MICROBIAL BIODIVERSITY

IN

TASMANIAN CAVES

Big Stalagmite, Entrance Cave, Tasmania. Photograph taken by Jodie van de Kamp.

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Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

Jodie Lee van de Kamp

25th August 2004

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ABSTRACT

Caves represent one of few remaining isolated planetary habitats, in terms of human impact and characterisation of microbial biodiversity. Caves are unique environments characterised by little or no light, low levels of organic nutrients, high mineral concentrations and a stable microclimate providing ecological niches for highly specialised organisms. Caves are not uniform environments in terms of geological and geochemical characteristics, as they can vary from one to the other, eg. rock type, method of formation, length, depth, number of openings to the surface, presence or absence of active streamways, degree of impact by human visitation etc. Furthermore, on a smaller scale, various microhabitats, with vast differences in community structure can exist within caves. Culture studies point to the dominance of actinomycetes in caves and reveals great taxonomic diversity within actinomycetes isolated. However it is widely accepted that only ~ 1 % of microbes are cultured in the laboratory. Culture-independent methods are being increasingly used to describe the composition of microbial communities and reveal significantly broader diversity than culture-based studies. Nevertheless, to date our knowledge of bacterial communities in caves is largely due to culture studies. Based on the literature available, this study was initially aimed at examining culturable vs. non-culturable diversity of actinomycetes in Entrance and Loons Caves and to gain an increased understanding of the composition of cave microbial communities employing classical isolation and advanced molecular detection methods.

As the study progressed the focus evolved as it became apparent that actinomycetes dominated only very specific habitats, the dry sediment in Entrance Cave, and represented only a minor fraction of the microbial biodiversity of most other microhabitats studied. Entrance Cave dry sediments and inactive (dry) speleothems produced a higher number of actinomycete isolates compared to saturated sediments and wet formations from Entrance and Loons Caves. This was reinforced by the actinomycetes being the second most abundant group (26.8%) detected in clone analysis of the dry Entrance sediment and low abundances (4-16%) detected in saturated sediments from both Entrance and Loons Caves. Sediment phylotypes and isolates identified in this study closely resemble species associated with oligotrophic, chemolithotrophic and heterotrophic lifestyles indicating that these communities survive by utilising a combination of metabolic pathways. Bacteria involved in the nitrogen and sulfur cycles were important members of all sediment communities along with hydrogen-oxidising bacteria. Pair-wise comparisons of sediment communities demonstrated that they were more similar to each other within individual cave systems, Entrance and Loons, rather than between microhabitat types (dry vs. wet sediment) though saturated sediment from Entrance Cave did show a higher degree of similarity in community composition to Loons Cave samples than the dry sediment from Entrance Cave. Saturated sediments were dominated by oligotrophs able to fix atmospheric gases, methanotrophs and had a high proportion of rare phylotypes most likely representing new

lineages related to microbes detected in anaerobic, anoxic environments, but low abundances of heterotrophic microbes.

Geomicrobiological activities are no longer underestimated since studies have shown that bacterial metabolism may lead to mineral precipitation or dissolution. Questions remain as to the identity of these microbes and whether they are actively involved in speleothem formation, or simply buried during mineral precipitation. Results demonstrated a marked difference between sediment communities and those associated with calcite speleothem and calcite mat samples. Results of ESEM and XRD analysis demonstrated that calcite speleothem samples ME3 and MX1 are true calcite moonmilk (mondmilch). Phylogenetic analyses and isolation results demonstrated the unique composition of the microbial communities associated with moonmilk deposits, predominantly composed of nitrogen-fixing β-Proteobacteria and psychrotrophic heterotrophic CFBs and to a lesser extent, heterotrophic actinomycetes. Despite XRD and ESEM analysis showing similar calcite composition and crystal morphology, phylogenetic results indicated that sample ME2 represented a very different microhabitat to moonmilk samples, dominated by oligotrophic α-Proteobacteria and heterotrophic actinomycetes composing 84.2% of the total diversity. Phylogenetic analyses and biodiversity indices reveal the striking similarities between moonmilk samples from both Entrance and Exit Caves and the uniqueness of the calcite mat in Entrance Cave. The one similarity in composition between all three calcite communities was the presence of members of the *Pseudonocardineae* in particular of the genus *Saccharothrix*, in all calcite samples.

16S rRNA gene sequencing of cave isolates detected high levels of diversity and novelty, particularly of moonmilk isolates. A total of two putatively novel genera (within the CFBs and *Actinobacteria*) and 18 putatively novel species (of genera: *Paracoccus, Actinoplanes/Couchioplanes, Micromonospora, Amycolatopsis, Saccharothrix, Bacillus, Paenibacillus, Methylobacterium, Porphyrobacter, Sphingomonas, Alcaligenes, Stenotrophomonas, Xanthomonas*) were identified.

This study represents the first reported culture-independent analysis of moonmilk microbial communities globally and of cave sediment communities in the Southern Hemisphere. Information gained from this study and the discovery of actively growing microbial communities appearing to precipitate $CaCO_3$ provides focus for important future studies and represents a unique opportunity to examine the nature and extent of complex microbe-mineral interactions in the formation of speleothems and implications for cave management. The biodiversity described acts as a baseline for assessing environmental impacts and to identify factors influencing microbial biodiversity.

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TABLE OF CONTENTS

SECTION 1:

LITERATURE REVIEW – MICROBIAL ECOLOGY OF CAVES

1.1 Microbial Ecology

1.1.1 Objectives of Microbial Ecology

Microbial ecology can be defined as investigating the impact of biodiversity on the structure and function of microbial communities and the ecosystem as a whole. According to Siering (1998) the questions directing much of the research in microbial ecology are theoretically quite simple: i) what are the numbers and identities of microorganisms in a given sample, ii) what are their activities and their role in ecosystem maintenance, iii) what genes are present to encode the activities of interest, iv) are the genes being expressed (i.e., transcribed), and are those transcripts translated and processed into active proteins, and v) what controls the rate of transcription and translation for environmentally significant genes, and can we measure these rates *in situ*? Much of the recent advances in the field of microbial ecology focuses on addressing the first question, microbial biodiversity, or, determining the identity of the organisms present in a given community.

Microbial biodiversity is greater than the diversity of any other group of organisms. Higher life forms rely on bacterial processes for their survival. Microorganisms are responsible for diverse metabolic functions that affect soil, plant and animal health, for example, nutrient cycling, organic matter formation and decomposition, soil structure formation, and plant growth promotion. Microbial biodiversity has received particular attention in areas where industrial applications are evident, such as for marine, medical, and food biotechnology, and where microbial activity has important implications for Earth's climate and for the bioremediation of polluted sites (Morris *et al.* 2002).

Different habitats may be characterised by a particular food source, substrate type, micro-climate, or a combination of these. Some organisms are entirely restricted to a certain

habitat whilst others, referred to as cosmopolitan species, range widely across a variety of habitats. Each of these environments/microhabitats has its own characteristics which preclude generalisations about the conditions of life in one being carried over to the others in most instances, and which select for bacteria adapted to their own micro-climate.
Enhancing knowledge of bacterial biodiversity and ecological function provides baseline information for conservation and sustainable development.

1.1.2 Methods in Microbial Ecology and Taxonomy

The study of microbial processes in an ecosystem is a multifaceted affair requiring attack from many angles and utilising a wide variety of techniques (Brown, 2000). Studies of biodiversity, characterising the composition of microbial communities in a given environment, can largely be a descriptive endeavour, but a necessary first step in determining the nature of biodiversity and its impact on ecological processes.

1.1.2.1 Biodiversity

Traditionally, microbial populations have been described in terms of isolating pure cultures and investigating a wide range of phenotypic traits, many of which are related to the practical interest in the habitat studied (eg. phenotypic characteristics of psychrophiles in Antarctica; Nichols *et al.* 1993, 1999). Biodiversity studies focusing on phylogenetic or taxonomic comparisons of microorganims reflect the historical tribulations surrounding the complexities of defining a bacterial species and the relatedness among individuals of different genotypes (Morris *et al.* 2002). Phylogenetic classification of bacteria is based on ancestral relationships (Woese, 1987). Surprisingly the term 'phylogeny' is rarely defined precisely (Young, 2001). A central outcome of phylogenetic classification is that taxa be monophyletic, ie. members of a taxon under consideration share the same common ancestor. A further requirement is that taxa sharing more recent common ancestry are considered to be more closely related to one another than they are to other taxa (Lincoln *et al.* 1998 in Young, 2001).

A study of microbial biodiversity publications by Morris & co-workers (2002), found that over the last 25 years, DNA-based characterisation techniques, in particular those based on targeted DNA sequences, have had the dominant role in studies of microbial relationships or in the search for new taxa, relative to other morphological or biochemical techniques. By the early 1980s, several studies had shown that ribosomal RNA (rRNA) held promise for phylogenetic reconstruction (Fox *et al.* 1980) and by the end of the decade, analysis of universally conserved nucleic acid sequences (particularly those of the small subunit rRNA gene) had become a powerful tool for microbial taxonomy, allowing identification of specific taxa on the basis of only a single gene sequence (Woese *et al.* 1990). In the 1990's, this approach had become the principal method of establishing phylogenetic relationships among the prokaryotes; today, it is more likely that a 16S rRNA gene sequence will be the first piece of data collected for unknown organisms, rather than a Gram stain (Lilburn & Garrity, 2004). Though rRNA methods are now commonplace it is worthwhile to quickly review the basis for this.

There are several reasons to focus on rRNAs to characterise microbial diversity and infer phylogenetic relationships. Olsen *et al.* (1986) summarised these as follows: i) rRNAs, as key elements of the protein-synthesising machinery, are present, and functionally and evolutionarily homologous, in all organisms, ii) rRNAs are ancient molecules, and conservation of function dictates conservation in overall structure thus, homologous rRNAs are readily identifiable by their size, iii) nucleotide sequences are also conserved allowing comparisons between different organisms and also providing convenient hybridisation targets for cloning and primer directed sequencing techniques, iv) rRNAs constitute a significant component of the cellular mass in actively growing cells (~10^4 ribosomes per actively growing *E. coli* cell; Siering, 1998) and are readily detected, isolated and sequenced from all types of organisms, v) rRNAs provide sufficient sequence information to permit statistically significant comparisons, vi) rRNA genes lack artefacts of lateral transfer between contemporaneous organisms. Thus, relationships between rRNAs reflect evolutionary relationships of the organisms. Conservation of function dictates a conservation of structure such that most of the rRNA molecule is conserved among the most divergent or organisms. Although different portions of the molecule evolve at different

rates resulting in hypervariable domains as well as highly conserved domains. Their resistance to evolutionary change allows the entire phylogenetic span of ancient and modern prokaryotes to be analysed simultaneously. However it has been shown that the resolution power of rRNA sequences is limited when closely related organisms that diverged at almost the same time are being examined (Woese, 1987; Fox *et al.* 1992).

It has been estimated that less than 1% of the total bacterial population in a given environment have been successfully isolated (Amann *et al.* 1995). The advent of culture-independent molecular methods, especially rRNA-based techniques, led to an explosion of microbial biodiversity papers starting in the late 1980s. Much of what is known is based on distinguishing different organisms as represented by their extracted and polymerase chain reaction (PCR) amplified nucleic acids without actually culturing them or having any direct knowledge of their morphology, physiology or ecology (Kemp & Aller, 2004).

PCR amplification of nucleic acids extracted from environmental samples (eg. soil, water, ice) is at present the most powerful cultivation-independent technique. PCR facilitates the sensitive and fast detection of low amounts of specific gene fragments. This is of particular importance to this study as subsurface environments are, in general, characterised by low biomass which releases low amounts of nucleic acids upon extraction (Chandler *et al.* 1998). Microbial diversity and identity can be estimated by cloning, sequencing and phylogenetic analysis of 16S rRNA amplified genes. Clone analysis, more often than not, results in sequences corresponding to previously uncharacterised and often unexpected lineages. An explosion of culture-independent studies of diversity in a wide range of microbial habitats in the past 15 years has resulted in a large database of more than 62 000 16S rRNA gene sequences providing a high resolution framework for phylogenetic analysis.

1.1.2.2 Community Fingerprinting

Community fingerprints, may be of use when trying to snapshot the diversity of a population or to follow changes in microbial communities that result from natural community succession, or environmental or anthropogenic perturbation. With specialised computer

software fingerprints can be databased and subjected to multivariate statistical analyses (eg. Röling *et al.* 2000; Dunbar *et al.* 2001). Computer assisted analysis allows the comparison of different profiles with each other and the establishment of relationships between fingerprints and environmental conditions (Röling & van Verseveld, 2002). Thus community fingerprinting is more efficient (eg. cost, time) than more detailed clone library analysis when attempting high throughput or comparisons of several communities. Several 16S rRNA gene-based techniques have been used to fingerprint microbial communities, examples of which include, denaturing or temperature gradient gel electrophoresis (DGGE/TGGE), terminal restriction fragment length polymorphism (T-RFLP), and fluorescent *in situ* hybridisation (FISH).

Although relatively new, DGGE/TGGE is an increasingly popular molecular tool to analyse general patterns of community diversity in microbial ecology. In DGGE/TGGE, 16S rRNA gene fragments are separated on the basis of differences in their melting behaviour resulting in a pattern of bands on a gel (Muyzer & Smalla, 1998). Theoretically, each band represents a unique sequence and therefore a unique species (Powell *et al.* 2003). In T-RFLP, fluorescently labelled PCR products are digested with restriction enzymes and separated using automated sequencing technology. T-RFLP offers some important advantages over other fingerprint techniques, its resolution is higher and direct reference can be made to the 16S rRNA gene sequence database (Tiedje *et al.* 1999; Marsh *et al.* 2000). The application of FISH to microbial systems provides a way to detect and enumerate microorganisms in natural systems without culturing (eg. Giovannoni *et al.* 1988; Delong *et al.* 1989; Amann *et al.* 1990, 1991). FISH is a technique whereby fluorescently labelled DNA probes are annealed to a target sequence in nucleic acids of fixed cells. Probes have been used capable of identifying bacteria at varying levels of taxonomic hierarchy.

1.1.2.3 Ecological Function

By phylogenetically aligning an organism to its next nearest cultivated relative, we may shed light on the metabolic and physiological processes that are occurring (Pace, 1997). However caution is advised when considering the results of these studies as comparisons can only be

made when there is a high degree of sequence similarity between the identified phylotypes and known cultivated species, although even closely related organisms can show distinct physiological differences (Achenbach & Coates, 2000). For many clone sequences, no closely related cultivated species are known and until recently, linking most 16S rRNA gene information to function and ecological processes was dependent on culturing studies. Relatively new nucleic acid-based techniques, such as stable isotope probing (Radajewski *et al.* 2000) and bromodeoxyuridine labelling (Urbach *et al.* 1999), are beginning to emerge in the literature, allowing specific microbial processes and functions to be related to individual members of microbial communities in a cultivation-independent manner (Röling & van Verseveld, 2002). These techniques rely on the synthesis of labelled DNA by microorganisms that grow in response to a specific stimulus and the subsequent separation of this labelled DNA from the pool of total DNA.

The use of biomarkers in combination with stable isotope analysis (eg. ^{13}C) is one example of these relatively new culture-independent approaches to function analysis in microbial ecology. Biomarkers are compounds that have a biological specificity in that they are produced only by a limited group of organisms (eg. fatty acids, ether lipids). Natural abundance isotope ratios of biomarkers can be used to study organic matter sources utilised by microbes in complex ecosystems and for identifying specific groups of bacteria like methanotrophs (Boschker & Middelburg, 2002). Addition of labelled substrates in combination with biomarker analysis enables direct identification of microbes involved in specific processes and also allows for the incorporation of bacteria into food web studies (Boschker & Niddelburg, 2002). Similarly, FISH performed with rRNA-targeted oligonucleotide probes and microautoradiography can be used to analyse structure and function of bacterial communities. Lee *et al.* (1999) demonstrated the potential of this method by visualising the uptake of organic and inorganic radiolabelled substrates in probe-defined microbial populations.

To understand the role of a microorganism in a geochemical process, detection and identification of the microorganism in an environment in which the process is occurring is essential. Although demonstrating the presence of an organism in an environment where the

process is occurring does not mean the detected organism is important in the process of interest (Siering, 1998). One ultimately needs to correlate the distribution and abundance of the organisms with the presence of the activity and the presence of any genes and gene products (functional genes) involved in the process. If functional genes known to be involved in a particular process have been identified, isolated, characterised and sequenced, it is possible to use this information to develop PCR primers for amplifying the gene of interest from indigenous bacteria in natural samples. Hutchens *et al.* (2004) used DNA-based stable isotope probing and functional gene analysis of groundwater and mat material from Movile Cave to identify methane-assimilating populations and results suggest that aerobic methanotrophs (*Methylomonas, Methylococcus, Methylocystis/Methylosinus* strains) actively convert CH_4 into complex organic compounds and thus help sustain a diverse community of microbes in this closed ecosystem. This richness of methanotrophs was not revealed by RFLP analysis of the 16S rRNA gene clone library alone, demonstrating the benefits of constructing both 16S rRNA gene and functional gene libraries (Hutchens *et al.* 2004). Probing also increased already existing knowledge of microbial diversity in Movile Cave to include relatives of the cultivated and uncultivated members of the alpha, beta and gamma Proteobacteria, members of the *Acidobacterium* division.

Amplifying and sequencing functional genes from organisms present in environmental samples allows us to investigate the distribution, evolutionary relationships, and diversity of functionally analogous genes (Siering, 1998). To prove a gene of interest is responsible for a process you must be able to detect expression of the gene *in situ* and correlate changes in gene expression with changes in the associated activity, for example detecting and quantifying the presence of particular messenger RNA (mRNA). This is often challenging due to the low quantities and very short lifespan of mRNA. Furthermore, gene expression studies require prior information, including transcript size and stability as well as expected levels of transcript present, which is not always available (Siering, 1998). Recent advances to increase detection sensitivities of gene expression rely on a form of PCR known as reverse transcriptase-PCR (RT-PCR). Reverse transcriptase is used to synthesise a single stranded DNA copy (cDNA) of the

RNA template then the complementary strand of the cDNA is synthesised and the double-stranded DNA molecule is subsequently amplified by normal PCR amplification.

1.1.3 Limitations of Methods

rRNA gene surveys have enormously extended the boundaries of microbial diversity, but caution should be exercised when relying entirely on such an approach. In a detailed culture-dependent survey of bacterial diversity in a wide range of deep-sea sediments, Li *et al.* (1999) isolated 75 different actinomycetes; however very few actinomycete sequences were cloned from these same samples in a later study (Colquhoun *et al.* 1998a,b, 2000).

The isolation of members of complex microbial communities as cultures also has significant advantages over culture-independent molecular approaches given the inability to identify with certainty the ecological, metabolic or physiological potential from novel molecular sequence data (Atalan *et al.* 2000). It is most probable that the inability of microbiologists to culture the majority of microbes in the laboratory results from the use of cultivation media that does not resemble natural conditions or perhaps that some strains are interdependent (Wagner *et al.* 1993). There is a trend emerging amongst microbial ecologists to continue to develop new culture methods and media to attempt to cultivate novel taxa from so-called "unculturable" groups of bacteria. In particular, Sait *et al.* (2002) and Joseph *et al.* (2003) had great success culturing from Australian soils numerous phylogenetically novel microbes (the "Ellin" isolates) belonging to previously uncultured groups using relatively simple cultivation methods. Regardless, it is indisputable that culture-independent studies based on obtaining 16S rRNA genes directly from the environment by broad-specificity primer PCR and cloning have greatly improved our understanding of microbial diversity.

PCR-based surveys also have a number of recognised, inherent limitations. The quality of extracted nucleic acids may be compromised by problems of shearing, degradation due to the presence of contaminating nucleases, or contamination with humics or other substances known to inhibit subsequent molecular biological manipulations. Techniques must be optimised for

each type of environmental sample. Unfortunately, most methods for the extraction of nucleic acids from environmental samples lack a quantitative component; little data exists on the efficiencies of bacterial lysis and how these lysis efficiencies are affected by the complex matrix of biological and non-biological material within different sample types (Siering, 1998).

Unfortunately PCR does not necessarily occur in an accurate and unbiased fashion. A primary concern in amplifying 16S rRNA genes from mixed samples is the formation of chimeric sequences from the artifactual joining of 16S rRNA gene sequences of two organisms (Liesack *et al.* 1991; Kopczynski *et al.* 1994) or from distinct copies of rRNA genes within the genome of a single organism (Wang & Wang, 1997). Such chimeric sequences occur at variable frequencies ranging from 4.1-20% (Robison-Cox *et al.* 1995) to 8.8-32% (Wang & Wang, 1997) and, therefore, should not be ignored. There are computational methods available to detect these artefacts (Robison-Cox *et al.* 1995; Komatsoulis & Waterman, 1997; Maidek *et al.* 1997), although all methods fail to detect some chimeras, especially those from closely related sequences, or misclassify non-chimeras as being chimeric. Hugenholtz & Hubert (2003) found during a recent collation within the public databases that, despite precautions taken, a surprising number of chimeric 16S rRNA gene sequences from molecular phylogenetic surveys were detectable. However, by being vigilant and using several available methods rather than a single method, such inaccuracies can be decreased.

A separate issue is PCR bias, that genes are not equally amplified from all organisms (Reysenbach *et al.* 1992; Suzuki & Giovannoni, 1996). This is one of the major drawbacks to developing quantitative PCR methods. Template bias is sometimes due to variable energetics in primer annealing and DNA denaturation due to G+C content in the template or primer DNA, in other instances causes for bias have not been identified (Suzuki & Giovannoni, 1996). Genome size and the number of different copies of rRNA genes within a given genome have also been shown to result in differential amplification of rRNA genes from mixed community DNA (Farrelly *et al.* 1995). These parameters are unknown for the majority of organisms present in a given sample, thus Farrelly *et al.* (1995) contended that it is impossible to accurately quantify compositions of microbial communities by analysing clone libraries from amplified 16S rRNA

genes. Clone library analysis provides useful phylogenetic information that is reflective of community composition and relative distributions of organisms. However, small sample sizes prevent adequate representation of microbial community phylotypes because of cost and labour limitations. Community fingerprinting methods can alleviate these issues.

Although useful for quick comparisons of multiple communities, the drawbacks to fingerprint-based methods include a lack of resolution provided by gel-based separation and also difficulty in assigning phylogenetic information to the complex banding patterns that are usually obtained. With fingerprinting techniques, phylogenetic inference is most effective when only a single bacterial division or smaller group is addressed and is far less useful when the entire bacterial community is profiled (Dunbar *et al.* 2001). A combination of the two methods, fingerprinting and detailed clone analysis would be a more comprehensive way to study community composition.

1.2 Caves

Spaces below the Earth's surface range in size from microfissures to hundreds of kilometres in length and theoretically most have no natural human-accessible entrances (Curl, 1966 in Northup & Lavoie, 2001). A cave is defined as any natural space below the surface that extends beyond the twilight zone and that is accessible to humans (Hill & Forti, 1986). Caves can be classified in several ways, particularly by the type of rock and method of formation (Palmer, 1991). The most common types of caves are those formed in carbonate rocks. Other types of caves are usually limited in extent and include those in gypsum, granite, quartz and sandstone.

1.2.1 Speleogenesis: Cave Formation

The birth of a cave system is referred to as speleogenesis (Ford & Cullingford, 1976). The gradual solution of carbonate rocks, usually taking several millions of years, results in a wide spectrum of landforms, collectively known as "karst" and caves are one of the most common examples of this process. Carbonate rocks, such as limestone, are derived from the accumulation of marine organisms (shells, corals etc) and as sediments on the sea floor. These marine sediments consolidate over a long period of time and may be subsequently uplifted forming parts of the landmass of many regions of the world. Carbonate rocks contain carbonate minerals such as calcium carbonate ($CaCO_3$), often enriched with magnesium or iron and that are easily dissolved by acids, even very weak solutions of acid.

Dissolution processes in carbonate rocks are due to the natural action of water. It occurs as: i) surface water run off, flowing over impervious cap rock that lies above the more porous carbonate rock then flowing into carbonate, (swallet); ii) from a surface stream draining another rock surface further upstream, then entering the carbonate rock, (streamsink); or iii) rain water seeping through forest mulch and soils into the carbonate rock below (percolation water) (Ford & Cullingford, 1976). These "charged" or "aggressive" waters are slightly acidic and penetrate through points of weakness in the rock (eg. cracks, joints, bedding planes). Run off water or

stream water also has a forceful action of erosion, corrosion and abrasion due to gravity, water mass or volume, and its sediment load of fine sands or gravels, which increases the magnitude of the dissolution process. The effect of seepage or percolating water is also aided by a number of factors. Rainwater contains dissolved carbon dioxide (CO_2) from the atmosphere forming a weak carbonic acid. This acidity is further strengthened by absorption of CO_2 from microbes and various humic or tannic acids from plant matter in the soil. Sulphuric acid sometimes derived from presence of sulphides in the soils, limestone or dolomite adds to the acidity of the water. As the acidic water reaches the water table, it stays in contact with the carbonate causing further dissolution of $CaCO_3$. This process is referred to as carbonic acid-driven speleogenesis. Limestone caves may also be derived from a second process referred to as sulfuric acid-driven speleogenesis. Hydrogen sulfide rises along fissures until it encounters the oxygenated zone and forms sulfuric acid that dissolves the surrounding carbonate rock (Hill, 1990).

1.2.2 Speleothems: Cave Decoration

A cave, at constant temperature and invaded by percolating solutions carrying various substances, forms an excellent environment for the slow deposition of minerals (Ford & Cullingford, 1976). One of the most commonly known aspects of caves is their visual beauty, due to their natural, internal formations, often referred to as cave decoration. These formations are secondary mineral deposits on the ceiling, floor and walls of a cave and are called "speleothems". Most caves have enough openings to allow air movement, which evaporates some of the moisture and allows the precipitation of carbonate minerals from the seeping waters to form speleothems. Their creation depends on a number of factors: i) amount of seepage waters entering the ground above the cave, ii) type of rocks in and around the cave, iii) type of dissolved materials contained in the water as it enters the cave, and iv) the cave environment, (eg. amount of moisture in the air, amount of air flow through the cave, cave temperature).

Formations are precipitated very slowly; it may take one hundred to one hundred and fifty years to form 2.5 cm of material and the slow growth and nearly constant conditions in

caves results in these mineral deposits displaying spectacular crystal development (Ford & Cullingford, 1976). The colouration of speleothems varies depending on the mineral composition of the carbonate rocks (eg. white or cream for almost pure $CaCO_3$, to yellowish or dark brown due to the presence of limonite, or red/orange hues from dissolved iron, or blue hues from manganese). The colour variations and the various crystal configurations create the beautiful wonderland of this subterranean world.

Hill & Forti (1986) recognised 38 "official" speleothem types, with numerous subtypes and varieties, (Eg. stalactites, stalagmites, flowstones, rimstone pools and moonmilk) and described over 250 different minerals found in caves. Of special interest is moonmilk, a widely distributed, secondary formation and refers to the very hydrated white spongy/pasty or powdery masses found coating walls and speleothems in caves. It is composed of several carbonate minerals, predominately calcite. The wet pasty forms of moonmilk are so striking that some special explanation for their origin seems to be necessary, since calcite in cave environments usually has a completely different habit, hard and crystalline (Ford & Cullingford, 1976).

1.2.3 Cave Environment

Cave environments are strongly buffered against daily, seasonal and long-term climate changes occurring on the surface providing stable, sheltered and moist refuges for organisms. The terrestrial cave environment is strongly zonal, with four major zones recognised; entrance, twilight, transition, and deep zone. The entrance zone is where the surface and underground environments meet. Beyond the entrance is the twilight zone where light still penetrates but progressively diminishes to zero. The transition zone is completely dark but the environmental effects from the surface are still felt. In the deep zone, environmental conditions are relatively stable, with fairly constant air and water temperatures (approximately the mean annual surface temperature) and the relative humidity near saturation resulting in an extremely low rate of

evaporation (Barr & Holsinger, 1985 in Eberhard, 1999). Note that conditions may be less stable surrounding active, surface-fed streamways or passages near internal cave entrances.

The extent of the different zones depends on the size, shape and location of the entrance(s), on the configuration of the cave passages and on the subterranean water/moisture supply (Howarth, 1988). The boundary between the transition and deep zones can be dynamic, changing on a seasonal or even daily basis, as air is pushed into, and pulled out of caves in response to changes in air density related to temperature and barometric fluctuations on the surface (Howarth, 1980). In temperate regions during summer, it is usually warmer outside the caves than inside, whereas in winter the reverse is true, generally resulting in a net movement of water vapour into caves during summer and out of caves during winter. Unlike the earth's surface, caves are not subject to the same weathering processes so what is found inside them often represents a different "snapshot" of the earth's history than would otherwise be available from the surface (http://www.speleonics.com.au; maintained by J. Rowling).

1.2.4 Speleology: Cave Study

The study of caves is called "speleology", and the study of life forms in caves, "biospeleology". The main focus of biospeleologists is the deep, dark zone, also referred to as the hypogean environment, due to the highly specialised organisms found there. Hypogean environments are not restricted to caves, but include any system of crevices and fissures deeper than the soil layer. In caves, the hypogean domain is most conveniently open to study by man. The hypogean domain may also be artificially penetrated for study particularly by mines and wells, both of which often yield hypogean organisms (Ford & Cullingford, 1976). These ecosystems are exposed to extreme environmental stresses and may be based on inorganic energy sources rather than sunlight. The limiting environmental characteristics of caves, little or no light, low levels of organic nutrients, high mineral concentrations and a stable microclimate, provide ecological niches for highly specialised organisms. Historically, macroscopic life was the primary source of interest for study in caves. However recently biospeleologists have turned

their attention to the microscopic life in these systems, revealing unique microbial ecosystems (eg. Cunningham *et al.* 1995; Sarbu *et al.* 1996; Jones 2001; Holmes *et al.* 2001; Schabereiter-Gurtner *et al.* 2002; Northup *et al.* 2003; Barton *et al.* 2004).

1.3 Microbial Biodiversity and Ecology of Caves

Caves are severely resource limited due to the absence of light that precludes primary production of organic material by photosynthetic organisms (Northup & Lavoie, 2001). Even so, microorganisms are widely distributed in caves and include bacteria, archaea, yeasts, fungi, and algae. Researchers proposed that the role of microbes in caves is to serve as a food source for higher trophic levels (eg. Dickson, 1979); however it was typically believed that microbes could not provide adequate energy to support a large and diverse ecosystem. In contrast, the work of Sarbu *et al.* (1996) in Movile Cave, Romania, and Vlasceanu *et al.* (2000) in Frasassi Caves, Italy, suggest that chemoautotrophic, sulfur-based microbial communities can generate enough energy as primary producers to sustain complex cave ecosystems. These caves receive little or no surface-derived organic material, but instead microbially reduced sulfur compounds in the cave waters provide the energy for carbon dioxide fixation (Mattison *et al.* 1998). The work in Movile Cave provided evidence of the first terrestrial microbial community known to be chemoautotrophically-based (Sarbu *et al.* 1996).

Culture-independent 16S rRNA gene sequence analyses have opened the way to study bacterial communities in environmental samples without prior cultivation and have revealed a significantly broader diversity than culture-based studies in many environments over the last 25 years (Amann *et al.* 1995; Head *et al.* 1998; Hugenholtz *et al.* 1998). Nevertheless, to date our knowledge of bacterial communities in caves has been largely due to culture studies (eg. Groth *et al.* 1999a; Laiz *et al.* 1999). As discussed in Section 1.1.2.3, phylogenetic analyses can be used to hint at the ecological functions of uncultivated phylotypes obtained from molecular analyses. The recent influx of molecular analyses of cave microhabitats (Eg. Holmes *et al.* 2001; Schabereiter-Gurtner *et al.* 2002; Northup *et al.* 2003; Barton *et al.* 2004; Chelius & Moore, 2004; Schabereiter-Gurtner *et al.* 2004) have attempted to do just this; elucidating the roles of bacteria in caves, how they survive, interact with, and affect, their environment.

1.3.1 *Chemolithoautotrophic Systems*

In cave ecosystems with little or no exogenous organic input, the rich variety of redox interfaces allows primary growth of chemolithotrophic (eg. ammonium-, nitrite-, sulfur-, manganese- or iron- oxidising) bacteria (Northup & Lavoie, 2001). Several studies of chemolithotrophic communities have been reported in the literature and have demonstrated that these bacteria play an important role in some cave ecosystems, acting as primary producers and supporting growth of heterotrophic microbes (eg. Sarbu *et al.* 1996). These subsurface microbial communities are based on chemolithoautotrophic energy processing where life does not depend directly upon energy and organic carbon from photosynthesis (Stevens & McKinley, 1995).

1.3.1.1 *Sulfur-based Systems*

Caves containing hydrogen sulfide-rich springs represent less than 10% of all known caves globally (Summers Engel *et al.* 2003). These caves serve as access points into sulfidic groundwater aquifers, typically associated with geothermal regions and oil-field basins, which play an important role in global sulfur cycling. The microbial communities colonising sulfidic cave habitats are of particular interest due to their chemolithoautotrophic metabolism that can sustain a high biomass and rich complex ecosystems in the subsurface (Sarbu *et al.* 1996; Angert *et al.* 1998; Hose, 1999) and their geomicrobiological impact, for example sulphuric acid-driven speleogenesis (Engel *et al.* 2001; Vlasceanu *et al.* 2000).

Frasassi Cave, Italy, and Cueva de Villa Luz, Mexico, are sulfidic cave systems where sulfuric acid drips from the walls and deadly levels of hydrogen sulfide and carbon monoxide are emitted from springs. Yet amidst these hostile conditions a rich and diverse ecosystem of invertebrates and microorganisms are alive and well. Biofilms consisting of extreme acidophiles grow on thick crusts of gypsum and elemental sulfur on the cave walls. Clone library analysis of the Frasassi biofilm revealed at least 2 strains belonging to the genera *Thiobacillus* and *Sulfobacillus* (Vlasceanu *et al.* 2000). An acid producing strain of *Thiobacillus* was also cultivated from the Frasassi biofilm. A defining feature of members of the *Thiobacillus* genus is their ability

to gain energy from the oxidation of reduced sulfur compounds and resulting production of sulfuric acid. Stable carbon isotope analysis revealed that the wall biofilms in Frasassi Cave are isotopically light. Terrestrial isopods living on the cave walls showed peculiar isotopic values markedly different from the rest of the invertebrates inhabiting the cave, implying that they are feeding on these biofilms (Vlasceanu *et al.* 2000). These results imply that the cave food-web is based on organic matter produced chemoautotrophically *in situ* by sulfur-oxidising microbes forming mats that cover the bottom and the mudbanks of the streams and the walls of the cave, similar to the microbial mats of the sulfidic springs of Movile Cave, Romania (Vlasceanu *et al.* 2000).

Microbially generated acid formed in the Frasassi biofilms diffuses through the gypsum to the carbonate surface or drips from the tips of the microbial biofilms onto exposed carbonate surfaces, causing rock dissolution (Vlasceanu *et al.* 2000). Summers-Engel *et al.* (2001) investigated microbial diversity in mats from hydrogen sulfide rich waters and cave wall biofilms in Cesspool Cave, Virginia, and pure cultures of *Thiobacillus* spp. isolated from this mat, demonstrated the ability to corrode $CaCO_3$ (Summers-Engel *et al.* 2001). Corrosion of $CaCO_3$ substrata causes subsequent gypsum precipitation. Substrate dissolution can be beneficial to microbes due to the release of nutrients such as nitrogen and phosphorus in oligotrophic habitats (Rogers *et al.* 1998), but rock dissolution can also be detrimental in the case of carbonates because of pH fluctuations, and in other rocks due to the release of toxic compounds, including aluminium or trace elements (Engel *et al.* 2001).

The formation of caves in limestone bedrock was traditionally considered to be driven by carbonic acid dissolution of carbonate, as discussed in Section 1.2.1. In contrast the formation of Carlsbad Cavern and Lechuguilla Cave, New Mexico, and Movile Cave, Romania, is inconsistent with this model of speleogenesis. Hill (1990) suggested that in caves where hydrogen sulfide-rich waters are present, the production and activity of sulfuric acid might be the primary cause of carbonate dissolution. Initially it was assumed to be a nonbiological process, the sulfuric acid resulting from the chemical oxidation of hydrogen sulfide. Parker & Jackson (1965), however, presented evidence that sulfuric acid production may be mediated by

Thiobacillus spp. Since then, several studies have confirmed the significant role of acid produced by sulfur-oxidising bacteria in the dissolution of limestone suggesting that the colonisation and metabolic activity of these bacteria may be enhancing cave enlargement (Engel *et al.* 2001; Vlasceanu *et al.* 2000).

1.3.1.2 Iron, Manganese, Nitrite, and other Systems

Microorganisms living at the interface between the host rock and cave passages can utilise reduced compounds in the host rock. Caves formed by the dissolution of limestone by carbonic acid are often enriched in iron, manganese and nitrogen and studies have yielded circumstantial evidence for chemolithoautotrophy by iron-, manganese- and nitrogen- oxidisers in these systems (Northup *et al.* 2000, 2003; Holmes *et al.* 2001). Unusual aquatic formations, mantles of mucus and biological material associated with crystalline material, in submerged passages of the Nullabor Caves, Australia contain a high proportion of phylogenetically novel sequence types and a high relative abundance (approximately 12%), of *Nitrospira* relatives, showing most similarity to autotrophic nitrite-oxidising bacteria (*Nitrospira moscoviensis*). Holmes *et al.* (2001) concluded that this community structure, the presence of nitrite in the water, and the apparent absence of aquatic macrofauna, indicate biochemically novel, chemoautotrophic communities dependant on nitrite oxidation.

Lechuguilla Cave, New Mexico, is an immense ancient cave in near pristine condition, an extremely low nutrient environment with, however, sulfur, iron, and manganese deposits harbouring diverse microbial life (Northup *et al.* 2003). 16S rRNA gene clone analysis of corrosion residues (ferromanganese deposits) showed the presence of known iron- and manganese- oxidising/reducing bacteria including phylotypes of the genera, *Hyphomicrobium, Pedomicrobium, Leptospirillum, Stenotrophomonas* and *Pantoea* (Northup *et al.* 2003). Black ferromanganese sediments in Vântului Cave, Romania, contain *Hyphomicrobium* spp., *Pedomicrobium fusiforme* and *Pedomicrobium mangancum*, known to mediate the oxidation and precipitation of manganese (Manolache & Onac, 2000). Northup *et al.* (2003) suggested that these diverse communities of microbes inhabiting ferromanganese deposits seem to exist by utilising

manganese and iron from the bedrock and that the ferromanganese deposits represent, at least in part, the end-product of microbially assisted dissolution and leaching of the underlying host carbonate, and enrichment of iron and manganese through microbial oxidation (Northup *et al.* 2000).

Literature on oligotrophic cave communities subsisting in regions of nutrient scarcity is still sparse and the majority of these investigations have concentrated on communities sustained by a specific and measurable energy input, whether from sulfide, nitrite or surface organic input (eg. Sarbu *et al.* 1996; Angert *et al.* 1998; Holmes *et al.* 2001). Barton *et al.* (2004) investigated microbial diversity in Fairy Cave, Colorado, looking at a wall community in the absence of observable energy sources. Their studies revealed a larger diversity in an oligotrophic environment than originally thought (phylotypes from 4 different divisions, *Proteobacteria, Actinobacteria, Cytophagales* and the low G+C Gram-positive bacteria). The limestone bedrock of Fairy Cave is almost pure $CaCO_3$, (>97.5%) with no significant reduced metal compounds available to act as electron donors and any metal ions that are present in the cave system were likely deposited by the rich mineral waters that formed the cave system (Barton *et al.* 2004). Metabolic analyses suggested that the community subsists using a complex metabolic network with input from trace organics within the environment or fixation of atmospheric gases using lithotrophic metabolism (Barton *et al.* 2004).

A common theme was observed in cultivated relatives of the cloned phylotypes from Fairy Cave, the fixation of atmospheric gases or the use of aromatic carbon compounds. The source of atmospheric gases is obvious, while the potential carbon sources may be the inorganic constituents of water filtering into the cave system. Previous research has suggested that cave waters contain dissolved organic matter from the soil, primarily phenolic compounds and lignin (Saiz-Jimenez & Hermosin, 1999). These compounds can be utilised as carbon sources by many of the species related to those found in Fairy Cave. Similar mechanisms of lithotrophy have been suggested for other cave systems (eg. Cunningham *et al.* 1995). Northup *et al.* (2000) also suggested that reduced metals, such as magnesium and iron, within the limestone matrix of

Lechuguilla Cave provide a sufficient source of electron donors for growth, which may further require the presence of atmospheric organic molecules as a carbon source.

In contrast to the molecular evidence, generally few chemolithoautotrophic bacteria in caves have been detected by cultivation as well as by PCR-based studies (Sarbu *et al.* 1996; Vlasceanu *et al.* 2000; Engel *et al.* 2001; Holmes *et al.* 2001). As discussed in Section 1.3.1, molecular analyses revealed unexpected dominance of mostly uncultured groups, (eg. *Epsilon* Proteobacteria in sulfidic springs of Lower Kane and Parker Caves). The majority of chemoautotrophic species isolated from caves belong to, the sulphur- and sulphide-oxidising genera, (*Thiobacillus, Thiosphaera, Thiothrix, Thiomicrospira, Beggiatoa, Achromatium, Sulfobacillus* and *Thioalcalovibrio*); the sulphate-reducing *Desulfovibrio* sp.; the iron-oxidising *Leptospirrillum ferrooxidans* and *Thiobacillus ferrooxidans*; the manganese- and iron-oxidising genus *Leptothrix* and nitrifiers such as *Nitrobacter* sp. (Schabereiter-Gurtner *et al.* 2002). Culture-independent analyses of Fairy Cave revealed a community distribution of phylotypes unique from previous observations in oligotrophic caves using cultivation, suggesting that many of the species identified are sufficiently adapted to the oligotrophic lifestyle and thus remain resistant to cultivation using standard techniques (Barton *et al.* 2004).

1.3.2 Heterotrophic Systems

Cave microbial communities usually rely on allochthonous input of organic matter transported from the surface (Groth *et al.* 1999a). In caves, animals and visitors can provide large amounts of organic input facilitating heterotrophic life (Hose *et al.* 2000; Groth & Saiz-Jimenez, 1999). Culture-dependent studies have focused on heterotrophic caves with allochtonous input of organic matter demonstrating that heterotrophic bacteria often dominate these communities (Groth & Saiz-Jimenez, 1999). Many microbes identified from deep caves are similar to surface forms and are probably transported into caves by water, air, sediment and animals (Saiz-Jimenez 2001; Schabereiter-Gurtner *et al.* 2002a, b). Actinomycetes are the most abundant

heterotrophic Gram-positive bacteria to be isolated from these caves particularly streptomycete, nocardioform and coryneform actinomycetes (Groth *et al.* 1999a).

