanin production on ISP#6. Negative results for arbutin and L-tyrosine degradation and a positive result for xanthine decomposition also contributed to this total. Differences in growth on carbon source plates were also shown, but contradictory results from two different references excluded the use of these results for comparison. Four differences were observed between the isolates and *S. cinnabarinus*,

including the ISP#5 and ISP#6 results, and the inability of *S. cinnabarinus* to degrade starch and a positive result for the xanthine test. For the comparison of *S. kunmingensis* against strains 18, 19 and 32, the results obtained during the project were used, despite there being contradictory results in other published work. Like strains 18 and 32 only, *S. kunmingensis* was able to reduce nitrate and decompose allantoin, but it shared a lack of urea decomposition with isolate 19. Two differences were found when comparing all three strains to *S. kunmingensis* – the inability of the latter to decompose starch and a lack of growth on sucrose.

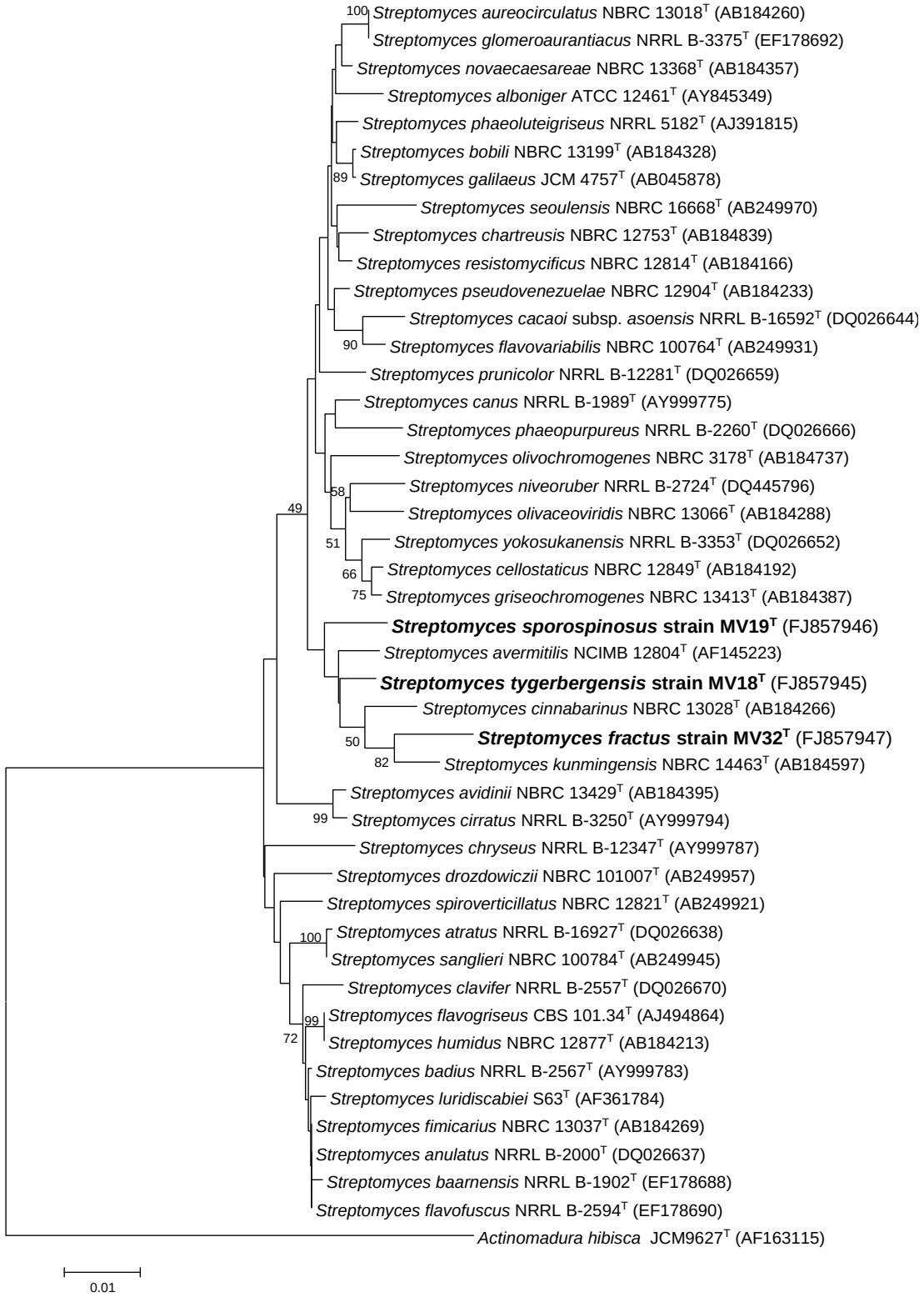

Figure 3.10: Neighbour-joining phylogenetic tree based on 1441 bp of 16S-rRNA gene sequence, showing the phylogenetic position of strains 18 (MIIX7C1), 19 (MIIX7C2) and 32 (MIIX7P4) amongst 41 of their closest phylogenetic relatives from the *Streptomyces* genus. GenBank sequence accession numbers are shown in parentheses. Bootstrap support for each node is indicated as a percentage, calculated from 1000 randomly re-sampled datasets (only values above 48% are shown). The scale bar indicates 1 nucleotide substitution per 100 nucleotides. *A. hibisca* JCM 9627^T was used as an outgroup.

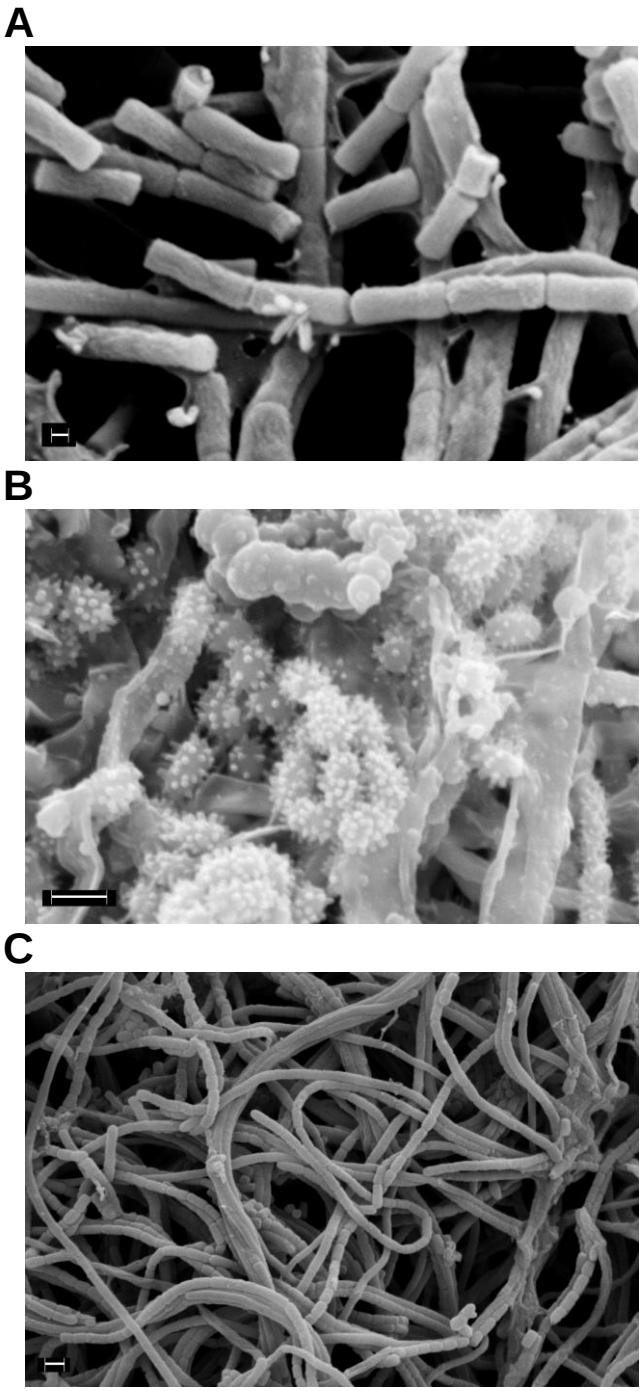

Figure 3.11: Scanning electron micrographs showing *Rectiflexibiles* spore chain morphologies and smooth spore surface ornamentations for both isolates 18 (A) and 32 (C), compared with the *Spirales* spore chains and spiny spore surface ornamentation exhibited by isolate 19 (B). All isolates were grown on ISP#4 for 14 days at 30°C. Bars represent 200nm (A) and 1µm (B & C).

Table 3.7: Physiological comparison between *Streptomyces* strains 18 (1), 19 (2) and 32 (3) and their closest phylogenetic relatives *S. avermitilis* MA-4680^T (4), *S. cinnabarinus* ISP 5467^T (5) and *S. kunmingensis* ATCC 35682^T (6).

	1	2	3	4	5	6
Spore surface ornamentation	smooth	spiny	smooth	smooth[b,c]	smooth[b,c]	N/D
Spore chain morphology	straight	spiral	straight	spiral[b,c]	flexuous[b,c]	sclerotia, loose spirals[a]
Aerial mycelium colour (ISP#4)	white	pale grey	white	grey[b,c] (oatmeal agar)	red[b,c] (oatmeal agar)	yellowish-white[a]
Substrate mycelium colour (ISP#4)	cream	brownish-cream	yellow-white	dark brown/tan	N/D	yellowish-tan/dark orange[a]
Production of diffusible pigment on:						
ISP#5	–	–	–	+[c] (yellow)?	+[c] (red)?	–
Production of melanin on:						
ISP#6	–	–	–	+[b,c]	+[b,c]	–
Reduction of:						
Nitrate	+	–	+	N/D	N/D	+
Decomposition of:						
Allantoin	+(w)	–	+	N/D	N/D	+
Arbutin	+	+	+	–[b]	+[b]	+
Gelatin	+	+	+	+[b,c]	N/D	+ (–[a])
Starch	+(w)	+	+	+[b,c]	–[b]	– (+[a])
L-Tyrosine	+	+	+	–[b]	N/D	+
Urea	+(w)	–	+	N/D	N/D	–
Xanthine	–	–	–	+[b,c]	+[b]	– (+[a])
Growth on sole carbon source:						
L-(+)-Arabinose	+	+	+	+[b,c]	–[b] +[c]	+(w)
Fructose	+	+	+	+[b,c]	–[b] +[c]	+
myo-Inositol	+	+	–	+[b,c]	–[b] +[c]	–
Sucrose	+	+	+	–[b] +[c]	+[b,c]	–
D-(+)-Xylose	+	+	+	+[b,c]	–[b] +[c]	+

Unless otherwise indicated, all results were obtained during this study. N/D = Not determined, +(w) = weakly positive; a = Ruan *et al*. (1985), b = Kim & Goodfellow (2002), c = Takahashi *et al*. (2002).

Based on the numerous differences between the isolates themselves and *S. avermitilis*, *S. cinnabarinus* and *S. kunmingensis*, DDH analysis was performed in various combinations for all strains. The decision of which combinations would be necessary was based on pairwise 16S-rRNA gene sequence alignments between each of the isolates and their closest relatives. The results for the pairwise alignments, as well as the final DDH similarities are combined into Table 3.8.

Table 3.8: Pairwise 16S-rRNA gene sequence alignment results and their associated DNA-DNA hybridisation similarities (in parentheses) for isolates 18, 19 and 32, against their closest phylogenetic neighbours.

	Strain 18	**Strain 19**	**Strain 32**
Strain 19	98.18% (**7.35** ± 6.01**%)**	N/A	97.1% (**18.35** ± 0.78**%**)
Strain 32	97.57% (**4.1** ± 2.40**%**)	N/A	N/A
***S. avermitilis* NRRL 8165^T**	96.62% (**19.45** ± 2.33**%**)	96.44%	95.03%
***S. cinnabarinus* NRRL B-12382^T**	97.36% (**4.7** ± 4.10**%**)	97.43% (**24.5** ± 0.99**%**)	97.15% (**10.35** ± 2.90**%**)
***S. kunmingensis* NRRL B-16240^T**	98.15% (**29.65** ± 3.75**%**)	97.46% (**1** ± 1.13**%**)	98.07% (**14.4** ± 0.85**%**)

N/A = Not applicable.

Table 3.8 clearly shows that all DDH values were well below 70% (the threshold value for the definition of bacterial species [Wayne *et al.*, 1987]) and thus, isolates 18, 19 and 32 can be considered to represent three new species within the genus *Streptomyces*. The names *Streptomyces tygerbergensis*, *Streptomyces sporospinosus* and *Streptomyces fractus* are proposed for strains 18, 19 and 32, respectively:

Streptomyces tygerbergensis: ty.ger.berg.en'sis. N.L. masc. adj., pertaining to Tygerberg, the name of the nature reserve from which it was isolated. *Streptomyces sporospinosus*: spo.ro.spi.no'sus. Gr. n. spora, seed, and in biology a spore; L. adj. spinosus, thorny, spiny; N.L. masc. adj. *sporospinosus*, spiny spore – pertaining to its spiny spore surface ornamentation. *Streptomyces fractus*: frac'tus. L. masc. adj., fractus, broken – a description of its ability to fragment in liquid culture.

3.4.2.6 Isolate 14

The GenBank BLAST analysis found strain 14 to be most closely related to *Streptomyces atratus* NRRL B-16927^T and *Streptomyces sanglieri* NBRC 100784^T (both 99% similar). Phylogenetic analysis showed that the type strains of *S. atratus*, *S. sanglieri*, *Streptomyces gelaticus* NRRL B-2928^T and *Streptomyces pulveraceus* NBRC 12783^T are the closest phylogenetic neighbours of isolate 14. As shown in Fig. 3.12, the bootstrap support for the separation of 14, *S. atratus* and *S. sanglieri* from the other two species is moderately high at 78%. Pairwise 16S-rRNA gene sequence comparisons between isolate 14 and its closest relatives showed that the sequence similarities to the type strains of *S. atratus*, *S. sanglieri*, *S. gelaticus* and *S. pulveraceus* were 99.73%, 99.66%, 99.32% and 99.45%, respectively.

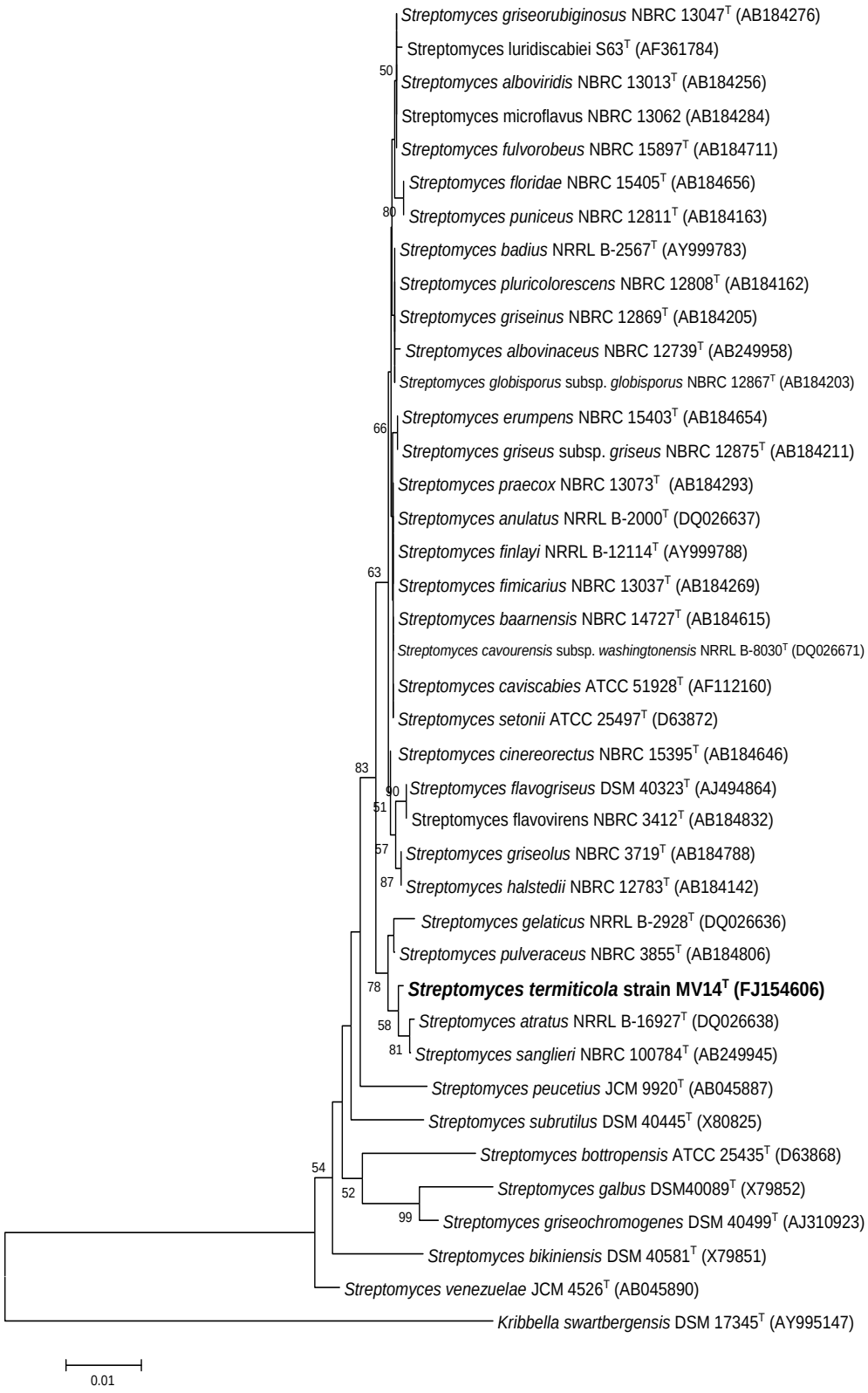

Figure 3.12: Neighbour-joining phylogenetic tree based on 1426 bp of 16S-rRNA gene sequence, showing the position of strain 14 (MIIC7P1) amongst its closest phylogenetic neighbours. GenBank sequence accession numbers are shown in parentheses. Bootstrap support for each node is indicated as a percentage, calculated from 1000 randomly re-sampled datasets (only values above 50% are shown). The scale bar indicates 1 nucleotide substitution per 100 nucleotides. *Kribbella swartbergensis* DSM 17345^T was used as an outgroup.

The chemotaxonomy results supported the placement of strain 14 within the *Streptomyces* genus, as the DAP isomer was found to be LL-DAP, and the whole cell sugar pattern had no diagnostic sugars (cell-wall Type I; Lechevalier & Lechevalier, 1970).

Isolate 14 shared the spiral spore chain morphology shown in Fig. 3.13 with *S. pulveraceus* and *S. sanglieri*, but not with *S. atratus* and *S. gelaticus*. Numerous physiological differences were found between strain 14 and *S. sanglieri* (see Appendix K and Table 3.9). These included the absence of diffusible pigments on ISP#5 and the absence of melanin production on ISP#7 for 14. Other differences included the inability of 14 to reduce nitrate and grow on sucrose as a sole carbon source, and its ability to produce H_2S. Differences between isolate 14 and both *S. atratus* and *S. sanglieri* included the ability of 14 to grow at 37^{o}C, as well as its inability to degrade allantoin, arbutin and urea. When comparing the growth of all three strains on sole carbon sources, isolate 14 was the only strain that was able to grow on L-(+)-arabinose and *myo*-inositol (weakly). It was also unable to grow on salicin. Comparisons with *S. gelaticus* and *S. pulveraceus* were more limited as these results were obtained from previous publications. However, 4 differences were shown between 14 and *S. gelaticus* in Table 3.9, including the inability of *S. gelaticus* to grow at 37^{o}C, a positive result for the decomposition of xanthine and growth on sucrose, as well as an inability to grow on D-(+)-raffinose, compared with 14. Five differences could be determined against *S. pulveraceus*. These were the production of melanin on ISP#6 and ISP#7 by *S. pulveraceus*, as well as the reduction of nitrate. *S. pulveraceus* was also able to grow on D-(−)-mannitol and salicin, whereas strain 14 could not.

Despite the high 16S-rRNA gene sequence similarity to its closest phylogenetic relatives, DDH analysis found isolate 14 to be a distinct species. When compared to *S. atratus* NRRL B-16927^{T}, the DNA reassociation value was found to be 35.45 ± 6.58%, and when compared to *S. sanglieri* DSM 41791^{T}, a value of 10.3 ± 0.42% was determined. These values are well below the 70% DNA reassociation cut-off point recommended by Wayne *et al.* (1987) for new genomic species. Based on the phylogenetic and phenotypic data presented, strain 14 is therefore proposed as a new species within the genus *Streptomyces* and is named *Streptomyces termiticola*:

Streptomyces termiticola: ter.mi.ti'co.la. L. n. termes -itis, a worm that eats wood, a woodworm, a termite; L. suff. –cola (from Latin noun incola), inhabitant, dweller; N.L. n. *termiticola*, termite-dweller.

Table 3.9: Morphological and physiological differences between isolate 14 (1) and *S. atratus* NRRL B-16927^T (2), *S. gelaticus* NRRL-B2928^T (3), *S. pulveraceus* NBRC 3855^T (4) and *S. sanglieri* DSM 41791^T (5).

	1	2	3	4	5
Spore chain morphology	spiral	loops[d]	straight[a]	spiral[c]	spiral[b]
Production of diffusible pigment on:					
ISP#5	–	–	–[a]	N/D	+
Production of melanin on:					
ISP#6	–	–	–[a]	+[a]	–
ISP#7	–	–	–[a]	+[a]	+
Growth at:					
37^{o}C	+	–	–[a]	N/D	–
Growth in presence of:					
5% NaCl	+	+(w)	N/D	N/D	+
Reduction of:					
Nitrate	–	–	N/D	+[c]	+
Production of:					
H_2S	+	+	N/D	N/D	–
Decomposition of:					
Allantoin	–	+	N/D	N/D	+
Arbutin	–	+	N/D	N/D	+
Cellulose	–	–	N/D	NG[c]	–
Urea	–	+	N/D	N/D	+
Xanthine	–	–	+[a]	N/D	–
Growth on sole carbon source:					
Glucose	+	+	N/D	+(w)[c]	+
L-(+)-Arabinose	+	–	N/D	+(w)[c]	–
Fructose	+	+(vw)	N/D	+[c]	+
D-(+)-Galactose	+	+	N/D	+[c]	+
myo-Inositol	+(w)	–	+[a]	+(w)[c]	–
D-(–)-Mannitol	–	–	–[a]	+(w)[c]	–
D-(+)-Raffinose	+	+	–[a]	+[c]	+
L-(+)-Rhamnose	+	+	+[a]	+[c]	+
Salicin	–	+	N/D	+[c]	+(w)
Sucrose	–	–	+[a]	–[a]	+
D-(+)-Xylose	+	+	N/D	+[c]	+

Unless otherwise indicated, all results were obtained during this study. N/D = Not determined; NG = No growth; +(w) = weakly positive; +(vw) = very weakly positive; a = Locci (1989); b = Manfio *et al.* (2003); c = Shibata *et al.* (1961); d = Shibata *et al.* (1962).

The new standards for the publication of new species re-emphasise the importance of chemotaxonomic data (Tindall *et al.*, 2009). This includes not only DAP and sugar analysis, but also an investigation of the fatty acid profile, the menaquinone profile and the phospholipids of a new species. These criteria have only recently been published and, prior to the new standards being implemented, the description of isolate 14 was ready for submission to the *International Journal of Systematic and Evolutionary Microbiology*. In order to now submit this paper, the full set of chemotaxonomic analyses will first have to be performed on isolate 14.

