TECHNISCHE UNIVERSITÄT MÜNCHEN

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Molekulare Identifizierung und *in situ* Nachweis von Prokaryonten in einer schwer zugänglichen Höhle

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Researchers are the aliens in these caves, unable to cope with extreme conditions in which the natives happily flourish.

Penelope Boston

Für meine Eltern.

INHALTSVERZEICHNIS

ABBILDUNGSVERZEICHNIS

TABELLENVERZEICHNIS

ABKÜRZUNGEN

bp	Basenpaare
bzw.	beziehungsweise
°C	Grad Celsius
ca.	cirka
CARD-FISH	*Catalyzed Reporter Deposition* FISH
cm	Zentimeter
Cy3	Cyanin 3 Farbstoff
Cy5	Cyanin 5 Farbstoff
d. h.	das heißt
DNS	Desoxyribonukleinsäure
et al.	*et alii* (und andere)
etc.	et cetera
E. coli	*Escherichia coli*
FISH	Fluoreszenz *In Situ* Hybridisierung
Fluos	5,(6)-carboxyfluoreszein-N-hydroxysuccinimidester
HRP	*Horse-Radish Peroxidase* (Meerrettich-Peroxidase)
km	Kilometer
LKC	*Lower Kane Cave*
m	Meter
µm	Mikrometer
mod.	modifiziert
MPN	*Most Probable Number*
OTU	*Operational Taxonomic Unit*
PCR	*Polymerase Chain Reaction* (Polymerase-Kettenreaktion)
RNS	Ribonukleinsäure
rRNS	ribosomale RNS
sp.	Spezies
spp.	Alle, bzw. mehrere Spezies einer Gattung
SRP	*Sulfate Reducing Prokaryotes* (sulfatreduzierende Prokaryonten)
u. a.	unter anderem
USA	*United States of America* (Vereinigte Staaten von Amerika)
usw.	und so weiter

VA	Virginia
WY	Wyoming
z. B.	zum Beispiel
z. T.	zum Teil

PUBLIKATIONEN

Die grundlegenden ermittelten Ergebnisse aus dieser Arbeit wurden mit den entsprechenden Diskussionen, Schlussfolgerungen, Material- und Methodenteilen im Detail in den unten aufgeführten Publikationen beschrieben. Die veröffentlichten Publikationen und die Beiträge der einzelnen Autoren wurden jeweils am Anfang des Kapitels der Anhänge A-C aufgelistet.

Anhang A **Lee, N.M., Meisinger, D.B., Aubrecht, R., Kovačik, L., Saiz-Jimenez, C., Baskar, S., Baskar, R., Liebl, W., Porter, M.L., and Engel, A.S.** 2012. Caves and karst environments. *In: Bell, E.M. (ed.) Life at Extremes; Environments, Organisms and Strategies for Survival. CAB International, Oxfordshire, UK, pp. 320-344, in Druck.*

Anhang B **Engel, A.S., Meisinger, D.B., Porter, M.L, Payn, R.A., Schmid, M., Stern, L.A., Schleifer, K.-H., and Lee, N.M.** 2010. Linking phylogenetic and functional diversity to nutrient spiraling in microbial mats from Lower Kane Cave (USA). *The ISME Journal 4: 98-110.*

Anhang C **Meisinger, D.B., Zimmermann, J., Ludwig, W., Schleifer, K.-H., Wanner, G., Schmid, M., Bennett, P.C., Engel, A.S., and Lee, N.M.** 2007. *In situ* detection of novel *Acidobacteria* in microbial mats from a chemolithoautotrophically based cave ecosystem (Lower Kane Cave, WY, USA). *Environmental Microbiology, vol 9 (6): 1523-1534.*

A. EINLEITUNG

Der Nachweis und die Charakterisierung von Mikroorganismen in außergewöhnlichen Ökosystemen sind heute von zunehmender Bedeutung für die Mikrobiologie. Man konnte faszinierende Adaptionen verschiedenster Lebensformen entdecken und neue Erkenntnisse bezüglich der Grenzen des Lebens gewinnen. Die Ergebnisse dieser Forschung trugen auch dazu bei, Licht in den Ursprung des Lebens zu bringen. Überdies ergaben sich auch Rückschlüsse auf die Möglichkeiten des Vorkommens von Leben auf anderen Planeten. Mikroorganismen wurden in verschiedensten extremen Ökosystemen entdeckt, z. B. in kochenden vulkanischen Gewässern, in der Antarktis oder in der Tiefsee (Stetter und Haber, 2011).

Der *subterrestrische* Bereich ist einer der am wenigsten erforschten außergewöhnlichen Ökosysteme auf unserem Planeten. Schätzungen gehen davon aus, dass hier bis zu 50% der mikrobiologischen Biomasse der Erde vorhanden sein könnten (Whitman et al., 1998; Fredrickson and Onstott, 2001). Die Bedingungen für Leben in dieser Region sind nicht einfach. Es herrschen extreme Lebensbedingungen wie Dunkelheit, niedriger Sauerstoffgehalt, geringeres Vorkommen von organischem Material, hohe Mineralienkonzentration, variabler Wassergehalt und zum Teil erhöhte Radioaktivität. Mit zunehmender Tiefe steigen zudem Temperatur und Druck. Bis Anfang des letzten Jahrhunderts war die Vorstellung vom Leben unter der Erde nur ein Phänomen in „Science Fiction"-Romanen (z. B. „Die Reise zum Mittelpunkt der Erde" von Jules Verne). Erst, als geologische Studien und Bohrungen in Ölfeldern und tiefen unterirdischen Sedimenten begonnen wurden, fanden Wissenschaftler auch dort (1,5 bis 2,8 km unter dem Meeresspiegel) Mikroben (Chivian et al., 2008; Roussel et al., 2008). Wie tief Mikroorganismen im *subterrestrischen* Bereich leben können, ist u. a. abhängig von der Temperatur, dem Wasservorkommen, bestimmten Energiequellen, der Porengröße des Gesteins und anderen chemisch-physikalischen Parametern (Colwell, 2001; Fredrickson and Onstott, 2001). Die *subterrestrischen* Habitate sind keineswegs monoton, sondern können je nach den geo-physikalischen-chemischen Verhältnissen sehr divers sein, wie z. B. *„deep subseafloor"*, Vulkane, Höhlen, Minen und Grundwassergebiete. Die Erforschung dieser Habitate kann auch wichtige Erkenntnisse für die Astrobiologie erbringen, da auf den die Erde umgebenden Planeten teilweise ähnlich radikale Bedingungen herrschen.

Aber auch für die Industrie ergeben sich mehrere neuartige, technische Anwendungsmöglichkeiten, z. B. hitzestabile Enzyme als hochspezifische und umweltfreundliche Katalysatoren. Zudem können hier Mikroorganismen mit weiteren wertvollen Eigenschaften vorkommen, die sich für Biotransformationen oder die Isolierung neuartiger Antibiotika eignen. Mit den zurzeit verfügbaren Kultivierungsmethoden können aus dem Boden nur ca. 0,3% der im Lichtmikroskop nachweisbaren Bakterien isoliert werden (Amann et al., 1995). Entsprechende Zahlen für den *subterrestrischen* Raum liegen nicht vor, aber sie bewegen sich sicherlich in einer ähnlichen Größenordnung.

Höhlen zeichnen sich häufig durch ausgeprägte Energie- und Nährstoffmangel aus. Deshalb bieten Höhlen eine einzigartige Gelegenheit, redox-abhängige biogeochemische Nährstoffkreisläufe und das Leben unter extremen Bedingungen zu studieren. Die Bedeutung von Mikroben für Höhlen wurde am Anfang der Höhlenforschung für nicht relevant gehalten. Aber seit dem Ende der 1970er Jahre gibt es zunehmende Hinweise, dass Mikroben eine wichtige Rolle bei der Entstehung und Weiterentwicklung von Höhlen spielen können (Anhang A). In einzelnen Höhlen konnten bis zu 10^6 Zellen pro Gramm Gestein nachgewiesen werden (Barton and Jurado, 2007). Die meisten Höhlen sind schwer zugänglich und können nur von Experten bzw. unter auffälligen körperlichen Anstrengungen erreicht werden. Die exakte Zahl der Höhlen auf unserem Planeten ist nicht bekannt, jedoch schätzt man, dass bis jetzt nur ca. zehn Prozent der auf der Erde vorkommenden Höhlen bekannt sind. Davon sind wiederum nur ca. zehn Prozent biologisch erforscht (Anhang A). Die Höhlen werden nach mehreren Parametern, z. B. Gesteins- und Bildungsart, klassifiziert. Die klassischen weltweit verbreiteten Kalkstein-Höhlen (z. B. *Altamira Cave* in Spanien) wurden durch allmähliche Auflösung des Gesteins durch kohlendioxidhaltiges Wasser gebildet, während Tunnelhöhlen auf Hawaii, USA durch fließende Lava geformt wurden (Northup and Lavoie, 2001). Die sulfidischen Höhlen (z. B. *Frasassi Cave* in Italien (Macalady et al., 2007), *Cueva de Villa Luz* in Tabasco, Mexiko (Hose and Pisarowicz, 1999) und *Lower Kane Cave* (LKC) in Wyoming, USA (Engel et al., 2003; Engel et al., 2004a)) wurden durch Einwirkung von Schwefelsäure gebildet (Northup and Lavoie, 2001). Sulfidische Höhlen wurden im letzten Jahrzehnt nicht nur geologisch untersucht, sondern auch biologisch, und zwar von der Fauna bis zur Mikrobiologie (z. B. *Frasassi Cave* in Italien (Macalady et al.,

2006; Macalady et al., 2007; Macalady et al., 2008), *Nullarbor Caves* in Australien (Holmes et al., 2001) und *Lower Kane Cave* (LKC) in Wyoming, USA (Engel et al., 2003; Engel et al., 2004a)). Im Rahmen der vorliegenden Arbeit wurde die *Lower Kane Cave* in Wyoming, USA zusammen mit Engel und Mitarbeitern detaillierter untersucht.

Die im Hinblick auf geologische Prozesse eher junge 10000 Jahre alte *Lower Kane Cave* (LKC) in Wyoming, USA wurde das erste Mal 1981 von dem Geologen Egemeier beschrieben. Die Höhle ist Bestandteil eines Multi-Höhlen-Systems entlang des *Bighorn Rivers* (Abbildung 1a und b). Die LKC besitzt eine horizontale Länge von 325 m sowie eine 180 m tiefe Strömungspassage mit <0,5 bis 2 m Breite. Die Höhle weist nur einen Eingang auf, der nur einen halben Meter hoch (Abbildung 1c und d; Engel, 2009a), und während des Anstiegs des *Bighorn Rivers*, im Spätfrühling, überflutet ist. Im Inneren der Höhle befindet sich keine natürliche Lichtquelle. Die jährliche Durchschnittshöhlenlufttemperatur beträgt 22°C. Innerhalb der Höhle befinden sich drei thermische Hauptschwefelquellen, *Fissure Spring*, *Upper Spring* und *Lower Spring*, deren jährliche Durchschnittswassertemperatur 21°C beträgt (Engel, 2009a). Die Höhle liegt in einem Gebiet mit geothermischer Aktivität, 130 km östlich vom *Yellowstone Nationalpark* und 120 km nördlich von Thermopolis (Anhang A, Abbildung 16.15, S. 87-88). Das *Bighorn Basin* beinhaltet natürliche extensive Ölfelder, chloroorganische Stoffe, sowie thermische und nichtthermische Quellen (Engel, 2009b). Für die Öffentlichkeit ist die Höhle nicht zugänglich und für Speläologen wird eine Genehmigung der dort ansässigen Behörde benötigt (Engel, 2009c). Für den Zugang, die Probeentnahmen und weitere wissenschaftlichen Untersuchungen in der Höhle ist eine spezielle Ausrüstung wie Helm, inklusive Lichtquelle, Gasmaske, Abfallbehälter etc. erforderlich (Elliot, 2006; Abbildung 1e und f).

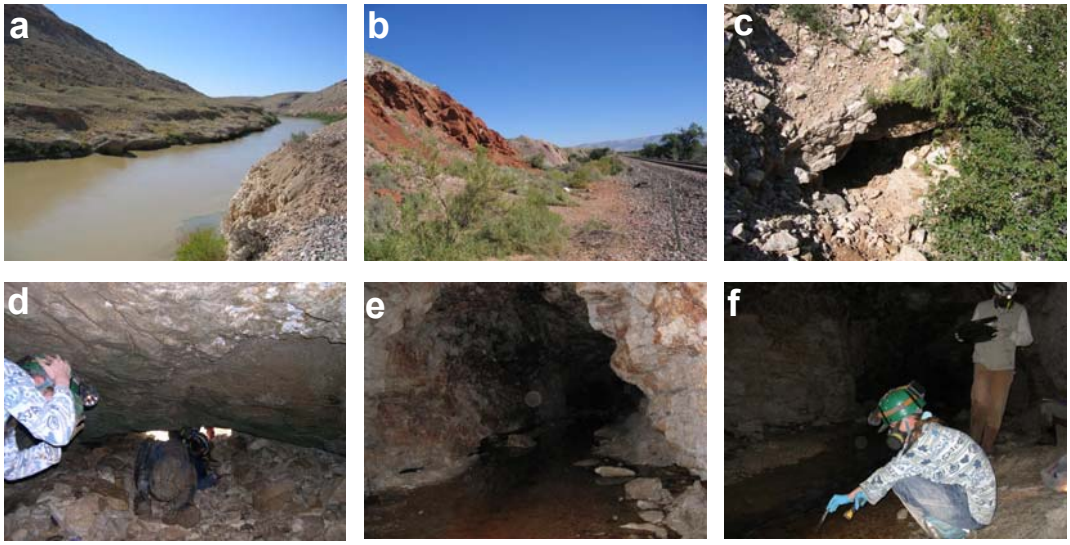

Abbildung 1: Bilder von einer Probenahme Aktion in der LKC im Jahre 2005
a: *Bighorn River*, WY, USA, **b:** Umgebung des *Bighorn Basin*, WY, USA, **c:** Höhleneingang der LKC, **d:** Schmaler Einstieg in dem Eingang der LKC, **e:** Im Inneren der LKC und **f:** Probenahme in der Höhle (Bilder von Meisinger D.B. und Lee N.M.).

Ein Aufenthalt in der Höhle ist höchstens für acht Stunden möglich, da die Höhlenatmosphäre hohe Konzentrationen toxischer, gasförmiger Schwefelverbindungen enthält. In dem offenen Strömungskanal wurde ein höherer gelöster Sauerstoff- als Sulfidgehalt gemessen, während tief in der Höhle nur noch gelöstes Sulfid vorhanden ist (Engel et al., 2004a). Das Grundwasser, das in die Höhle gelangt, besteht aus einem Gemisch aus Kalzium-Bikarbonat-Sulfat und enthält nicht nur Schwefelwasserstoff, sondern auch Spuren von radioaktivem Uran- und Vanadium (Egemeier, 1981; Engel et al., 2004a).
Diese nahezu unberührte Höhle besitzt mit seinen komplexen mikrobiellen Matten ein in sich abgeschlossenes Ökosystem, in dem es keine wetterbedingten Einflüsse gibt und in dem keine Pflanzen oder höhere Tiere vorkommen – mit Ausnahme einzelner Schneckenarten (Engel et al., 2004a).

Durch Untersuchungen von Engel und Mitarbeitern konnte nachgewiesen werden, dass Bakterien an der Entstehung sulfidischer Höhlen wie z. B. der LKC beteiligt sind (Engel et al., 2004a). Entlang der LKC befinden sich auf dem Boden, auf den Wandflächen und im fließendem Gewässer sowie in Quellen von tieferliegendem Grundwasser verschiedene Arten von mikrobiellen Matten (Biofilmen) von einem bis zu 20 Metern Länge (Abbildung 2; Engel et al., 2003; Engel et al., 2004a). Die

mikrobiellen Matten enthalten *chemolithoautotrophe* Mikroorganismen (Sarbu et al., 1996; Engel et al., 2004a), die reduzierte Schwefelverbindungen (z. B. Schwefelwasserstoff (H_2S)) oder elementaren Schwefel als Elektronendonator verwenden und als Endprodukt Schwefelsäure (H_2SO_4) produzieren. Die Schwefelsäure greift die geologische Matrix, das Kalziumkarbonat ($CaCO_3$), an und verwandelt dieses in Kalziumsulfat ($CaSO_4$, Gips). Der Gips löst sich im Grundwasser aus dem Gestein und formt kleine Löcher, die mit der Zeit immer größer werden (Northup and Lavoie, 2001; Engel et al., 2004a+b; Spiegel Online, 2004).

Die chemische Reaktionsgleichung lautet wie folgt (Engel et al., 2004b):

$$H_2SO_4 + CaCO_3 + H_2O \rightarrow CaSO_4 \times 2\ H_2O + CO_2$$

In den bisherigen mikrobiologischen Studien (Engel et al., 2003; Engel et al., 2004a) konnte festgestellt werden, dass die mikrobiellen Matten in der Höhle sehr komplexe und vielfältige Ökosysteme darstellen. Die mikrobiellen Matten in den fließenden Gewässern bestehen aus verschiedenen Arten von fadenförmigen und netzbildenden Mikroorganismen sowie verschiedenen anderen Morphotypen wie Stäbchen und Kokken. Diese mikrobiellen Matten zeichnen sich durch unterschiedliche Farben aus (z. B. weiß, grau-braun, schwarz oder rot), die sehr wahrscheinlich auf unterschiedliche Redoxphasen verschiedener Mineralien zurückzuführen sind (Abbildung 2a-f). Engel und Mitarbeiter untersuchten vor allem die weißen Matten, da dies die häufigste Art des Biofilms in der LKC ist.

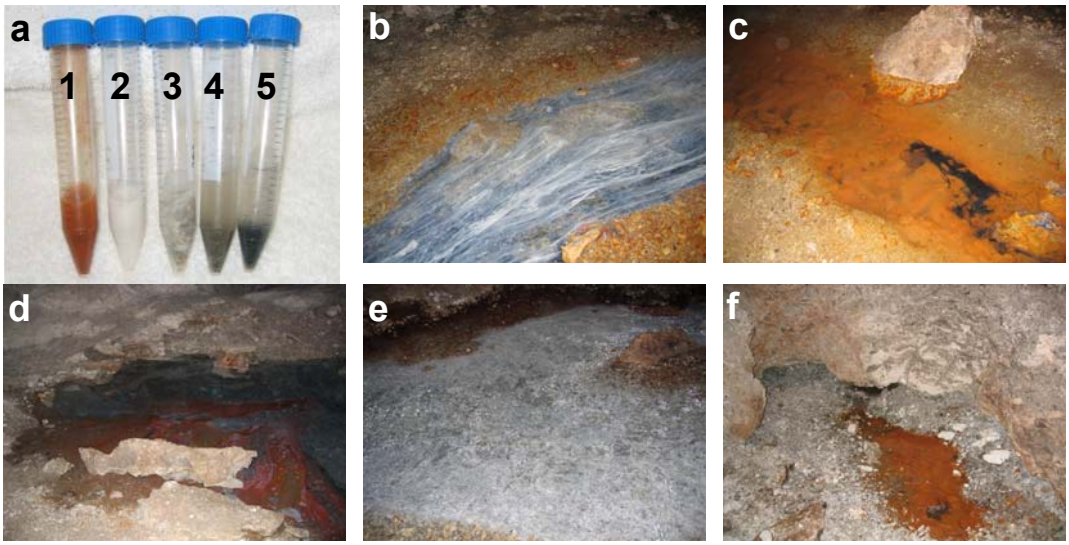

Abbildung 2: Bilder von verschiedenen mikrobiellen Matten
a: Ethanol-fixierte Proben von fünf verschiedenen mikrobiellen Matten in LKC. 1: rote Matte (f), 2: weiße Matte (b), 3: grau-weiße Matte (e), 4: grau-braune Matte (d) und 5: schwarze Matte (c); **b:** weiße Matte in 118 m; **c:** schwarze Matte in 138 m; **d:** grau-braune Matte in 189 m; **e:** grau-weiße Matte in 203 m; **f:** rote Matte in 216 m (Bilder von Meisinger D.B.).

Generell besteht die Mattenstruktur in dem oberen, *aeroben* Bereich (3-5 mm) aus weißen Fäden (aufgrund von Schwefelablagerungen), während im darunterliegenden, *anaeroben* Bereich Strukturen aus grauen Fäden dominieren (Engel et al., 2004a). Erste Untersuchungen mit Hilfe von 16S-rRNS Genklonierungen und Gensonden an über sieben verschiedenen weiße Matten entlang der Höhle ergaben, dass meistens bis zu sechs verschiedene Bakteriengruppen vorkommen. Bei diesen Gruppen handelt es sich um *Beta*-, *Gamma*-, *Delta*-, *Epsilonproteobacteria*, *Acidobacteria* und *Bacteroidetes/Chlorobi*. Besonders in dem oberen, *aeroben* bis 5 mm tiefen Bereich dominierten bis zu ca. 70% nicht kultivierbare Vertreter der Klasse *Epsilonproteobacteria* und ca. zwölf Prozent kultivierbare Vertreter von *Thiothrix* spp. der Klasse *Gammaproteobacteria* (Engel et al., 2003; Engel et al., 2004a). Die Diversität und die Funktion der oberen Schicht der mikrobiellen Matten, die sich am Nährstoffkreislauf beteiligen, sind weitestgehend erforscht. Dagegen ist über die mikrobielle Diversität und den Nährstoffkreislauf innerhalb der verschiedenen unteren Schichten und entlang des Stromverlaufs bisher nur sehr wenig bekannt.

Das Ziel der Arbeit war es, in Zusammenarbeit mit Frau Engel und ihren Mitarbeitern, an verschiedenen Stellen in der LKC Proben aus den Matten zu nehmen und mit mikrobiologischen Methoden ergänzende Kenntnisse zur mikrobiellen Diversität zu erhalten. Dabei sollte von einer Probe aus dem *anaeroben* Bereich einer Matte eine 16S-rRNS Datenbank erstellt und an verschiedenen Proben aus dem *aeroben* und *anaeroben* Bereich die Fluoreszenz *in situ* Hybridisierung (FISH) mit spezifischen rRNS-gerichteten Oligonukleotidsonden durchgeführt werden. Anschließend sollten diese Ergebnisse mit den in Zusammenarbeit mit Engel und Mitarbeitern erhaltenen geochemischen Daten diskutiert werden. Zudem sollte die mikrobielle Diversität global mit anderen Höhlen im Rahmen einer Inventuranalyse verglichen werden.

B. MATERIAL UND METHODEN

Die Lage und der Aufbau der Höhle *Lower Kane Cave* (LKC) in Wyoming, USA wurde in der Publikation im Anhang B dargestellt. Die Probennahme sowie die Verarbeitung der Proben wurden in der Publikation im Anhang C detailliert beschrieben.

Sämtliche molekularbiologischen Analysen, basierend auf DNS Extraktion, *in vitro* Amplifikation, Klonierung sowie Sequenz- und Phylogenieanalyse wurden in der Publikation im Anhang C aufgelistet. Die Erstellung und Bearbeitung des phylogenetischen Stammbaums anhand der 16S-rRNS Gensequenzen aus verschiedenen Höhlen wurden im Anhang A beschrieben. Die Informationen der Inventur, die zur Erstellung der Weltkarte der jeweiligen Höhlen zusammengestellt wurden, sind in Anhang A zu finden.