Organic input may also be dissolved in the seepage/dripping waters or as particulate organic matter carried in by active or periodic flooding of a subterranean streamway (Schabereiter *et al.* 2002). High sulphate and nitrate concentrations have been found in dripping waters in Tito Bustillo and other Spanish and Italian caves (Hoyos *et al.* 1999) which, in addition to the concentrations of iron, manganese and other elements found in cave rocks, supports heterotrophic bacteria involved in the nitrogen, sulphur, iron and manganese cycles. Laiz *et al.* (1999) investigated the microbial diversity of dripping waters of Altamira Cave, Spain. Water communities were not dominated by actinomycetes but contained low proportions of Gram-positive bacteria, and were mainly composed of Gram-negative rods and cocci (*Enterobacteriaceae* and *Vibrionaceae;* genera *Aeromonas* and *Acinetobacter*). Compounding this, in an earlier study of dripping waters in Altamira Cave carried out by Somavilla *et al.* (1978) *Bacillus* and *Pseudomonas* appeared to be the most abundant genera, followed by *Flavobacterium* and *Erwinia.* In comparison, isolations from ceiling rock of Altamira Cave resulted mainly in Gram-positive *Streptomyces* spp. The absence of culturable actinomycetes in dripping waters agrees with the observations of Kölbel-Boelke *et al.* (1988). They found very few actinomycetes in 60 water and sediment samples clearly demonstrating that dripping water communities are very different to those of cave rock though both are heterotrophic based systems.

Wind Cave, South Dakota, is a heterotrophic detritus-based limestone cave. Clone library analysis by Chelius & Moore (2004) illustrated that Gamma Proteobacteria and *Acidobacteria* predominated water-saturated sediments in the dark zone. Furthermore, most of the microbial sequences were not related to known chemolithoautotrophs, therefore it was concluded that this particular community is likely detritus-based, where allochthonous energy and carbon are transported into the cave by infiltrating waters. Although some clones resembled sequences from other caves, they found that no cave-specific bacterial community was evident. Clones mostly resembled those from soil, freshwater, plant associated and polluted environments (Chelius & Moore, 2004). Conversely, culture studies of the same sediments from

Wind Cave produced representatives of only the actinomycetes and Proteobacteria (Alpha, Beta and Gamma) though clone analysis indicated that these were relatively minor components of the microbial community (Chelius & Moore, 2004). Most isolates were related to other cultivated members and sequences retrieved from soil and various polluted environments. It is important to note that Wind Cave is also a show cave, impacted by humans and lighted for tours, but this study still represents baseline data. Although in general molecular analyses reveal them as relatively minor representatives of cave communities, actinomycetes are still the most dominant group of bacteria isolated from caves (Schabereiter-Gurtner *et al.* 2002; Chelius & Moore, 2004).

1.3.3 Actinomycetes in Caves

Results of studies in caves of China, Korea, Northern Spain, and Southern Italy have demonstrated that actinomycetes are not only the most abundant bacteria isolated from these caves, but also reveal a great taxonomic diversity (Groth *et al.* 1999a,b; Groth & Saiz-Jimenez, 1999). Several new species of actinomycetes have been described from hypogean environments (Lee *et al.* 2000a,b,c; Lee *et al.* 2001) including three new genera, *Knoellia sinensis* gen. nov., sp. nov. and *Knoellia subterranean* sp. nov, and *Beutenbergia cavernae* gen. nov., sp. nov., isolated from sediment sampled from Reed Flute Cave, China (Groth *et al.* 2002, 1999b); *Hongia koreensis* gen. nov., sp. nov., isolated from sediment in a gold mine cave of Korea (Lee *et al.* 2000). Three novel species were also described from the gold mine cave in Korea, *Pseudonocardia kongjuensis* sp. nov. and *Saccharothrix violacea* sp. nov., and *S. albidocapillata* comb. nov (Lee *et al.* 2000, 2001). However, little has been published about the cave environments that these novel species have been described from.

Caves are not uniform environments in terms of geological and geochemical characteristics, as they can vary from one to the other, eg. rock type, method of formation, length, depth, number of openings to the surface, presence or absence of active streamways, degree of impact by human visitation etc. Furthermore, on a smaller scale, various microhabitats, with vast differences in community structure can exist within caves. It seems

fairly widely accepted that dry cave substrate typically yields a higher proportion of actinomycetes than does dripping water and wet sediment (Kolbel-Boelke *et al.* 1988; Laiz *et al.* 1999). Somavilla *et al.* (1978) did not culture actinomycetes from the air of Altamira and La Pasiega Caves, whereas Arroyo & Arroyo (1996) found actinomycetes from contact plates from the floor, walls, and ceilings of the same cave.

Limestone caves and lava tube caves often contain wonderful displays of filamentous actinomycetes that may cover entire ceilings and walls of caves giving a 'silvered' appearance. Probably many of the discrete lichen-like colonies frequently noted on walls and formations in the dark zone may be actinomycetes of the genus *Streptomyces* since they often have the powdery appearance and characteristic earthy odour common to cultures of this genus. It has also been suggested that the abundant *Streptomyces* in caves is probably responsible for the earthy smell of caving (Caumartin, 1963 in Ford & Cullingford, 1976). *Streptomyces* and *Nocardia* are the most common, and abundant, groups isolated from caves (Arroyo & Arroyo, 1996). *Streptomyces* species are particularly abundant though this may be due to their easy growth in the laboratory.

The majority of the work on actinomycetes in hypogean environments has been conducted in Altamira, Tito Bustillo, La Garma, and Llonin caves, Spain, and Grotta dei Cervi, Italy all of which have spectacular galleries with paleolithic rock art paintings (Groth & Saiz-Jimenez, 1999; Groth *et al.* 1999a, 2001; Laiz *et al.* 1999, 2000). Groth *et al.* (1999a) reviewed the growth of actinomycetes on the ceiling and walls in Altamira and Tito Bustillo caves isolating approximately 350 strains. Actinomycete growth was distributed all over the caves and could be observed on the active stalactites, on upper and lower parts of the rock wall and in the cave soils. Large parts of the cave's rock surfaces were covered by macroscopic colonies (1-2 mm) visible to the naked eye and direct isolation from these colonies resulted solely in *Streptomyces xanthophaeus*. However culture-independent DGGE analyses detected 14 separate bands representing other species, most of them closely related to uncultured bacteria affiliated with Proteobacteria, *Acidobacteria*, *Cytophaga-Flavobacteria-Bacteroides* group (CFBs) and *Actinobacteria* (Schabereiter-Gurtner *et al.* 2002).

Samples of active stalactites, wall concretions and rocks from the walls and ceilings of the galleries have been investigated and a high number of isolates obtained. Most abundant were genera of the actinomycetes, particularly *Streptomyces* especially from rock walls and soils. Other genera isolated included *Nocardia, Nocardioides, Saccharothrix, Amycolatopsis, Brevibacterium, Rhodococcus, Aureobacterium,* and members of the family *Micrococcaceae*. However, in stalactites, the most abundant species isolated belonged to the low G+C Gram-positive bacteria of the genus *Bacillus*, although the most conspicuous and visible to the naked eye were actinomycetes of the genera, *Agromyces, Amycolatopsis, Arthrobacter, Nocardiopsis, Rhodococcus,* and *Streptomyces* (Groth *et al.* 2001). These microorganisms are able to colonise the bare rock surfaces utilising organics in dripping water. Apart from published novel species from caves, most other papers characterise cave strains to the genus level only as they use morphological and biochemical means of identification rather than phylogeny. At present it is therefore difficult to make comparisons at the species level between cave environments.

Culture-dependent studies have focused on typical heterotrophic microbes from the surface and have mostly come from so called "show caves" open to the public and which are heavily impacted by humans. There is an apparent correlation between the number of visitors and diversity of bacteria. The higher the number of visitors the higher the diversity of isolated strains, as indicated by the data obtained in Tito Bustillo and Altamira caves (Groth *et al.* 1999a). Altamira Cave revealed a great taxonomic diversity with predominant isolates belonging to *Streptomyces, Nocardia, Nocardioides, Saccharothrix, Amycolatopsis, Brevibacterium, Rhodococcus, Aureobacterium,* and members of the family *Micrococcaceae* (Groth *et al.* 1999a). Caves with restricted access, Llonín and La Garma, yielded lower diversity. This increasing diversity is likely associated with lighting, which promotes the growth of phototrophic microorganisms, and also the introduction of organic matter by visitors into the ecosystem (Ariño & Saiz-Jimenez 1996). Thus one could argue that it is the public nature of these caves that tend to heterotrophy dominated by actinomycetes, rather than it being a general trend in cave systems with allochthonous input of organic matter. However, Grotta dei Cervi shows similar colonisation patterns to Altamira Cave, in spite of the fact that this cave was discovered more recently in 1970

(91 years later than Altamira) and visitation is restricted to scientific purposes (as in Llonin and La Garma), the biodiversity was surprisingly high. This is perhaps due to the appreciably high input of organic matter present in Grotta dei Cervi in the form of bat guano, promoting heterotrophy and dominance of actinomycetes.

The study of cultivated microbes in these caves has revealed only a minor and not very representative proportion of the cave microbial populations. Gram-positive bacteria identified in Llonin, La Garma, Altamira, and Tito Bustillo Caves by culture-independent techniques is relatively low (<30 %), though Gram-positive bacteria, and in particular actinomycetes were the dominating isolates obtained from cultivation (eg. Groth & Saiz-Jimenez, 1999; Schabereiter-Gurtner *et al.* 2002). More recently DGGE community fingerprinting combined with phylogenetic analyses used to investigate samples from paintings and surrounding rock in Altamira and Tito Bustillo revealed greater taxonomic diversity detecting unknown and unexpected bacterial groups, particularly the *Proteobacteria, Acidobacteria* division, CFBs, actinomycetes, green non-sulfur bacteria and *Planctomycetes*. DGGE analysis of paintings in Llonin and La Garma caves (Schabereiter-Gurtner *et al.* 2004) also illustrated a high biodiversity of chemolithotrophic, as well as heterotrophic, bacteria; the most abundant groups found were the *Proteobacteria*, actinomycetes and *Acidobacteria*. This data compared to results from Altamira and Tito Bustillo caves revealed similarities in the bacterial community components, especially in the high abundance of the *Acidobacteria* and *Rhizobiaceae*, and ammonia- and sulfur-oxidisers (Schabereiter *et al.* 2002). Which is interesting in that Llonin and La Garma are restricted visitor access for research purposes only whereas Altamira and Tito Bustillo are open to the public. These studies have revealed diverse and unknown microbial colonisation on the paintings in contrast to previous culture-dependant investigations.

In the past, the study of microbial communities and biogeochemical processes in hypogean environments is mainly related to the fact that microbes affect cultural heritage properties that humans wish to protect (Groth & Saiz-Jimenez, 1999) and we owe much of our initial knowledge of cave microbiota to these studies. The role of actinomycetes in the deterioration of paintings and frescoes in hypogean environments (not just caves, but crypts,

tombs and underground churches) has been emphasised by many investigations (eg. Monte & Ferrari, 1993; Groth & Saiz-Jimenez, 1999; Groth *et al.* 1999a). Actinomycetes are known to destroy wall paintings by the excretion of organic and inorganic metabolic products (Schabereiter-Gurtner *et al.* 2004). The first actinomycetes identified as degraders of rock art were *Streptomyces rectus flexibilis, S. griseolus, S.cinereoruber, S.vinaceus, S.albus* and *Nocardia* sp. (Giacobini *et al.* 1988).

Though the role of actinomycetes in rock art is highly recognised, it is interesting that they haven't been isolated from works of art, except those located in hypogean environments (eg. caves, crypts, grottos and tombs) (Giacobini *et al.* 1988). Atlanterra Shelter, Spain, contains rock art paintings made with iron oxides. The shelter is exposed to terrestrial environmental fluctuations. The bacteria isolated from Atlanterra Shelter seem to constitute a homogenous community with abundance of *Bacillus* strains, very different to actinomycete dominated communities found in rock art paintings from karstic hypogean environments (Groth & Saiz-Jimenez, 1999; Laiz *et al.* 1999; Gonzalez *et al.* 1999). All isolated *Bacillus* strains were able to reduce hematite which is significant due to the fact that Fe(III)-(hydr)oxides are the most abundant pigments in rock art. This work demonstrates that actinomycetes are not alone in their role as biodeteriogens of rock art, however they do seem to be the dominant group in hypogean environments, perhaps indicating favourable selective pressures in the cave environment.

A number of actinomycetes isolated from caves have the ability to produce various types of crystals. Studies in Altamira and Tito Bustillo Caves demonstrate that the host-rock, cave formations and rock art are coated by dense networks of bacteria, mainly actinomycetes and these bacteria can induce constructive (calcification, crystalline precipitates) and destructive (irregular etching, spiky calcite) fabrics. Because of this ability it has been proposed that these bacteria and others are directly or indirectly involved in constructive biomineralisation processes in caves (Laiz *et al.* 1999; Barton *et al.* 2001; Canaveras *et al.* 2001; Groth *et al.* 2001; Jones, 2001). Little is known concerning the distribution, population dynamics, growth rates and biogeochemical processes of actinomycetes in caves, in spite of the fact that they seem to constitute a significant part of the "culturable" microbial population of these habitats. A

prerequisite for the study of the role of actinomycetes in biogeochemical processes is the isolation and identification of these organisms (Groth *et al.* 1999a).

1.3.3.1 Actinobacteria

Stackebrandt *et al.* (1997) proposed a new hierarchic classification system, *Actinobacteria* classis nov. for the actinomycete line of descent, wholly defined by the phylogenetic analysis of small subunit 16S rRNA gene sequences. The *Actinobacteria* is comprised of high-G+C content Gram-positive bacteria with a common ancestry and includes Subclasses *Acidimicrobidae, Rubrobacteridae, Coriobacteridae, Sphaerobacteridae* and *Actinobacteridae*. The Order *Actinomycetales* (actinomycetes) is within the Subclass *Actinobacteridae*. It is important to note here that quite often the actinomycetes are referred to simply as *Actinobacteria*, which, although fundamentally correct, is misleading, as the Class *Actinobacteria* encompasses a broader range of taxa than the *Actinomycetales* alone. For the purpose of this study the term actinomycete(s) will be used to describe only members of the Class *Actinobacteria*, Subclass *Actinobacteridae*, Order *Actinomycetales*.

1.3.3.2 Actinomycetes

Actinomycetes are Gram-positive bacteria which form branching hyphae at some stage of their development and may produce a spore bearing mycelium (McCarthy & Williams, 1990). They are aerobic saprophytes and are widely distributed in nature (Goodfellow & Williams, 1983) mainly found in soil where they manufacture enzymes which degrade complex molecules and play a major role in decomposition of organic matter (Lechevalier & Lechevalier, 1985). These organisms are selected for in environments characterised by oligotrophic conditions, low water activities and high concentrations of $CaCO_3$. Hyphal actinomycetes are typically slow growing and their spores can remain viable for a number of years in unfavourable conditions; the exact length of time for which they can survive is uncertain. Although predominantly soil bacteria, actinomycetes have been isolated from a wide variety of environments, including

freshwater, lake sediments, rivers, streams, marine environments, salt marshes, fodder and related materials, and air (Loyd, 1969; Cross, 1981; Hirsch & McCann-McCormick, 1985; Labeda & Shearer, 1990). Actinomycetes have also been isolated from extreme environments such as; ice, sediments and air in Antarctica, and, as discussed previously, rock surfaces and sediments in cave environments (Eg. Cameron *et al.* 1976; Groth *et al.* 1999a,b).

1.3.3.3 Actinomycete Taxonomy

Over 150 genera of actinomycetes have been isolated from soils. The exact composition and phylogenetic boundaries of the actinomycetes has remained open to question and modification due to continued development and application of new taxonomic classifications. Early attempts at taxonomic classification of actinomycetes were based on morphological and pigmentation characteristics of the sporing bodies and substrate mycelia, which is a useful but arbitrary approach to classification and not based on the phylogenetic relationships between different species (Williams *et al.* 1983). Variation in biochemical and physiological properties were incorporated into actinomycete taxonomy, however these new data alone could not be used to devise a satisfactory phylogenetically based taxonomy (Embley & Stackebrandt, 1994). The rich chemical, morphological and physiological diversity of phylogenetically closely related genera of actinomycetes makes the description of families and higher taxa so broad that they become meaningless for the description of the enclosed taxa (Stackebrandt *et al.* 1997).

The application of molecular techniques based on variations in nucleic acid sequences between different bacteria, especially 16S rRNA gene sequencing, has had a dramatic impact on actinomycete systematics. It was soon discovered that some morphological characteristics given greater weight in earlier studies, such as the ability to form spores, were not reliable in a phylogenetic system of classification (Stackebrandt *et al.* 1981). Almost any description based on morphology or physiology would have exceptions and actinomycete taxonomy now relies heavily on molecular comparisons (Ensign, 1992). The only phenetic characteristics shared by all members of the actinomycetes is a relatively high level of guanine (G) and cytosine (C) as a

percentage of total DNA (>55%) (Goodfellow, 1989). Actinomycete taxonomy is still under development and more taxonomic information needs to be collected in all fields in order to develop a phylogenetic system of classification with confidence (Holloway, 1997). To determine a phylogenetic classification of actinomycete which is both true and practical it is necessary to employ a polyphasic approach, employing a combination of molecular, chemical and numerical taxonomic methods (Murray *et al.* 1990).

1.3.3.4 Actinomycete Ecology

As soil bacteria, actinomycetes contribute significantly to the turnover of complex biopolymers, such as lignocellulose, hemicellulose, pectin, keratin and chitin (Williams *et al.* 1984). Additionally nitrogen-fixing actinomycetes of the genus *Frankia* have one of the broadest host ranges known, forming root nodule symbioses in more than 200 species of flowering plants (Huss-Danell *et al.* 1997). Actinomycetes can be recovered from most soils in relatively high numbers although this may not give an accurate picture of proportions of active bacteria in the soil because most of the colonies are probably isolated from spores (Williams, 1978). *Streptomyces* and *Arthrobacter* are ubiquitous in soil and are the most numerous of the actinomycetes (Goodfellow & Williams, 1983). The next most common actinomycetes are, in descending order, members of the genera *Micromonospora, Actinoplanes, Actinomadura,* and *Nocardia* (Lechevalier & Lechevalier, 1985).

Although soil is the main habitat of the actinomycetes, they can be isolated from humans, animals, plants, waste water, food products, stones, buildings and works of art (eg. Groth & Saiz-Jimenez, 1999). Despite intensive studies there are still many gaps in our knowledge of the role played by actinomycetes in soil processes (Goodfellow & Williams, 1983). Caves are unique environments characterised by little or no light, low levels of organic nutrients, and a stable, but cool to cold, microclimate. Russell, (1990), hypothesised that it is not necessary for a microbe to function at optimal rates as long as it can compete effectively in its

particular environment. It may be quite advantageous for cave bacteria to metabolise submaximally and have long generation times in nutrient poor environments.

Actinomycetes are well known for their ability to grow on nutrient poor media (Lechevalier & Lechevalier, 1985) and streptomycetes can exist for extended periods of time as arthrospores that germinate in the occasional presence of nutrients (Goodfellow & Williams, 1983). Low temperatures are not a limiting factor for actinomycete growth. Suzuki *et al.* (1997) described an obligately psychrophilic actinomycete (*Cryobacterium psychrophilum*), and Xu *et al.* (1996) reported actinomycete populations in cool areas of China, with average temperatures of 5° C or below 0° C, where *Streptomyces* spp. constituted up to 97% and 83%, respectively, of the total heterotrophic count. Some were psychrophiles with an optimum growth temperature of 10–15° C. Groth & Saiz-Jimenez (1999) suggested that growth of actinomycetes in hypogean environments might result from the association of two factors: low temperatures and high relative humidity. These environmental conditions, together with nutrient availability and nature of organic matter are recognised to be important factors controlling the activity of actinomycetes in caves.

1.4 Geomicrobiology

Geomicrobiology is the term given to studies of the microbe-mineral interface, including microbial weathering and sedimentation processes, microbial roles in formation and degradation of minerals, mineralisation of organic matter, subsurface microbiology, biogeochemical cycling of elements, and bioremediation. Microorganisms are important active and passive promotors of redox reactions that influence geological formations (Ehrlich, 1999). There is extensive literature demonstrating the influence of microorganisms in mineral formation from non-cave environments for a wide variety of minerals including, carbonates, oxides, phosphates, sulfides, and silicates (Fortin *et al.* 1997). Bacteria may produce minerals as a result of growth. Cell walls have chemically reactive sites that bind dissolved mineral-forming elements allowing nucleation and growth of crystals from an oversaturated solution to occur (Groth *et al.* 2001). Alternatively, mineral precipitation may result from metabolic activities of bacteria. Bacterial activity may simply trigger a change in solution chemistry that leads to oversaturation and mineral precipitation. In biological processes, oversaturation is considered an important prerequisite for the precipitation of minerals from solution (Fortin *et al.* 1997). Although Gonzalez-Munoz *et al.* (1996), suggested that this is merely incidental and the critical point is the participation of cellular membranes in inducing nucleation. Caves can be used as experimental study systems for geomicrobiology, not because they are strange, but because they are simple and often locally abundant, allowing for replicate studies (Northup & Lavoie, 2001). While geomicrobiology in general has received substantial interest in the last decade, one unresolved issue is the involvement of microbial activity in the dissolution of, or formation of speleothems in caves (Barton *et al.* 2001).

1.4.1 Geomicrobiology in Caves

Caves are nutrient-limited environments containing a variety of redox interfaces and they provide an accessible window into subsurface environments in which to study precipitation and dissolution processes and products (Northup & Lavoie, 2001). A variety of

precipitation and dissolution processes results in the deposition of carbonate speleothems, silicates, iron and manganese oxides, sulfur compounds and nitrites and the break down of limestone walls resulting in corrosion residues. Geomicrobiological activities in caves are no longer underestimated since studies have shown that bacterial metabolism can affect these mineral precipitation and dissolution processes (Cañaveras *et al.* 2001; Northup & Lavoie (2001). Studies of microorganisms in caves have been predominantly descriptive, as illustrated in Section 1.3, with only a few experimental studies reported although increased interest in microbe-mineral interactions in caves is emerging.

Microbially influenced corrosion or dissolution of mineral surfaces can occur through mechanical attack, the secretion of enzymes, and organic and mineral acids (eg. Sulfuric acid). Microbially mediated reactions can generate considerable acidity that can dissolve cave walls and speleothems. Possible microbially influenced corrosion include limestone corrosion residues composed of iron and manganese oxides and clays (eg. Lechuguilla and Spider Caves, New Mexico; Northup & Lavoie, 2001; Northup *et al.* 2003), and sulfuric acid speleogenesis and cave enlargement (eg. Movile Cave, Romania, and Cueva de Villa Luz, Mexico; Vlasceanu *et al.* 2000). Microbially induced mineralisation is documented in the formation of carbonates, moonmilk, silicates, clays, iron and manganese oxides, sulfur, and saltpeter. For example, sulfate generated by sulfur/sulfide-oxidising bacteria can be used as an electron-acceptor by sulfate reducers. This reaction produces bicarbonate that can complex with calcium, resulting in the precipitation of calcite in the form of subaqueous mantles (eg. Weebubbie Cave, Nullabor, Australia) (Contos *et al.* 2001). There is no clear idea as to the significance of biological involvement in speleothem formation, however, there are clues.

Studies of cave geomicrobiology are largely still qualitative in nature. Barton *et al.* (2001) and Jones (2001) offered critical guidelines for the biogenicity of 'objects' visualised in cave deposits: they must, be found in a liveable environment, show complex form, show representations by numerous specimens, be members of a multicomponent assemblage, show morphological variability, reproduction by biological means, exhibit a range of degradation, organic residues and exhibit biogenic isotopic features. Various microbiological techniques have

been used to illustrate that microbes are present in most spelean environments and commonly modify the composition of the fluids and/or influence precipitation of various minerals, including calcite (e.g. Melim *et al.* 2001). Classical isolation combined with molecular phylogenetic techniques reveals the presence of microbial communities associated with speleothems (Cañaveras *et al.* 1999). Enrichment experiments with microorganisms cultured from cave environments have aided in identifying dissolution and precipitation abilities of these cave microbes (eg. Groth *et al.* 1999a) and stable isotope techniques has provided information on the microbial contribution to processes of mineral formation (eg. Hose *et al.* 2000) and ecosystem bioenergenetics (eg. Sarbu *et al.* 1996).

Most bacteria in nature live as part of dynamic metabolically interactive assemblages, commonly referred to as biofilms, found covering most solid substrates (rocks, plants, man-made structures) (Douglas & Douglas, 2000). The primary techniques for examination of biological material on mineral surfaces are transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy and environmental scanning microscopy (ESEM) (Siering, 1998). Previous studies by Ray *et al.* (1997) and Douglas & Douglas, (2000) have shown the worth of ESEM for investigations of microbe-mineral relationships in natural microbial communities. Though SEM has been an important tool used to study cave microbial carbonates (Northup & Lavoie, 2001), ESEM allows the viewing of fully hydrated specimens that have not undergone structural or chemical alterations imposed by the extensive procedures necessary for viewing biological specimens in high vacuum necessary for conventional SEM. Besides allowing visualisation of microorganisms in their natural form and as intact assemblages, ESEM also detects elements, especially those lighter that Si, which tend to be lost or masked by the processes used to prepare samples for conventional SEM (Douglas & Douglas, 2000).

The biogenicity of mineral-associated, purportedly biological features can be questionable and extremely difficult to resolve (Barton *et al.* 2001). Microbial activity has been directly or indirectly linked to the formation of many different minerals, however most geomicrobiology studies have focused their attention on the microbiological processes that are

associated with the development of carbonate deposits. Even with this focus, our knowledge of the microbial involvement in these processes has been limited by, i) the fact that there are few studies that have approached the issue from a geological perspective, ii) the fact that many geological studies of older deposits assume abiogenicity, iii) the fact that most geologists lack formal microbiological training, and iv) the scale of observation (Jones, 2001).

$CaCO_3$ speleothems predominate in most caves, and microbial studies have been conducted on stalactites, stalagmites, helictites, moonmilk, pool fingers and cave pearls. Microorganisms have been found fossilised in carbonate speleothems (Jones & Motyka, 1987; Polyak & Cokendolpher, 1992; Jones & Kahle, 1995; Melim *et al.* 2001). Fungi, algae and bacteria have all been implicated in the precipitation of carbonate dripstone in caves (Went, 1969; Danielli & Edington, 1983). There is much evidence for rich and diverse chemoautotrophic and heterotrophic communities in caves (eg. Angert, *et al.* 1998; Sarbu, *et al.* 1996), it remains unclear however, what role, if any, these communities play in speleothem formation

1.4.2 Microbially Mediated CaCO3 Precipitation

As some of the most abundant minerals on earth, carbonates are ubiquitous and highly reactive components of natural environments. Carbonate minerals play important parts in global carbon cycling, alkalinity generation, cycling of major and trace elements, and transfer of matter among oceans the continents and the atmosphere (Warren *et al.* 2001). Understanding carbonate precipitation has wide ranging implications from interpretation of biogeochemical cycles, potential impact of increased atmospheric concentrations of CO_2 or reactive transport of radionuclides and trace metals in contaminated aquifers.

Bacterial precipitation of $CaCO_3$ has been reported in a variety of environments including hot springs, tidal mats and caves. It has been known since Boquet *et al.* (1973) that most heterotrophic soil bacteria can induce $CaCO_3$ precipitation. Phillips & Self (1987) demonstrated that in soils with a high calcite concentration needle fibre-calcite formed within fungal mycelia and also encrusted rod-shaped microbes. Chafetz (1994) reported research carried out in the

field and laboratory conditions to demonstrate beyond doubt that $CaCO_3$ precipitation occurring within microbial mats was a process controlled by living bacteria and does not occur when the bacteria are dead, even in the presence of other living microorganisms.

Bacteria and fungi can precipitate $CaCO_3$ extracellularly through a variety of processes that include photosynthesis, ammonification, denitrification, sulfate reduction, and aerobic sulfide oxidation (Ehrlich, 1996; Castanier *et al.* 1999; Riding, 2000). Castanier *et al.* (1999) proposed biologically mediated or active precipitation of $CaCO_3$ where carbonate particles are produced by ionic exchanges through the cell membrane of heterotrophic bacteria in an environment enriched in organic matter. Initially, this involves the adsorption of Ca2+ and Mg2+ ions to negatively charged cell surfaces and the cell then acts as a nucleation site. Subsequent $CaCO_3$ precipitation may be active or purely inorganic. Riding (2000) noted that microbial production of extracellular polymeric substances (EPS), which trap sediments, is often critical to the creation of microbial carbonates. Terrestrial oncoids (microbially formed carbonate constructions from dolestones, Cayman Islands) developed when calcifying filaments and spores trapped and bound detritus within the associated mucus (Jones, 1991). These resemble cave pearls, a speleothem that has been suggested to have a microbial association during formation (Gradzinski, 1997).

More recent studies have attempted to identify the factors that control the contribution of microorganisms to carbonate precipitation. Further progress in this field has been made in non-cave environments. Van Lith *et al.* (2003) found that only pure cultures of metabolising sulphate-reducing bacteria, isolated from hypersaline lagoons in Brazil, induced calcium-dolomite and high magnesium-calcite precipitates indicating that the carbonate nucleation takes place in the locally changed microenvironment around the bacterial cells. Dittrich *et al.* (2004) showed that picocyanobacteria were involved in fast and effective calcite precipitation in an oligotrophic lake. Whether by saturation or nucleation they observed small calcite crystals produced by eukaryotic picoplankton whereas cyanobacterial picoplankton produced micritic carbonate indicating that different cells may induce very different, distinct precipitation processes.

As previously discussed, geological formations in caves (speleothems) like stalagmites and stalactites, are mineral depositions formed by precipitation of carbonates from ground water. Extensive documentation of microbial precipitation of $CaCO_3$ exists in non-cave literature, biogenic carbonates in particular have been studied since the late 19th century (eg. stromatolites; Chafetz & Buczynski, 1992). Microorganisms are believed to affect carbonate precipitation both through affecting local geochemical conditions and by serving as potential nucleation sites for mineral formation (McGenity & Sellwood, 1999). In natural environments, the primary means by which microorganisms promote $CaCO_3$ precipitation is by metabolic processes that increase alkalinity (Fujita *et al.* 2000). However, investigators have not established whether cave carbonate material has a similar origin.

Some of the most intriguing work on cave fungi associated with speleothem formation was conducted by Went (1969). The author made the interesting discovery that the growth of stalactites in Lehman Caves, Eastern Nevada, was associated with a fungus, *Cephalosporium lainellaecola.* This discovery was made using a special microscope mounted horizontally on an adjustable bracket sliding along a vertical steel bar so that stalactites could be observed *in situ* within the cave. He found that fungal hyphae occurred in a drop of water at the end of a straw stalactite and that strings of tiny calcite crystals tended to form along them. The hyphae not only functioned as crystallisation nuclei but also prevented the crystals from being removed with the falling drops. Perhaps actinomycete filaments act in the same way. Actinomycetes isolated from either dripping waters or rock in Altamira Cave showed the ability to produce crystals and therefore could play a role in the deposition of $CaCO_3$ polymorphs on the rock surface (Laiz *et al.* 1999). Although there is no known role for $CaCO_3$ in bacterial metabolism, certain organisms precipitate calcite during their growth (Buczynski & Chafetz, 1990). Groth *et al.* (2001), found that 45% of isolates from stalagmites in Grotta dei Cervi, Porto Badisco, Italy were able to precipitate $CaCO_3$ in culture medium. Organisms such as *Achromatium oxaliferum* contain internal calcite inclusions during growth (Head *et al,* 1996). There is also an established role for bacteria in the nucleation of $CaCO_3$ precipitation for stromatolite formation (Ehrlich 1999; Laval *et al.* 2000).

Various experiments have shown that bacteria may be replaced or encrusted by inorganic materials resulting in the fossil preservation of bacterial morphology. There are reports of bacteria preserved in carbonate rocks (Folk 1993; Jones 1995; Trewin & Knoll 1999) and a few reports of iron-oxidising bacteria preserved in carbonate speleothems in caves (Polyak & Cokendolpher, 1992). However, although microfossils have been identified in carbonate speleothems, no direct connection with active precipitation processes in the formation of these features has been demonstrated. Bacterially induced changes in solution chemistry can be a passive process (eg. stromatolites; Chafetz & Buczynski, 1992). Evidence that microbes play a role in the formation of cave carbonates is still largely circumstantial and based on their physical presence. The question still remains whether the organisms identified are actively involved in speleothem formation, or simply buried during mineral precipitation (Polyak & Cokendolpher, 1992). Of special interest is the speleothem moonmilk. As discussed in Section 1.2.2, the wet pasty forms of moonmilk are so striking that some special explanation for their origin seems to be necessary. Cañaveras *et al.* (1999) suggested that bacteria present in caves may play a role in the formation of moonmilk deposits as microbial communities predominantly composed of different species of the genus *Streptomyces* were found in association with hydromagnesite and needle-fiber aragonite deposits in Altamira Cave.

1.4.3 Moonmilk

Moonmilk is a widely distributed, secondary formation and refers to the very hydrated white spongy/pasty or powdery masses found coating walls and speleothems in caves. It is not a mineral, it is a speleothem. It is often described as having a cottage cheese-like consistency and may be composed of several carbonate minerals. The historical term Mondmilch (= calcite moonmilk) is related to the proper type locality, the cave Mondmilchloch from South-Pilatus near Lucerne, Switzerland. Mondmilch was first mentioned by Agricola (1546, p. 194) and described by Gesner (1555) after visiting the cave Mondmilchloch (Fischer, 1988). This was without consideration of the actual mineral composition of the deposits. Moonmilk became well

known throughout Europe and used as a medication (Scheuchzer, 1752; in Fischer, 1988). In the 16th and 17th centuries, physicians in Europe used dried moonmilk from caves as a dressing for wounds. Apparently moonmilk would stop the bleeding and act as a dehydrating agent. However, some also believed it had curative properties. It is rather interesting that modern research or theories have discovered bacteria associated with moonmilk include actinomycetes that, as previously discussed, possess antibiotic properties.

Many descriptions of moonmilk are not only related to calcite growth, although the original term mondmilch from the cave Mondmilchloch refers to calcite precipitation and does not represent the phenomenon for speleothems in general. Hill & Forti (1986) suggested that texture rather than composition is implied by the term 'mondmilch'. Fischer (1988) defined mondmilch as a calcite microcrystalline or needle-crystalline speleothem with a minimum calcite content of 90 % weight, for the purpose of distinguishing true calcite mondmilch from other carbonate speleothems (< 90 % weight calcite) and other subterranean deposits, (eg. ferromanganese, sulfates, phosphates, silicates). The mineralogy and crystallography of potential mondmilch samples can be easily proved using X-Ray Diffraction Analysis (XRD) and scanning electron microscopy (SEM) methods (Fischer, 1988). Numerous synonyms in different languages exist for mondmilch, including the English version moonmilk.

True calcite moonmilk (mondmilch) has been found in many caves all over the world and appears to be particularly abundant in caves of cool temperature and high humidity. In warmer semi-arid regions limestones contain significant amounts of magnesium and moonmilk deposits may consist of a number of magnesium minerals including hydromagnesite, magnesite, huntite or dolomite (Moore & Nicholas, 1964). In Australian caves moonmilk has been documented in a variety of forms including, thin dry wall coatings, white cheese-like pasty forms at the bottom of rimstone pools or wall niche deposits, stalactites, cauliflower-like deposits and fluffy fungus-like forms in Jenolan Cave, NSW (http://www.speleonics.com.au; maintained by J. Rowling). The origin of moonmilk deposits is highly contested among the literature. Hill & Forti (1986) cite four main theories as to the origin of moonmilk: i) freezing in ice caves, ii) precipitation from groundwater in which there is an agent which prevents the

crystals from growing large, (the theory preferred by Hill & Forti), iii) a disintegration product of bedrock, and iv) a by-product of the life cycle of various microorganisms. Each of these theories taken individually has its pros and cons as an explanation for the development of moonmilk.

White (1976) suggested that moonmilk in alpine caves may be precipitated chemically at low temperatures as hydrated carbonates, however these forms are only stable at low temperatures. Also freezing in alpine icy caves does not explain the occurrence of temperate and tropical moonmilk. Rapid precipitation of substances will generally result in small crystal size and this occurs near cave entrances where precipitation is due to both outgassing CO_2 and evaporation of water (http://www.speleonics.com.au; maintained by J. Rowling). It also occurs where gypsum is being produced. However, this does not explain the pasty textured moonmilk or that speleothems in cave entrances are hard and crystalline and quite unlike moonmilk.

Moonmilk is usually considered a depositional product. However, Hill & Forti (1986) suggested it can also form by corrosion processes. It is suggested that moonmilk could be a product of microbial metabolism which could biochemically corrode underlying bedrock. There are of course various types of bedrock and numerous disintegration processes that can occur within caves and though calcite deposits may be formed, they do not have the pasty texture that moonmilk is famous for. One common cause of bedrock disintegration is bat guano (http://www.speleonics.com.au; maintained by J. Rowling). It has been suggested that the thin films of moonmilk may be a result of bat guano bedrock disintegration, however this would not explain the moonmilk films in Entrance Cave, Tasmania, Australia, as there are no bats inhabiting Tasmanian caves due to cool air temperatures. Gradzinski *et al.* (1997) concluded that moonmilk deposits from several caves in Poland might be the result of microbial degradation of the host rock, as well as, or in place of microbially mediated precipitation of calcite. Conversely, in Spider Cave, New Mexico, SEM pictures of the microcrystals that make up moonmilk do not show the evidence of weathering that would be expected if microbial corrosion routinely resulted in moonmilk production (Northup & Lavoie, 2001).

It is generally accepted that moonmilk might be the by-product of the life-cycle of various microbes, although the question remains whether the organisms identified are actively involved or simply buried during mineral precipitation. A number of organisms have been isolated from cave environments and shown to precipitate $CaCO_3$ in the laboratory (Danielli & Edington, 1983; Groth *et al.* 2001). One very interesting point, a thick wall-niche deposit of moonmilk in Jenolan Cave is recorded as having been damaged by a person's handprint to at least 1 cm depth, yet 8 years later the print is/was no longer visible (http://www.speleonics.com.au; maintained by J. Rowling). Given the long length of time for usual speleothem growth (1 inch in 100-150 years; Section 1.2.2) this prompts the question as to whether the moonmilk was "alive" or not. Putative cells and an organic matrix can be frequently seen in moonmilk samples with SEM or in thin sections, but not in all cases. A wide range of microbes, particularly bacteria, streptomycetes but also fungi, algae and protozoa, can be cultured from moonmilk often in high densities (Northup *et al.* 2000).

Williams (1959) inoculated moonmilk samples from several caves in South Wales into various nutritional media and isolated eight species of heterotrophic bacteria belonging to the genera *Bacillus, Micrococcus, Bacterium,* and *Streptomyces.* In one culture a Gram-negative rod, a thiobacteria, was detected with the ability to produce $CaCO_3$ in crystalline forms similar to those found in moonmilk. Danielli & Edington (1983) isolated a wide range of colony types (mostly Gram-negative cells) from moonmilk collected from caves in Wales and calcite precipitation was a common factor of these isolates. These authors suggested that cells were using the organic salt anion for energy and dumping the calcium as a waste product. When the calcium exceeded the solubility threshold, precipitation resulted. $CaCO_3$ encrusted cells then served as a nucleation site for further crystal formation. Gradzinski *et al.* (1997) proposed stages in the progressive formation of moonmilk where cells and an organic matrix first provide a structural framework; then, active bacterial cells are calcified and the extracellular organic matrix fills the remaining space with calcite. Although there is no known benefit of $CaCO_3$ precipitation in bacterial metabolism, detoxification of calcium has been suggested (Northup & Lavoie, 2001).

A very interesting cave phenomena usually associated with moonmilk deposits is the white/grey silvered films which are covered in reflective dots and usually occur on walls and ceilings within the Twilight to Dark Zones of limestone caves and lava tube caves (Jones, 1995) Although there are no published works that this author could find, it seems to be widely accepted that the white/grey coating is colonies of actinomycetes. The surface of these colonies is hydrophobic but the filamentous structures are hydrophilic and the droplets are attached to the end of these filaments. Lake & Rowling (pers. comm. J. Rowling, Aristocrat Technologies Australia, 2004) collected some liquid from colonies at Jenolan Caves and investigated the mineral aspects of the liquid, and found that it was almost entirely calcite. These investigators postulated that perhaps these actinomycetes contributed to moonmilk deposits. The ability to form $CaCO_3$ polymorphs seems widely distributed among environmental actinomycetes; 19 out of 31 cave strains isolated and tested by Laiz *et al.* (1999) produced a considerable amounts of crystals in both solid and liquid media.

Microbial precipitation does not explain all forms of moonmilk. It is likely that there may be abiotic forms as well. An extensive survey of moonmilk deposits from high-altitude caves in the Italian Alps revealed no evidence of microbial involvement in calcite precipitation. A review of factors contributing to the formation of moonmilk deposits in these alpine caves includes elevation and temperature, along with surface cover of soils and conifer forests and with low discharge rates of seepage water and high humidity (Borsato *et al.* 2000). However, the majority of samples were from fossil deposits (Borsato *et al.* 2000). Given the wide variability of minerals that may form moomilk it is not surprising that several mechanisms, biotic and abiotic, have been proposed for its formation, one or more of which may be involved in the deposition of moonmilk in a particular form or particular type of cave.

Biotic and abiotic hypotheses for the formation of moonmilk do not need to be mutually exclusive (Northup & Lavoie, 2001). Given the variety of mineral types involved and the range of physicochemical conditions, microbes are clearly involved in the formation of moonmilk by dissolution or by serving as nucleation sites in some cases, but they may play a minor or negligible role in other cases. Friedman & Sanders (1978) noted that "Purely inorganic chemical

reactions can take place only where simple organisms are totally absent. At the surface of the earth, environments devoid of such organisms are uncommon." That same observation is true for subsurface environments. Studies of dissolution and precipitation of carbonates, moonmilk, silicates, clays, iron and manganese oxides, sulfur, and saltpetre in caves span only a few decades. A variety of organisms with biogenic potential have been discovered and some fascinating systems and environments have been described from caves. These studies provide insights into biomineralisation in general, and in the formation of speleothems in particular (Northup & Lavoie, 2001).