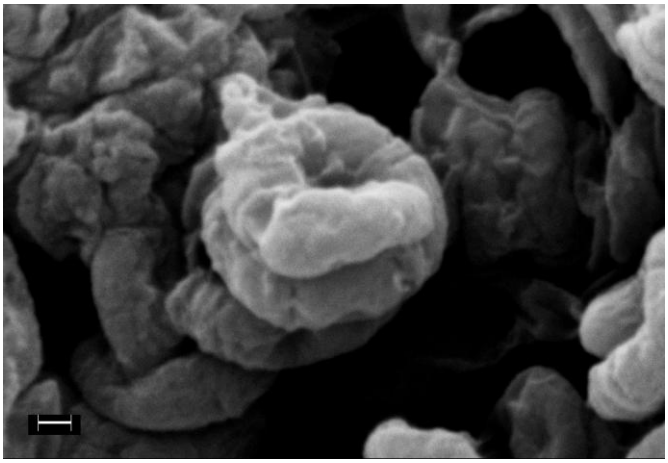

Figure 3.13: Scanning electron micrograph of *Streptomyces* isolate 14 (grown on ISP#2 at 30°C for 14 days) showing its smooth spore surface ornamentation and *Spirales* spore chain morphology. The bar represents 300nm.

3.4.3 Strain de-replication

The use of three restriction enzymes for the de-replication of the *M. viator* hindgut actinobacterial isolates proved to be very useful in discriminating potentially novel strains from those that had already been investigated further. As can be seen in Appendix L, the *Hpa*II enzyme was only able to separate the isolates into 5 unique patterns, making it useful only for separation of strains into major groups. Enzyme *Cfo*I provided the best resolution across all isolates, confirming the phylogenetic separation shown already for the sequenced isolates. A total of 20 unique digestion patterns could be discerned using this enzyme. *Hinf*I was fairly limited in the number of patterns that were observed (a total of 12 patterns), but its usefulness was mainly due to its ability to separate isolates with the same *Hpa*II and *Cfo*I patterns, and also to provide unique combinations of patterns with the other two enzymes in order to find strain diversity that was not evidenced by the individual enzymes alone.

Strain de-replication based on restriction endonuclease patterns has been used successfully on many occasions and even has applications in clinical settings, such as when the enzymes *Hae*III and *Hpa*II were used by Hall *et al.* (2001) in their study to confirm placement of 176 fresh and 299 stored clinical isolates within the *Actinomyces* genus. A similar method was used by Lanoot *et al.* (2005) in their investigation of 463 *Streptomyces* and *Kitasatospora* type strains, where they made use of 16S-rRNA gene restriction fragment length polymorphism fingerprinting to delineate the strains being compared (combining restriction digestion patterns using enzymes *Bst*UI and *Hae*III). They concluded from their results that this method had a higher resolution than that of 16S-rRNA gene sequencing, thereby

providing a potential tool for reducing the number of DNA-DNA hybridisations necessary for discovering potentially new species within the *Streptomyces* genus.

For the work shown in Appendix L, as with Lanoot *et al.* (2005), a good correlation between the restriction enzyme profiles and the phylogeny revealed by 16S-rRNA gene sequencing was found. This only applied to the first 19 isolates shown in Appendix L, as these were the only isolates that had been sequenced. Novel patterns for all three enzymes were obtained for isolates 5 and 33, with the rest of the members of their groups sharing the same patterns as isolates 5 and 33, respectively. The same applied for both *Nocardia* isolates. Strain 14 was the only other isolate to have a unique pattern for all three enzymes. Isolate 13 had unique *Cfo*I and *Hinf*I patterns and was separated from the similar 16 and 37 isolates due to their different *Hpa*II patterns. Isolates 18 and 19 also had unique *Cfo*I and *Hinf*I patterns, whereas strain 32 shared its *Hinf*I pattern with isolate 19, but was separated from the other two because of a unique *Cfo*I pattern. Isolates 2 and 7 were found to be unique from all other sequenced isolates due to novel *Cfo*I and *Hinf*I patterns for strain 2 and a unique *Cfo*I pattern only for isolate 7. All of these separations agree with the separations and similarities shown in the phylogenetic trees shown in sections 3.4.1 and 3.4.2.

In terms of identifying which isolates should be investigated further as potentially novel species, the following observations were made. In some cases, as with the sequenced isolates, some of the isolates had completely unique enzyme profiles for two or all three enzymes and, as such, were immediately identified as potentially novel isolates. In some cases, only a unique pattern for one enzyme would separate an isolate from all others. The last group of isolates that were judged to be worthy of further work were those that did not have unique patterns, but had unique combinations of enzyme patterns that had not been seen for any other isolate. For the Originals isolates, all had the same *Hpa*II pattern as isolate 14. Isolate 4 was the only one to have two unique patterns. Eight isolates had a single unique pattern, comprising isolates 9 and 25 which were separated because of their *Hinf*I pattern, and 8, 10, 15, 23, 29 and 30 because of a unique *Cfo*I profile. Isolates 17, 24 and 32 were identified based on a unique combination of patterns – for example 17 had pattern Cf10 combined with Hi5, whereas 24 had the combination Cf32 and Hi5. The Duplicates isolates had a mix of Hp5 and Hp14 patterns for the *Hpa*II digests. Only one isolate out of all the other unsequenced Originals and the Duplicates had three unique enzyme patterns – isolate 51. No Duplicates had two unique patterns, but isolates 50 and 60 were both separated from the others because of their single unique *Cfo*I patterns. Seven isolates were dereplicated based on novel combinations, namely, isolates 39D, 40, 45, 49, 52, 62 and 64.

A total of 12 Originals and 10 Duplicates isolates were therefore identified as being different enough to warrant further investigation into their potential as a new *Streptomyces* species. Work on these isolates should follow in order of the uniqueness of their patterns. Investigation should start with those that had two or three unique enzyme patterns, followed by those with a novel pattern for only one enzyme, and lastly for those with unique enzyme pattern combinations. Therefore, the original strain de-replication based on colonial colour and morphology was not that sensitive, as it missed some of the strain diversity.

This strain de-replication method was carried out in order to help determine the next step in this work, by using a quick restriction endonuclease-based process that was able to identify those isolates that might be novel, without having to resort to the full 16S-rRNA gene sequencing option. This method therefore provides a simple and effective way of screening a fairly large quantity of isolated strains for novelty. In this instance, the availability of already sequenced strains allowed the effectiveness of the de-replication method to be assessed – for example, the *Hpa*II digests were able to separate Groups 5 and 33 from each other, confirming the separation already made from the sequencing data. This same enzyme and the specific patterns obtained for isolates 13, 16 and 37 was also able to confirm the separation of all three isolates from all other strains, as well as being able to separate 13 from strains 16 and 37. The *Cfo*I and *Hinf*I enzymes were also able to confirm separation of isolate 32 from 18 and 19, whereas all three enzymes in combination were able to confirm that strains 2 and 7 were also unique from all other isolates (a result that, without the use of the de-replication method, would only have been possible with the use of 16S-rRNA gene sequencing).

3.5 References

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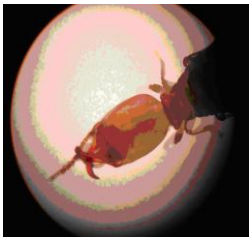
CHAPTER 4



CULTURE-INDEPENDENT ANALYSIS OF THE ACTINOBACTERIAL DIVERSITY IN THE HINDGUT OF *M. VIATOR*

CHAPTER 4

CULTURE-INDEPENDENT ANALYSIS OF THE ACTINOBACTERIAL DIVERSITY IN THE HINDGUT OF *M. VIATOR*

CONTENTS

CHAPTER 4

CULTURE-INDEPENDENT ANALYSIS OF THE ACTINOBACTERIAL DIVERSITY IN THE HINDGUT OF *M. VIATOR*

4.1 ABSTRACT

A total of 326 16S-rRNA gene clones was obtained for the culture-independent analysis of the actinobacterial diversity in the colon and paunch (P3b) hindgut regions of *M. viator* (both workers and soldiers) and the soil surrounding the mound from which the specimens were collected. PCR amplification using the actinobacterial-specific 16S-rRNA gene primers S-C-Act-0235-a-S-20-F and S-C-Act-0878-a-A-19-R, and subsequent ARDRA analysis of the products using the restriction enzymes *Alu*I and *Rsa*I revealed 31 unique patterns from the paunch, 55 from the colon and 49 from the surrounding soil. Fifteen of these patterns were common to two or more of the isolation locations. A total of 222 of the 326 clones was sequenced, (58, 83 and 81 clones from the paunch, colon and surrounding soil samples, respectively).

A calculation of the coverage index as well as rarefaction analysis for each sample revealed that the majority of the diversity from all samples was represented in the clone libraries. The Shannon-Wiener diversity index revealed that the surrounding soil sample (3.69) was the most diverse of all three, followed closely by the colon (3.29) and lastly the paunch sample (3.14). However, according to a summary of the BLAST analyses against the GenBank database, the colon was found to have the highest number of unique clones, with 67% of these having less than 97% sequence similarity to all other GenBank sequences.

The clone diversity within each sample (revealed from the sequencing and BLAST results) was summarised in pie chart format, and was used in the comparison of the samples. The paunch sample was dominated by clones with no nearest cultured relative, as well as three filamentous actinobacterial families – *Thermomonosporaceae* (15.52% of all clones from the paunch sample), *Streptomycetaceae* (10.34%) and *Micromonosporaceae* (8.62%). In contrast, the colon sample was dominated by members of the family *Propionibacteriaceae* (46.99%). The surrounding soil sample was dominated by three main groups – 23.46% uncultured

bacteria, 12.35% unknown (clones with no specific taxonomic designation) and 13.56% *Nocardioidaceae*. In comparison to both the paunch and colon, the surrounding soil showed much greater filamentous actinobacterial diversity, with 13 families, including 21 genera (11 of which were filamentous groups), as opposed to the 10 families and 10 genera (3 filamentous) discovered for the paunch, and 14 families and 20 genera (5 filamentous) identified from the colon.

A phylogenetic analysis was also performed, involving the generation of a neighbour-joining tree based on 435 bp of common 16S-rRNA gene sequence, showing a selection of the total diversity of all clones, with all relevant genus type species as well as a selection of termite-specific sequences obtained from the GenBank database. For the non-filamentous actinobacteria, there was a distinct separation of the colon and paunch clones from their surrounding soil counterparts, whereas for the filamentous actinobacteria, there appeared to be less obvious clustering of the different samples into separate groups. The phylogenetic results also served to emphasise the wide diversity of actinobacteria associated with the hindgut and surrounding soil of the *M. viator* termite.

A separate phylogenetic tree was generated to include the culture-independent streptomycete clones as well as the cultured *Streptomyces* representatives and their closest relatives. Here, a clear separation of colon and paunch clones from surrounding soil streptomycete clones lent support for the proposal of a termite hindgut-specific actinomycete population.

4.2 INTRODUCTION

The discovery of new microbes and the characterisation of their functions are some of the main goals in the study of microbial diversity. Traditionally, estimation of microbial diversity was based solely on the cultivation and subsequent characterisation of culturable strains. However, there is an inherent bias associated with culture-based studies, as it has been estimated that 99% of bacteria cannot be cultured under standard laboratory conditions (Babalola *et al.*, 2009). It is therefore a widely accepted fact that culture-based techniques only allow for the isolation of a small fraction of the bacteria present within any environment being sampled (Cardenas & Tiedje, 2008; Xin *et al.*, 2008; Babalola *et al.*, 2009). Recent advances in molecular biology, in particular metagenomic studies of environmental samples, have allowed microbial ecologists to access previously uncharacterised microbial diversity. Metagenomics now provides a gene-based exploration of the community as a whole, while isolation and cultivation continue to provide an important function in the testing of metabolic activities and allow for detailed genomic studies of individual members of these bacterial communities. The use of the 16S-rRNA gene in particular has led to great advances in the understanding of population structures within natural microbial communities (Cardenas & Tiedje, 2008; Xin *et al.*, 2008; Babalola *et al.*, 2009).

It is important to recognise the advantages and limitations of both cultivation-based and culture-independent approaches for studying the actinobacterial diversity in various environments (Xin *et al.*, 2008). Metagenomic studies are also prone to the bias that is introduced by differences in DNA extraction efficiency, PCR bias because of differences in primer binding, and the bias caused by sequence heterogeneity due to different copy numbers of 16S-rRNA genes. One of the limitations that is shared by both culture-based and culture-independent techniques is that they are unable to determine the role that each phylotype plays within a specific environmental niche (Schmitt-Wagner *et al.*, 2003a; Babalola *et al.*, 2009). However, despite these limitations, the PCR-based approach remains one of the most powerful tools for determining the phylogenetic diversity within complex microbial communities (Xin *et al.*, 2008).

The problems with cultivation from unusual or extreme environments are particularly relevant to the study of the termite gut. Termite gut bacteria require strict environmental conditions for their survival and reproduction, and therefore culturing techniques are often inadequate for their study. Also, comparison of the microbial communities between different termite species by cultivation-based methods alone is less informative, as these methods are only able to reveal a small proportion of each community (Brauman *et al.*, 2001; Fisher *et al.*, 2007). Culture-independent studies have provided an alternative, as these methods provide an

effective tool for the study of the species richness found in the termite gut. Analyses of 16S-rRNA gene sequence and fingerprinting data have proven particularly useful in providing a steady flow of information on the species richness of the gut bacteria in a wide variety of termite species For example, recent examinations of the gut microbiota of one of the most well studied termite genera, *Reticulitermes*, revealed that the majority of the recovered 16S-rRNA gene sequences represented novel and as yet uncultured species (Brauman *et al.*, 2001; Fisher *et al.*, 2007).

Various culture-independent analyses have been performed on a wide range of different termite guts, ranging from the phylogenetically higher termites, to the lower termites, and also from a range of diets, including soil and wood-feeders. It has been shown that the termite hindgut is a highly complex and compartmentalised system, containing an abundance and wide diversity of microbes, each involved in some way in the metabolic activities taking place there (Schmitt-Wagner *et al.*, 2003a).

In this chapter, the actinobacterial diversity found inside the colon and paunch (P3b) hindgut regions of *M. viator* was assessed in comparison to that found in the surrounding soil environment, using a culture-independent approach. This metagenomic study served to complement the culture-based analysis of the actinomycetes found within the same gut system, described in previous chapters.

4.3 MATERALS AND METHODS

4.3.1 Sample collection and termite dissection

Three separate sampling sites were chosen for the culture-independent analysis – paunch, colon, as well as a composite soil sample from the area surrounding the termite mound. Twenty *M. viator* termites (combining worker and soldier representatives) were collected from Tygerberg Nature Reserve, Cape Town, as well as ~1g of soil from each of 10 sample sites in a perimeter, with a radius of 1 metre, around the colony mound on the 11 April 2008. Each soil sub-sample was collected from a depth of approximately 1cm.

The same termite dissection protocol described in section 2.3.2 was used, except that all colon and paunch regions were combined and suspended in separate microfuge tubes containing 500µl phosphate buffer (10 mM $Na_2HPO_4.2H_2O$, 1.8 mM KH_2PO_4; pH 7.0).

4.3.2 DNA extraction, 16S-rRNA gene PCR amplification and cloning

0.25g of soil was taken from each soil sub-sample and combined to provide the composite surrounding soil sample. This composite sample, as well as the 500µl colon and paunch suspensions was used for DNA extraction using the ZR Soil Microbe DNA Kit™ (Zymo Research). This was followed by DNA purification using a Wizard® DNA Clean-up System (Promega). The soil sample had to be divided into two tubes during DNA purification, but the DNA was combined afterwards into one 100µl volume. The paunch and colon samples were purified separately and the DNA re-dissolved in the recommended 50µl volume.

General bacterial primers F1 and R5 were used for the initial 16S-rRNA gene amplification as described by Cook & Meyers (2003), followed by a previously described 'touchdown', nested PCR protocol on the amplified DNA using specific actinobacterial 16S-rRNA gene primers S-C-Act-0235-a-S-20-F and S-C-Act-0878-a-A-19-R that amplify a ~640 bp stretch of the 16S-rRNA gene (Stach *et al.*, 2003). These primers were chosen because of their frequent citation in the literature, as well as their proven ability to amplify sequences from almost all actinomycetes. The PCR samples were set up in 50µl volumes containing 1.5mM $MgCl_2$, 0.5µM of each primer, 150µM of each dNTP, 0.5U Super-Therm *Taq* DNA polymerase (JMR Holdings, U.S.A.) and 100ng DNA template. The PCR protocol consisted of an initial denaturation at 95°C for 4min, followed by denaturation at 95°C for 45s, annealing at 72°C for 45s and extension at 72°C for 1min. During the first 10 cycles, the annealing temperature was decreased by 0.5°C per cycle. The program for the next 15 cycles was 95°C for 45s, 68°C for 45s and 72°C for 1min, followed by a 5min extension at 72°C. All PCR reactions were performed in a Techne TC-512 gradient thermal cycler.

PCR products were electrophoresed in 1% agarose gels containing 0.8µg/ml ethidium bromide in 1xTAE buffer, before being excised from the gel and purified using a Talent TA050CLN Cleanmix Kit (Talent, Italy). This DNA was then cloned using the pGEM®-T Easy Vector System (Promega). *Escherichia coli* DH5α competent cells were used for all transformations. All putative recombinant clones (identified by blue-white screening) were screened for inserts by colony PCR in 25µl volumes, using a KAPA2G Robust PCR Kit (KapaBiosystems, Cape Town, South Africa), including 0.5U KAPA2G Robust DNA polymerase and 0.5µM of each of primers S-C-Act-0235-a-S-20-F and S-C-Act-0878-a-A-19-

R. The colony PCR products were run on 1% agarose gels containing 0.8µg/ml ethidium bromide in 1xTAE buffer, at 85V for 1-2 hours before visualisation using a GelDoc (BioRad).

4.3.3 Clone de-replication/ARDRA

Amplified ribosomal DNA restriction analysis (ARDRA) was performed in order to group all clones from the clone libraries generated for all three sample sites and minimise the number of strains selected for sequencing. The clone library de-replication followed the same protocol as that used for the de-replication of the Originals and Duplicates strains, described in section 3.3.2. However, the restriction enzymes used here were *Alu*I and *Rsa*I. Originally *Hinf*I was also to be included as part of the analysis but, after an initial pilot study using all three enzymes, it was determined that the use of *Alu*I and *Rsa*I only was sufficient for the purpose of de-replication. *Alu*I patterns could be used to assign the clones to major groupings, and *Rsa*I patterns were most useful for differentiating novel clones within the large *Alu*I groups. All digests were run on 1.5% agarose gels containing 0.8µg/ml ethidium bromide in 1xTAE buffer. Clone restriction patterns were assigned by eye to *Alu*I and *Rsa*I groups based on unique banding patterns.

4.3.4 Sequencing and phylogenetic analysis

Based on the results obtained from the clone library de-replication, a number of clones were selected for sequencing. All PCR product purifications and subsequent sequencing were performed as described in section 2.3.7.3. However, only the S-C-Act-0235-a-S-20-F primer was used for sequencing of the clones, resulting in a short ~640 bp region of the 16S-rRNA gene sequence that was sufficient for BLAST analysis to determine the clones' taxonomic affiliations.

All neighbour-joining (Saitou & Nei, 1987) phylogenetic trees were generated using the MEGA version 4 software program (Tamura *et al.*, 2007), based on common regions of the 16S-rRNA gene sequence and were generated in order to determine the phylogenetic associations between various combinations of *M. viator* cultured strains and cloned sequences from all three samples, as well as sequences obtained from the GenBank database for published type strains and termite associated uncultured strains.

4.3.5 Statistical analyses

The majority of the statistical results were generated based on the number of confirmed insert-containing clones obtained during cloning, and the associated ARDRA patterns that were found for each of these clones. All calculations and plots were performed for all three sample sites – paunch, colon and surrounding soil.

The coverage index (*C*) (Taton *et al.*, 2003) was calculated using the following equation:

$$C = (1-n/N) \times 100$$

where *n* is the number of phylotypes (unique clone ARDRA patterns) composed of a single clone and *N* is the total number of clones.

The Shannon-Wiener diversity index (*H'*) (Good, 1953; Taton *et al.*, 2003) was calculated as follows:

$$H' = -\sum (p_i \times \ln p_i)$$

where p_i is the relative abundance of each species (clone) calculated as the proportion of individuals of a given species to the total number of individuals in the community: $p_i = n_i/N$ (n_i = number of clones in each ARDRA pattern, N = total number of clones).

Two sets of rarefaction curves (Gotelli & Colwell, 2001) were plotted manually using Microsoft Excel for all three sample sites. The first involved a comparison of the number of clones versus the number of unique ARDRA patterns obtained for all clones within each sample. The second plot was generated with the number of sequenced clones from each sample site plotted against the number of those clones with less than 97% sequence similarity to all sequences within the GenBank database.

4.4 RESULTS AND DISCUSSION

4.4.1 Cloning results and ARDRA de-replication

A total of 326 clones was obtained from the three samples investigated during the culture-independent analysis. The majority of the insert-containing clones was from the colon, followed by the surrounding soil (SS) sample and lastly the paunch (see Table 4.1 for details). All were subjected to ARDRA de-replication, with the result that for *Alu*I digestion, 34, 12 and 14 unique colon, paunch and SS patterns were identified, respectively. Three different

*Alu*I groups included more than 30 clones, namely, AC5 (51 clones), AC1 (38) and AC10 (34) (see Appendix M). The remaining clones fell within groups ranging from single clone representatives to those with numerous members. As a result, it was determined that the use of *Alu*I was important in the initial clone separation. *Rsa*I was used in the next stage of the ARDRA de-replication, as the patterns obtained for this enzyme enabled confirmation of particular clones within the larger *Alu*I groups, or separation into their own *Rsa*I sub-group, thereby identifying a unique clone. Some *Rsa*I groups, such as RC5 (28 members), still contained high numbers of matching clones (see Appendix N for details), but a total of 55, 31 and 49 unique *Rsa*I patterns was identified for the colon, paunch and SS samples, respectively. This final separation formed the basis of the de-replication of all clones into their respective ARDRA patterns, and resulted in the identification of 135 unique phylotypes (see Table 4.1). A similar ARDRA approach was used by Fisher *et al.* (2007) in their investigation of the diversity of the gut bacteria inside *R. flavipes*, and they found 261 different ARDRA profiles from the 512 clones analysed.

Table 4.1: Metagenomic analysis summary including species richness (number of phylotypes), as well as coverage and diversity indices of the actinobacterial communities found within the different sample sites.

Sample site	No. clones obtained	No. phylotypes (unique ARDRA patterns)	No. clones sequenced	Coverage index (*C*)	Shannon Diversity index (*H'*)
Paunch	80	31	58	94.72	3.14
Colon	137	55	83	89.44	3.29
Surrounding soil	109	49	81	87.58	3.69
Total	326	135	222		

4.4.2 Clone sequencing, rarefaction analysis and coverage and diversity indices

All samples were calculated to have coverage indices above 80%, with the coverage index for the paunch sample highest at 94.72 (see Table 4.1 for details). These values indicated that the bacterial diversity within the clone libraries for each sample was sufficiently covered (any value greater than 60% is considered to be good coverage).

The larger ARDRA pattern groups often contained representative clones from all three sample sites (see Appendices M and N). Multiple clones from a particular group were chosen for sequencing, so as to provide a complete representation of the clones obtained in each one of the 135 unique ARDRA patterns. As shown in Table 4.1, 58 paunch clones were chosen

for sequencing, along with 83 and 81 clones from the colon and SS samples, respectively, making a total of 222 sequenced clones from the original 326.

Appendix O provides a table of all the results obtained after the sequence for each clone was submitted for BLAST analysis against the GenBank database. A summary of the sequence similarities recorded for each clone is provided in Table 4.2. The majority of the clones from the paunch sample fell within the 97-99% sequence similarity range, with 40% of the clones showing less than 97% sequence similarity. By comparison, the clones from the colon sample demonstrated a much greater level of apparent species richness, with 67% of the clones from this sample having less than 97% sequence similarity to all sequences within the GenBank database. However, 31 of these colon clones had the same BLAST match indicating that these particular clones are most probably representatives of the same abundant bacterium (it should be noted that these 31 clones did *not* have identical sequences; most of them fell within the three largest ARDRA groups, AC1, AC5 and AC10, but some had unique ARDRA patterns). Therefore in terms of the abundance of unique clones, the colon was higher than the other two samples, but in terms of actual species richness, the colon did not appear to be the most diverse sample. Only 33% of the colon clones fell within the 97-99% sequence similarity range. The SS sample had the greatest number of clones with 100% matches to the database sequences, and a similar number of clones falling within the <97% and 97-99% sequence similarity ranges, as that found for the paunch sample.