Die mikroskopischen Methoden, basierend auf Oligo-FISH Technik, die Konstruktion neuer Sonden sowie das Mikroskopieren mittels eines konfokalen Laser-Scanning-Mikroskops wurden in der Publikation im Anhang C aufgelistet. Die CARD-FISH Technik wurde in der Publikation im Anhang B beschrieben.

C. ERGEBNISSE

C1. Genbankstudie aus dem *anaeroben* Bereich der Mattenprobe 203g aus der *Lower Kane Cave* (LKC)

→ siehe auch Anhang B und C

Im Rahmen dieser Arbeit wurde der tiefere, *anaerobe* Bereich der Mattenprobe 203g (graue Fäden; 2 cm unterhalb des oberen, *aeroben* Bereichs, der aus weißen Fäden besteht; Abbildung 3) zum ersten Mal auf seine mikrobielle Diversität untersucht. Das Ziel war, die vorangegangenen Studien über den oberen, *aeroben* Bereich zu ergänzen (Mattenproben 203f und 203y; Engel et al., 2004a), um somit ein vollständigeres Bild der gesamten Biodiversität der weißen mikrobiologischen Matten in der LKC zu erhalten.

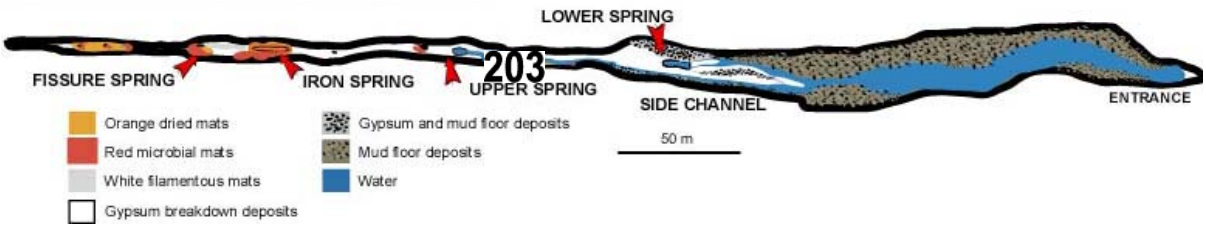

Abbildung 3: Schematische Darstellung der LKC mit der Position der Matte an der Stelle 203 m
Die schwarze Zahl 203 in der Abbildung gibt die Position in Metern der weißen mikrobiellen Matte (203g) an, die in dieser Arbeit mittels einer Genbankstudie untersucht wurde (vom hinteren Teil der Höhle zum Eingang; Abbildung von Engel, www.geo.utexas.edu/chemhydro/lowerkane/cavedesc.htm, mod.).

Hierfür wurden drei verschiedene DNS-Extraktionen ausgetestet und vier verschiedene Primerpaare zur Amplifikation von 16S-rRNS Genfragmenten von *Bacteria* und *Archaea* zur Erstellung von vier 16S-rRNS Genbanken benutzt. Über 800 Klone wurden durch Klonierung erhalten und diese wurden anschließend mittels M13 PCR und Enzymverdau genauer untersucht.

Von diesen 800 Klonen wurden 346 Klonen ausgewählt, die alle mittels der verwendeten Methoden identifizierbare einzelne Gruppen repräsentierten. Basierend auf einer 98% Sequenzidentität konnten 122 *„Operational Taxonomic Units“* (OTU) identifiziert werden. Davon zeigten nur sieben Prozent der OTUs >98% Sequenzähnlichkeit zu den nächsten Verwandten. Zehn einzelne mittels des Software Pakets ARB (Ludwig et al., 2004) berechnete 16S-rRNS Bäume der

verschiedene einzelnen Gruppen sind im Anhang B, ergänzendes Material, Abbildungen SM1-10, S. 121-130 dargestellt. Diese Klone konnten 16 taxonomisch anerkannten *Phyla* innerhalb der Bakterien und dem *Phylum Euryarchaeota* zugeordnet werden (Tabelle 1; detaillierte Ergebnisse in Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118). Zur Zuordnung der OTUs wurde anschließend ein phylogenetischer Stammbaum mit 135 repräsentativen Klonen und relevanten Referenzsequenzen (mit einer Sequenzlänge über 1100 bp) errechnet (Anhang B, Abbildung 2, S. 99). Bei der Berechnung des Stammbaumes fielen ein paar Klone von zwei Gruppen (unklassifizierte Candidate Divisions und *Verrucomicrobia*) in eine zusätzliche Gruppe, die im Baum als *„basal“* gekennzeichnet wurde. Fast 70% der Klone waren zu Vertretern von folgenden fünf taxonomischen Hauptgruppen verwandt: *Deltaproteobacteria*, *Chloroflexi*, *Gammaproteobacteria*, *Bacteroidetes/Chlorobi* und *Firmicutes*. Die zwei dominanten Gruppen gehörten zur Klasse *Deltaproteobacteria* und zum *Phylum Chloroflexi*. In diesen zwei Gruppen zeigten nur drei Prozent der Klone über 99% Sequenzähnlichkeit zu bekannten kultivierbaren Vertretern. Nur zwei Prozent der Klone waren auch verwandt zu Klonen, die in einer vorherigen Studie der LKC (Engel et al., 2003; Engel et al., 2004a) wie auch in der sulfidischen Höhle *Frasassi Cave* in Italien (Macalady et al., 2007) beschrieben worden sind. Hierzu gehörten überwiegend bisher unkultivierbare Vertreter der Gruppen *Acidobacteria*, *Bacteroidetes/Chlorobi*, *Gamma-*, *Delta-* und *Epsilonproteobacteria*.

Tabelle 1: Taxonomischer Überblick der *anaeroben* Genbankstudie

Taxonomischer Überblick der ermittelten 16S-rRNS Gensequenzen und deren Häufigkeit in der weißen Mattenprobe 203g (detaillierte Ergebnisse in Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118).

Häufigkeit (%)	Taxonomische Einteilung	Similarität (%)	Nächster Verwandte	Isolierungsquellen der nächsten Verwandten
	Bakterien			
24	***Deltaproteobacteria***	87-97	unkultivierbare Vertreter und kultivierbare Vertreter *Chondromyces pediculatus, Desulfomonile liminaris, Desulfobacca acetoxidans*	*Frasassi Cave*, Mangroven- und Graslandboden, Meer- und Seesediment, Schlamm, Biofilm der Rhizosphäre, hypersaline mikrobielle Matte
14	***Chloroflexi***	90-98	unkultivierbare Vertreter und kultivierbare Vertreter *Leptolinea tardivitalis*	Mangrovenboden, Schlamm, Biofilm der Rhizosphäre, Abwasser, Antarktische Eisseesediment, Teeröl kontaminiertes Gewässersediment, Sediment, Boden
12	***Gammaproteobacteria***	89-97	unkultivierbare Vertreter	LKC, Süßwassersediment, Trinkwasser, Schlamm, Reisfeld
9	***Bacteroidetes/Chlorobi***	89-100	unkultivierbare Vertreter	LKC, *Frasassi Cave*, Schlamm, Süßwasser, Seesediment, kontaminierte Sediment
7	***Firmicutes***	92-99	unkultivierbare Vertreter	Meeresbodensediment
6	***Betaproteobacteria***	87-99	unkultivierbare Vertreter	Schlamm, Oberflächenwasser, Biofilm der Rhizosphäre, Graslandboden, Boden
5	***Planctomycetes***	87-97	unkultivierbare Vertreter	hypersaline mikrobielle Matte, Sediment, Abwasser, Korallengewebe, Hochlandstrom
5	***Verrucomicrobia***	75-98	unkultivierbare Vertreter	*Frasassi Cave*, Boden, Meer-, See- und Flusssediment, Schlamm
5	**unklassifizierte Candidate Divisions**	84-98	unkultivierbare Vertreter	Boden, hypersaline mikrobielle Matte, Sediment, kontaminierter Sediment
3	***Acidobacteria***	87-99	unkultivierbare Vertreter	*Frasassi Cave*, heiße Quellen, Hochlandstrom, Abwasser, See- und Canyonsediment
2	***Alphaproteobacteria***	92-99	unkultivierbare Vertreter	Teeröl kontaminierter Boden
2	***Epsilonproteobacteria***	86-95	unkultivierbare Vertreter	LKC, *Frasassi Cave*, kontaminierte Sedimente
1	***Actinobacteria***	98	unkultivierbare Vertreter	Seesediment, Abwasser
1	***Fibrobacter***	91-95	unkultivierbare Vertreter	hypersaline mikrobielle Matte, Meeresbodensediment
1	***Nitrospirae***	99	unkultivierbare Vertreter	Trinkwasser
<1	***Spirochaetes***	95	unkultivierbare Vertreter	Schlamm
	Archaeen			
1	***Euryarchaeota***	93-94	unkultivierbare Vertreter	Süßwassersediment

Nach der allgemeinen Diversitätsstudie folgte eine detailliertere Untersuchung zu Vertretern des *Phylums Acidobacteria*, das 1997 zum ersten Mal von Ludwig et al. beschrieben wurde. Bis heute sind nur wenige kultivierbare Vertreter bekannt, obwohl in *terrestrischen* und *subterrestrischen* Systemen zahlreiche neue 16S-rRNS Gensequenzen nachgewiesen werden konnten, die für eine eindeutige Verwandtschaft zu *Acidobacteria* sprechen (Ward et al., 2009). Im *anaeroben* Bereich der Mattenprobe 203g konnten drei Prozent der Genbank dem *Phylum Acidobacteria* zugeordnet werden. Anhand der 16S-rRNS Gensequenzen konnten sie in sieben OTUs mit einer internen Sequenzähnlichkeit von 81-100% unterschieden werden. Die Mehrheit dieser OTUs gehören zu den Untergruppen 7 und 8 (gemäß der Unterteilung von Zimmermann et al., 2005). Eine OTU entspricht der Untergruppe 6 und eine weitere ist am nächsten mit der Untergruppe 10 verwandt. Die repräsentativen 16S-rRNS Klone von *Acidobacteria* sind in der Tabelle 1 zusammengefasst und der dazugehörige 16S-rRNS Baum ist im Anhang C, Abbildung 2, S. 136 sowie Anhang B, ergänzendes Material, Abbildung SM1, S. 121 wiedergegeben.

C2. Phylogenetischer Vergleich der Genbankstudien an den *aeroben* bzw. *anaeroben* Bereichen der weißen Matten

→ siehe auch Anhang B

Zwischen den zehn Proben aus dem *aeroben* (Abbildung 4a; Engel et al., 2004a) und dem einem *anaeroben* Bereich der weißen Matten entlang der LKC (Abbildung 4b), konnten zwei signifikante Unterschiede bezüglich der 16S-rRNS Gendiversität festgestellt werden:

1) Die Diversität war im *anaeroben* Bereich des Standorts 203g wesentlich höher als im *aeroben* Bereich der zehn weißen Mattenproben von verschiedenen Standorten. Im *anaeroben* Bereich konnten 16 Bakteriengruppen ermittelt werden, während es im *aeroben* Bereich nur sechs Bakteriengruppen waren. Alle ermittelten Bakteriengruppen wurden aus dem *aeroben* Bereich (Engel et al., 2004a) auch im *anaeroben* Bereich des Standorts 203g gefunden. Der *anaerobe* Bereich enthielt

zusätzlich eine große Vielfalt an Vertretern aus verschiedenen *Phyla* wie *Actinobacteria*, *Alphaproteobacteria*, *Chloroflexi*, *Fibrobacter*, *Firmicutes*, *Nitrospirae*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia*, unklassifizierte Candidate Divisions, und *Euryarchaeota* unter den Archaeen.

2) Die *aeroben* und *anaeroben* Bereiche in den weißen Mattenproben wurden von völlig verschiedenen Bakteriengruppen dominiert. Im *aeroben* Bereich dominierten die *Epsilonproteobacteria* mit einem Anteil von 68% an der gesamten Genbank, während er im *anaeroben* Bereich der Anteil nur bei zwei Prozent lag. Von den vielen Bakteriengruppen die im *anaeroben* Bereich vorkommen sind drei Gruppen besonders zu erwähnen:

- die *Deltaproteobacteria*, die 24% der Genbank ausmachte. Ihr Anteil im *aeroben* Bereich betrug dagegen nur ein Prozent.
- die *Chloroflexi,* mit einem Anteil von 14%. Im *aeroben* Bereich konnten dagegen keine *Chloroflexi* nachgewiesen werden (Engel et al., 2004a).
- die *Gammaproteobacteria* machten zwölf Prozent der Genbank aus dem *anaeroben* Bereich aus. Im *aeroben* Bereich wurde ein ähnlich hoher Anteil gefunden.

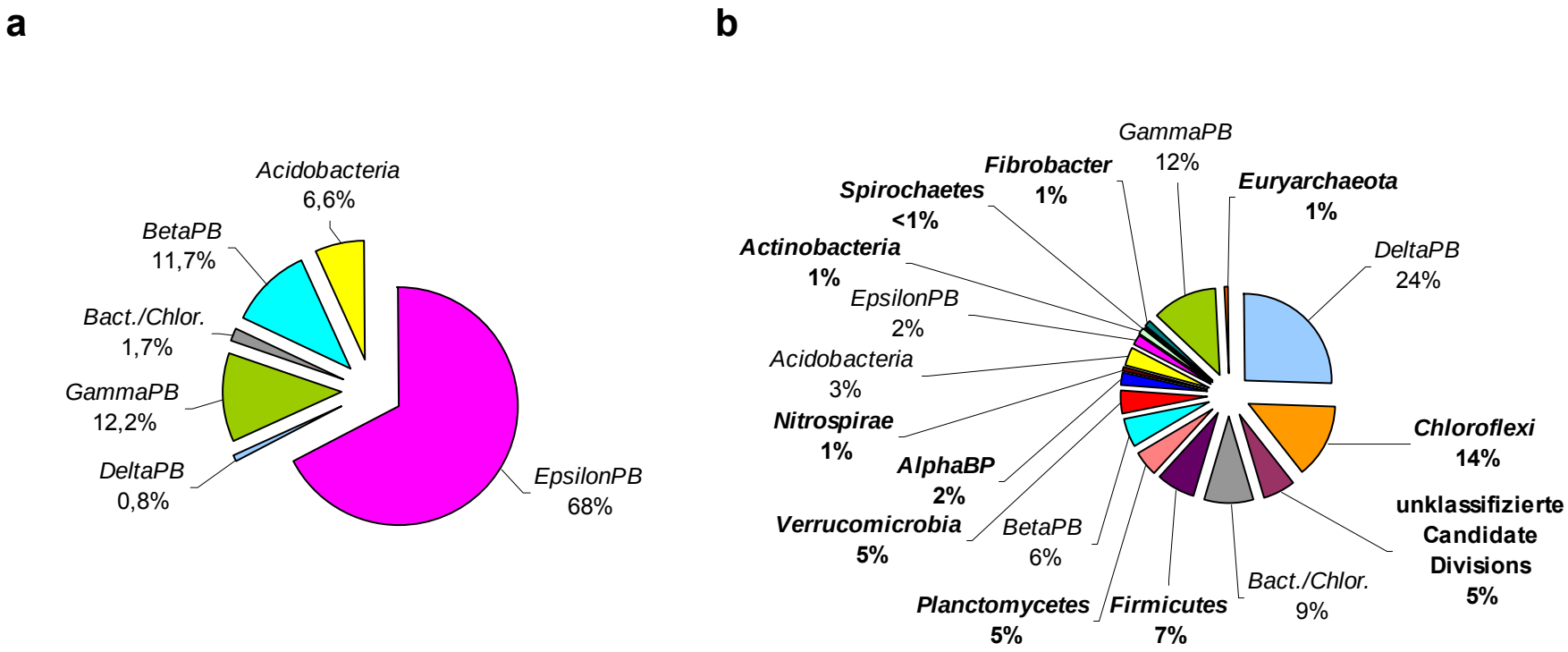

Abbildung 4: Prozentuale Verteilung der einzelnen *Phyla* im *aeroben* und *anaeroben* Bereich
Die Kreisdiagramme geben die prozentuale Verteilung der einzelnen *Phyla* der ermittelten 16S-rRNS Gensequenzen aus den *aeroben* (a; aus zehn Probestandorten; Daten von Engel et al., 2004a) und dem *anaeroben* Bereich (b; aus einem Probestandort; Anhang B) der mikrobiellen Matte vom Standort an der Stelle 203 m. Die neuen Gruppen, die nur im *anaeroben* Bereich erfasst werden konnten, sind fettgedruckt (b).

AlphaPB: *Alphaproteobacteria*, ***BetaPB:*** *Betaproteobacteria*, ***GammaPB:*** *Gammaproteobacteria*, ***DeltaPB:*** *Deltaproteobacteria*, ***EpsilonPB:*** *Epsilonproteobacteria*, ***Bact./Chlor.:*** *Bacteroidetes/Chlorobi*

C3. Nachweis von verschiedenen Bakteriengruppen mittels Fluoreszenz *in situ* Hybridisierung (FISH) in unterschiedlichen Mattenproben

→ siehe auch Anhang B

Die Fluoreszenz *In Situ* Hybridisierung (FISH) wurde benutzt, um die Verteilung, die Morphologie und Assoziation der Mikroorganismen in Mattenproben nachzuweisen. Das Ziel war es, die Ergebnisse der Genbanken zu ergänzen und etwaige Abweichungen festzustellen (Juretschko et al., 2002).

FISH wurde mit 29 verschiedenen 16S- oder 23S-rRNS-gerichteten Oligonukleotidsonden (Anhang C, Tabelle 1, S. 138; Anhang B, ergänzendes Material, Tabelle SM1, S. 114) durchgeführt. Dabei wurden 30 verschiedene PFA- und Ethanol-fixierte Mattenproben analysiert, die aus *aeroben* und *anaeroben* Bereichen (Abbildung 5 und Tabelle 2) von verschiedenen Standorten innerhalb von 123-248 m entlang der LKC stammten.

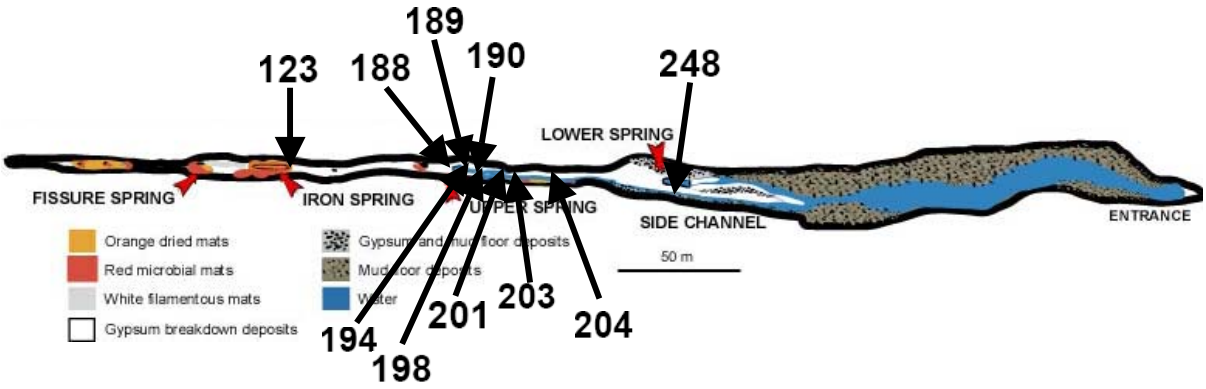

Abbildung 5: Schematische Darstellung der LKC mit den Positionen verschiedener Matten
Die schwarzen Zahlen in der Abbildung geben die Position in Metern der untersuchten mikrobiellen Matten an (vom hinteren Teil der Höhle zum Eingang; Abbildung von Engel, www.geo.utexas.edu/chemhydro/lowerkane/cavedesc.htm, mod.).

Abhängig vom Mattentyp wurden für 18 der 30 Proben Fluoreszenzsignale mit mehreren verschiedenen Zellen mit der allgemeinen Sonde für die Domäne *Bacteria* EUB338-I erhalten (Anhang B, Tabelle 1, S. 101). Von den 29 getesteten Oligo-FISH Sonden zeigten zehn gruppenspezifische Sonden, je nach Sonde, starke oder schwache Signale einzelner Zellen (Anhang B, Tabelle 1, S. 101). Die gesamte

prozentuale Verbreitung der ermittelten Gruppen in den *aeroben* und *anaeroben* Bereichen der Matten ist im Anhang B, ergänzendes Material, Abbildung SM11, S. 131 dargestellt. Die Ergebnisse können wie folgt zusammengefasst werden:

- Ein Nachweis von Bakterien mit Hilfe von FISH war in den meisten Proben möglich, wobei im Allgemeinen prozentual mehr Zellen in den Proben aus dem *aeroben* als dem *anaeroben* Bereich detektiert werden konnten. Nur sieben Proben waren wegen zu hoher Autofluoreszenz oder auf Grund der Verunreinigung mit anorganischem Material für eine FISH-Analyse nicht geeignet.
- Verschiedene Zellmorphotypen wie Kokken, lange und kurze Stäbchen und verschiedenartige Fäden konnten mikroskopisch nachgewiesen werden. Auffallend ist, dass in allen Proben verschiedene Arten von fadenförmigen Bakterien dominierten.
- In den meisten Fällen reagierten die fadenförmigen Organismen mit Gensonden gegen vier Gruppen innerhalb der *Proteobacteria* (*Alpha*-, *Gamma*-, *Delta*- *Epsilonproteobacteria)* gegen das *Phylum Bacteroidetes*. Mittels eines unten dargestellten abweichenden FISH-Protokolls, wurde eine Reaktion gegen das *Phylum Chloroflexi* detektiert (Abbildung 6, Reihe I-VI).
- In über 50% (Anteil der jeweiligen Gruppe) der 30 untersuchten Proben konnten folgende drei Gruppen nachgewiesen werden (70% *Gammaproteobacteria*, 60% *Acidobacteria* und 60% *Epsilonproteobacteria*). Sieben Gruppen konnten in weniger als 50% (Anteil der jeweiligen Gruppe) der Mattenproben nachgewiesen werden (40% *Alpha*- und 40% *Deltaproteobacteria*, 40% *Firmicutes*, 30% *Bacteroidetes/Chlorobi*, 30% *Betaproteobacteria*, 10% *Actinobacteria* und 10% *Planctomycetes*). Eine ausführliche Liste hierzu ist im Anhang B, Tabelle 1, S. 101 zu finden.
- Mit den Sonden für die drei Bakteriengruppen *Chloroflexi* (CFX1223 und GNSB941), *Nitrospira* (Ntspa662 und Ntspa712) und *Verrucomicrobia* (EUB338-III) und den Sonden für *Archaea* (Arch915 und Arch344) konnte allerdings bei keiner der 30 Mattenproben ein zuverlässiges Sondensignal mittels Oligo-FISH erhalten werden. Jedoch konnte *Chloroflexi* mittels einer weiteren Hybridisierungstechnik der *„Catalyzed Reporter Deposition“* FISH

(CARD-FISH; Pernthaler et al., 2002), nachgewiesen werden. Hierbei wurde die mit Meerrettich-Peroxidase (HRP) markierte Sonde CFX1223 (Anhang C, Tabelle 1, S. 138) verwendet. Vorläufige Ergebnisse zeigten, dass positive Signale in fadenförmigen Strukturen (Abbildung 6, Reihe VI) mit dieser Sonde in sechs Mattenproben (LKC1, 3, 11, 24, 27 und 29) aus dem *aeroben* und elf aus dem *anaeroben* Bereich (LKC4, 5, 7, 8, 10, 12, 14, 15, 19, 25 und 26) detektiert werden konnten (Meisinger et al., Manuskript unter Vorbereitung).