1.5 *Significance*

1.5.1 Biodiversity and Conservation Value

Biodiversity is the variety of all life forms: the different plants, animals and microorganisms, their genes and the ecosystems to which they belong. Australia is one of the most biologically diverse countries in the world with a large portion of its species found nowhere else in the world (1/5 of the world's diversity). Biodiversity underpins the processes that make life possible. Healthy ecosystems are necessary for maintaining and regulating atmospheric quality, climate, fresh water, marine productivity, soil formation, cycling of nutrients and waste disposal. Thus we depend on biodiversity for our survival and quality of life.

At the 1992 Earth Summit in Rio de Janeiro, world leaders agreed on a comprehensive strategy for "sustainable development", meeting our needs while ensuring that we leave a healthy and viable world for future generations (Department of Environment and Heritage, Australian Biological Resources Study website; http://www.deh.gov.au/biodiversity/abrs/). One of the key agreements adopted at Rio was the Convention on Biological Diversity setting commitments to sustainable development. The two main goals established by the Convention were the conservation of biodiversity and the sustainable use of its components. The most significant impediment to the conservation and management of biodiversity is our lack of knowledge of it and the effects of human population and activities on it. Accordingly, a taxonomic perspective is necessary to conserve biodiversity and achieve sustainable development.

A taxonomic perspective includes providing underlying taxonomic knowledge of biodiversity and the environmental factors influencing species distribution in microhabitats. Providing baseline information on the composition and distribution of cave microbial communities is essential to aid the conservation of cave microbial communities from human impacts.

1.5.2 *Bioprospecting*

A critical element in drug discovery based on microbial extracts is the isolation of unexploited groups of microorganisms that are at the same time good producers of secondary metabolites. Together with their importance in soil ecology, actinomycetes are best known as a source of antibiotics. This became apparent in 1940, following Selman Waksman's seminal discovery of actinomycin (Waksman & Woodruff, 1940) and was fully realised by the 1980s when actinomycetes accounted for almost 70% of the world's naturally occurring antibiotics (Okami & Hotta, 1988). Actinomycetes, represent an important source of biologically active compounds whose members have unparalleled ability to produce diverse secondary metabolites. These molecules present original and unexpected structure and are selective inhibitors of their molecular targets (Donadio *et al.* 2002). Thus actinomycetes are a group of high economic, social and health significance.

In the past two decades there has been a decline in the discovery of new lead compounds from common soil-derived actinomycetes as culture extracts yield unacceptably high numbers of previously described metabolites (Mincer *et al.* 2002). Natural products continue to be a potent source of novel drugs and other bioactive compounds despite the emergence of combinatorial chemistry. The important attributes of natural products are their molecular diversity, still very much greater than that of combinatorial libraries, and their biological functionality (Nisbet & Moore, 1997). For this reason cultivation of rare or novel actinomycete taxa has become a major focus in the search for the next generation of pharmaceutical agents (Bull *et al.* 2000). The pharmaceutical industry has a strong interest in the acquisition of novel actinomycete biodiversity in the search for new lead compounds. There is strong incentive therefore to discover novel microbes whether it is done by exploiting molecular biology and/or by exploring unusual biotopes (Colquhuon *et al.* 2000). Due to this interest significant biodiversity has been targeted and described from accessible environments. Williams *et al.* (1993) stated that one approach to the isolation of novel actinomycetes is to concentrate on understudied environments or substrates while using appropriate selective isolation techniques or to investigate habitats in which one or more of the environmental factors (eg. temperature,

pH, aeration, or osmotic stress) are extreme. This has lead to the strategic targeting of extreme or unusual ecosystems. The importance of this area of research has been recognised by the international research community, for example by recent EU funding of a new initiative at the University of Newcastle upon Tyne ("New Approaches to the Discovery of Novel Bioactive Compounds from Natural Actinomycete Communities").

Caves are unique ecosystems exposed to extreme environmental stresses. The limiting environmental characteristics of caves, little or no light, low levels of organic nutrients, high mineral concentrations and a stable microclimate, provide ecological niches for highly specialised and very diverse microbiota. Preliminary investigations of microbes isolated from the most remote and least human-impacted regions of Lechuguilla Cave, New Mexico, have highlighted their potential as sources of anti-cancer treatments, because of their ability to kill breast cancer cells (Northup & Mallory, 1998). Novel actinomycetes isolated from caves represent an important, potentially valuable biotechnological resource for the screening and discovery of novel bioactive compounds due to their origin from a unique and as yet poorly studied environment.

1.5.3 Bioremediation

Microbial biodiversity is a reservoir of resources that remains relatively untapped. Microbes are the only life-forms that have been encountered in the deeper regions of the earths crust. Subsurface microbes with novel metabolic properties may be of potential value to industry for applications in bioremediation and biotechnology (eg. Gold, 1992; Boone *et al.* 1995; Stevens & McKinley 1995; Bale *et al.* 1997; Krumholz *et al.* 1997, 1999; Chandler *et al.* 1998; Whitman *et al.* 1998; Kieft *et al.* 1999; Takai & Horikoshi, 1999). In spite of recent findings, many of these microbial habitats remain poorly characterised mainly due to difficulties associated with access and sampling. Caves provide an accessible point of entry to the shallow subsurface.

Throughout the world, organic and inorganic substances leach into the subsurface as a result of human activities and accidents, for example agricultural pesticides, landfill leachate.

There, the chemicals pose direct or indirect threats to the environment and to increasingly scarce drinking water resources. At many contaminated sites the subsurface is able to attenuate pollutants that, potentially, lowers the costs of remediation. Natural attenuation comprises a wide range of processes of which the principle mediators are the microbiological component, which is responsible for intrinsic bioremediation, and can decrease the mass and toxicity of the contaminants by transforming or mineralising pollutants and is, therefore the most important (Christensen *et al.* 2001; Röling & van Verseveld, 2002). Of particular relevance is the ability of subsurface microbes to induce formation of $CaCO_3$ minerals which presents an opportunity to develop and *in situ* bioremediation techniques for groundwater contaminated with divalent metals or radionuclides (Fujita *et al.* 2000). Reliance on intrinsic bioremediation requires methods to monitor the process. Knowledge of the subsurface geology and hydrology, microbial ecology and degradation processes can be used to monitor the potential and capacity for intrinsic bioremediation in the subsurface.

1.5.4 Biodeterioration & Biomineralisation Processes

1.5.4.1 Palaeolithic Frescoes and Rock Art in Hypogean Environments

It is now well recognised that wall paintings can be severely damaged by microbial growth (Ciferri, 1999). It has been reported in the literature that pigment formation, crystal growth and other types of biodeterioration processes related to microbial activity affect rock paintings and frescoes in cave environments. In studies on the bacterial community associated with such deterioration, members of the actinomycetes both previously cultured and novel, are frequently cultivated (Sorlini *et al.* 1987; Weirich, 1989; Petushkova *et al.* 1990; Altenburger *et al.* 1996, 2002; Rölleke *et al.* 1996; Groth *et al.* 1999a; Wieser *et al.* 1999; Gurtner *et al.* 2000; Heyrman & Swings, 2001; Gurtner *et al.* 2001; Heyrman *et al.* 2002). Studies in Altamira and Tito Bustillo Caves, Spain, demonstrate that rock art paintings are coated by dense networks of bacteria, mainly actinomycetes. Identified damage includes: i) covering (scattered coloured spots, whitish powdery patinas, staining) of paintings by the microbial communities themselves and/or by

their metabolic activity (including biofilms and bio-induced precipitates); ii) chemical alteration, such as microbial mediated dissolution; and iii) mechanical alteration, such as rock substrate breakdown.

Bacteria can use organic compounds from the paint layer as growth substrates, producing acids, which cause discolouration of the paint or changes in its consistency. For example, iron-enriched pigments in rock art act as a substrate for attachment and a mineral supply for growth. In favourable conditions the bacteria present can change the colour of the paintings from the reddish yellow hues characteristic of iron pigments to a dark yellowish colour as a result of microbial metabolism. As noted previously in Section 1.4, some cave bacteria may play an important role in the precipitation and/or deposition of $CaCO_3$ speleothems. Many of the actinomycetes isolated from caves are able to precipitate $CaCO_3$ crystals. These bacteria can induce constructive (calcification, crystalline precipitates) and destructive (irregular etching, spiky calcite) fabrics on the paintings and/or surrounding rock. Microbes can penetrate into the painting and its bedrock resulting in mechanical destruction of the cultural heritage (dissolution, etching of the host rock). In a study by Cañaveras *et al.* (1999) a *Streptomyces xanthophaeus* strain isolated from Tito Bustillo Cave walls was inoculated onto stalactite slices which showed pitting formation after only three months of culture in the laboratory illustrating a bacterially mediated calcite dissolution process. Because of this ability it has been proposed that these bacteria and others are directly or indirectly involved in constructive and destructive biomineralisation processes in caves (Laiz *et al.* 1999).

1.5.4.2 Monuments

Interestingly, a group of geomicrobiologists in Spain are following a unique view of biomineralisation processes by suggesting using bacterially induced carbonate mineralisation as a novel and environmentally friendly strategy for conservation of ornamental stone monuments. Increasing environmental pollution in urban areas has been endangering the survival of

carbonate stones in monuments and statuary for many decades. Numerous conservation treatments have been applied for the protection and consolidation of these works of art. Most of them, however, either release dangerous gases during curing or show very little efficacy. There have been a number of studies looking at biomineralisation processes, particularly bio-mediated calcite precipitation, for monumental stone conservation (Di Bonaventura *et al.* 1999; Tiano *et al.* 1999; Urzi *et al.* 1999), for example, *Myxococcus xanthus* –induced $CaCO_3$ precipitation efficiently protects and consolidates porous ornamental limestone. (Rodriguez-Navarro *et al.* 2003). Calcite-precipitating cave isolates have the potential to contribute in this area.

1.5.5 Management Issues

Cave environments are generally quite stable. Diurnal changes have little effect on the cave microclimate. Similarly, seasonal variations in temperature and humidity are relatively minor. Air movement is regulated largely by cave morphology and if present, by the active watercourse. There are low numbers of macroscopic living organisms in caves, mostly insects and spiders. In such a stable environment, microbial growth is the main threat to the preservation of the cave environment. The effects of microbial growth are exacerbated by human impact both on the external cave environment (eg. pollution, changed land use) and by visiting the caves. Visitation produces a more direct and pronounced effect. Visitors produce variations in environmental conditions and increase microbial dispersal and colonisation, Humans can introduce foreign organisms from the surface environment that can establish in caves and they leave behind organic material (lint, hair, skin flakes etc) that provide a rich nutrient source for the proliferation of micro-organisms.

There are implications for Heritage Management in the case of hypogean environments containing Palaeolithic rock art. Pigment formation, crystal growth and other types of biodeterioration processes related to microbial activity affect rock paintings and frescoes in cave environments. These bacteria induce constructive effects such as calcification, crystalline

precipitates, covering) and/or destructive fabrics such as irregular etching, spiky calcite, substrate break-down and dissolution.

There are also implications for cave management issues include the impacts of changes in hydrology, cave sediment contamination on speleothems, and tourist cave lighting upon the natural microbial communities existing within cave microhabitats. Whether microbial communities are actively or passively involved in speleothem formation, disruptions to the natural communities will have an effect on the health and continued formation of speleothems and cave systems.

1.6 Conclusion

Cave environments represent one of few remaining isolated planetary habitats, in terms of human impact and the characterisation of novel microbial diversity. In the past, the study of microbial communities and biogeochemical processes in hypogean environments is mainly related to the fact that microbes affect cultural heritage properties that humans wish to protect and we owe much of our initial knowledge of cave microbiota to these studies. These studies may not necessarily reflect the biodiversity in 'natural' cave systems ie. those that are not heavily impacted by tourism. Compounding this, culture-based studies often have no 16S rRNA gene sequence data for isolates. Most published studies use morphological and biochemical means of identification, rather than phylogeny, to characterise cave strains to the genus level only. Thus it is difficult to make detailed comparisons at the species level between cave environments. Sequence data is available for described novel species from caves, however, little has been published about the cave environments that these novel species were isolated from.

It is widely accepted that only ~ 1 % of microbes are cultured in the laboratory. Culture-independent methods are being increasingly used to describe the composition of microbial communities and reveal significantly broader diversity than culture-based studies. Nevertheless, to date our knowledge of bacterial communities in caves is largely due to culture-based studies. The past decade has seen a rapid increase in published investigations of microbial ecology in caves. However, the diverse range of types of caves (Eg. sulfur caves, carbonate caves, aquatic caves, tourist/show caves, restricted access caves) and microhabitats (Eg. acidic biofilms on walls, filamentous microbial mats in sulfur waters, aquatic microbial mantles, Palaeolithic rock art, cave walls, ferromanganese deposits, sediments) studied and the geographic separation of sites (Romania, Italy, Australia, Mexico, Spain, North America) makes it difficult to draw many comparisons or conclusions about cave microbial diversity (Eg. Sarbu *et al.* 1996; Angert *et al.* 1998; Vlasceanu *et al.* 2000; Holmes *et al.* 2001; Summers-Engel *et al.* 2001; Schabereiter-Gurtner *et al.* 2002, 2004; Northup *et al.* 2003; Chelius & Moore, 2004; Barton *et al.* 2004). Despite this recent expansion of our knowledge, literature on cave microbial communities, their distribution and

taxonomic diversity, is limited and restricted to only a few caves world-wide, predominately in the northern hemisphere. In the southern hemisphere investigations of microbial diversity in caves is represented by only one publication. Holmes *et al.* (2001) investigated microbial diversity in unusual aquatic formations, mantles of mucus and biological material associated with crystalline material in submerged passages in the Nullabor Caves, Australia; a very unique microhabitat thus most likely not representative of general cave microbial biodiversity in the southern hemisphere. Molecular techniques are only recently being applied to geomicrobiological questions in hypogean environments (Eg. ferromanganese residues in Lechuguilla Cave; Northup *et al.* 2003), and as yet there are no published culture-dependent reports of microbial communities associated with moonmilk deposits.

The description of the composition of microbial communities is an important starting point in studies of microbial biodiversity and sets the stage for fundamental studies concerning how these populations function (Morris *et al.* 2002). The microbial diversity of as yet poorly studied environments is being increasingly explored by molecular detection methods (eg. Eppard *et al.* 1996; Rheims *et al.* 1996, 1998; Sarbu *et al.* 1996; Vlasceanu *et al.* 2000; Holmes *et al.* 2001; Summers-Engel *et al.* 2001; Schabereiter-Gurtner *et al.* 2002, 2004; Northup *et al.* 2003). While molecular methods are valuable tools in characterising the microflora, isolation and culturing are still required for describing the microbial diversity, especially in the case of novel taxa (Palleroni, 1997).

SECTION 2:

MICROBIAL BIODIVERSITY IN TASMANIAN CAVES

Chapter 1: Introduction

Some of the deepest, longest and most beautiful caves in Australia are found in Tasmania. Tasmanian caves are of mixed character (wet/muddy vs. dry) and range from commercially used caves to new or unexplored caves. Due to our southerly latitude, the caves in Tasmania are colder and wetter than elsewhere in Australia with temperatures ranging as low as 4-7 °C. An interesting point to note is that there are no bats in Tasmanian caves, probably due to the cool air temperature in these caves. The Ida Bay Karst area is located in southern Tasmania, mostly within the Tasmanian Wilderness World Heritage Area (Figure 1.1). Most of the karst retains native vegetation cover, which is wet sclerophyll forest and rainforest. The Ida Bay Karst developed in Ordovician Gordon limestone from 510 to 439 million years ago (Mya), outcropping between 50 and 300 m above sea level. Cave development is substantial, with more than 140 cave entrances and in excess of 20 km of mapped passage, and predominantly $CaCO_3$ speleothems (Eberhard, 1999). The extensive cave systems in this region have a long and complex history of development, with Cainozoic (65 MYA to present) cold climate change exerting a major influence (Goede, 1968; Kiernan, 1982). Environmental conditions within the cave systems are thought to have changed little since they formed, except for periodic glacial sediment inclusion.

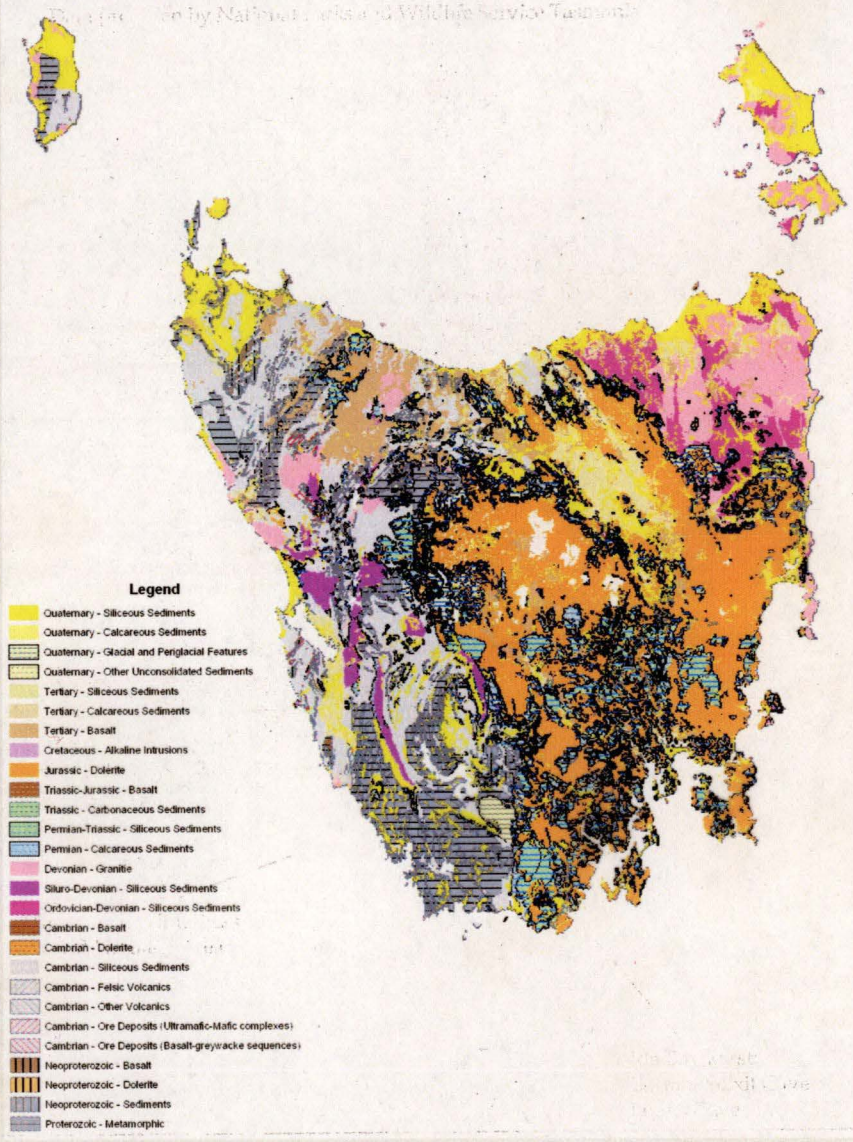
Legend
Quaternary - Siliceous Sediments
Quaternary - Calcareous Sediments
Quaternary - Glacial and Periglacial Features
Quaternary - Other Unconsolidated Sediments
Tertiary - Siliceous Sediments
Tertiary - Calcareous Sediments
Tertiary - Basalt
Cretaceous - Alkaline Intrusions
Jurassic - Dolerite
Triassic-Jurassic - Basalt
Triassic - Carbonaceous Sediments
Permian-Triassic - Siliceous Sediments
Permian - Calcareous Sediments
Devonian - Granitic
Siluro-Devonian - Siliceous Sediments
Ordovician-Devonian - Siliceous Sediments
Cambrian - Basalt
Cambrian - Dolerite
Cambrian - Siliceous Sediments
Cambrian - Felsic Volcanics
Cambrian - Other Volcanics
Cambrian - Ore Deposits (Ultramafic-Mafic complexes)
Cambrian - Ore Deposits (Basalt-greywacke sequences)
Neoproterozoic - Basalt
Neoproterozoic - Dolerite
Neoproterozoic - Sediments
Proterozoic - Metamorphic

Figure 1.1: Map of Tasmania depicting the World Heritage Area and Ida Bay karst region. Entrance-Exit Cave system and Loons Cave are located in the Ida Bay karst. Overlay detailing geology of Tasmania, including calcerous sediments of the Ida Bay karst region.

Data provided by National Parks and Wildlife Service Tasmania.

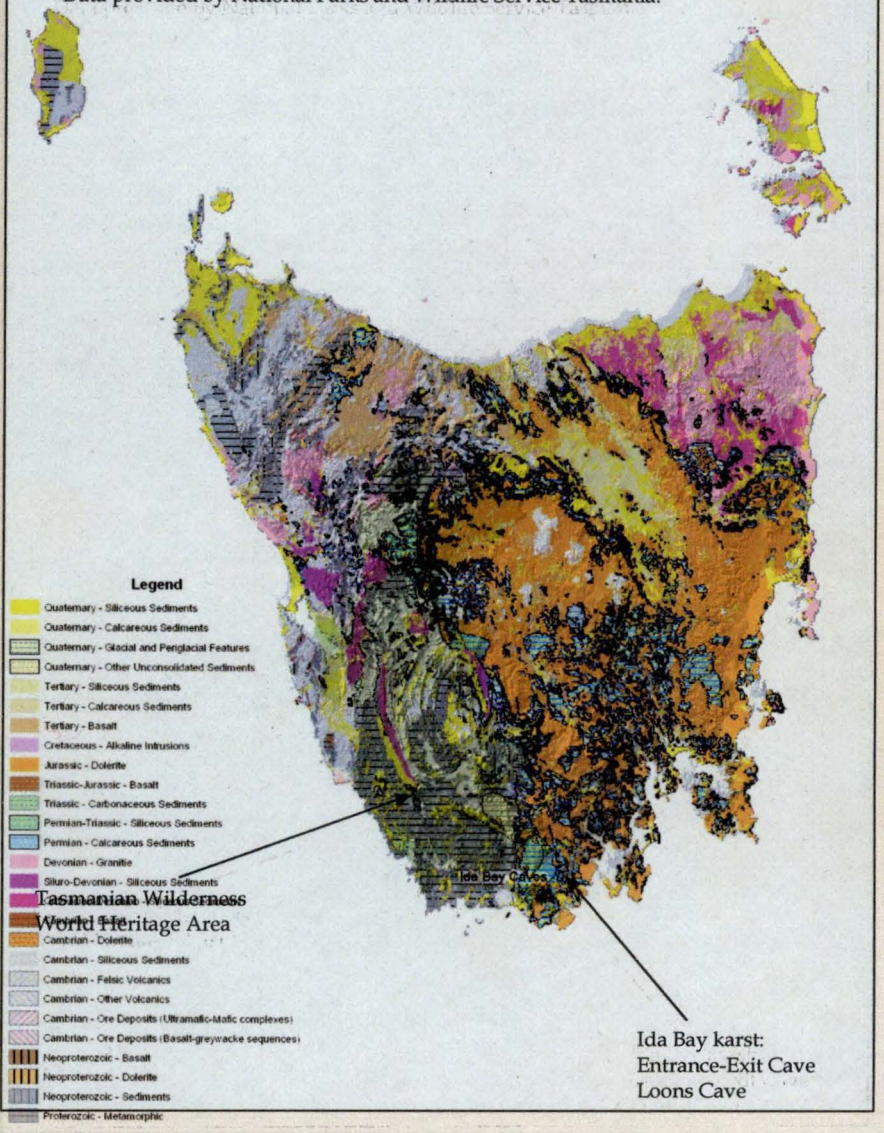

Figure 1.1: Map of Tasmania depicting the World Heritage Area and Ida Bay karst region. Entrance-Exit Cave system and Loons Cave are located in the Ida Bay karst. Overlay detailing geology of Tasmania, including calcerous sediments of the Ida Bay karst region.

Data provided by National Parks and Wildlife Service Tasmania.

The biological importance of the Ida Bay caves has been recognised for more than 100 years, beginning with an article published in *Scientific American* describing the spectacular glow worm display in Entrance Cave (Anon. 1895 in Eberhard, 1999). Over the years, many rare and endemic obligate cave fauna have been discovered and described from Ida Bay caves resulting in this region being widely recognised as containing one of the more diverse and significant assemblages of cave fauna in Australia's temperate zone (Richards & Ollier, 1976).

The Entrance-Exit Cave System is a site of high biological significance, the most outstanding biological feature of the caves being the glow worm display. The Entrance Cave subsystem, in particular, is the type locality for many obligate cave dwelling fauna (Richards & Ollier, 1976). Near the entrance and extending for some distance into Entrance Cave there is a very significant cave fill deposit consisting of a conglomerate of rounded boulders and pebbles set in a fine matrix and thoroughly indurated. This deposit has not been studied in detail but an intelligent guess is that it is sediment of glacial times, when solifluction was prevalent but running water was much reduced (Richards & Ollier, 1976). The significant feature is that this deposit extends to roof level which possibly means it blocked the cave completely at one stage allowing 'evolution' in isolation and has since been largely removed by subsequent stream action (Richards & Ollier, 1976).

Loons Cave, although very close in proximity, is a very different system to the Entrance-Exit Cave System. Loons Cave essentially consists of a single, narrow, low energy stream passage that appears to be fed primarily by waters of seepage origin not a streamway originating from the surface (Household & Spate, 1990). The cave is reasonably well decorated with speleothems that are generally massive and robust. Loons Cave is commonly used as an "outdoor experience" locality for school and recreational groups and is therefore a site of high human impact in contrast to the majority of the Entrance – Exit Cave system.

Deposits of moonmilk are a common feature of many Tasmanian caves (Goede, 1988). Despite their abundance they are amongst the least studied and understood of any of the cave

deposits. Very large moonmilk deposits are evident in Exit Cave occurring as a uniform or botryoidal layer that covers stalactites, cave walls, ceilings and floors. Entrance Cave is also known to have moonmilk deposits although on a much smaller scale than Exit Cave. Within the Entrance Chamber of Entrance Cave, just beyond the cave mouth, large white mats with silvered droplets (similar to those described in Section 1.4.3) are visible on the ceiling rock. These have been anecdotally described as being actinomycete colonies by enthusiastic cavers, though this has previously not been investigated. The white mats are visible past the Twilight Zone of the cave and in the Dark Zone, however not to the same extent. Although there are no moonmilk speleothems in Entrance Cave *per se*, it was discovered during the course of this study that there are large deposits of moonmilk beneath the sediment throughout the cave.

The focus of this research was the characterisation of microbial biodiversity from Tasmanian caves (Entrance-Exit Cave system and Loons Cave) in 3 microhabitats; sediments, speleothems and moonmilk deposits. Isolation of pure cultures reveals only a minor fraction (~ 1%) of the actual biodiversity in an environment. Culture-independent 16S rRNA gene sequence analyses have opened the way to study bacterial communities in environmental samples without prior cultivation and reveal a significantly broader diversity than culture-based studies (Amann *et al.* 1995; Head *et al.* 1998; Hugenholtz *et al.* 1998). Bacterial diversity in Tasmanian caves have not been investigated using culture-independent techniques and to date there is no published culture-independent study on moonmilk worldwide. Thus classical isolation and molecular detection methods (DGGE, 16S rRNA gene clone library analysis) were used to compare culturable vs. non-culturable biodiversity, particularly of the actinomycetes who appear to dominate isolations from culture–based studies of heterotrophic cave systems. To expand our knowledge of cave microbial diversity, phylogenetic analysis was used to determine diversity at the species level and to infer ecological function where possible. The biodiversity described acts as a baseline for assessing environmental impacts and also identifying factors influencing microbial diversity.

Chapter 2: Materials and Methods

2.1 Site description and sample collection

2.1.1 Entrance-Exit Cave System

The Entrance Cave subsystem has a simple cave opening where Mystery Creek goes underground and is located approximately 2 km from Ida Bay, along the South Lune Road. The cave follows the course of Mystery Creek entering the north side of Marble Hill, also known as Caves Hill, at an elevation of 115 m (Richards and Ollier, 1976). The cave floor is a riverbed, covered with large boulders and cobbles. Water and nutrients are contributed to the lower level passages by the active inflow stream Mystery Creek, whereas the upper level passages are dry. Mystery Creek re-emerges via a non-negotiable route into Exit Cave and a subterranean section of the D'Entrecasteaux River draining out of the south side of Marble Hill. Exit Cave is the longest cave in Australia with greater than 15 km of passages, generally large sized. Mystery Creek is also an important inflow stream to the Exit Cave subsystem, contributing water and nutrients. There are also many smaller feeder passages in the Exit Cave subsystem with low energy streams and more than 100 other known caves in the Ida Bay karst that are predominantly vertical shaft caves on the slopes of Marble Hill and connect with the Exit Cave subsystem at depth.

2.1.2 Loons Cave

Loons Cave essentially consists of a single, narrow, low energy stream passage that appears to be fed primarily by waters of seepage origin (Household & Spate, 1990). The natural, undisturbed substrate in this stream consists of a lightly cemented veneer of pebbles overlying a deep unconsolidated mass of fine clay sediment. The effect of repeated trampling on this sensitive veneer has caused its breakage and collapse into the underlying soft sediments,

resulting in the formation of deep muddy pools. Parts of the stream substrate remain in original, pristine condition where it crosses underneath sections of passage inaccessible to people.

2.1.3 Sample Collection

Samples were collected from Entrance, Exit and Loons caves, concentrating on three microhabitats; floor sediments, speleothems and moonmilk deposits. Sites were chosen with minimal contamination factors and to reduce impact of our sampling to a minimum. Samples were collected to the side of the main paths to avoid contamination from trampling of cavers and 'clean' speleothems and moonmilk were chosen with no visible human impact or handling (e.g. mud smears, hand prints etc.). Samples were collected under the provisions of permit number ES 01147 issued by National Parks and Wildlife Service, Tasmania.

Sediment sampling consisted of collecting approximately 10 g/sample using a sterile teaspoon and placing into individual sterile plastic bags. Sterile swabs (EUROTUBO® Collection Swabs; I.A.S.A) moistened with sterile double distilled water (ddH_2O) were used to sample from speleothems. To collect moonmilk deposits, ME1 and 3, in Entrance Cave, the upper layer of sediment was scraped away with a sterile teaspoon and sterile 15 mL falcon tubes (REDLINE Scientific Pty. Ltd.) were inserted into the deposit. The tubes were withdrawn from the deposit approximately half full and capped immediately. Similarly, samples were collected from moonmilk speleothems in Exit Cave, MX1, by inserting sterile 15 mL falcon tubes into the formation till they hit the 'hard' speleothem surface, withdrawing and capping immediately. Samples of the white mat, ME2, in Entrance Cave were collected by inserting glass slides between the mat and mud or substrate rock. The slides were placed on wet tissue paper within closed petri dishes to keep them hydrated. Samples were transported to the laboratory on ice and stored at 4 °C until processed. Sample locations and descriptions are listed in Table 2.1.

Table 2.1: Identity, location and description of samples collected from Entrance, Exit and Loons Caves

Sample*	Cave and sample location	Description
SE1	Entrance Cave, Big Stalagmite Cavern; Dark Zone	Dry sediment from indentation, 1.2 ms above floor, Big StalStalagmite.
SE2	""	Wet sediment from front drainage region of flow form by Big Stalagmite
SL1	Loons Cave, "Tarpit", Dark Zone	Dry sediment from left hand sidewall deposit above flood zone.
SL2	""	Wet sediment from bottom of 1 m deep permanent mudhole.
SPE3	Entrance, Big Stalagmite; Dark Zone	Swab, droplet on shelf roof, right hand side of passage.
SPE5	""	Swab, wet flow form, right corner of passage entry.
SPE7	""	Swab, Big Stalagmite, dry surface, 1.5 m above floor.
SPE10	Entrance, Big Flow form; Twilight Zone	Swab, moonmilk mat on cave roof.
SPE12	Entrance, left hand platform; Twilight Zone	Swab, old dry flow form.
SPL2	Loons, First Aven; Dark Zone	Swab, large drip stone under aven.
SPL3	Loons, dry platform past first Aven; Dark Zone	Swab, red droplet on fungal mycelia.
SPL6	Loons, Lower entrance crawl; Twilight Zone	Swab, sloping surface among small stalagmites.
SPL8	Loons, Sump; Dark Zone	Swab, cream flowstone surface.
SPL9	""	Swab, carrot stalactite.
SPL12	""	Swab, cream flowstone pools.
ME1	Entrance Cave, Cave Mouth; Light Zone	Moonmilk beneath sediment of boulder
ME2	Entrance Cave, Entrance Chamber; Twilight Zone	White mat on ceiling mud and rock
ME3	Entrance Cave, Second Chamber; Dark Zone	Moonmilk beneath sediment cave floor
MX1	Exit Cave, Ballroom Chamber; Dark Zone	Stalactite with thick coating of moonmilk

* Samples catalogued using the following code: the first character(s) represent the microhabitat (S = sediment, SP = speleothem, M = moonmilk), the last character represents the cave (E = Entrance, X = Exit, L = Loons). Number is indicative of the site that samples were collected from. All samples collected by Jodie van de Kamp and Dr David Nichols, with base support from Dr. Kevin Sanderson during 2001 and 2002 Permit number ES 01147 issued by National Parks and Wildlife Service, Tasmania.

2.2 *Microscopy and Mineralogy*

2.2.1 *ESEM and X-Ray Elemental Microanalysis*

ESEM was used to visualise microbes within the moonmilk matrix. Fresh, unfixed samples were viewed by ESEM approximately 4 h after collection. Small pieces of moonmilk were removed from the glass slides or falcon tubes using a sterile scalpel blade and placed on aluminium SEM stubs for viewing by ESEM 2020 (Phillips, Australia). The elemental composition of specimens was obtained by means of X-Ray Microanalysis (pers. comm. David Steele, University of Tasmania, 2002).

2.2.2 *X-Ray Diffraction Analysis*

Mineralogical compositions of moonmilk were determined by X-Ray Diffraction (XRD) Analysis. Moonmilk samples were prepared by drying, grinding to <~10-75 μm and pressing into a 25 mm diameter aluminium sample holder. The samples were run on an automated Philips X-Ray Diffractometer system: PW 1729 generator, PW 1050 goniometer, PW 1710 microprocessor, with nickel-filtered copper radiation at 40 kV/30 mA, a graphite PW 1752 monochromator, sample spinner and a PW 1711 sealed gas filled proportional detector. The PW 1710 system is driven by software packages, "Visual XRD v 2.6" (Diffraction Technology, Australia) and "PW 1710 for Windows" (CSIRO, Australia), with plotting software, "XPLOT for Windows" (CSIRO, Australia) and "Traces v 5.1" (Diffraction Technology, Australia). Interpretation was mostly by manual methods. Samples were calibrated with an internal standard of natural quartz. The semi-quantitative mineralogy was determined by manual search-match methods using a series of prepared standards (pers. comm. Ralph Bottril, Mineral Resources Tasmania, 2003).

2.3 *Isolation and Identification of Microbes*

2.3.1 *Isolation and culturing of microbes*

Microbes were isolated from sediments using selective isolation procedures developed by the Antarctic Microbiology Group (University of Tasmania). Approximately 5 g of sediment was transferred into a sterile petri dish and left open in a laminar flow (Gelman Sciences, Australia) overnight to dry. Sediments were ground to an even consistency using a sterile mortar and pestle and then divided into two equal portions by weight; one untreated control sample (overnight drying and incubation at room temperature for 2 h; OD) and one treated sample (overnight drying and subjected to a heat treatment of 70 °C for 2 h; ODΔ). Samples were transferred to individual McCartney bottles containing 9 mL of sterile dd H_20 and placed on a tube roller (Luckham Ltd.) for 30 min to mix.

Microbes were isolated from moonmilk using a modified version of an isolation procedure developed by Olivier Braissant (pers. comm. Université de Neuchâtel, Germany, 2002). Similarly to sediments, up to 5 g of moonmilk (moonmilk being very light in comparison to sediments) was weighed into petri dishes, dried overnight, ground, and divided into four equal portions by weight. Samples were subjected to one of four different treatments by transferring to individual McCartney bottles containing either: 1) 5% acetic acid (CH_3COOH) in 0.01 M $MgSO_4.7H_2O$; 2) 1% acetic acid in 0.01M $MgSO_4.7H_2O$; 3) 1 mM Ethylenediaminetetraacetic Acid (EDTA); or 4) 0.1 mM EDTA. Samples were then placed on a tube roller for 30 min to mix.

Dilution series to 10^{-3} were prepared for sediment and moonmilk samples (initial bottle 10^0) and 0.1 mL of each dilution spread plated in duplicate on selective media that favours the growth of actinomycetes; Starch-Casein Agar (SC) (Kuster & Williams, 1964), Arginine-Vitamin Agar (AV) (Nonomura & Ohara, 1969), Marine Agar (MA) (Oxoid 2216) and R2A Agar (R2A) (Oxoid CM 906) and non-selective agar for moonmilk samples only; 1/2 strength Tryptone Soya Agar (1/2 TSA) (Oxoid CM 129) (see Appendix 1 for culture media recipes and preparation). Swab samples were directly streaked onto the above selective media immediately on return

from sampling trips. Plates were left to dry in a laminar flow for 30 min. Plates were sealed with O_2 permeable parafilm (American National Can™, USA) and duplicates incubated at 25 °C (within optimal temperature range for isolation of actinomycete) and 10 °C (representing the cave environment) for 2–4 wk or until there was sufficient growth of colonies. After incubation, actinomycete-like colonies were selected from the primary plates and sub-cultured on Oatmeal Agar (OA) (Williams & Wellington, 1982) (see Appendix 1). For moonmilk samples, non-actinomycete-like colonies were also selected and subcultured on 1/2 TSA. Secondary plates were incubated at 25 °C for approx. 1 wk. All further sub-culturing was conducted as described until pure isolates were obtained. Isolates were cryopreserved (see Appendix 2 for protocol) in replicate for long-term preservation and future use.

2.3.2 *16S rRNA gene sequencing and phylogenetic analysis of isolates*

2.3.2.1 *Extraction of nucleic acids and purification*

Genomic DNA was extracted using a method modified from Marmur (1961). Culture biomass was harvested by scraping with a sterile loop. Cells were resuspended in sterile 1.5 mL microcentrifuge tubes (Eppendorf; Greiner Bio-one) with 400 µL saline-EDTA (pH 8) and vortexed (MT 17 Vortex; CHILTERN) to mix. 50 µL of lysozyme (40 mg mL^{-1}; AMRESCO) was added and the tubes incubated for 30 min at 55 °C in a M20 waterbath (LAUDA). 20 µL of proteinase K (10 mg mL^{-1}; SIGMA) was added and the tubes again incubated for 15 min at 55 °C and 20 µL of 25% (w/v) sodium dodecylsulphate (SDS) (SIGMA) for a further 30 min at 55 °C. Tubes were mixed by vortexing between each incubation step. Samples were then subjected to a freeze/thaw step by incubation at –20 °C overnight and thawing at 55 °C for 30 min. Cell debris was separated from aqueous DNA solution by centrifugation at 14000 rpm x 5 min, 4 °C in a bench top Eppendorf Centrifuge 5417 R (Laboratory Supply Australia Pty. Ltd.). The supernatant (approx. 400 µL) was transferred to a new sterile microcentrifuge tube. DNA was extracted twice by adding an equal volume of 25:1 (vol/vol) chloroform-isoamyl alcohol

(SIGMA), followed by vortexing and centrifugation at 14000 rpm x 10 min, 4 °C. The aqueous phase was transferred to a new, sterile microcentrifuge tube each time. DNA was further purified using the Prep-a-Gene® DNA Purification Kit (Bio-Rad) reagents and protocol. DNA products were stored at -20 °C.

2.3.2.2 Agarose gel electrophoresis

To analyse extracted nucleic acids they were fractionated by electrophoresis through 1.0-1.5% (w/v) agarose (AMRESCO) gels with 0.5 μg/mL ethidium bromide (EtBr) in Tris-acetate EDTA buffer (40 mM Tris-acetate; 1 mM disodium EDTA; pH 8) (TAE), in a mini-gel apparatus (Horizon 58, Horizontal Gel Electrophoresis, BRL). 5 μL of DNA product was mixed with 3 μL of 6x gel loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol FF; 40% sucrose) and loaded into the gel. To determine the size of nucleic acid fragments, samples were run alongside 5 μL of the DNA molecular weight marker HyperLadder I (Bioline). Electrophoresis was carried out using a Power Pack 300 power supply (Bio-Rad) at 80 V for 30 min. The DNA/EtBr complex was visualised under short wavelength ultra-violet radiation on an electronic ultraviolet light transilluminator (Ultra. Lum. Inc.).

2.3.2.3 Determination of DNA concentration

The concentration of DNA and PCR solutions (DNA_{conc}) was determined by measuring absorbance at 260 nm using a spectrophotometer (Pharmica) and calculated using the following equation:

$$DNA_{conc} (mg\ mL^{-1} = \mu g\ \mu L^{-1}) = (A_{260} \times 50\ \mu g\ mL^{-1} \times D) / 1000\ \mu g\ mL^{-1}$$

Where D = dilution factor

2.3.2.2 16S rRNA gene PCR amplification and purification

The 16S rRNA gene fragment was amplified by Polymerase Chain Reaction (PCR) from extracted genomic nucleic acids using two universal primers, 10 forward and 1500 reverse (Stackebrandt *et al.* 1991) (Table 2.2). These primers were used as they gave thorough coverage of the three hypervariable regions in the 16S rRNA gene fragment (Stackebrandt *et al.* 1991). PCR was performed using the HotStarTaq™ PCR Master Mix Kit (QIAGEN) reagents and protocol. PCR reactions consisted of:

HotStarTaq Master Mix	25 µL
Primer 5′ (50 pmol)	2 µL
Primer 3′ (50 pmol)	2 µL
Q-Solution*	2.5 µL
Template DNA^	2 µL
ddH_2O to total volume	50 µL

* Q-Solution changes the melting behaviour of DNA and was used for PCR reactions that did not work well under standard conditions.
^ Amount of template DNA added to PCR mix varied depending on the concentration of the DNA, however in most cases 2 µL was sufficient.

PCR reactions were carried out in a PTC – 200 Peltier Thermal Cycler (MJ Research) using the following parameters:

Initial activation step:	15 min	95 °C
3-step cycling:		
Denaturation:	1 min	94 °C
Annealing:	1 min	52 °C
Extension:	3 min	72 °C
Number of cycles:	30	
Final extension:	10 min	72 °C*

*The final extension step is prolonged to 10 min to allow full extension of any partly amplified DNA fractions.