Table 4.2: Summary of the clone sequencing results, providing the number of clones from each sample that fell within particular similarity percentiles, according to the BLAST analyses against the GenBank database.

Sample site	**< 97%**	**97 – 99%**	**> 99%**	Total
Paunch	23 (40%)	33 (57%)	2 (3%)	58
Colon	56 (67%)	27 (33%)	0	83
Surrounding soil	33 (41%)	45 (55%)	3 (4%)	81

Note: The percentage of the total number of clones from within each sample is provided in parentheses.

Rarefaction analysis is used to compare and standardise species richness computed from samples with different 'population' sizes, and was therefore particularly useful for comparison of the colon, paunch and SS samples. The slope at the end of the rarefaction curve indicates the rate at which new phylotypes/diversity would be discovered if more sampling were to be done and whether new taxa can be expected if additional clones were to be analysed (Schmitt-Wagner *et al.*, 2003a; Yang *et al.*, 2005). The rarefaction curves in Fig. 4.1A, which were based on the number of unique ARDRA patterns, all showed an

exponential slope, indicating for all three samples that sampling of the clones for new phylotypes had not yet reached saturation, and that further sampling would identify additional unique clones. However, the data in Fig. 4.1B, which was based on the number of sequenced clones with less than 97% similarity (and was therefore more appropriate for the samples being analysed), suggested a completely different result. Here the slopes for all three sample sites were reaching saturation. For both paunch and SS samples, the slopes indicated that the total actinobacterial diversity of these samples had been sufficiently sampled (i.e. no additional diversity would have been uncovered if more clones were to be analysed). The result for the colon suggested that more sampling should be done to reveal additional actinobacterial diversity.

Overall, the rarefaction data, in combination with the coverage estimates, suggested that further sampling was not necessary as the library adequately represented the actinobacterial diversity and, according to the sequence-based rarefaction curve, little further novel diversity would be discovered if more clones were to be sampled from the paunch and surrounding soil samples (the rarefaction curve for the colon indicated that a significant amount of additional actinobacterial diversity remains to be identified in this region of the *M. viator* hindgut).

The Shannon-Wiener diversity index reflects the relative abundance of the different phylotypes in each sample site. The values calculated for each sample supported the suggestion that the colon sample was more diverse than the paunch, with a value of 3.29 calculated for the colon compared to the 3.14 obtained for the paunch. Therefore, according to these values, the diversity of the samples was lowest in the paunch, with the colon intermediate between the paunch and the surrounding soil (which had the highest diversity index of all three samples; Table 4.1). Both the coverage and diversity indices were calculated based only on ARDRA patterns (i.e. no calculations were performed using the sequencing results).

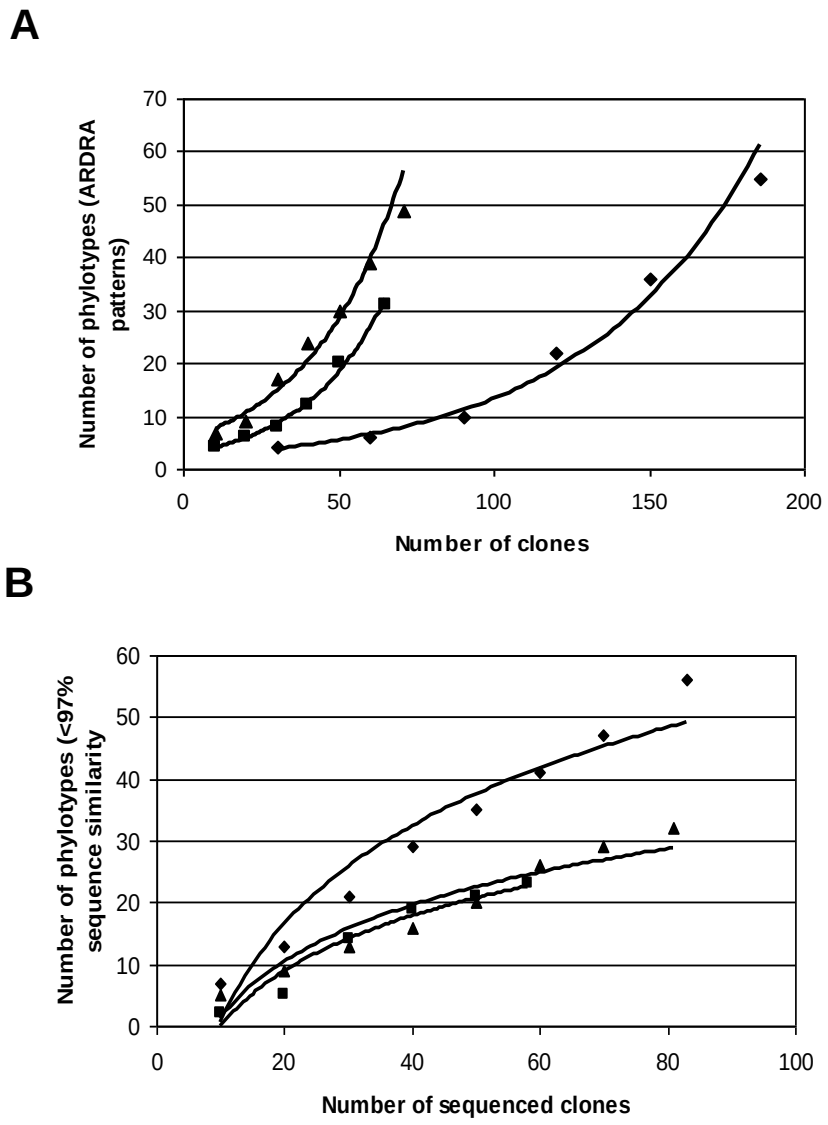
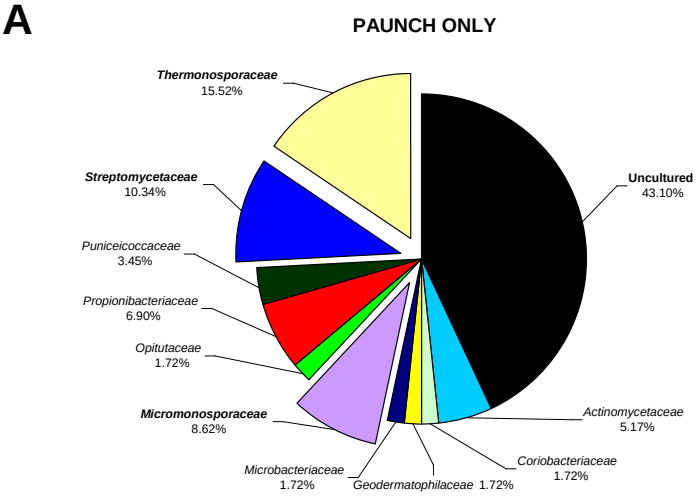


Figure 4.1: Rarefaction analysis of the clone libraries generated from the different sample sites: ♦ = colon, ■ = paunch, ▲= surrounding soil. The slope in A shows the rate at which new phylotypes (in the form of unique ARDRA patterns) were discovered at the point where sampling was stopped. B shows the same information, but is based on the number of sequenced clones with less than 97% BLAST sequence similarity.

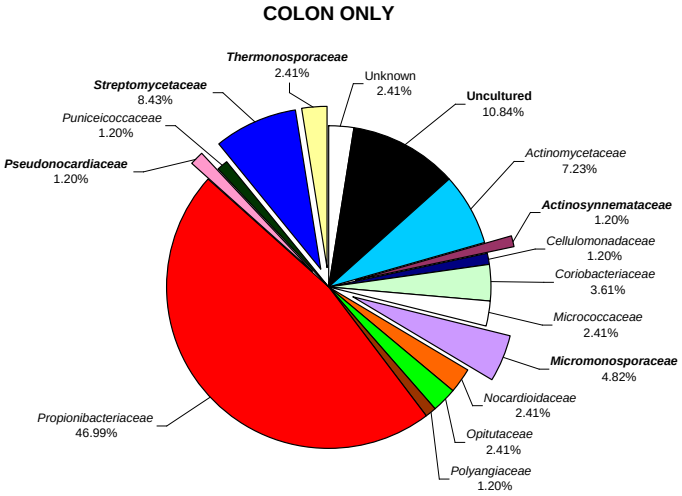
4.4.3 Clone diversity

Comparative sequence analysis revealed the presence of bacteria from numerous actinobacterial families among the hindgut (colon and paunch) microbiota of *M. viator*, as well as in the mound's surrounding soil. The following section is a comparison of these three samples, making use of the summaries of the diversity and taxonomy shown in Fig. 4.2 and Table 4.3, as well as the detailed sequencing results presented in Appendix O.

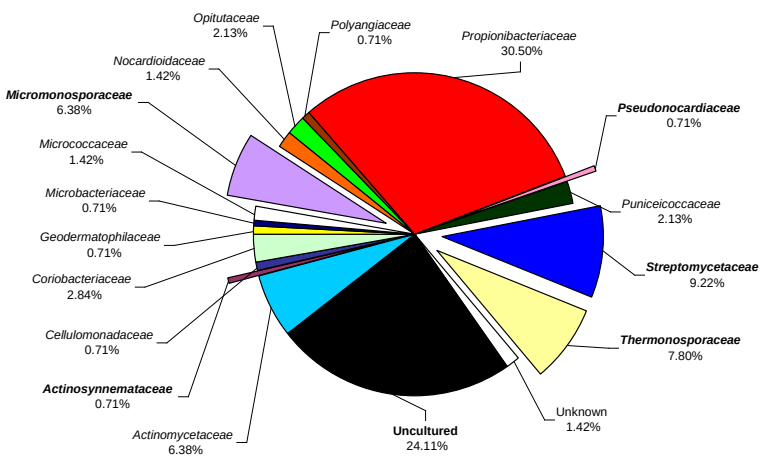
A


PAUNCH ONLY
Thermonosporaceae 15.52%
Streptomycetaceae 10.34%
Puniceicoccaceae 3.45%
Propionibacteriaceae 6.90%
Opitutaceae 1.72%
Micromonosporaceae 8.62%
Microbacteriaceae 1.72%
Geodermatophilaceae 1.72%
Coriobacteriaceae 1.72%
Actinomycetaceae 5.17%
Uncultured 43.10%

B


COLON ONLY
Thermonosporaceae 2.41%
Unknown 2.41%
Streptomycetaceae 8.43%
Uncultured 10.84%
Puniceicoccaceae 1.20%
Actinomycetaceae 7.23%
Pseudonocardiaceae 1.20%
Actinosynnemataceae 1.20%
Cellulomonadaceae 1.20%
Coriobacteriaceae 3.61%
Micrococcaceae 2.41%
Micromonosporaceae 4.82%
Nocardioidaceae 2.41%
Propionibacteriaceae 46.99%
Opitutaceae 2.41%
Polyangiaceae 1.20%

C


COLON AND PAUNCH
Opitutaceae 2.13%
Polyangiaceae 0.71%
Propionibacteriaceae 30.50%
Nocardioidaceae 1.42%
Micromonosporaceae 6.38%
Pseudonocardiaceae 0.71%
Micrococcaceae 1.42%
Puniceicoccaceae 2.13%
Microbacteriaceae 0.71%
Geodermatophilaceae 0.71%
Streptomycetaceae 9.22%
Coriobacteriaceae 2.84%
Thermonosporaceae 7.80%
Cellulomonadaceae 0.71%
Actinosynnemataceae 0.71%
Unknown 1.42%
Actinomycetaceae 6.38%
Uncultured 24.11%

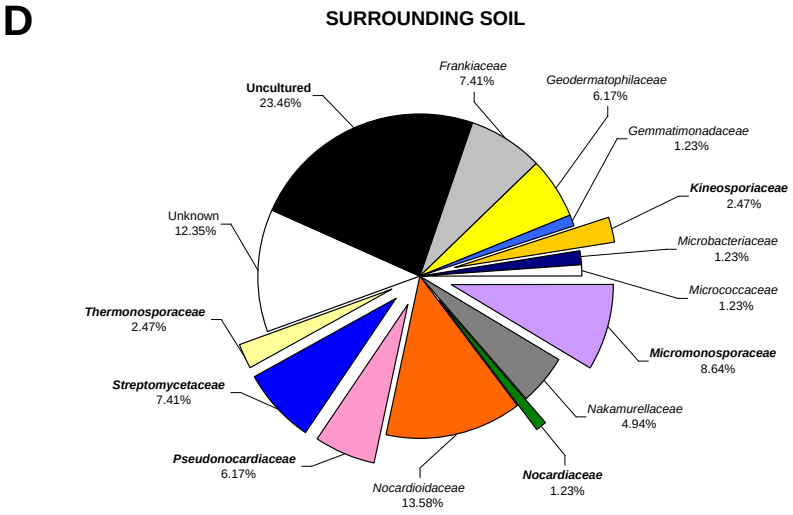


Figure 4.2: Clone diversity found within the paunch (A) and colon (B) of the *M. viator* hindgut, as well as in the surrounding soil (D), and reflected as a percentage composition of the total clones found within each sample. All filamentous actinobacterial groups have been highlighted in bold and sit outside of the main body of each chart.

4.4.3.1 Paunch

Whereas the colon clones were dominated by *Propionibacteriaceae*, in the paunch this group only represented 6.9% of the diversity, and was dominated by uncultured clones that have no nearest cultured relative. The presence of a large percentage of uncultured clones (see Fig. 4.2A) was not surprising as this trend had been reported elsewhere, where the majority of the phylotypes identified in metagenomic studies fell into uncultured classes (Babalola *et al.*, 2009).

The 43.1% matches to cloned sequences of uncultured bacteria (paunch; Fig. 4.2A) was mostly made up of uncultured *Acidobacteria*. Molecular phylogenetic work has also revealed the presence of this group during other surveys of termite gut microbes, including other phyla not well represented in culture such as *Verrucomicrobia*, *Planctomyces* and the *Elusimicrobia* (Wertz & Breznak, 2007). Surprisingly, also included in the *M. viator* paunch results were a number of uncultured *Treponema* sp. clones, which have been shown to be a major component of other termite gut samples (Ottesen *et al.*, 2006). An investigation of the gut of the termite *R. santonensis* showed that 9% of all assigned clones in the clone library could be assigned to this *Spirochaetes* group and, of these, the majority were sourced from the hindgut fluid and protozoa found there. This is to be expected as many species from this genus (*Treponema*) have been identified as obligate symbionts of the protozoa in the termite gut (Yang *et al.*, 2005; Fisher *et al.*, 2007). Another interesting component of this large

'uncultured' group included clones that were found to be closely related to *Candidatus* 'Tammella caduceiae' clones, as well as one clone related to an uncultured *Fibrobacteres* clone originating from a metagenomic study on the hindgut of a wood-feeding higher termite. The Tammella clones were generated as part of a study on the symbionts of a termite gut flagellate. These results support the suggestion that there is a significant proportion of the termite hindgut community that remains largely uncultured, hampered mainly by the general difficulty in culturing these organisms (Ikeda-Ohtsubo & Brune, 2009). However, Ikeda-Ohtsubo & Brune (2009) also suggest that the elucidation of their identity and phylogenetic affiliation has been substantially improved due to the use of culture-independent techniques using molecular markers. From the results presented here, it appears that there are a number of *Actinobacteria* that form part of this recalcitrant bacterial community.

Compared to the colon, Fig. 4.2A shows that the filamentous actinomycetes in the paunch made up a much larger proportion of the clone diversity. Despite the fact that there are no *Pseudonocardiaceae* or *Actinosynnemataceae* present in the paunch, the proportions of most other common groups were substantially higher. The *Thermomonosporaceae* made up 15.52% of the total clone diversity within the paunch, compared with 2.41% in the colon. The *Streptomycetaceae* represented 10.34% (8.43% in the colon), and the *Micromonosporaceae* diversity (8.62%) was almost double that found in the colon (4.82%). The *Actinomycetaceae*, which are represented by clones related to an *Actinomyces howellii* strain, were less abundant (5.17%) when compared with the 7.23% diversity shown by the colon. The other non-filamentous actinobacterial clones (from five families) made up 10.33% of the total clonal diversity – two families not seen in the colon were *Microbacteriaceae* (represented by the genus *Agromyces*) as well as *Geodermatophilaceae* (represented by *Blastococcus*).

4.4.3.2 Colon

The greatest proportion of the colon clone sequences was made up by the *Propionibacteriaceae* (46.99%; Fig. 4.2B). This group also made up the highest diversity with respect to the number of genera, including six different genera (see Table 4.3 for details). However, it must be noted that the vast majority of this group was made up of clones that all had the same BLAST hit result (closely matching a cultured *Propionicella superfundia* strain – see Appendix O) and may therefore represent clones of the same dominant microbe.

Other groups that made up a high proportion of the colon clones included the *Streptomycetaceae* (8.43%), *Actinomycetaceae* (7.23%), as well as uncultured clones with no nearest cultured representative. The *Streptomycetaceae* mainly represented clones related to a

strain of *Streptomyces globisporus* subsp. *globisporus*, whereas the *Actinomycetaceae* were represented by clones related to the same *A. howellii* strain mentioned for the paunch clones.

Fig. 4.2B shows that other filamentous actinobacteria represented in the colon included *Micromonosporaceae* (4.82%, represented by the genus *Micromonospora*), *Thermomonosporaceae* (2.41%, genus *Actinomadura*), *Actinosynnemataceae* (1.2%, genus *Actinokineospora*) and *Pseudonocardiaceae* (1.2%, genus *Pseudonocardia*). Together, the filamentous actinobacteria represented 18.06% of the total clone diversity. The remaining colon clones made up a 14.44% proportion of the total, and represented some lesser-known actinobacterial families, including genera such as *Eggerthella*, *Cellulomonas*, *Rothia* as well as the genus *Chondromyces* which falls within the class *Deltaproteobacteria*.

Despite the fact that the *Propionibacteriaceae* may only be represented by one microbe, when compared to the paunch results, the colon definitely showed more actinobacterial diversity, with 14 families and a total of 20 genera, compared to the 10 families and 10 genera found in the paunch sample (see Table 4.3 for details).

It is also clear that, whereas the non-filamentous actinobacteria dominate in the colon, the filamentous actinobacteria form the dominant component of the paunch. Previous studies on the diversity and community structure of soil-feeding and wood-feeding termites have shown that the different gut compartments (which provide distinct physicochemical environments) also harbour distinct microbial communities and are not merely a reflection of what is found in the soil (Schmitt-Wagner *et al.*, 2003a; 2003b; Yang *et al.*, 2005) This also appears to be the case for the actinobacterial population inside the hindgut of *M. viator*, with respect to the colon and paunch regions.

Table 4.3: Summary of the taxonomy of the clones discovered within each sample site, including suborder, family and representative genera.

PAUNCH ONLY

Suborder	Family	Genus
Actinomycineae	*Actinomycetaceae*	*Actinomyces*
Coriobacterineae	*Coriobacteriaceae*	*Eggerthella*
Frankineae	*Geodermatophilaceae*	*Blastococcus*
Micrococcineae	*Microbacteriaceae*	*Agromyces*
Micromonosporineae	*Micromonosporaceae*	*Micromonospora*
*Opitutales**	*Opitutaceae**	*Opitutus**
Propionibacterineae	*Propionibacteriaceae*	*Propionicimonas*
*Puniceicoccales**	*Puniceicoccaceae**	*Coraliomargarita**
Streptomycineae	*Streptomycetaceae*	*Streptomyces*
Streptosporangineae	*Thermomonosporaceae*	*Actinomadura*

COLON ONLY

Suborder	Family	Genus
Actinomycineae	*Actinomycetaceae*	*Actinomyces*
Coriobacterineae	*Coriobacteriaceae*	*Atopobium, Eggerthella*
Micrococcineae	*Cellulomonadaceae* *Micrococcaceae*	*Cellulomonas* *Rothia*
Micromonosporineae	*Micromonosporaceae*	*Micromonospora*
*Opitutales**	*Opitutaceae**	*Opitutus**
Propionibacterineae	*Nocardioidaceae* *Propionibacteriaceae*	*Friedmanniella* *Brooklawnia, Microlunatus, Propionicella, Propionicimonas, Propioniferax, Tessaracoccus*
Pseudonocardineae	*Actinosynnemataceae* *Pseudonocardiaceae*	*Actinokineospora* *Pseudonocardia*
*Puniceicoccales**	*Puniceicoccaceae**	*Coraliomargarita**
*Sorangiineae**	*Polyangiaceae**	*Chondromyces**
Streptomycineae	*Streptomycetaceae*	*Streptomyces*
Streptosporangineae	*Thermomonosporaceae*	*Actinomadura*

SURROUNDING SOIL

Suborder	Family	Genus
Corynebacterineae	*Nocardiaceae*	*Actinopolymorpha*
Frankineae	*Frankiaceae* *Geodermatophilaceae* *Kineosporiaceae* *Nakamurellaceae*	*Frankia* *Blastococcus, Geodermatophilus* *Kineococcus* *Humicoccus, Saxeibacter*
?	*Gemmatimonadaceae**	*Gemmatimonadetes**
Micrococcineae	*Microbacteriaceae* *Micrococcaceae*	*Leifsonia* *Arthrobacter*
Micromonosporineae	*Micromonosporaceae*	*Actinoplanes, Dactylosporangium, Salinispora, Virgisporangium*
Propionibacterineae	*Nocardioidaceae*	*Friedmaniella, Marmoricola, Nocardioides*
Pseudonocardineae	*Pseudonocardiaceae*	*Amycolatopsis, Streptoalloteichus*
Streptomycineae	*Streptomycetaceae*	*Streptomyces*
Streptosporangineae	*Thermomonosporaceae*	*Actinomadura*

Note: Those genera highlighted in blue represent filamentous actinomycete groups. Non-actinobacterial groups are marked by asterisks.

4.4.3.3 Surrounding soil

Compared to the combined pie chart results for the colon and paunch shown in Fig. 4.2C, the surrounding soil sample showed a vast difference in composition. In the surrounding soil sample (Fig. 4.2D) there was an explosion of diversity, specifically of the filamentous actinobacterial genera. The three largest components of the SS sample were made up of the following: uncultured clones (23.46%), including various undefined actinobacterial clones; a 12.35% unknown component that encompassed uncultured clones for which there was no genus assignment, as well as no closely related and cultured relative; and 13.58% *Nocardioidaceae*, composed of three genera – two non-filamentous (*Friedmanniella* and *Marmoricola*) and one filamentous representative (*Nocardioides*).

In a similar study comparing the bacterial communities of the gut of the soil-feeding termite *C. niokoloensis* with that of its mound and surrounding soil, Fall *et al.* (2007) also found evidence of the genus *Nocardioides* in their clone library prepared from the hindgut. Of the 72 different actinobacterial clones that they obtained, they found 37 phylotypes. These phylotypes were later classified into 16 different *Actinobacteria* families. The mound material clones were affiliated to four main families, namely *Nocardioidaceae*, *Rubrobacteraceae*, *Streptosporangiaceae* and *Acidothermaceae*. For the surrounding soil sample, the *Dermabacteraceae* family was dominant, as were the genera *Geodermatophilus* and *Micromonospora*. The five termite gut clones were affiliated with three families – *Coriobacteriaceae*, *Promicromonosporaceae* and *Nocardioidaceae*. *Nocardia* and *Cellulomonas* genera were also found in the gut, but there were no *Streptomyces* clones. However, much like the SS sample for *M. viator*, their mound material library showed the *Streptomyces* prominence that is typical of the soil environment (Fall *et al.*, 2007). There did not appear to be much similarity between the diversity of actinobacteria found within the gut and surrounding soil of *M. viator* and *C. niokoloensis*, but that is to be expected as *M. viator* is a lower termite, whereas *C. niokoloensis* is a higher termite, and they have different feeding habits.