Alle Ergebnisse dieser FISH Analysen von Mattenproben der LKC aus 13 *aeroben* und 17 *anaeroben* Bereichen sind in Tabelle 3 zusammengefasst.

Tabelle 2: Überblick und Beschreibung aller 30 Mattenproben von beiden Bereichen

Überblick und Beschreibung aller 30 Mattenproben aus *aeroben* und *anaerobe*n Bereichen (persönliche Mitteilung von Engel A.S.), die in dieser Arbeit zu FISH-*Screening* benutzt wurden.

Probe	Höhlenposition[a]	Mattentyp	*aerob*	*anaerob*	mit FISH nachweisbar[b]
LKC1	248 m, LS	weiße Fäden	+	-	+
LKC2	248 m, LS	graue Fäden	-	+	-
LKC3	248,1 m, LS	weiße Matte mit gelben Ablagerungen	+	-	+
LKC4	248,3 m, LS	grau-braune Fäden	-	+	+
LKC5	248,4 m, LS	grau-braune und orange Matte unterhalb weißer Fäden	-	+	+
LKC6	188 m, US	roter Biofilm	+	-	-
LKC7	188,5 m, US	oranger Biofilm und Sediment	-	+	+[d]
LKC8	189 m, LS	schwarzer Biofilm in der Nähe vom roten Biofilm	-	+	+[d]
LKC9	190 m, US	graues Sediment	-	+	-
LKC10	190 m, US	weiße Fäden	-	+	+
LKC11	194 m, Zufluss	weiße filamentöse Matten	+	-	+
LKC12	194,5 m, Zufluss	Zentrum des Kanals, graue Fäden	-	+	+
LKC13	194,7 m, Zufluss	Nordseite vom Kanal, weiße Fäden	+	-	+
LKC14a	198,1 m, Zufluss	Südseite vom Kanal, graue & weiße Fäden	-	+	+
LKC14b	198,2 m, Zufluss	Südseite vom Kanal, graue & weiße Fäden	-	+	+
LKC15	198,5 m, Zufluss	Zentrum des Kanals, grauer Boden	-	+	+
LKC16	198,7 m, Zufluss	Nordseite, weiße Fäden	+	-	+
LKC17	201 m, Kanal	Mattennetz, obere Komponente	+	-	+
LKC18	201 m, Kanal	unter LKC17, graue Matte	-	+	+
LKC19	201,3 m, Kanal	Mitte des Kanals, Bodenmatte mit vielen Methanblasen	-	+	+
LKC20 (203f[c])	203,5 m, Kanal	Nordseite, weiße Oberflächenfäden	+	-	+
LKC21	203 m, Kanal	weißes Netz, obere Komponente	+	-	+
LKC22 (203g[c])	203 m, Kanal	~2 cm unterhalb der weißen Matte, grau-braune Matte und graue Fäden	-	+	-
LKC23	203 m, Kanal	~2 cm unterhalb der weißen Matte, grau-braune Matte und graue Fäden	-	+	-
LKC24 (203y[c])	203,3 m, Kanal	weiße Matte mit gelben Ablagerungen	+	-	+
LKC25	203,5 m, Kanal	Gallertartige grau-braun weiße Matte	-	+	+
LKC26	203,7 m, Kanal	~2 cm unterhalb von LKC25, grau-braune Matte mit Blasen	-	+	+
LKC27	204 m, Kanal	Ende der Matte, gallertartige orange, weiße und gelbe Matte mit Blasen	+	-	+
LKC28	123 m, FS	graues Sediment auf dem Quellenboden	+	-	+
LKC29	123 m, FS	weiße Fäden, die im Wasser schwimmen	+	-	+

a: Standort in Meter entlang des Höhlenflusses und die drei Hauptquellen: LS: *Lower Spring*; US: *Upper Spring*; FS: *Fissure Spring* (siehe Abbildung 5)

b: genauere Details siehe Tabelle 3

c: f: weiße Fäden; g: graue Fäden; y: gelblich-weiße Matte

d: nur mit CARD-FISH nachweisbar

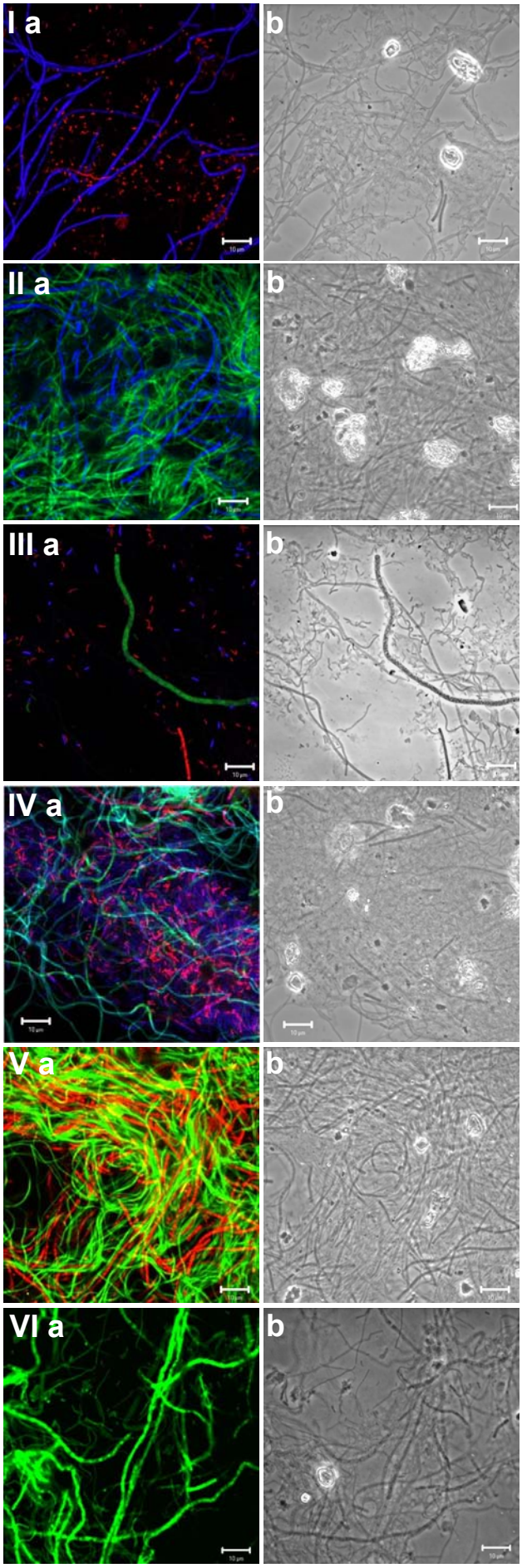

Abbildung 6: Beispiele von FISH-Bildern

Visualisierung von *Bacteroidetes*, *Chloroflexi*, *Alpha-*, *Beta-*, *Gamma-* und *Deltaproteobacteria* mittels Oligo-FISH (Reihe I-V) und CARD-FISH (Reihe VI) in drei verschiedenen Mattenproben am Anfang (LKC1), Mitte (LKC11) und Ende (LKC29) der LKC vom Eingang aus (siehe Abbildung 5). Maßstab: 10 µm.

Reihe I: Mattenprobe LKC29 **a:** Cy3-markierter 16S-rRNS Sondenmix CF319a,b,c (Manz et al., 1996) für *Bacteroidetes* (rot) und Cy5-markierte 16S-rRNS Sonde LKC1006 (Engel et al., 2003) für *Epsilonproteobacteria* (blau), **b:** Phasenkontrastbild.

Reihe II: Mattenprobe LKC11 **a:** Fluos-markierte 16S-rRNS Sonde LKC1006 (Engel et al., 2003) für *Epsilonproteobacteria* (grün) und Cy5-markierte 16S-rRNS Sonde Alf1b+968 (Manz et al., 1992; Neef et al., 1998) für *Alphaproteobacteria* (blau),
b: Phasenkontrastbild.

Reihe III: Mattenprobe LKC29 **a:** Cy3-markierter Sondenmix DELTA495a,b,c (16S-rRNS; Loy et al., 2002) für *Deltaproteobacteria* (rot); Cy5-markierte 23S-rRNS Sonde Bet42a (Manz et al., 1992) für *Betaproteobacteria* (blau) und Fluos-markierte 23S-rRNS Sonde Gam42a (Manz et al., 1992) für *Gammaproteobacteria* (grün),
b: Phasenkontrastbild.

Reihe IV: Mattenprobe LKC11 **a:** Überlagerung von drei Bildern: Cy3-markierter Sondenmix DELTA495a,b,c (16S-rRNS; Loy et al., 2002) für *Deltaproteobacteria* (rot); Cy5-markierter (16S-rRNS; Daims et al., 1999) Sondenmix EUB338-I,II,III für Bakterien (blau) und Fluos-markierte 16S-rRNS Sonde LKC1006 (Engel et al., 2003) für *Epsilonproteobacteria* (grün), **b:** Phasenkontrastbild.

Reihe V: Mattenprobe LKC11 **a:** Überlagerung von zwei Bildern: Cy3-markierter Sondenmix DELTA495a,b,c (16S-rRNS; Loy et al., 2002) für *Deltaproteobacteria* (rot) und Fluos-markierte 16S-rRNS Sonde LKC1006 (Engel et al., 2003) für *Epsilonproteobacteria* (grün), **b:** Phasenkontrastbild.

Reihe VI: Mattenprobe LKC1 **a:** HRP Sonde (mit ALEXA 488 markiert) CFX1223 (Björnsson et al., 2002) für *Chloroflexi* (grün), **b:** Phasenkontrastbild.

C4. FISH-Analyse von *Acidobacteria* in unterschiedlichen Mattenproben

Die Verteilung von *Acidobacteria* wurde mittels FISH näher studiert, da aufgrund der Genbankstudie möglicherweise neue Vertreter innerhalb der *Acidobacteria* vorkommen.

C4.1 Konstruktion neuer 16S-rRNS Oligonukleotidsonden für *Acidobacteria*

→ siehe auch Anhang C

In der 16S-rRNS Genbank konnte eine größere Diversität (vier Untergruppen: 6, 7, 8, 10) als in der 23S-rRNS Genbank ermittelt werden. Deshalb wurden in dieser Arbeit für *Acidobacteria* auch 16S-rRNS Gensonden erstellt. Zwölf neue 16S-rRNS Oligonukleotidsonden für die verschiedenen 16S-rRNS Klonsequenzen innerhalb der *Acidobacteria* konnten gegen diese vier Untergruppen *in silico* konstruiert werden (Anhang C, Tabelle 1, S. 138; Anhang C, Abbildung 2, S. 136). Die Spezifität der neuen 16S-rRNS Sonden für *Acidobacteria* wurde mit sechs weißen mikrobiellen Mattenproben (LKC11, 13, 15, 20, 21, 29) getestet. Deren Biomasse wies wenig Fluoreszenzhintergrund auf und es war aufgrund einer vorherigen Studie mittels 23S-rRNS Sonden bekannt (Zimmermann, 2002), dass diese Proben ausreichend *Acidobacteria*-Zellen enthalten. Allerdings konnte nur eine der vier Untergruppen, nämlich Untergruppe 7, erfolgreich mit den Sonden SS_subd7_UB55-585 und SS_subd7_UB55-632 nachgewiesen werden (Abbildung 7). Die anderen Sonden gegen die Untergruppen 6, 8 und 10 ergaben entweder keine oder nur fragwürdige Signale (Anhang C, Tabelle 1, S. 138).

C4.2 Nachweis von *Acidobacteria*

→ siehe auch Anhang C

Obwohl die *Acidobacteria* nur drei Prozent der Klone der Genbank aus dem *anaeroben* Bereich in der weißen Matte des Standorts 203g darstellten, konnten sie dennoch in vielen anderen Mattenproben (acht Mattenproben aus dem *aeroben* und fünf aus dem *anaeroben* Bereich) mittels 16S- und 23S-rRNS gerichteter Oligonukleotidsonden und FISH (Oligo-FISH) detektiert werden. Von den sieben verschiedenen OTUs innerhalb der *Acidobacteria*, die mittels der Genbank identifiziert wurden, konnten nur zwei unterschiedliche morphologische Gruppen detektiert werden, nämlich die Morphotypen A und B (Zimmermann, 2002; Anhang C, Abbildung 4, S. 139). Morphotyp A war ~8 µm lang und hatte einen Durchmesser von ~0,3 µm (Zimmermann, 2002). Diese langen Stäbchen gehörten der Untergruppe 7 an (Anhang C, Abbildung 2, S. 136). Morphotyp B hatte einen ähnlichen Durchmesser und war mit einer Länge von ~2,5 µm wesentlich kürzer (Zimmermann, 2002). Diese kurzen Stäbchen gehörten zur Untergruppe 8 (Anhang C, Abbildung 2, S. 136). Beide Morphotypen A und B traten immer in Clustern auf (Zimmermann, 2002). Ergänzend zu den Ergebnissen von Zimmermann, 2002 über das Vorkommen von Morphotyp A und B in nur fünf Mattenproben (LKC11, 14, 15, 21, 29) wurde das Vorkommen beider Morphotypen in dieser Arbeit in weiteren 25 Mattenproben untersucht. Dabei wurden die 23S-rRNS Gensonden (Anhang C, Tabelle 1, S. 138) auf verschiedenen hierarchischen Ebenen verwendet. Aufgrund der größeren Diversität in der 16S-rRNS Genbank wurden auch die neu entwickelten 16S-rRNS Gensonden eingesetzt, um die größtmögliche Diversität zu erfassen. Die 23S-rRNS Gensonden zeigten jedoch stärkere Signale als die 16S-rRNS Gensonden.

Die Ergebnisse der fünf Mattenproben von Zimmermann, 2002 konnten größtenteils bestätigt werden. Nur die Ergebnisse von zwei Mattenproben LKC14 und LKC15 waren nicht wiederholbar (Tabelle 3). Drei verschiedene Verteilungsmuster der zwei Morphotypen A und B konnten nach dem kompletten *Screening* in den jeweiligen Mattenproben beobachtet werden:

1) *Vorkommen von Morphotyp A:* Die langen Stäbchen der Untergruppe 7 konnten in zwei *aeroben* (LKC24 und 29) und drei *anaeroben* Bereichen (LKC14, 15 und 19) mit der 23S-rRNS Sonde LS_subd7_ALP761 nachgewiesen werden (Anhang C, Abbildung 4A, S. 139).

2) *Vorkommen von Morphotyp B:* Die kurzen Stäbchen der Untergruppe 8 konnten nur im *aeroben* Bereich der Mattenprobe LKC13 mit den 23S-rRNS Sonden LS_subd8_SGB1223 und LS_subd8_SGB1420 detektiert werden (Anhang C, Abbildung 4B, S. 139).

3) *Gleichzeitiges Vorkommen von Morphotyp A und B:* In fünf *aeroben* (LKC1, 11, 20, 21 und 29) und zwei *anaeroben* Bereichen (LKC4 und 5) konnten einzelne Zellen beider Untergruppen 7 und 8, die in Clustern vorkamen, erfasst werden (Anhang C, Abbildung 4A+B, S. 139). Die Zellen der Untergruppe 7 konnten häufiger in den verschiedenen Mattenproben nachgewiesen werden als die Zellen der Untergruppe 8.

Sämtliche Zellen, die mit den Gensonden gegen *Acidobacteria* hybridisierten, traten immer zusammen mit einzelnen Fäden der Klassen *Epsilonproteobacteria* (Zimmermann, 2002; Anhang C, Abbildung 5, S. 140; Abbildung 8a), *Gammaproteobacteria* oder mit beiden Gruppen auf (Abbildung 8b). Das gemeinsame Auftreten mit den *Gammaproteobacteria* konnte im Rahmen dieser Arbeit nachgewiesen werden. Alle Ergebnisse des FISH-*Screenings* von Mattenproben der LKC aus acht *aeroben* und fünf *anaeroben* Bereichen sind in Tabelle 3 zusammengefasst.

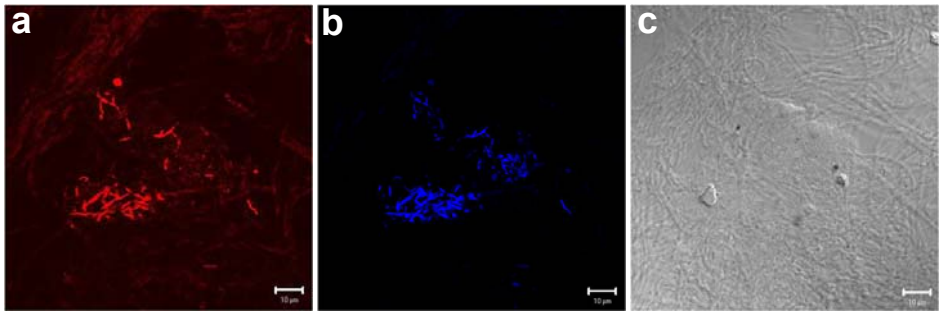

Abbildung 7: Oligo-FISH Bilder von einer spezifischen Sonde
Oligo-FISH Bilder mit der fixierten Mattenprobe LKC20 und der neukonstruierten 16S-rRNS Sonde SS_LKC22_UB55_585 für den Klon SS_LKC22_UB55 bei der *E. coli* Position 588 der Untergruppe 7 von *Acidobacteria*.

a: Cy3-markierte 16S-rRNS Sonde SS_LKC22_UB55_588, **b:** Cy5-markierten *Phylum*sonden Hol1400 (16S-rRNS; Zimmermann, 2002) und Hol189 (23S-rRNS; Zimmermann, 2002) für *Acidobacteria*, **c:** Phasenkontrastbild. Maßstab: 10 µm.

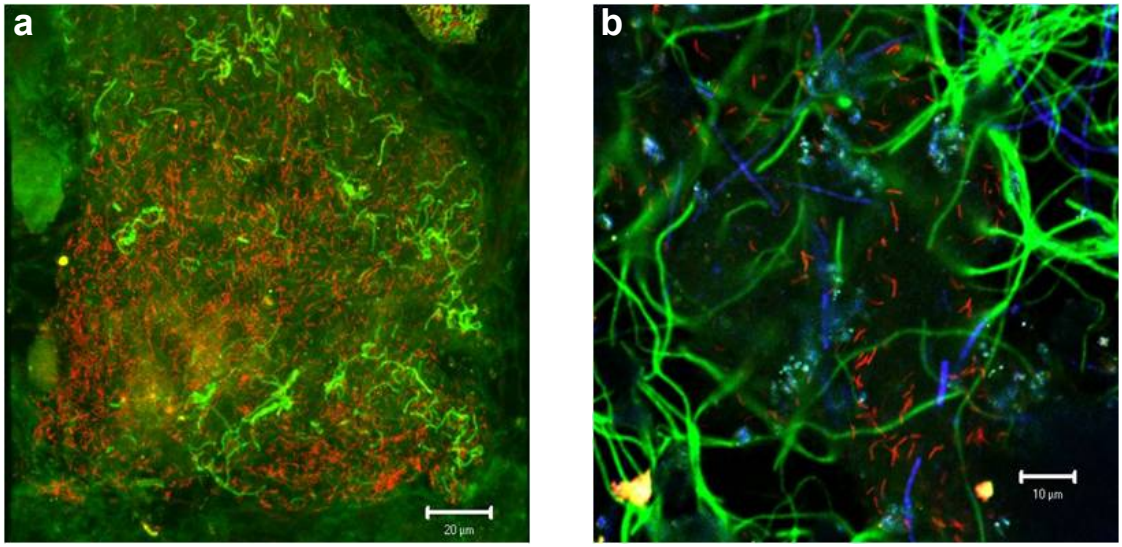

Abbildung 8: Oligo-FISH Bilder einzelner *Acidobacteria* Zellen eingebettet mit *Epsilon*- und/oder *Gammaproteobacteria*
a: Mattenprobe LKC20 mit der Sonde 23S-rRNS ALP761 (Zimmermann, 2002) für die Untergruppe 7 der *Acidobacteria* (rot) und 16S-rRNS Sonde EPSY549 (Lin et al., 2006) für *Epsilonproteobacteria* (grün). Maßstab: 20 µm.
b: Mattenprobe LKC11 mit den *Phylum*sonden (Hol189 (23S-rRNS)/Hol1400 (16S-rRNS); Zimmermann, 2002) für *Acidobacteria* (rot), 23S-rRNS Sonde Gam42a+Bet42a comp (Manz et al., 1992) für *Gammaproteobacteria* (blau) und 16S-rRNS Sonde LKC1006 (Engel et al., 2003) für *Epsilonproteobacteria* (grün). Maßstab: 10 µm.

Tabelle 3: Oligo- und CARD-FISH Ergebnisse von 30 Mattenproben

Phyla / Matten-probe (Meter)	*Acidobacteria* Untergruppe 7[a]	*Acidobacteria* Untergruppe 8[a]	*Actino-bacteria*[a]	*Bacter-oidetes*[a]	*Firmi-cutes*[a]	*Plancto-mycetes*[a]	APB[a]	BPB[a]	GPB[a]	DPB[a]	EPB[a]	*Chloro-flexi*[b]
LKC1 (248 m)	+	+	-	+	-	-	+	+	+	+	+	+
LKC2 (248 m)	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	n.d.
LKC3 (248,1 m)	-	-	-	-	+	-	-	+	+	-	+	+
LKC4 (248,3 m)	+	+	-	-	+	-	+	-	+	+	+	+
LKC5 (248,4 m)	+	+	-	-	-	-	-	-	+	+	+	+
LKC6 (188 m)	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	n.d.
LKC7 (188,5 m)	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	+
LKC8 (189 m)	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	+
LKC9 (190 m)	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	n.d.
LKC10 (190 m)	-	-	-	-	-	-	-	-	+	-	-	+
LKC11 (194 m)	+[c]	+[c]	-	+	+	-	+	-	+	+	+	+
LKC12 (194,5 m)	-	-	-	-	-	-	-	-	-	-	-	+
LKC13 (194,7 m)	-	+	-	-	+	+	+	-	+	+	+	-
LKC14a (198,1 m)	+[c]	-[d]	-	-	+	-	+	-	+	+	+	+
LKC14b (198,2 m)	-	-	-	-	-	-	-	-	+	+	+	n.d.
LKC15 (198,5 m)	+[c]	-[d]	-	-	-	-	+	-	+	+	-	+
LKC16 (198,7 m)	-	-	-	-	-	-	+	+	+	+	-	n.d.
LKC17 (201 m)	-	-	-	-	+	-	+	-	+	-	-	n.d.
LKC18 (201 m)	-	-	-	-	-	-	-	-	+	-	-	-
LKC19 (201,3 m)	+	-	-	-	-	-	-	-	+	-	-	+
LKC20 (203,5 m)	+	+	-	+	-	-	-	+	+	-	+	-
LKC21 (203 m)	+[c]	+[c]	-	-	+	-	+	-	-	+	-	-
LKC22 (203 m)	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	-
LKC23 (203 m)	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	Autofl.	n.d.
LKC24 (203,3 m)	+	-	+	+	+	+	+	+	+	+	+	+
LKC25 (203,5 m)	-	-	-	+	+	-	-	+	-	-	+	+
LKC26 (203,7 m)	-	-	-	-	-	-	-	-	+	-	-	+
LKC27 (204 m)	-	-	-	-	-	-	+	-	-	-	+	-
LKC28 (123 m)	-	-	-	-	-	-	-	-	-	-	-	-
LKC29 (123 m)	+[c]	+[c]	-	+	+	+	-	-	+	+	+	+

a: Oligo-FISH *Screening*
b: CARD-FISH *Screening* nur bei *Chloroflexi*
c: Bestätigung der Ergebnisse von Zimmermann, 2002

d: positive Ergebnisse von Zimmermann, 2002 nicht wiederholbar
n.d.: nicht durchgeführt, **Autofl.:** Autofluoreszenz; **+:** positives Signal, **-:** kein Signal
Felder grau unterlegt: Mattenproben aus *anaerobem* Bereich
APB: *Alphaproteobacteria*; **BPB:** *Betaproteobacteria*; **GPB:** *Gammaproteobacteria*; **DPB:** *Deltaproteobacteria*; **EPB:** *Epsilonproteobacteria*

C5. Vergleich der Genbankstudie mit FISH-Ergebnissen

→ siehe auch Anhang B

Der Vergleich von den Ergebnissen die mit den Genbankstudien und mit dem FISH-*Screening* erhalten wurden, diente dazu:

i) die Verteilung und Morphologie von den Gruppen, die in den verschiedenen Genbanken identifiziert wurden, mittels FISH zu visualisieren;
ii) Unterschiede zwischen *aeroben* und *anaeroben* Bereichen aufzuschlüsseln;
iii) methodische Diskrepanzen und ihren Einfluss auf die Interpretation der Ergebnisse zu erkennen.