PCR fragments were purified using the Prep-a-Gene® DNA Purification Kit (Bio-Rad) reagents and protocol. PCR products were electrophoresed as described previously to ensure fragments of the correct size were obtained, and to determine quantity and quality. PCR products were stored at -20 °C.

2.3.2.3 16S rRNA gene sequencing

PCR products were sequenced directly using the CEQ 2000 Dye Terminator Cycle Sequencing (DTSC) Quick Start Kit (Beckman Coulter) reagents and modified protocol. For initial identification of microbes universal primer 519 forward (Stackebrandt *et al.* 1991) (Table 2.2) was used for amplification. To obtain full sequence information of selected isolates, universal primers 10 forward and 1500 reverse were also used. Sequence reactions consisted of:

DTCS Quick Start Master Mix	2 µL
Primer (5 pmol)	1 µL
Template PCR*	X µL
ddH_2O to total volume	10 µL

*According to Template Preparation Table in CEQ 2000 DTSC protocol.

Amplification parameters were:

Denaturation:	96 °C	20 sec
Annealing:	50 °C	20 sec
Extension:	60 °C	4 min
Number of cycles:	35	

Amplification reactions were purified by ethanol (EtOH) precipitation according to the CEQ 2000 DTCS protocol. Subsequent electrophoresis and analysis was performed using an automated CEQ™ 2000XL Genetic Analysis System (Beckman Coulter). In most cases, 16S rRNA gene fragment sequences spanned nucleotide positions 519–1540 (*E.coli* equivalent). Entire 16S rRNA gene sequences spanning nucleotide positions 10-1540 were obtained for novel isolates.

Table 2.2: Primers used for PCR amplification and sequencing of 16S rRNA gene fragments.

Primer (Reference)	Binding Region*	Primer Sequence (5' to 3')
10 (f) (Stackebrandt *et al.* 1991).	10-29	GAG TTT GAT CCT GGC TCA G
1500 (r) (Stackebrandt *et al.* 1991).	1520-1540	AGA AAG GAG GTG ATC CAG CC
519 (f) (Stackebrandt *et al.* 1991).	519-536	CAG CMG CCG CGG TAA TAC
1392 (r) with GC clamp (Ferris *et al.* 1996)	1406-1392	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAC GGG CGG TGT GTA C
907 (f) (Santegoeds *et al.* 1998)	907-926	GGC AGT TAA GGA AAC TCA AA
pUC/M13 (f) (Promega)	N/A	GTA AAA CGA CGG CCA GT
pUC/M13 (r) (Promega)	N/A	CAG GAA ACA GCT ATG AC

* Number is based on the *Escherichia coli* numbering system from Brosius *et al.* 1981

2.3.2.4 *Phylogenetic Analysis*

Sequence electrophoretograms were examined using the program CHROMAS (http://www.technelysium.com.au/chromas.html) in order to resolve any ambiguous base positions. 16S rRNA gene sequences were initially analysed using the National Center for Biotechnology Information (NCBI) database, Genbank, BLAST tool (http://www.ncbi.nlm.nih.gov/blast/blast.cgi; Altschul *et al.* 1997) to identify related sequences available in public databases and to determine phylogenetic groupings of sequences. For phylogenetic analysis, sequences were aligned using the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; Hall, 2001) for comparison with validly described and published sequences of representative members of the actinomycete obtained from NCBI GenBank. Distance matrices and phylogenetic dendrograms using the neighbour-joining method were generated using programs DNADIST and NEIGHBOUR of the PHYLIP 3.573c package (Felsenstein, 1993).

2.4 *Molecular Analysis of Sediments and Moonmilk*

2.4.1 *Extraction and purification of nucleic acids from environmental samples*

2.4.1.1 *Sediments*

Total nucleic acids was extracted from sediment following a method modified from Purdy *et al.* (1996). Approximately 0.5 g of sediment was aseptically transferred to a 2 mL screw-cap microcentrifuge tube (Astral Scientific) containing 0.5 g of 0.1 mm diameter zirconia-silica beads (Biospec Products) and suspended in 700 µL 120 mM sodium phosphate buffer (pH 8.0) (Na_2HPO_4), 500 µL Tris-equilibrated phenol (pH 8.0) (AMRESCO), 50 µL 20% (w/v) SDS and 1 % acid-washed polyvinyl-polypyrrolidone (PVP) (AMRESCO). To lyse the soil microbes, the sample was disrupted in a mini-beadbeater (Biospec Products) at 3 800 rpm for 3 x 30 sec pulses, with a 30 sec incubation on ice between pulses. Cell debris was separated from aqueous DNA solution by centrifugation at 12000 rpm x 2 min, 4 °C. The supernatant was transferred to a new sterile microcentrifuge tube and incubated on ice. The pellet was resuspended in 700 µL 120 mM Na_2HPO_4 Buffer (pH 8.0) to extract residual nucleic acids from the sample. Cell disruption and centrifugation was repeated as described and the supernatant removed and pooled with the first extraction. Nucleic acids were precipitated by adding 0.1 volumes of 3M sodium acetate (pH 4.6) (NaOAc) and 2 volumes of cold absolute EtOH followed by incubation at –20 °C for at least 30 min, preferably overnight. The supernatant was removed after centrifugation at 14 000 rpm x 30 min, 4 °C. The DNA pellet was washed twice in 300 µL cold 70% EtOH, with further centrifugation at 14 000 rpm x 5 min, 4 °C. After removing the supernatant the pellet was allowed to air dry in a laminar flow hood and subsequently resuspended in 40 µL sterile ddH_2O.

2.4.1.2 *Moonmilk*

Nucleic acids were extracted by a procedure developed for this study, modified from Miller *et al.* (1999); the Phosphate, SDS, Chloroform-Bead Beater method (PSC-B) (pers. comm. Susan Turner, University of Auckland, New Zealand, 2003). Approximately 0.5 g of moonmilk was aseptically transferred to a 2 mL screw-cap microcentrifuge tube containing 0.5 g of 0.1 mm

diameter zirconia-silica beads (Biospec Products) and 300 µL 100 mM Na_2HPO_4 (pH 8.0) and resuspended by vortexing. 30 µL lysozyme (50 mg mL^{-1}) was added and the tubes incubated at 37 °C for 30 min, followed by a further incubation at 65 °C for 60 min to enhance lysis of the cells. Following incubation, 300 µL of SDS lysis buffer (100 mM NaCl, 500 mM Tris pH8, 10% SDS) was added and the tubes inverted to mix, followed by adding 300 µL chloroform-isoamyl alcohol (24:1; v/v) (SIGMA). Samples were mechanically lysed by bead-beating at 4 000 rpm for 2 x 40 sec pulses, with a 40 sec incubation on ice between pulses. Cell debris was pelleted from aqueous DNA solution by centrifugation at 12000 rpm x 5 min, 4 °C. The supernatant, approximately 650 µL, was transferred to a new sterile microcentrifuge tube with 360 µL 7M ammonium acetate (NH_4OAc). Tubes were inverted to mix and centrifuged at 12000 rpm x 5 min, 4 °C to separate the phases. The clear supernatant (approximately 580 µL) was transferred to a new sterile microcentrifuge tube and the lower organic phase, with the SDS forming a gel-like substance, discarded. 0.54 volumes (approx. 315 µL) of isopropanol (SIGMA) was added and the tubes incubated at room temperature for 15 min. After incubation tubes were centrifuged at 12000 rpm x 5 min, 4 °C to pellet the DNA. The supernatant was discarded and the pellet washed twice with 1 mL 70% EtOH, centrifugation at 12000 rpm x 5 min, 4 °C, and the supernatant discarded. The pellet was allowed to air dry in a laminar flow hood before resuspending in 50 µL sterile ddH_2O. Additional purification of sediment and moonmilk DNA samples was performed using the CHROMA SPIN™ Columns DNA Purification Kit (CLONTECH Laboratories Inc.) reagents and protocol. DNA quality and quantity was analysed as described in Section 2.3.2.3.

2.4.2 DGGE

DGGE was conducted on four sediment samples and three moonmilk samples (SE1, SE2, SL1 and SL2; ME2, ME3 and MX1; refer to Table 2.1) in accordance with a protocol developed by Powell *et al.* (2003). A standard control mix consisting of 5 ng μL^{-1} each of genomic DNA extracts from four strains grown routinely in our laboratory and chosen because they denatured at a range of different denaturant concentrations was also used as a control and for comparisons between gels. The 16S rRNA gene fragment was amplified by PCR using the Advantage® 2 Polymerase Mix (CLONTECH Laboratories Inc.) reagents and protocol with Universal primers 907 forward (Santegoeds *et al.* 1998) and 1392 reverse (Ferris *et al.* 1996) with a GC clamp (Ferris *et al.* 1996).

Reactions consisted of:

10 x Buffer	5 µL
50 x dNTP Mix (10 mM each)	1 µL
Primer 5′ (10 pmol)	1 µL
Primer 3′ (10 pmol)	1 µL
50 x Advantage 2 Polymerase Mix	1 µL
Template DNA^	1 µL
ddH_2O to total volume	50 µL

^ Amount of template DNA added to PCR mix varied depending on the concentration of the DNA, however in most cases 1 µL was sufficient.

The touchdown thermal cycling parameters were:

Initial denaturation step:	5 min	94 °C
1st 3-step cycling:		
Denaturation:	1 min	94 °C
Annealing:	1 min	65 °C
(decreasing by 1 °C each cycle)		
Extension:	3 min	72 °C
Number of cycles:	10	
2nd 3-step cycling:		
Denaturation:	1 min	94 °C
Annealing:	1 min	55 °C
Extension:	2 min	72 °C
Number of cycles:	20	
Final extension:	4 min	72 °C*

*The final extension step is prolonged to 4 min to allow full extension of any partly amplified DNA fractions.

DGGE was conducted using a D-Code Universal Mutation Detection System (Bio-Rad). Half the volume of PCR products were run on 6% (w/v) acrylamide gels with a denaturing gradient of 20-80% (where 100% dentaurant is 7 M urea and 40% formamide). Gels were run at 80 V for 16 h at 60 °C in 1 x TAE (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA). Standards were run on either side of the gel and the outside lanes were not used. In order to obtain even heat distribution throughout the tank, the entire tank was placed on a magnetic stirring plate. Gels were stained in 1:1000 Sybergold (Molecular Probes) in the dark with gentle shaking for approximately 20 min. Gels were washed once with deionised H_2O and destained with deionised H_2O for 20 min before viewing on a UV transilluminator (UVP Inc.). Single bands were excised from the gel using a sterile scalpel blade and resuspended in ddH_2O in sterile microcentrifuge tube for 16S rRNA gene sequence analysis. Gel photos were scanned in and viewed with the UTHSCSA ImageTool program, developed at the Health Science Centre (University of Texas, San Antonio, TX, USA) and available on the internet (ftp://maxrad6.uthscsa.edu). Best banding patterns were obtained by enhancing the contrast and greyscale of the images. The 16S rRNA gene fragment was amplified and purified from

eluted bands as previously described in Section 2.3.2.4 using the HotStarTaq™ PCR Master Mix Kit (QIAGEN) reagents and protocol with the exception that DGGE primers 907 (f) and 1392 (r) with a GC clamp were used, and 1 µL of the eluted DGGE band was directly added to the PCR mix. DGGE PCR products were directly sequenced as described in Section 2.3.2.4 using DGGE primer 907 (f) and subjected to phylogenetic analysis as described in Section 2.3.2.5.

2.4.3 Clone Library Analysis

2.4.3.1 16S rRNA gene PCR amplification, ligation and clone library construction.

Clone libraries were generated from four sediment samples and three moonmilk samples (SE1, SE2, SL1 and SL2; ME2, ME3 and MX1; refer to Table 2.1). The 16S rRNA gene fragment was amplified as described for DGGE analysis (Section 2.4.2) with universal primers, 519 forward and 1500 reverse (Stackebrandt *et al.* 1991) (Table 2.2). Reactions consisted of:

10 x Buffer	5 µL
50 x dNTP Mix (10 mM each)	1 µL
Primer 5′ (50 pmol)	1 µL
Primer 3′ (50 pmol)	1 µL
50 x Advantage 2 Polymerase Mix	1 µL
Template DNA^	1 µL
ddH_2O to total volume	50 µL

^ Amount of template DNA added to PCR mix varied depending on the concentration of the DNA, however in most cases 1 µL was sufficient.

Thermal cycling parameters were:

Initial denaturation step:	15 min	95 °C
3-step cycling:		
Denaturation:	1 min	94 °C
Annealing:	1 min	50 °C
Extension:	1 min	72 °C
Number of cycles:	30	
Final extension:	5 min	72 °C*

*The final extension step is prolonged to 5 min to allow full extension of any partly amplified DNA fractions.

PCR fragments were purified using the UltraClean™ PCR Clean-up DNA Purification Kit (MoBio Laboratories Inc.) and analysed for size and concentration as described in Section 2.3.2.3.

16S rRNA gene PCR fragments were ligated using the pGEM® -T Easy Vector System I Kit (Promega) reagents and protocol. Ligation reactions were subjected to an overnight incubation at 4 °C to produce the maximum number of transformants. Transformation of ligation products was performed using the Epicurian Coli® XL2-Blue Ultracompetent Cells (Stratagene) reagents and protocol. Transformants were screened using blue-white colony colour selection. Aliquots (50 µL and 100 µL) of the transformation mixture were plated on Luria Broth agar plates containing 100 µg mL^{-1} ampicillin (SIGMA) (LB-Amp) and coated with 100 µL 0.1 M iso-propyl-beta-D-thio-galactopyranoside (120 mg mL^{-1}) (IPTG) (SIGMA) and 20 µL 5-bromo-4-chloro-3-indoyl-beta-D-thio-galactopyranoside (50 mg mL^{-1}) (X-gal) (SIGMA) (see Appendix 1). Plates were incubated overnight for 16-20 h at 37 °C. Colonies containing recombinant plasmids with the 16S rRNA gene fragment appear white, whereas colonies containing un-recombinant colonies appear blue. Approximately 150 white colonies from each library were sub-cultured to LB-Amp plates and re-incubated overnight at 37 °C.

2.4.3.2 Restriction Fragment Length Polymorphism screening and 16S rRNA gene sequencing of clones

Recombinant plasmids were extracted and purified from transformed cells using the UltraClean™ Mini Plasmid Prep Kit (Mo Bio Laboratories Inc.) reagents and protocol. Plasmids were electrophoresed in a 1 % (w/v) agarose gel, 80 V x 40 min (see Section 2.3.2.2) to confirm they contained the 16S rRNA gene insert. Recombinant plasmid DNA were confirmed by correlation of their position on the gel with a plasmid known to contain the correct size insert. Plasmids containing an insert of the correct size were further screened by Restriction Fragment Length Polymorphism (RFLP) analysis. Restriction digests were performed on plasmids by separate incubation with the restriction nucleases *Hinf*1 (New England Biolabs) and *Rsa*1 (New England Biolabs) and accompanying buffers (New England Biolabs) at 37 °C for 3-4 h. Digests were fractionated by electrophoresis on 3% (w/v) agarose gels, 100 V x 3 h, (see Section 2.3.2.2) resulting in characteristic banding patterns allowing the diversity and abundance of cloned

phylotypes to be approximated. Clones exhibiting diverse banding patterns (including 2-4 duplicate clones possessing the same RFLP pattern) were selected at random for sequencing. Clones were sequenced as described in Section 2.3.2.4 with the exception that plasmid templates were subjected to a pre-heat treatment and primers pUC/M13 forward and reverse were used for amplification (Promega) (see Table 2.2). Binding sites for these primers are located on the pGEM® -T Vector, positioned either side of the insert. The pre-heat treatment consisted of diluting the template with water to the appropriate concentration, heating to 96 °C for 1 min in a PTC – 200 Peltier Thermal Cycler (MJ Research), and cooling to room temperature before adding the remainder of the sequencing-reaction components. In most cases, 16S rRNA gene clones were entirely sequenced with the sequences spanning nucleotide positions 519 – 1540 (*E. coli* equivalent).

2.4.4 *Phylogenetic and biodiversity analysis*

Phylogenetic analysis was conducted as described in Section 2.3.2.4 with the exception that the Ribosomal Database Project II (RDP) CHIMERA-CHECK program (http://rdp.cme msu edu/; Maidak *et al.* 2001) was used to detect PCR-amplified hybrid sequences. In addition, potential chimeras were determined from inconsistencies in branching order. Chimerical clones detected were not included in subsequent phylogenetic or biodiversity analyses. For calculation of diversity indices, the libraries were normalised to 50 clones using the rarefaction method (Simberloff, 1972) by utilising the program RAREFACT.FOR written by C. J. Krebs (University of British Columbia) and which is available through the internet (http://www2.biology.ualberta.ca/jbrzusto/rarefact.php).

Estimates of Diversity (H') were determined using the Shannon-Weaver (or Shannon-Weiner) Index (Krebs, 1989). H' is given by the formula:

$$H' = \frac{n \log n - \sum_{i=1}^{k} N \log N}{n}$$

where k is the total number of unique phylotypes, n is the total number of clones and N is the number of observations of each phylotype (i).

Measures of dominance concentration were determined using the Simpson Index (SI') (Krebs, 1989). SI' is given by the formula:

$$SI' = \sum_{i=1}^{k} \frac{Ni\,(Ni - 1)}{n\,(n - 1)}$$

Equitability indices (J') were based on Shannon-Weaver index data. J' is given by the formula:

$$J' = \frac{H'}{H\,max}$$

Where $H\,max$ is equal to $\log k$.

Biodiversity coverage (C) (Mullins *et al.* 1995) was derived by the formula:

$$C = 1 - \left(\frac{n_1}{N}\right)$$

Where n_1 is the number of phylotypes containing only one clone, and N is the total number of clones.

Pairwise comparisons of clone libraries were carried out using the Similarity Coefficient (S) (Odum, 1971). S is derived from the formula:

$$S = \frac{2C}{A + B}$$

Where A and B are the number of phylotypes in libraries A and B respectively, and C is the number of shared phylotypes.

Chapter 3: Results and Discussion

3.1 Microscopy and Mineralogy

Putative moonmilk samples were collected from an extensive speleothemic deposit in the dark zone of Exit Cave (MX1) and white mat-like material on the ceiling rock of Entrance Cave within the twilight zone (ME2). During the course of this study large moonmilk-like deposits were found beneath sediment in Entrance Cave (ME1, ME3) and analysed for comparison. ESEM with X-Ray Microanalysis was employed to investigate the microbe-mineral interface of moonmilk samples.

The samples collected from the ceiling of Entrance Cave (ME2) exhibited distinct, isolated areas of thin white material on the muddy rock surface (Figure 3.2; A). X-Ray microanalysis of this material (Figure 3.1; A, B) revealed high levels of silicon and aluminium suggesting a clay-type mud and high levels of carbon and oxygen suggesting areas of organic material. Figure 3.2 (B) illustrates that the isolated areas of white material on the mud surface from ME2 contained a crystalline character associated with biological growth of hyphal material. In the dark zone of Lechuguilla Cave $CaCO_3$-mineralised organic filaments have been reported (Cunningham *et al.* 1995). High magnification of the mat demonstrated the presence of hyphae-forming microorganisms with segmented hyphae of width 0.5-1 μm, consistent in size and morphology with filamentous actinomycetes. Non-biological (crystalline) structures were evident both beneath the mat of hyphal growth and also encrusting individual hyphae. Putative cells and an organic matrix can be frequently seen in moonmilk samples with SEM or in thin sections, but not in all cases (Northup *et al.* 2000). Biological material or cells were not evident in ESEM analysis of moonmilk samples ME3 or MX1, though CYBR staining confirmed the presence of DNA in the samples (data not shown).

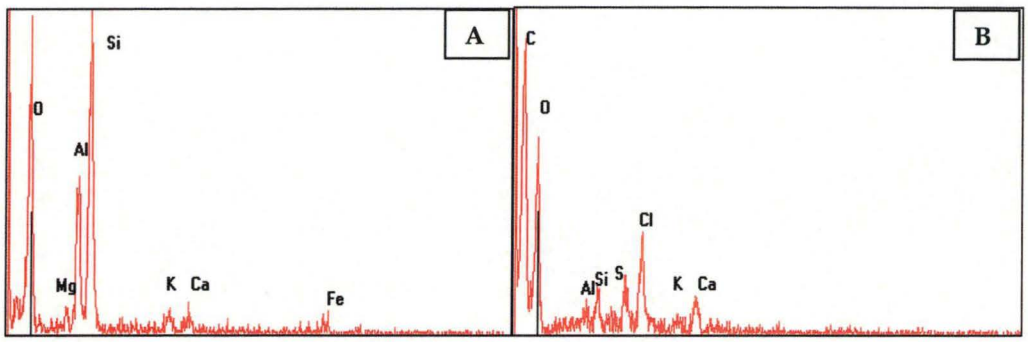

Figure 3.1: X-Ray microanalysis spectra of sample ME2 from the ceiling rock in the twilight zone of Entrance Cave. (A) Spectra of mud containing high levels of silicon, oxygen and aluminium consistent with a clay-type mud. (B) Spectra of mat sample ME2, illustrating the presence of organic material, indicated by high levels of carbon and oxygen.

For the purpose of distinguishing mondmilch from other carbonate speleothems, Fischer (1988) defined true calcite moonmilk as a calcite microcrystalline or needle-crystalline speleothem with a minimum calcite content of 90 % weight. ESEM of samples MX1 and the crystalline areas of ME2 (Figure 3.3) shows the needle-fibre crystalline characteristics of the calcite (confirmed by X-Ray microanalysis, data not shown). XRD studies revealed that the mineralogical composition of moonmilk samples from both Entrance Cave and Exit Cave were almost identical (Table 3.1). Moonmilk samples consisted predominantly (85-100%) of calcite (CaO_3), with trace amounts of quartz, mica (clay, most likely illite) and hydrated iron oxide, goethite [∝-FeO(OH)].

Table 3.1: X-Ray Diffraction analysis of moonmilk samples from Entrance Cave, ME2 and ME3, and Exit Cave, MX1. Approximate mineralogy recorded as % weight.

Sample	Calcite	Quartz	Mica*	Goethite
ME2	85	3	10	2
ME3	100			
MX1	100			

* Probably illite.
Note: Peak overlap may interfere with identifications and quantifications. Minerals present in trace amounts may not be detected.

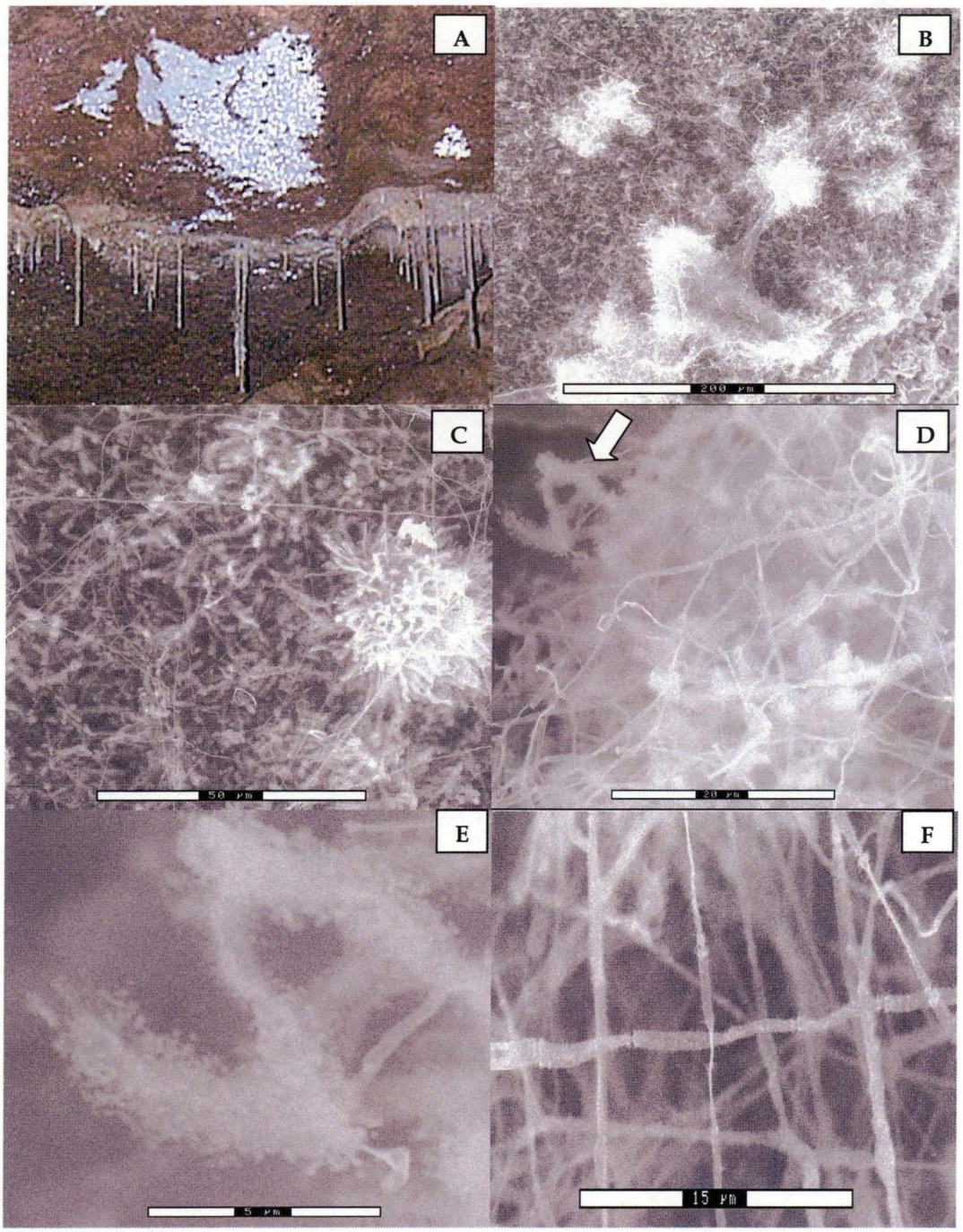

Figure 3.2: Photographs and ESEM pictures of cave sample ME2 showing biological hyphal material and calcite encrusted hyphae.
(A) White mat with reflective droplets on ceiling of Entrance Cave (ME2). B-F: ESEM images of sample ME2. (B) Mat of microbial growth on the ceiling. (C) Clumps of hyphae encrusted with calcite and uncalcified hyphae on the surrounding mud. ESEM images of calcite encrusted microbial filaments at high magnification. (D) Detailed view illustrating different degrees of encrustation exhibited by hyphae. (E) Detailed view of calcite encrusted hyphae. (F) Segmented hyphae, width approx. 0.5-1 µm.

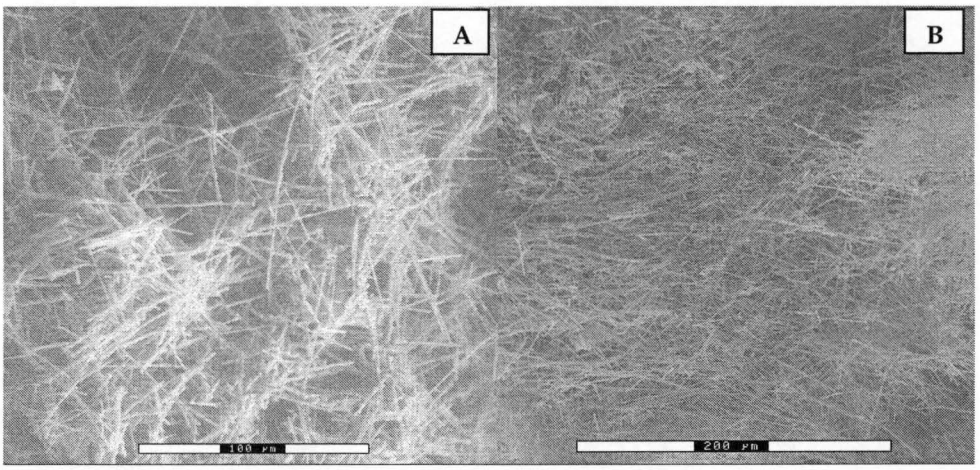

Figure 3.3: ESEM images of moonmilk samples illustrating microcrystalline, needle-fibre form of $CaCO_3$ crystals (confirmed by X-Ray microanalysis, data not shown).
(A) Sample ME2 from Entrance Cave. (B) Sample MX1 from Exit Cave.

XRD and ESEM results indicate that samples ME1, 3 and MX1 are true calcite moonmilk (98-100% $CaCO_3$). Sample ME2 had a slightly lower calcite composition (85%). The thin, mat-like nature of this sample from the ceiling of Entrance Cave made it difficult to collect samples from just the white material and inevitably some of the clay layer (2-3mm thick) on the ceiling was collected too, perhaps accounting for the higher clay content (10 %) of this sample

3.2 *Method Development for Calcite Moonmilk Samples*

It has previously been suggested that DNA extraction from environmental samples containing high levels of $CaCO_3$ is problematic (Guthrie *et al.* 2000; Northup, pers. comm. 2001). Initial clone analysis of samples ME2, ME3 and MX1, that have high calcite content (85-100%) as demonstrated by XRD analysis, (Section 3.1) resulted in a single phylotype most closely related to γ-Proteobacteria, *Pseudomonas fluorescens*. Isolations also proved to be problematic initially producing almost pure cultures of *Bosea thiooxidans*. Though these organisms were dominant components of the calcite-based microbial communities (Table 3.2), ESEM results depicting hyphal organisms indicated that these results were not necessarily representative of the true diversity. DNA extraction methods and cultivation procedures rely on the bacterial cells being readily released from their environmental matrix. Current DNA extraction protocols for

molecular analyses are poorly adapted for lithic or encrusted microbial communities due mostly to the hard, usually cemented nature of the mineral matrix (Wade & Garcia-Pichel, 2003). Guthrie *et al.* (2000) suggested that as DNA was released from coral matrices it was adsorbed by the calcite minerals resulting in very low quantities of DNA being recovered. A significant portion of this study was directed at method development to enhance DNA extraction and cultivation procedures for calcite cave samples.

A comparison of three DNA extraction protocols was undertaken: a modified protocol from Purdy *et al.* (1996) utilised for cave sediments in this study, the protocol from Guthrie *et al.* (2000) which was successful for coral samples, and a modified protocol from Miller *et al.* (1999), the Phosphate, SDS, Chloroform-Bead Beater method (PSC-B) which was successful with pure opal-A silica sinter samples (pers. comm. Dr. Susan Turner, University of Auckland, 2003). DGGE analysis of PCR amplified 16S rRNA gene from DNA product of the three extraction protocols was used to determine which method was most appropriate. PCR product resulting from the modified PSC-B DNA extraction displayed the highest degree of diversity in the banding pattern for all samples (Figure 3.4). Thus this protocol was utilised for further clone library analysis.

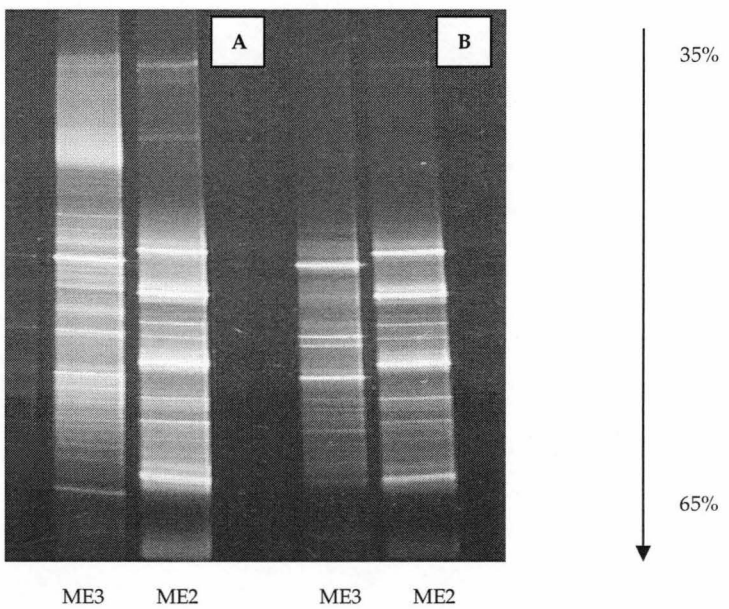

Figure 3.4: 16S rRNA gene DGGE community fingerprint of Entrance Cave moonmilk samples, ME2 and ME3. (A) DNA extraction using PSC-B method. (B) DNA extraction using Guthrie *et al.* (2000) coral method. Illustrates the greater diversity of banding patterns for DNA extracted using PSC-B method.

Microbes were isolated from moonmilk using a modified version of an isolation procedure developed by Olivier Braissant (pers. comm. Université de Neuchâtel, Germany, 2002). Calcite samples were subjected to one of five different treatments to dissolve the carbonate and free bacterial cells for cultivation:

1) 5% acetic acid (CH_3COOH) in 0.01 M $MgSO_4.7H_2O$
2) 1% acetic acid in 0.01M $MgSO_4.7H_2O$
3) 1 mM Ethylenediaminetetraacetic Acid (EDTA)
4) 0.1 mM EDTA
5) ddH_2O (control)

The 1% acetic acid and 1mM EDTA treatments produced the greatest number of different colony morphology types on primary isolation plates (data not shown). The 0.1 mM EDTA and ddH_2O treatments resulted in far fewer colonies and only a limited number of colony morphologies on isolation plates, indicating that these treatments did not sufficiently separate the cells from the calcite matrix. The 5% acetic acid treatment produced the least number of colonies on primary plates possibly due to the acid being bacteriocidal at this concentration. It is recognised that the application of EDTA and acetic acid solutions may have introduced unknown degrees of bias to the resulting isolations. However, as it was necessary to dissolve the carbonate to obtain greater diversity in isolations, this bias was unavoidable.

3.3 *Phylogenetic Diversity Overview*

16S rRNA gene clone libraries were constructed from DNA extracted from four sediment samples (SE1, SE2, SL1 and SL2) and three moonmilk samples (ME2, ME3 and MX1). Libraries were constructed with universal primers. Approximately 100-120 clones per library were screened by analysis of RFLP patterns and selected representatives of novel RFLP patterns were sequenced. Sequences greater than 500 base pairs (bp) were included in phylogenetic analysis. Groups of two or more highly related sequences (≥ 98% sequence identity) were considered to belong to the same sequence type designated a phylotype. From a total of 488 nonchimeric clones analysed from seven libraries, 148 phylotypes were defined affiliated with the domain *Bacteria*. A total of 43 phylotypes were found in two or more libraries. Table 3.2 provides a summary of the representative sequences and their phylogenetic affiliations. The majority of clones fell into three major phylogenetic groups: the Proteobacteria (dominating all samples), the high G+C Gram-positive *Actinobacteria*, and the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group. DGGE and subsequent 16S rRNA gene sequencing of bands was used to analyse moonmilk samples for comparison to clone library results. DGGE was also applied to sediment samples however the greater species diversity from sediments made the accurate defining of individual bands for sequencing difficult, a common result for sediment samples. Common banding patterns between samples indicate common community representatives. A total of six major bands present in moonmilk samples were sequenced and phylogenetically aligned with the α-Proteobacteria, *Actinobacteria* and CFBs.

Cultures were isolated from four sediment and three moonmilk samples and from swabs of speleothems in Entrance and Loons Caves to investigate culturable diversity (see Table 2.1 for sample locations). Sediment sites were chosen away from main pathways in the dark zone of the caves and covering a range of sediment types from two caves of different character: Entrance Cave (SE1 - dry sediment, SE2 – saturated sediment) and Loons Cave (SL1 – dry sediment, SL2 – saturated sediment). Moonmilk samples were chosen to include two cave types (Entrance and Exit) and cover a range of forms, speleothemic (MX1), mat-like (ME2) and floor

deposit (ME3). Selective procedures and media favouring actinomycete growth were applied to sediment and speleothem samples whereas non-selective procedures and media were used for moonmilk samples. Gross morphology was used to discard duplicate cultures and isolates displaying novel morphology were identified using 16S rRNA gene sequencing and phylogenetic analysis. Groups of two or more highly related sequences (≥ 97.5% identical) were considered to belong to the same species in accordance with the definition of a bacterial species (Goebel & Stackebrandt, 1994; Vandamme *et al.* 1996). A total of 302 isolates belonging to 39 genera were sequenced, mostly belonging to the order *Actinomycetales*. Table 3.3 summarises the phylogenetic affiliations of representative isolates. The majority of actinomycete isolates from all samples belonged to the *Streptomycineae, Pseudonocardineae, Corynebacterineae* and *Micrococcineae.* Isolates from moonmilk samples belonged to the *Actinomycetales, Firmicutes,* Proteobacteria and CFB groups.

Table 3.2: Summary of Phylotype* Abundance and Phylogenetic Affiliations from Cave Microhabitats

PHYLOTYPE[A]	NEAREST TAXON (% IDENTITY)[B]	ABUNDANCE IN MICROHABITAT[C]						
		SE1	SE2	SL1	SL2	ME2	ME3	MX1[D]
BACTERIA								
α-Proteobacteria								
Caulobacterales	***Caulobacteraceae***							
ME30021	*Nitrobacteria hamadaniensis* AY569007 (93.9%)						1	
MON0045	*Brevundimonas alba* AJ227785 (94.6%)				1	1		
CAV0001	*Brevundimonas alba* AJ227785 (98.2%)					7	1	2
DMON1	*Brevundimonas alba* AJ227785 (98.2%)					√	√	√
Rhizobiales	***Beijerinckiaceae***							
MX10051	*Methylocella palustris* Y17144 (89.1%)							4
	Bradyrhizobiaceae							
ME20020	*Bradyrhizobium japonicum* AF363150 (98.2%)					3		
CAV0002	*Bosea thiooxidans* X81044 (99.8%)			2	4	14		1
DMON2	*Bosea thiooxidans* X81044 (99.8%)					√	√	√
MX10048	*Bosea thiooxidans* X81044 (90%)							1
SL20043	*Afipia massiliensis* AY029562 (95.1%)				2			
MX10021	*Afipia* genosp 9 U87780 (99.2%)							1
CAV0008	*Rhodopseudomonas palustris* D12700 (93.7%)			2	2			1
	Brucellaceae							
SL20011	*Ochrobactrum anthropi* U70978 (94.2%)				1			
	Hyphomicrobiaceae							
ME20041	*Hyphomicrobium sulfonivorans* AF235089 (93.8%)					1		
SED0019	*Hyphomicrobium vulgare* X53182 (91.9%)		1	1	1			
SL10054	*Devosia riboflavina* AY512822 (99.6%)			1	2			
	Methylobacteriaceae							
SE10044	*Methylobacterium extorquens* L20847 (91.1%)	1						
	Phyllobacteriaceae							
SE20001	*Phyllobacterium myrsinacearum* AJ011330 (99.2%)	2	1		1			
ME20015	*Aminobacter nigataensis* AJ011761 (96.1%)					1		
	Rhizobiaceae							
MX10017	*Rhizobium giardinii* U86344 (99.1%)							1
Rhodobacterales	***Rhodobacteriaceae***							
SL20056	*Rhodobacter azotoformans* D70846 (97%)				1			
MX10016	*Rhodobacter sphaeroides* D16424 (98.4%)							1
SE10043	*Amaricoccus macauensis* U88042 (85.7%)	1						
SPE008	*Paracoccus solventivorans* AY014175 (??%)							
Sphingomonadales								
SE10056	*Sphingomonas aerolata* AJ429240 (97.5%)	1						
MON0003	*Sphingomonas phyllosphaerae* AY453855 (97.1%)					1		1
CAV0009	*Sphingopyxis alaskensis*AF378795 (94.2%)	1	2		2	5		1
DMON3	*Sphingopyxis alaskensis*AF378795 (93%)					√	√	√
β-Proteobacteria								
Burkholderiales	***Acaligenaceae***							
SL20039	*Derxia gummosa* (91.6%)		1	3				
SE20024	*Bordetella pertussis* AF366576 (91.4%)		1					
	Burkholderiaceae							
SL10008	*Burkholderia sordidicola* AF512827 (92.9%)			4				
SL10014	*Limnobacter thiooxidans* AJ289885 (89.2%)		1	2				
CAV0003	*Pandorea apista* AF139172 (93.1%)			2	1			1
	Commamonadaceae							
CAV0004	*Hydrogenophaga defluvii* AJ585993 (94.3%)				1		2	1
SL20003	*Hydrogenophaga palleronii* AF019073 (98.7%)				1			
MX10008	*Delftia tsuruhatensis* AY302438 (97.3%)							1
MON0010	*Polaromonas vacuolata* U14585 (95 7%)						1	3
SL10033	*Variovorax paradoxus* AJ420329 (99.3%)			1				
SL20010	*Acidovorax valerianellae* AJ431731 (96.1%)				1			
SE20028	*Ottowia thiooxydans* AJ537466 (92.4%)		1					
	Oxalobacteraceae							
CAV0005	*Janthinobacter agaricidamnosum* Y08845 (98.3%)			2	2		4	3
MON0015	*Massilia timonae* U54470 (97.5%)						5	4
CAV0006	*Duganella violaceusniger* AY376163 (97%)				1		5	5
ME30010	*Oxalobacter formigenes* U49758 (96 3%)						1	1
CAV0021	*Herbaspirillum frisingense* AJ238358 (??)		2				3	1

Continued on next page

PHYLOTYPE[A]	NEAREST TAXON (% IDENTITY)[B]	ABUNDANCE IN MICROHABITAT[C]						
		SE1	SE2	SL1	SL2	ME2	ME3	MX1[D]
Hydrogenophilales	***Hydrogenophilaceae***							
SL10010	*Thiobacillus denitrificans* AJ243144 (94.5%)			2				
Methylophilales	***Methylophilaceae***							
SL00008	*Methylophilus leismgeri* AF250333 (97.8%)			2	5			
SE20011	*Methylophilus freyburgensis* AJ517772 (93.3%)		8					
SL00038	*Methylovorus mays* AY486132 (94.3%)			3	8			
Nitrosomonadales	***Nitrosomonadaceae***							
SL20020	*Nitrosospira briensis* AY123800 (90.2%)				4			
Unclassified								
SED0039	*Thiobacter subterraneus* AB180657 (89%)		1	1				
δ-Proteobacteria								
Desulfuromonadales	***Desulfuromonadaceae***							
MON0018	*Desulfuromonas thiophila* Y11560 (90%)					1	1	1
	Geobacteraceae							
SE10098	*Geobacter pelophilus* U96918 (91%)	1						
Desulfoarculales	***Desulfoarculaceae***							
SL0043	*Nitrospina gracilis* L35504 (92%)		2	2	1			
γ-Proteobacteria								
Acidithiobacillales	***Acidothiobacillaceae***							
SE10004	*Acidothiobacillus ferroxidans* AJ457808 (98%)	2						
Alteromonadales	***Alteromonadaceae***							
SED0012	*Marinobacterium georgiense* AB021408 (99%)	1		1				
Chromatiales	***Chromatiaceae***							
SED0017	*Nitrosococcus oceani* AF363287 (91%)	3			1			
SL10022	*Nitrosococcus oceani* AF363287 (90%)				1			
SED0010	*Thiocapsa roseoperscina* Y12303 (93%)	3			1			
SE10089	*Thiobacillus prosperus* AY034139 (94%)	1						
	Ectothiorhodospiraceae							
SE10058	*Thioalkalivibrio thiocyanodenitrificans* AY360060 (92%)	6						
Enterobacterales								
SED0008	*Photorhabdus luminescens* D78005 (95%)		9	9	2			
Legionellales								
SE10003	*Legionella londiniensis* Z49728 (94%)	1						
Methylococcales								
SE10021	*Methylococcus capsulatus* X72770 (90%)	1						
ME30011	*Methylococcus capsulatus* X72770 (91%)						1	
Pseudomonadales								
CAV0011	*Pseudomonas fluorescens* AF094729 (98%)		1	3	2		4	3
SE20012	*Pseudomonas putida* AF094743 (98%)		2					
SE20021	*Pseudomonas anguilliseptica* X99540 (98%)		1					
MX10050	Uncultured bacterium clone C11-K11 AJ421116 (95%)							1
	Moraxellaceae							
ME30060	*Acinetobacter johnsonii* Z93440 (94%)						2	
Thiotrichales	***Thiotrichaceae***							
SE20006	Achromatium oxaliferum *L48227 (93%)*				1			
Xanthomonadales								
SE10045	*Lysobacter gummosus* AB161361 (97%)	2						
SE10044	*Luteimonas mephitis* AJ012228 (97%)	1						
SL10051	*Frauteria aurantia* AJ010481 (95%)			1				
SED0009	*Hydrogenocarbophaga effusa* AY363244 (93%)		1	2	1			
CAV0030	*Pseudoxanthomonas mexicana* AF273082 (96%)			1			1	1
Actinobacteria								
Actinobacterideae								
Actinomycetales	***Corynebacterineae***							
ME20019	*Nocardia carnea* X80607 (99%)	3	2			2		
ME20104	*Nocardia corynebacteroides* X80615 (94%)					2		
	Micrococcineae							
MEX005	*Arthrobacter chlorophenolicus* AF102267 (96%)							
DMON4	*Arthrobacter chlorophenolicus* AF102267 (96%)					√	√	√
CAV0027	*Arthrobacter pascens* X80740 (99%)	1	2				2	2
CAV0046	*Arthrobacter oxydans* X83408 (99%)				1		2	

Continued on next page.