Of the filamentous groups represented in the SS sample, the proportion of *Micromonosporaceae* was similar to that shown in the combined colon and paunch chart. The *Streptomycetaceae* and *Thermomonosporaceae* made up a smaller proportion of the clone diversity, decreasing to 7.41% and 2.47%, respectively. The decrease in the *Thermomonosporaceae* component in the SS sample was quite noticeable. Conversely, there was a substantial increase in the proportion of *Pseudonocardiaceae* in the SS sample (up from 0.71% to 6.17%). The *Actinosynnemataceae* were not well represented in the soil sample, but two new families, *Kineosporiaceae* (represented by the genus *Kineococcus*) and

Nocardiaceae (genus *Actinopolymorpha*) were detected. An increase in diversity was seen in the SS results (see also Table 4.3), compared with the combined colon and paunch sample, especially for the *Micromonosporaceae* and *Pseudonocardiaceae* families, with some of the rarer genera being found in the SS analysis. The *Micromonosporaceae* family was now represented by four different genera (*Actinoplanes*, *Dactylosporangium*, *Salinispora* and *Virgisporangium*, but not *Micromonospora*) and the *Pseudonocardiaceae* now included the *Amycolatopsis* and *Streptoalloteichus* genera, but not *Pseudonocardia*.

Table 4.3 shows that only five families of filamentous actinobacteria were present in the gut samples, with quite different percentage compositions, when compared to the SS sample. This difference in composition was also shown for the non-filamentous components. The *Microbacteriaceae* and *Micrococcaceae* were now represented by different genera. The greatest increase in diversity was due to representatives of the suborder *Frankineae*, including four families and a total of six genera. Families *Frankiaceae* (genus *Frankia*), *Geodermatophilaceae* (*Blastococcus* and *Geodermatophilus*), filamentous group *Kineosporiaceae* (*Kineococcus*) as well as *Nakamurellaceae* (*Humicoccus* and *Saxeibacter*) all fall within this suborder and in total made up 20.99% of the SS sample. The unexpected presence of *Gemmatimonadetes* clones in the SS sample is undoubtedly an artefact, as other studies have also reported amplification of this non-actinobacterial group when the same actinobacterium-specific primers utilised in this work were used during PCR amplification (Babalola *et al.*, 2009). This fact, as well as the amplification of other non-actinobacterial groups during this work, suggests that the actinobacterium-specific primers may only preferentially amplify actinobacteria and are not in fact specific to this group.

In summary, for the SS sample, the filamentous component made up 28.39% of the total diversity, almost exactly the same as that of the non-filamentous at 28.38%. By comparison, the combined gut samples were composed of 24.82% filamentous and 49.66% non-filamentous – almost double the composition. This result is almost certainly a result of the microaerobic/anaerobic conditions found within the termite hindgut and the aerobic conditions found near the soil surface. In the paunch and colon, the *Propionibacteriaceae* and *Actinomycetaceae* represent anaerobic groups and form a large portion (36.88%) of the gut microbiota. However, it is also clear that the aerobic filamentous actinobacterial representatives are thriving in both gut regions and this is most likely as a result of growth and interaction with the microaerobic layer found next to the gut wall.

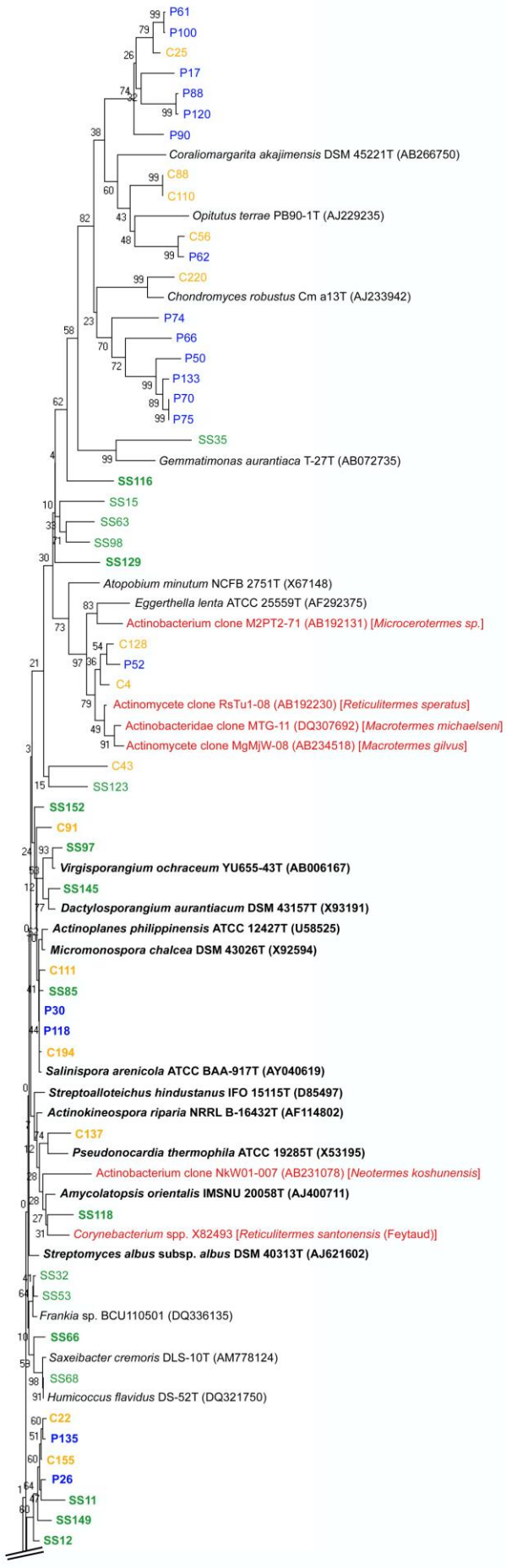
The effect that termites have on the soil and microbial communities found therein, and *vice versa*, is closely linked to their feeding habits and the types of colonies that they build (Fall *et al.*, 2007).

Presumably, all components of the surrounding soil would pass through the gut system of *M. viator* during the construction of its mounds, yet there are clear differences in the diversity and composition of both the hindgut community and that found in the surrounding soil. It therefore seems possible from the comparison of the colon and paunch sample with the surrounding soil sample, that there is some form of selection process that occurs in the hindgut of *M. viator*, which may relate to the specific conditions found in this unique environment. This same situation has already been shown by Fall *et al.* (2007) for the soil-feeding *C. niokoloensis*. They found that there was a major shift in bacterial community structure between the different gut regions analysed and the mound material, and they suggested that this observed shift was as a result of the shift in conditions, from an environment that is almost anoxic and half-liquid (in the gut) to one that is oxic, solid and rich in organic matter (mound soil). They also suggest that the dominance of *Actinobacteria* in the mound may even be helped or maintained by the presence of the termites (Fall *et al.*, 2007). Based on T-RFLP patterns, Schmitt-Wagner *et al.* (2003b) also found that the microbial community changed significantly even between the rectal contents and the nest material (which is constructed from soil and faeces in *Cubitermes* spp.) and suggested that, in terms of bacterial communities, the mound material was more closely related to the surrounding soil than to that found inside the gut.

4.4.4 Phylogenetic analysis

4.4.4.1 Composite clone tree

The phylogenetic tree shown in Fig. 4.3 includes a total of 92 clones, made up of 27 paunch clones, 28 colon clones and 37 surrounding soil clone sequences. All of these clones were chosen from the individual sample trees (marked in bold) shown in Appendices P, Q and R, to represent the full diversity discovered from all three samples. Also included were 39 genus type strains plus a *Frankia* sp. (chosen to represent this genus) selected from all of the genera discovered during analysis of the clones from the colon, paunch and SS samples (Table 4.3). Thirteen sequences identified as termite-specific actinobacterial clones and downloaded from the GenBank database, were also included in the final composite neighbour joining tree, making up a total of 144 partial 16S-rRNA gene sequences.


P61
P100
C25
P17
P88
P120
P90
Coraliomargarita akajimensis DSM 45221T (AB266750)
C88
C110
Opitutus terrae PB90-1T (AJ229235)
C56
P62
C220
Chondromyces robustus Cm a13T (AJ233942)
P74
P66
P50
P133
P70
P75
SS35
Gemmatimonas aurantiaca T-27T (AB072735)
SS116
SS15
SS63
SS98
SS129
Atopobium minutum NCFB 2751T (X67148)
Eggerthella lenta ATCC 25559T (AF292375)
Actinobacterium clone M2PT2-71 (AB192131) [Microcerotermes sp.]
C128
P52
C4
Actinomycete clone RsTu1-08 (AB192230) [Reticulitermes speratus]
Actinobacteridae clone MTG-11 (DQ307692) [Macrotermes michaelseni]
Actinomycete clone MgMjW-08 (AB234518) [Macrotermes gilvus]
C43
SS123
SS152
C91
SS97
Virgisporangium ochraceum YU655-43T (AB006167)
SS145
Dactylosporangium aurantiacum DSM 43157T (X93191)
Actinoplanes philippinensis ATCC 12427T (U58525)
Micromonospora chalcea DSM 43026T (X92594)
C111
SS85
P30
P118
C194
Salinispora arenicola ATCC BAA-917T (AY040619)
Streptoalloteichus hindustanus IFO 15115T (D85497)
Actinokineospora riparia NRRL B-16432T (AF114802)
C137
Pseudonocardia thermophila ATCC 19285T (X53195)
Actinobacterium clone NkW01-007 (AB231078) [Neotermes koshunensis]
Amycolatopsis orientalis IMSNU 20058T (AJ400711)
SS118
Corynebacterium spp. X82493 [Reticulitermes santonensis (Feytaud)]
Streptomyces albus subsp. albus DSM 40313T (AJ621602)
SS32
SS53
Frankia sp. BCU110501 (DQ336135)
SS66
Saxeibacter cremoris DLS-10T (AM778124)
SS68
Humicoccus flavidus DS-52T (DQ321750)
C22
P135
C155
P26
SS11
SS149
SS12

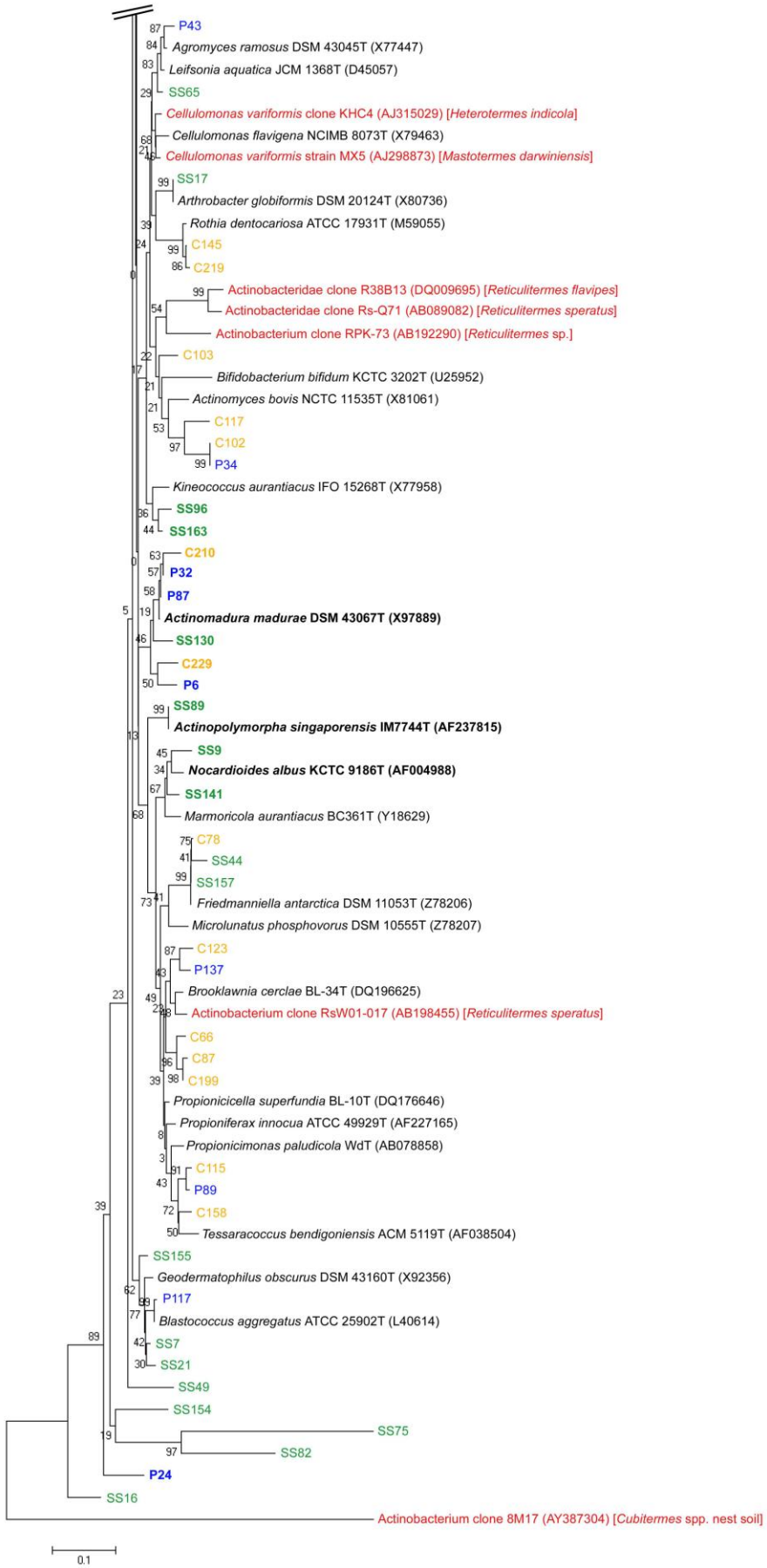

P43
Agromyces ramosus DSM 43045T (X77447)
Leifsonia aquatica JCM 1368T (D45057)
SS65
Cellulomonas variformis clone KHC4 (AJ315029) [Heterotermes indicola]
Cellulomonas flavigena NCIMB 8073T (X79463)
Cellulomonas variformis strain MX5 (AJ298873) [Mastotermes darwiniensis]
SS17
Arthrobacter globiformis DSM 20124T (X80736)
Rothia dentocariosa ATCC 17931T (M59055)
C145
C219
Actinobacteridae clone R38B13 (DQ009695) [Reticulitermes flavipes]
Actinobacteridae clone Rs-Q71 (AB089082) [Reticulitermes speratus]
Actinobacterium clone RPK-73 (AB192290) [Reticulitermes sp.]
C103
Bifidobacterium bifidum KCTC 3202T (U25952)
Actinomyces bovis NCTC 11535T (X81061)
C117
C102
P34
Kineococcus aurantiacus IFO 15268T (X77958)
SS96
SS163
C210
P32
P87
Actinomadura madurae DSM 43067T (X97889)
SS130
C229
P6
SS89
Actinopolymorpha singaporensis IM7744T (AF237815)
SS9
Nocardioides albus KCTC 9186T (AF004988)
SS141
Marmoricola aurantiacus BC361T (Y18629)
C78
SS44
SS157
Friedmanniella antarctica DSM 11053T (Z78206)
Microlunatus phosphovorus DSM 10555T (Z78207)
C123
P137
Brooklawnia cerclae BL-34T (DQ196625)
Actinobacterium clone RsW01-017 (AB198455) [Reticulitermes speratus]
C66
C87
C199
Propionicicella superfundia BL-10T (DQ176646)
Propioniferax innocua ATCC 49929T (AF227165)
Propionicimonas paludicola WdT (AB078858)
C115
P89
C158
Tessaracoccus bendigoniensis ACM 5119T (AF038504)
SS155
Geodermatophilus obscurus DSM 43160T (X92356)
P117
Blastococcus aggregatus ATCC 25902T (L40614)
SS7
SS21
SS49
SS154
SS75
SS82
P24
SS16
Actinobacterium clone 8M17 (AY387304) [Cubitermes spp. nest soil]
0.1

Figure 4.3: Neighbour-joining phylogenetic tree based on 435bp of 16S-rRNA gene sequence, showing the position of all colon (orange), paunch (blue) and surrounding soil (green) clones amongst all relevant actinobacterial genus type strains, as well as a selection of termite-associated GenBank sequences (shown in red with the termite host indicated in square brackets). All filamentous actinobacteria are highlighted in bold. GenBank sequence accession numbers are shown in parentheses. Bootstrap support for each node is indicated as a percentage, calculated from 1000 randomly re-sampled datasets. The scale bar indicates 1 nucleotide substitution per 10 nucleotides.

Overall, the non-filamentous *Actinobacteria* appeared to cluster at the topmost and bottommost edges of the tree, while the majority of the filamentous forms occupied the centre of Fig. 4.3. At the top of Fig. 4.3, there did appear to be a distinct separation of the hindgut specific non-filamentous clones from the surrounding soil representatives. Besides the *Coraliomargarita*, *Opitutus* and *Chondromyces* clones, the colon and paunch clones encompassed the uncultured *Acidobacteria* (clones P61, P100 and C25) as well as the *Treponema* (P88 and P120) and *Candidatus* Tamella (e.g. P74) clusters from the paunch sample. The *Eggerthella* (C128 and P52) and *Atopobium* (C4) clones appeared to be most closely related to four of the termite specific clones from three separate termite genera.

The 10 filamentous actinobacterial genera shown in the top half of Fig. 4.3, as well as the three genera in the bottom half, emphasised the wide diversity of these bacteria found within the hindgut of *M. viator*, as well as the soil environment in which these termites live. There did not appear to be the same distinct separation of hindgut clones from SS clones as was demonstrated for the non-filamentous forms. However, for those genera where more than one clone representative was found, there was some degree of separation. This was most easily seen for the *Streptomyces* genus clones at the very bottom of the top half of Fig. 4.3, where the mix of hindgut clones (C22, P135, C155 and P26) was more closely related to each other than to their SS counterparts (SS11, SS149 and SS12). A similar situation was seen for the *Actinomadura* clones shown in the bottom half of Fig. 4.3, where the colon and paunch clones were separated phylogenetically from the SS130 *Actinomadura* representative.

The genus *Cellulomonas* is discovered often in termite culture-independent investigations. The very top of the bottom half of Fig. 4.3 shows clustering of a *Cellulomonas variformis* clone as well as a strain of this species (originating from two separate termite genera) with the type strain of the *Cellulomonas* genus, *Cellulomonas flavigena*. Fall *et al.* (2007) also found evidence of this group inside the hindgut of the termite *C. niokoloensis*. *Z. angusticollis* has also been shown to harbour this group of actinobacteria in its gut bacterial community (Wenzel *et al.*, 2002).

Another interesting trend from Fig. 4.3 that has been shown previously in other termite specific culture-independent work is the clustering of termite specific lineages. Schmitt-Wagner *et al.* (2003a) showed in their phylogenetic analysis of two *Cubitermes* termite species that many clones grouped into distinct clusters that only contained sequences derived from termites. In Fig. 4.3 the clustering of three termite-derived clone sequences (clones R38B13, Rs-Q71 and RPK-73) is even more specific, as all clones were isolated from *Reticulitermes* termite species and appear to be most phylogenetically related to the *Actinomyces* clones obtained from the colon and paunch (C117, C102 and P34 and C103) of *M. viator*. Also, clustering of clones C4, C128 and P52 with three termite-derived clone sequences (RsTu1-08, MTG-11 and MgMjW-08) further demonstrates this point. This trend of the closest relatives of a given clone falling within lineages from closely related termite species adds support for the proposed coevolution of gut microbiota with their termite hosts (Hongoh *et al.*, 2005; Yang *et al.*, 2005).

The base of the phylogenetic tree covers a wide range of non-filamentous actinobacterial diversity, including amongst others, genera such as *Brooklawnia*, *Tessaracoccus*, *Geodermatophilus* and *Blastococcus*. Another cluster within this portion of Fig. 4.3 includes representative clones (C123, P137, C87, C199, C115, P89 and C158) from three genera of the *Propionibacteriaceae* family – *Propionicicella*, *Propioniferax* and *Propionicimonas*. The possibility that the *Propionibacteriaceae* clones from the colon sample are all most probably representatives of the same bacterium is supported by the individual sample tree shown in Appendix Q, as their clustering at the top of the tree and almost imperceptible separation suggest very close phylogenetic affiliation. Clustered within this large group in Fig. 4.3 is the termite-specific clone RsW01-017, which was discovered during an investigation of the hindgut of the termite *R. speratus* (Nakajima *et al.*, 2005). As with the dominance of the *Propionibacteriaceae* in the colon library of *M. viator*, the RsW01-017 phylotype was also found to be an abundant part of the hindgut community of *R. speratus*, in particular that of the gut wall libraries, consisting of 16 clones. Later FISH analysis, using a RsW01-017-specific probe, found the signal for this particular phylotype to be evenly distributed around the circumference of the paunch wall (Nakajima *et al.*, 2005).

The remaining portion of Fig. 4.3 was made up of a mixture of distantly related filamentous (P24 - *Actinomadura*) and non-filamentous (SS82 - *Saxeibacter* and SS16 - *Frankia*) clones; all with less than 94% sequence similarity to their closest BLAST hits (see Appendix O for details). The most distantly related was one of the termite-specific clones, sourced from the nest material of a *Cubitermes* species.

4.4.4.2 *Streptomyces* phylogenetic tree

Fig. 4.4 was generated from a combination of the *Streptomyces*-related culture-independent clone sequences from the colon, paunch and SS, as well as all cultured *Streptomyces* strains and their closest phylogenetic relatives (as determined in Chapter 3), in order to determine the phylogenetic relationships amongst all of the *Streptomyces* representatives discovered from both the culture-based and culture-independent approaches.

Except for clones SS51 and SS164, all other surrounding soil clones clustered away from the paunch and colon clones, supporting the proposal that the streptomycetes in the *M. viator* gut are different from those found in the surrounding soil. By comparison, the mixing of the clones from the paunch and colon in the top half of Fig. 4.4 seems to suggest that within the gut, the streptomycetes are closely related. The majority of these clones appeared to be most closely related to the cultured isolates 18, 19 and 32, as well as their published *Streptomyces* relatives.

None of the clones from the culture-independent analysis appeared to be related to the large group of rugose-spored streptomycetes isolated during the initial isolation work, or to *Streptomyces* strain 7. The only clone that was found to cluster within this particular group within the phylogenetic tree was clone SS164, which was found to be most closely related to the unique 13, 16 and 37 cultured strains. Clustering of isolates 13, 16 and 37 in their own clade also seemed to support their uniqueness, compared with all other streptomycete isolates and clones. The failure to detect any close phylogenetic relationship between the cultured streptomycetes and those *Streptomyces* clones generated during the culture-independent analysis is noteworthy, as it suggests that both methods were not exhaustive enough. Sequencing of more clones or the isolation of more *Streptomyces* isolates may have resolved this issue.

The majority of the SS clones, as well as a single paunch clone (P26) formed outgroups of these two major clusters within Fig. 4.4, again supporting the separation of gut specific clones and *Streptomyces* representatives isolated from the SS.
Other, related work has also shown a pronounced difference amongst the microbiota between the gut and the surrounding soil, as well as amongst the different gut compartments of a soil-feeding termite (Schmitt-Wagner *et al.*, 2003a).

Figure 4.4: Neighbour-joining tree of the cultured streptomycetes isolated from the colon and paunch of *M. viator*, as well as all cloned *Streptomyces* sequences generated from the two gut regions as well as the surrounding soil. The result was based on 605bp of 16S-rRNA gene sequence, including those published species that were found to be most closely related to the cultured isolates. Bootstrap support above 45% for each node is indicated and is reflected as a percentage, calculated from 1000 randomly re-sampled datasets. The bar represents 2 nucleotide substitutions per 100 nucleotide positions.

Overall, when comparing all three samples, it is clear that there are obvious differences in the actinobacterial diversity and composition within the hindgut of *M. viator*, compared with the surrounding soil material (which is ingested by the termites in the construction of their mounds), suggesting that there may be some form of selection process occurring in the termite hindgut. It must be noted that comparison between the different samples is limited by a lack of biological replicates.

In comparison to the culture-based analysis in *M. viator*, where only *Streptomyces* and *Nocardia* species were isolated, it is clear that the cultured diversity is a gross under-representation of the true gut diversity. Based on this finding, attempts should now be made to access this untapped diversity and, judging by the fact that most of the cultured isolates that have been investigated further appear to be novel species, the chances are high that this unaccessed diversity could also represent novel species from other, rarer genera.