In der Tabelle 4 sind von den acht verschiedenen mikrobiellen Matten bei verschiedenen Höhlenpositionen (123, 190, 194, 198, 201, 203, 204, 248) die ermittelten FISH-Ergebnisse zusammengefasst.

Tabelle 4: Oligo- und CARD-FISH Ergebnisse verschiedener mikrobieller Matten

Position der Matten (m) / Taxonomie	123	190	194	198	201	203	204	248
Acidobacteria	a	-	a	an	an	a	a	a/an
Actinobacteria	-	-	-	-	-	a	-	-
Bacteroidetes/Chlorobi	a	-	a	-	-	a/an	-	a
Chloroflexi *	a	an	a/an	an	an	a/an	-	a/an
Firmicutes	a	-	a	an	a	a/an	-	a
Planctomycetes	a	-	a	-	-	a	-	-
Alphaproteobacteria	-	-	a	a/an	a	a	a	a/an
Betaproteobacteria	-	-	-	a	-	a/an	-	a
Gammaproteobacteria	a	an	a	a/an	a/an	a	-	a/an
Deltaproteobacteria	a	-	a	a/an	-	a	-	a/an
Epsilonproteobacteria	a	-	a	an	-	a/an	a	a/an

a: *aerobe* Bereiche; **an:** *anaerobe* Bereiche
***:** Ergebnisse anhand CARD-FISH. Die Ergebnisse mit Oligo-FISH waren alle negativ.

Je nach Bakteriengruppe gab es verschiedene Verteilungsmuster zwischen den *aeroben* und *anaeroben* Bereichen:

1. Alle sechs Gruppen *Acidobacteria*, *Bacteroidetes*, *Beta-*, *Gamma-*, *Delta-* und *Epsilonproteobacteria* wurden bei vier von zehn *aeroben* Mattenproben

detektiert (Engel et al., 2004a). Diese sechs Gruppen konnten auch mittels FISH in zwölf von den 30 untersuchten *aeroben* Mattenproben erfasst werden.

2. Darüber hinaus konnten in neun untersuchten Mattenproben aus *aeroben* Bereichen noch fünf weitere Gruppen (*Actinobacteria*, *Alphaproteobacteria*, *Chloroflexi*, *Firmicutes*, *Planctomycetes*) mit FISH detektiert werden, die mit den *aeroben* Genbanken (Engel et al., 2004a) nicht erfasst werden konnten. Die fünf Gruppen konnten aber mit der Genbank aus dem Bereich der *anaeroben* Mattenprobe 203g detektiert werden (Tabelle 5).

Tabelle 5: Vergleich der Ergebnisse aus der Genbankstudie und FISH-Analysen

Methode / Organismen	***aerobe* Bereiche**		***anaerobe* Bereiche**	
	16S[a]	**FISH[b]**	**16S[c]**	**FISH[b]**
Bakterien	+	+	+	+
Acidobacteria	+	+	+	+
Actinobacteria	-	+	+	-
Bacteroidetes/Chlorobi	+	+	+	+
Chloroflexi	-	+	+	+
Fibrobacter	-	n.d.	+	n.d.
Firmicutes	-	+	+	+
Nitrospirae	-	-	+	-
Planctomycetes	-	+	+	-
Alphaproteobacteria	-	+	+	+
Betaproteobacteria	+	+	+	+
Gammaproteobacteria	+	+	+	+
Deltaproteobacteria	+	+	+	+
Epsilonproteobacteria	+	+	+	+
Spirochaetes	-	n.d.	+	n.d.
Verrucomicrobia	-	-	+	-
unklassifizierte Candidate Divisions	-	n.d.	+	n.d.
Archaeen	n.d.	-	n.d.	-
Euryarchaeota	-	n.d.	+	n.d.

a: Ergebnisse bezüglich der Mattenproben 124f, 127f, 190f, 195f, 198f, 203f, 203w, 203y, 248f und 248y (Engel et al., 2004a)
b: FISH-Ergebnisse bezüglich 23 untersuchten Mattenproben aus *aeroben* bzw. *anaeroben* Bereichen
c: Ergebnisse der Mattenprobe 203g *anaerober* Bereich
n.d.: nicht durchgeführt
+: Erhalt von 16S-rRNS Gensequenzen bzw. positives Signal bei FISH
-: kein Erhalt von 16S-rRNS Gensequenzen bzw. kein Signal bei FISH

3. Von der Genbank aus dem *anaeroben* Bereich der Mattenprobe 203g konnten neun Gruppen (*Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, *Epsilonproteobacteria*) auch mittels FISH in acht weiteren *anaeroben* Bereichen detektiert werden.

4. Sieben Gruppen (*Actinobacteria*, *Nitrospirae*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia*, unklassifizierte Candidate Divisions, Archaeen) konnten nur in der Genbank aus der Mattenprobe 203g erfasst werden (Tabelle 5), die mittels FISH nicht detektiert werden konnten.

5. In den Genbanken aus den *aeroben* Bereichen und in FISH-Analysen dominierten *Epsilon*- und *Gamma*- sowie z. T. *Beta*- bzw. *Alphaproteobacteria*. In der Genbank aus dem *anaeroben* Bereich der Mattenprobe 203g dagegen dominierten *Deltaproteobacteria* und *Chloroflexi* und z. T. *Gammaproteobacteria*. Im Gegensatz dazu wurden mittels FISH vor allem neben fadenförmige *Deltaproteobacteria* und *Gammaproteobacteria* auch *Epsilonproteobacteria* nachgewiesen (Tabelle 6).

Tabelle 6: Ergebnisse über die Diversität und der dominanten Gruppen

Methode / Parameter	***aerobe* Bereiche**		***anaerobe* Bereiche**	
	16S[a]	**FISH[b]**	**16S[c]**	**FISH[b]**
Diversität (*Phyla*)	6	11	17	9
dominante Gruppen	1) *EpsilonPB* 2) *GammaPB* 3) *BetaPB*	1) *EpsilonPB* 2) *GammaPB* 3) *AlphaPB*	1) *DeltaPB* 2) *Chloroflexi* 3) *GammaPB*	1) *GammaPB* 2) *DeltaPB* 3) *EpsilonPB*

a: Ergebnisse bezüglich der Mattenproben 124f, 127f, 190f, 195f, 198f, 203f, 203w, 203y, 248f und 248y (Engel et al., 2004a)
b: FISH-Ergebnisse (nur fadenförmige Zellen) bezüglich 23 untersuchten Mattenproben aus *aeroben* und *anaeroben* Bereichen
c: Ergebnisse der Mattenprobe 203g *anaerober* Bereich
***AlphaPB*:** *Alphaproteobacteria*; ***BetaPB*:** *Betaproteobacteria*; ***GammaPB*:** *Gammaproteobacteria*; ***DeltaPB*:** *Deltaproteobacteria*; ***EpsilonPB*:** *Epsilonproteobacteria*

D. DISKUSSION

D1. Die Biodiversität in der sulfidischen *Lower Kane Cave*

→ siehe auch Anhang B und C

Das Ziel dieser Arbeit war es, die Biodiversität in der *Lower Kane Cave* in USA detaillierter zu untersuchen. Zusätzlich zu den bereits früher untersuchten oberen, *aeroben* Bereichen (Engel et al., 2004a), sollte auch von einem tieferen, *anaeroben* Bereich eine 16S-rRNS Genbank erstellt werden. Des Weiteren wurde die Mikroskopietechnik FISH zum *in situ* Nachweis von Bakterien in verschiedenen Proben entlang der LKC verwendet. Die Ergebnisse aus den kulturvierungs-unabhängigen Analysen ergaben neue Hinweise zur Diversität und Verteilung der Mikroorganismen innerhalb der LKC. Im Rahmen dieser Arbeit wurde erstmals phylogenetisch die Diversität im *anaeroben* Bereich der mikrobiellen Matte bei der Position 203 m analysiert und mit der Diversität von dem *aeroben* Bereich verglichen. Während im *aeroben* Bereich in den 16S-rRNS Genbanken von Engel et al. (2004a) nur insgesamt sechs Bakteriengruppen detektiert wurden, konnten mindestens 16 Bakteriengruppen und eine Archaeengruppe im *anaeroben* Bereich nachgewiesen werden.

Der Schwefelkreislauf ist ein weitverbreiteter Prozess, der außer in Höhlen auch in vielen Ökosystemen vorkommt. Verschiedene Mikroorganismen sind bei den *aeroben* und *anaeroben* Prozessen des Schwefelkreislaufes beteiligt. Schwefel- und sulfidoxidierende Bakterien sind *chemolithoautotroph* und benutzen reduzierte Schwefelverbindungen als Elektronendonatoren, um durch Oxidation zu Sulfat Energie zu erzeugen. Sulfatreduzierende Prokaryonten (*„Sulfate Reducing Prokaryotes“*, SRP), verwenden Sulfat bzw. Schwefel als Elektronenakzeptor (Sulfat/Schwefelatmung), und bilden Schwefelwasserstoff (Reineke und Schlömann, 2007). In der LKC konnten mindestens zwei Hauptgruppen von Schwefelbakterien identifiziert werden: die Schwefeloxidierer und die Sulfatreduzierer. Im oberen, *aeroben* Bereich dominierte die Bakteriengruppe Schwefeloxidierer (*Epsilon-* und *Gammaproteobacteria*) deutlich über alle anderen Gruppen (>70% der mikrobiellen Gesellschaft, basierend sowohl auf Genbankstudien als auch auf FISH-Analysen). Hinsichtlich einer bestimmten Bakteriengruppe im *anaeroben* Bereich gab es keine

deutliche Dominanz. Die identifizierbare funktionelle Hauptgruppe im tieferen, *anaeroben* Bereich ist die der Sulfatreduzierer innerhalb der *Deltaproteobacteria*. Die Gruppe *Deltaproteobacteria* betrug prozentual 24% in der *anaeroben* Genbankstudie und von dieser Gruppe war die Hälfte der Klone ähnlich zu kultivierbaren SRPs. Inwieweit es sich bei der anderen Hälfte innerhalb der *Deltaproteobacteria* im *anaeroben* Bereich auch um SRPs handelt, konnte mit den verwendeten Methoden nicht nachgewiesen werden. Mittels FISH konnte die Gruppe *Deltaproteobacteria* in 40% von zwölf untersuchten Mattenproben in beiden Bereichen nachgewiesen werden. Das Vorhandensein und die Funktion der SRP wurden nicht nur mit 16S-rRNS Genbanken und FISH Studien mit rRNS-gerichteten Sonden nachgewiesen. Sie wurden sowohl durch MPN auf verschiedenen spezifischen Medien als auch einem Funktionsgenansatz basierend auf *dsrAB* Gene bestätigt (unveröffentlichte Daten; neun *dsrAB* Gensequenzen veröffentlicht auf http://www.ncbi.nlm.nih.gov/ unter den Sequenznummern AM490772-AM490780). Basierend auf Kultivierungsstudien mittels verschiedener Medien (Anhang B, Abbildung 4, S. 102) konnten auch andere funktionelle Bakteriengruppen wie gärende Bakterien oder eisenreduzierende Bakterien nachgewiesen werden. Jedoch war es in dieser Arbeit nicht möglich, die Zuordnung der Gensequenzen von der Genbankstudie zu den funktionellen Gruppen zuzuordnen, da das 16S-rRNS Gen in der Regel keine Rückschlüsse auf die Funktion zulässt.

Aufgrund der vorliegenden Ergebnisse in der Genbankstudie kann jedoch eindeutig gezeigt werden, dass die Diversität im *anaeroben* Bereich größer ist als im *aeroben* Bereich. Dies mag jedoch z. T. auch daran liegen, dass im Vergleich zu den früheren Studien (Engel et al., 2004a) hier in dieser Arbeit vier verschiedene 16S-rRNS Primerpaare statt einem Primerpaar benutzt wurden, und damit eine höhere Diversität erhalten werden konnte. In dieser Arbeit wurden beim FISH-*Screening* im *aeroben* Bereich fünf weitere Gruppen erfasst, die mit den *aeroben* Genbanken aus früheren Arbeiten von Engel nicht nachgewiesen werden konnten. Dies deutet daraufhin, dass die Diversität im *aeroben* Bereich bisher unterschätzt wurde. Die unterschiedlichen Ergebnisse bezüglich der geringeren mikrobiellen Diversität im *aeroben* Bereich der Matten aus der LKC könnten folgende Gründe haben:

- Bei der DNS-Extraktion, PCR und Erstellung der Genbank können folgende Faktoren eine ganzheitliche Erfassung der tatsächlichen Diversität beeinflussen: unvollständige Extraktion der DNS, die Benutzung verschiedener Primerpaare und die spezifischen PCR Reaktionsbedingungen.

- Mit der FISH-Methode können z. T. Bakteriengruppen erfasst werden, die in Genbanken nicht nachweisbar waren.

Im *aeroben* Bereich dominieren jedoch eindeutig die *chemolithoautotrophe* Schwefeloxidierer. Als Primärproduzenten bilden sie - wie im *chemolithoautotrophen* Ökosystem der hydrothermalen Quellen (*black smokers*; Brazelton et al., 2006; Zhou et al., 2009) - die Grundlage für das mikrobielle Ökosystem in der LKC. Diese Schwefeloxidierer können so die Entwicklung einer komplexeren *anaeroben* Bakteriengemeinschaft ermöglichen (Newbold et al., 1981). Außerdem ist von früheren Studien von Engel und ihren Mitarbeitern bekannt, dass in der LKC *chemolithoautotrophe*, schwefeloxidierende Bakterien, die zu den *Epsilon-* und *Gammaproteobacteria* gehören, am Entstehungsprozess der Höhle beteiligt sind.

Es muss jedoch erwähnt werden, dass auch in der vorliegenden Studie trotz mehrerer Genbanken von 300 Klonen eine Sättigungskurve der Diversität nicht erreicht werden konnte (siehe Verteilungskurve im Anhang B, Abbildung 3, S. 100). Allerdings gibt es auch einschränkende Faktoren, die eine umfassende Erfassung der Diversität mit Hilfe von FISH beeinflussen können, wie z. B. starke Autofluoreszenz, schlecht zu suspendierende Proben, niedrige Ribosomenkonzentrationen oder geringe Zelldichte. Für zukünftige Studien müssten auch andere Methoden wie quantitative PCR, Pyrosequenzierung und Metagenomics eingesetzt werden.

D2. Übersicht zur Diversität im *anaeroben* Bereich und möglicher Funktionen

→ siehe auch Anhang B und C

D2.1 Die polyphyletische Gruppe der Schwefeloxidierer

Zu der polyphyletischen Gruppe der Schwefeloxidierer gehören diverse Vertreter der drei Gruppen *Epsilon*-, *Gamma*- und *Betaproteobacteria*. Im Rahmen dieser Arbeit konnte nur ein geringer Anteil (zwei Prozent) diverser Vertreter der *Epsilonproteobacteria* im *anaeroben* Bereich gefunden werden. Die Hälfte der *Epsilonproteobacteria* aus dem *anaeroben* Bereich der LKC war nicht nur zu einem bereits bekannten Klon LKC1.57.5 aus den *aeroben* Bereich (Engel et al., 2003; Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118) zu 95% verwandt, sondern auch zu Klonen aus der *Frasassi Cave* in Italien (Macalady et al., 2008).
In dieser Arbeit konnten auch in den 16S-rRNS Genbanken aus dem *anaeroben* Bereich fadenförmige und schwefeloxidierende *Gammaproteobacteria* der Gattung *Thiothrix* spp. erhalten werden. 14 Klone waren zu 96% ähnlich zu einem bereits bekannten *Thiothrix* Klon LKC3_22.33 (Engel et al., 2004a; Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118) aus der LKC. Drei weitere Klone waren 90% zu *Thiothrix* sp. Klon Run-S43 (Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118) verwandt. Die 16S-rRNS Gensequenzen dieser 17 Klone waren 90-97% ähnlich zu dem kultivierbaren *Thiothrix unzii* (L79961). Diese Klone weisen daher mit hoher Wahrscheinlichkeit auf das Vorhandensein von Schwefeloxidierern hin. *Gammaproteobacteria* wurde in beiden Genbanken (*aerob* und *anaerob*) mit derselben Häufigkeit (zwölf Prozent) erfasst. Das weist darauf hin, dass sie in beiden Bereichen eine zentrale Rolle spielen.
Betaproteobacteria konnten auch in dieser Arbeit mit der 16S-rRNS Genbank (fünf Prozent) aus dem *anaeroben* Bereich nachgewiesen werden. Die Klone aus der LKC zeigten aber keine Ähnlichkeit zu der bekannten *Betaproteobacteria* Gattung *Thiobacillus* spp. aus früheren Analysen. Die erhaltenen Klone waren zu 77-92% ähnlich zu dem kultivierbaren Cholesterin oxidierenden *Sterolibacterium denitrificans* (AJ458502).
Die Klone der *Betaproteobacteria* und die restlichen Klone der *Gamma*- und *Epsilonproteobacteria* ähnelten aufgrund ihrer 16S-rRNS Sequenz Stämmen, die in

anderen Ökosystemen (z. B. kontaminierte Sedimente, Trinkwasser, Schlamm, Reisfeld; Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118) gefunden wurden. Ob es sich bei diesen Organismen auch um Schwefeloxidierer handelt, konnte mit den 16S-rRNS Genbanken und FISH nicht nachgewiesen werden. Die Schwefeloxidierer (*Beta*-, *Gamma*-, *Epsilonproteobacteria*) konnten auch in sulfidischen Höhlen z. B. in der *Frasassi Cave* in Italien (Macalady et al., 2008) und in der *Movile Cave* in Rumänien (Chen et al., 2009; Porter et al., 2009) gefunden werden.

Die fadenförmigen *Epsilonproteobacteria* dominierten zusammen mit den fadenförmigen *Gammaproteobacteria* sowohl in der Genbank als auch mit FISH (70% in den Mattenproben; Anhang B, Tabelle 1, S. 101). Die FISH-Analysen zeigten, dass die *Epsilonproteobacteria* in engen räumlichen Kontakt mit Einzelzellen aus den Gruppen der *Beta*-, *Delta*-, *Gammaproteobacteria*, *Acidobacteria* und *Bacteroidetes* vorkommen. Die fadenförmigen *Betaproteobacteria*, die zu 30% in der LKC erfasst wurden, waren mit *Gamma*- und *Deltaproteobacteria* assoziiert. Möglicherweise handelt es sich hier um eine Art von Syntrophie.

D2.2 Die polyphyletische Gruppe der Sulfatreduzierer

→ siehe auch Anhang B

Zu der polyphyletischen Gruppe der Sulfatreduzierer gehören diverse Vertreter innerhalb der Klasse *Deltaproteobacteria,* der *Phyla Firmicutes, Nitrospirae* und einige Archaeen. Sie sind in der Lage, in Abwesenheit von Sauerstoff Sulfat als Elektronenakzeptor zu verwenden.

In den Genbanken aus dem *aeroben* Bereich der LKC konnten einzelne Sulfatreduzierer (z. B. die Klone LKC3 190.28, LKC2 190.37, LKC3 190.63, LKC3 190.75 und LKC3 102.22) der Gattung *Desulfocapsa* spp. innerhalb der *Deltaproteobacteria* erfasst werden (Engel et al., 2004a). Mit einem Anteil von weniger als ein Prozent kann es sich aber auch um eine Kontamination aus dem *anaeroben* Bereich handeln. In der 16S-rRNS Genbank aus dem *anaeroben* Bereich waren dagegen die *Deltaproteobacteria* mit einem Anteil von 24% die dominante Gruppe (siehe Kapitel D1). Die 16S-rRNS Sequenzen von zehn Klonen zeigten 92-96% Ähnlichkeit zu drei kultivierbaren sulfatreduzierenden Vertretern von

Desulfomonile limimaris, Chondromyces pediculatus und *Desulfobacca acetoxidans* (Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118). Die Mehrheit der Klone von *Deltaproteobacteria* in der 16S-rRNS Genbank aus dem *anaeroben* Bereich war ähnlich zu Sequenzen aus der sulfidischen Höhle *Frasassi Cav*e in Italien und aus anderen Ökosystemen (z. B. Boden und Sedimente; Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118).

Die *Phyla Nitrospirae*, *Euryarchaeota* (jeweils ein Prozent) und *Firmicutes* (sieben Prozent) konnten erst mit dieser Arbeit in der LKC isoliert werden. Aber kein Klon dieser Gruppen zeigte Ähnlichkeit zu bekannten Sulfatreduzierern. Zwar wurden in anderen sulfidischen Höhlen z. B. in der *Movile Cave* in Rumänien (Chen et al., 2009; Porter et al., 2009) auch Vertreter von *Nitrospirae* und *Euryarchaeota* gefunden, jedoch unterschieden sie sich in ihrer Sequenz von den Klonen aus der LKC. Dies könnte daran liegen, dass diese Bakteriengruppen durch die räumliche Trennung eine voneinander unabhängige Entwicklung vollzogen haben. Dabei könnte es unter dem jeweiligen Selektionsdruck eine optimale Anpassung an die Gegebenheiten der jeweiligen Höhlen gegeben haben, so dass eine genetische Variation in den jeweiligen Gensequenzen auftrat. *Firmicutes* konnten auch an Höhlenwände und in Höhlensedimenten identifiziert werden (Laiz et al., 2003; Schabereiter-Gurtner et al., 2004; Cacchio et al., 2004; Chelius and Moore, 2004; Ikner et al., 2007; Portillo et al., 2009).

Die 16S-rRNS Gensequenzen der Klone von *Nitrospirae* zeigten 99% Ähnlichkeit zu Klonen, die aus Trinkwasser isoliert wurden (Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118). Außerdem waren die 16S-rRNS Gensequenzen der Klone dieser Gruppe 98% ähnlich zu dem kultivierbaren *chemolithoautotrophen*, nitrit-oxidierenden *Nitrospira moscoviensis* (X82558). Engel und ihre Mitarbeiter haben durch MPN Untersuchungen Hinweise erhalten, dass die Nitratreduktion mit der Sulfidoxidation verbunden ist.