PHYLOTYPE[A]	NEAREST TAXON (% IDENTITY)[B]	ABUNDANCE IN MICROHABITAT[C]						
		SE1	SE2	SL1	SL2	ME2	ME3	MX1[D]
SL20016	*Arthrobacter psychrolacticus* AF134183 (98%)				1			
MON021	*Knoellia subterranea* AJ294413 (99%)	3	1				1	2
	Pseudonocardineae							
ME20021	*Pseudonocardia asaccharolytica* Y08536 (95%)					3		
SL10013	*Actinobispora alanniphila* AF325726 (96%)			1				
ME20012	*Amycolatopsis fastidiosa* AJ400710 (96%)					1		
ME20103	*Amycolatopsis sulphurea* AJ293756 (96.5%)					2		
ME20081	*Saccharothrix coeruleofusca* AF114805 (95%)					1		
CAV0010	*Saccharothrix texasensis* AF350247 (97%)	2	1	1		4		
MON0007	*Saccharothrix cryophilis* AF114806 (95%)					4	2	3
DMON5	*Saccharothrix cryophilis* AF114806 (95%)					√	√	√
MON0018	*Lentzea albidocapillata* X84321 (96%)					1		
SE10086	*Lechevalieri aerocolonigenes* AY196703 (88%)	1						
SL20046	*Kibdelosporangium phillipinense* AJ512464 (95%)				1			
	Propionibacterineae							
CAV0023	*Propionibacterium acnes* AB042288 (98%)			1				1
SED0051	*Nocardioides fulvus* AF005016 (94%)	1		2				
MX10002	*Nocardioides* sp. LMG20237 AJ316318 (92%)							1
MX10032	*Pimelobacter simplex* Z78212 (98%)							1
	Micromonosporineae							
SL10009	*Micromonospora echinoaurantiaca* X92618 (98%)			1				
ME20061	*Actinoplanes cyaneus* AB036997 (95%)					1		
MX10039	*Virgosporangium ochraceum* AB006167 (91%)							2
	Frankineae							
SE0098	*Frankia* sp.	2	2					
SE10039	*Blastococcus saxobsidens* AJ316570 (90%)	1						
	Streptomycineae							
SL10019	*Streptomyces caviscabies* AF112160 (99%)	2		1			1	
ME20022	*Streptomyces subrutilis* X80825 (97%)		1			2		
ME30039	*Streptomyces sanglieri* AY094364 (98%)					1		
ME20033	*Streptomyces violaceoruber* AF503492 (98%)					1		
SE00050	*Streptomyces yunnanensis* AF346818 (91%)	1	1					
CAV0015	*Streptomyces macrosporus* Z68099 (90%)			1				2
ME20041	*Streptomyces rutgersensis* Z76688 (99%)					1		
SE10028	*Streptomyces galilaeus* AB045878 (98%)	1						
SL10055	*Kitasatospora mediocidica* U93324 (97%)			1				
Rubrobacterideae								
ME30059	*Thermoleophilum minutum* AJ458464 (89%)						1	
ME30009	*Thermoleophilum album* AJ458463 (93%)						1	
Sphaerobacterideae								
SE10001	*Sphaerobacter thermophilus* AJ420142 (90%)	1						
Unclassified *Actinobacteria*								
SE10060	Candidatus *Microthrix parvicella* X89774 (91%)	2					1	
Firmicutes								
SL10009	*Ruminococcus flavefaciens* X85097 (91%)			2				
MX10063	*Bacillus subtilus* AB042061 (99%)		1					1
ME20098	*Sporosarcina ureae* AF202057 (98%)					2		
Cytophaga-Flavobacteria-Bacteroides								
Bacteroidetes								
Flavobacteriales	***Flavobacteriacea***							
MX10045	*Cryomorpha ignava* AF170738 (92%)							1
SL10003	*Flavobacteria ferrugineum* M62798 (96%)			1				
SL10020	*Flavobacterium columnare* M58781 (93.3%)			1				
CAV0015	*Flavobacteria limicola* AB075230 (98%)	1	2				6	8
ME30007	*Flavobacteria limicola* AB075230 (93%)						2	
CAV0018	*Flavobacteria leeana* AB180738 (98%)	2	3	1	1			10
DMON6	*Flavobacteria leeana* AB180738 (98%)					-	√	√
CAV0030	Antarctic bacterium R-7933 AJ440987 (97.1%)		1					1
Sphingobacteriales	***Sphingobacteriaceae***							
MON0015	*Pedobacter cryconitis* AJ438170 (97%)						2	1
ME30041	*Sphingobacterium faecium* AJ438176 (93.4%)						3	
Bacteroidales								

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PHYLOTYPE[A]	NEAREST TAXON (% IDENTITY)[B]	ABUNDANCE IN MICROHABITAT[C]						
		SE1	SE2	SL1	SL2	ME2	ME3	MX1 [D]
SL0019	*Flexibacter tructuosus* M58789 (92%)			1	1			
SL10002	Uncultured bacterium clone C44K17 AJ297617 (92.9%)			1				
CAV0026	Bacteroidetes bacterium Mo-0.2plat-K3 AJ622888 (90.9%)	1				1	1	
Acidobacteria								
ME20061	Uncultured bacterium DA008 Y12597 (97%)					1		
MON0045	Uncultured bacterium DA008 Y12597 (94%)						2	2
ME20013	Uncultured bacterium DA008 Y12597 (90%)					2		
ME20020	Bacterium Ellin6075 AY234727 (94%)					1		
ME20050	Bacterium Ellin6075 AY234727 (91%)					1		
SL20005	Bacterium Ellin6071 AY234723 (95%)				1			
SL10020	Bacterium Ellin5289 AY234640 (89%)			1				
Planctomycetales								
SE10039	*Planctomyces brasiliensis* AJ231190 (90%)	5						
CAV0062	*Planctomyces maris* AJ231184 (90.4%)	4	2	1		1		
SED0061	*Pirellula staleyi* AJ231183 (96%)	2	1	2	2			
SE0051	*Pirellula* sp. X81947 (88 8%)	3	1					
SED0047	*Planctomycete* str.292 AJ231182 (87.2%)	2	1	1				
SL10061	BacteriumDR2A-7G19 AB127858 (91.2%)			1				
ME30013	*Gemmata*-like str. Cjuq14 AF239693 (81.9%)						2	
***Chloroflexi* (green nonsulfur)**								
ME20011	*Caldilinea aerophila* AB067647(86.4%)					2		
SE0037	*Caldilinea aerophila* AB067647 (90%)	1	1					
MX10044	*Dehalococcoides ethenogenes* AF004928 (95.6%)							1
MX10041	*Anaerolinea thermophila* AB046413 (91.9%)							1
ME30006	Unidentified bacterium strain BD3-16 AB015556 (86.9%)						1	
Verrucomicrobia								
SE10094	Uncultured verrucomicrobium DEV010 AJ401127 (92%); *Verrucomicrobia spinosum* X90515 (86.5%)	2						
SE10006	*Opitutus* sp VeSm13 X99392 (91.8%)	1						
OP10								
SL20004	Uncultured bacterium SJA-176 AJ009504 (86%)				1			
SL20017	Uncultured bacterium GC55 AJ271048 (90.2%)				1			
Gemmatimonadetes								
SL20096	*Gemmatimonas aurantiaca* AB072735 (87 6%)				3			
SL20036	Bacterium Ellin 5301 AY234652 (87.8%)				2			
ARCHAEA								
Crenarchaeota								
SL20017	Uncultured archaeon WSB-11 AB055993 (91.8%) *Desulfurococcus amylolyticus* AF250331 (75%)				1			
Total: # phylotypes (# clones)		**40 (72)**	**34 (60)**	**39 (68)**	**39 (68)**	**31 (71)**	**29 (61)**	**40 (75)**

*Phylotypes represented in sediment and moonmilk samples (CAV) Phylotypes represented in sediments of Entrance and Loons Caves (SED).
Phylotypes represented in more than one moonmilk sample (MON) DGGE bands from moonmilk (DMON); presence (√), absence (-).
[A] A unique sequence or group of highly related sequences (> 98% identical) considered to belong to the same sequence type.
[B] Inferred from direct sequence comparison to representative sequences on GENBANK. Accession numbers given
[C] No of clones in phylotype represented in each microhabitat studied based on direct sequence comparisons or inferred from RFLP patterns
[D] Microhabitats represented by samples. SE1 (dry sediment, Entrance Cave), SE2 (wet sediment, Entrance Cave), SL1 (dry sediment, Loons Cave), SL2 (wet sediment, Loons Cave), ME2 (calcite mat, Entrance Cave), ME3 (moonmilk, Entrance Cave), and MX1 (moonmilk, Exit Cave).

Proteobacteria

The Proteobacteria were the most commonly sampled group (35.2-76.5% of clones) present within the cave samples. Representatives of the alpha (α), beta (β), gamma (γ) and delta (δ) subclasses were detected in varying proportions in the clone libraries. No epsilon (ε) Proteobacteria clones were detected in this study.

α-Proteobacteria

A total of 81 clones representing 24 phylotypes were affiliated with the α-Proteobacteria and representatives were detected in all libraries (Table 3.2). Three DGGE bands (DMON1, 2 and 3) affiliated with the α-Proteobacteria were present in all 3 moonmilk samples. Isolates from sediment, speleothems and moonmilk were also affiliated with the α-Proteobacteria. Figure 3.5 displays an evolutionary distance dendrogram of representatives of the α subclass and associated cave clones, DGGE bands and isolates.

The most pronounced clade was the *Rhizobiales* consisting of 15 phylotypes from all samples affiliated with *Beijerinckiaceae, Bradyrhizobiaceae, Brucellaceae, Hyphomicrobiaceae, Methylobacteriaceae, Phyllobacteriaceae* and the *Rhizobiaceae*. The most dominant phylotype present in high numbers in both Loons sediments and all moonmilk samples (CAV0002) was most closely related to *Bosea thiooxidans* (99.8% sequence similarity), a thiosulfate oxidiser (Das *et al.* 1996). The isolation of a strain of *Bosea thiooxidans* and the presence of 14 clones of this phylotype in the Entrance mat material indicates that this is a major component (19.71%) of the total microbial community. The *Bosea thiooxidans* phylotype was also detected in all moonmilk samples by DGGE analysis (band DMON1). A number of clones were affiliated with methylotrophic taxa (phylotypes MX10051, ME20041, SED0019, SE10044) including representatives of the genera *Methylobacterium, Methylocella,* and *Hyphomicrobium*. Phylotypes ME20041 and SED0019 formed a deep lineage within the *Hyphomicrobiaceae*. A novel pink-pigmented *Methylobacterium* sp. was isolated from moonmilk and phylotypes branching with genera *Methylobacterium* and *Methylocella* were detected in Entrance sediment and moonmilk from Exit. Phylotype MX10051, consisting of four clones, formed a deep branching lineage

within the *Beijerinckiaceae* affiliated loosely with *Methylocella paulstris* (89.1% sequence similarity). *M. palustris* is a methanotrophic acidophile isolated from peat wetlands (Dedysh *et al.* 2003). Other members of the *Beijerinckiaceae* are free-living aerobic nitrogen-fixing bacteria (eg. *Beijerinckia*) which grow well in acidic soils. Sediment phylotypes were also affiliated with nitrogen-fixing bacteria including those usually associated with plant nodules (eg. *Rhizobium, Bradyrhizobium*) (Young & Haukka, 1996).

The second clade of interest is the *Caulobacterales*. Phylotype SL20021 was affiliated with *Nitrobacter* sp., a facultative nitrifying chemolithotroph (Zare *et al.* 2003; published in database only), detected in saturated sediment from Loons but not detected in dry sediment from Loons or Entrance samples. Phylotypes affiliated with *Brevundimonas* sp. were detected in all moonmilk samples. MON0045 was most closely related to *Brevundimonas alba* (98.2%), a prosthecate oligotroph (Abraham *et al.* 1999), and present in particularly high numbers in sample ME2 (~10% of total community). Prosthecae are narrow extensions of the bacterial cell wall containing cytoplasm and it has been proposed that these structures confer a variety of benefits to aerobic heterotrophic bacteria including mechanisms for attachment to solid substrates and enhanced respiration and nutrient uptake (Hedlund *et al.* 1996). *Brevundimonas alba* was also present in the DGGE analysis (band DMON2) and isolated from all moonmilk samples, reinforcing its ubiquity in moonmilk.

Members of the *Sphingomonadales* were detected in sediments and moonmilk samples. Particularly, phylotype CAV0009 most closely related to *Sphingopyxis alaskensis* (94.2%) was detected in all samples except for SL1 and was detected in DGGE analysis (DMON3). Putatively novel members of the genus *Sphingomonas* and *Sphingopyxis* were also isolated from sediments (SEE005) and moonmilk (MAE322). Members of the *Sphingomonadales* are oligotrophic and found in nutrient limited subsurface environments where they metabolise a large number of aromatic compounds (Fredrickson *et al.* 1995 Balkwill *et al.* 1997; Barton *et al.* 2004). Such metabolic diversity has led to the identification of members of this genus in numerous starved environments including distilled waters and oligotrophic marine ecosystems (Balkwill *et al.*

1997). A novel *Porphyrobacter* sp. was isolated from moonmilk (MEE338). Members of the *Porphyrobacter* are aerobic and photosynthetic bacteria.

Three phylotypes and one isolate clustered within the *Rhodobacterales* lineage. Two phylotypes from moonmilk (MX10016) and Loons sediment (SL20056) were affiliated with phototrophic *Rhodobacter* sp. A third phylotype SE10043 from Entrance sediment was loosely affiliated (85.7% sequence similarity) with members of the genera *Amaricoccus,* isolated from activated sludge. A novel methylotroph from the *Rhodobacterales* lineage, *Paracoccus* sp., was isolated from a speleothem in Entrance Cave. *Paracoccus* sp. can utilise methylamine and methyl formamide (Urakami *et al.* 1990).

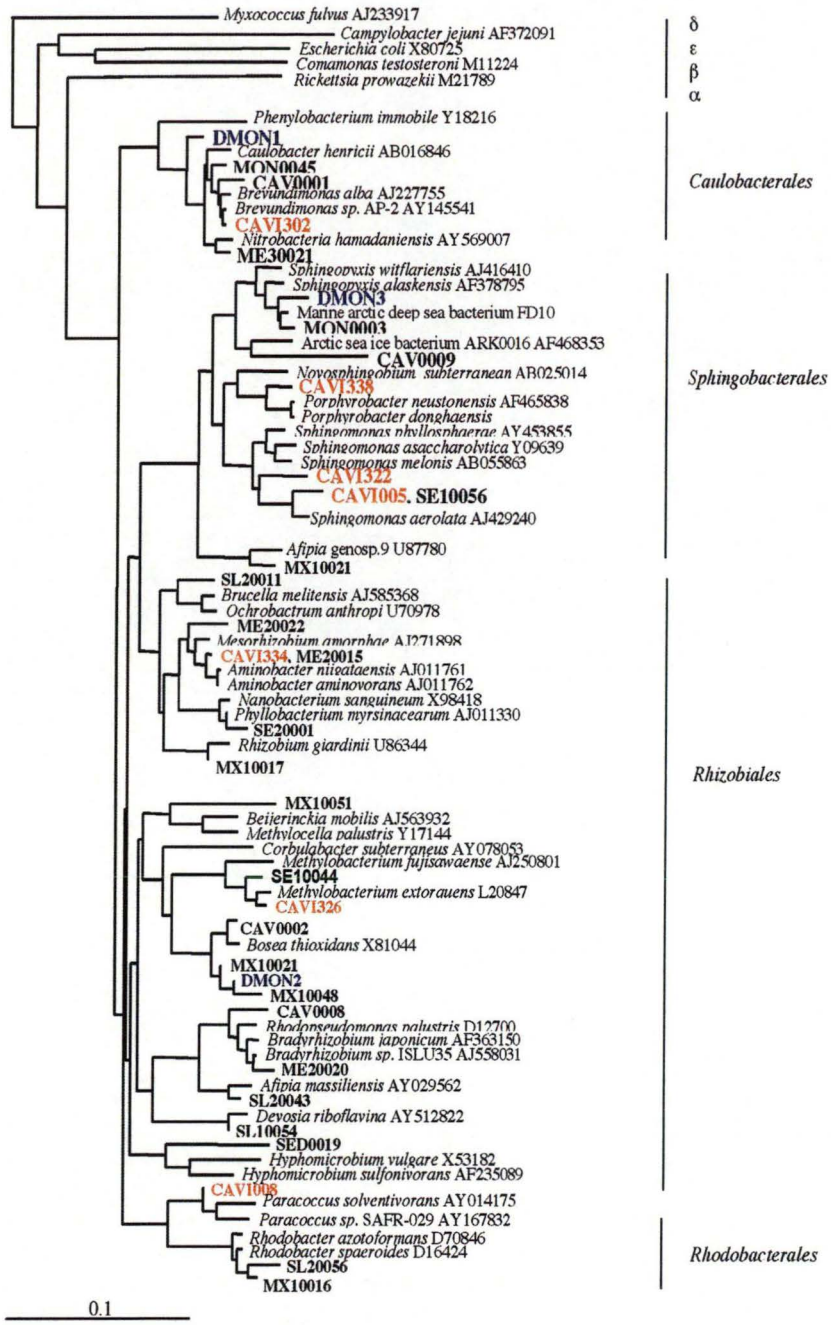


Figure 3.5: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the α-*Proteobacteria*. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Thermoproteus tenax* was used as the outgroup species. The scale bar indicates 10% sequence divergence.
Colour Code: **Black** = Clone sequences, **Blue** = DGGE sequences, **Brown** = Isolate sequences.

β- Proteobacteria

Clones affiliated with the β-Proteobacteria were the most abundant group detected in this study (102 sequences) and were distributed fairly evenly between sample sites SE2, SL1, SL2, ME3 and MX1 contributing approximately 25-34% to the total diversity sampled (Table 3.2). In comparison however, no β-Proteobacteria were detected from sites SE1 or ME2.

Phylotypes affiliated with the β-Proteobacteria are depicted in Figure 3.6, clustering with known chemolithotrophs, particularly hydrogen utilising bacteria, thiosulfate oxidisers, and nitrogen-fixing bacteria. Most phylotypes clustered within the Order *Burkholderiales*. Several sequences obtained from both sediment and moonmilk were closely related (94-99.3% sequence similarity) to members of the *Commamonadaceae*, particularly the Acidovorax group, including the genera *Acidovorax, Variovorax, Polaromonas*, and *Hydrogenophaga*. DGGE analysis also detected a member of the *Hydrogenophaga* in ME3 and MX1 (DMON4). A novel *Acaligenes* sp. (MEE109) was isolated from moonmilk. Members of the *Commamonadaceae* and *Acaligenaceae* are aerobic chemoorganotrophs and some strains are capable of chemolithoautotrophy utilising hydrogen as an energy source. Nitrogen-fixation has been reported for some genera, eg. *Burkholderia, Derxia* and *Hydrogenophaga* (Willems *et al.* 1991). Phylotypes from sediment and moonmilk were distantly related to members of thiosulfate oxidising genera *Thiobacillus, Limnobacter, Ottowia* and *Delftia* (eg. Spring *et al.* 2001). A number of clones from moonmilk samples were distributed within five phylotypes affiliated with the *Oxalobacteraceae*, showing close relationships (>96%) with the genera *Janthinobacter, Massilia, Duganella, Oxalobacter* and *Herbaspirillum*. A number of members of the Oxalobacter group are nitrogen-fixing bacteria associated with plants (Valverde *et al.* 2003). Members of one genus *Duganella* are also reported to have chitinolytic properties, most likely associated with the breakdown of organic matter. The *Oxalobacteriaceae* appear to be a dominant component of the true calcite moonmilk microbial communities sampled accounting for 24% and 18% of samples ME3 and MX1, respectively. Further evidence of this is the presence of DGGE band DMON6 clustering with *Janthinobacter* phylotypes.

Phylotypes affiliated with the *Methylophilales* dominated the sediment samples, particularly the saturated sediments from Entrance and Loons Cave. SE20011, most closely related to *Methylophilus freyburgensis* (93.3% sequence similarity) accounts for 13% of the sampled microbial community in saturated sediment from Entrance Cave. Members of the *Methylophilus* genus are methanol utilising. SL00038 most closely related to *Methylovorus mays* (94.3% sequence similarity) accounts for 18% of the observed microbial community in saturated sediment from Loons Cave. Members of the *Methylovorus* are aerobic obligate methylotrophs associated with plants (Doronina *et al.* 2000). A single phylotype (SL20020) from saturated Loons sediment grouped with the ammonia-oxidising species *Nitrosospira briensis* (90.2%).

δ- Proteobacteria

Clones affiliated with the δ-Proteobacteria were detected in all samples. This phylum encompasses sulfate- and sulfide-reducers that are morphologically diverse and obligate anaerobes. Six clones were distributed among three phylotypes (Table 3.2), thus the δ-Proteobacteria were a minor, though ubiquitous component of the microbial communities sampled. Two types of sulfate-reducers are recognised, those species that reduce sulfate to hydrogen sulfide (H_2S) (eg. *Desulfovibrio, Desulfomonas, Desulfotomaculum, Desulfobulbus*) and those that reduce sulfate to sulfide (eg. *Desulfobacter, Desulfococcus, Desulfosarcina, Desulfonema*). Two phylotypes formed separate deep branching lineages within the *Desulfuromonadales* (Figure 3.7). MON0018 was detected in all moonmilk samples and represents a putatively novel lineage forming a monophyletic clade with the genus *Desulfuromonas* (90% sequence similarity to *Desulfuromonas thiophila*). Members of this genus are obligate sulfate-reducers and widespread in terrestrial and aquatic environments that become anoxic as a result of microbial decomposition processes (Finster *et al.* 1997). Phylotype SED0098 present in sediment samples SE2, SL1 and SL2 were affiliated with sulfur- and iron- reducing members of the *Geobacteraceae*. A third phylotype SE10043 detected in sample SE1, formed a deep branching lineage within the *Desulfoarculaceae*. The closest cultured relative to this clone was nitrite-oxidiser *Nitrospina gracilis*.

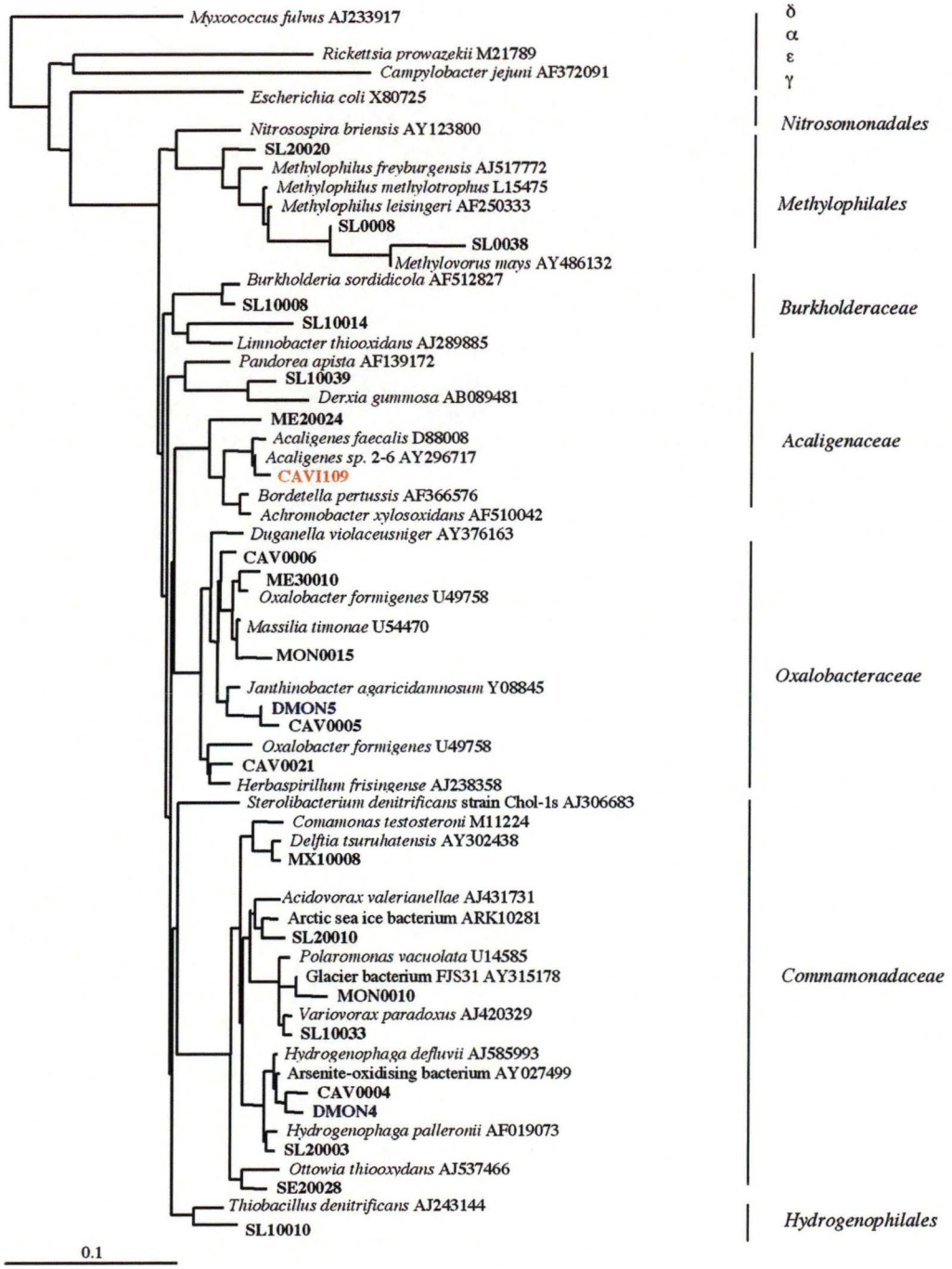


Figure 3.6: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *β-Proteobacteria*. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Thermoproteus tenax* was used as the outgroup species. The scale bar indicates 10% sequence divergence.
Colour Code: **Black** = Clone sequences, **Blue** = DGGE sequences, **Brown** = Isolate sequences.

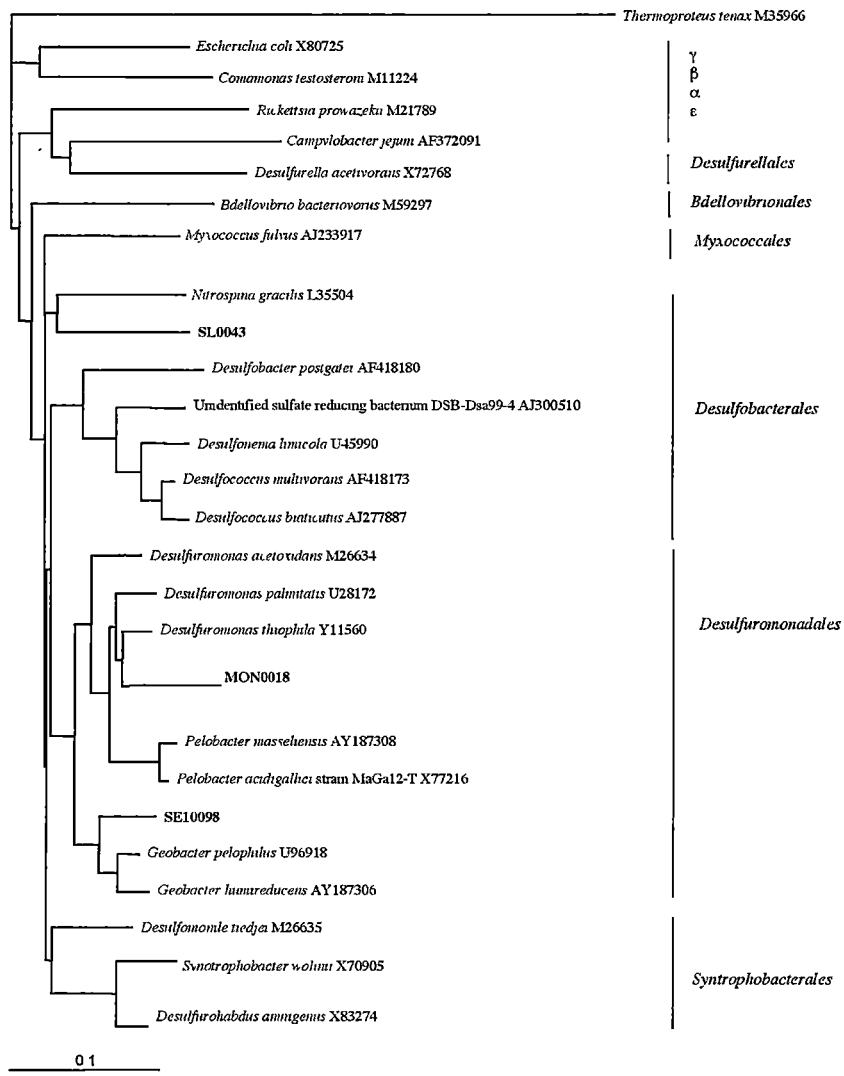


Figure 3.7: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the δ-Proteobacteria The dendrogram was constructed from an alignment of 1000 nucleotide positions Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method *Thermoproteus tenax* was used as the outgroup species The scale bar indicates 10% sequence divergence

γ-Proteobacteria

A total of 77 clones in 22 phylotypes were affiliated with the γ-Proteobacteria. Figure 3.8 illustrates the phylogenetic distribution of γ-phylotypes. The γ-Proteobacteria dominated Entrance sediments SE1 and SE2 representing 29.6% and 26.2%, respectively, of the diversity sampled and also represented a significant component of sample SL1 (21.8%) (Table 3.2). Several sequences from Entrance sediment SE1 clustered within the Order *Chromatiales*, whose members

are predominantly phototrophic and includes sulfur-, H_2S- and thiosulfate- and nitrite-oxidising autotrophic genera *Nitrosococcus, Thioalkalivibrio, Thioploca, Beggiatoa*. Some cultured representatives are capable of utilising atmospheric CO_2 as a carbon source for growth in dark conditions. SE1 clones affiliated with the *Chromatiales* represent 19% of total diversity sampled thus inferring that these are a dominant component of the community. SE10058, consisting of six clones, was affiliated with *Thioalkalivibrio thiocyanodenitrificans* (92% sequence similarity) an obligate sulfur-oxidising/nitrifying chemolithoautotroph. Two phylotypes distantly related to autotrophic denitrifyer species *Nitrosococcus oceani* (90-91% sequence similarity) were detected in both Loons and Entrance sediment. Phylotype SED0010, also detected in both Entrance and Loons sediment clustered with *Thiocapsa roseoperscina*, a thiosulfate-oxidiser.

The Pseudomonads (*Pseudomonadales* and *Xanthomonadales*) are a diverse group of aerobic chemoheterotrophs that never show fermentative metabolism. Some members are chemolithotrophic using H_2 and CO as sole electron donors and some members can use nitrate as an electron donor. Within the *Pseudomonadales*, a number of sequences, distributed in four phylotypes, from sediments and moonmilk clustered with the genus *Pseudomonas*, most closely related to members of the fluorescent sub-group (*P. fluorescens, P.putida,* and *P.aeruginosa*) and a single phylotype from moonmilk clustered with *Acinetobacter*. Pseudomonads have simple nutritional requirements, the most striking feature being a versatile metabolic lifestyle and the ability to metabolise a range of substrates including numerous aromatic compounds as the sole carbon and energy source.

Several clones were distributed amongst five phylotypes showing high sequence similarity (95-97%) with denitrifying genera of the *Xanthomonadales*, (*Lysobacter, Luteimonas, Frauteria, Hydrogenocarboniphaga, Pseudoxanthomonas*) and a novel *Xanthomonas* sp. was isolated from moonmilk. *Xanthomonadales* are also ecologically important in soil and water and are probably responsible for degradation of many soluble compounds derived from the breakdown of plant and animal materials in oxic environments (eg. *Lysobacter* sp. can lyse both bacteria and fungi through array of lytic enzymes). A second novel Xanthomonad was isolated from moonmilk, the closest cultured relative being *Stenotrophomonas maltophilia*, which is also the

closest relative of clones of novel iron-oxidising bacteria (Emerson & Moyer, 1997). Rice *et al.* (1995) also found that *S.maltophilia* studied in biofilms showed exceptionally adhesive and corrosive properties.

Phylotype SED0008 from sediment samples SE2, SL1 and 2, clustered with the Enteric bacteria, a homogenous, facultatively aerobic, group within γ-Proteobacteria. This phylotype was numerically significant in that it contained nine clones from SE2 and SL1, and 2 clones from SL2. Phylogenetically, it was most closely related to both *Photorhabdus luminescens* and *Escherichia coli* strain 5.1. *P.luminescens* is a symbiotic bacteria and *E.coli* is able to grow on a wide variety of carbon and energy sources.

Other minor components of the γ-Proteobacteria clones include, a phylotype (SE20006) closely related to *Achromatium oxaliferum* (93%) a sulfur-oxidiser that has sulfur and calcite inclusions within the cell, detected in Entrance sediment. Phylotypes, SE10021 and ME30011, were distantly affiliated (90-91%) with *Methylococcus capsulatus*, a methane dependant bacteria. SE10004 was closely related to *Acidothiobacillus ferroxidans* (98%) a ubiquitously distributed chemolithotroph that derives energy from reduced sulfur compounds or by oxidising ferrous iron to ferric iron (Kelly & Wood, 2000). *A.ferroxidans* is also capable of autotrophic growth by CO_2 fixation. No γ–Proteobacteria clones were detected in sample ME2 or in DGGE analysis.

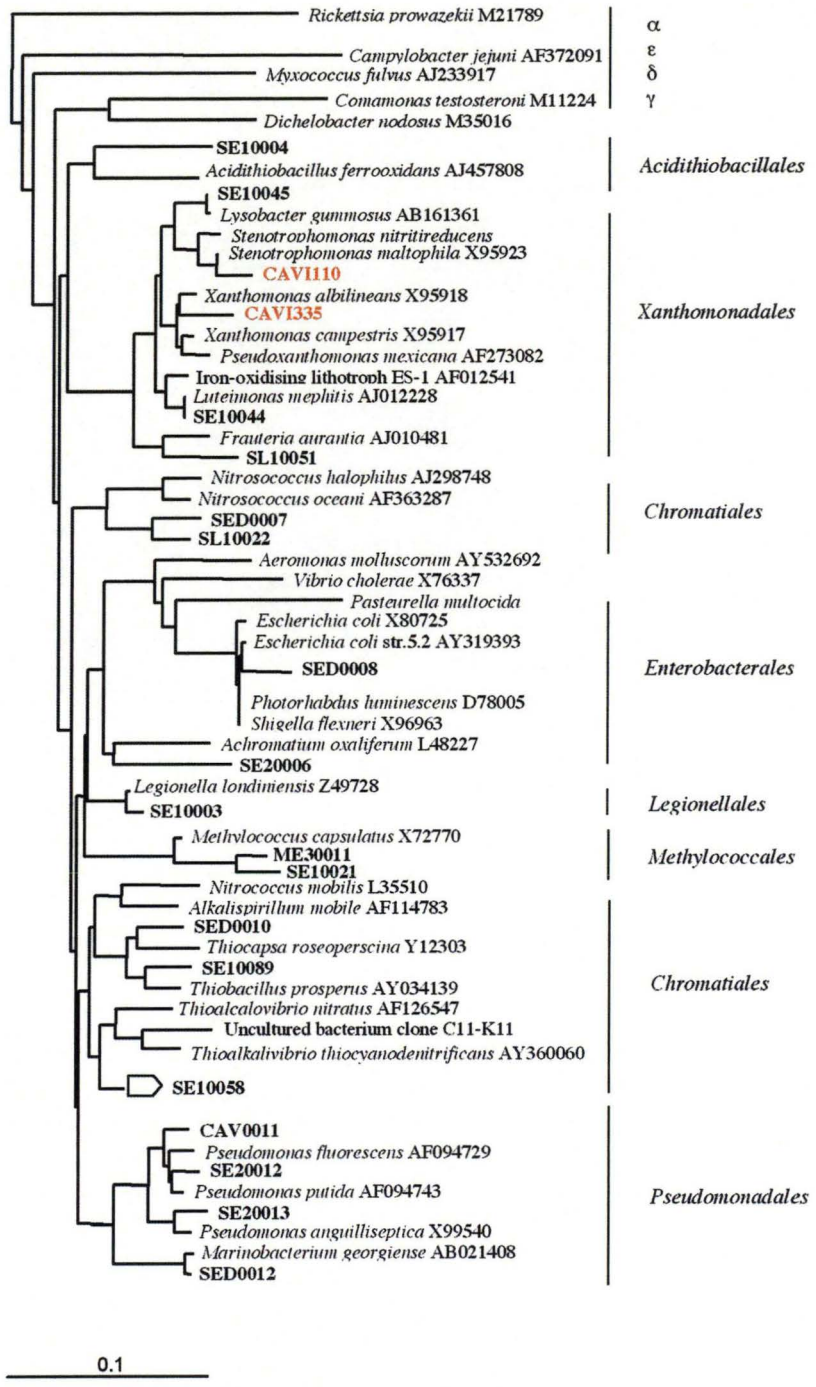


Figure 3.8: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the γ-Proteobacteria. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Thermoproteus tenax* was used as the outgroup species. The scale bar indicates 10% sequence divergence.
Colour Code: **Black** = Clone sequences, **Brown** = Isolate sequences.

Actinobacteria

The *Actinobacteria* were the second most commonly sampled group overall behind the β-Proteobacteria though not always the second most abundant group in individual libraries. Unlike the β-Proteobacteria, phylotypes affiliated with the *Actinobacteria* were detected in all sediment and moonmilk samples. A total of 85 clones were distributed among 37 phylotypes illustrating the broad diversity of *Actinobacteria* sampled in this study (Table 3.2). Particularly, the *Actinobacteria* were the second most abundant group in sediment sample SE1 and mat sample ME2, both from Entrance Cave, composing 36.6% and 26.8%, respectively, of the total sampled clonal diversity. DGGE analysis revealed two *Actinobacteria* taxa in moonmilk samples, DMON6 and DMON7 (Table 3.2). Isolations from sediments, speleothems and moonmilk samples were dominated by *Actinobacteria* resulting in cultured representatives from 14 genera, including one putatively novel genus and five putatively novel species (Table 3.3).

The *Pseudonocardineae* dominated the clone libraries and revealed great diversity. A total of ten phylotypes were detected (Figure 3.9) and were particularly abundant in calcite sample ME2 with 6 phylotypes consisting of 15 clones. Several sequences from sediment and moonmilk were affiliated with the genus *Saccharothrix* most closely related to various described species. 17% of the total diversity sampled in ME2 were affiliated with *Saccharothrix* species illustrating the dominance of this taxa in the calcite samples. DGGE analysis also revealed the presence of *Saccharothrix* sp. in calcite moonmilk samples (DMON5). *Saccharothrix* sp. were isolated from moonmilk and sediment, including *S.albidocapillate, S.cryophilus* and *S.violacea. S. violacea* is a chemoorganotrophic strict aerobe that was isolated from soils inside a gold mine cave in Korea (Lee *et al.* 2000) and has been detected in other caves (Schabereiter-Gurtner *et al.* 2002, 2004; Northup *et al.* 2003). A novel *Amycolatopsis* sp. was isolated from sediment from Entrance Cave. Clones and isolates affiliated with the genera *Micromonospora, Couchioplanes* and *Actinoplanes* were also present from sediments and moonmilk. Sequences clustering within the *Propionibacterineae* were detected in sediment and moonmilk samples (Figure 3.9). A phylotype closely related to *Propionibacterium acnes* (98%), a common human skin commensal, is probably a contaminant. Clones related to *Nocardioides fulvus* (94% sequence similarity) and *Pimelobacter*

simplex (98%) were detected in sediment and calcite samples. Members of the *Nocardioides* are oligotrophic and able to support growth on a wide variety of substrates (Yoon *et al.* 1999). A single sequence only distantly related to *Blastococcus saxobsidens* (90%) within the *Frankineae* was detected in Entrance Cave sediment. Several members of the *Frankineae* including *Blastococcus,* have been isolated from monuments. Several sequences detected in Entrance sediments were distantly related to the genus *Frankia*. *Frankia* sp., are nitrogen-fixing bacteria usually associated with plants.