4.5 References

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CHAPTER 5

GENERAL DISCUSSION

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The more 'primitive' lower termites harbour eukaryotic flagellates in their hindguts and the majority of the work on lower termites focuses on these flagellates and their interactions with the prokaryotes that also live in the termite gut environment. The presence of flagellates in the hindgut of higher termites has been lost over evolutionary time (Brauman *et al.*, 2001) and all work on these higher termites focuses on the bacterial component of the hindgut community, as there is no eukaryotic population. As a result of this, comparatively little focus is placed on the bacterial communities residing inside the hindgut of lower termites. In general, those papers that do investigate the microbiology of the lower termite gut cover the full spectrum of prokaryotes found there (Varma *et al.*, 1994; Brune, 2006; Ohkuma, 2008). To my knowledge, this is the first work to focus specifically on the actinobacterial community (particularly the filamentous actinomycetes) that lives in the hindgut system of a lower termite, in this case, from an endemic South African termite, *Microhodotermes viator*.

The *M. viator* gut is unique amongst lower order termites, as has been described by Noirot (1995). The distinctive separation of the two regions of the paunch into P3a and P3b that is shown in Fig. 2.3, as well as the lack of flagellates found in region P3b (as discovered by Noirot (1995) and shown during this work), suggest that the gut of *M. viator* resembles more the complex compartmentalisation of the higher termites, as opposed to the typically simpler construction of the lower termite hindgut. In fact, except for one family, all higher termite lineages show a trend for elongation and increased compartmentalisation of the hindgut, especially for the soil-feeding groups (Brune, 2006).

Although there is little data to support the suggestion, it is possible that the specific environmental conditions to which this particular termite is exposed (conditions which are found nowhere else) have, over evolutionary time, modified the structure of the gut to allow the termite to adapt to and thrive under these conditions. Is it also possible that *M. viator* could be both a wood- and soil-feeder? The distribution of *M. viator* is confined to the Cape Provinces of South Africa. This area covers two main vegetation types – Karooveld (characterised by hot, dry conditions with little water and comparatively little vegetation); and Fynbos (a large abundance and diversity of vegetation that grows under conditions of very low soil nutrient quality). In order to survive under both conditions, evolution of both

wood/plant-based feeding as well as soil-feeding would provide a distinct advantage for the termite. When the vegetation is sparse (Karooveld), soil-feeding would be the dominant source of nutrition, whereas in areas where the soil nutrient quality is poor (Fynbos), the termites could survive mostly using their wood-feeding ability. The structure of the gut may have evolved accordingly, as the first dilation of the paunch (P3a), which harbours the flagellate population common to all lower order termites, would allow for the processing of plant matter/lignocellulosics into food for their host. The second paunch dilation (P3b) which has no flagellates, but has a large population of actinobacteria (in particular filamentous actinomycetes, as shown by the results from this work), would take on the role of extracting nutrients from the soil.

A great deal of information has been uncovered during the investigation of the *M. viator* hindgut, including some interesting characteristics exhibited by the actinomycetes that were isolated during the culture-based component of the project. Some of the data, such as the antibacterial activities of the isolates is preliminary, meaning that there is huge scope for future work based on what has already been determined. It would be interesting to investigate whether the *in vitro* antibacterial activities described in Chapter 2 can also be proven *in vivo*, thereby providing evidence that the actinomycetes inside the termite hindgut could have a more profound effect on the diversity and structure of the gut community than was previously thought possible.

As shown in Fig. 2.5 and Table 2.2, some of the actinomycete isolates were found to produce strong antibacterial activity and, even if the suggestion made above that the antibiotics produced by the actinomycete population have an effect on the rest of the gut community is unfounded, there is still the possibility that the antibiotics produced by these organisms inside the termite gut could contribute to a general defence against pathogenic bacterial infection within this system. The presence of fungi inside the gut of *M. viator* has been demonstrated (Fig. 2.4), but the lack of actual mycelial growth as evidenced by the scanning electron microscopy results could be explained by the production of fungistatic compounds by this same gut actinomycete population. These proposals would have to be proven *in situ*, but very little is known about the insect immune system, and an investigation of the potential contribution by the gut actinomycetes would be very interesting.

Even if the actinomycete population in the *M. viator* hindgut community is not responsible for structuring the microbial community as a whole, as suggested above, the work presented in this project suggests that there is some form of selection process occurring in this particular gut system. Evidence of this is provided by the differences observed between the diversity of

actinobacteria discovered during the culture-independent investigation of the gut samples in comparison to the surrounding soil sample. It is possible that the gut system of *M. viator* selects for specific groups of bacteria/actinobacteria in order to outcompete any pathogens. The specific selection of actinomycetes for their ability to produce antibiotics has been documented previously in the insect world – much like the Beewolf wasps select for their antennal commensals, and the Attine ants select for *Pseudonocardia* symbionts (Kaltenpoth, 2009). Their amazing ability to utilise a wide range of carbon and nitrogen sources, as well as their extraordinary ability to produce an extensive selection of secondary metabolites, makes the actinobacteria the perfect choice for protective symbioses. Also, actinobacteria are regularly isolated from a wide variety of environmental sources, particularly from soil and are frequently encountered by insects (Kaltenpoth, 2009).

The ability of some actinobacteria to form spores would provide *M. viator* with the perfect built-in selection mechanism. In the soil, the ability to produce spores allows actinobacteria to survive unfavourable conditions and, although not to the degree that endospores allow other bacteria to survive, actinobacterial spores provide a greater degree of tolerance to environmental stresses such as desiccation or low pH than vegetative cells (Kaltenpoth, 2009). In the lower termite, this characteristic may allow the actinomycetes to survive passage through the anterior gut regions (and associated digestive enzymes) to allow their establishment in the oxygenated hindgut paunch and colon regions.

The interactions described above do not include the vast number of potential nutritional interactions that occur within the termite hindgut system. Actinobacteria are fairly slow-growing bacteria that are generally not fastidious in their nutrient requirements and are therefore cheap to maintain as symbionts. The role of actinobacteria in termite nutrition has been linked to their ability to degrade complex polymers such as lignocellulose, their metabolic versatility allowing them to access nutrient sources that are not readily available to the termite or to other bacteria. However, the specific contributions of the many different individual groups of bacteria that make up the termite community to their host's nutrition are still poorly understood (Kaltenpoth, 2009).

It has been shown from this work that there is a portion of the actinomycete community isolated from the hindgut of *M. viator* that is able to degrade both CMC and xylan and this may contribute to the nutrition of the host *in situ*. Future work investigating whether the actinomycete community is actively involved in this process would go some way towards understanding their role in the termite gut.

In terms of culture-based approaches to understanding the diversity within the termite gut, there has been a renewed interest in finding novel methods to capture more of the microbial functional diversity. These novel methods have demonstrated that culturing of the previously unculturable components of the bacterial community is often made possible by mimicking the niche conditions found in their host environment. In contrast to what was done before, the novel isolation conditions try to reproduce the natural environment in terms of nutrients (concentration and composition), pH, oxygen levels and associations with other microbes (Cardenas & Tiedje, 2008). One such example is the use of gellan gum instead of the traditional agar used as a solidifying agent in media. Gellan gum produces a clearer plate that is more stable under a wider range of temperature and pH conditions. In terms of oxygen levels, microaerophilic conditions have proven to be very useful in the recovery of termite-specific gut bacteria (Cardenas & Tiedje, 2008).

The presence of an abundant population of filamentous actinobacteria inside the hindgut of *M. viator* is undeniable. The scanning electron micrographs in Chapter 2 clearly show their presence amongst the other bacteria found near the gut wall, and their presence was confirmed by both the culturing of these organisms from both the paunch and colon regions, as well as the culture-independent amplification of their 16S-rRNA genes.

By far the greatest amount of information about the diversity of bacteria inside the termite gut has been revealed by cultivation-independent means. Studies involving the amplification and sequencing of 16S-rRNA genes extracted from the guts of *Reticulitermes* species (one of the most well studied termite genera), have shown that the majority of the recovered sequences represent novel and as-yet-uncultured species. As such there is often very little overlap between the results obtained using the two different techniques, emphasising the need to incorporate both in any investigation of the bacterial communities found inside this unique environment (Brauman *et al.*, 2001; Brune, 2006; Kurtböke & French, 2007).

The work presented here emphasises this point as, unlike in the culture-independent metagenomic portion of this project, almost all of the actinobacteria isolated during the culture-based component were *Streptomyces* species. This trend has been observed in other ‘extreme’ environments, such as in an Antarctic soil sample investigation (Babalola *et al.*, 2009). Also, a comparison of the culture-based strain sequences with those generated during the culture-independent analysis (shown in Fig. 4.4) showed that the isolated streptomycetes were more closely related to the culturable, validly published species, whereas the majority of the phylotypes identified in the metagenomic analysis were related to each other, and according to Fig. 4.3, to other uncultured actinobacterial clones.

An important issue to take note of is that the sampling for the culture-based and culture-independent work did not occur at the same time, limiting the direct comparability of the two sets of data. However, termites were sampled from exactly the same mound at the same time of year. At the time of sampling for the culture-based work, the only aim was to determine whether any actinomycetes could be isolated from the hindgut of *M. viator*. It was only later, during the evolution of the project, that it was determined that a culture-independent analysis would provide a more complete picture of the actinomycete diversity found within this never-before-investigated termite species. This in no way detracts from the fact that both sets of information are important, or that the metagenomic analysis revealed a vast actinobacterial diversity that was missed in the culture-based portion of this work.

The dominance of certain phylotypes revealed during the culture-independent analysis in the different gut regions is noteworthy - in particular the presence of a large percentage of *Propionibacteriaceae* clones in the colon clone library. Nakajima *et al.*, (2005) also noted a particular abundance of *Actinobacteria* (16%) associated with the gut wall libraries whereas this group was far less abundant (1.1%) in the gut lumen of *R. speratus*. A particular *Propionibacteriaceae* clone, RsW01-017 made up the greatest portion of this sample. In contrast, *Spirochaetes* and the Termite Group 1 (*Elusimicrobia*) phylum were abundant in the gut lumen and whole gut, but showed low clone numbers in the gut wall library. From these results, it was determined that these abundant phylotypes probably represented the major bacterial components of these different sample sites (Nakajima *et al.*, 2005). FISH analysis showed the RsW01-017 clone to be rod-shaped, colonising the surface of the paunch wall in a uniform sheet-like pattern, much like that found for other work in *R. flavipes*. The presence of *Propionibacteriaceae* was also found in the paunch clone libraries of *M. viator* but, as is shown in Fig. 4.2, to a much lesser degree than that for the colon sample. The scanning and transmission electron microscopy results from Chapter 2 showed an abundance of rod-shaped bacteria lining the paunch wall, much like that shown by Nakajima *et al.* (2005), suggesting that these clones may have been sourced from this wall-associated group of bacteria.

It may also therefore be possible that the dominance of the *Propionibacteriaceae* clones in the *M. viator* gut is related to surface area. The total surface area inside the paunch is much smaller than that found in the colon (in relation to the actual gut volume for the different regions) and this could explain why this actinobacterial group did not dominate in the paunch, but showed much greater representation in the colon sample. This finding would also suggest that the large group of *Propionibacteriaceae* clones discovered inside the hindgut of *M. viator* represent facultative anaerobes or must at least be aerotolerant, in order to thrive in the oxygen rich environment that is the gut periphery. Most members of this family are non-

motile and are either aerobes or facultative anaerobes (Nakajima et al, 2005), thereby supporting the hypothesis described above. Interestingly, the members of this family are also known to accumulate polyphosphate in their cells, similar to that which was seen in some of the transmission electron micrographs (Fig. 2.18) during the morphological examination of the *M. viator* hindgut.

Because of the unique nature of the termite gut, in terms of the flow of oxygen and the presence of both oxygenated and anaerobic habitats within the same system, future work could involve an assessment of the culturable anaerobic component of the *M. viator* hindgut, in order to complement the aerobic culture-based work already done. The fact that anaerobic groups such as the actinomycete genus *Actinomyces* were discovered during the metagenomic analysis of the hindgut suggests that there is a wealth of information relating to the anaerobic portion of the bacterial diversity inside the *M. viator* gut that is yet to be discovered.

A phylogenetic analysis based on the phylotypes obtained from the mound soil and the surrounding soil of the *C. niokoloensis* termite, showed a clear separation of the clones from the different sample sites. Interestingly, although the termite gut and surrounding soil did not share any common actinobacterial phylotypes, nearly all of the phylotypes from the termite gut were also found in the mound soil. Overall, Fall *et al.* (2007) were able to demonstrate that the termite mound harbours its own specific bacterial community, in terms of structure and diversity, which is distinct from the surrounding soil and is characterised by the dominance of actinobacteria, which they suggested probably originate from the termite gut. It would be interesting to look at these changes within the *M. viator* system, to see whether the mound soil is more like the gut or the surrounding soil in terms of its bacterial diversity, or whether there is some intermediate bacterial diversity that results from the activities of the *M. viator* termite and its effects on the immediate environment. Again, a culture-based analysis in combination with a culture-independent assessment would probably provide a much clearer picture of what is occurring in the system as a whole.

One of the many questions that is often asked when working on the bacterial communities within termite guts is whether any portion of these communities is only found within a specific termite, or group of termites, suggesting a host specific population. Previous comparisons of gut bacterial diversity have indicated that these communities are conserved significantly within termite genera, forming lineages from all bacterial groups that are unique to termites. This conservation across gut communities, as well as their uniqueness, suggests that the diversity is endemic (autochthonous) and does not represent the diversity that is found in the surrounding environments (Ohkuma, 2008).

A study performed by Fisher *et al.* (2007) showed that over 90% of the 16S-rRNA gene sequences obtained from the gut of *R. flavipes*, that shared >90% sequence similarity with other sequences from a BLASTN search, only did so with uncultivated termite gut bacteria. As such, it appeared that there was a much higher similarity between sequences obtained from termite guts, compared with those that were found in other environments, thereby providing evidence for the possible co-evolution of termite hosts and their gut bacteria.

Wertz & Breznak (2007) made use of 16S-rRNA gene clone libraries prepared from *R. flavipes* gut contents, termite nest soil and non-termite-associated forest soil to show that a particular group of strains were autochthonous members of the *R. flavipes* gut community. A similar approach was attempted during the analysis of the *M. viator* hindgut community, except that the clone libraries for this work compared the paunch, colon and surrounding soil. As with the Wertz & Breznak (2007) strains, there did appear to be differences in the phylogenetic relatives of the streptomycetes obtained from within the *M. viator* hindgut and those obtained from the surrounding soil (as shown in Fig. 4.3 and 4.4 and discussed in Chapter 4). This therefore lends support for the proposal that there is a specific streptomycete community residing inside the gut of *M. viator*.

The work presented in this thesis has only just begun to scratch the surface of understanding the complex bacterial community that makes up the hindgut of *M. viator* as well as the functioning of the specific communities, in particular the actinobacteria, within this unique environment. Future metagenomic work related to that already done could involve investigating the actinobacterial diversity within other regions of the *M. viator* gut, such as the midgut. Diversity indices and sequence based analyses could determine whether there is an increase in bacterial diversity from the midgut to the hindgut, similar to that shown by Yang *et al.* (2005) during their investigation of the bacterial community structure inside the gut of *R. santonensis*. It would also be interesting to compare the diversity and structuring of the communities within specific regions, by making comparisons of the gut wall versus the lumen, as was done by Nakajima *et al.* (2005) inside the gut of *R. speratus*.

The benefit of using terminal restriction fragment length polymorphism (T-RFLP) analysis in combination with clonal analysis has been emphasised by Schmitt-Wagner *et al.* (2003). In their work on the bacterial community structure inside the gut of *Cubitermes* spp., a correlation between the T-RFLP patterns and the clones from the clone libraries was established, confirming the diversity evidenced by clonal analysis, and also confirming that all hindgut sections harboured distinct bacterial communities.

Another technique that was not within the scope of this project, but has exciting implications for the future study of the ecology inside the termite gut, is the use of microfluidics. This technique involves the manipulation of small volumes and, by working at a dimension relevant to microbes, allows for the study of specific microbes via isolation, gene amplification or genome reconstruction. In particular, microfluidics has allowed for individual members of termite hindgut flora to be sorted and screened by multiplex PCR and, by targeting the 16S-rRNA gene as well as functional genes, has made it possible to link phylogeny with function (without the need for cultivation) (Cardenas & Tiedje, 2008).

The study of cultured isolates remains the easiest method to characterise an organism's function in a particular environment and evaluate its biotechnological potential. As such, with sufficient intensive study, ingenuity and patience, there appears to be no obstacle to making most of the formerly unculturable microbes culturable (Cardenas & Tiedje, 2008).

To exploit the wide diversity of actinobacteria found inside the hindgut of *M. viator* and to explore the full potential of this work for application in biotechnology, a synergistic approach should be adopted. The metagenomic data and some of the culture-based work has provided insight into the possible physiological roles of the different phylotypes and cultured strains identified from all of the different sample sites. This knowledge can be used to guide subsequent culture-based isolation schemes, for example, by applying more targeted media for the isolation of the observed diversity.

Our understanding of the biology of the termite gut community in general remains poor – mainly due to the vast complexity of the system, but also due to the difficulty of cultivating (and thereby studying) the majority of its members. Recent culture-independent analyses, including the work shown here, have revealed the unexpected and impressive nature of this environment (Ohkuma, 2008) and findings suggest that the great majority of the bacteria found in the gut are members of uncultured lineages that are unique to termites. Therefore, it is only through the synthesis of these two valuable techniques – the culture-based and the culture-independent – that a complete understanding of the functioning of the termite gut community will ever be achieved.

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APPENDICES

APPENDICES

CONTENTS

Appendix A: List of 'Original' isolates and their respective 'Duplicates'

Original isolate code	Duplicate code/s
M375C1	None
M375P1	MIIC75P3, MIIC6P3, MIIG65C1, MIIX75C3, MIIX65C8
M375P2	None
M375P3	None
MIIC75C1	MIIC7C2, MIIC7C3, MIIC65C2, MIIC65C4, MIIC75P4, MIIX75C2, MIIX7C3, MIIX65C5, MIIX6C3 MIIX75P4, MIIX7P1
MIIC75C2	None
MIIC75C3	MIIC75P5
MIIC7C1	None
MIIC7C4	MIIC65C6, MIIC6C2, MIIC75P2, MIIC7P2, MIIC7P3, MIIC65P2, MIIC65P3, MIIC65P5, MIIC6P2
MIIC7C5	None
MIIC7C6	MIIC65C3, MIIC75P1, MIIC65P1
MIIC65C7	None
MIIC6C1	None
MIIC7P1	None
MIIC65P4	None
MIIC6P1	MIIX7C5, MIIX6P2
MIIX75C1	MIIX7C7, MIIX65C1, MIIX65C7, MIIX75P1
MIIX7C1	None
MIIX7C2	None
MIIX7C4	None
MIIX7C41	None
MIIX65C2	None
MIIX65C3	MIIX65P3
MIIX65C4	MIIX65C6, MIIX6C1, MIIX75P2, MIIX75P5, MIIX65P1
MIIX65C41	None
MIIX6C2	None
MIIX75P3	None
MIIX75P6	None
MIIX75P7	None
MIIX7P2	None
MIIX7P3	None
MIIX7P4	None
MIIX7P41	None
MIIX7P42	None
MIIX65P2	None
MIIX65P4	None
MIIX65P41	None
MIIX6P1	None
MIIC75C21	None

*Bacterial codes in bold represent isolates identified and subcultured after four weeks growth, on their respective media at 30°C.

Appendix B: Screening of Original isolates for antibacterial and degradation activities

Strain no.	Bacterial isolation codes	Antibacterial activity				Degradation activity	
		E. coli ATCC 25922	*S. aureus* ATCC 25923	*E. faecium* Van A	*M. aurum* A+	CMC	Xylan
1	M375C1	–	+	–	–	+	–
2	M375P1	+	–	–	+	+	–
3	M375P2	–	+	–	–	+	–
4	M375P3	–	+	+	+	+	–
5	MIIC75C1	–	+	+	+	+	+
6	MIIC75C2	–	+	+	+	+	+
7	MIIC75C3	–	+	–	–	+	–
8	MIIC7C1	–	+	+	+	+	–
9	MIIC7C4	–	–	–	+	+	–
10	MIIC7C5	–	+	–	–	+	–
11	MIIC7C6	–	+	+	+	+	–
12	MIIC65C7	–	–	–	–	–	–
13	MIIC6C1	–	+	–	+	NG	NG
14	MIIC7P1	–	–	–	+	+	–
15	MIIC65P4	–	+	+	–	–	–
16	MIIC6P1	–	–	–	+	ND	ND
17	MIIX75C1	–	+	–	+	+	–
18	MIIX7C1	–	–	–	+	+(w)	+(w)/ –
19	MIIX7C2	–	+	–	+	+	–
20	MIIX7C4	–	–	–	–	–	–
21	MIIX7C41	–	+	–	–	+	–
22	MIIX65C2	–	–	–	+	+	–
23	MIIX65C3	–	+	+	+	+	–
24	MIIX65C4	–	+	+	+	+	–
25	MIIX65C41	–	+	+	+	+(w)	–
26	MIIX6C2	–	+	+	+	+	+
27	MIIX75P3	–	–	–	–	+	–
28	MIIX75P6	–	–	–	+	+	–
29	MIIX75P7	–	+	+	+	+	–
30	MIIX7P2	–	+	+	+	+	–
31	MIIX7P3	–	+	–	+	+	–
32	MIIX7P4	–	–	–	+	+(w)	–
33	MIIX7P41	–	+	+	+	+	–
34	MIIX7P42	–	+	+	+	+	–
35	MIIX65P2	–	+	+	+	+	–
36	MIIX65P4	–	+	+	+	+	–
37	MIIX65P41	–	+	–	+	+	–
38	MIIX6P1	–	+	+	+	+	–
39	MIIC75C21	–	+	–	+	+	–

* ND = Not determined; NG = No growth. For antibacterial activity: + = activity against a test strain, – = no activity against a test strain. For degradation activity: + = shows degradation activity on that medium, +(w) = weak degradation activity, – = no degradation activity.

Appendix C: Screening of Duplicate isolates for degradation activities

Strain no.	Bacterial isolation code	Degradation activity	
		CMC	Xylan
39D	MIIG65C1	+	–
40	MIIC7C2	+	+
41	MIIC7C3	+	+
42	MIIC65C2	+	+
43	MIIC65C3	+	–
44	MIIC65C4	+	+
45	MIIC65C6	+	–
46	MIIC6C2	+	–
47	MIIC75P1	–	–
48	MIIC75P2	+(w)	–
49	MIIC75P3	+	–
50	MIIC75P4	+	+
51	MIIC75P5	+	–
52	MIIC7P2	+(w)	–
53	MIIC7P3	+	–
54	MIIC65P1	+	–
55	MIIC65P2	+	–
56	MIIC65P3	+	–
57	MIIC65P5	+	–
58	MIIC6P2	ND	ND
59	MIIC6P3	+	–
60	MIIX75C2	+	+
61	MIIX75C3	+	–
62	MIIX7C3	+	+
63	MIIX7C5	ND	ND
64	MIIX7C7	+	–
65	MIIX65C1	+	–
66	MIIX65C5	+	+
67	MIIX65C6	+	–
68	MIIX65C7	+	–
69	MIIX65C8	+	–
70	MIIX6C1	+	–
71	MIIX6C3	+	+
72	MIIX75P1	+(w)	–
73	MIIX75P2	+	–
74	MIIX75P4	+	+
75	MIIX75P5	+	–
76	MIIX7P1	+	+
77	MIIX65P1	+	–
78	MIIX65P3	+	–
79	MIIX6P2	ND	ND

* ND = Not determined; + = shows degradation activity on that medium; +(w) = weak degradation activity; – = no degradation activity.