Die ermittelten 16S-rRNS Gensequenzen der *Firmicutes* Klone waren dagegen eher zu Sequenzen verwandt, die aus Meeresbodensediment isoliert wurden und zu Stämmen, die dechlorieren können (Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118). Die ermittelten 16S-rRNS Sequenzen von *Firmicutes* zeigten nur 82-88% Ähnlichkeit zu dem nächsten kultivierbaren Vertreter *Bacillus licheniformis* (AF426473).

Die ermittelten 16S-rRNS Gensequenzen der Gruppe *Euryarchaeota* waren eher ähnlich zu Vertretern von *Euryarchaeota*, die aus methanogenen Süßwassersedimenten isoliert wurden. Die geringe Ähnlichkeit von 77% zu dem nächsten kultivierbaren *Euryarchaeota Natronomonas pharaonis* (D87971) weist darauf hin, dass es sich bei den beiden Klonen (100% Sequenzsimilarität) in der LKC um neue bisher unbekannte *Euryarchaeota* handelt.

Mit FISH konnte nicht nur die Gruppe *Deltaproteobacteria* zu 40% in der LKC erfasst werden, sondern auch das *Phylum Firmicutes* (Anhang B, Tabelle 1, S. 101). Daraus lässt sich schließen, dass diese Gruppe wahrscheinlich auch eine wesentlich bedeutendere Rolle am Kreislauf aufweist, als bisher angenommen wurde. Mit den beiden *Nitrospira* Sonden Ntspa662 und Ntspa712 sowie den beiden *Archaea* Sonden Arch34 und Arch915 (Anhang B, Tabelle 1, S. 101) konnten keine oder nur unschlüssige FISH Signale erhalten werden. Dies könnte daran liegen, dass die Zelldichte zu gering war, um sie mit FISH zu erfassen. Ein weiterer Grund könnte auch ein zu geringer Ribosomengehalt sein. Die verwendeten Sonden Arch344 und besonders Arch915 hatten außerdem unspezifische Sondensignale zu einigen *Gammaproteobacteria* Fäden in den Mattenproben. Zudem zeigten etliche 16S-rRNS Gensequenzen, die mit dem *Archaea*-spezifischen Primerpaar A112f-A934b erhalten wurden, Ähnlichkeiten zu Vertretern innerhalb der *Gammaproteobacteria* Gattung *Thiothrix* spp. Dies ist im Einklang mit früheren Studien, die darauf hinweisen, dass die Sonde Arch915 und das Primerpaar A112f-A934b, auch nicht *Archaea*-spezifische Ergebnisse erbrachten (Amann and Fuchs, 2008).

D2.3 Das *Phylum Acidobacteria*

→ siehe auch Anhang B und C

Die bisherigen Studien hatten den Schwerpunkt auf einige der fadenförmigen schwefeloxidierenden Spezies aufgrund ihrer offenbaren Dominanz gelegt. In dieser Arbeit wurden nähere Studien über eine nicht fadenförmige Gruppe von Bakterien, das *Phylum Acidobacteria* erstellt. Die *Acidobacteria* kommen mit einem Anteil von drei Prozent in der 16S-rRNS Genbank aus dem *anaeroben* Bereich vor. *Acidobacteria* wurde zuletzt taxonomisch in 26 Untergruppen eingeteilt (Barns et al.,

2007). Sieben neue OTUs konnten innerhalb der vier bekannten Untergruppen (6, 7, 8, 10) identifiziert werden. Zwei 16S-rRNS Gensequenzen von *Acidobacteria* waren 99% ähnlich zu Sequenzen, die aus einer anderen sulfidischen Höhle *Frasassi Cave* (Macalady et al., 2006) isoliert wurden. Zwei weitere 16S-rRNS Gensequenzen zeigten 95% Ähnlichkeit zu Sequenzen, die aus heißen Quellen (Hall et al., 2008; Lau et al., 2009) erhalten wurden. *Acidobacteria* wurden nicht nur in sulfidischen Höhlen (Macalady et al., 2006), sondern auch in nicht sulfidischen Höhlen (z. B. *Wind Cave* in South Dakota, USA (Chelius and Moore et al., 2004), *Altamira Cave* in Spanien (Zimmermann et al., 2005; Portillo et al., 2009)) gefunden.

Acidobacteria konnte mittels FISH (in 60% der Mattenproben) fast überall mit 23S-rRNS und den neu konstruierten 16S-rRNS Gensonden, sowohl an Bereichen mit hohen als auch mit niedrigen gelösten Sulfid- und Sauerstoffgehalt, in der LKC nachgewiesen werden. Die detaillierte Quantifizierung der *Acidobacteria* Zellen mit FISH war aber nicht möglich, da einige Mattenproben hohe Autofluoreszenz zeigten bzw. wegen der großen Menge an stark fluoreszierenden fadenförmigen *Epsilon-* und *Gammaproteobacteria* Zellen nicht erkennbar waren.
Die *Acidobacteria* der Untergruppen 7 und 8 kamen in allen Mattenproben mit FISH in dieser Arbeit immer zusammen mit *Epsilon-* und/oder *Gammaproteobacteria* vor. Engel und ihre Kollegen (Engel et al., 2003; Engel et al., 2004a) fanden heraus, dass diese zwei dominanten Proteobakterien Kohlenstoffisotopwerte hatten, die auf einen *chemolithoautotrophen* Metabolismus hinwiesen. Die *Acidobacteria* könnten möglicherweise als *Chemoorganotrophe* auf Abfall- oder Stoffwechselprodukten der *Chemolithotrophen* wachsen. Die Beziehung und die Funktion der *Acidobacteria* mit den dominanten *Epsilon-* und *Gammaproteobacteria* ist allerdings noch unbekannt.

D2.4 Sonstige Bakterien mit unbekannter Funktion

→ siehe auch Anhang B

Chloroflexi ist ein tiefverzweigtes *Phylum* und besitzt eine breite metabolische Diversität. Innerhalb des *Phylums Chloroflexi* ist bekannt, dass manche Vertreter spezielle Abbauwege besitzen. Vertreter der *Chloroflexi* können nicht nur *anoxygen photoautotroph* wachsen, sondern auch *photoheterotroph*. Bei Lichtmangel ist

bekannt, dass einige Vertreter auch *chemoorganotroph* wachsen können (Garrity and Holt, 2001). In der LKC wurde *Chloroflexi* in früheren Analysen (*aerober* Bereich) nicht gefunden. Erst im Rahmen dieser Arbeit konnte die Gruppe *Chloroflexi* in der 16S-rRNS Genbank aus dem *anaeroben* Bereich mit einem relativ hohen Anteil von 14% nachgewiesen werden. Obwohl die ermittelten 16S-rRNS Gensequenzen von *Chloroflexi* auch von der sulfidischen Höhle *Frasassi Cave* in Italien (Engel et al., 2007) und der schwefelwasserstoffführenden *Zodletone* Quelle in Oklahoma, USA (Elshahed et al., 2003) isoliert wurden, waren die Klone aus der LKC eher ähnlich zu Vertretern, die aus Boden, Sediment oder Schlamm (90-98% Sequenzsimilarität; Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118) isoliert wurden und ähnlich zu kultivierten *anaeroben*, fadenförmigen Stämmen (88-90% Sequenzsimilarität). Aufgrund ihrer entfernten Beziehung zu einer großen Anzahl bisher nicht kultivierbarer *Chloroflexi*, die in *anaeroben* Lebensräumen gefunden wurden, und zu kultivierbaren, nicht *obligat anaeroben* Vertretern der Gattungen *Leptolinea* und *Caldilinea* in der Unterklasse I und VI der *Chloroflexi* (Kohno et al., 2002; Yamada et al., 2005), liegt es nahe, dass die Vertreter der in der LKC gefundenen, neuen *Chloroflexi* Gruppe *anaerob* wachsen können. Diese fadenförmigen *Chloroflexi* konnten auch mit CARD-FISH (Pernthaler et al., 2002) in 60% der Mattenproben nachgewiesen werden. Welche Funktion diese *Chloroflexi* in der LKC ausüben, ist allerdings unbekannt.

Eine 16S-rRNS Gensequenz aus dem *anaeroben* Bereich zeigte 100% Ähnlichkeit zu einem *Sphingobacteria* spp. Klon LKC3_102b.33 (Engel et al., 2004a) innerhalb dem *Phylum Bacteroidetes*. Welche Funktion diese Gruppe tatsächlich in der LKC hat, ist noch nicht bekannt. Die restlichen Klone dieser Gruppe zeigten Sequenzähnlichkeiten zu entsprechenden Klonen aus See, Schlamm, mit Teeröl kontaminierte Gewässer (Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118) und sulfidischen Höhlen wie *Frasassi Cave* in Italien (Macalady et al., 2008). Die erhaltenen Klone waren zu 75-90% ähnlich zu den kultivierbaren *Bacteroides putredinis* (L16497). Da unter 90% kein eindeutiger Vergleich mit den nächsten kultivierbaren Spezies (>99% Sequenzsimilariät) gemacht werden konnte, dürfte es sich vermutlich bei diesen Organismen in der LKC um neue Vertreter handeln. Mit FISH konnten *Bacteroidetes* in 30% der Mattenproben erfasst werden (Anhang B,

Tabelle 1, S. 101). Auch hier ist nichts über ihre Funktion in sulfidischen Höhlen bekannt.

In den sulfidischen Höhlen z. B. *Movile Cave* in Rumänien (Porter et al., 2009), *Cesspool Cave* in Virginia, USA (Engel et al., 2001) und *Parker Cave* in Kentucky, USA (Angert et al., 1998) konnten *Bacteroidetes* in großer Anzahl zusammen mit *Beta-*, *Gamma-*, *Delta-* und *Epsilonproteobacteria* erhalten werden (Porter et al., 2009). In nicht sulfidischen Höhlen (z. B. *Kartchner Cave* in Arizona, USA (Ikner et al., 2007)) konnte diese Gruppe auch nachgewiesen werden. Über ihre Funktion dieser Gruppe ist nur wenig bekannt. Es gibt Hinweise bzw. Hypothesen, dass Vertreter dieses *Phylums* an der Gärung oder am Metallkreislauf beteiligt sein können (Angert et al., 1998; Schabereiter-Gurtner et al., 2004; Chelius and Moore, 2004; Macalady et al., 2006; Ikner et al., 2007; Chen et al., 2009).

Die restlichen sieben Gruppen (*Actinobacteria*, *Alphaproteobacteria*, *Fibrobacter*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia* und mehrere unklassifizierte Candidate Divisions) konnten erst im Rahmen dieser Arbeit in der 16S-rRNS Genbank aus dem *anaeroben* Bereich der LKC mit einem Anteil von einem bis fünf Prozent entdeckt werden.

Nur die 16S-rRNS Gensequenzen von zwei Klonen der Gruppe *Verrucomicrobia* waren 98% ähnlich zu einem Klon einer anderen sulfidischen Höhle (*Frasassi Cave* in Italien). Die restlichen Klone zeigten eher Ähnlichkeiten zu Klonen aus Boden, Sedimenten verschiedener aquatischer Ökosystemen, Abwasser, Korallengewebe, Hochlandstrom und Schlamm (Anhang B, ergänzendes Material, Tabelle SM2, S. 115-118). Zu kultivierbaren Vertretern der jeweiligen Gruppe ergab sich nur eine Ähnlichkeit von 76-91%. Nur die 16S-rRNS Gensequenzen der Klone der Gruppe *Alphaproteobacteria* zeigten eine höhere Sequenzähnlichkeit mit 87-97% zu dem kultivierbaren, *methanotrophen* Bakterium *Methylocystis echinoides* (AJ458502; Bowman et al., 1993). Wegen der nahen Verwandtschaft kann es sich hier auch um *methanotrophe* Organismen handeln. Methan kommt ja nach den Untersuchungen von Engel et al. (2004a) im Strömungskanal der LKC vor.

Von den sieben Gruppen konnten mit FISH nur Vertreter der drei Gruppen *Actinobacteria* (zehn Prozent), *Planctomycetes* (zehn Prozent) und *Alphaproteobacteria* (40%) nachgewiesen werden (Anhang B, Tabelle 1, S.101). Die Gruppe *Verrucomicrobia* konnte mit FISH nicht detektiert werden. Vermutlich lag die

Menge der Zellen unter der Detektionsgrenze bzw. war der Ribosomengehalt zu niedrig. Für drei Gruppen *Fibrobacter*, *Spirochaetes* und mehrere unklassifizierte Candidate Divisions wurde kein FISH-*Screening* durchgeführt, da es zum Zeitpunkt dieser Arbeit noch keine geeigneten FISH Gensonden gab.

Alphaproteobacteria, Planctomycetes und *Verrucomicrobia* konnten auch in zwei anderen sulfidischen Höhlen (z. B. *Frasassi Cave* in Italien und *Movile Cave* in Rumänien (Macalady et al., 2007; Chen et al., 2009; Porter et al., 2009)) gefunden werden. *Fibrobacter*, *Actinobacteria* und mehrere unkultivierbare Vertreter von Candidate Divisions konnten nur in der sulfidischen Höhle *Frasassi Cave* in Italien (Macalady et al., 2007; Macalady et al., 2008) isoliert werden. Die Gruppe *Spirochaetes* konnte auch in den sulfidischen Höhlen *Cave of Acquasanta Therme* in Italien (Jones et al., 2010) und *Movile Cave* in Rumänien (Chen et al., 2009) erfasst werden. In anderen nicht sulfidischen Höhlen konnten diese Gruppen mit Ausnahme von *Fibrobacter* ebenfalls nachgewiesen werden (Schabereiter-Gurtner et al., 2002; Barton et al., 2004; Chelius and Moore, 2004; Portillo et al., 2009).

D3. Phylogenetischer Vergleich der 16S-rRNS Gensequenzen in bisher untersuchten Höhlen

→ siehe auch Anhang A

Global gesehen, sind wie in der Einleitung und im Anhang A schon erwähnt, nur ca. zehn Prozent aller Höhlen auf der Welt biologisch beschrieben. Von diesen sind wiederum nur ca. zehn Prozent molekularbiologisch auf ihre mikrobiologische Diversität untersucht worden. Die Inventurstudie (Stand Februar 2011) in dieser Arbeit von über 73 Höhlen (z. B. sulfidische Höhlen, Eis-, Lava-, Orthoquarzit- und Kalksteinhöhlen) von verschiedenen Regionen der Erde (Anhang A, Abbildung 16.15, S. 87-88) ergab, dass nur weniger als ein Prozent aller bisher ermittelten 16S-rRNS Gensequenzen aus Höhlen stammen (http://www.arb-silva.de, April 2011, ca. 1,9 Millionen 16S-rRNS Gensequenzen). Reinkulturen wurden nur aus 14 Höhlen isoliert und charakterisiert. Das waren nur drei Prozent aller ermittelten 16S-rRNS Gensequenzen aus Höhlen. Dies zeigt noch mal sehr deutlich, wie wenig von dem Ökosystem Höhle bekannt ist. Von drei Höhlensystemen *El Zacaton sinkhole* in

Mexiko (Kalksteinlöcher), *Frasassi Cave system* in Italien (sulfidische Höhle) und der *Lower Kane Cave* (WY) in USA (sulfidische Höhle) wurden über 1000 16S-rRNS Gensequenzen erfasst. Über 17 verschiedene taxonomische Gruppen wurden allein in den Höhlensystemen *Lower Kane Cave* (WY) in USA, *El Zacaton sinkhole* (Mexiko), *Frasassi Cave system* in Italien und den *Lava Caves* und *Tubes* (Hawaii) in USA ermittelt.

Der im Anhang A (Abbildung 16.15, S. 88) abgebildete phylogenetische Stammbaum beruht auf aus 28 Höhlen ausgewählten 373 16S-rRNS Gensequenzen, die jeweils ≥1400 Basenpaaren aufwiesen. Darunter waren auch fünf sulfidische Höhlen (*Cesspool Cave* (VA) und *Lower Kane Cave* (WY) in USA, *Cave of Acquasanta Therme* und *Frasassi Cave system* in Italien sowie *Movile Cave* in Rumänien). Es wurden nur Bakteriensequenzen berücksichtigt, da die 16S-rRNS Gensequenzen der Archaeen unter 1400 Basenpaare lagen. Insgesamt konnten 23 taxonomische Gruppen erfasst werden. 48% der 373 16S-rRNS Gensequenzen gehören zum *Phylum Proteobacteria*, wobei die *Gammaproteobacteria* Gruppe mit 14% die größte Gruppe darstellen.

Mit der vorliegenden Arbeit gehört die LKC zu einer der am besten untersuchten Höhlen bezüglich der Diversität im Bereich der Prokaryonten (Porter et al., 2009; Engel, 2010; Anhang A, Abbildung 16.15, S. 87-88). Trotz den vielen Beschränkungen aufgrund möglicher methodischer Schwächen und Ungenauigkeiten (siehe Kapitel D1) sowie verschiedenen Methoden (DNS-Extraktion, Primer usw.) und andere Gegebenheiten (unterschiedliche Zugänge der Höhlen, Vorhanden sein von Kontaminationen usw.) erlaubt diese Studie gewisse generelle Aussagen:

- Die mikrobiellen Matten in den bis jetzt erforschten aktiven sulfidischen Höhlen zeigen zum Teil ähnliche mikrobielle Zusammensetzungen, wobei die Dominanz der Hauptgruppen allerdings in den einzelnen Höhlen unterschiedlich ist (Chen et al., 2009). Eine häufig dominante Gruppe sind die *Proteobacteria*, insbesondere die *Epsilon-, Gamma-* und *Betaproteobacteria* (Anhang A, Abbildung 16.15, S. 88; Porter et al., 2009). Viele Vertreter dieser Klassen sind an der Schwefeloxidation beteiligt (Engel, 2010). In den

sulfidischen Höhlen konnten bisher mindestens 20 verschiedene Bakteriengruppen sowie *Cren-* und *Euryarchaeota* erfasst werden.

- Die Diversität in Höhlen mit Wandmalereien aus der frühen Steinzeit, die für Besucher jahrelang geöffnet waren, zeigten zum Teil ähnliche Bakteriengemeinschaften mit ebenso unterschiedlichen Dominanzen der Gruppen wie in den sulfidischen Höhlen (Anhang A, Abbildung 16.15, S. 88). Im Vergleich zu den sulfidischen Höhlen konnte allerdings die Gruppe *Fibrobacter* nicht nachgewiesen werden. Dafür fand man andere Gruppen z. B. *Gemmatimonadetes* (Zhou et al., 2007), *Deinococcus-Thermus* (Spear et al., 2007), *Fusobacteria* (Portillo et al., 2009) und mehrere Candidate Divisions. Insgesamt konnten in den nicht sulfidischen Höhlen mehr Bakteriengruppen sowie *Cren-* und *Euryarchaeota* (mindestens 33 verschiedene Gruppen) als in den sulfidischen Höhlen nachgewiesen werden.

Die Ergebnisse in dieser Arbeit aus der sulfidischen *Lower Kane Cave* können somit helfen, neue Erkenntnisse bezüglich der Grenzen bzw. der Entstehung des Lebens in extremen Ökosystemen beizutragen und zu ergänzen. Zudem können diese Forschungen auch Rückschlüsse auf die Möglichkeiten des Vorkommens von Leben auf anderen Planeten bieten.

D4. Schlussfolgerung und Ausblick

Schwer zugängliche sulfidische Höhlen wie die hier untersuchte *Lower Kane Cave* in Wyoming (USA) bieten eine einzigartige Gelegenheit, geobiologische Interaktionen von Mikroorganismen, die möglicherweise an der Höhlenentstehung beteiligt sind, zu studieren und zu verstehen. Die Erstellung von 16S-rRNS Genbanken und die Anwendung von FISH mit spezifischen rRNS-gerichteten Gensonden ergaben Hinweise, dass in der LKC eine Reihe bisher unbekannter Bakterien und Archaeen vorkommen. Im Unterschied zu den 16S-rRNS Genbank Studien von Engel et al., (2004a) aus dem *aeroben* Bereich der Matten wurde unter Verwendung zusätzlicher Primerpaare im *anaeroben* Bereich eine wesentlich höhere Diversität erhalten. Mit

Hilfe von FISH-Analysen konnte allerdings gezeigt werden, dass die Diversität qualitativ in beiden Bereichen ähnlich hoch ist. In beiden Bereichen bestehen aber deutliche quantitative Unterschiede. So dominieren im *aeroben* Bereich die Schwefeloxidierer und im *anaeroben* Bereich die Sulfatreduzierer.

Um aber in der Zukunft den kompletten zusammenhängenden Nährstoffkreislauf und die funktionelle Beziehung aller beteiligten Mikroorganismen in den *aeroben* und *anaeroben* Bereichen in der LKC zu verstehen, bedarf es weiterer mikrobiologischer und molekularbiologischer Untersuchungen. Ein wichtiger Schritt wäre die Kultivierung von Isolaten aus der LKC, um weitere Informationen über ihre Stoffwechselaktivitäten und ihr genetisches Potential zu erhalten. Die physiologischen Eigenschaften der Isolate ließen wichtige Rückschlüsse auf ihre ökologische Bedeutung und ihre Rolle im Nährstoffkreislauf zu. Weiterhin ist nicht nur eine qualitative Aussage von spezifischen Gruppen in der LKC von Bedeutung, sondern auch eine quantitative Auswertung. Diese könnten mit weiteren Methoden wie z. B. einer quantitativen PCR oder NanoSIMS mit verschiedenen Proben entlang der Höhle durchgeführt werden. Funktionelle oder sequenzorientierte metagenomische DNS-Analysen mit Hilfe schneller und leistungsfähiger Sequenzierungsverfahren wären ebenfalls sehr hilfreich. Mit diesen Verfahren könnte man über die ökologische Bedeutung der verschiedenen Mikroorganismen in der LKC erfahren.

E. ZUSAMMENFASSUNG

E.1 Zusammenfassung

Höhlen stellen ein extremes, abgeschlossenes Habitat dar, das sich durch fehlendes Licht und Nährstofflimitation auszeichnet. Sie bieten daher hervorragende Möglichkeiten, um geobiologische Interaktionen und die Entwicklung des Lebens unter extremen Bedingungen im Untergrund zu studieren. Leider ist wenig über dieses Ökosystem bekannt. So ergab eine Inventurstudie, dass bisher nicht einmal ein Prozent von den auf der Erde vorkommenden Höhlen biologisch erforscht wurde.

Das Ziel dieser Arbeit war, mittels kulturvierungs-unabhängiger Ansätze wie Nukleinsäure-basierten Methoden (PCR und 16S-rRNS Genbanken) und mikroskopischer Identifizierung von Einzelzellen (FISH) die mikrobielle Diversität einer schwer zugänglichen sulfidischen Höhle, der *Lower Kane Cave* (LKC) in Wyoming, USA zu untersuchen. Die sulfidhaltigen Wasserströme entlang der LKC fördern das Wachstum von umfangreichen (1-20 m lang), einzigartigen mikrobiellen Matten. In früheren Studien, die sich in erster Linie auf die oberen, *aeroben* Bereiche der Matten beschränkten, konnten nur sechs Entwicklungslinien von Bakterien nachgewiesen werden. Dabei dominierten zwei Gruppen von fadenförmigen, schwefeloxidierenden *Epsilon-* und *Gammaproteobacteria* (>80%). In der vorliegenden Arbeit wurde der *anaerobe* Bereich einer Matte in der LKC detailliert auf seine mikrobielle Diversität untersucht. Hierzu wurden zunächst 16S-rRNS Genbanken erstellt. Die Analyse dieser Genbanken ergab eine wesentlich höhere Diversität (17 identifizierbare Gruppen von Prokaryonten) als im *aeroben* Bereich der Matte. Bei der Mehrheit der sequenzierten Klone handelt es sich um bisher unbekannte 16S-rRNS Sequenzen.