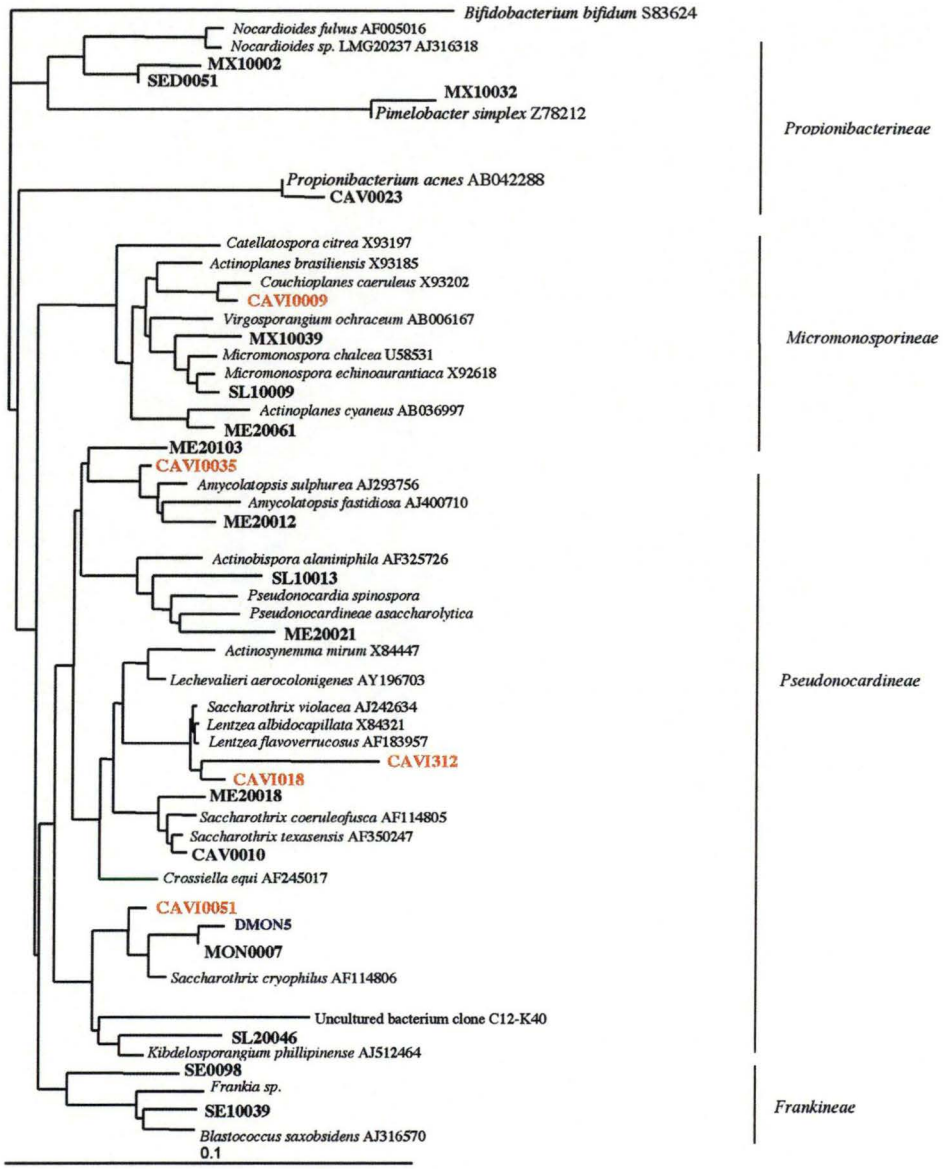


Figure 3.8: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *Actinomycetales*. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Bifidobacterium bifidum* was used as the outgroup species. The scale bar indicates 10% sequence divergence.
Colour Code: **Black** = Clone sequences, **Blue** = DGGE sequences, **Brown** = Isolate sequences

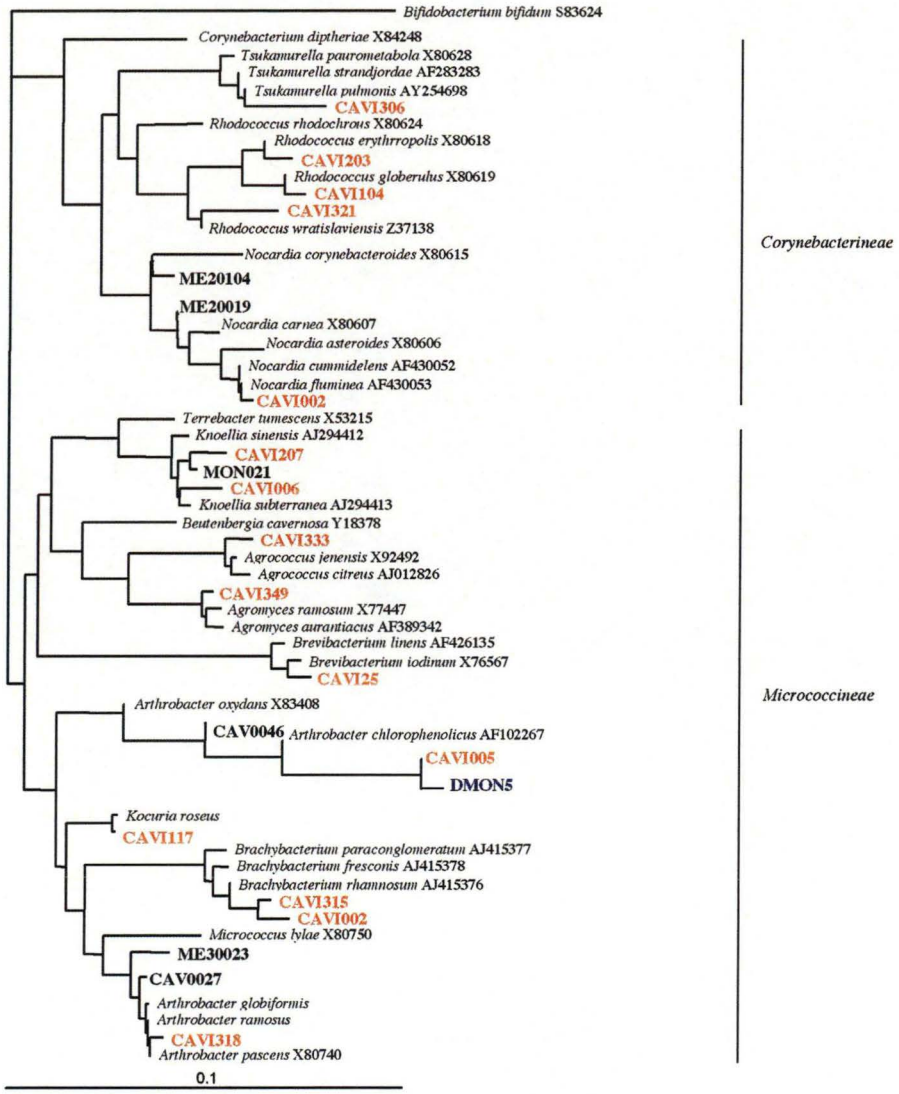


Figure 3.10: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *Micrococcineae* and *Corynebacterineae*. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Bifidobacterium bifidum* was used as the outgroup species. The scale bar indicates 10% sequence divergence. Colour Code: **Black** = Clone sequences, **Blue** = DGGE sequences, **Brown** = Isolate sequences

Figure 3.10 is a phylogenetic dendrogram of the *Microccineae* and *Corynebacterineae*. These bacteria are among the most common organisms isolated from caves. Several strains of *Arthrobacter* were isolated from cave sediment and moonmilk. One group of *Arthrobacter* moonmilk isolates were related to *Arthrobacter chlorophenolicus* (96% similarity). This is a putatively novel cave species that was also represented in DGGE analysis (DMON4). *Arthrobacter* is one of the main genera of *Micrococcineae*, and consists mainly of soil organisms.

Arthrobacter sp. are remarkably resistant to desiccation and starvation, despite not forming spores and demonstrate considerable nutritional versatility including the ability to decompose a variety of organic compounds. Members of the *Arthrobacter* have previously been observed in caves demonstrating survival by means of nitrogen fixation or the use of organic substrates as the sole source of carbon and energy, and remain resistant to prolonged periods of nutrient limitation (Barton *et al.* 2004). A phylotype very closely related to *Knoellia subterranea* (99%) was detected in sample ME3 and was also isolated from sample ME2. *Knoellia sinensis* and *Knoellia subterranea*, were recently isolated from sediment in Reed Flute Cave in China (Groth *et al.* 2002). Two phylotypes affiliated with the genus *Nocardia* were detected in sample ME2, ME20019 being almost identical to *N.carnea* (99%) and ME20104 being more distantly related to *N.corynebacteroides* (94%) perhaps representing a novel species of the *Nocardia.* Coryneform bacteria, *Nocardia* and *Rhodococcus*, are soil organisms sometimes utilising hydrocarbons. Species of these genera are known to degrade organic matter and are able to decompose environmentally hazardous chemical compounds. Several *Nocardia* and *Rhodococcus* sp. were isolated from all sediment and moonmilk samples and although *Rhodococcus* sp. were not detected in culture-independent analyses. Members of the genus *Rhodococcus* show a remarkable degree of metabolic diversity and currently are used as whole-cell biocatalysts in several industrial processes (Hughes *et al.* 1998).

Phylotypes affiliated with the *Streptomyces* were ubiquitous in cave samples (Figure 3.11). Members of the *Streptomyces* dominated isolations from sediment and moonmilk accounting for approximately 60% of isolates obtained. These isolates represented 10 species of *Streptomyces* (Table 3.3) The most common species isolated were *S. subrutilus* and *S. caviscabies*. *S. subrutilus* was detected in all sediment, speleothem and moonmilk samples and clones clustering with this lineage were detected in Entrance sediment and calcite mat material, ME2. *S. caviscabies* was isolated from all samples except for ME2. It was also detected in Loons sediment and moonmilk samples. The genus *Streptomyces* encompasses a large number of recognised species. *Streptomyces* are the most common soil bacteria along with the *Arthrobacter*. Members of the *Streptomyces* favour alkaline to neutral, well drained soils such as sandy loams or soils

covering limestone. Limestone caves and lava tube caves often contain wonderful displays of filamentous actinomycetes that may cover entire ceilings and walls of caves giving a 'silvered' appearance (similar to sample ME2). Probably many of the discrete lichen-like colonies frequently noted on walls and formations in the dark zone may be *Streptomyces* species since they often have the powdery appearance and characteristic earthy odour common to cultures of this genus. Several of the isolates from sediments and moonmilk in this study had this powdery appearance and earthy odour. It has also been suggested that the abundant *Streptomyces* in caves is probably responsible for the earthy smell of caving (Caumartin, 1963 in Ford & Cullingford, 1976).

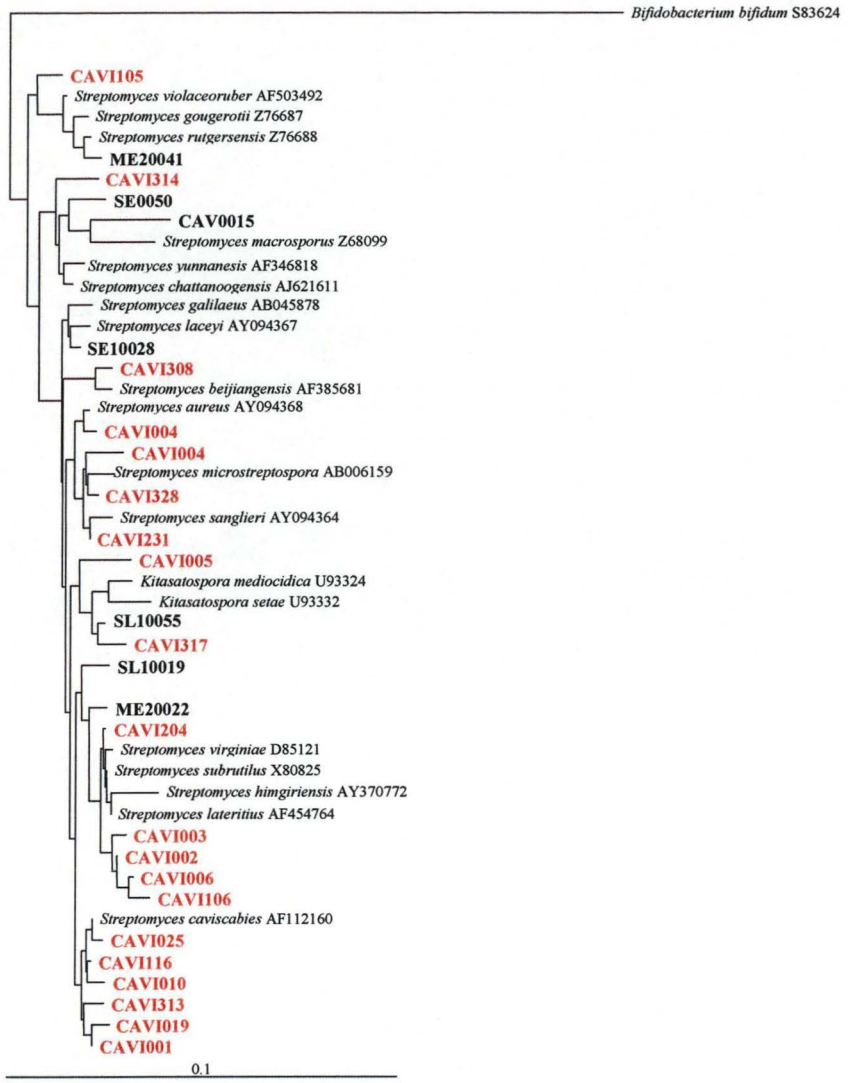


Figure 3.11: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *Streptomycineae*. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Bifidobacterium bifidum* was used as the outgroup species. The scale bar indicates 10% sequence divergence.
Colour Code: **Black** = Clone sequences, **Brown** = Isolate sequences.

Four phylotypes were detected clustering within the *Actinobacteria* but not affiliated with the *Actinomycetales*. Figure 3.12 is a phylogenetic dendrogram of *Actinobacteria* subclasses *Rubrobacterideae* and *Sphaerobacterideae* and unclassified *Actinobacteria*. Described members of the *Rubrobacterideae* are largely thermophilic (eg. *Thermoleophilum minutum, Thermoleophilum album, Rubrobacter radiotolerans*). Two clones, from moonmilk samples, were loosely affiliated with members of the thermophilic genus *Thermoleophilum* (89-93%) forming a monophyletic radiation within the *Rubrobacterideae* and perhaps representing cold-adapted members of this taxa. The *Rubrobacterideae* are a broad monophyletic group within the *Actinobacteria* consisting of to date largely uncultivated organisms (Rheims *et al.* 1996). Culture-independent studies have detected members of this group as ubiquitous and an ecologically significant radiation of the *Actinobacteria*, inhabiting a diverse array of environments including peat bog (Rheims *et al.* 1996), forest soil (Liesack & Stackebrandt, 1992), geothermal soil (Fuhrman *et al.* 1993), paddy and soybean fields (Ueda *et al.* 1995) and marine habitats (Fuhrman *et al.* 1993). Within the unclassified *Actinobacteria*, 2 clones from Entrance sediment, SE1, were distantly related to Candidatus *Microthrix parvicella* (91%). *Microthrix parvicella* is a filamentous organism isolated from an activated sewage treatment plant. One sequence, also from SE1, was loosely affiliated with thermophile *Sphaerobacter thermophilus*.

As confirmed in this study, actinomycetes are the most common and abundant group isolated from caves samples and are detected consistently, though in moderate numbers, in culture-independent studies. *Streptomyces* species are particularly abundant and in some cases, can be found as apparently monospecific colonies (Arroyo & Arroyo, 1996). A number of actinomycetes isolated from caves have the ability to produce various types of crystals. Studies in Altamira and Tito Bustillo Caves demonstrate that the host-rock (bedrock), cave formations and rock art paintings are coated by dense networks of bacteria, mainly actinomycetes and these bacteria can induce constructive (calcification, crystalline precipitates) and destructive (irregular etching, spiky calcite) fabrics. Because of this ability it has been proposed that these bacteria and others are directly or indirectly involved in constructive biomineralisation processes in caves (Laiz *et al.* 1999; Barton *et al.* 2001; Canaveras *et al.* 2001; Groth *et al.* 2001; Jones, 2001). Little is

known concerning the distribution, population dynamics, growth rates and biogeochemical processes of *Actinobacteria* in caves, in spite of the fact that they seem to constitute a significant part of the "culturable" microbial population of these habitats. A prerequisite for the study of the role of actinomycetes in biogeochemical processes is the isolation and identification of these organisms (Groth *et al.* 1999a).

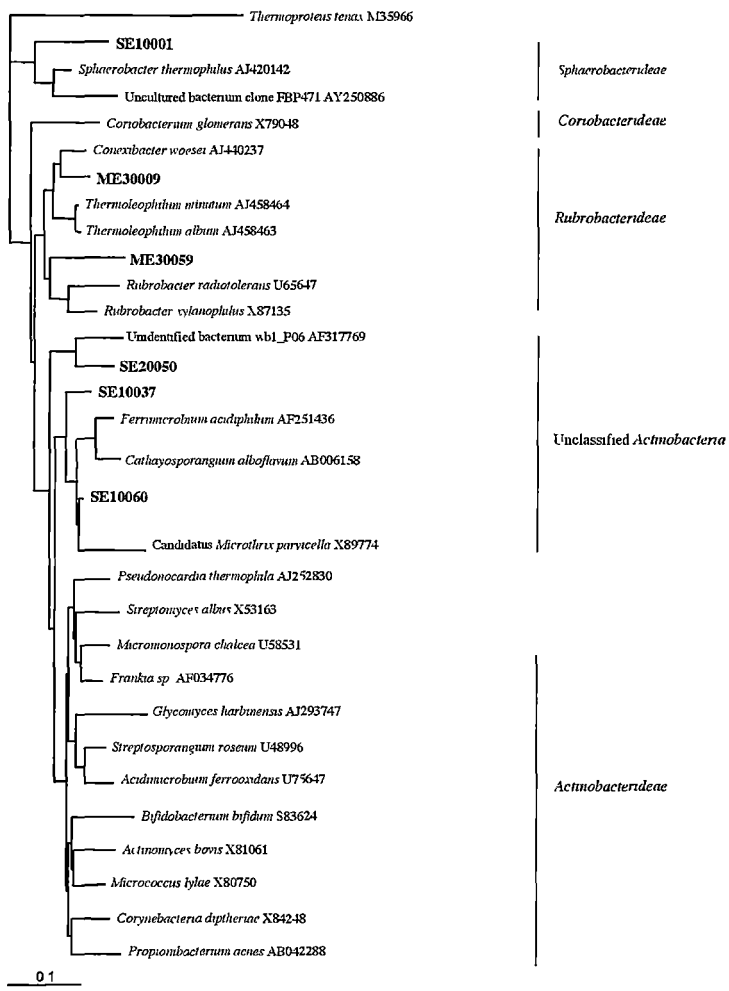


Figure 3.12: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *Actinobacterideae* The dendrogram was constructed from an alignment of 1000 nucleotide positions Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method *Thermoproteus tenax* was used as the outgroup species The scale bar indicates 10% sequence divergence

Firmicutes

Few clones in this study were affiliated with the *Firmicutes* (low G+C Gram-positive bacteria). A total of five sequences distributed in three phylotypes were detected (Table 3.2). In contrast, 17 moonmilk isolates distributed across seven strains were identified as members of the genera *Bacillus, Paenibacillus* and *Sporosarcina*. Figure 3.13 illustrates the phylogenetic relationships of phylotypes and cave isolates to cultivated members of the *Firmicutes*. Two phylotypes were detected in moonmilk samples. MX10063 was closely related (99%) to *Bacillus subtilus*, also isolated from samples ME3 and MX1. Remaining *Bacillus* species isolated include *B. simplex, B. pumilus, B. indicus*, and *B. mycoides*, cultured from all moonmilk samples (Figure 3.13). *Bacillus* sp. are aerobic, endospore forming and mainly found in soil. ME20098 was affiliated with *Sporosarcina ureae* and strains of this microbe were isolated from sample ME2 and ME3. Members of the genus *Sporosarcina* are strictly aerobic. *S. ureae* is common in soils with urea input and is perhaps an important ecological degrader of urea. A single phylotype affiliated with *Firmicutes* was detected in sediment, SL10009, showing a distant relationship (91%) to *Ruminococcus flavescians*, usually detected as a symbiont in the gut of animals.

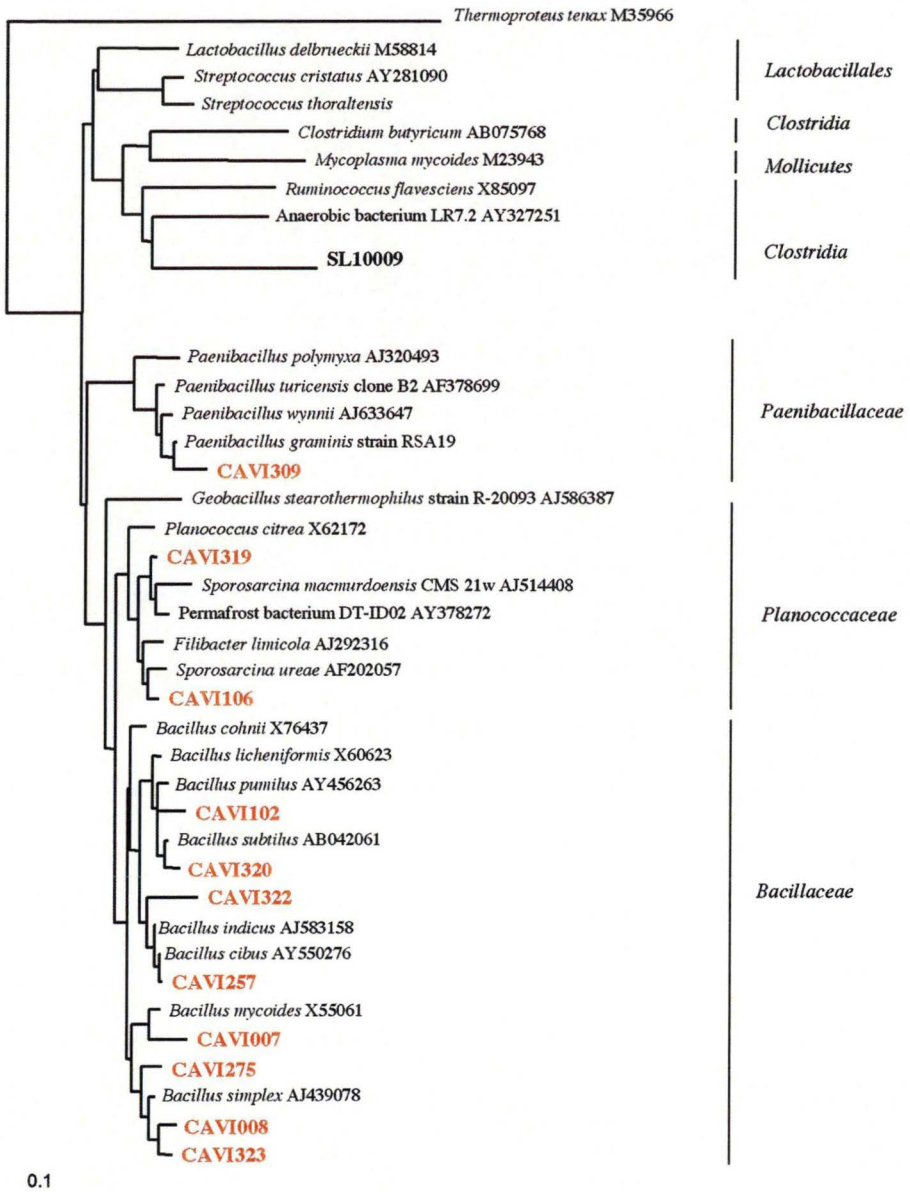


Figure 3.13: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *Firmicutes*. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Thermoproteus tenax* was used as the outgroup species. The scale bar indicates 10% sequence divergence.
Colour Code: **Black** = Clone sequences, **Brown** = Isolate sequences.

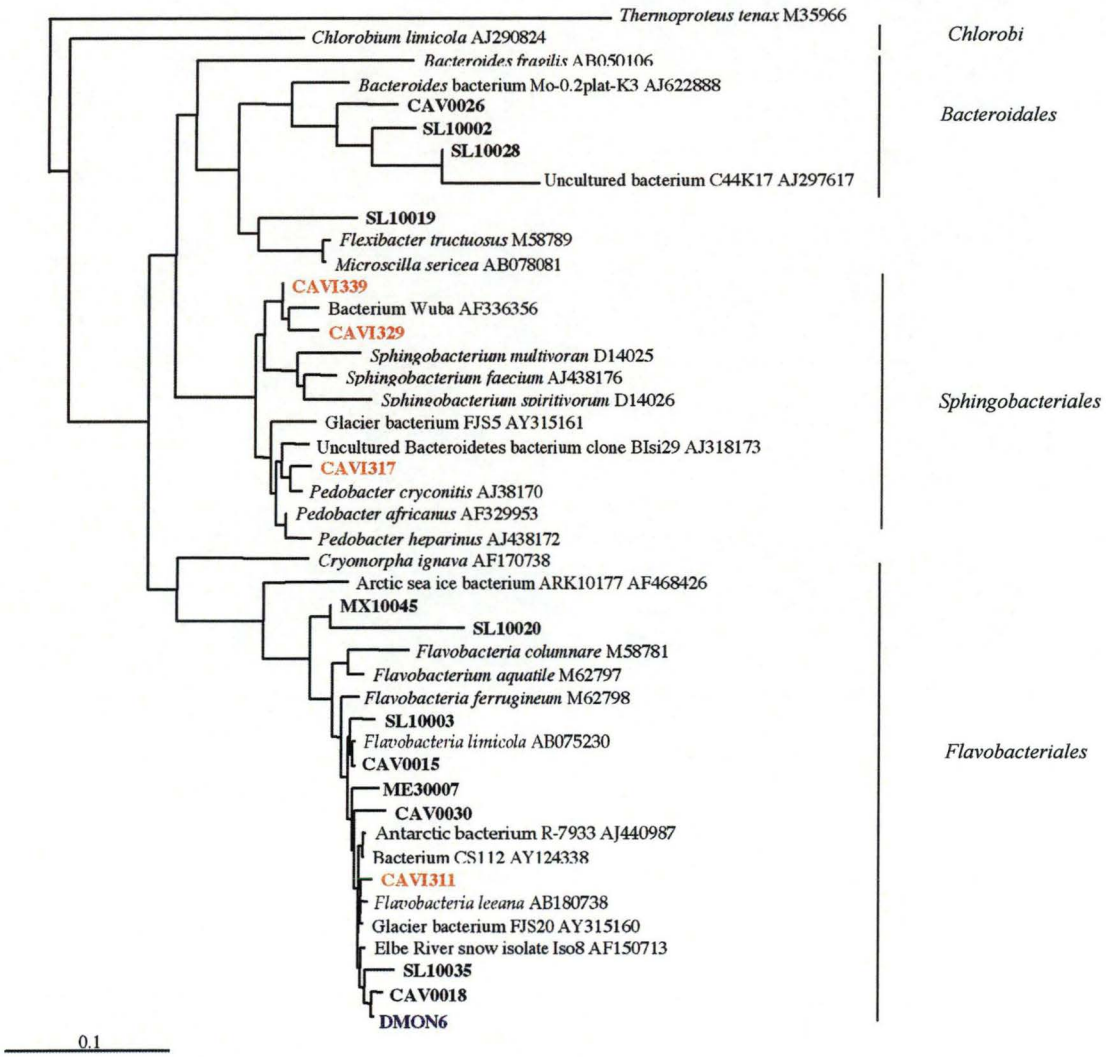


Figure 3.14: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *Cytophaga-Flexibacter-Bacteroides* group. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Thermoproteus tenax* was used as the outgroup species. The scale bar indicates 10% sequence divergence.
Colour Code: **Black** = Clone sequences, **Blue** = DGGE sequences, **Brown** = Isolate sequences.

CFB Group

A total of 65 clones distributed in 14 phylotypes were affiliated with the CFBs (Table 3.2). The CFBs were the second most abundant major phyla detected in moonmilk samples ME3 and MX1. Several of these sequences clustered with psychrophilic *Flavobacteriaceae* (Figure 3.14) that are represented by various aerobic and heterotrophic genera. Several sequences from both samples ME3 and MX1 were closely related to *Flavobacteria limicola* (98%) a psychrophilic, organic polymer degrader (Tamaki *et al.* 2003). This phylotype was present in DGGE analysis

and represented 10-12% of the total diversity sampled in the moonmilk clone libraries, demonstrating its dominance in these habitats. A novel *Flavobacteria* sp. was isolated from moonmilk sample ME3 clustering with the *F. leeana*-like sequences. A single sequence MX10045 showed distant similarity (92%) to *Cryomorpha ignava* a cold-adapted, strict aerobe isolated from Antarctic quartz stone subliths (Bowman *et al.* 2003). Phylotypes ME30011 and MON0015 from moonmilk clustered with psychrophilic members of the *Sphingobacteriales*, genera *Sphingobacterium* and *Pedobacter* (Figure 3.14). Phylotype ME30011 represented by three clones was related to *Pedobacter cryconitis*, a facultative psychrophile isolated from an alpine glacier (Margesin *et al.* 2003). Phylotype MON0015 was affiliated with *Sphingobacterium faecium*. A number of uncultured glacier and sub-glacial sediment clones (FJS and FX clone groups) clustered with moonmilk phylotypes identified in this study, inferring the presence of cold-adapted taxa in these samples.

Three phylotypes clustered within the *Bacteroides* group. SL0019 branched with *Flexibacter tructuosus* (92% sequence similarity). Phylotype CAV0026, detected in sediment and moonmilk samples from Entrance Cave, is distantly related to uncultured Bacteroides bacterium Mo-0.2plat-K3, detected in freshwater. Phylotype SL10028 was not closely affiliated with any described taxa, however it clustered with a group of uncultured bacterial clones from Palaeolithic rock art in Spanish and Italian caves within the *Bacteroides* clade (Figure 3.14). The *Bacteroides* group includes a mixture of physiological types such as strictly anaerobic *Bacteroides* and aerobic gliding bacteria such as *Flexibacter*. Bacteria with gliding motility have no flagella but are able to move when in contact with surfaces.

Acidobacteria

A total of 11 clones affiliated with the *Acidobacteria* were detected in the cave samples. Most clones form a monophyletic clade within sub-Phylum A of the *Acidobacteria* showing varying degrees of similarity to uncultured bacterium DA008 (90-94%), a clone from grassland soils (Figure 3.15). These sequences were retrieved from moonmilk samples, particularly sample ME2 (5 clones). The remaining sequences were affiliated with Ellin isolates from Australian soils

(Sait *et al.* 2002; Joseph *et al.* 2003) within sub-Phyla C and D. Though the Ellin group represents cultured members, these have not been described to date thus no information is available about their physiology or metabolism. The *Acidobacteria* are a relatively cosmopolitan group, widely distributed in the environment though in general are highly correlated with the soil habitat. (Hugenholtz *et al.* 1998). The division was defined by Ludwig *et al.* (1997) on the basis of cloned 16S sequences from soil, freshwater sediments and activated sludge in many geographic locations and its members are thought to be ecologically significant in many ecosystems. However it is a poorly studied division thus far, consisting of only a few cultured representatives: *Acidobacterium capsulatum* an acidophilic chemoorganotroph from acid mineral environment (Kishimoto *et al.* 1991), *Geothrix fermentans* an iron-reducing bacteria from a hydrocarbon contaminated aquifer (Coates, 1999), and *Holophaga foetida* a homoacetogenic bacterium degrading methoxylated aromatic compounds (Liesack *et al.* 1994).

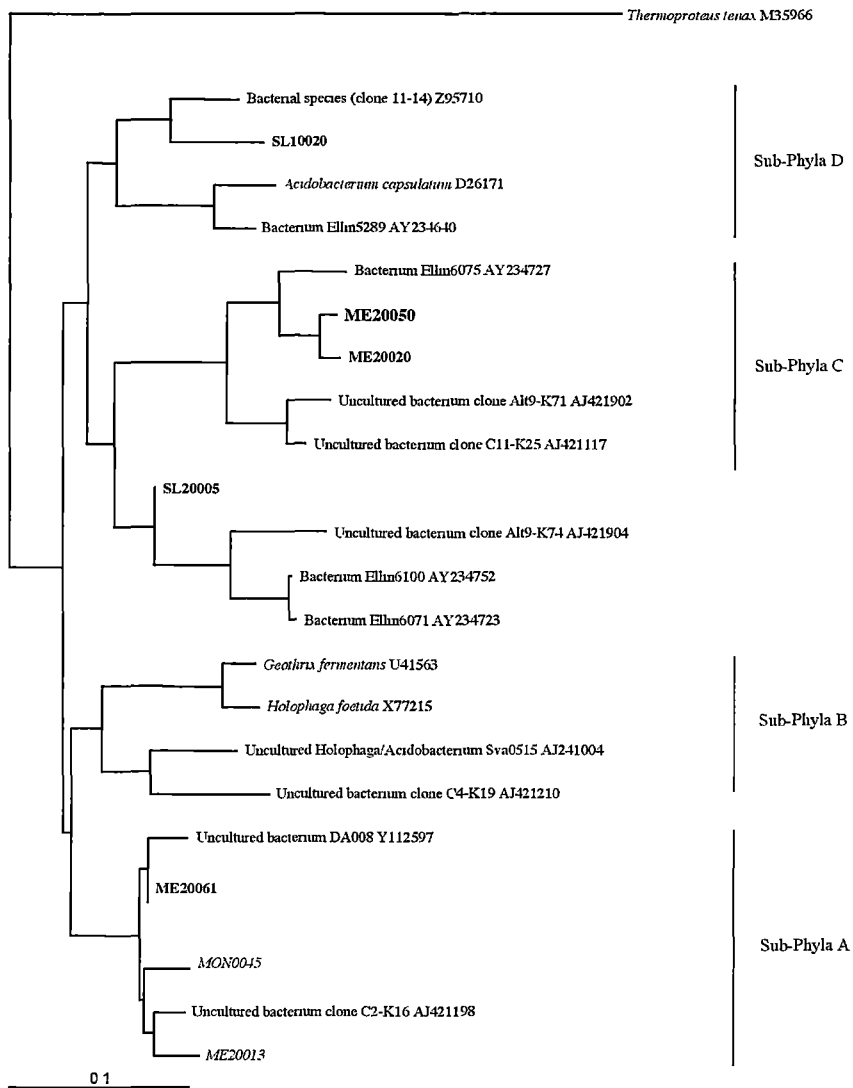


Figure 3.15: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *Acidobacteria* The dendrogram was constructed from an alignment of 1000 nucleotide positions Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method *Thermoproteus tenax* was used as the outgroup species The scale bar indicates 10% sequence divergence

Planctomycetales

A total of 31 clones affiliated with the *Planctomycetales* were detected in sediment and moonmilk samples (Figure 3.16). The majority of these clones belonged to six deeply branching phylotypes within the genera *Planctomyces, Pirellula* and *Gemmata,* showing distant (87.2-90% similarity) relationships to cultured members. This is not suprising as the intralineage phylogenetic depth of the *Planctomycetales* was recently expanded to 30.6% (Chouari *et al.* 2003). One phylotype (SED0047) detected in all sediments, was closely related to *Pirellula staleyi* (96%). Four cultured genera, consisting of seven species overall, have been described to date, *Planctomyces, Pirellula, Gemmata* and *Isophaera* (eg. Schlesner, 1986; Giovannoni *et al.* 1987; Schlesner 1989). All these organisms are aerobic chemoheterotrophs. Knowledge of this group is limited because of the relatively few species that have been obtained in pure culture. Membership of the planctomycete group has been extended not only to chemoorganotrophs and obligate or facultative aerobes but also to obligate anaerobes, autotrophs and phototrophs, demonstrating diverse metabolic properties within this line of descent (Fuerst, 1995; Miskin *et al.* 1999). For example, a planctomycete was found to be responsible for anaerobic oxidation of ammonia (Strous *et al.* 1999). All *Planctomycetales* were originally isolated from aquatic habitats as diverse as acid bogs and sewage treatment plants though culture-independent studies have revealed the presence of *Planctomycetales* in more diverse environments including marine, sediment, anoxic bioreactors, anoxic sediments and caves (DeLong *et al.* 1993; Godon *et al.* 1997; Holmes *et al.* 2001; Tay *et al.* 2001; Chouari *et al.* 2003). The *Planctomycetales* were a significant component of Entrance Cave dry sediment being the third most abundant group detected in sample SE1 (22.5%) whereas in all other samples they constituted a relatively minor component of the community (1-8%).

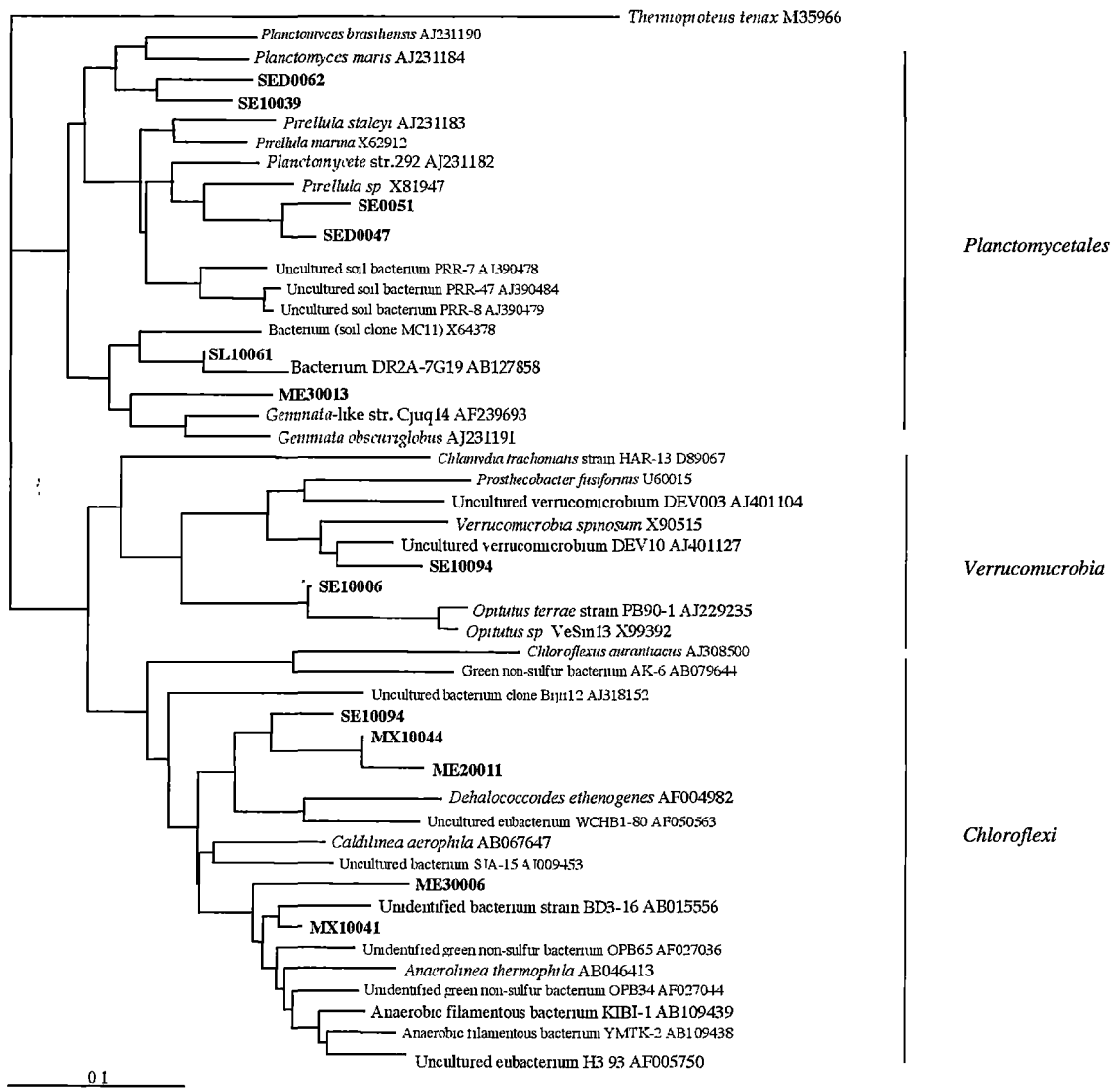


Figure 3.16: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *Planctomycetales*, *Verrucomicrobia* and *Chloroflexi* divisions The dendrogram was constructed from an alignment of 1000 nucleotide positions Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method *Thermoproteus tenax* was used as the outgroup species The scale bar indicates 10% sequence divergence

Chloroflexi (green non-sulfur bacteria)

Clones affiliated with the *Chloroflexi* (green non-sulfur) bacteria were detected in Entrance Cave sediments and moonmilk samples (Figure 3.16). Members of the *Chloroflexi* are filamentous and exhibit gliding motility. *Chloroflexus*, though a phototroph, can grow chemoorganotrophically in the dark under aerobic conditions. Many members of this group digest cellulose or chitin and are widespread in soil and water. Two phylotypes detected in moonmilk samples (ME30006 and MX10041) were deeply branched within an, until recently,

uncultivated lineage of *Chloroflexi* bacteria, sub-phyla I. Sekiguchi *et al.* (2003) described *Anaerolinea thermophila* and *Caldilinea aerophila*, thin filamentous thermophilic microbes found in abundance in methanogenic granular sludges, representing this lineage. Sub-phyla I is the most diverse of divisions in the *Chloroflexi* with sequences derived from hot springs, subsurface environments, aerobic and anaerobic waste water treatment sludges and contaminated aquifers, which hints at its ecological and physiological breadth (Chandler *et al.* 1998; Hugenholtz *et al.* 1998; Sekiguchi *et al.* 2003). A novel lineage represented by two phylotypes detected in moonmilk samples (ME20011 and MX10044) forms a monophyletic clade with sub-phyla II representatives. *Dehalococcoides ethenogenes* an anaerobe isolated from activated sludge, able to reductively dechlorinate tetrachloroethane, a common contaminant of groundwater is the most closely related cultured representative of this group (Maymo-Gatell *et al.* 1999).