Appendix D:
Complete physiological comparison between *Nocardia* isolates 12, 20 and *N. nova* ATCC 33726^T

Test	12 MIIC65C7	20 MIIX7C4	*N. nova* ATCC 33726^T
Aerial mycelium colour (ISP#4)	bald?	white	N/D
Substrate mycelium colour (ISP#4)	yellow-brown	yellow-brown	N/D
Production of diffusible pigment on:			
ISP#5	–	–	N/D
Production of melanin on:			
ISP#6	–	–	N/D
ISP#7	–	–	N/D
Growth at:			
37^{o}C	+	+	+^{c,f}
45^{o}C	–	–	–^{b,c,e,f}
Growth in presence of:			
3% NaCl	+	+	N/D
5% NaCl	+	+	N/D
7% NaCl	+(w)	+(w)	N/D
Reduction of:			
Nitrate	+	+	+^{b,e,f}
Production of:			
H_2S	+(w)	+/–	N/D
Decomposition of:			
Allantoin	–	–	N/D
Adenine	–	NG	–^{b,c,e}
Arbutin	–	+(w)	+^{b,e}
Casein	–	–	–^{a,b,c,e}
Cellulose	–	–	N/D
Esculin	+	+	–^{a}/+^{b,e}
Gelatin	–	–	–^{a}
Guanine	–	–	N/D
Hypoxanthine	–	–	–^{a,b,c,e}
Starch	–	–	N/D
L-Tyrosine	N/D	–	–^{a,b,c,e}
Urea	–	–	+^{a,b,e,f}/–^{c}
Xanthine	–	–	–^{a,b,c,e}
Xylan	–	–	N/D
Growth on sole carbon source:			
Glucose	+	+	+^{a,c,f}
L-(+)-Arabinose	–	–	–^{a,c,f}
Fructose	–	–	+^{f}
D-(+)-Galactose	–	+/–	+^{a,c,d}/–^{f}
myo-Inositol	–	–	–^{a,c,f}/+^{d}
D-(–)-Mannitol	–	–	–^{a,f}/+^{b,e}
D-(+)-Raffinose	–	–	–^{a}
L-(+)-Rhamnose	–	–	–^{a,b,c,e,f}/+^{d}
Salicin	+/–	+/–	N/D
Sucrose	–	–	–^{a,f}/+^{d}
D-(+)-Xylose	–	–	–^{a,f}

NG = No growth; N/D = Not determined; +(w) = weakly positive; a: Yassin & Brenner (2005); b: Zhang *et al*. (2003); c: Kageyama *et al*. (2004); d: Kim *et al*. (2002); e: Cui *et al*. (2005); f: Tsukamura (1982). All other results were determined during this project.

Appendix E:

Complete physiological comparison between *Streptomyces* isolate 2 and its closest phylogenetic relatives, *S. costaricanus* NBRC 100773^T, *S. griseofuscus* NBRC 12870^T and *S. murinus* NBRC 12799^T

Test	2 M375P1	*S. costaricanus* NBRC 100773^T	*S. griseofuscus* NBRC 12870^T	*S. murinus* NBRC 12799^T
Spore surface ornamentation	smooth	smooth[a]	N/D	N/D
Spore chain morphology	spiral	spiral[a]	spiral[a]	spiral[a]
Aerial mycelium colour (ISP#4)	white	grey-brown[a]	pale greyish red-brown[a]	grey[a]
Substrate mycelium colour (ISP#4)	pale yellow-brown	yellow[a]	pale greyish brown[a]	greyish yellow[a]
Production of diffusible pigment on:				
ISP#5	–	yellow[a]	–[a]	yellow[a]
Production of melanin on:				
ISP#6	–	–[a]	–[a]	–[a]
ISP#7	–	–[a]	–[a]	–[a]
Growth at:				
37^{o}C	+	N/D	N/D	N/D
45^{o}C	+	N/D	N/D	N/D
Growth in presence of:				
3% NaCl	+	N/D	N/D	N/D
5% NaCl	+	–[a]	–[a]	+[a]
7% NaCl	+	–[a]	–[a]	+[a]
Reduction of:				
Nitrate	–	N/D	N/D	N/D
Production of:				
H_2S	–	N/D	N/D	N/D
Decomposition of:				
Allantoin	–	N/D	N/D	N/D
Adenine	+	N/D	N/D	N/D
Arbutin	+	N/D	N/D	N/D
Casein	+	N/D	N/D	N/D
Cellulose	–	N/D	N/D	N/D
Esculin	+	N/D	N/D	N/D
Gelatin	+	N/D	N/D	N/D
Guanine	–	N/D	N/D	N/D
Hypoxanthine	+	N/D	N/D	N/D
Starch	–	N/D	N/D	N/D
L-Tyrosine	+	N/D	N/D	N/D
Urea	–	N/D	N/D	N/D
Xanthine	–	N/D	N/D	N/D
Xylan	–	N/D	N/D	N/D
Growth on sole carbon source:				
Glucose	+	+[a]	+[a]	+[a]
L-(+)-Arabinose	–	–[a]	+[a]	+(w)[a]
Fructose	+	+[a]	+[a]	+[a]
D-(+)-Galactose	+	N/D	N/D	N/D
myo-Inositol	–	N/D	N/D	N/D
D-(–)-Mannitol	+	+[a]	+[a]	+[a]
D-(+)-Raffinose	+(w)	–[a]	+(w)[a]	+(w)[a]
L-(+)-Rhamnose	–	–[a]	+(w)[a]	+(w)[a]
Salicin	+(w)	+[a]	–[a]	+[a]
Sucrose	–	–[a]	+(w)[a]	+(w)[a]
D-(+)-Xylose	+	+[a]	+[a]	+[a]

N/D = Not determined; +(w) = weakly positive; a = Esnard *et al.* (1995).

Appendix F:

Complete physiological comparison between *Streptomyces* isolate 7 and *S. achromogenes* subsp. *rubradiris* NBRC 14000^T, *S. nodosus* NRRL B-2371^T and *S. rameus* LMG 20326^T

Test	7 MIIC75C3	*S. achromogenes* subsp. *rubradiris* NBRC 14000^T	*S. nodosus* NRRL B-2371^T	*S. rameus* LMG 20326^T
Spore surface ornamentation	smooth	smooth[a]	N/D	smooth[a]
Spore chain morphology	spiral	spiral[a]	spiral[b]	spiral[a]
Aerial mycelium colour (ISP#4)	rose/pink	grey[a]	grey[b]	grey[a]
Substrate mycelium colour (ISP#4)	reddish-brown	yellow-brown[a]	green[b]	N/D
Production of diffusible pigment on:				
ISP#5	–	–[a]	N/D	N/D
Production of melanin on:				
ISP#6	–	–[a]	–[b]	+[a]
ISP#7	–	–[a]	–[b]	+[a]
Growth at:				
37^{o}C	+	N/D	+[b]	N/D
45^{o}C	–	–[a]	–[b]	N/D
Growth in presence of:				
3% NaCl	+(w)	N/D	N/D	N/D
5% NaCl	+(w)	N/D	N/D	N/D
7% NaCl	+(vw)	–[a]	N/D	N/D
Reduction of:				
Nitrate	+	–[a]	+[b]	N/D
Production of:				
H_2S	+	+[a]	–[b]	N/D
Decomposition of:				
Allantoin	–	N/D	N/D	N/D
Adenine	?	N/D	N/D	N/D
Arbutin	+	+[a]	N/D	N/D
Casein	+	N/D	N/D	N/D
Cellulose	–	N/D	N/D	N/D
Esculin	+	N/D	N/D	N/D
Gelatin	+	N/D	N/D	N/D
Guanine	–	N/D	N/D	N/D
Hypoxanthine	+	N/D	N/D	N/D
Starch	+	N/D	+[b]	N/D
L-Tyrosine	+	N/D	N/D	N/D
Urea	–	N/D	N/D	N/D
Xanthine	–	+[a]	N/D	N/D
Xylan	–	N/D	N/D	N/D
Growth on sole carbon source:				
Glucose	+	N/D	+[b]	N/D
L-(+)-Arabinose	+	N/D	–[b]	+[a]
Fructose	+	N/D	+[b]	+[a]
D-(+)-Galactose	+	N/D	N/D	+[a]
myo-Inositol	–	+[a]	+[b]	–[a]
D-(–)-Mannitol	+(vw)	+[a]	+[b]	+[a]
D-(+)-Raffinose	+(w)	+[a]	–[b]	+[a]
L-(+)-Rhamnose	+(vw)	+[a]	+[b]	–[a]
Salicin	–	N/D	N/D	+[a]
Sucrose	–	+[a]	–[b]	+[a]
D-(+)-Xylose	+	N/D	+[b]	+[a]

N/D = Not determined; ? = Inconclusive result (test must be repeated); +(w) = weakly positive; +(vw) = very weakly positive; a = Locci (1989); b = Trejo & Bennett (1963).

Appendix G:

Complete physiological comparison between *Streptomyces* isolates 33, 35, 36 and 38 and three close phylogenetic relatives, *S. javensis* NBRC 100777^T, *S. violaceusniger* NBRC 13459^T and *S. yogyakartensis* NBRC 100779^T

Test	33 MIIX7P41	35 MIIX65P2	36 MIIX65P4	38 MIIX6P1	*S. javensis* NBRC 100777^T	*S. violaceusniger* NBRC 13459^T	*S. yogyakartensis* NBRC 100779^T
Spore surface ornamentation	rugose	rugose	rugose	rugose	rugose[c]	rugose[a]	rugose[c]
Spore chain morphology	spiral	spiral	spiral	spiral	spiral[c]	spiral[a]	spiral[c]
Aerial mycelium colour (ISP#4)	dark grey/black	dark grey/black	dark grey/black	dark grey	grey (ISP#3)[c]	white-grey[a]	grey (ISP#3)[c]
Substrate mycelium colour (ISP#4)	cream	cream	yellowish-cream	cream	greyish yellow (ISP#3)[c]	greyish yellow[a]	greyish yellow (ISP#3)[c]
Production of diffusible pigment on:							
ISP#5	–	–	–	NG	yellow (ISP#3)[c]	–[a]	yellow (ISP#3)[c]
Production of melanin on:							
ISP#6	–	–	–	–	–[c]	N/D	–[c]
ISP#7	–	–	–	NG	–[c]	N/D	–[c]
Growth at:							
37^{o}C	+	+	+	–	N/D	N/D	N/D
45^{o}C	–	–	–	–	–[c]	+[b]	+[c]
Growth in presence of:							
3% NaCl	–	+	+	–	N/D	N/D	N/D
5% NaCl	–	+(w)	–	–	N/D	N/D	N/D
7% NaCl	–	–	–	–	N/D	N/D	N/D
Reduction of:							
Nitrate	–	–	–	+	–[b]	+[b]	–[c]
Production of:							
H_2S	–	+	–	–	–[b]	–[b]	–[b]
Decomposition of:							
Allantoin	NG	NG	NG	NG	+[c]	–[b]	+[c]
Adenine	+	+	+	+	+[c]	+[b]	+[c]
Arbutin	+	+	+	+	+[b]	+[b]	+[b]
Casein	+	+	+	+	+[c]	+[b]	+[c]
Cellulose	–	–	–	–	N/D	N/D	N/D
Esculin	+	+	+	+	+[c]	+[b]	–[c]
Gelatin	+	+	+	+	+[b]	+[b]	+[b]
Guanine	–	–	–	–	–[b]	–[b]	–[b]
Hypoxanthine	+	+	+	+	+[b]	+[b]	+[b]
Starch	+	+	+	+	+[b]	+[b]	+[b]
L-Tyrosine	+	+	+	+	+[c]	+[b]	+[c]
Urea	NG	NG	NG	NG	+[b]	+[b]	+[b]
Xanthine	–	–	–	–	–[b]	–[b]	–[b]
Xylan	–	–	–	–	+[c]	+[b]	+[c]
Growth on sole carbon source:							
Glucose	+	+	+	+	+[b]	+[b]	+[b]
L-(+)-Arabinose	+	+	+	–	+[b]	–[b]	+[b]
Fructose	+	+	+	+	+[b]	+[b]	+[b]
D-(+)-Galactose	+	+	+	+	+[b]	+[b]	+[b]
myo-Inositol	+	+	+	+	+[b]	+[b]	+[b]
D-(–)-Mannitol	+	+	+	+	+[b]	+[b]	+[b]
D-(+)-Raffinose	+	+	+	+	+[b]	+[b]	+[b]
L-(+)-Rhamnose	+	+	+	+	+[b]	+[b]	+[b]
Salicin	+	+	+	+	+[b]	+[b]	+[b]
Sucrose	+	+	+	–	+[c]	–[b]	+[c]
D-(+)-Xylose	+	+	+	+	+[b]	–[b]	+[b]

NG = No growth; N/D = Not determined; +(w) = weakly positive; a = Goodfellow *et al.* (2007); b = Saintpierre *et al.* (2003); c = Sembiring *et al.* (2000).

Appendix H:

Complete physiological comparison between *Streptomyces* isolates 5, 6, 26 and 39 and their closest phylogenetic relative *S. malaysiensis* DSM 41687^T

Test	5 MIIC75C1	6 MIIC75C2	26 MIIX6C2	39 MIIC75C21	*S. malaysiensis* DSM 41687^T
Spore surface ornamentation	rugose	rugose	rugose	rugose	rugose[a]
Spore chain morphology	spiral	spiral	spiral	spiral	spiral[a]
Aerial mycelium colour (ISP#4)	dark grey	dark grey	dark grey	white	grey/smoky black[a]
Substrate mycelium colour (ISP#4)	yellow-brown	pale yellow-brown	pale yellow-brown	yellow-brown	brown/grey or yellow/grey[a]
Production of diffusible pigment on:					
ISP#5	–	–	–	–	+[a]
Production of melanin on:					
ISP#6	–	–	–	–	–
ISP#7	–	–	–	–	–/+[a]
Growth at:					
37^{o}C	+	+	+	+	+[a]
45^{o}C	–	–	–	–	–[a]
Growth in presence of:					
3% NaCl	+	+	+	+(w)	+[a]
5% NaCl	+	+	–	–	–[a]
7% NaCl	–	–	–	–	N/D
Reduction of:					
Nitrate	+	+	+	+	N/D
Production of:					
H_2S	+	+	–	–	–[a]
Decomposition of:					
Allantoin	NG	NG	NG	NG	–[c]
Adenine	+	+	+	+	+
Arbutin	+	+	+	+	–[c]
Casein	+	+	+	+	+[a]
Cellulose	–	–	–	–	–
Esculin	–	+	+	+	+[b]
Gelatin	+	+	+	+	N/D
Guanine	–	–	–	–	–
Hypoxanthine	+	+	+	+	+
Starch	+	+	+(w)	+(w)	+[a]
L-Tyrosine	+	+	+	–	+
Urea	NG	NG	NG	NG	+[c]
Xanthine	–	–	–	–	–
Xylan	–	–	–	–	–
Growth on sole carbon source:					
Glucose	+	+	+	+	+
L-(+)-Arabinose	+	+	+	+	+
Fructose	+	+	+	+	+
D-(+)-Galactose	+	+	+	+	+
myo-Inositol	+(w)	+(w)	+(w)	+	+
D-(–)-Mannitol	+	+	+	+	+
D-(+)-Raffinose	+(w)	+(w)	+(w)	–	+(w)
L-(+)-Rhamnose	+	+	+	+(w)	+
Salicin	+(w)	+(w)	+(w)	–	+(w)
Sucrose	–	–	–	–	–
D-(+)-Xylose	+	+	+	+	+

NG = No growth; N/D = Not determined; +(w) = weakly positive; a = Al-Tai *et al.* (1999); b = Goodfellow *et al.* (2007); c = Saintpierre *et al.* (2003). All other results were determined during this project.

Appendix I:

Complete physiological comparison between *Streptomyces* isolates 13, 16 and 37 and *S. sodiiphilus* YIM 80305^T

Test	13 MIIC6C1	16 MIIC6P1	37 MIIX65P41	*S. sodiiphilus* YIM 80305^T
Spore surface ornamentation	hairy	smooth	smooth	smooth
Spore chain morphology	spiral	straight?	straight	straight to flexuous
Aerial mycelium colour (ISP#4)	bald	bald	bald	grey-white (ISP#2, pH 9)
Substrate mycelium colour (ISP#4)	yellow-brown	yellow-brown	yellow-brown	N/D
Production of diffusible pigment on:				
ISP#5	–	–	–	NG
Production of melanin on:				
ISP#6	–	–	–	–
ISP#7	–	–	–	–
Growth at:				
37^{o}C	+(w)	+(w)	+	N/D
45^{o}C	–	–	–	N/D
Growth in presence of:				
3% NaCl	+(w)	–	–	N/D
5% NaCl	–	–	–	N/D
7% NaCl	–	–	–	N/D
Reduction of:				
Nitrate	–	–	–	+
Production of:				
H_2S	–	–	–	–
Decomposition of:				
Allantoin	–	NG	NG	N/D
Adenine	–	–	–	N/D
Arbutin	+	+	+	N/D
Casein	+	+	+	N/D
Cellulose	–	–	–	N/D
Esculin	+	+	+	N/D
Gelatin	+	+	+	+
Guanine	–	–	–	N/D
Hypoxanthine	+(w)?	+(w)	–	N/D
Starch	+	+	+	–
L-Tyrosine	+	+	+	N/D
Urea	–	NG	NG	–
Xanthine	–	–	–	N/D
Xylan	–	–	–	N/D
Growth on sole carbon source:				
Glucose	+	+	–?	–
L-(+)-Arabinose	–	–	–?	–
Fructose	+(w)	+	–?	–
D-(+)-Galactose	+	+	–?	–
myo-Inositol	+	+(w)	–?	–
D-(–)-Mannitol	+	+	–?	–
D-(+)-Raffinose	+	+	–?	–
L-(+)-Rhamnose	+	+	–?	+
Salicin	+/–	+	–?	N/D
Sucrose	+	+	–?	–
D-(+)-Xylose	+	+	–?	–

All *S. sodiiphilus* results were taken from Li *et al.* (2005). N/D = Not determined; NG = No growth; ? = Result uncertain; +(w) = weakly positive.

Appendix J:

Complete physiological comparison between *Streptomyces* isolates 18, 19 and 32 vs *S. avermitilis* MA-4680^T, *S. cinnabarinus* ISP 5467^T and *S. kunmingensis* ATCC 35682^T

Test	18 MIIX7C1	19 MIIX7C2	32 MIIX7P4	*S. avermitilis* MA-4680^T	*S. cinnabarinus* ISP 5467^T	*S. kunmingensis* ATCC 35682^T
Spore surface ornamentation	smooth	spiny	smooth	smooth[b,c]	smooth[b,c]	N/D
Spore chain morphology	straight	spiral	straight	spiral[b,c]	flexous[b,c]	sclerotia, loose spirals[a]
Aerial mycelium colour (ISP#4)	white	pale grey	white	grey[b,c] (oatmeal agar)	red[b,c] (oatmeal agar)	yellowish-white[a]
Substrate mycelium colour (ISP#4)	cream	brownish-cream	yellow-white	dark brown/tan	N/D	yellowish-tan/dark orange[a]
Production of diffusible pigment on:						
ISP#5	–	–	–	+[c] (yellow)?	+[c] (red)?	–
Production of melanin on:						
ISP#6	–	–	–	+[b,c]	+[b,c]	– (–[a])
ISP#7	–	–	–	N/D	N/D	– (–[a])
Growth at:						
37°C	+	+	+	+[b]	N/D	+(w) (+[a])
45°C	–	–	–	–[b] (50 °C)	N/D	– (–[a] 42 °C)
Growth in presence of:						
3% NaCl	+	+	+	N/D	N/D	+
5% NaCl	+	+	+	+[c]	N/D	+
7% NaCl	+	+(w)	+	N/D	N/D	+
Reduction of:						
Nitrate	+	–	+	N/D	N/D	+ (+[a])
Production of:						
H_2S	+	+	+(vw)	+[c]	N/D	+
Decomposition of:						
Allantoin	+(w)	–	+	N/D	N/D	+
Adenine	+	+	+	+[b,c]	+[b]	+ (+[a])
Arbutin	+	+	+	–[b]	+[b]	+
Casein	+	+	+	+[b,c]	N/D	+ (+[a])
Cellulose	–	–	–	–[b,c]	N/D	–
Esculin	+	+	+	N/D	N/D	+ (+[a])
Gelatin	+	+	+	+[b,c]	N/D	+ (–[a])
Guanine	–	–	–	N/D	N/D	–
Hypoxanthine	+	+	+	+[c]	N/D	+ (+[a])
Starch	+(w)	+	+	+[b,c]	–[b]	– (+[a])
L-Tyrosine	+	+	+	–[b]	N/D	+ (+[a])
Urea	+(w)	–	+	N/D	N/D	– (–[a])
Xanthine	–	–	–	+[b,c]	+[b]	– (+[a])
Xylan	–	–	–	N/D	N/D	–
Growth on sole carbon source:						
Glucose	+	+	+	+[b,c]	+[c]	+ (+[a])
L-(+)-Arabinose	+	+	+	+[b,c]	–[b] +[c]	+(w) (+[a])
Fructose	+	+	+	+[b,c]	–[b] +[c]	+ (+[a])
D-(+)-Galactose	+	+	+	N/D	N/D	+
myo-Inositol	+	+	–	+[b,c]	–[b] +[c]	– (–[a])
D-(–)-Mannitol	+	+	+	+[c]	+[c]	+ (+[a])
D-(+)-Raffinose	+	+	+	+[c]	+[c]	+ (+[a])
L-(+)-Rhamnose	+	+	+	+[b,c]	+[c]	+ (+[a])
Salicin	+(vw)	+	+	N/D	N/D	+(w)
Sucrose	+	+	+	–[b] +[c]	+[b,c]	– (–[a])
D-(+)-Xylose	+	+	+	+[b,c]	–[b] +[c]	+ (+[a])

Unless otherwise indicated, all results were obtained during this study. N/D = Not determined; +(w) = weakly positive; +(vw) = very weakly positive; a = Ruan *et al*. (1985); b = Kim & Goodfellow (2002); c = Takahashi *et al*. (2002).

Appendix K:

Complete physiological comparison between *Streptomyces* isolate 14 and its closest phylogenetic relatives *S. atratus* NRRL B-16927^T, *S. gelaticus* NRRL B-2928^T, *S. pulveraceus* NBRC 3855^T and *S. sanglieri* DSM 41791^T

Test	14 MIIC7P1	*S. atratus* NRRL B-16927^T	*S. gelaticus* NRRL B-2928^T	*S. pulveraceus* NBRC 3855^T	*S. sanglieri* DSM 41791^T
Spore surface ornamentation	smooth	smooth[d]	smooth[a]	smooth[a]	smooth[b]
Spore chain morphology	spiral	loops[d]	straight[a]	spiral[c]	spiral[b]
Aerial mycelium colour (ISP#4)	white	grey (glucose-asparagine)[d]	N/D	light greyish olive (glucose-asparagine)[c]	grey (ISP#5)[b]
Substrate mycelium colour (ISP#4)	yellow-brown	black (glucose-asparagine)[d]	yellow-brown[a]	orange (glucose-asparagine)[c]	reddish-orange (ISP#5)[b]
Production of diffusible pigment on:					
ISP#5	–	–	–[a]	N/D	+
Production of melanin on:					
ISP#6	–	–	–[a]	+[a]	–
ISP#7	–	–	–[a]	+[a]	+
Growth at:					
37^{o}C	+	–	–[a]	N/D	–
45^{o}C	–	–	–[a]	N/D	–
Growth in presence of:					
3% NaCl	+	+	N/D	N/D	+
5% NaCl	+	+(w)	N/D	N/D	+
7% NaCl	–	–	–[a]	N/D	–
Reduction of:					
Nitrate	–	–	N/D	+[c]	+
Production of:					
H_2S	+	+	N/D	N/D	–
Decomposition of:					
Allantoin	–	+	N/D	N/D	+
Adenine	+	+	N/D	N/D	+
Arbutin	–	+	N/D	N/D	+
Casein	+	+	N/D	N/D	+
Cellulose	–	–	N/D	NG[c]	–
Esculin	+	+	N/D	N/D	+
Gelatin	+	+	N/D	+[c]	+
Guanine	–	–	N/D	N/D	–
Hypoxanthine	+	+	N/D	N/D	+
Starch	+	+	N/D	+[c]	+
L-Tyrosine	+	+	N/D	N/D	+
Urea	–	+	N/D	N/D	+
Xanthine	–	–	+[a]	N/D	–
Xylan	–	–	N/D	N/D	–
Growth on sole carbon source:					
Glucose	+	+	N/D	+(w)[c]	+
L-(+)-Arabinose	+	–	N/D	+(w)[c]	–
Fructose	+	+(vw)	N/D	+[c]	+
D-(+)-Galactose	+	+	N/D	+[c]	+
myo-Inositol	+(w)	–	+[a]	+(w)[c]	–
D-(–)-Mannitol	–	–	–[a]	+(w)[c]	–
D-(+)-Raffinose	+	+	–[a]	+[c]	+
L-(+)-Rhamnose	+	+	+[a]	+[c]	+
Salicin	–	+	N/D	+[c]	+(w)
Sucrose	–	–	+[a]	–[a]	+
D-(+)-Xylose	+	+	N/D	+[c]	+

Unless otherwise indicated, all results were obtained during this study. N/D = Not determined; NG = No growth; +(w) = weakly positive; +(vw) = very weakly positive; a = Locci (1989); b = Manfio *et al.* (2003); c = Shibata *et al.* (1961); d = Shibata *et al.* (1962).