Die *Deltaproteobacteria*, die im *aeroben* Bereich nur in geringen Mengen vorkommen, bildeten im *anaeroben* Bereich eine der größten Gruppen. Hierbei muss hervorgehoben werden, dass Vertreter dieser Gruppe in der Lage sind, unter *anaeroben* Bedingungen Sulfat zu reduzieren. Eine weitere große Gruppe, die in dieser Arbeit erstmals in der LKC beschrieben wurde, waren mehrere fadenförmige Vertreter der *Chloroflexi.* Daneben scheinen auch die nicht fadenförmigen Vertreter des *Phylums Acidobacteria*, die stets vergesellschaftet mit Schwefeloxidierern vorkamen, eine gewisse Rolle im Ökosystem der LKC zu spielen. Allein von diesem

Phylum konnten sieben bisher unbekannte *„Operational Taxonomic Units*“ (OTUs) nachgewiesen werden.

FISH-Analysen wurden entlang der LKC an verschiedenen Mattenproben aus den *aeroben* und *anaeroben* Bereichen durchgeführt. Dabei war die ermittelte Diversität in beiden Bereichen qualitativ sehr ähnlich. Im *anaeroben* Bereich wurden neun Gruppen aus den 16S-rRNS Genbanken mit FISH bestätigt. Im *aeroben* Bereich dagegen wurden fünf weitere Gruppen erfasst, die mit entsprechenden 16S-rRNS Genbanken bisher nicht erhalten wurden. Die spezifische Gruppe *Acidobacteria* wurde mit FISH fast überall in der LKC gefunden. Für diese Gruppe wurden neue 16S-rRNS gerichtete Oligonukleotidsonden entwickelt.

E.2 Summary

Caves are unique portals to the subsurface. They represent a hardly explored, isolated, and extremely nutrient-limited ecosystem in complete darkness. They offer fascinating opportunities to study geobiological interactions and the development of life under extreme conditions. Unfortunately, our knowledge about this ecosystem is extremely scarce. A recent, global inventory study showed that less than one percent of all caves on our planet have been geobiologically examined.

The goal of this thesis was to explore the microbial diversity of sulfidic Lower Kane Cave (LKC) in Wyoming, USA using nucleic acid based methods (PCR and 16S-rRNA clone libraries) as well as different whole cell-targeted microscopy-based methods (FISH). The sulfidic compounds emerging from the groundwater and distributed in the stream along the LKC are believed to be the main nutrient source supporting the growth of overwhelming (1-20 m long) microbial mats. Previous studies which focused on the upper aerobic portions identified merely six phyla of bacteria with two dominating groups of filamentous chemolithoautotrophic sulfur-oxidizing species, *Epsilon-* and *Gammaproteobacteria,* accounting for approximately 80% of all species. A special focus was laid on the microbial diversity of the anaerobic portion of one of the mats in the LKC by using 16S-rRNA clone libraries. Larger 16S-rRNA gene diversity was found in this portion (17 identifiable groups of prokaryotes) compared to the aerobic portion, including various novel but not culturable strains.

One group, the *Deltaproteobacteria*, which had previously been detected only in small amounts in the aerobic portion, was identified as one of the largest groups in the anaerobic portion, most likely because at least some of its representatives are capable of reducing sulfate. The second largest group in the anaerobic portion that was identified for the first time in LKC, were several novel, unculturable filamentous species affiliated to the phylum *Chloroflexi*. In addition, a non-filamentous group of bacteria belonging to the phylum *Acidobacteria* was studied in more detail. Seven hitherto unknown operational taxonomic units (OTUs) could be affiliated to this phylum. They were often observed to be closely associated with filamentous species such as the sulfur-oxidizing filamentous bacteria and may play a certain role within the ecosystem of LKC.

A whole FISH screening was applied to different mat samples from aerobic and anaerobic portions within LKC. In these experiments, the diversity was found to be similar in both portions. Nine groups of bacteria detected by 16S-rRNA clone libraries in the anaerobic portion were also confirmed by FISH. In contrast to the aerobic portion, five novel groups were detected, which were not received by previously published 16S-rRNA clone libraries. One specific group, the *Acidobacteria*, was found to be ubiquitous in the LKC. For this group novel 16S-rRNA gene probes were constructed.

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ANHÄNGE

Anhang A

Caves and karst environments

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16 Caves and Karst Environments

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16.1 Introduction

Caves have played a fascinating role throughout the history of our planet and of our culture in different ways. Our first associations with caves centre on how they served as primitive dwelling sites for animals and humans, and as settings for mysterious fantasies and myths (Fig. 16.1). Today, scientific exploration has added another aspect to our connection to caves by revealing a plethora of unexpected insights into diverse disciplines, including the natural sciences (geology, palaeontology, climatology, physics, chemistry, biology and cosmology), medical sciences and engineering, as well as social disciplines such as archaeology, theology, and the cultural history of mankind (Fig. 16.2). The foundation for this is the extreme nature of caves, characterized by a lack of light and geographic isolation, but also by nutrient limitation and a range of extreme redox conditions. In recent years, a variety of different types of caves and intriguing cave creatures have been discovered. Therefore, cave-based sciences play an important role in enhancing our understanding of the history of our planet and also form a foundation for exploring novel concepts about the boundaries of life and the evolution of extreme dark life ecosystems on Earth, as well in other parts of the Universe (Krajick, 2001; NOVA, 2002; SPACE/Malik and Writer, 2005; Forti, 2009).

16.2 Description of Caves: Definition, Distribution and Biogeochemistry

16.2.1 Introduction

Many different definitions have been used to describe a cave or a cavern. The most general way to describe a cave, irrespective of its geological history and location, is to

Fig. 16.1. Entrance zone of a cave: Cascade Caverns, Carter Caves State Resort Park, Kentucky, USA. Depending on the entrance morphology, sunlight only penetrates a limited distance into the cave. © Annette S. Engel.

simply define it as a natural cavity in a rocky environment where at least some part of it is in total darkness. The science of exploration of caves and various karst features is termed as speleology (Gunn, 2004). Speleology is a broad interdisciplinary science since a multitude of parameters and disciplines needs to be considered for a thorough exploration of the development of caves and their various impacts on their surroundings worldwide throughout history.

Caves are developed in soluble rocks and constitute a characteristic feature of karst (carbonate rock, such as limestone and dolomite) and pseudokarst (non-carbonate rocks) landscapes that covers roughly 15–20% of the Earth's ice-free land surface (Ford and Williams, 2007). Although caves are found in various regions of the Earth and at all latitudes, caves are generally not interconnected over large physiographic provinces, limited by the extent of rock type. Caves come in a wide range of shapes and sizes, from micro-fissures to caverns several thousands of metres deep and high, and hundreds of kilometres in length. Only a few of these are accessible to humans. In fact, many cave openings consist only of microscopic fractures. Despite numerous caving activities all over the world, it is estimated that, even in well explored areas like Europe and North America, so far only 50% of all caves in these regions have been accessed; globally, only ~10% of all caves have been discovered (Eavis, 2009; Engel, 2011). The subsurface can be regarded as one of the least explored environment types on Earth, second only to the deep oceans. Caves often serve as the only available natural entrances and connections to the subsurface, offering windows into this vast and unexplored habitat.

Because of the increasing interest in cave sciences and all the promising prospects they offer, plus the development of improved caving technology that allows access to even the most difficult caves, many novel types of caves have been discovered during the last decade (Eavis, 2009). Son Doong Cave, Vietnam, found in 2009, contains the largest discovered cavern to date, measuring up to

(a)

(b)

Fig. 16.2. (a) Grotta dei Cervi, Italy (discovered in February 1970), with black Neolithic wall paintings, made with bat guano. Photographer Cesareo Saiz-Jimenez. (b) Castle in front of cave, Predjamski Grad, Slovenia. © Annette S. Engel.

140 by 140 m and 4.5 km in length. The deepest cave, Krubera Cave, Georgia, near the Black Sea, was discovered in 2001 and extends to a depth of –2191 m into the subsurface. Qaqa Mach'ay Cave is the highest cave known, and was discovered in Peru in 2004 at an altitude of nearly 5000 m. Several fascinating underwater caves have also been described, such as those in the Yucatan Peninsula, Mexico, and the Nullarbor Plain,

Australia; each area contains hundreds of kilometres of submerged passageways. However, even caves that have been known about and explored for many years, e.g. Mammoth Cave, Kentucky, USA, also continue to reveal astonishing insights. Due to natural karstification as well as continued exploration, Mammoth Cave is now estimated to cover a distance of around 600 km, and as such represents the longest cave known on Earth (for a summary of spectacular cave types, see NSS GEO2 committee, 2011).

However, it is not only the size and depth of caves that are impressive (Figs 16.3 and 16.4), many of their contents undisputedly continue to capture our imagination and curiosity, e.g. their spectacular speleothems (cave rock and mineral formations), such as stalactites, stalagmites and giant crystals that may be over 70 m in length, and fascinating creatures belonging to unique, extreme ecosystems (Taylor, 1999; Krajick, 2001; Culver and Pipan, 2009; Eavis, 2009).

Fig. 16.3. Examples of spectacular constructions in different types of caves. (a) The large room of Škocjan Caves Regional Park, Slovenia, a UNESCO site. This photo highlights the massive size that speleothems can reach (people for scale in the lower left). Photographer: Annette S. Engel. (b) Unique formations of unknown nature (possibly biospeleothems) inside a sandstone cave in Venezuela (Aubrecht *et al*., 2008). Photographer Jan Schloegl and Roman Aubrecht. (c) Entrances to caves can be very small and embedded in water; Cascade Caverns, Carter Caves State Resort Park, Kentucky, USA. Photographer Annette S. Engel. (d) Example of different colour formations due to various microbiological redox processes. Sulfidic spring in a cave with toxic hydrogen sulfidic gases, Lower Kane Cave, Wyoming, USA. Photographer: Annette S. Engel.

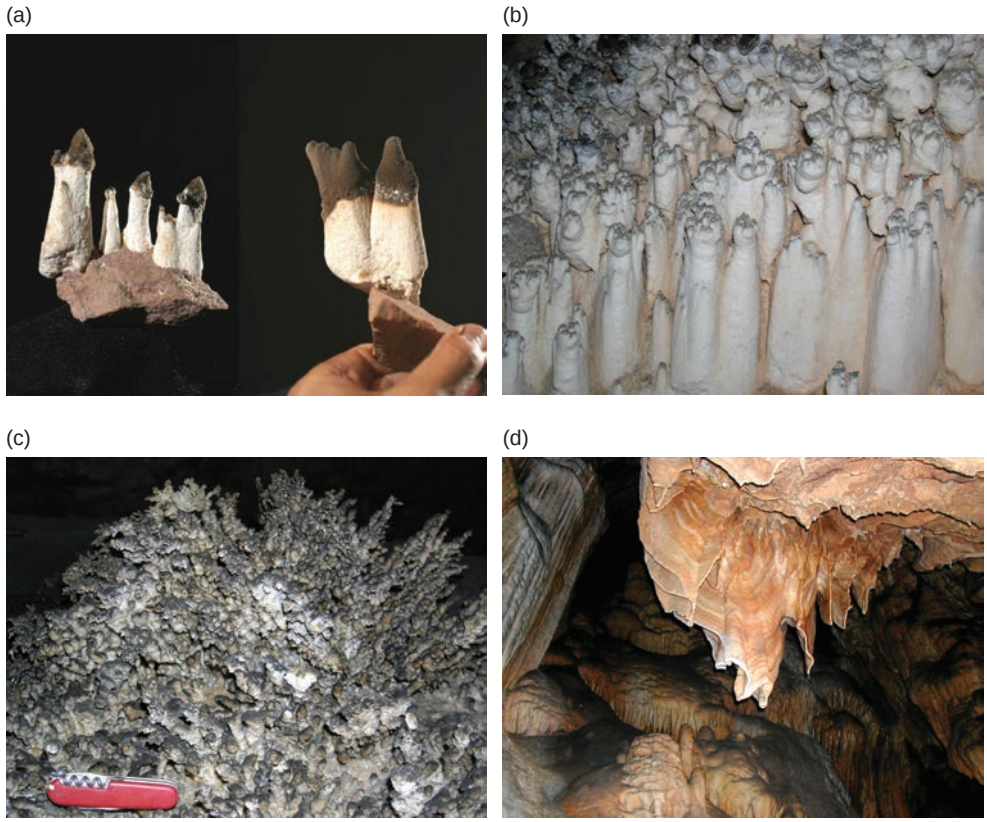


Fig. 16.4. (a–d) Different types of speleothems and biospeleothems in a sandstone cave in Venezuela. © Roman Aubrecht and Lubomir Kovacik.

16.2.2 Classification and formation of caves

Caves are classified using several criteria (Northup and Lavoie, 2001; Gunn, 2004; Engel, 2011): the solid rock/bedrock type, the proximity to the groundwater table, the overall passage morphology and organization (i.e. size, length, depth, routes of fluid flow, etc.) and the speleogenetic history (related to the origin and development of caves). So far, at least 250 different minerals have been described from karst and pseudokarst settings (Hill and Forti, 1997). However, the most common rock types are calcareous rocks (e.g. limestone, which underlies about 15% of Earth's surface) and basaltic rock (e.g. lava tubes). Other less common rock types include other volcanic deposits, gypsum, granite, quartzite, sandstone, salt and even ice.

Although new caves are constantly being formed somewhere on Earth, the majority of the caves so far described and explored have most likely persisted for longer periods, from thousands to millions of years. Irrespective of their speleogenetic histories, all caves are constantly changing over time, either by natural karstification processes or simply due to continued exploration. Depending on the proximity to the groundwater table, two different general modes of cave formation can be discerned: (i) epigenic caves, as the most commonly described caves, are related to surface processes by formation at or proximal to the groundwater table, and are critically dependent on the hydrological conditions of the region; and (ii) hypogenic caves, which are related to subsurface processes because they form by the action of rising fluids, such as

water or gases, at or below the water table (Engel, 2011). The most commonly described hypogenic caves to date are sulfidic caves, which are formed by a combination of abiotically and microbially produced sulfuric acid (Engel, 2007). Irrespective of the cave's relation to surface or subsurface processes, the character of the void spaces and the fluid flow patterns are crucial to the continuous evolution of the cave, as these produce the foundation for all flow systems and circulations relevant for the various speleogenetic processes.

Depending on rock type, and geochemical and geophysical (including climatic) conditions, different speleogenetic processes may take place (Northup and Lavoie, 2001; Gunn, 2004; Engel, 2011).

1. Solubilization of the host rock and the precipitation of minerals, which may eventually initiate the formation of speleothems. Speleothems are secondary mineral deposits and may contain a number of different minerals or unconsolidated materials such as clays and organic matter. So far, at least 38 different types of speleothems have been described (e.g. stalactites, stalagmites, helictites, cave pearls, curtains of dripstone, flowstone, rimstone, pool fingers, etc.), based on the formation mechanism and dominant (bio)-geochemical reactions (Hill and Forti, 1997; Boston *et al.*, 2001; Self and Hill, 2003; Melim *et al.*, 2009; Lavoie *et al.*, 2010; see the website of the NOAA Paleoclimatology Speleothem (2011) for information about the value of speleothems for paleoclimatology research; Fig. 16.4). Examples of caves containing a variety of different speleothem types include Mammoth Cave, Kentucky, USA, and Castañar de Ibor Cave, Spain.
2. Solubilization of the host rock from sulfuric acid-driven speleogenesis, which may dissolve calcareous rocks to generate significantly large caverns and often are associated with active microbiological colonization if reactive solutions are still present (Engel, 2007). Examples of caves formed from sulfuric acid speleogenesis include Carlsbad Cavern, New Mexico and Lower Kane Cave, Wyoming, USA, as well as Movile Cave, Romania.
3. Volcanism leading to lava flows that produce lava tube caves (Halliday, 2004), which first may result in the sterilized surfaces after a volcanic eruption that may then however be colonized through time. Examples of extensive lava tube caves have been described for the Kilauea Volcano, Hawaii, USA.
4. Physical weathering by water, which results in the formation of sea (littoral) caves due to the constant pounding of waves and digging out of seashore cliffs (Bunnell, 2004).
5. Anchialine caves along the coast are formed by the constant solubilization of host rock at a freshwater–saline water interface.
6. Ice (glacier) caves are formed by streams that erode tunnels under and through glaciers (Gunn, 2004; Fig. 16.5).

(a)

(b)

Fig. 16.5. (a) Isfjellelva Cave, Vestre Torellbreen, Svalbard, © Stanislav Rehak. (b) Tone Cave, Tonebreen Glacier, Svalbard, © Stanislav Rehak.

16.2.3 The cave environment

Several parameters contribute to the formation of the cave environment (Northup and Lavoie, 2001; Gunn, 2004; Barton and Northup, 2007; Engel, 2011). Intergranular spaces, pores, joints, fractures and fissures, and dissolutionally enlarged conduits and cave passages form a porosity and permeability continuum that can be colonized by organisms. An opening connected to the surface can have three major zones based on light penetration and intensity: (i) the entrance zone, which is exposed to full sunlight and experiences the daily light cycle; (ii) the twilight zone; and (ii) the dark zone, where no light penetrates (Fig. 16.6). Different types of life, and subsequently adaptations expressed by that life, are generally correlated to these zones because of specific physico-chemical and geochemical conditions related to photic gradients. Entrance and twilight zone conditions are tolerable by a wide variety of organisms, from insects to vertebrates, with little modification to their overall lifestyle.

In contrast to surface habitats, conditions in the deeper parts of caves are generally more stable (i.e. stable temperature throughout the year), but however stable, the conditions can also be considered more extreme simply because it is constantly dark in this zone, thereby making photosynthesis impossible. As such, deep cave environments are generally considered to be extremely oligotrophic (nutrient poor) because many of the resources needed for surface-based ecosystems, such as light and organic matter, are limited. Some systems with direct hydrological connections to the surface may occasionally be subject to catastrophic events, such as floods (Fig. 16.7), whereas caves in deserts may undergo long periods of drought, and lava tube systems may experience renewed volcanic activity. For hypogenic caves, there may also be a range of hazardous conditions, including toxic concentrations of inorganic compounds such as sulfur, heavy metals, lethal gases, or radioactivity (Fig. 16.8). Depending on the rock type, caves can also have extreme pH and redox gradients, such as the interface between

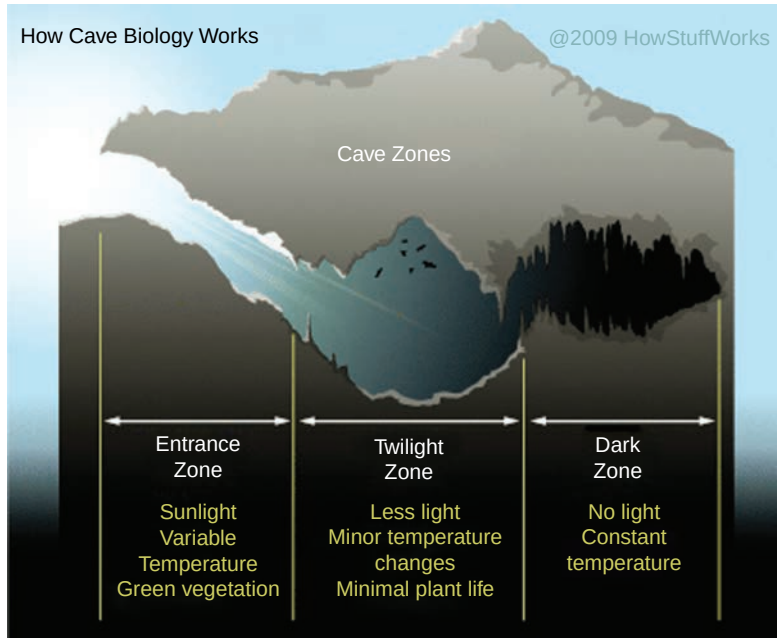


Fig. 16.6. The three zones (entrance, twilight, dark) of a cave (graph from www.howstuffworks.com).

Fig. 16.7. Some cave systems with direct hydrological connections to the surface may occasionally be subject to catastrophic events, such as floods. Here, scientists wade through a flooded cave to reach their sampling site. © Natuschka M. Lee.

Fig. 16.8. Castañar de Ibor, Spain, is a cave with extremely high radon (^{222}Rn) concentrations reaching up to 50,462 Bq m^{-3}. Cigna (2005) studied 220 caves around the world and calculated an annual average value of 2500 Bq m^{-3}, well below the range of Castañar de Ibor Cave. © Sergio Sanchez-Moral.

the host rock and cave passage atmosphere, or rock and water. The rock itself may contain various reduced compounds that create significantly different redox gradients within short distances. These redox-variable environments play a crucial role in the development of complex micro- and macro-biological communities that each have some, and probably different, impact on the speleogenetic history for a cave system (Northup and Lavoie, 2001; Gunn, 2004; Engel, 2011).

16.3 The Biology of Caves

16.3.1 History of biological cave research

The first biological studies in caves were initiated in the Middle Ages in Europe and China. In the centuries that followed, the biology of caves only evoked interest among a limited group of specialists (Culver and Pipan, 2009; Romero, 2009; Engel, 2010). In the late 1970s to early 1980s dark life ecosystems were discovered at hydrothermal vent systems in the Atlantic Ocean (see also Lutz, Chapter 13, this volume), providing insights into the evolution of life adapted to extreme and dark conditions. Shortly after this marine discovery the extreme terrestrial dark life ecosystem in the Movile Cave in Romania was described (Sarbu *et al.*, 1996). Both habitats (vents and caves) revealed astonishingly rich ecosystems of various faunal species, geochemically fuelled by novel species of microorganisms. From this time forward, the modern era of extreme environment research in caves began. With the further development of caving technology and molecular biological tools that enabled the exploration of previously inaccessible caves and of unculturable, novel species, the number of discoveries of extreme ecosystems in different types of caves has consistently increased. Based on the investigations conducted to date, it has become obvious that caves serve as excellent model systems for several fundamental biological disciplines, in particular geobiology and astrobiology (Taylor, 1999; Boston *et al.*, 2001; Engel and Northup, 2008; see also Gómez *et al.*, Chapter 26, this volume). Therefore, cave research has been used to enhance our understanding of the mechanisms of biological adaptation to extreme conditions, the interactions between organisms and minerals, the role of inorganic matter in different dark environments, the evolution and speciation of biological systems under extreme conditions, and also led to various biotechnological applications (i.e. screening of novel enzymes or pharmaceutical research).