Verrucomicrobia

Two sequences from the Entrance Cave dry sediment sample (SE10094 and SE10006) were phylogenetically affiliated with the *Verrucomicrobia* (Figure 3.16). The division *Verrucomicrobia* contains very few cultured representatives but a large number and diverse range of clones from extremely diverse environments including forest soil (Liesack & Stackebrandt, 1992), soybean and rice paddy fields (Ueda *et al.* 1995); lake (Hiorns *et al.* 1997); and marine (Fuhrman *et al.* 1993) environments. This diversity prompted Hugenholtz *et al.* (1998) to declare they represent a ubiquitous branch of the domain *Bacteria*. SE10094 was most closely related to uncultured verrucomicrobium clone DEV10 (92% sequence similarity). The only cultured member of this lineage is *Verrucomicrobium spinosum* (86.5% sequence similarity to SL10094), an aerobic oligotrophic and chemoheterotrophic prosthecate bacteria (Staley, 1968). SE10006 was phylogenetically affiliated with a relatively newly described genus of the *Verrucomicrobia*, *Opitutus* (Chin *et al.* 2001). SE10006 was most closely related to *Opitutus* sp. VeSm13 (91.8%) a novel obligately anaerobic ultramicrobium isolated from anoxic rice paddy soil (Janssen *et al.* 1997).

The *Verrucomicrobia* are often a numerically abundant component of soil microbial communities; Buckley & Schmidt (2001) found that the *Verrucomicrobia* contributed ~1.9 % of diversity in 85 soil samples analysed. However no clones affiliated with *Verrucomicrobia* were detected in sediment samples SE2, SL1 or SL2, or any moonmilk samples. Statistically significant variations in verrucomicrobial rRNA gene abundance can be explained by changes in soil moisture content (Buckley & Schmidt, 2001), perhaps explaining the absence of *Verrucomicrobia* in the more saturated sediments and moonmilk samples in this study.

OP10

Phylotypes SL20004 and SL20017 were affiliated with Candidate division OP10 (Figure 3.17). Clone analysis of sediments from Opal Pool, a hot spring in Yellowstone National Park, yielded representatives of 12 novel lineages designated the OP 1-12 Candidate Divisions (Hugenholtz, 1998). The Loons Caves phylotypes are deep branching representing putatively novel lineages. SL20004 is most closely related to uncultured bacterium SJA-176 (86%) and SL20017 is most closely related to uncultured bacterium GC55 (90.2%) detected from a full-scale activated sludge plant (Dalevi *et al.* 2001). Candidate Division OP10 consists entirely of environmental sequence data with no reported cultivated members to date thus nothing is known of their metabolic or physiological activities. OP10 phylotypes have been detected in hydrocarbon contaminated soil suggesting that this lineage may represent an ecologically significant group (Hugenholtz *et al.* 1998).

Gemmatimonadetes

Two phylotypes, SL20096 (three clones) and SL20036, detected in Loons Cave sediment samples were phylogenetically affiliated with the *Gemmatimonadetes* (Figure 3.17). The *Gemmatimonadetes* is a new phylum consisting of one described species, *Gemmatimonas aurantiaca* (Zhang *et al.* 2003) and numerous environmental sequences. *G. aurantiaca* is a Gram-negative aerobic polyphosphate-accumulating microbe. Despite there being only one described species, recently Joseph *et al.* (2003) were able to isolate a number of *Gemmatimonadete* species from

Australian soils (Ellin isolates). Prior to the description of *G. aurantiaca* the environmental clones in this phylum were designated the candidate division BD and have been found in soils and activated sludge (Hugenholtz *et al.* 2001), deep sea sediments (Li *et al.* 1999) and Antarctic sediment (Bowman & McCuaig, 2003). SL10036 and SL20033 were only distantly related to *G. aurantiaca* (86.5-87.5% sequence similarity) and formed a deeply branching monophyletic clade within the *Gemmatamonadetes* most likely representing a new group. This low sequence identity is not uncommon in this phylum. Environmental sequence data suggests that members of this phylum are widespread in nature and have a phylogenetic breadth (19% 16S rRNA gene sequence divergence) that is greater than well-known phyla such as the *Actinobacteria* (18% divergence) (Zhang *et al.* 2003).

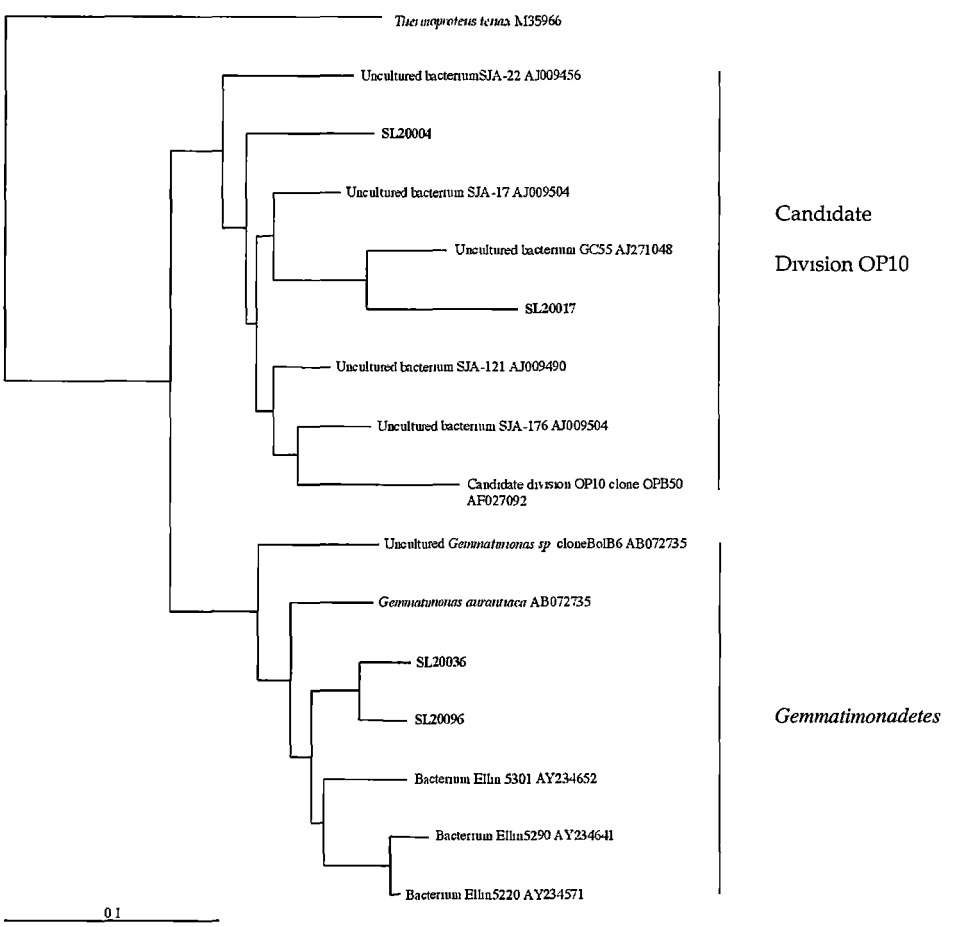


Figure 3.17: Phylogenetic dendrogram illustrating the evolutionary relationship between cave taxa and members of the *Gemmatimonadetes* and Candidate Division OP10. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Thermoproteus tenax* was used as the outgroup species. The scale bar indicates 10% sequence divergence.

Nitrospira

One phylotype from saturated Loons sediment, SL20060, was similar to *Nitrospira* sp. All known members of *Nitrospira* are obligate nitrite-oxidising chemolithoautotrophs (Ehrlich *et al.* 1995). Sequence information for clone SL20060 was only 300 bp in length and not included in further phylogenetic analysis.

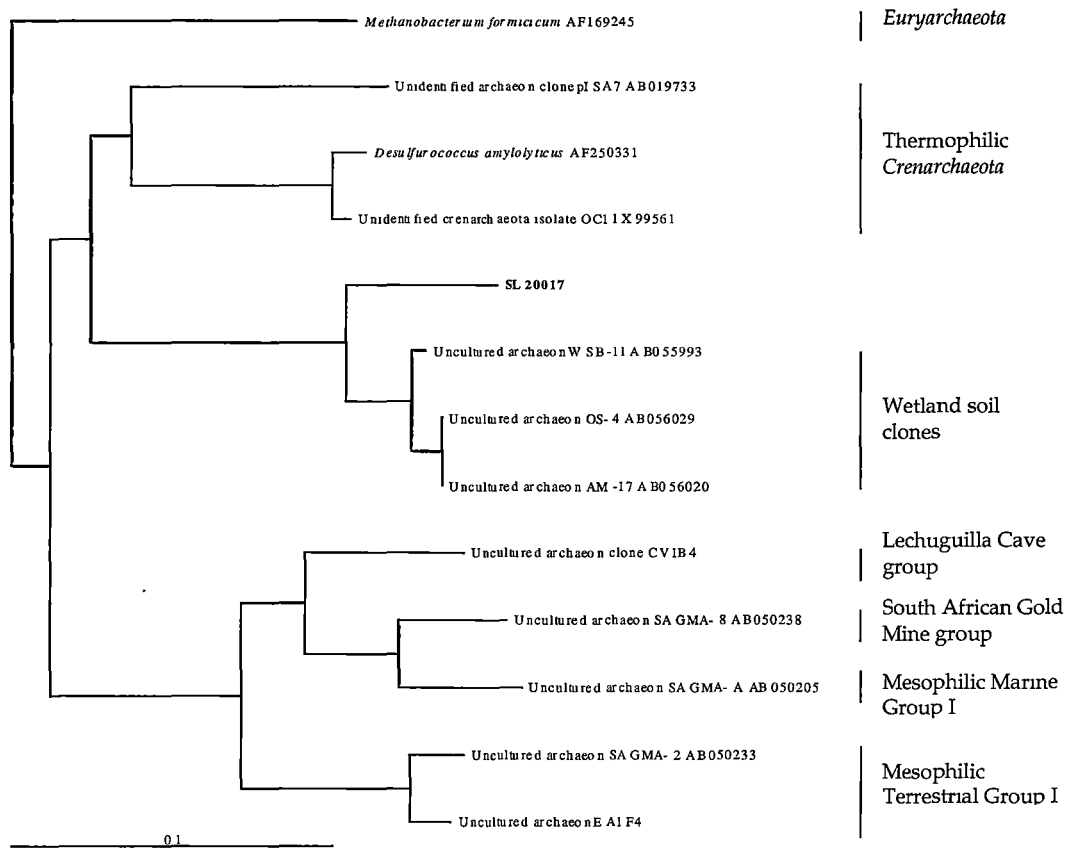


Figure 3.18: Phylogenetic dendrogram illustrating the evolutionary relationship between cave clone SL20017 and members of the *Crenarchaeota*. The dendrogram was constructed from an alignment of 1000 nucleotide positions. Distances were calculated in DNADIST and trees were inferred by the neighbour-joining method. *Methanobacterium formicicum* was used as the outgroup species. The scale bar indicates 10% sequence divergence.

Archaea

One archaeal clone sequence, SL20017, was detected from saturated sediment from Loons Cave (Figure 3.18). SL20017 is a deeply branching novel lineage only distantly related to cultured *Crenarchaeota, Desulfurococcus amylolyticus*; (75% sequence similarity) and most closely related to uncultured archaeon clones, WSB-11 (91.8%), OS-4 and AM-17 from wetland soils (published only in datebase; Utsumi *et al.* 2001). All cultured *Crenarchaeota* including, *Desulfurococcus amylolyticus*, are extreme thermophiles found in high temperature environments (>80 °C). In recent years, nonthermophilic *Crenarchaeota* sequences have been detected from low to moderate temperature (1.5 to 32 °C) terrestrial and aquatic environments (-1.5 to 32 °C). Mesophilic *Crenarchaeota* sequences were first reported from the Pacific Ocean (Fuhrman *et al.*

1992). To date there have been no reports of successful laboratory pure cultivation of mesophilic *Crenarchaeota* and nothing is known of their physiology and biochemistry (Northup *et al.* 2003). There are four main clusters of mesophilic *Crenarchaeota* (Marine Group I, Freshwater cluster, Terrestrial Group and FFSB cluster) that form a distinct lineage from thermophilic *Crenarchaota*. Recently it has been proposed that a fifth cluster within the mesophilic *Crenarchaeota* clade is distinct and unique to the subsurface environment (three clones from South African gold mine waters; SAGMA clones) (Takai *et al.* 2001).

Clone SL20017 and the wetland soil clones do not branch with mesophilic sequences but rather form a separate clade more closely related to thermophilic *Crenarchaeota*. Almost all of the cultivated thermophilic *Crenarchaeota* are obligate anaerobes with sulfur-dependent metabolisms (Buckley *et al.* 1998). The great phylogenetic distance of SL20017 to any cultured members of the *Crenarchaeota* makes it difficult to infer any metabolic properties of this clone.

There have been few reports of archaeal cave communities at circumneutral pH, though those reported are suprisingly abundant and diverse including representatives of mesophilic *Crenarchaeota* most closely related to organisms from marine or soil habitats, and 'Group 2' *Euryarchaeota* (Mattison *et al.* 1998; Northup *et al.* 2003; Chelius & Moore, 2004). Overall, reported *Crenarchaeota* sequences detected in cave environments cluster with those found in waters from a South African gold mine (SAGMA clones) (Takai *et al.* 2001) even though they have been detected from very different microhabitats, ferromanganese corrosion residues in Lechuguilla Cave, New Mexico (Northup *et al.* 2003) and saturated sediment from Wind Cave, South Dakota (Chelius & Moore, 2004). SL20017 does not align closely with any SAGMA or cave archaeal sequences. To draw too many conclusions on archaeal diversity on the basis of one clone sequence is too presumptive. This study would benefit from further culture-independent investigations employing *Archaea*-specific primers, as used in the previously mentioned cave studies, to target the archaeal portions of the microbial community.

Table 3.3: Taxonomic affiliations of cave isolates as determined by 16S rRNA gene sequencing. Presence of an isolate in a microhabitat represented as (+)

Isolate	Taxonomic Identification or Nearest Cultivated Neighbour for Putatively Novel Species (%16S rRNA gene sequence similarity)	SEE	SPE	SEL	SPL	ME2	ME3	MX1*
	ACTINOBACTERIA							
	Micrococcineae							
CAVI333	*Agrococcus jenensis* (98.43%)							+
CAVI349	*Agromyces ramosum* (98.79%)						+	
CAVI005	*Arthrobacter chlorophenolicus* (97.5%)	+	+	+	+	+	+	+
CAVI318	*Arthrobacter pascens* (99.72%)	+				+	+	
CAVI315, 002	*Brachybacterium paraconglomeratum* (98.69%)						+	+
CAVI125	*Brevibacterium iodinum* (98.65%)						+	
CAVI207	*Knoellia sinensis* (98.40%)	+	+				+	+
CAVI006	*Knoellia subterranean* (98.34%)		+			+		+
CAVI117	*Kocuria rosea* (98.53%)						+	
	Corynebacterineae							
CAVI002	*Nocardia flumina* group (99.68%)	+	+	+	+	+	+	+
CAVI203	*Rhodococcus erythropolis* (99.42%)							
CAVI104	*Rhodococcus globerulus* (99.27%)						+	+
CAVI321	*Rhodococcus wratislavensis* (98.51%)					+		
CAVI306	*Tsukamurella pulmonis* (98.00%)						+	
	Pseudonocardineae							
CAVI0035	*Amycolatopsis* sp. nov. (*A. sulphurea* 96.90%)	+						
CAVI018	*Saccharothrix* sp. nov. (*S albidocapillata* 96.89%)					+		
CAVI0051	*Saccharothrix cryophilus* (98.23%)		+			+		+
CAVI312	Gen. Nov. [*Saccharothrix violacea*(92.94%); *Lentzea flavoverrucosispora* (92.93%)]						+	
	Micromonosporineae							
CAVI0009	*Couchioplanes caeruleus* (96.65%); *Actinoplanes brasiliensis* (96.95%)	+						
CAVI0023	*Micromonospora* sp. nov. (*M purpureochromgenes* 96.82%)	+						
	Streptomycineae							
CAVI004	*Streptomyces aureus* (99.50%)				+			
CAVI308	*Streptomyces beijiangensis* (99.15%)						+	
CAVI025, 116, 010,	*Streptomyces caviscabies* (99.08-99.82%)	+	+		+		+	+
CAVI313, 019, 001	*Streptomyces caviscabies* (97.76%)	+		+			+	
CAVI314	*Streptomyces chattanoogensis* (98.34%)						+	
CAVI004	*Streptomyces* sp. nov. (*S.clavuligerus* 97.37%)		+					
CAVI328	*Streptomyces microstreptospora* (99.10%)						+	
CAVI231	*Streptomyces sanglieri* (98.43%)					+		
CAVI204, 003, 002, 006, 106	*Streptomyces subrutilus* (98.02-99.86%)	+	+	+	+	+	+	+
CAVI105	*Streptomyces violceoruber* (98.18%)							
CAVI005, 317	*Kitasatospora mediocidica* (97.57%)	+					+	
	Firmicutes							
CAVI322	*Bacillus* sp. nov. (*B cohnii* 94.62%)						+	
CAVI102	*Bacillus* sp nov. (*B. pumilus* 97.5%)							+
CAVI257	*Bacillus cibus* (99%)					+		
CAVI007	*Bacillus mycoides* (97.5%)				+			
CAVI008, 323, 275	*Bacillus simplex* (98.25%)				+	+	+	+
CAVI320	*Bacillus subtilus* (98.75%)						+	
CAVI309	*Paenibacillus* sp. nov. (*P. graminis* 97.47%)							+
CAVI319	*Sporosarcina* sp. nov. (*S. macmurdoensis* 97.34%)						+	
CAVI106	*Sporosarcina ureae* (98.93%)							+
	CFBs							
CAVI311	*Flavobacterium leeana* (97.35%)						+	+
CAVI120	*Flavobacterium psychrolimnae* (98.20%)						+	+
CAVI329	Gen. Nov. [*Sphingobacterium multivorum* (94.18%); *Pedobacter cryconitis* (95.15%)]						+	
CAVI339	Gen. Nov. [*Sphingobacterium multivorum* (95.90%), *Pedobacter cryconitis* (96.49%)]						+	
CAVI317	*Pedobacter cryconitis* (98.36%)						+	
	α-Proteobacteria							

Continued on next page

CAVI334	*Aminobacter niigataensis* (99.31%)			+	
CAVI210	*Bosea thiooxidans* (99.67%)		+		
CAVI302	*Brevundimonas alba* (99.07%)		+	+	
CAVI326	*Methylobacterium* sp. nov. (*M. fujisawaense* 95.45%)			+	+
CAVI005	*Sphingomonas* sp. nov. (*S. aerolata* 97.34%)	+			
CAVI322	*Sphingomonas* sp. nov. (*S. melonis* 96.03%)			+	
CAVI008	*Paracoccus* sp. nov. (*P solventivorans* 96.13%)	+			
CAVI338	*Porphyrobacter* sp. nov. (*P nuestonensis* 97.20%)			+	
	β-Proteobacteria				
CAVI109	*Acaligenes* sp. nov. (*A. faecalis* 96.05%)			+	+
CAVI111	*Acaligenes* sp. nov. (*A. faecalis* 97.22%)			+	
	γ-Proteobacteria				
CAVI110	*Stenotrophomonas* sp. nov. (*S maltophila* 97.21%)			+	
CAVI335	*Xanthomonas* sp. nov (*X campestris* 95.12%)			+	

* Microhabitats represented by samples: SEE (sediment, Entrance Cave), SPE (speleothems, Entrance Cave), SEL (sediment, Loons Cave), SPL (speleothems, Loons Cave), ME2 (calcite mat, Entrance Cave), ME3 (moonmilk, Entrance Cave), and MX1 (moonmilk, Exit Cave).

3.4 *Isolation of Novel Cave Microbes*

Cultures were isolated from sediment, speleothem and moonmilk samples from Entrance-Exit Caves and Loons Cave to investigate culturable diversity (discussed in Section 3.2 and 3.5) and to determine the novelty of cave microbes. Caves are unique ecosystems exposed to extreme environmental stresses. The limiting environmental characteristics of caves, little or no light, low levels of organic nutrients, high mineral concentrations and a stable microclimate, provide ecological niches for highly specialised and very diverse microbiota. Thus, this study attempted to identify putatively novel cave microbiota. In accordance with the definition of a bacterial species, cave isolates with ≥ 97.5% 16S rRNA gene sequence similarity to validly described microorganisms were considered to belong to the same species (Stackebrandt & Goebel, 1994; Vandamme *et al.* 1996). Table 3.3 summarises the phylogenetic affiliations of representative isolates including putatively novel species and genera. A total of two putatively novel genera and 18 putatively novel species were identified.

Sediment and speleothem isolations were selective for actinomycetes, accordingly, actinomycetes dominated the culture collection. However, three non-actinomycete isolates were identified including a putatively novel *Paracoccus* sp. isolated from a speleothem in Entrance Cave. The majority of cave isolates from sediments and speleothems in both Entrance and Loons Caves proved to be cosmopolitan members of the actinomycetes, particularly of the genera *Streptomyces, Arthrobacter* and *Nocardia.* A number of novel actinomycete isolates were detected

from Entrance Cave sediments. One novel species, CAVI009 is phylogenetically most closely related to species from two genera, the *Actinoplanes* and *Couchioplanes* (Table 3.3). The boundaries of these genera is not clearly defined, *Actinoplanes brasiliensis* clusters with *Couchioplanes caeruleus* rather than with other members of the *Actinoplanes* and isolate CAVI009 clusters within this clade (Figure 3.9b). Further characterisation of this novel cave isolate represents an opportunity to clarify the taxonomic positions of these species. Novel *Micromonospora* sp. and *Amycolatopsis* sp. were also isolated from Entrance sediments and a novel *Saccharothrix* sp. was isolated from a speleothem in Entrance Cave.

Moonmilk isolations (which were non-selective) produced the most novelty, particularly from the *Firmicutes*, *Proteobacteria*, and CFBs. This result is not surprising given the uniqueness of the moonmilk habitat and the paucity of published studies of moonmilk microbes. Several isolates from all moonmilk samples belonged to the Gram-positive *Firmicutes*, particularly of the genus *Bacillus* (Table 3.3). There were four isolates deemed putatively novel species: two *Bacillus* sp. most closely related to *B. cohnii* and *B. pumilus*, a novel *Paenibacillus* sp. and a novel *Sporosarcina* sp. most closely related to *Sporosarcina macmurdoensis*. The novel *Sporosarcina* sp. was closely related to an uncultured permafrost bacteria (Figure 3.14), indicating that this isolate is most likely a cold-adapted bacterium. Of the Proteobacteria, eight novel species were isolated (Table 3.3). From the α-Proteobacteria a novel *Methylobacterium* sp., *Porphyrobacter* sp., and two *Sphingomonas* sp., were detected. Two novel *Alcaligenes* sp. of the β-Proteobacteria and a novel *Stenotrophomonas* sp. and *Xanthomonas* sp. of the γ-Proteobacteria were also isolated. Demonstrating the novelty of the moonmilk cultures, members of two putatively novel genera were isolated. From sample ME3 two isolates, CAVI339 and CAVI329, most likely representing individual species (>2.5% sequence dissimilarity), clustered on a distinct branch within the *Sphingobacteriales* of the CFBs (Figure 3.14). An uncultured bacterium detected in a karstic aquifer also branched within this clade. Isolates CAVI339 and 329 showed only 94-95% sequence similarity to *Pedobacter* sp. and *Sphingobacterium* sp., indicating that these two isolates from moonmilk may represent two species of a new genera within this lineage. Sample ME2, the calcite mat, produced an isolate, CAVI218 that clustered within the *Pseudonocardineae* but was

only distantly related to described members (Figure 3.9). CAVI218 showed the highest similarity (~92%) to *Saccharothrix violacea* and *Lentzea flavoverrucosispora* most likely representing a new genus within this lineage.

The definition of a bacterial species used in this study (≥ 97.5% sequence similarity) is considered by some to be too conservative, especially in the case of highly related species of genera like *Streptomyces*. Also, 16S rRNA gene results are not sufficient alone to define a new bacterial genus or species. Further morphological, biochemical and physiological testing, and further genetic characterisation (% G+C, DNA:DNA hybridisation) is needed to validly describe these putatively novel cave microbiota.

3.5 *Differences in Microhabitat Community Structure*

Measures of diversity were determined followed normalisation of the clone libraries using the rarefaction method. Indices indicating biodiversity coverage (C), diversity (Shannon-Weaver index H'). dominance (Simpson index SI'), and eveness (J') are displayed in Table 3.4. Biodiversity coverage (C) (Mullins *et al.* 1995) measures the portion of a clone library of infinite size that would be sampled by the smaller clone library obtained. The coverage of biodiversity was quite high for all libraries, ranging from 67.6 to 81.3% and particularly high for calcite-based samples (76.1-81.3%).

Table 3.4: Biodiversity indices for cave sediment and moonmilk samples.

Sample	C	H′	SI′	J′
SE1	71.8%	1.527	0.022	0.953
SE2	70.5%	1.400	0.030	0.914
SL1	70.5%	1.501	0.025	0.944
SL2	67.6%	1.494	0.025	0.939
ME2	76.1%	1.325	0.058	0.889
ME3	77.4%	1.379	0.024	0.943
MX1	81.3%	1.467	0.044	0.993

Estimates of Diversity (*H′*) were determined using the Shannon-Weaver (or Shannon-Weiner) Index (Krebs, 1989). This index measures the average degree of uncertainty (synonymous with diversity) of predicting the species (or phylotype) of a given individual picked at random from a community. Diversity measures were high for all samples illustrating the diverse nature of cave microbial communities. Diversity was higher for the dry sediments from Entrance and Loons Caves (1.527 and 1.501 respectively) than the wet sediments (1.400 and 1.494 respectively). Dry cave substrate typically yields a higher proportion of *Actinobacteria* than does dripping water and wet sediment (Kolbel-Boelke *et al.* 1988; Laiz *et al.* 1999). Perhaps the discrepancy in diversity measures may reflect the absence of high numbers of *Actinobacteria* from wet sediment. Dominance values were fairly low for all samples (0.022 to 0.058) and consequently eveness values were high (0.889 to 0.993). Measures of dominance concentration were determined using the Simpson Index (*SI′*) (Krebs, 1989). This index is based on the probability of drawing a pair of individuals of the same species from a sample. Equitability indices (*J′*) were based on Shannon-Weaver index data. This index measures the eveness with which individuals are distributed among the species present in a sample. Though all dominance values were comparatively low, the highest values were seen for the calcite mat sample ME2

(0.058), reflecting the high percentage of clones distributed among a few phylotypes (eg. *Bosea* sp., *Brevundimonas* sp.; Table 3.2)

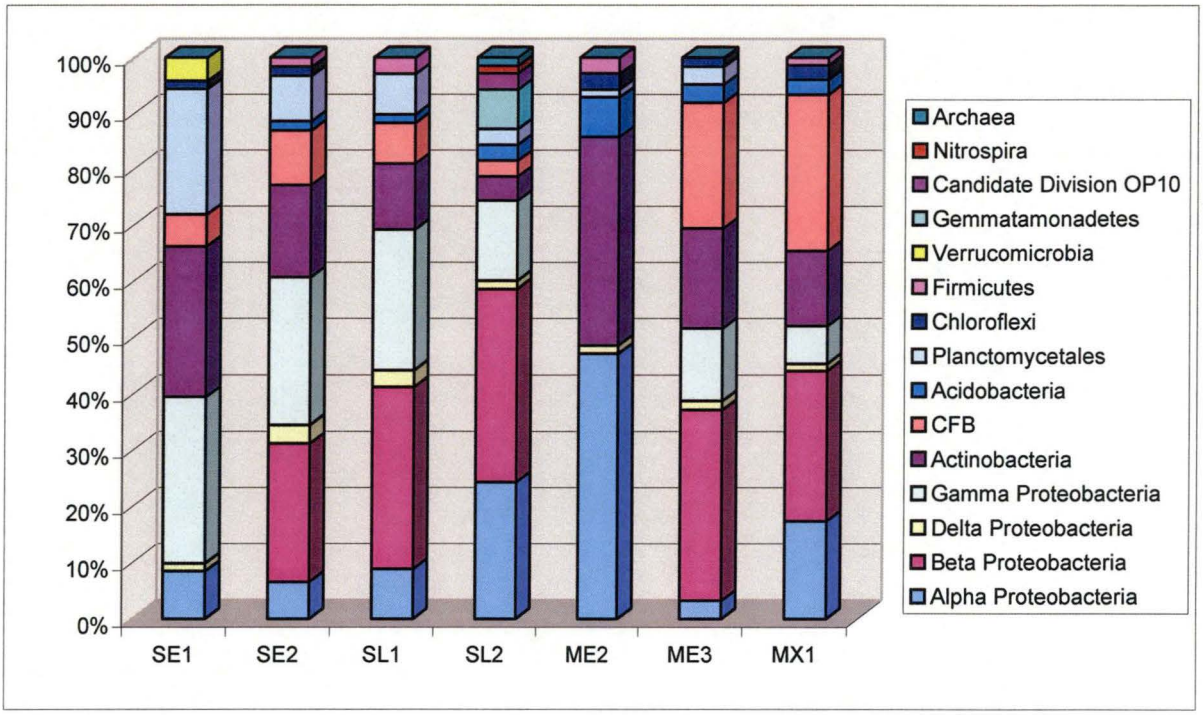
There are many ecological diversity measures, but their suitability for use with highly diverse bacterial communities is unclear and seldom considered (Hill *et al.* 2003). Inherent limitations of molecular techniques, including extraction efficiency and PCR bias, mean that measures of abundance, diversity and richness may not be strictly accurate reflections of the actual community structure. Species abundance models are useful, irrespective of coverage, because they address the whole distribution of a sample, aiding comparison by revealing overall trends as well as specific changes in particular abundance classes. Bengtsson (1998) cautions that it is naïve to contemplate that one single number – species richness, a diversity, the number of functional groups, or connection – can capture the complex relationships between many species and the functions performed by these interactions in soil. The indices may reveal more if applied to smaller, more homogenous habitats where a reasonable level of coverage may be obtained. However, any estimates of microbial diversity must acknowledge the inability of microbiologists to satisfactorily define a bacterial species.

A graph of the distribution or abundance of major cloned phylogenetic groups (Figure 3.19) further highlights the similarities and differences in community composition over the range of samples, microhabitats, examined. Examination of the rRNA genes recovered from soil microbial communities at diverse sites reveal that eight bacterial groups are present in the majority of soil microbial communities: the α-, β-, and γ-Proteobacteria, the *Actinobacteria*, the CFBS, the *Acidobacteria*, the *Planctomycetales* and the *Verrucomicrobia* (Buckley & Schmidt, 2001). Cave sediment samples in this study were characterised by the presence of these typical terrestrial bacteria though in different abundances to other terrestrial environments (Figure 3.19). All clonal samples are dominated by the super-phyla Proteobacteria (39.4-77% of total diversity), however there are marked differences in the distribution of diversity between the α-, β-, γ- and δ-divisions. Sample SE1 showed striking differences to samples SE2, SL1 and SL2. The latter were dominated by the β- (24.6 %, 32.4% and 34.8%) and γ- (26.2%, 25% and 14.1%) Proteobacteria whereas SE1 was dominated by the γ-Proteobacteria (29.6%) and the β-

Proteobacteria were not detected. The second striking difference is the abundance of *Actinobacteria* (26.8%) and *Planctomycetales* (22.5%) in sample SE1 compared to their comparatively lower presence in other sediments. Sample SE1 is a dry sediment sample from Entrance Cave and whereas samples SE2 and SL2 are saturated sediment samples which is the most probable explanation for the differences in community structure. Sample site SL1, though above the water line and chosen for its dry nature, is still likely to be more hydrated than sample SE1 due to the 'wet' nature of Loons Cave. The saturated sediment from Loons is more diverse than all other samples with members of 12 major phylogenetic groups being detected in comparison to 8-9 in other cave samples (Figure 3.19). A number of minor components detected in sample SL2 were not detected in other samples (eg. *Gemmatimonadetes*, Candidate Division OP10, *Nitrospira* and *Crenarchaeota*). These tended to be 'rare' phylotypes consisting of one clone and representing putatively novel lineages within divisions with few or no cultivated representatives (Figure 3.17, Figure 3.18). Members of these divisions are often detected in saturated, anoxic environments (eg. wetland soils, deep sea sediments, activated sludge).

Sample ME2, from the ceiling rock of Entrance Cave, displayed a very different community composition to all other samples, in particular, very different to calcite moonmilk samples ME3 and MX1. The α-Proteobacteria and *Actinobacteria* completely dominated the diversity sampled from ME2 (47.1% and 37.1% respectively). Within these major phyla, clones are distributed between few phylotypes within the genera *Brevundimonas* and *Bosea* and *Saccharothrix* (Table 3.2) illustrating the simplicity of this mat-like microbial community.

Figure 3.19: Comparison of community structure between cave microhabitats.



The abundances of various cloned prokaryote groups in sediment and moonmilk samples from Entrance-Exit Cave system and Loons Cave calculated as percent of total clones. SE1 - dry sediment, Entrance Cave. SE2 - saturated sediment, Entrance Cave. SL1 - dry sediment, Loons Cave. SL2 - saturated sediment, Loons Cave. ME2 - calcite mat, Entrance Cave. ME3 - moonmilk, Entrance Cave.
MX1 - moonmilk, Exit Cave.

Calcite moonmilk samples ME3 and MX1 show remarkable similarities in community structure (Figure 3.19) though being derived from geographically separated sites in Entrance and Exit caves. Also, their habit is quite different, ME3 was sampled from a moonmilk deposit beneath sediment, whereas MX1 was sampled from a moonmilk-coated stalactite. Both samples are dominated by the β-Proteobacteria (33.9% and 26.7% respectively), particularly the *Oxalobacteriaceae* (Figure 3.6) whereas no β-Proteobacteria taxa were detected in sample ME2. Surprisingly, the second most abundant group in ME3 and MX1 were the CFB (22.6% and 28%), reinforced by the isolation of a number of *Flavobacteriaceae* moonmilk samples (Figure 3.15) however the CFB group were present in very low numbers in the mat material ME2 (1.4%). The fundamental difference between cave sediments and moonmilk samples was that the CFBs were

a comparatively minor component of cave sediment microbial diversity (2.8-9.8%). The *Actinobacteria*, historically thought to be a major component of moonmilk based on culture-dependent and microscopical studies, were the third most abundant clonal group (17.7% and 13.3%) though dominating the isolations (Table.3.3). The single similarity between sample ME2 and ME3 and MX1 is the dominance of phylotypes affiliated with the *Pseudonocardineae* and *Micrococcineae* (Table 3.2). The γ-Proteobacteria were present in moderate numbers in sample ME3 and MX1 (12.9% and 6.7%) however not detected in sample ME2, again highlighting the differences between ME2 and moonmilk samples.

Pairwise comparisons of clone libraries were carried out using the Similarity Coefficient (*S*) (Odum, 1971) which illustrates that the biodiversity of all samples overlap, ie. share phylotypes, to some extent (Table 3.5). Moonmilk samples ME3 and MX1 showed the highest similarity of comparisons (0.493). These samples shared 17 phylotypes reflecting the similarity of moonmilk microbial community composition in both Entrance and Exit Cave. The lowest similarity values were seen between sample ME2 and the Loons Cave sediment (0.086), sharing only three phylotypes. Sample ME2 was also distinct from Entrance Cave sediment (0.141 and 0.154) and moonmilk samples (0.133 and 0.141), highlighting the unique nature of the microbial community inhabiting the calcite mat. Sediment samples from Entrance Cave, SE1 and SE2 shared a high number of phylotypes (15) as reflected by the high comparison value (0.405). Similarly Loons Cave sediments shared 15 phylotypes reflected in the high similarity value (0.385). Similarity values between Entrance and Loons Cave sediments are much lower indicating that the microbial communities are more similar within the individual cave systems than they are between similar habitats.

Table 3.5: Pairwise comparisons of cave sediment* and moonmilk* clone library phylotype composition.

	SE1	SE2	SL1	SL2	ME2	ME3	MX1
SE1	1.000						
SE2	0.405	1.000					
SL1	0.177	0.356	1.000				
SL2	0.152	0.247	0.385	1.000			
ME2	0.141	0.154	0.086	0.086	1.000		
ME3	0.203	0.159	0.118	0.147	0.133	1.000	
MX1	0.100	0.162	0.228	0.228	0.141	0.493	1.000

* SE1 – dry sediment, Entrance Cave. SE2 – saturated sediment, Entrance Cave. SL1 – dry sediment, Loons Cave. SL2 – saturated sediment, Loons Cave. ME2 – calcite mat, Entrance Cave. ME3 – moonmilk, Entrance Cave. MX1 – moonmilk, Exit Cave.

3.6 Culturable vs. Non-culturable Diversity

It is widely recognised that culture-dependent techniques are limited and it has been estimated that less than 1% of the microorganisms in an environment are readily cultivated in the laboratory using standard techniques (Amann *et al.* 1995). The use of culture-independent molecular techniques to identify unculturable microbial species has vastly expanded our knowledge and understanding of microbial diversity (Pace, 1997). However, phylogenetic information does not necessarily impart information on the functional potential or *in situ* activities of microorganisms demonstrating an apparent need for characterising novel genera in pure culture to understand their functional role in the ecosystem. *Actinobacteria* are the most abundantly isolated group of bacteria from almost all published cave culture studies (except sulfur caves) of sediments, rock, speleothems and rock art, seeming to demonstrate their dominance in these habitats (Groth *et al.* 1999a; Groth & Saiz-Jimenez, 1999, Chelius & Moore, 2004). However, the apparent dominance of *Actinobacteria* in these habitats appears to be an artefact of culture-dependent studies. It has repeatedly been demonstrated in culture-independent studies of the same sites, that *Actinobacteria* do not dominate, and are a minor, to moderate at best, component of the community (Groth *et al.* 1999a; Groth *et al.* 2001; Schabereiter-Gurtner *et al.* 2002a, b; Chelius & Moore, 2004). The discrepancies between culture-dependent and culture-independent studies in relation to actinomycete abundance are not

restricted to cave environments. Li *et al.* (1999) isolated 75 different actinomycetes from marine samples; however very few actinomycete sequences were cloned from these same samples in a later study (Colquhoun *et al.* 2000; 1998a,b). Thus, this study endeavoured to determine how consistent concurrent culture-dependent versus culture-independent results were within the *Actinomycetales*, rather than at the whole community level, by selectively isolating these bacteria from sediments and speleothems from Entrance and Loons Caves.

Actinomycetes were cultivated from all sediments using the selective procedures detailed in Section 2. Primary plates were dominated by actinomycete-like colonies with diverse morphologies and 16S rRNA gene sequencing revealed that isolates were distributed over eight genera. Approximately 60% of isolates were of the genus *Streptomyces* belonging to only a few species (Figure 3.11). *Nocardia* sp. were the next most abundantly isolated. Other genera isolated but in relatively low numbers were, *Arthrobacter, Knoellia, Micromonospora, Couchioplanes/Actinoplanes, Amycolatopsis* and *Saccharothrix*. In contrast, though phylotypes affiliated with all these genera were detected in sediments, the relative abundances were not the same (Table 3.2). Most obviously, phylotypes affiliated with *Streptomyces* did not account for 60% of actinomycete diversity. Other taxa, not detected by cultivation, were detected in the clone analysis, eg. *Frankia* sp., *Blastococcus* sp., and *Rhodococcus*. These results demonstrate that culture studies of cave sediments, which we know are not representative at the whole community level, do not give a true representation of the actinomycete diversity either. Thus the observed cultured actinomycete diversity is more likely due to the ease with which members of the *Actinomycetales* can be cultured, eg. *Streptomyces* generally being the most easily cultivated actinomycete and thus represented by 60% of isolation results in this study.

There are very few published culture studies of moonmilk samples and no culture-independent reports. Thus non-selective procedures were used to investigate culturable vs. non-culturable diversity at the whole community level of moonmilk samples. Mostly novel isolates of the α-, β- and γ-Proteobacteria and CFBs and previously described *Firmicutes* dominated the culture collection of samples ME3 and MX1, rather than actinomycetes. Information on moonmilk microbial communities is scarce though isolations from moonmilk from several caves

in South Wales produced eight species of heterotrophic bacteria belonging to the genera *Bacillus*, *Micrococcus*, *Bacterium*, and *Streptomyces* and moonmilk consisting of a silicate gel in Nikitsky Catacomb, Moscow, produced *Flavobacterium* sp., *Alcaligenes* sp. and *Arthrobacter* sp. (Williams, 1959; Semikolennykh, 1997). Speleothem dripping waters are probably a similar microhabitat to the very hydrated nature of moonmilk. Laiz *et al.* (1999) investigated the microbial diversity of dripping waters of Altamira Cave, Spain. Water communities were not dominated by actinomycetes but contained low proportions of Gram-positive bacteria, and were mainly composed of Gram-negative rods and cocci (*Enterobacteriaceae* and *Vibrionaceae*; genera *Aeromonas* and *Acinetobacter*). Compounding this, in an earlier study of dripping waters in Altamira Cave carried out by Somavilla *et al.* (1978) *Bacillus* and *Pseudomonas* appeared to be the most abundant genera, followed by *Flavobacterium* and *Erwinia*. The absence of culturable actinomycetes in dripping waters agrees with the observations of Kölbel-Boelke *et al.* (1988). They found very few actinomycetes in 60 water samples clearly demonstrating that dripping water communities are very different to those of cave rock though both are heterotrophic based systems. This trend was reflected in the clone library analysis; moonmilk libraries being dominated by β-Proteobacteria and CFB clones, accounting for more than 50% of the diversity sampled in total (Table 3.2, Figure 3.19). The *Actinobacteria* were far less dominant, (13-17% of diversity). An anomaly in clone and DGGE analysis was the absence of sequences related to *Bacillus* species, though these were cultivated in great numbers from all moonmilk samples. PCR bias against Gram-positive, low G+C bacteria (*Firmicutes*) and the work of Laiz *et al.* (2003) who found that *Bacillus* species were not easily separated in DGGE analyses because of co-migration of bands which may explain this anomaly.

In contrast to moonmilk samples ME3 and MX1, isolations from sample ME2 were dominated by actinomycetes. This is not surprising given the abundance of clones affiliated with the *Actinobacteria* (37.1%) and the networks of hyphal organisms visualised with ESEM. Which further demonstrates the uniqueness of the microbial community in the calcite mat in Entrance Cave. The one common trend between the culture-dependent and culture-independent studies of the calcite mat and moonmilk microbial communities is the striking consistency between

isolations, clone phylotypes and DGGE phylotypes of the *Actinomycetales* (as previously demonstrated for sediment samples) and the CFBs and α-Proteobacteria. *Saccharothrix cryophilus, Arthrobacter chlorophenolicus, Brevundimonas alba, Bosea thiooxidans* and *Sphingomonas* sp. were detected in the isolations, clone libraries and DGGE analysis of all three samples. *Flavobacteria leeana*-like microbes were detected in isolations, clone libraries and DGGE analysis of moonmilk samples ME3 and MX1 (CFBs were only a minor component of sample ME2).