Appendix L:

De-replication of the Original and Duplicate isolates, showing whether a unique banding pattern was obtained after digestion with the enzymes *Hpa*II, *Cfo*I and *Hinf*I, as well as which isolates are considered to be unique overall, and therefore worthy of further work.

Isolate	Unique banding pattern			Worthy of further work	Isolate	Unique banding pattern			Worthy of further work
	***Hpa*II**	***Cfo*I**	***Hinf*I**			***Hpa*II**	***Cfo*I**	***Hinf*I**	
5	√ Hp5	√ Cf5	√ Hi5	N/A	39D	× Hp5	× Cf4	× Hi2	√
6	× Hp5	× Cf5	× Hi5	N/A	40	× Hp5	× Cf23	× Hi9	√
26	× Hp5	× Cf5	× Hi5	N/A	41	× Hp5	× Cf23	× Hi9	×
39	× Hp5	× Cf5	× Hi5	N/A	42	× Hp5	× Cf23	× Hi9	×
33	√ Hp33	√ Cf33	√ Hi33	N/A	43	× Hp14	× Cf7	× Hi33	×
35	× Hp33	× Cf33	× Hi33	N/A	44	× Hp5	× Cf23	× Hi9	×
36	× Hp33	× Cf33	× Hi33	N/A	45	× Hp14	× Cf4	× Hi9	√
38	× Hp33	× Cf33	× Hi33	N/A	46	× Hp14	× Cf32	× Hi5	×
12	√ Hp12	√ Cf12	√ Hi12	N/A	47	× Hp14	× Cf7	× Hi33	×
20	× Hp12	× Cf12	× Hi12	N/A	48	× Hp14	× Cf32	× Hi5	×
14	√ Hp14	√ Cf14	√ Hi14	N/A	49	× Hp14	× Cf14	× Hi2	√
13	× Hp33	√ Cf13	√ Hi13	N/A	50	× Hp5	√ Cf50	× Hi9	√
16	× Hp14	× Cf13	× Hi13	N/A	51	√ Hp51	√ Cf51	√ Hi51	√
37	× Hp14	× Cf13	× Hi13	N/A	52	× Hp14	× Cf14	× Hi5	√
18	× Hp14	√ Cf18	√ Hi18	N/A	53	× Hp14	× Cf14	× Hi5	×
19	× Hp14	√ Cf19	√ Hi19	N/A	54	× Hp14	× Cf7	× Hi33	×
32	× Hp14	√ Cf32	× Hi19	N/A	55	× Hp14	× Cf14	× Hi5	×
2	× Hp14	√ Cf2	√ Hi2	N/A	56	× Hp14	× Cf14	× Hi5	×
7	× Hp14	√ Cf7	× Hi33	N/A	57	× Hp14	× Cf14	× Hi5	×
1	× Hp14	× Cf7	× Hi33	×	59	× Hp14	× Cf14	× Hi2	×
3	× Hp14	× Cf7	× Hi33	×	60	× Hp5	√ Cf60	× Hi5	√
4	× Hp14	√ Cf4	√ Hi4	√	61	× Hp14	× Cf2	× Hi2	×
8	× Hp14	√ Cf8	× Hi5	√	62	× Hp5	× Cf50	× Hi4	√
9	× Hp14	× Cf19	√ Hi9	√	64	× Hp14	× Cf2	× Hi5	√
10	× Hp14	√ Cf10	× Hi33	√	65	× Hp14	× Cf2	× Hi5	×
11	× Hp14	× Cf10	× Hi33	×	66	× Hp5	× Cf60	× Hi5	×
15	× Hp14	√ Cf15	× Hi5	√	67	× Hp14	× Cf7	× Hi33	×
17	× Hp14	× Cf10	× Hi5	√	68	× Hp14	× Cf2	× Hi5	×
21	× Hp14	× Cf7	× Hi33	×	69	× Hp14	× Cf2	× Hi2	×
22	× Hp14	× Cf5	× Hi5	×	70	× Hp14	× Cf23	× Hi4	×
23	× Hp14	√ Cf23	× Hi4	√	71	× Hp5	× Cf60	× Hi5	×
24	× Hp14	× Cf32	× Hi5	√	72	× Hp14	× Cf2	× Hi5	×
25	× Hp14	× Cf5	√ Hi25	√	73	× Hp14	× Cf7	× Hi33	×
27	× Hp14	× Cf5	× Hi25	×	74	× Hp5	× Cf60	× Hi5	×
28	× Hp14	× Cf23	× Hi5	√	75	× Hp14	× Cf2	× Hi5	×
29	× Hp14	√ Cf29	× Hi5	√	76	× Hp5	× Cf60	× Hi5	×
30	× Hp14	√ Cf30	× Hi5	√	77	× Hp14	× Cf7	× Hi33	×
31	× Hp14	× Cf23	× Hi4	×	78	× Hp14	× Cf2	× Hi5	×
34	× Hp14	× Cf8	× Hi5	×					

N/A = Not applicable

Appendix M: *Alu*I digest groupings, indicating the pattern name, number of clones included within the specific pattern/group and a list of the clones included within each group. Clone source identification: C = colon, P = paunch and SS = surrounding soil.

Group **(No. of clones)**	Included clones
AC1 **(38)**	C1,C18,C27,C31,C48,C49,C52,C53,C103,C108,C134,C138,C145,C149,C150,C156, C159,C164,C172,C188,P53,SS12,SS16,SS17,SS18,SS31,SS43,SS51,SS85,SS97,SS109 SS113,SS128,SS135,SS142,SS155,SS161,SS163
AC3 **(2)**	C3,C179
AC4 **(3)**	C4,C160,C220
AC5 **(51)**	C5,C6,C7,C15,C17,C19,C23,C40,C42,C44,C47,C51,C54,C64,C67,C78,C86,C88,C90, C92,C96,C97,106,C109,C113,C117,C133,C147,C151,C154,C163,C178,C180,C185, C192,C213,C227,P10,P12,P47,P83,P84,P85,P97,P100,P101,P138,SS89,SS94,SS99, SS157
AC8 **(4)**	C8,C26,P6,SS6
AC10 **(34)**	C10,C12,C59,C63,C66,C94,C95,C101,C126,C152,C174,C175,C176,C218,C219,C225, C226,P31,P43,P58,P89,P104,P112,P117,P137,SS53,SS65,SS103,SS105,SS125,SS127, SS136,SS138,SS149
AC13 **(1)**	C13
AC21 **(29)**	C21,C57,C98,C158,C193,C232,P4,P14,P26,P38,P39,P40,P42,P73,P86,P122,SS7,SS46,SS61, SS70,SS72,SS81,SS101,SS108,SS110,SS114,SS124,SS139,SS152
AC22 **(1)**	C22
AC24 **(6)**	C24,C72,C205,C210,C212,C215,SS15
AC25 **(1)**	C25
AC33 **(20)**	C33,C77,C91,C111,C120,C127,C189,C194,P15,P29,P30,P103,P111,P116,P118,P124,SS29,S S34,SS71,SS91,SS145
AC43 **(1)**	C43
AC55 **(17)**	C55,C102,C110,C141,C181,C196,C209,C222,P81,P108,SS8,SS67,SS147,SS153,SS156,SS15 8,SS166
AC56 **(1)**	C56
AC60 **(1)**	C60
AC61 **(1)**	C61
AC80 **(1)**	C80
AC87 **(3)**	C87,C182,C229
AC112 **(2)**	C112,SS10
AC114 **(1)**	C114
AC115 **(2)**	C115,P8
AC123 **(1)**	C123
AC125 **(6)**	C125,C128,C224,P32,P52,P87
AC132 **(1)**	C132
AC137 **(1)**	C137
AC155 **(1)**	C155
AC165 **(1)**	C165
AC166 **(1)**	C166
(AC1?) AC188 **(8)**	C188,SS60,SS75,SS87,SS95,SS112,SS120,SS151
AC197 **(15)**	C197,C199,C202,C203,SS20,SS21,SS41,SS48,SS49,SS56,SS58,SS115,SS117,SS133, SS143
AC206 **(4)**	C206,SS55,SS137,SS141
AC215 **(1)**	C215
(AC5?) AC227 **(4)**	C227,P25,P61,P80
AP17 **(8)**	P17,P50,P62,P70,P74,P120,P133,SS102
AP24 **(14)**	P24,P55,P56,P60,P67,P71,P107,P109,P110,P113,SS13,SS68,SS107,SS159
AP34 **(9)**	P24,P54,P91,P114,P119,P123,SS44,SS59,SS98

(AC1?) AP53 **(3)**	P53,SS66,SS111
AP59 **(2)**	P59,P88
AP66 **(1)**	P66
AP68 **(2)**	P68,P102
AP75 **(1)**	P75
AP90 **(1)**	P90
AP93 **(1)**	P93
(AP17?) AP120 **(2)**	P120,SS116
AP128 **(1)**	P128,P135
ASS9 **(1)**	SS9
ASS11 **(1)**	SS11
ASS22 **(1)**	SS22
ASS32 **(4)**	SS32,SS38,SS96,SS154
ASS35 **(1)**	SS35
ASS39 **(1)**	SS39
ASS63 **(3)**	SS63,SS130,SS164
ASS82 **(1)**	SS82
ASS90 **(1)**	SS90
ASS118 **(1)**	SS118
ASS123 **(1)**	SS123
ASS129 **(1)**	SS129
ASS140 **(1)**	SS140
ASS150 **(1)**	SS150

Appendix N: *Rsa*I digest groupings, indicating the pattern name, number of clones included within the specific pattern/group and a list of the clones included within each group. Clone source identification: C = colon, P = paunch and SS = surrounding soil.

Group **(No. of clones)**	Included clones
RC1 **(14)**	C1,C18,C27,C31,C48,C49,C108,C134,C138,C149,C156,C159,C164,C172
RC3 **(1)**	C3
RC4 **(2)**	C4,C220
RC5 **(28)**	C5,C6,C7,C17,C19,C23,C40,C42,C44,C47,C54,C64,C67,C86,C90,C92,C96,C97,C106,C109,C113,C133,C147,C151,C163,C180,C192,C227
RC8 **(2)**	C18,C26
RC10 **(17)**	C10,C59,C63,C66,C94,C95,C101,C126,C152,C174,C175,C176,C218,C225,C226,P104,P137
RC12 **(9)**	C12,P31,P58,P112,P117,SS53,SS105,SS127,SS136
RC13 **(1)**	C13
RC15 **(2)**	C15,C51
RC21 **(21)**	C21,C57,C193,P4,P14,P73,P86,P122,SS7,SS46,SS61,SS70,SS72,SS81,SS101,SS108, SS110,SS114,SS124,SS139,SS152
RC22 **(1)**	C22
RC24 **(2)**	C24,C212,C215
RC25 **(1)**	C25
RC33 **(2)**	C33,SS71
RC43 **(1)**	C43
RC55 **(1)**	C55
RC56 **(1)**	C56
RC60 **(1)**	C60
RC61 **(1)**	C61
RC72 **(1)**	C72
RC78 **(9)**	C78,C185,P12,P47,P83,P84,P87,P100,SS99
RC80 **(1)**	C80
RC87 **(2)**	C87,C182
RC88 **(1)**	C88
RC91 **(4)**	C77,C91,C127,C189,C194
RC98 **(3)**	C98,C158,C232
RC102 **(7)**	C102,C141,C196,SS8,SS147,SS152,SS158
RC103 **(4)**	C103,SS17,SS128,SS155
RC110 **(1)**	C110
RC111 **(1)**	C111
RC112 **(1)**	C112
RC115 **(1)**	C115
RC117 **(1)**	C117
RC120 **(1)**	C120
RC123 **(1)**	C123
RC125 **(3)**	C125,C224,P52
RC128 **(1)**	C128
RC132 **(1)**	C132
RC137 **(1)**	C137
RC154 **(1)**	C154
RC155 **(1)**	C155
RC160 **(1)**	C160
RC165 **(1)**	C165
RC166 **(1)**	C166
RC178 **(1)**	C178
RC179 **(1)**	C179
RC181 **(1)**	C181
RC188 **(12)**	C52,C53,C188,P53,SS16,SS18,SS31,SS43,SS87,SS135,SS151,SS161
RC197 **(4)**	C197,C199,C202,C203

RC205 **(2)**	C205,C210
RC206 **(1)**	C206
RC209 **(2)**	C209,C222
RC213 **(1)**	C213
RC219 **(1)**	C219
RC229 **(1)**	C229
RP6 **(1)**	P6
RP8 **(1)**	P8
RP10 **(3)**	P10,P101,SS94
RP15 **(6)**	P15,P29,P103,P111,P116,P124
RP17 **(1)**	P17
RP24 **(10)**	P24,P55,P56,P60,P67,P71,P107,P109,P110,P113
RP25 **(3)**	P25,P61,P80
RP26 **(5)**	P26,P38,P39,P40,P42
RP30 **(4)**	P30,P118,SS29,SS145
RP32 **(2)**	P32,P87
RP34 **(3)**	P34,P54,P91
RP43 **(2)**	P43,SS65
RP50 **(1)**	P50
RP62 **(1)**	P62
RP66 **(1)**	P66
RP68 **(2)**	P68,P102
RP70 **(1)**	P70
RP74 **(1)**	P74
RP75 **(1)**	P75
RP81 **(2)**	P81,P108
RP85 **(1)**	P85
RP88 **(1)**	P88
RP89 **(2)**	P89,SS138
RP90 **(1)**	P90
RP93 **(1)**	P93
RP114 **(2)**	P114,P119
RP120 **(1)**	P120
RP123 **(1)**	P123
RP128 **(2)**	P128,P135
RP133 **(1)**	P133
RP138 **(1)**	P138
RSS6 **(1)**	SS6
RSS9 **(1)**	SS9
RSS10 **(1)**	SS10
RSS11 **(1)**	SS11
RSS12 **(1)**	SS12
RSS13 **(4)**	SS13,SS68,SS107,SS159
RSS15 **(1)**	SS15
RSS20 **(3)**	SS20,SS115,SS143
RSS21 **(7)**	SS21,SS41,SS48,SS49,SS58,SS117,SS133
RSS22 **(1)**	SS22
RSS32 **(1)**	SS32
RSS34 **(1)**	SS34
RSS35 **(1)**	SS35
RSS38 **(1)**	SS38
RSS39 **(1)**	SS39
RSS44 **(3)**	SS44,SS59,SS98
RSS51 **(2)**	SS51,SS142
RSS55 **(1)**	SS55
RSS56 **(1)**	SS56
RSS60 **(1)**	SS60
RSS63 **(1)**	SS63

RSS66 **(1)**	SS66
RSS67 **(3)**	SS67,SS156,SS166
RSS75 **(3)**	SS75,SS95,SS120
RSS82 **(1)**	SSS82
RSS89 **(1)**	SS89
RSS90 **(1)**	SS90
RSS91 **(1)**	SS91
RSS96 **(1)**	SS96
RSS97 **(4)**	C150,SS85,SS97,SS109
RSS102 **(1)**	SS102
RSS111 **(1)**	SS111
RSS112 **(1)**	SS112
RSS113 **(1)**	SS113
RSS116 **(1)**	SS116
RSS118 **(1)**	SS118
RSS123 **(1)**	SS123
RSS125 **(1)**	SS125
RSS129 **(1)**	SS129
RSS130 **(1)**	SS130
RSS137 **(1)**	SS137
RSS140 **(1)**	SS140
RSS141 **(1)**	SS141
RSS149 **(1)**	SS149
RSS150 **(1)**	SS150
RSS154 **(1)**	SS154
RSS157 **(1)**	SS157
RSS163 **(2)**	C145,SS163
RSS164 **(1)**	SS164

Appendix O: Complete list of the sequencing results obtained after sequencing of all clones from all three sample sites, including the sequence similarity to each clone's closest BLAST hit, as well as its nearest identified or cultured relative.

Sample origin	Clone	Closest BLAST hit	Similarity (%)	Closest identified/cultured relative	Similarity (%)
Colon	MVC1	Uncultured *Actinobacteridae* clone LrhB52	96%	*Propionicicella superfundia* strain BL-10T	95%
	MVC3	*Streptomyces* sp. 213101	99%	*Streptomyces globisporus* subsp. *globisporus* isolate XSD-114	99%
	MVC4	Uncultured *Actinobacteridae* clone Rs-J59	93%	*Atopobium vaginae*	90%
	MVC5	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC8	Uncultured *Actinobacteridae* clone LrhB52	96%	*Propionicicella superfundia* strain BL-10T	95%
	MVC10	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	95%
	MVC12	Iron-reducing enrichment clone Cl-A3	98%	*Propionicimonas* sp. F6	97%
	MVC13	Uncultured bacterium clone BCf3-22	92%	*Actinomyces howellii* strain NCTC 11636	91%
	MVC15	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC21	*Streptomyces* sp. 213101	99%	*Streptomyces globisporus* subsp. *globisporus* isolate XSD-114	99%
	MVC22	*Actinomycetales* bacterium JH105	99%	*Streptomyces globisporus* subsp. *globisporus* isolate XSD-114	99%
	MVC24	Uncultured *Actinobacteridae* clone Rs-J59	96%	None	N/A
	MVC25	Uncultured *Acidobacteria* clone M1PT4-70	96%	None	N/A
	MVC26	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC27	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC33	*Micromonospora auratinigra* strain HBUM49317	99%	*Micromonospora arenae* strain MuizA5S (Intereresting hit)	99%
	MVC43	Uncultured bacterium clone 2005-MA-24-090607	97%	Uncultured *Candidatus Microthrix* sp. clone BL030B78	97%
	MVC48	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	95%
	MVC51	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC52	*Streptomyces* sp. VC-YC6673	99%	*Streptomyces amakusaensis* strain NBRC 12835	99%
	MVC55	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propioniferax innocua*	94%
	MVC56	Uncultured *Verrucomicrobia* clone Cc3-002	93%	*Coraliomargarita akajimensis*	83%
	MVC59	Uncultured *Actinobacteridae* clone LrhB52	94%	*Propionicicella superfundia* strain BL-10T	88%

	MVC60	Uncultured *Actinomycetales* clone TUM-Mbac-TR1-B1-K2-124	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC61	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	95%
	MVC66	Uncultured bacterium clone BOf2-16	95%	*Brooklawnia cerclae* strain BL-34	94%
	MVC72	Uncultured *Actinobacteridae* clone Rs-J59	98%	None	N/A
	MVC77	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC78	Uncultured bacterium clone 2-4H	99%	*Friedmanniella antarctica* strain AA-1042	99%
	MVC80	Uncultured *Actinobacteridae* clone LrhB52	96%	*Propionicicella superfundia* strain BL-10T	95%
	MVC87	Uncultured *Actinobacteridae* clone LrhB52	90%	*Propionicicella superfundia* strain BL-10T	88%
	MVC88	Uncultured *Opitutus* sp. clone AMBD11	88%	*Opitutus* sp. strain VeCb1	86%
	MVC91	*Micromonosporaceae* bacterium UMM518	95%	Actinokineospora diospyrosa strain NRRL B-24047	94%
	MVC97	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC98	Uncultured bacterium clone N1903_51	97%	*Propionicimonas paludicola* strain RR22	96%
	MVC102	Uncultured bacterium clone BCf3-22	91%	*Actinomyces howellii* strain NCTC 11636	91%
	MVC103	*Cellulomonas* sp. strain d20	94%	*Isoptericola variabilis* strain TPID6	94%
	MVC110	Uncultured *Opitutus* sp. clone AMBD11	86%	*Opitutus* sp. VeSm13	84%
	MVC111	*Micromonosporaceae* bacterium 211018	98%	*Micromonospora tulbaghiae* strain TVU1	98%
	MVC112	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	95%
	MVC115	Uncultured bacterium clone N1903_51	97%	*Propionicimonas paludicola* strain RR22	95%
	MVC117	Uncultured actinobacterium clone NkW01-002	92%	*Actinomyces howellii* strain NCTC 11636	91%
	MVC120	Uncultured *Actinobacteridae* clone LrhB52	94%	*Propionicicella superfundia* strain BL-10T	93%
	MVC123	Uncultured bacterium clone BOf2-16	96%	*Propionibacteriaceae* bacterium SH081	94%
	MVC125	Uncultured *Actinobacteridae* clone Rs-J59	96%	None	N/A
	MVC127	*Micromonosporaceae* bacterium 211018	94%	*Micromonospora auratinigra* strain HBUM49317	94%
	MVC128	Uncultured bacterium clone COB P3-21	95%	*Eggerthella* sp. YY7918	90%
	MVC132	Uncultured *Actinobacteridae* clone Rs-J59	98%	None	N/A
	MVC137	*Pseudonocardia* sp. GMKU095	98%	*P. asaccharolytica* (2nd hit)	96%
	MVC145	*Rothia aeria* strain Lan	99%	*Rothia aeria* strain SMC-A2662	99%
	MVC150	Iron-reducing enrichment clone Cl-A3	97%	*Microlunatus* sp. J109	95%

	MVC154	Uncultured bacterium clone BCf3-22	91%	*Actinomyces howellii* strain NCTC 11636	90%
	MVC155	*Streptomyces* sp. VC-YC6673	97%	*Streptomyces amakusaensis* strain NBRC 12835	97%
	MVC158	*Tessaracoccus flavescens* strain SST-39T	93%	*Tessaracoccus bendigoensis*	93%
	MVC160	Uncultured bacterium clone COB P3-21	95%	Uncultured *Coriobacteriales* bacterium clone BLUC-G	89%
	MVC165	Uncultured bacterium clone N1903_51	97%	*Propionicimonas* sp. F6	96%
	MVC166	*Tessaracoccus flavescens* strain SST-39T	93%	*Tessaracoccus bendigoensis* (second hit)	93%
	MVC178	Uncultured bacterium clone BCf3-22	92%	*Actinomyces howellii* strain NCTC 11636	91%
	MVC179	Uncultured *Actinobacteridae* clone: Rs-J59	97%	None	N/A
	MVC181	Uncultured bacterium clone TD47	96%	*Propionicicella superfundia* strain BL-10T	94%
	MVC182	Uncultured bacterium clone 2-4H	99%	*Friedmanniella antarctica*	98%
	MVC185	Uncultured bacterium clone N1903_51	83%	*Propionicimonas paludicola* strain RR22	82%
	MVC188	*Streptomyces* sp. VC-YC6673	99%	*Streptomyces amakusaensis* strain NBRC 12835	99%
	MVC193	*Streptomyces* sp. 213101	99%	*Streptomyces globisporus* subsp. *globisporus* isolate XSD-114	99%
	MVC194	*Micromonospora tulbaghiae* strain TVU1	99%	*Micromonospora auratinigra* strain HBUM49317 (2nd hit)	99%
	MVC196	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC197	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC199	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	95%
	MVC202	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC205	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	94%
	MVC206	Uncultured *Actinobacteridae* clone LrhB52	94%	*Propionicicella superfundia* strain BL-10T	94%
	MVC209	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	95%
	MVC210	Uncultured *Actinobacteria* clone AKYG1141	94%	*Actinomadura chibensis* strain HBUM174700	94%
	MVC212	Uncultured *Actinobacteridae* clone LrhB52	95%	*Propionicicella superfundia* strain BL-10T	95%
	MVC213	Uncultured bacterium clone BCf3-22	92%	*A. howellii*	91%
	MVC215	Uncultured *Actinobacteridae* clone Rs-J59	98%	None	N/A
	MVC219	*Rothia aeria* strain Lan	99%	*Rothia aeria* strain SMC-A2662 (4th hit)	99%
	MVC220	Uncultured bacterium clone ORSFAB_d11	96%	*Chondromyces lanuginosus* strain KYC2904	92%