16.3.2 Life in caves

All kinds of life forms (i.e. viruses, bacteria (including cyanobacteria), fungi, algae, protists, plants, animals) have been found in caves (Culver and Pipan, 2009; Romero, 2009), in either an active or fossilized state and in a range of different types of habitats (i.e. rocks, springs, pools, cave walls (Fig. 16.9), or even dispersed in the air). Depending on the environmental conditions, cave organisms can be either motile or sessile, and can be directly or indirectly associated with other organisms in symbiotic associations (e.g. parasitic or mutualistic). In general, ecological interactions profoundly influence the development and maintenance of cave ecosystem dynamics and food webs. Such interactions can include how chemolithoautotrophic microorganisms form the base for some subsurface ecosystems, whereby rich and diverse higher level organisms are sustained, but also can include how bat guano in some caves provides a significant energy and nutrient source for many other organisms (Figs 16.10 and 16.11b).

Depending on the location and the conditions in the cave, organisms may be either non-residents or permanent residents (Culver and Pipan, 2009; Romero, 2009). Non-resident organisms, referred to as accidentals, enter a cave occasionally via wind, water (groundwater, sea spray, rain water), or air, as sediment or spores, or can even be carried into a cave by other animals. Depending on their actions and length of stay in the cave, they may have a profound

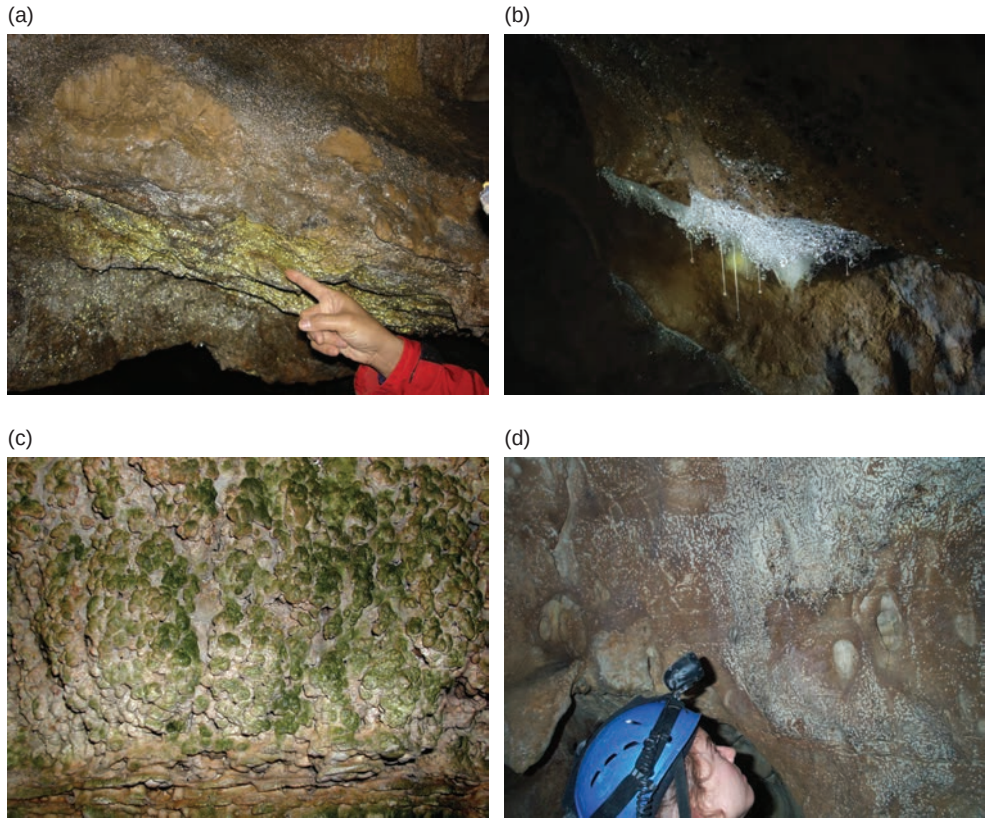


Fig. 16.9. Microbial growth on walls in caves: (a) colonies of white and gold actinomycetes and other bacteria on cave wall, Slovenia, © Annette S. Engel; (b) fungal snottiness from Sharps Cave, West Virginia, USA, © Megan Porter; (c) cyanobacterial growth, Green Cave Bojnice, Slovakia, © Lubomir Kovacik; (d) biovermiculations on the cave wall in the Frasassi Caves, Italy. These features, composed of clays and organic matter, are thought to be formed by microbial and nematode activity, © Annette S. Engel.

influence on the resident populations. The mechanism for the origin of permanent residents is still under debate; the most plausible explanation is that they originally descended from the surface, and entered the subsurface either accidentally or were forced underground by catastrophic events. Once below, they adapted to the conditions.

Depending on the available energy source over time and the survival ability of the species, different types of adaptations in response to the cave environment are likely to have taken place for at least certain species. A good example of this are microbial species that adapted metabolically to a strict chemolithoautotrophic life style, living off inorganic compounds present in the rocks and groundwater, such as metals, sulfur and methane (Engel, 2010). Other examples include higher organisms that adapted to the subterranean environments via various metabolic and morphological adaptations, such as pigment loss, eye loss, wing loss, reduction in size, development of more sensitive sensory organs, limb extension, reduction in metabolic rates and increased longevity (Culver and Pipan, 2009; Romero, 2009; Fig. 16.11a–d). Interestingly, a broad taxonomic range of organisms obligately adapted to the subsurface, in either terrestrial (troglobionts) or aquatic (sytgobionts) habitats, share this suite of characters, referred to as troglomorphy. These obligate subsurface organisms generally also have

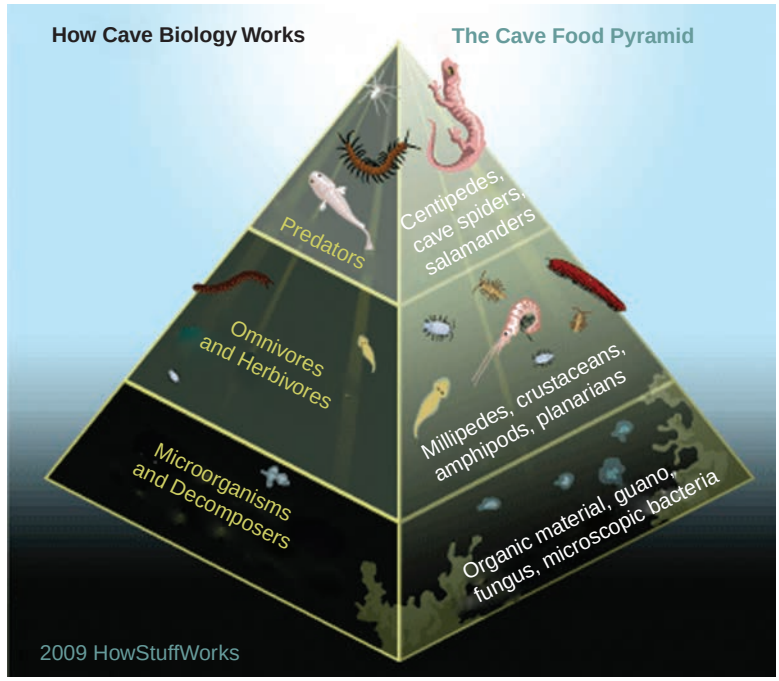


Fig. 16.10. The cave food pyramid (graph from www.howstuffworks.com).

limited possibilities for dispersal, which can further constrain genetic populations to local, and rarely regional, hydrostratigraphic regions.

Viruses

Viruses are the most abundant type of 'biological entity' on Earth, being found wherever there is life, and have probably existed since the first cells evolved. Viruses are capable of infecting all types of organisms, from prokaryotes to plants and animals. It is thus evident that viruses have had and still have a strong impact on virtually all evolutionary and ecological processes (Abedon, 2008; Forterre, 2010). Unfortunately, there have been no detailed, holistic studies on the ecology of viruses in caves or on the role of viruses or the mechanisms of their impact. There are few, sporadic reports of high abundances of viruses in various extreme environments or caves, with at least three related categories of case studies pointing toward the potentially high significance of viruses in caves: (i) many prokaryotes in various types of extreme ecosystems are in general attacked by viruses (e.g. see examples in Rainey and Oren, 2007); (ii) large numbers of novel viruses have been detected in the subsurface and are thus postulated to play a crucial role in the subsurface (e.g. Kyle *et al.*, 2008); and (iii) several infectious viruses have been reported from caves, in particular from animals like insects or mammals residing in caves. A classic example of this comes from bats: the animals may themselves be attacked by viruses, such as the West Nile virus, or alternatively serve as significant reservoirs of viruses that infect humans and other animals, e.g. emerging zoonotic viruses, such as lyssaviruses, filoviruses and paramyxoviruses (Quan *et al.*, 2010). Furthermore, most of the outbreaks of hemorrhagic fever caused by the lethal Marburg virus in humans were associated with visits to caves and mines in Africa (Kuzmin *et al.*, 2010). The reason for this is unknown because the source of the initial infection is unclear, but a reasonable lesson

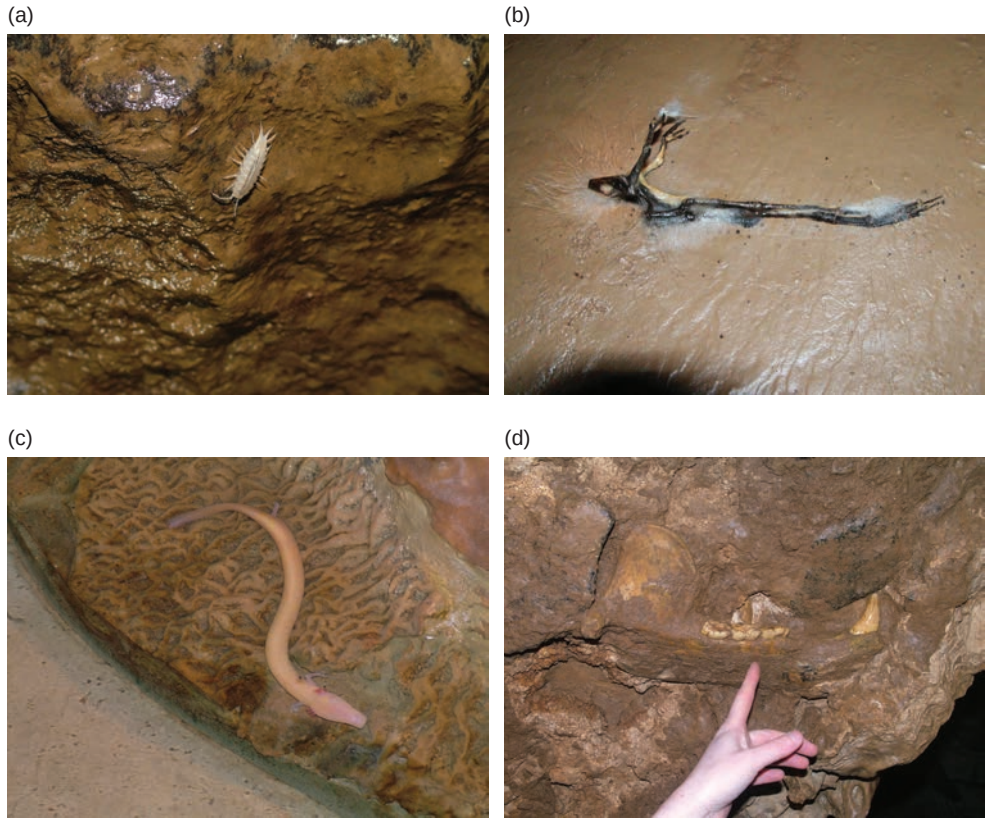


Fig. 16.11. Examples of cave animals: (a) the amphibious non-pigmented isopod *Titanethes albus,* from Planinska Jama, Slovenia, © Megan Porter; (b) dead cave frog overgrown by fungi, Photographer Peter Luptacik, © Elsevier; (c) the blind amphibious, non-pigmented, cave salamander *Proteus anguinus*, Slovenia, © Megan Porter; (d) jaw of the Late Pleistocene cave bear *Ursus spelaeus,* Krizna Jama, Slovenia, © Megan Porter.

to be learned from this case is that the evolution of a rather special gene pool (in this case viral) in isolated portions of caves may have profound impacts on other organisms once they enter these isolated areas. Thus, research into the unique, isolated ecosystems presented by caves may yield many interesting and relevant insights into a number of other biological disciplines.

Bacteria and Archaea

The prokaryotes, which comprise the domains Bacteria and Archaea, are the most abundant group of organisms on our planet. It is well understood that these microbial groups have played a key role in the development of our planet since the early beginnings. The first microbiological studies in caves were performed in the late 1940s using predominately microscopy and enrichment techniques, and these approaches continued for almost three decades. The research revealed few spectacular insights. For example, these studies demonstrated that microbes were prevalent but not as diverse as in surface habitats, and that several geological processes, such as speleothem formation, cave deposits such as saltpetre and moonmilk (Fig. 16.12), iron oxidation (Fig. 16.13) and sulfur oxidation, may be microbially mediated (Northup and Lavoie, 2001; Barton and Northup, 2007; Engel and Northup, 2008; Engel, 2010). Unfortunately, quantitative and undisputable evidence for these hypotheses was missing from these earlier studies.

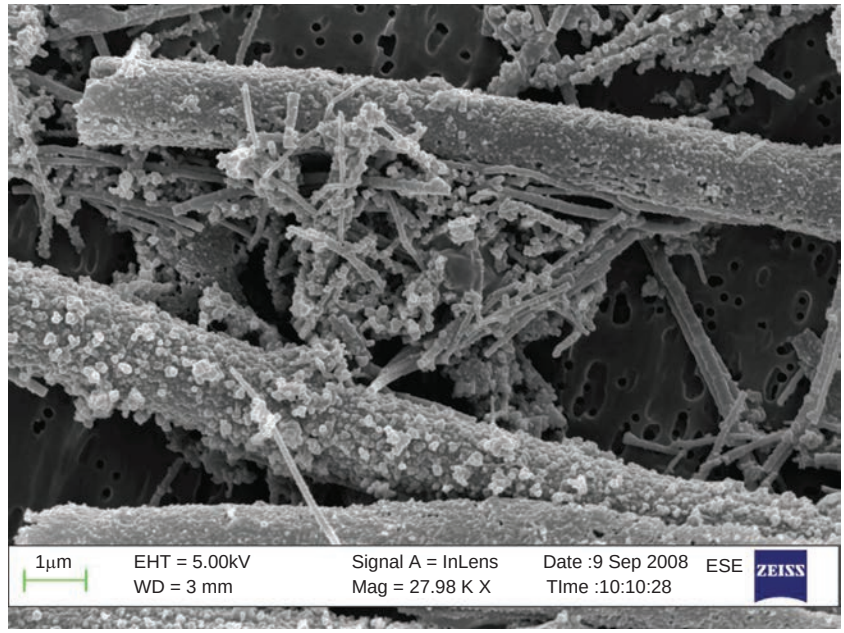
Fig. 16.12. Moonmilk in Altamira Cave, Spain, most likely of biological origin (Cañaveras *et al.*, 2006).

Furthermore, because standard microbiological approaches are only capable of identifying culturable species and these showed that several identified cave microbes were similar to surface-derived groups, e.g. from soils, it was assumed that the microbes identified in the caves had merely been transported into the cave. The conclusion was, therefore, that no unique cave microbes existed.

Fortunately, with the development of molecular and other analytical techniques since the 1980s, the exploration of non-culturable organisms has become possible. Despite the fact that only ca. 10% of all caves discovered so far have been biologically explored, many different types of bacterial and archaeal groups have now been detected in caves (Engel, 2010). Evidence has mounted that these cave microbes may indeed be unique and genetically divergent from surface groups, which has important implications regarding the role of microbes in distinct geochemical and geobiological processes. Consequently, several hypotheses have been proposed that address questions related to surface–subsurface linkages, biogeography and endemism, as well as microbial adaptation mechanisms to extreme conditions without light. One hypothesis being evaluated suggests that older caves may serve as a long-term reservoir of microbes in the subsurface and thus offer unique possibilities to explore the vast unknown biodiversity of the subsurface. In this way, cave research provides analogues for marine and deep-sea hydrothermal vent systems and possible life on other planetary bodies (Northup and Lavoie, 2001; Barton and Northup, 2007; Engel and Northup, 2008; Engel, 2010).

Many different types of biogeochemical reactions driven by microorganisms have been observed from distinct ecological cave zones (e.g. ammonification, denitrification, nitrification, sulfate reduction, anaerobic sulfide oxidation, metal oxidation, metal reduction, methane cycling, photosynthesis; Northup and Lavoie, 2001; Barton and Northup, 2007; Engel and Northup, 2008; Engel, 2010). Depending on the rock type, the concentration and nature of electron donors and acceptors, availability of oxygen and flux of organic material derived from

(a)



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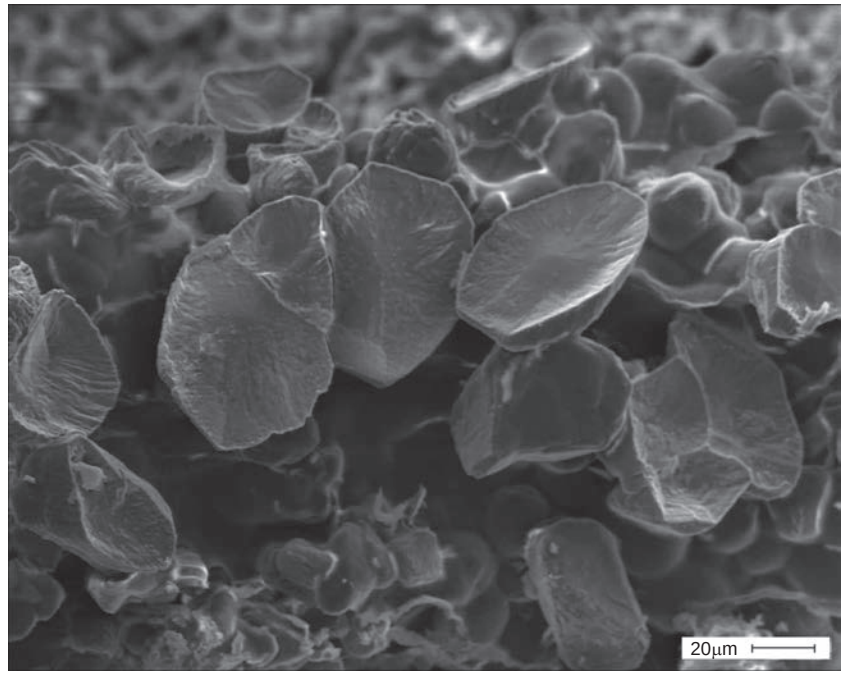


Fig. 16.13. Precipitates of biological origin produced by various cave bacterial species: (a) biogenic iron precipitated by a dense microbial community on *Leptothrix* sheaths, Borra caves, India; (b) calcium carbonate precipitate. Calcite crystals precipitated *in vitro* by *Bacillus pumilis* isolated from Sahastradhara caves, Dehradun, India (Baskar *et al.*, 2006). © Sushmitha Baskar and Ramanathan Baskar.

the surface, chemolithoautotrophs and heterotrophs play different ecological roles. Extreme environmental conditions also influence the metabolism of oligotrophic, acidophilic, thermophilic and/or sulfidophilic species. Different types of growth patterns may be observed, including single-celled, planktonic life stages to impressive aggregates, such as biofilms, forming either massive microbial mats on cave springs or pools (Fig. 16.14), or microbial draperies ('snottites') on cave wall surfaces (Fig. 16.9).

An important question that is being investigated by current research is what the

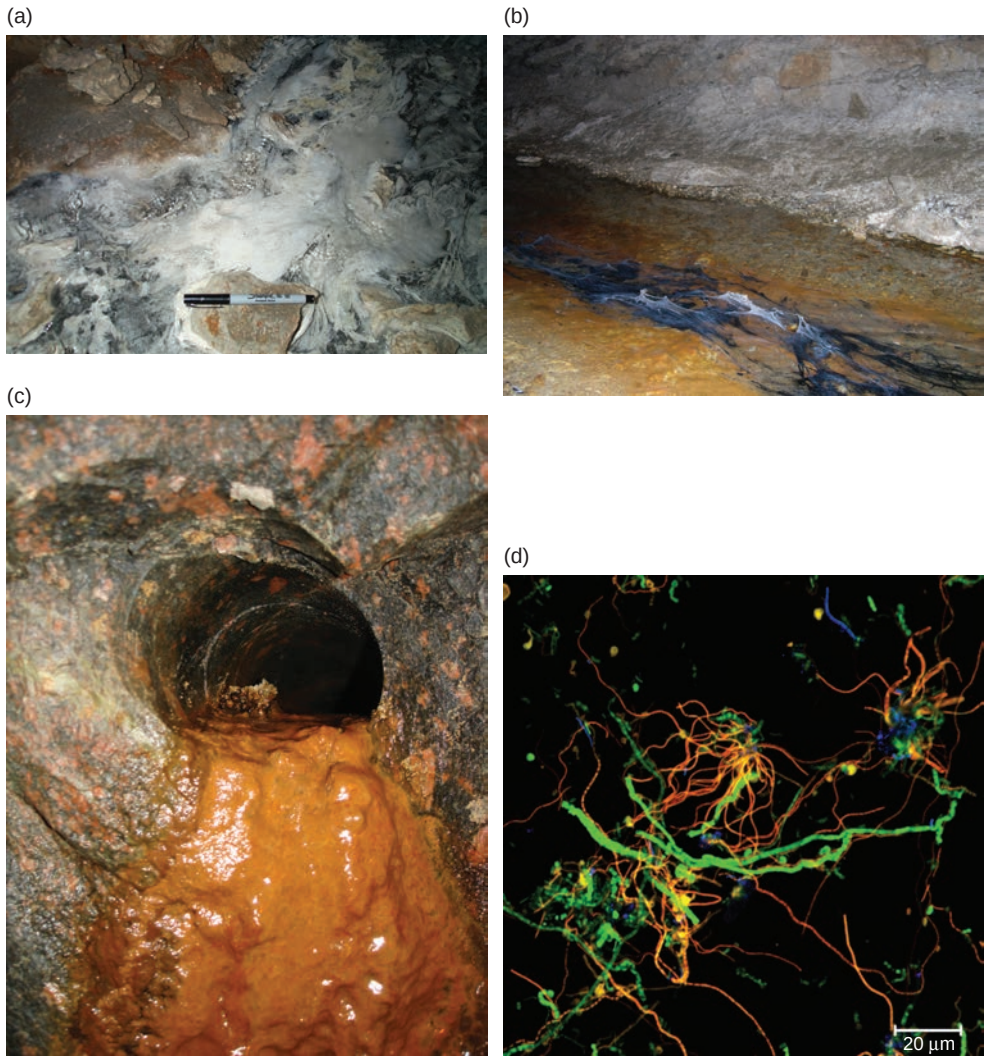


Fig. 16.14. Microbial mats in caves: (a) white filamentous microbial mats in the sulfidic stream of the Pozzo di Cristali in the Frasassi Caves, Italy, © Annette S. Engel; (b) black microbial mats in the sulfidic stream of the Lower Kane Cave, Wyoming, USA, © Natuschka M. Lee; (c) orange microbial mat with iron oxidizing bacteria growing on the flow water dripping out from a drill hole (the subsurface hard rock laboratory, Äspö, Sweden), © Natuschka M. Lee; (d) fluorescence *in situ* hybridization of different types of S-oxidizing filamentous bacteria (novel Epsilonproteobacteria, *Thiothrix* and other unknown species) in a microbial mat in Lower Kane Cave, Wyoming, USA, © Natuschka M. Lee.

source and transportation modes for microorganisms are that bring cells into, distribute cells throughout, and carry cells out of cave environments. Possible inoculation sources include soil, water (i.e. groundwater, sea spray and rainwater), plants, animals, deep-seated fluids circulating in sedimentary basins, and possibly even the rock itself. Microbial transport is itself linked to various circulation systems, such as vertical and horizontal fluid flow, which affects retention in zones of slow movement and circulation. Slow movement is strongly influenced by sorption onto biofilms that form on nearly every solid and semi-solid surface (e.g. rocks or the shells of macroorganisms; Northup and Lavoie, 2001; Barton and Northup, 2007; Engel and Northup, 2008; Engel, 2010).