Most published studies of isolations in caves have been performed using standard isolation procedures and incubation at 28 °C (Groth *et al.* 1999a, 2001; Laiz *et al.* 1999, 2000). However, the constant low temperatures throughout the year in most studied caves suggests the possibility of an indigenous pychrophilic microflora, adapted to low temperatures, that could be overlooked using standard microbiological procedures with incubation at higher temperatures. Laiz *et al.* (2003) isolated bacteria from sediments of Tito Bustillo, Llonín and La Garma Caves at a variety of temperatures from 5 – 45° C and investigated their temperature ranges for growth. They found that isolated bacteria were psychrotrophs (Morita, 1975) or psychrotolerants, as most of them could grow at 5° C. No isolates had an optimum growth temperature below 20° C and therefore could not be considered true psychrophiles. The main difference in diversity of isolated bacteria with the use of different isolation temperatures concerned the recoverability of actinomycetes (Laiz *et al.* 2003). For example, at 13° C only six actinomycete strains were isolated from Tito Bustillo though the diversity of non- actinomycete sp. increased in comparison to isolations at higher temperatures. At 28° C the number of actinomycete strains isolated was tripled, indicating that the isolation of actinomycetes diversity is temperature-dependent. Thus Laiz *et al.* suggests the need to use low temperatures to detect maximum diversity of culturable bacteria other than actinomycetes and higher temperatures to detect maximum diversity of actinomycetes. Isolations in this study were carried out at 25 °C to detect maximum diversity of actinomycetes and at 10 °C to mimic the cave environment. Similarly to the results of Laiz *et al.* (2003), most actinomycete diversity was detected in isolations from the calcite sample ME2 incubated at 25 °C whereas, at 10 °C only two colony morphologies were detected, one identified as *Bosea thiooxidans*. Conversely, there was no detectable difference between

incubation temperatures and colonies isolated for moonmilk samples ME3 and MX1; *Proteobacteria*, CFBs and *Firmicutes* dominated all isolations reinforcing their dominance in moonmilk samples.

Through the course of isolation studies of moonmilk samples, a flaw was detected in earlier sediment and speleothem culture studies. Hyphal soil actinomycetes often show distinctive morphologies that can aid in identification to the generic level. These morphological characteristics include presence of aerial spores or mycelia that aids detection of "actinomycete-like" colonies on primary plates. Culture studies of sediments and speleothems were aimed at sub-culturing only actinomycete-like colonies whereas culture studies of moonmilk samples were aimed at sub-culturing all different colonies. 16S rRNA gene sequencing revealed that many moonmilk colonies deemed "non-actinomycete-like" were actually actinomycetes (in particular members of the *Micrococcineae* and *Corynebacterineae*). Thus, a portion of the culturable actinomycete diversity may have been over-looked in isolations from cave sediments and speleothems.

The well recognised discrepancies between culturable and non-culturable diversity has limited our understanding of species diversity in natural bacterial communities. Plating leads to an overestimation of the number of spore-forming bacteria with respect to quiescent vegetative forms; the later are less easily cultured but readily detected by culture-independent techniques (Laiz *et al.*, 2003). Members of the *Actinobacteria* have established a wide ecological distribution and survive long periods of nutrient deprivation by producing endospores, which allows their ready cultivation in favourable conditions and the presence of available nutrients. However, the majority of oligotrophic organisms don't employ such sophisticated techniques to survive extreme nutrient deprivation, but simply grow and reproduce continuously at an exceedingly slow rate (Koch, 1997). A problem with cultivation of oligotrophs therefore comes with the assumption that the rate-limiting step in bacterial growth is simply nutrient availability, and not the ability of the cell itself to grow. The sudden addition of excess nutrients through cultivation methods to organisms adapted to nutrient limitation, may result in rapid cell death via osmotic swelling (Koch, 1997) thus many of these oligotrophic species cannot be easily cultivated using

standard techniques. Thus microbiologists need to work on developing culturing methods that better mimic the *in situ* chemical and physical parameters faced by microbes in the real world. This limitation has been partially overcome by the advances of culture-independent techniques which have revealed surprisingly high levels of novel biodiversity. Some of these groups previously undetected by cultivation have emerged as numerically abundant and seemingly important ecological groups (eg. *Acidobacteria*). The limited number or absence of cultivated members of these groups restricts our understanding of their role in the environment. Parallel studies of laboratory cultures strongly complement molecular ecological investigations. Recent advances by Sait *et al.* (2002) and Joseph *et al.* (2003) have shown that many of the previously uncultured lineages can be isolated using relatively simple media (Eg. the Ellin isolates of the *Acidobacteria, Verrucomicrobia, Gemmatimonadetes* and novel members of already well characterised phyla, *Proteobacteria* and *Actinobacteria*).

3.7 Metabolic/Ecological Comparisons

By phylogenetically aligning an organism to its next nearest cultivated relative, we may shed light on the metabolic and physiological processes that are occurring (Pace 1997). However such comparisons can only be made when there is a high degree of sequence similarity between the identified phylotypes and known cultivated species (Achenback & Coates, 2000). Caves are severely resource limited due to the absence of light that precludes primary production of organic material by photosynthetic organisms (Northup & Lavoie, 2001). In cave ecosystems with little or no exogenous organic input, the rich variety of redox interfaces allows primary growth of chemolithotrophic (eg. ammonium-, nitrite-, sulfur-, manganese- or iron- oxidising) bacteria (Northup & Lavoie, 2001). Chemolithotrophs are physiologically united by their ability to utilise inorganic electron donors as energy sources. Most chemolithotrophs are also capable of autotrophic growth. The best studied chemolithotrophs are those capable of oxidising reduced sulfur and nitrogen compounds and the hydrogen-oxidising bacteria.

Chemolithotrophic growth on reduced sulfur compounds is a property of a diverse group of bacteria many of which were identified in clone analysis of both sediment and moonmilk samples in this study, particularly members of the *Chromatiales* that dominated the dry sediment from Entrance Cave. Sulfur-oxidisers mostly oxidise reduced sulfer compounds like sulfide and thiosulfate. The calcite mat-like material from Entrance Cave was dominated by a phylotype closely related to *Bosea thiooxidans*, thiosulfate-oxidiser, which was also isolated from this site. Sulfur-oxidising bacteria play a role in the dissolution of limestone in caves with hydrogen-rich waters, contributing to cave enlargement. The extent to which bacteria contribute to the corrosion of limestone and to the enlargement of existing caves remains uncertain. The sulfide needed by sulfur-oxidisers can be derived from sulfate- or sulfur-reducing bacteria associated with sulfur-oxidising bacteria. The δ-Proteobacteria consists of sulfate and sulfur-reducing bacteria. These microbes are obligately anaerobic and morphologically diverse. They are widespread in terrestrial and aquatic environments that become anoxic as a result of microbial decomposition processes, for example, *Desulfovibrio* species are common in waterlogged soils like Loons sediments, containing abundant organic material and sufficient levels of sulfate. The activity of sulfate-reducing bacteria leads to the production of large amounts of H_2S. Some sulfate-reducers also display the ability to grow chemolithotrophically using ferrous iron at acid pH, eg. *Acidithiobacillus ferrooxidans*, sequences related to this species were detected in Entrance sediment.

A number of the Proteobacteria are nitrifying bacteria able to grow on reduced inorganic nitrogen compounds. No chemolithotroph is known to carry out the complete oxidation of ammonia to nitrate thus nitrification of ammonia in nature results from the sequential action of two separate groups of organisms: the ammonia-oxidising bacteria, nitrosifyers (eg. *Nitrosomonas* sp., *Nitrosococcus* sp., *Nitrosospira* sp.) and the nitrite-oxidising bacteria nitrifyers (eg. *Nitrobacter* sp., *Nitrospina* sp., *Nitrococcus* sp., *Nitrospira* sp.). Most nitrofyers are obligate chemolithotrophs and able to grow when provided with CO_2 as the sole carbon source. Nitrifyers are wide spread in soil and water though usually more abundant in neutral or alkaline habitats as acidity results in inhibition of nitrification. High identity values

with cultivated members of these groups, particularly members of the Pseudomonads and *Xanthomondales*, may indicate that bacteria detected in all cave sediments and moonmilk analysed in this study may play a role in the nitrogen cycle. Clone analysis also revealed affiliations with aerobic nitrogen-fixing bacteria in cave sediments, including plant-associated genera (eg. *Rhizobium, Bradyrhizobium, Frankia*) and free-living genera (eg. *Derxia* and *Beijerinckia*). Interestingly, plant-associated nitrogen-fixers of the *Oxalobacteriaceae* and *Burkholderiaceae* were particularly dominant in Loons sediments and moonmilk speleothems. The waterway in Loons Cave is thought to be primarily fed by seepage waters through the ceiling rock. Filtration waters are also involved with the development of speleothems. The significant presence of plant-associated nitrogen-fixers in these samples may be a result of bacteria filtering with seepage waters into the cave systems from the surface soils.

Chemolithotrophic hydrogen-oxidising bacteria, including representatives in the genera *Pseudomonas, Paracoccous* and *Acaligenes, Hydrogenophaga, Acidovorax* and *Arthrobacter*, are capable of growing with H_2 as the sole electron donor and O_2 as the electron acceptor. Most hydrogen-oxidising bacteria are facultative and can also grow as chemoorganotrophs with organic compounds as energy sources. This represents the major distinction between hydrogen-oxidisers and the nitrifyers and sulfur bacteria as most representatives of these groups are obligately chemolithotrophic and growth does not occur in the absence of the inorganic energy source. By contrast, the hydrogen chemolithotrophs can switch between chemolithotrophy and chemoorganotrophy and presumably do so in nature as nutritional conditions warrant. Several sequences obtained from both sediment and moonmilk were related to hydrogen-oxidising bacteria, particularly of the Acidovorax group and novel *Paracoccus* and *Acaligenes* species were isolated from a speleothem and moonmilk, respectively.

Early researchers proposed that the role of microbes in caves is to serve as a food source for higher trophic levels (Dickson, 1979). However it was typically believed that microbes could not provide adequate energy to support a large and diverse ecosystem. The work of Sarbu *et al.* (1996) in Movile Cave, Romania, and by Vlasceanu *et al.* (2000) in Frasassi Caves, Italy, suggest that chemoautotrophic, sulfur-based microbial communities can generate enough energy as

primary producers to sustain complex cave ecosystems. Thus it is proposed that the chemolithotrophic and oligotrophic bacteria identified in this study may support the abundant heterotrophic microbial life detected in all samples. However, heterotrophic cave microbial communities usually rely on allochthonous input of organic matter transported from the surface (Groth *et al.* 1999a). Animals and visitors can provide large amounts of organic input facilitating heterotrophic life. Organic input may also be dissolved in the seepage/dripping waters or as particulate organic matter carried in by active or periodic flooding of a subterranean streamway (Schabereiter *et al.* 2002). Previous research has suggested that cave waters contain dissolved organic matter from the soil, primarily phenolic compounds and lignin (Saiz-Jimenez & Hermosin, 1999). These compounds can be utilised as carbon sources by many of the species related to those identified in this study. High sulphate and nitrate concentrations have been found in dripping waters in Tito Bustillo and other Spanish and Italian caves (Hoyos *et al.* 1999) which, in addition to the concentrations of iron, manganese and other elements found in cave rocks, probably supports heterotrophic bacteria including members of the *Actinobacteria*, that were dominant members of the dry sediment and calcite mat microbial communities in Entrance Cave, and members of the *Flavobacteriaceae* that were dominate community components of moonmilk samples.

In oligotrophic environments there are no obvious sources of exogenous energy sources (eg. surface organics, sulfide or nitrite). A common theme was observed in cultivated relatives of identified phylotypes in this study: the fixation of atmospheric gases or the use of aromatic carbon compounds. Within the α-Proteobacteria phylotypes related to species able to fix atmospheric gases (eg. *Sphingomonadales, Brevundimonas* sp., *Hyphomicrobium* sp. and *Methylobacterium* sp.), were particularly abundant in the calcite mat from Entrance Cave and were also detected in Loons sediments and moonmilk samples. Several sequences showing high sequence similarity with oligotrophic bacteria of the *Sphingomonadales* were detected in the calcite mat from Entrance Cave. Methylotrophs are chemoorganotrophs that utilise carbon compounds more reduced than CO_2, are widespread in aquatic and terrestrial environments and include representatives of the genera *Methylobacterium, Methylocella,* and *Hyphomicrobium.*

Hutchens *et al.* (2004) used DNA-based stable isotope probing and functional gene analysis of groundwater and mat material from Movile Cave to identify methane-assimilating populations and results suggest that aerobic methanotrophs (*Methylomonas, Methylococcus, Methylocystis/Methylosinus* strains) actively converted CH_4 into complex organic compounds and thus helped sustain a diverse community of microbes in this closed ecosystem. *Hyphomicrobium* spp. are able to use atmospheric methyl-halides as their sole source of carbon and energy and are also able to oxidise manganese (McAnulla *et al.* 2001). A novel pink-pigmented *Methylobacterium* sp. was isolated from moonmilk. *Methylobacterium* carry out Type I formaldehyde assimilation and this activity has previously been described in oligotrophic bacterial communities living on limestone masonry (Hanson & Hanson, 1996). The source of atmospheric gasses is clear, while the potential carbon sources may be the organic constituents of water filtering into the cave system. Northup *et al.* (2000) suggested that reduced metals such as magnesium and iron within the limestone matrix of Lechuguilla Cave provide sufficient source of electron donors for growth, which may further require the presence of atmospheric organic molecules as a carbon source. Similar mechanisms of lithotrophy have been suggested in other cave systems (Cunningham *et al.* 1995). However, moonmilk samples from Entrance and Exit Caves are almost pure $CaCO_3$ (~98-100%) with no significant presence of reduced metal compounds available to act as electron donors. Similarly, the Leadville limestone bedrock of Fairy Cave, Colorado, is almost pure $CaCO_3$ (97.5%). Barton *et al.* (2004) suggested that any metal ions present in Fairy Cave were likely deposited by the rich mineral waters that formed the cave system.

The physiological response of bacteria to temperature is critical for the regulation of biogeochemical processes. Moonmilk samples were found to harbour an abundant microflora of phylotypes and isolates closely related to described psychrotrophs which is not surprising given the near constant cold temperatures (7-10 °C) in Tasmanian caves. As discussed previously, Laiz *et al.* (2003) investigated temperature ranges of cave microbiota finding that though they were able to grow at 5 °C, growth optima were above 20 °C which indicates psychrotolerant growth, not true psychrophilic growth. There are conflicting views as to the effect of cold environmental

temperatures on the *in situ* chemolithotrophic metabolic rates of psychrotolerant bacteria. Zhang *et al.* (1999) found that as a physiological adaptation of natural microbial populations to the permanently cold deep Pacific marine sediments and Alaskan tundra permafrost, reduction of ferric iron utilising organic acids or hydrogen as electron donors, was fastest at 10 °C than at 25 °C, indicating that microbial iron reduction is likely widespread in cold environments. Conversely, sulfate-reducing bacterium *Desulfobacterium autotrophicum* responded to low temperatures by reducing metabolic activities, which agrees with *in situ* activities measured in field studies and was suggested to reflect a common physiological principle of psychrotolerant bacteria (Rabus *et al.* 2002). Arnosti *et al.* (1998) found that rates of organic carbon mineralisation were always higher at temperatures above ambient environmental temperatures in Arctic and temperate sediments. However, as the mean environmental temperature dropped, the optimal temperature also dropped, suggesting that organic carbon turnover in the cold Arctic was not actually intrinsically slower than in temperate environments. One study of metabolic activities related to *in situ* temperatures for cave microbiota demonstrated that carbon utilisation was found to be more efficient at lower temperatures (13 °C) suggesting that these bacteria were adapted to live at lower temperatures than their optimal (Laiz *et al.* 2003). It is widely accepted that psychrotrophs are able to metabolise at lower than optimum temperatures and thus are able to continue growth in cold environments. Whether these organisms are metabolising at optimum rates at *in situ* temperatures remains unclear.

3.8 Comparison with other Cave Environments

Literature on cave microbial communities, their taxonomic diversity and distribution, is limited and restricted to a few caves worldwide. Culture-independent analyses have opened the way to study microbial diversity in environmental samples without prior cultivation more often than not revealing surprising diversity. Nevertheless, until recently, our knowledge of bacterial communities in caves has been largely due to culture-dependent studies. The beginning of this century has seen an influx of culture-independent diversity analyses of cave environments

enormously increasing our knowledge of cave microbial diversity. However, with the diverse range of types of caves (eg. sulfur caves, carbonate caves, aquatic caves, tourist/show caves, restricted access caves) and microhabitats (eg. acidic biofilms on walls, filamentous microbial mats in sulfur waters, aquatic microbial mantles, Palaeolithic rock art, cave walls, ferromanganese deposits, sediments) studied and the geographic separation of sites (Romania, Italy, Australia, Mexico, Spain, North America) it can be difficult to draw comparisons or conclusions about cave microbial diversity (eg. Sarbu *et al.* 1996; Angert *et al.* 1998; Vlasceanu *et al.* 2000; Holmes *et al.* 2001; Summers-Engel *et al.* 2001; Schabereiter-Gurtner *et al.* 2002, 2004; Northup *et al.* 2003; Chelius & Moore, 2004; Barton *et al.* 2004).

BLAST comparisons of sequences obtained in this study to the GENBANK database consistently yielded high similarity with several DGGE sequences from the *Proteobacteria, Actinobacteria,* CFBs, *Acidobacteria, Planctomycetales* and *Chloroflexi,* detected in the analysis of rock art and surrounding cave surfaces in Spanish and Italian caves (Schabereiter-Gurtner *et al.* 2002, 2004). Universal primers 341f and 907r were used in the DGGE studies by Schabereiter-Gurtner & colleagues whereas primers 519f and 1492r were used in this study, thus there was only a 3-400 bp overlap between sequences obtained in either studies. Determining exact phylogenetic relationships between the rock art phylotypes and the clones from this study was often difficult and the DGGE sequences were removed from subsequent phylogenetic analysis.

Chelius & Moore (2004) stated that because few exhaustive studies of microbial community structure in caves have been conducted, habitat-specific trends are difficult to detect. Although some clones they obtained from saturated cave sediments in Wind Cave resembled sequences from other caves, they found that no cave-specific bacterial community was evident. Clones mostly resembled those from soil, freshwater, plant associated and polluted environments and most isolates were related to other cultivated members and sequences retrieved from soil and various polluted environments (Chelius & Moore, 2004). Similarly, aside from the cave rock art DGGE sequences, few cave sequences resulted from BLAST searches of the clones from this study. However, rather than being a function of the paucity of studies, it's hypothesised that perhaps these trends are difficult to elucidate due to the overwhelming

quantities of data available. With the explosion of culture-independent studies in the past 15 years, to date, more than 62 000 sequences are available from public databases. Thirty seven division-level lineages have been detected (Hugenholtz *et al.* 1998), almost a third of which are not represented by cultured microorganisms. This provides a high resolution framework for the assignation of novel sequences obtained in 16S rRNA gene libraries constructed from environmental diversity surveys but perhaps disguises the phylogenetic groupings consistently found in caves. Results from this study indicate that there are some general and more specific trends apparent in cave samples.

There is much evidence for rich and diverse chemoautotrophic and heterotrophic microbial communities in caves. Several studies of chemolithotrophic cave microbial communities that do not depend directly upon energy and organic carbon from photosynthesis, have been reported and its been demonstrated that these bacteria play an important role in some cave ecosystems, acting as primary producers and supporting growth of heterotrophic microbes (eg. Sarbu *et al.* 1996). High sulfate and nitrate concentrations have been found in dripping waters in Spanish and Italian caves (Hoyos *et al.* 1999; Van Grieken *et al.*, 1999; Holmes *et al.* 2001) which, in addition to the concentrations of iron, manganese and other elements found in the cave fully support the finding of bacteria involved in the nitrogen, sulphur, iron and manganese cycles. Members of the *Proteobacteria* dominate all culture-independent analyses of cave environments, including this study. Sulfur- and sulfide- oxidisers, iron- and manganese-oxidisers, sulfate-reducers and nitrifiers and denitrifyers appear abundant in caves. Nullabor Caves were found to have a high abundance of *Nitrospira* clones suggesting chemoautotrophic communities dependent on nitrite-oxidation (Holmes *et al.* 2001). A number of clones grouping with *Nitrospira* sp. were also found in ferromanganese deposits in Lechuguilla (Northup *et al.* 2003) and in Llonin, La Garma and Tito Bustillo Caves in association with Palaeolithic rock art (Schabereiter *et al.* 2002, 2004). Schabereiter-Gurtner *et al.* (2002) found ammonia oxidisers such as *Nitrosospira* sp., and *Nitrosococcus* sp., in Tito Bustillo Cave. Clone library analysis by Chelius & Moore (2004) illustrated that γ-Proteobacteria and predominated water-saturated sediments in the dark zone of Wind Cave. A number of alpha phylotypes which grouped into two 'fixer'

clades are common in caves (eg. *Chromatiales, Hyphomicrobium, Chelatobacter* and *Rhizobium*). These phylotypes are related to species able to fix atmospheric gases. Members of the *Comamonadaceae*, particularly of the Acidovorax group have been detected in high numbers in ferromanganese residues of Lechuguilla and cave rock art (Northup *et al.* 2003; Schabereiter *et al.*, 2004) and were particularly dominant in Loons Cave sediment and moonmilk.

The *Acidobacteria* have been detected as one of the most abundant groups of microbes in recent culture-independent analyses of a number of cave rock art sites and saturated sediment in Wind Cave (Schabereiter-Gurtner *et al.* 2002, 2004; Chelius & Moore, 2004). Clones affiliated with the *Acidobacteria* were detected in most samples in this study (except for SE1) but in low numbers (1.5-3.2%). Both Wind Cave and the cave rock art sites are 'show' caves open to the public. With few cultured representatives of this group, little is known about ecology of *Acidobacteria*. Perhaps the increased colonisation of *Acidobacteria* in show caves compared to more restricted access caves is due to the anthropogenic impacts and increased nutrient load connected with visitors to the caves. Minor representations of the *Planctomycetales*, the *Chloroflexi* (green non-sulfur), particularly of the *Dehalococcoides* lineage, the *Verrucomicrobia* and the *Gemmatimonadetes* (previously Candidate division BD), have been detected in a variety of cave environments (aquatic formations in Nullabor caves, Holmes *et al.* 2001; rock art and cave walls in Altamira and Tito Bustillo Caves, Schabereiter *et al.* 2002, 2004; saturated sediments in Wind Cave, Chelius & Moore, 2004) mostly displaying low similarities to known, cultivated relatives of these groups suggesting new lineages. One point of interest is the high numbers (previously unreported for cave sediments) of the *Planctomycetales* (22.5%) in sediment sample SE1 from Entrance Cave. The *Planctomycetales* were considered to be of limited environmental importance, but molecular microbial ecology has demonstrated that these bacteria are ubiquitous, metabolically diverse and constitute a representative part of the natural bacteria population in diverse environments (Hugenholtz *et al.* 1998).

A significant result of this study is the abundance of cloned members of the CFBs in moonmilk samples ME3 and MX1 (Figure 3.19). As previously stated, the CFBs, particularly *Flavobacteriaceae*, were the second most abundant phyla in sampled moonmilk diversity. DGGE

and isolation results also confirm the dominant presence of these microbes (Figure 3.14). CFBs have rarely been identified in previous cave studies (cultured or uncultured), and when present are as minor components showing low similarity identities with known *Cytophagales* members (Schabereiter-Gurtner *et al.* 2002; Chelius & Moore, 2004; Barton *et al.* 2004). Previous studies identifying CFBs in caves have been based on sediment samples. Similarly, sediments from Entrance and Loons Caves also displayed low CFB abundances (2.8-9.8%). Due to the absence of any published culture-independent analysis of moonmilk, it is impossible to determine whether the dominance of CFB phylotypes is a general trend. Although isolations from moonmilk consisting of a silicate gel in Nikitsky Catacomb, Moscow, produced *Flavobacterium* sp., (Semikolennykh, 1997).

Culture-dependent studies have focused on caves with allochtonous input of organic matter demonstrating that heterotrophic bacteria dominate these microbial communities (Groth & Saiz-Jimenez, 1999). Actinomycetes are the most abundant bacteria to be isolated from heterotrophic cave systems, and have demonstrated great taxonomic diversity. Interestingly, in most papers the recognition of this biodiversity, that is the identification of cave isolates, has been through chemotaxonomic analysis only to the genus level. This has resulted in a lack of 16S rRNA gene sequences of cave isolates in public databases, making it difficult to compare and contrast biodiversity at the species level between other studied cave systems. Nevertheless we can still determine trends at the genus level.

The most abundant actinomycetes isolated from caves are the streptomycete, nocardioform and coryneform actinomycetes (eg. Groth *et al.* 1999a; Chelius & Moore, 2004). Samples of sediments, active stalactites, wall concretions and rocks from the walls and ceilings of various caves have been investigated and a high number of isolates obtained. Most abundant were the genera, *Streptomyces, Nocardia, Nocardioides, Brevibacterium, Rhodococcus,* and members of the family *Micrococcaceae.* Similarly, in this study, members of the genera *Streptomyces, Nocardia, Arthrobacter* and *Rhodococcus* were repeatedly cultivated in high numbers from sediment, speleothem and moonmilk samples from Entrance, Exit and Loons Caves (Table 3.3). Also isolated in this study, though less frequently, were *Agromyces* sp., *Agrococcus* sp., *Knoellia*

sp., *Brevibacterium* sp., and *Brachybacterium* sp. (Table 3.3). Members of the *Pseudonocardineae*, particularly of the genera, *Lentzea*, *Saccharothrix*, and *Amycolatopsis*, were the most abundantly isolated and culture-independently detected group of *Actinobacteria* from walls and rock art in La Garma and Llonin caves (Schabereiter-Gurtner *et al.* 2004) and also from calcite-based samples in this study.

Many microbes identified from deep caves are similar to surface forms and are probably transported into caves by water, air, sediment and animals (Groth *et al.* 1999a; Groth *et al.* 2001; Saiz-Jimenez 2001; Schabereiter-Gurtner *et al.* 2002a, b; Chelius & Moore, 2004) as reflected by the high sequence identity of isolates and clones to already cultured and cosmopolitan representatives of this group. However, recent findings and the results of this study have revealed the presence of actinomycete species so far only detected in caves. *Knoellia sinensis* and *Knoellia subterranean*, isolated from sediment in Reed Flute Cave in China (Groth *et al.* 2002) was isolated and clones detected in cave sediment from Entrance Cave and all moonmilk clone libraries. *Saccharothrix violacea* isolated from a gold mine cave in China (Lee *et al.* 2000) was detected in clone analysis and isolated from sediment and moonmilk samples from Entrance Cave and DGGE analysis of moonmilk and has also been detected in ferromanganese deposits in Lechuguilla and cave rock art. Recent studies have emphasised the unique nature of the bacterial and archaeal assemblages found in geographically separated and distinct 'types' of caves (Holmes *et al.* 2001; Schabereiter-Gurtner *et al.* 2002, 2004; Northup *et al.* 2003; Barton *et al.* 2004; Chelius & Moore, 2004). For example, a large proportion of the *Crenarchaeota* sequences detected in Lechuguilla Cave ferromanganese deposits and saturated sediments from Wind Cave closely resembled sequences from a South African gold mine (SAGMA clones which showed great phylogenetic diversity to uncultivated members) (Takai *et al.* 2001; Northup *et al.* 2003; Chelius & Moore, 2004). Given the distance between North America and South Africa, it is unlikely that the archaeal assemblages are as similar by chance or by recent colonisation. A more plausible explanation is that the archaeal clones isolated from these sites evolved within a common subsurface environment, a conjecture supported by the geologic history of the respective regions and recent work on SAGMA clones (Chelius & Moore, 2004). Thus, in the

same way members of the genera *Knoellia, Lentzea* and *Saccharothrix* may represent indigenous cave microbiota present in a wide variety of global subterranean environments. Although it is not clear whether these trends represent convergent evolution at different geographical sites or whether these taxa represent remnants of ancient forms that existed when the continents were joined as the global super-continent, Pangea which formed over 600 million years ago (Mya) and began to separate approximately 400 Mya.

Chapter 4: Concluding Remarks

Over the last 15 years it has definitely been established that large and diverse microbial populations are active to great depths in the terrestrial subsurface and below the sea floor. One of the most compelling questions is the mechanism of supply of nutrients to subsurface populations. Both heterotrophic organisms that consume deeply buried ancient carbon and chemolithotrophic organisms that harness geochemical energy of reactive rocks have been documented in the subsurface. However abundant organisms do not exist everywhere in the subsurface, making it clear that a better understanding of the ecology of the subsurface ecosystems is needed to predict the abundance and significance of the subsurface biosphere.

Caves are not uniform environments in terms of geological and geochemical characteristics, as they can vary from one to the other, eg. rock type, method of formation, length, depth, number of openings to the surface, presence or absence of active streamways, degree of impact by human visitation etc. Furthermore, on a smaller scale, various microhabitats, with vast differences in community structure can exist within caves. Despite many recent advances in the field, literature on cave microbial diversity is still scarce and very few general trends have been detected. Literature on microbial diversity in caves has indicated that actinomycetes dominate culture studies. The majority of the work on actinomycetes in hypogean environments has been conducted in Altamira, Tito Bustillo, La Garma, and Llonin caves, Spain, and Grotta dei Cervi, Italy all of which have spectacular galleries with Palaeolithic rock art paintings, and more recently, in Wind Cave, Dakota, all of which are open to the public (Groth & Saiz-Jimenez, 1999; Groth *et al.* 1999a, 2001; Laiz *et al.* 1999, 2000; Chelius & Moore, 2004). Culture-independent analyses in all of these show caves indicate that actinomycetes are not the most dominant member of the microbial communities.

Based on the literature available, this study was initially aimed at detecting novel actinomycete diversity. However, as the study progressed the focus evolved as it became apparent that actinomycetes dominated only very specific habitats, the dry sediment and the calcite mat in Entrance Cave, and represented only a minor fraction of most other microhabitats

studied. Entrance Cave dry sediments and inactive (dry) speleothems produced a higher number of actinomycete isolates compared to saturated sediments and wet formations from Entrance and Loons Cave which was reinforced by the actinomycetes being the second most abundant group (26.8%) detected in clone analysis of the dry Entrance sediment and low abundances (4-16%) detected in saturated sediments from both Entrance and Loons caves. Many actinomycetes are obligate aerobes and prefer moderate levels of moisture rather than waterlogged soils (Williams *et al.* 1972) and it seems fairly widely accepted that dry cave substrate typically yields a higher proportion of actinomycetes than does dripping water and wet sediment (Kolbel-Boelke *et al.* 1988; Laiz *et al.* 1999). Actinomycetes dominated isolations from the calcite mat in Entrance, and were a major component of the clone analysis, which is not surprising given the results of ESEM studies showing extensive networks of hyphal organisms that compose the mat. Moonmilk samples in comparison were not dominated by actinomycetes. As a result of this work, it is hypothesised that the dominance of actinomycetes in the cave literature is due to the caves that these culture studies have been conducted in being show caves open to the public, providing increased allochtonous input, together with the organics available in the paint layers of rock art, leading to a proliferation of heterotrophic organisms. Secondly, it may be a function of the ease, rather than dominance, with which some actinomycetes can be cultured in the laboratory compared to other indigenous cave bacteria.

The Entrance-Exit Cave System and Loons Cave offer contrasting opportunities with regard to the search for novel microbial biodiversity, as do sediment samples versus moonmilk. Sediment phylotypes and isolates identified in this study closely resemble species associated with oligotrophic, chemolithotrophic and heterotrophic lifestyles indicating that these communities survive by utilising a combination of metabolic pathways. Bacteria involved in the nitrogen and sulfur cycles were important members of all sediments along with hydrogen-oxidising bacteria. These oligotrophic and chemolithotrophic members of sediment communities probably provide energy for the heterotrophic members of the community. Pair-wise comparisons of sediment communities demonstrated that they were more similar to each other within individual cave systems, Entrance and Loons, rather than between microhabitat types

(dry vs. wet sediment). Indicating that the type of cave system does have an effect on the observed biodiversity, probably a reflection of differing factors such as nutrient supply. For example, in Entrance Cave there is the active inflow stream, Mystery Creek, which would bring exogenous inputs of nutrients into the system whereas Loons is fed primarily by ultra-filtered seep waters. The wet sediment from Entrance Cave did show a higher degree of similarity in community composition to Loons Cave samples than the dry sediment from Entrance Cave indicating that the water content of the sediment also has an affect on the distribution. Saturated sediments were dominated by oligotrophs able to fix atmospheric gases and methanotrophs and had a high proportion of rare phylotypes most likely representing new lineages related to microbes detected in anaerobic, anoxic environments, but low abundances of heterotrophic microbes.

Results also demonstrated a marked difference between sediment communities and calcite communities. One of the more significant findings in this study was the work with moonmilk. Results of ESEM and XRD analysis demonstrated that samples ME3 and MX1 are true calcite moonmilk (mondmilch). Phylogenetic analyses and isolation results demonstrated the unique composition of the microbial communities associated with moonmilk deposits. These were predominantly composed of nitrogen-fixing β-Proteobacteria and psychrotrophic heterotrophic CFBs and to a lesser extent, heterotrophic *Actinobacteria*. This study also revealed the dominant presence of cold-adapted (psychrotrophic) aerobic heterotrophic CFBs in moonmilk samples, indicating that this bacterial community survives utilising nitrite and organic material probably dissolved in the cave dripping waters. Phylogenetic analyses and biodiversity indices reveal the striking similarities between moonmilk samples from both Entrance and Exit Caves and the uniqueness of the calcite mat in Entrance Cave. Despite XRD and ESEM analysis showing similar calcite composition and crystal morphology, phylogenetic results indicated that sample ME2 represented a very different microhabitat to moonmilk samples. The mat-like material of site ME2 was dominated by oligotrophic α-Proteobacteria and *Actinobacteria* composing 84.2% of the total diversity. Though mostly a heterotrophic, community, members of the genus *Saccharothrix*, present in high numbers in the calcite mat, are

chemoorganotrophic. Metabolic analyses suggested that the community subsists using a complex metabolic network with input from trace organics within the environment or fixation of atmospheric gases using lithotrophic metabolism.

ESEM investigations of the ceiling rock and moon milk illustrated networks of hyphal bacteria involved with $CaCO_3$ crystallisation. The one significant similarity between the moonmilk samples and mat material was the dominance of *Saccharothrix* sp. in the clone library, DGGE analysis and isolation results for all samples indicating that members of this genus are a dominant member of cave calcite microhabitats. Cañaveras *et al.* (1999) isolated a *Saccharothrix* sp. from calcite which grew very slowly and formed a deep black soluble pigment on oatmeal agar and developed a white aerial mycelium which turned grey with age. This morphology is similar to a culture isolated from samples ME2, ME3 and MX1 and identified as *Saccharothrix cryophilis*. A clone phylotype and DGGE band also present in all samples were most closely related to this organism also perhaps indicating that these organisms are involved in calcite precipitation.

This study represents the first reported culture-independent analysis of moonmilk microbial communities globally and of cave sediment communities in the Southern Hemisphere. Studies of microbial biodiversity are a fundamental starting point for further research into ecosystem function. This project has provided critical baseline information on the composition and distribution of microbial communities in a variety of cave microhabitats and provides a focus for future studies of ecological function such as the microbial contribution to geochemical cycling and mineral precipitation and deposition in the subsurface biosphere. This study has taken significant steps in identifying the microorganisms present in cave environments, and this research now needs to be taken to the next level, ie. what are these bacteria doing and what role do they play in the ecosystem? Cross-disciplinary studies are needed which correlate the presence and distribution of microorganisms with a comprehensive analysis of the habitat at the scale in which microbes function to attempt to understand the daunting complexities of the interactions between microbes and minerals.

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APPENDICES

Appendix 1: Media Preparation and Recipes

Media Preparation

Culture media was prepared as per manufacturers instructions unless otherwise stated. Addition of supplements was as per manufacturers instructions or as described in this section. Where necessary pH was modified by the addition of 0.1M NaOH or 0.1M HCl as required. All water used in the preparation of media was prepared by glass distillation of tap water. Sterilisation was by autoclaving at 121°C at 15 psi for 20 min (unless otherwise specified) or in the case of non-sterile, heat sensitive supplements by filter sterilisation. After autoclaving, agar medium was cooled to 55 °C prior to pouring plates. Media were stored at 4 °C for up to 4 wk. Media containing antibiotics was stored in the dark at 4 °C for up to 2 wk.

Culture Media

Starch-Casein Agar (SC); (Kuster & Williams, 1964).

10 g soluble starch, 0.3 g casein, 2 g KNO_3, 2 g NaCl, 2 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4.7H_2O$, 15 g agar, 1000 mL distilled water, adjust to pH 7.0-7.2, autoclave for 20 min at 121 °C, cool to ~55 °C, add 10 mL Nystatin.

Arginine -Vitamin Agar (AV) (Nonomura & Ohara, 1969).

0.3 g L-Arginine, 1 g glucose, 1 g glycerol, 0.3 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.3 g NaCl, 15 g agar, 1000 mL distilled water, autoclave for 20 min at 121 °C, cool to ~55 °C, add 10 mL Nystatin, 5 mL vitamin solution, 5 mL mineral solution.

Marine Agar (MA)

37.4 g Marine broth (Oxoid 2216), 15 g agar, 1000 mL distilled water, autoclave for 20 min at 121 °C, cool to ~55 °C, add 10 mL Nystatin.

R2A Agar (R2A)

18.1 g R2A agar (Oxoid CM 906), 1000 mL distilled water, autoclave for 20 min at 121 °C, cool to ~55 °C, add 10 mL Nystatin.

1/2 Strength Tryptone Soya Agar (1/2 TSA)

15 g TSA Broth (Oxoid CM 129), 15 g agar, 1000 mL distilled water, autoclave for 20 min at 121 °C, cool to ~55 °C, add 10 mL Nystatin.

Oatmeal Agar (OA) (Williams & Wellington, 1982).

20 g commercial blended oats, 800 mL distilled water, autoclave for 30 min at 121 °C, cool to room temperature with occasional vigorous shaking; add 1 g yeast extract, 1 mL mineral solution, 15 g agar, 200 mL distilled water, autoclave for 20 min at 121 °C, cool to ~55 °C, add 10 mL Nystatin.

Luria Broth Agar with Ampicillin (LB-AMP)

10 g Bacto®-tryptone, 5 g Bacto®-yeast extract, 5 g NaCl, 15 g agar, 1000 mL distilled water, adjust to pH 7.0, autoclave for 20 min at 121 °C, cool to ~55 °C, add ampicillin to a final concentration of 100 $\mu g\ mL^{-1}$.

Supplements

Nystatin
0.1 g Nystatin (SIGMA), 20.0 mL Methanol, filter sterilise and store at 4 °C.

AV and OA Mineral Solution
2.0 g $Fe_2(SO_4).H_2O$, 0.2 g $CuSO_4.5H_2O$, 0.2 g $ZnSO_4.7H_2O$, 0.2 g $MnSO_4.7H_2O$, 200 mL distilled water, filter sterilise and store at 4 °C.

AV Vitamin Solution
0.1 g Thiamine Hydrochloride, 0.1 g Riboflavin, 0.1 g Nicotimamide, 0.1 g Pyridoxine Hydrochloride, 0.1 g Inositol, 0.1 g Calcium Pantotheenate, 0.1 g Para-Aminobenzoic Acid, 0.05 g Biotin, 200 mL distilled water, filter sterilise and store at 4 °C.

0.1 M iso-propyl-beta-D-thio-galactopyranoside (IPTG)
1.2 g IPTG, 50 mL ddH_2O, filter-sterilise and store at 4 °C.

5-bromo-4-chloro-3-indoyl-beta-D-thio-galactopyranoside (X-Gal)
100 mg X-Gal, 2 mL N,N'-dimethyl-formamide, cover with foil and store at –20 °C.

Appendix 2: Cryopreservation Protocol

Protocol for Freeze-Drying Bacterial Cultures
(pers. comm. Carol Mancuso Nichols, University of Tasmania, 2003)

1) Subculture pure isolate onto solid media and incubate long enough to ensure a good cover of growth.

2) Prepare labels with Strain Identification on one side and date on the other. Make sure ink will not run when wet. Prepare 4-5 ampoules for each strain.

3) Place these labels into glass ampoule (Borosilicate - approx 8mm diam X 105 mm long). Plug ampoule with small piece of cotton wool, rolled for easy removal and replacement.

4) Place ampoules into paper Sterilope for autoclaving and autoclave at 121 °C for 20 min.

5) Prepare 50 ml 1/2 strength seawater (25 ml deionized water + 25 ml seawater (or equivalent). Autoclave at 121 °C for 20 min. Prepare 50 ml 20% (w:v) solution of skim milk in deionized water and autoclave at 108 °C for 30 min. After cooling combine the solutions.

6) Aseptically, using the skim milk solution, prepare a suspension of cells from agar plate. Add 0.5 ml to each ampoule and replace cotton plug. Try to avoid dripping suspension down the inside of the ampoule, if possible.

7) Place ampoule into circular rack for vacuum centrifugation [Speedivac Centrifigal Freeze Drier, Model 5PS, Edwards High Vacuum, Ltd, Sussex, England], which spins the ampoules that are placed under a glass dome. Turn on vacuum. The centrifugation keeps the liquid milk/cells from boiling up under vacuum.

8) After 2 hrs, turn off centrifuge and continue drying for a further 6-8 hrs.

9) When the contents of the ampoules is dried, remove ampoules. Using air/gas mix, draw out ampoules in flame so that the section of the ampoule below 2 from the top and above 5-6 cm from the bottom is a narrow capillary.

10) Carefully place ampoule on the 48 port manifold of the freeze-drier. Turn on vacuum. Leave for 1-2 hrs to ensure vacuum.

11) Holding ampoule at the bottom, use flame to seal ampoule at the narrow section while pulling it gently away from the manifold. Ensure the system is still under vacuum before proceeding. Use flame to round any sharp/pointy ends on the ampoule.

12) Open ampoule and add 0.5 ml liquid media. Streak onto agar plate to check for viability and purity.

Bacterial cultures stored at -70°C. Cultures maintained in duplicate, with one set held for subculture purposes only. Vials were placed at -20°C for 24 hrs before transfer to -70°C for storage up to 7 years.