	MVC222	Uncultured *Actinobacteridae* clone LrhB52	95%	Actinobacterium MH1-1	94%
	MVC224	Uncultured *Actinobacteridae* clone Rs-J59	96%	*Eggerthella* sp. YY7918	91%
	MVC227	Uncultured *Actinobacteridae* clone LrhB52	95%	Actinobacterium MH1-1	94%
	MVC229	Uncultured bacterium clone 8F319	98%	*Actinomadura spadix* strain NBRC 14099	95%
	MVC232	Uncultured bacterium clone N1903_51	97%	*Propionicimonas paludicola* strain RR22	96%
Paunch	MVP4	*Streptomyces* sp. 213101	98%	*Streptomyces anulatus* strain HBUM174601	98%
	MVP6	Uncultured bacterium clone A55_D21_H_B_E02	98%	*Actinomadura livida* strain IMSNU 22191T	94%
	MVP8	Uncultured *Actinobacteria* bacterium clone AKYG1141	99%	*Actinomadura chokoriensis*	98%
	MVP10	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
	MVP12	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
	MVP15	*M.melanospora*	99%	*Micromonospora tulbaghiae* strain TVU1	99%
	MVP17	Uncultured *Acidobacteria* clone M1PT4-70	91%	***Candidatus Tammella caduceiae* clone CcCv-02**	86%
	MVP24	Uncultured *Actinobacteria* clone AKYG1141	94%	*Actinomadura macra* strain NBRC 14102	93%
	MVP25	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
	MVP26	*Streptomyces* sp. HBUM87074	97%	*Amycolatopsis coloradensis* strain HBUM173696	96%
	MVP30	*Micromonospora auratinigra* strain HBUM49317	99%	*Micromonospora echinofusca* strain HBUM175187 (2nd hit)	99%
	MVP31	Uncultured bacterium clone N1903	96%	*Propionicimonas paludicola*	96%
	MVP32	Uncultured *Actinobacteria* clone AKYG1141	98%	*Actinomadura chokoriensis*	98%
	MVP34	Uncultured bacterium clone BCf3-22	91%	*Actinomyces howellii*	90%
	MVP38	Uncultured *Actinobacteria* clone AKYG1141	99%	*Actinomadura chokoriensis*	98%
	MVP42	Uncultured *Actinobacteria* clone AKYG1141	99%	*Actinomadura chokoriensis*	99%
	MVP43	*Agromyces neolithicus* strain 23-23	99%	Rhizosphere soil bacterium isolate RSI-19 (3rd hit)	98%
	MVP50	Uncultured bacterium clone Tc-61	93%	*Candidatus Tammella caduceiae* clone CcCv-02	91%
	MVP52	Uncultured *Actinobacteridae* clone Rs-J59	97%	*Eggerthella* sp. YY7918	90%
	MVP53	*Actinomycetales* bacterium JH105	100%	*Streptomyces* sp. 213101 (2nd hit)	100%
	MVP54	Uncultured bacterium clone BCf3-22	91%	*Actinomyces howellii* strain NCTC 11636	91%
	MVP61	Uncultured *Acidobacteria* clone M1PT4-70	95%	None	N/A
	MVP62	Uncultured *Verrucomicrobia* clone Cc3-002	89%	*Opitutus* sp. SA-9	84%

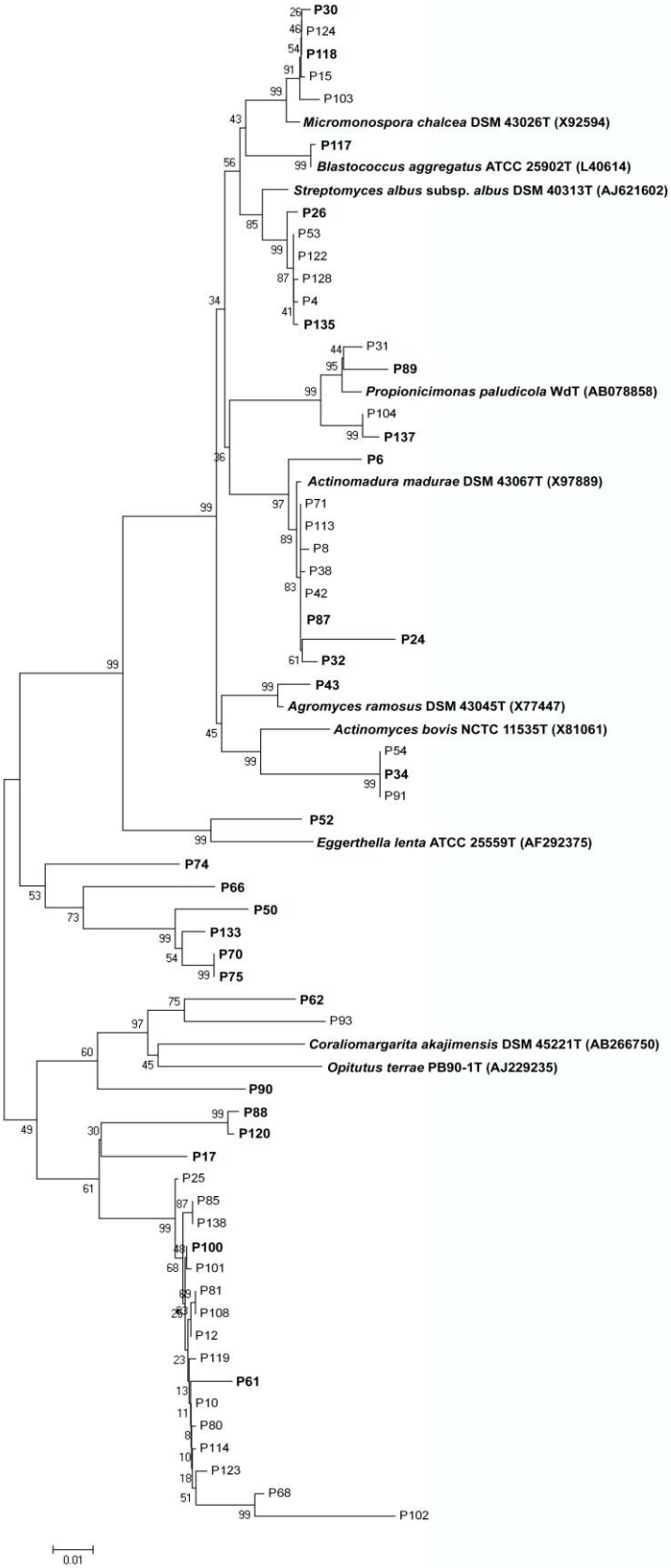
MVP66	Uncultured bacterium clone M1NP1-08	93%	None	N/A
MVP68	Uncultured *Acidobacteria* clone M1PT4-70	93%	None	N/A
MVP70	Uncultured bacterium clone Tc-61	90%	*Candidatus Tammella caduceiae* clone CcCv-02	89%
MVP71	Uncultured *Actinobacteria* bacterium clone AKYG1141	99%	*Actinomadura chokoriensis*	99%
MVP74	Uncultured candidate division TG3 bacterium clone M2PB4a-070	89%	**Uncultured *Fibrobacteres* bacterium clone 290cost002-P3L-1553**	88%
MVP75	Uncultured bacterium clone Tc-61	91%	*Candidatus Tammella caduceiae* clone CcCv-02	89%
MVP80	Uncultured *Acidobacteria* clone M1PT4-70	95%	None	N/A
MVP81	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
MVP85	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
MVP87	Uncultured *Actinobacteria* clone AKYG1141	99%	*Actinomadura chokoriensis*	99%
MVP88	Uncultured *Treponema* sp. clone M2PB4-19	86%	Uncultured *Treponema* sp. clone 290cost002-P3L-1791 (5th hit)	86%
MVP89	Iron-reducing enrichment clone Cl-A3	96%	*Propionicimonas* sp. F6	95%
MVP90	Uncultured *Acidobacteria* clone M1PT4-70	87%	*Coraliomargarita akajimensis*	83%
MVP91	Uncultured bacterium clone BCf3-22	91%	*Actinomyces howellii* strain NCTC 11636	91%
MVP93	Uncultured bacterium clone RsaP110	85%	*Coraliomargarita akajimensis*	84%
MVP100	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
MVP101	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
MVP102	Uncultured *Acidobacteria* clone M1PT4-70	88%	None	N/A
MVP103	*Micromonospora* sp. JH48	98%	*Micromonospora* sp. 31106 (2nd hit)	98%
MVP104	Uncultured bacterium clone BOf2-16	98%	*Propionibacteriaceae* bacterium SH081	95%
MVP108	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
MVP113	Uncultured *Actinobacteria* clone AKYG1141	99%	*Actinomadura chokoriensis*	99%
MVP114	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
MVP117	*Blastococcus* sp. YIM 65287	99%	*Blastococcus* sp. BC521 (2nd hit)	99%
MVP118	*Micromonospora auratinigra* strain HBUM49317	99%	*Micromonospora echinofusca* strain HBUM175187	99%
MVP119	Uncultured *Acidobacteria* clone M1PT4-70	97%	None	N/A
MVP120	Uncultured *Treponema* sp. clone 290cost002-P3L-1791	86%	Uncultured bacterium clone RsaHf365 (2nd hit)	86%
MVP122	*Streptomyces* sp. 213101	99%	*Streptomyces globisporus* subsp. *globisporus* isolate XSD-114	99%
MVP123	Uncultured *Acidobacteria* clone 290cost002-P3L-1601	96%	None	N/A
MVP124	*Micromonospora tulbaghiae* strain TVU1	100%	*Micromonospora auratinigra* strain HBUM49317 (2nd hit)	100%
MVP128	*Actinomycetales* bacterium JH105	99%	*Streptomyces* sp. 213101 (2nd hit)	99%

	MVP133	Uncultured bacterium clone M2PB4a-100	91%	*Candidatus Tammella caduceiae* clone CcCv-02	89%
	MVP135	*Actinomycetales* bacterium JH105	99%	*Streptomyces* sp. 213101 (2nd hit)	99%
	MVP137	Uncultured bacterium clone BOf2-16	97%	*Propionibacteriaceae* bacterium SH081	94%
	MVP138	Uncultured *Acidobacteria* clone: M1PT4-70	97%	None	N/A
Surrounding	MVSS6	Uncultured bacterium ARFS-34	98%	*Frankia* sp. BCU110501	98%
soil	MVSS7	*Geodermatophilus obscurus obscurus*	98%	*G. obscurus* strain DSM43162 (2nd hit)	98%
	MVSS8	*Friedmanniella* sp. EL-17a	95%	*Friedmanniella capsulate* (6th hit)	94%
	MVSS9	Uncultured bacterium clone FFCH10216	98%	*Nocardioides* sp. MSL 23	98%
	MVSS10	Uncultured actinobacterium clone GASP-WC1W1_A02	99%	None	N/A
	MVSS11	Uncultured bacterium clone FFCH12143	92%	*Streptomyces flavidovirens* strain IFO 13039	92%
	MVSS12	Uncultured actinobacterium clone NPK-42	96%	*Streptomyces bungoensis* strain 173014	96%
	MVSS13	Uncultured bacterium isolate BF0001B070	99%	*Humicoccus flavidus* strain DS-52	99%
	MVSS15	Uncultured bacterium clone FFCH12550	92%	Unknown Actinomycete (MC 19)	91%
	MVSS16	Uncultured actinobacterium clone 4S2_F09	94%	Frankia sp. strain FCe42	93%
	MVSS17	*Arthrobacter sulfonivorans* strain Asd MW-A1	99%	*Arthrobacter oryzae* strain Asd M5-8 (2nd hit)	99%
	MVSS20	Uncultured actinobacterium clone NPK-96	89%	None	N/A
	MVSS21	Uncultured bacterium clone FFCH2585	99%	*Blastococcus jejuensis* strain KST3-10	99%
	MVSS22	Uncultured actinobacterium clone A23YD23RM	99%	*Virgosporangium ochraceum* strain YU793-41	98%
	MVSS29	Uncultured bacterium clone FCPT586	96%	None	N/A
	MVSS32	*Actinomycetales* str. Ellin143	99%	*Frankia* sp. BCU110501	97%
	MVSS34	Uncultured actinobacterium clone GASP-KA1W3_B01	99%	None	N/A
	MVSS35	Uncultured *Gemmatimonadetes* clone AMLC7	89%	*Gemmatimonadetes* bacterium Ellin7146	86%
	MVSS38	Uncultured actinobacterium clone: Dolo_16	97%	*Streptoalloteichus* sp. IM-5630	95%
	MVSS39	Uncultured *Gemmatimonadetes* bacterium clone Elev_16S_693	96%	None	N/A
	MVSS44	*Friedmanniella* sp. FB1	95%	*Friedmanniella spumicola* (4th hit)	95%
	MVSS45	Uncultured bacterium clone RT_17	99%	None	N/A
	MVSS49	Uncultured *Blastococcus* sp. clone GASP-WC2S3_C10	91%	*Blastococcus* sp. CNJ868 PL04	90%
	MVSS51	*Actinomycetales* bacterium TLI156	96%	*Streptomyces shiodaensis* strain NBRC 13835	96%

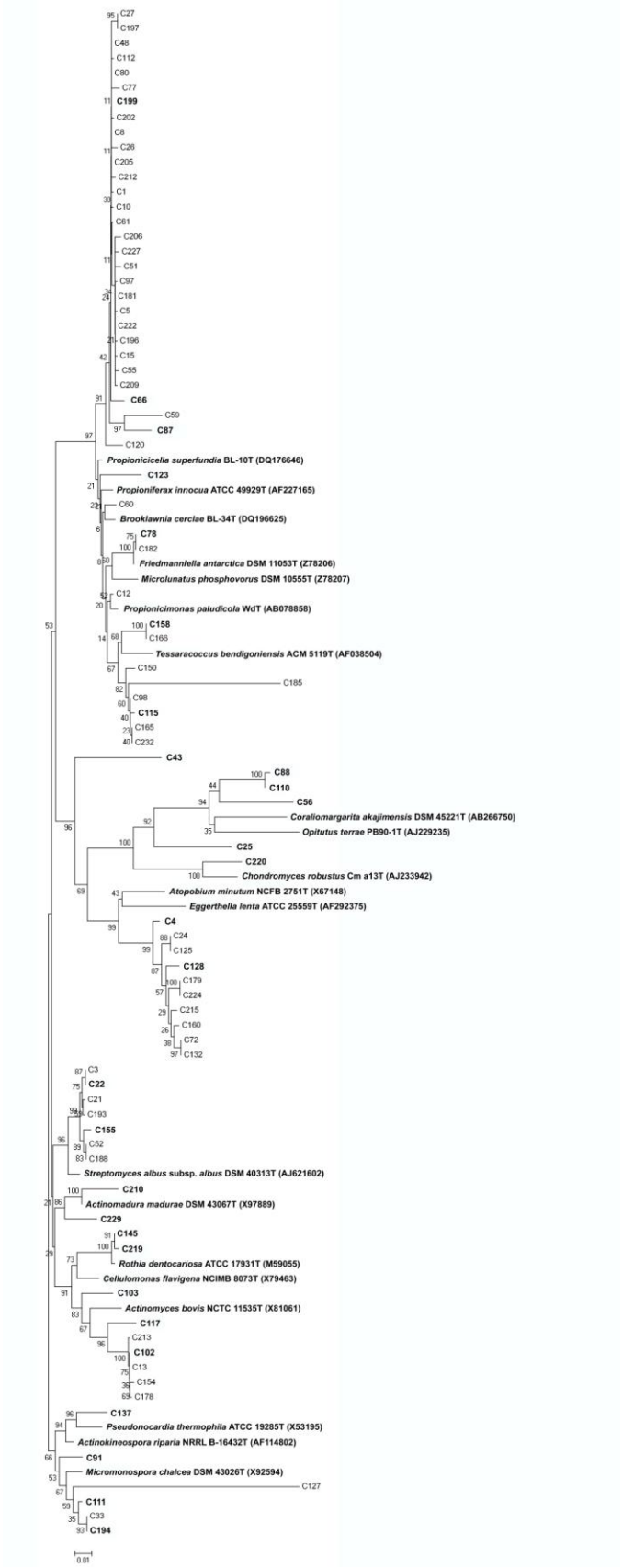
	MVSS53	Uncultured bacterium clone E18	98%	*Frankia* sp. BCU110501	98%
	MVSS55	Uncultured *Gemmatimonadetes* clone GASP-MA1W1_E01	98%	None	N/A
	MVSS56	Uncultured bacterium clone M6A-658	97%	*Acidimicrobidae* bacterium Ellin7143	97%
	MVSS59	Uncultured bacterium clone 2-4H	98%	*Friedmanniella antarctica*	98%
	MVSS60	Uncultured bacterium clone RT_17	99%	None	N/A
	MVSS63	Uncultured organism clone ctg_CGOGA19	95%	None	N/A
	MVSS65	*Leifsonia kribbensis* strain MSL-13	99%	*Agrococcus* sp. DNPRC1034 (3rd hit)	99%
	MVSS66	Uncultured actinobacterium clone Dolo_16	97%	*Streptoalloteichus* sp. IM-5630	95%
	MVSS67	Uncultured bacterium clone FFCH10604	96%	Bacterium Ellin5273	93%
	MVSS68	Uncultured bacterium isolate BF0001B070	99%	*Humicoccus flavidus* strain DS-52	99%
	MVSS71	Uncultured bacterium clone JEG.a3	99%	None	N/A
	MVSS75	Uncultured soil bacterium clone Tc119-H02	88%	None	N/A
	MVSS82	Uncultured bacterium isolate N93-C07	87%	*Saxeibacter cremoris* strain DLS-10T	82%
	MVSS85	Uncultured actinobacterium clone A6YD13RM	98%	*Salinispora* sp. NPS-14029	95%
	MVSS89	*Actinopolymorpha rutilus* strain YIM 45725	99%	*Actinosingapura polymorpha* (3rd hit)	99%
	MVSS90	Uncultured actinobacterium clone JCMYJSC33	98%	*Nocardioides* sp. MTD22	98%
	MVSS91	*Actinomadura longicatena* strain IMSNU 22180T	99%	*Actinomadura glomerata* strain IMSNU 22179T (3rd hit)	98%
	MVSS94	*Nocardioides* sp. MTD22	98%	*Nocardioides* sp. MSL 22 (8th hit)	98%
	MVSS95	Uncultured actinobacterium clone GASP-WC1W1_A02	99%	None	N/A
	MVSS96	Uncultured *Cellulomonadaceae* clone Plot03-2H04	99%	*Kineococcus* sp. S2-20	96%
	MVSS97	Uncultured bacterium clone FFCH9435	99%	*Virgosporangium aurantiacum* strainYU438-5	97%
	MVSS98	Uncultured actinobacterium clone HJH2SS58	96%	None	N/A
	MVSS99	*Marmoricola* sp. Ellin111	97%	*Nocardioides* sp. MSL 22 (6th hit)	97%
	MVSS102	Uncultured *Gemmatimonadetes* clone GASP-WC1S1_D09	91%	None	N/A
	MVSS111	Uncultured bacterium clone FCPT586	87%	None	N/A
	MVSS112	*Amycolatopsis coloradensis* strain HBUM173696	96%	*Streptomyces* sp. VC-YC6673 (2nd hit)?	96%
	MVSS113	Uncultured *Gemmatimonadetes* clone GASP-WC2S3_D02	95%	None	N/A
	MVSS115	Uncultured bacterium clone RT_17	99%	None	N/A
	MVSS116	Uncultured bacterium clone FFCH2528	90%	*Streptomyces flavorectus* strain NBRC 13672	90%
	MVSS118	*Amycolatopsis saalfeldensis* strain HKI0474	94%	*Amycolatopsis* sp. GY077 (4th hit)	94%
	MVSS120	Uncultured bacterium clone M6A-658	99%	*Acidimicrobidae* bacterium Ellin7143	99%

	MVSS123	Uncultured soil bacterium clone 4S2_E02	94%	*Frankia* sp. strain Agb1-9	87%
	MVSS125	Uncultured bacterium clone M6A-658	96%	*Acidimicrobidae* bacterium Ellin7143	96%
	MVSS129	Uncultured bacterium clone FFCH12658	93%	*Dactylosporangium* sp. KB2-4	92%
	MVSS130	*Actinomadura longicatena* strain IMSNU 22180T	99%	*Actinomadura glomerata* strain IMSNU 22179T (3rd hit)	98%
	MVSS133	Uncultured bacterium clone FFCH11334	99%	None	N/A
	MVSS136	Uncultured bacterium clone FCPT426	99%	*Blastococcus* sp. CNJ868 PL04	98%
	MVSS137	Uncultured actinobacterium clone JCMYJSC33	98%	*Nocardioides* sp. MTD22	98%
	MVSS138	Uncultured bacterium clone FFCH9435	98%	*Virgosporangium aurantiacum* strain YU438-5	96%
	MVSS140	Uncultured bacterium clone FFCH2528	93%	*Streptomyces* sp. L-2-16	93%
	MVSS141	Uncultured actinobacterium clone MNPK-60	98%	*Nocardioidaceae* str. Ellin101	98%
	MVSS142	Uncultured bacterium clone FCPT588	100%	Bacterium Ellin5273	95%
	MVSS143	Uncultured bacterium clone FFCH8308	96%	None	N/A
	MVSS145	Uncultured bacterium clone KC-26	98%	*Dactylosporangium* sp. KB2-	97%
	MVSS149	*Amycolatopsis coloradensis* strain HBUM173696	96%	*Streptomyces* sp. VC-YC6673 (2nd hit)?	96%
	MVSS150	Uncultured *Gemmatimonadetes* clone A23YN16RM	99%	Bacterium Ellin5301	94%
	MVSS152	*Actinomycetales* bacterium JH37	94%	*Actinoplanes teichomyceticus* strain DSM 43866T	94%
	MVSS154	Uncultured *Cellulomonadaceae* clone Plot03-2H04	95%	*Micrococcineae* str. Ellin184	93%
	MVSS155	Uncultured bacterium clone FCPT426	99%	*Geodermatophilaceae* bacterium CIBE-G8	98%
	MVSS156	Uncultured bacterium clone FFCH10604	96%	Bacterium Ellin5273	94%
	MVSS157	Uncultured bacterium clone 2-4H	100%	*Friedmanniella antarctica*	99%
	MVSS158	*Nocardioides* sp. MTD22	100%	*Nocardioides* sp. MSL 22 (7th hit)	99%
	MVSS159	Uncultured bacterium isolate BF0001B070	99%	*Humicoccus flavidus* strain DS-52	99%
	MVSS161	Uncultured bacterium ARFS-34	98%	*Frankia* sp. BCU110501	98%
	MVSS163	Uncultured bacterium clone MSB-2B5	98%	*Kineococcus* sp. S2-20	96%
	MVSS164	*Streptomyces lazureus* strain NBRC 13384	98%	*Streptomyces lactamdurans* (2nd hit)	98%
	MVSS166	Uncultured bacterium clone FFCH10604	96%	Bacterium Ellin5273	93%

Appendix P: Unrooted neighbour-joining phylogenetic tree based on 561bp of 16S rRNA gene sequence, showing the position of all paunch clones amongst relevant actinobacterial genus type strains. GenBank sequence accession numbers are shown in parentheses. Bootstrap support for each node is indicated as a percentage, calculated from 1000 randomly re-sampled datasets. The scale bar indicates 1 nucleotide substitution per 100 nucleotides.



Appendix Q: Neighbour joining phylogenetic tree of the colon clones as well as all relevant actinobacterial genus type strains. The tree was based on 633bp of 16S rRNA gene sequence and bootstrap support, shown at all nodes, was calculated from 1000 randomly resampled datasets. GenBank sequence accession numbers are shown in parentheses. The bar represents 1 nucleotide substitution per 100 nucleotides.



Appendix R: Unrooted neighbour-joining phylogenetic tree based on 540bp of 16S rRNA gene sequence, showing the phylogenetic position of all surrounding soil sample clones amongst all relevant genus type strains. GenBank sequence accession numbers are shown in parentheses. Bootstrap support for each node is indicated as a percentage, calculated from 1000 randomly re-sampled datasets. Bar indicates 5 nucleotide substitutions per 100 nucleotides.