As in other ecosystems, biogeochemical processes in caves are controlled by a complex interaction between geochemistry, geophysics and system ecology (for a more detailed review, see Northup and Lavoie, 2001; Barton and Northup, 2007; Engel and Northup, 2008; Engel, 2010). This makes it difficult to distinguish between abiotically and biogenically driven processes in the cave environment. None the less, microbes have been shown to influence many geochemical processes in caves at various stages of speleogenesis, because microbes are considered to be agents of *concentration*, whereby they localize the accumulation of inorganic minerals (e.g. $CaCO_3$ deposits such as moonmilk, or FeS_2 formation from sulfate reduction); *dispersion*, whereby they initiate the solubilization, mobilization and dispersion of insoluble minerals (e.g. Fe(III)-oxide reduction); *fractionation*, whereby they preferentially use one component in a mixture, resulting in the fractionation of elements and isotopes; and *reduction*, whereby they form new compounds due to the use of certain other compounds (e.g. acids from respiration (H_2CO_3), from S_0 oxidation (H_2SO_4), or H_2S production from sulfate reduction) (Ehrlich, 1996).

With this understanding, the focus of previous research has been to distinguish what effects and interactions microbes have on precipitation and dissolution processes in caves. Microbes may promote these processes in either an active (e.g. by enzymes) or passive way. Organisms (live or dead) or their products, such as extracellular substances (EPS), serve as nucleation sites for chemical reactions by sorbing various compounds (e.g. metals to amphoteric functional groups such as carboxyl, phosphoryl and amino constituents, or on to negatively charged cell wall surfaces, sheaths or capsules). These biochemical functional groups provide additional sites for chemical interactions, reduce activation energy barriers, change the system pH, or remove solutes from solution by causing solid phases, like minerals, to precipitate. Precipitation processes result in the formation of different types of products of various sizes, from microscopic structures to large speleothems, and compositions (e.g. calcium carbonate (Fig. 16.13) such as moonmilk (Fig. 16.12) or silicates and clays, iron and manganese oxides, sulfur compounds, or nitrates such as saltpetre). Microbially influenced dissolution and corrosion processes can be mediated by iron-, manganese- and sulfur-oxidizing bacteria, occurring via mechanical attachment and secretion of exoenzymes or from organic or mineral acids (e.g. sulfuric acid) that generate considerable acidity (Northup and Lavoie, 2001; Barton and Northup, 2007; Engel and Northup, 2008; Engel, 2010).

The number of bacterial and archaeal 16S rRNA gene sequences retrieved by standard clone libraries and pyrosequencing from various caves thus far constitute only a small fraction of all 16S rRNA gene sequences retrieved from the environment on a global basis (Engel, 2010). Despite this fact, several interesting insights have been gained. Approximately half of the bacterial phyla, and less than half of the archaeal phyla, are identifiable to a certain extent. The rest of the 16S rRNA gene sequences so far retrieved from cave and karst environments represent novel, so far unculturable species with unknown function. However, certain patterns can be discerned within a number of known phylogentic groups. For instance, some epigenic caves appear to contain *Deltaproteobacteria, Acidobacteria,*

Nitrospira and *Betaproteobacteria*, whereas some hypogenic caves, such as sulfidic caves, appear to be dominated either by *Epsilonproteobacteria*, or *Gammaproteobacteria* and *Betaproteobacteria* in microbial mats, or by *Acidimicrobium, Thermoplasmales, Actinobacteria*, bacterial candidate lineages, and some Archaea in 'snottites' on cave wall surfaces (Engel, 2010; Fig. 16.15).

Along with this, several different bacterial genera, such as *Firmicutes, Bacillus, Clostridium* and various enteric bacteria/human indicator bacteria have been discovered in cave systems that are believed to result from some kind of contamination via, for example, local wastewater treatment plants, storm events, or visitors. Fortunately, many of these microbial contaminants have a low persistence in the cave environment, indicating that caves may have the potential to recover from short-lived, occasional contamination events. Nevertheless, there are also a number of caves, especially caves with valuable rock-art paintings, that have had enormous difficulty recovering from invasive species, such as heterotrophic bacteria, phototrophs and fungi, spread by improper cave management, insects, or animals (Section 16.4).

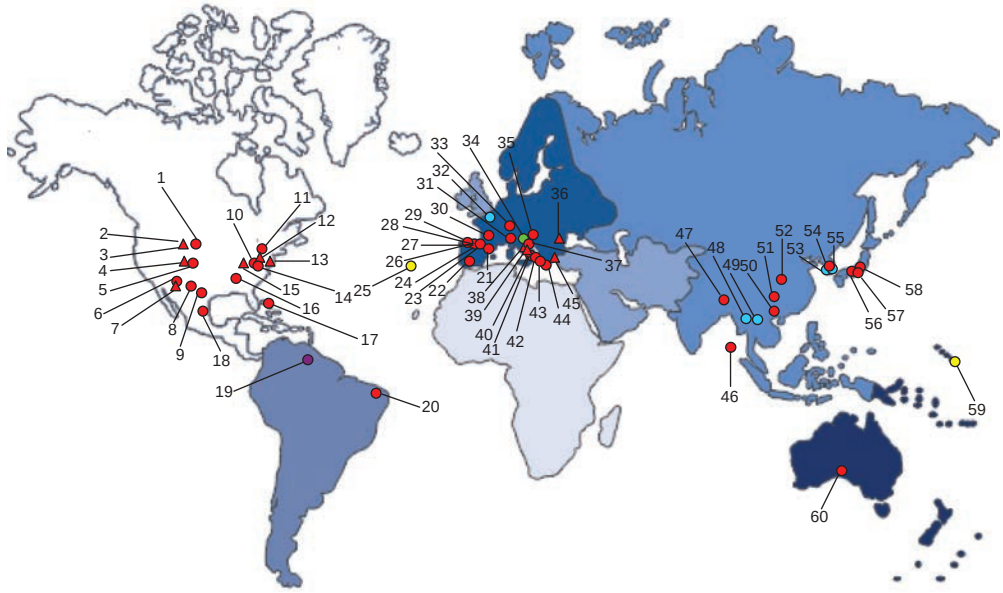
Further research is needed to resolve the questions remaining about the presence of endemic and invasive microbial species in caves and their possible roles. Despite all developments in terms of molecular and microbial analytical tools, only a fraction of the real taxonomic and functional diversity of Bacteria and Archaea in caves has been thoroughly and appropriately described. This is due to several methodological problems, ranging from inadequate sampling technology, insufficient distribution data, and the lack of appropriate methodology to address a research question. Testing specific hypotheses requires modifications to traditional approaches using more holistic, full-cycle methods, in order to more truly identify, quantify and determine the function and activity of culturable, as well as unculturable, species and thus provide correlations and evidence of the role of microbes in different types of speleogenetic processes.

Eukaryotes

Many different types of eukaryotes have been detected in caves worldwide, from single-celled species, such as different types of Protozoa, to a large variety of multicellular species ranging from fungi, invertebrates (flatworms, annelids, millipedes, centipedes, diplurans, insects, collombolans, spiders, mites, crustaceans, scorpions) and vertebrates (amphibians, reptiles, fish, mammals such as bats). For extensive details, see Culver and Pipan (2009). Actively growing plants, algae, or microscopic phototrophs are only found in cave entrances, in streams or other locations where sufficient amounts of light are available, whether natural or artificial due to cave lighting, where as little as 1 µmol photon m^{-2} may support growth (Grobbelaar, 2000).

Depending on the location within a cave zone, adaptation level and residence time in the caves, organisms may be classified as trogloxenes – terrestrial organisms that use the cave merely for occasional shelter, such as bats – or troglophiles – terrestrial organisms that may complete their life cycle in the cave but are still able to survive outside the caves. Many troglophiles therefore maintain at least some of their original senses. Stygoxenes are aquatic trogloxenes and stygophiles are aquatic troglophiles. Trogloxenes and troglophiles are usually found in the entrance or the twilight zone (Howarth, 1980; Culver and Pipan, 2009).

Troglobites and stygobites, obligate terrestrial and aquatic subsurface-dwellers, respectively, have developed astonishing adaptation mechanisms to life in caves (Culver and Pipan, 2009). Troglobites are generally exclusively found in the deeper parts of the caves where it is permanently dark and the humidity often high (up to 95–100%). Aquatic stygobites are generally geographically more wide-ranging than troglobites, and stygobites are more commonly found in caves with tropical and subtropical climates than troglobites (Lamoreux, 2004). Both groups have developed impressive morphological adaptations, as well as physiological mechanisms, to survive e.g. darkness, high humidity and limited food supplies. Typical food



cave type (simplified):	
Main categories – symbol code:	
○ non-sulfidic	△ sulfidic cave
Mineralogy – colour code:	
ice ●	orthoquartzite ●
lava ●	miscellaneous ●
limestone ●	

number	cave name, country	seq./diversity[a]	number	cave name, country	seq./diversity[a]
1	Wind Cave, SD, USA	83/14	31	Swallow hole, Yverdon-les-Bains, Switzerland[b]	134/>10
2	Hellespont Cave, WY, USA	11/1	32	Scladina case (cave layer), Belgium	63/10
3	Lower Kane Cave, WY, USA[b,] (→ Fig. 16.13b, d)	>1500/>17	33	Herrenberg Cave, Germany[c]	9/3
4	Glenwood Springs Cave, CO, USA	334/14	34	Alpine Ice Caves, Werfen, Austria[b, c]	2/1
5	Fairy Cave, CO, USA	37/6	35	Domice Cave/Domica Cave, Slovakia	52/3
6	Kartchner Cave, AZ, USA[b, c]	164/10	36	Movile Cave, Romania[b]	466/8
7	Millipede Cave, AZ, USA	>12/1	37	Pajsarjeva jama Cave, Slovenia[b]	50/11
8	Carlsbad Lechuguilla Cave and Spider Cave, NM, USA[b]	46/9	38	Frasassi Cave system, Italy (→ Fig. 16.?; 16.13a)	1039/>18
9	Hinds Cave, TX, USA	50/4	39	Cave of Acquasanta Therme, Italy[b]	173/11
10	Mammoth Cave, KY, USA[b]	254/10	40	Saint Callixtus Catacombs, Rome, Italy[b]	8/1
11	Tytoona Cave, PA, USA	13/7	41	Grotta Azzurra of Palinuro Cape, Italy[b]	39/10
12	Parker Cave, KY, USA	17/4	42	Grotta dei Cervi, Italy[b, c] (→ Fig. 16.2)	2/1
13	Cesspool Cave, VA, USA[b]	129/>6	43	Cave Koutouki, Attika, Greece[c]	1/1
14	Limestone Cave, KY, USA	4/2	44	Cave Kastria, Peloponnese, Greece	
15	Big Sulfur Cave, KY, USA	>109/1	45	Shallow-water Cave, Paxos, Greece	148/9
16	Blowing Spring Cave, AL, USA	9/4	46	Lime Cave, Baratang, India[b, c]	1/1
17	South Andros Black Hole Cave, Bahamas[b, c]	3/1	47	Meghalaya Caves, India[c]	3/2
18	El Zacaton sinkhole, Mexico[b]	1589/27	48	Thai Cave, Thailand[b, c]	9/1
19	Roraima Sur Cave, Venezuela[b]	42/9	49	Pha Tup Cave Forest Park, Thailand	2/1
20	Gruta da Caridade, Rio Grande do Norte, Brazil	2/1	50	Reed Flute Cave, Guilin, Guangxi, China[b, c]	2/1
21	Cave Papellona, Barcelona, Spain[b, c]	1/1	51	Niu-Cave, China	142/14
22	Ardales Cave, Spain (→ Fig. 16.15)	19/7	52	Heshang Cave, China	111/13
23	Santimamiñe Cave, Spain	10/10	53	Cave Apatite DepositsSouth Korea	65/7
24	Covalanas, Cantabria and La Haza Caves, Spain	325/3	54	Pseudo-limestone Cave, South Korea	2/1
25	Lava Tubes, Azores, Terceira, Portugal	515/>10	55	Gold Mine Caves, Kongju, South Korea[b, c]	4/2

Fig. 16.15. 16S rRNA phylogenetic tree.

26	Altamira Cave, Spain[b, c] (→ Fig. 16.11)	>613/12	56	Ryugashi Cave, Shizuoka, Japan	1/1
27	Monedas and Chufin Caves, Spain	128/10	57	Cave Sabichi, Ishigakijima, Japan[b]	1/1
28	Llonin Cave und La Garma Cave, Spain	85/10	58	Jomon and Joumon Limestone Caves, Gifu, Japan[b, c]	9/2
29	Tito Bustillo Cave, Spain (→ Fig. 16.16)	41/10	59	Lava Caves and Tubes, HI, USA[b]	302/>18
30	Lascaux Cave, France	681/>4	60	Nullarbor Caves, Australia	52/8

a: The ratio demonstrates the total amount of 16S rRNA gene sequences from the domains Bacteria and Archaea *versus* the amount of identifiable phyla. This information is based on ~ 8,000 partial 16S rRNA gene sequences retrieved from the Gene bank (http://www.ncbi.nlm.nih.gov/) in February 2011. A detailed list of the sequences and the references can be downloaded from the website xxx.

b: A representative selection of these sequences (> 1,400 nucleotides) were used to calculate an overall 16S rRNA phylogenetic tree (figure (see figure 16.15b/website) showing all so far retrieved 16S rRNA gene sequences from different parts of the world.

c: This study also describes 16S rRNA gene sequences from isolates.

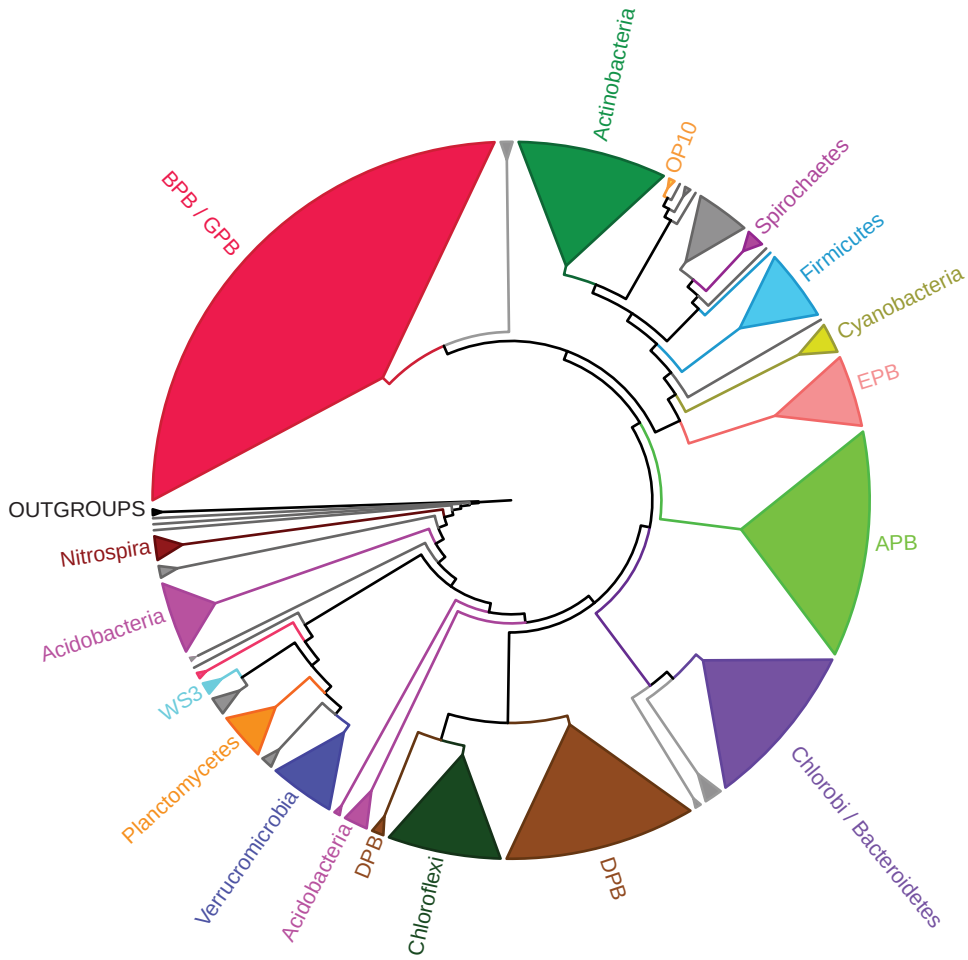


Fig. 16.15. Continued.

sources may include microorganisms, other animals, faeces (e.g. bat guano), carcasses from trogloxenes, or other matter, such as twigs and plant residues delivered into the cave by other animals or aquatic streams. Because of their low metabolic rates, sometimes sedentary lifestyle and infrequent reproduction, trogoblites and stygobites usually live longer than many non-cave species. Several animal categories, such as carabid

beetles, gastropods, collembolans and spiders are found in many caves worldwide, while some animal species have so far only been detected in certain caves, e.g. scorpions are mostly found in Mexican caves. The most common troglobite so far described is the carabid beetle of the subfamily Trechinae, which measures approximately 5–7 mm (Barr and Holsinger, 1985). The largest troglobite found so far is the cave salamander (*Proteus anguinus*), commonly referred to as the 'Olm', that can measure up to 30 cm in length (Krajick, 2007; Fig. 16.11c).

To date, nearly 8000 species of troglobites have been described (Krajick, 2007). However, since approximately only 10% of the caves thought to exist worldwide have been discovered, it should be anticipated that many more species and interesting ecological interactions remain to be discovered. A striking example for this is the unknown diversity of micro-eukaryotes in caves. Currently, 18S rRNA gene sequence data suggest that most of the micro-eukaryotes so far are not identifiable (Engel, 2010). Their role is unknown, but the limited knowledge retrieved suggests that at least some of the micro-eukaryotes may have a severe impact on speleothems and deterioration of valuable cave walls and paintings. Different types of other ecological functions can be ascribed, depending on the species and activity level. Some eukaryotic structures, such as fungal hyphae, may serve passively as nuclei for crystallization or as sites for attachment of crystals and thus, indirectly, contribute to a speleological process (Figs 16.11b and 16.16).

Fig. 16.16. Photo of fungi (*Beauveria felina*) on rodent excrement, Ardales Cave, Spain. © Cesareo Saiz-Jimenez.

16.4 Natural and Anthropogenically Disturbed Caves and the Future of Cave Preservation

Unfortunately, our chance of realizing the true biodiversity of caves is endangered by many factors, including the environmental problems caused by mining, drilling, pumping of aquifers, contamination, or invasions by other organisms, including humans via tourism and even research activities. Many cave environments appear to be endangered by several parameters, ranging from climatic catastrophes, through different types of environmental pollution. Even an ecosystem impacted by a minor disturbance, such as a natural flood (Fig. 16.7), can become susceptible to additional, more severe disturbance, like disease or being out competed for nutrients and resources by invading species.

In general, microbial colonization of a cave is a natural process that has occurred a long time before a cave's discovery. However, as soon as a cave, with its already established ecosystem that is finely balanced in terms of ecological interactions and environmental conditions such as nutrient input, is opened and connected with the exterior environment, the ecosystem becomes subjected to an unaccustomed input of abundant organic matter, or may be impacted by invading communities coming from the surface. This can lead to significant food web changes because the newcomers may exert an enormous pressure on the original inhabitants. In the worst-case scenario, the newcomers may even displace the original populations and communities. There are several dramatic examples of this (Saiz-Jimenez, 2010).

The strongest disturbance recorded so far has been commercial cave mass tourism, which commenced during the second half of the 19th century. At that time, minimal consideration was given to conservation. As a consequence, practices adopted for

visits to caves often resulted in irreversible damage caused by thousands of visitors, for example, in the form of lint, litter, and even increased carbon dioxide (from exhaled breath) and altered temperature levels (due to human body heat) in passages with low circulation. In addition, destructive construction works have removed tonnes of rocks and other materials from the topsoil and cave entrances to allow access for tourists to many caves worldwide. Lastly, the introduction of artificial lighting in some tourist caves, sometimes left illuminated all day, is sufficient to turn the darkness into a terrarium in some caves and support the growth of various phototrophs and plants that would not otherwise be able to survive. One example of this is the green alga, *Bracteacoccus minor*, that now occurs on the wall paintings in the Lascaux Cave, France. For the most part, the ancient rock-art paintings in the Lascaux Cave and others in Europe are not only threatened by algae, but also by fungal species introduced and spread by human activities. Due to this, a number of caves had to be closed for several years to treat invading organisms with various biocides (e.g. the Lascaux Cave, France; for more detailed information see review by Saiz-Jimenez, 2010; Fig. 16.17).

Another striking example of the impact of disturbance on cave ecosystems is the unexpected outcome of the occurrence of the fungal species *Geomyces destructans*, in some caves in the USA. Although still under investigation, it is highly probable the *G. destructans* is a natural fungal species to humid, cool subsurface environments. In North America, its link to the 'white nose syndrome', which has been associated with the sudden deaths of more than a million bats in the USA, is mysterious (Blehert *et al*., 2009; Fig. 16.18). Strikingly, the first discovery was made in 2006 in a heavily visited, commercial cave in Schoharie County, New York, USA. After this, the white-nose syndrome fungal infection spread astonishingly fast to over 100 other caves throughout the North American continent – including caves and mines that are not generally accessible to humans. The event, and more significantly the death of so many different valuable bat species, has had several serious consequences: it has led to

Fig. 16.17. Example of an invasive species on the ground in a cave, caused by artificial lighting. This promotes the growth of calcifying cyanobacteria (*Scytonema julianum*) and algae in tourist caves, as shown in Tito Bustillo Cave, Spain (Saiz-Jimenez, 1999). © Cesareo Saiz-Jimenez.

(a)

(b)

Fig. 16.18. Cave bats infected by the 'white nose syndrome', caused by the fungus *Geomyces destructans*. (a) Close-up of little brown bat's nose with fungus, New York, USA. Photo courtesy Ryan von Linden, New York Department of Environmental Conservation (www.fws.gov/whitenosesyndrome/images/3842close-upofnosewithfungus.jpg). (b) Fungus on wing membrane of little brown bat, New York, USA. Photograph courtesy Ryan von Linden, New York Department of Environmental Conservation (www.fws.gov/whitenosesyndrome/images/3845Fungusonwingmembrane.jpg).

the near extinction of several species and has had a tremendous negative ecological impact on agriculture owing to the fact that bats consume enormous numbers of insects. The United States Forest Service has estimated that about 1.1 million kg of insects will go uneaten in the most heavily impacted regions, and it is likely that insect infestation will become a great financial burden on the agricultural sector. Without a natural regulator of insect population, the use of insecticides is likely to increase, which will in turn increase existing environmental problems (for more information see the USDA website on the white nose syndrome). Clearly, research into the biology of caves is not only a matter of exploring unique and extreme ecosystems, but is also fundamental to our understanding of the delicate ecological balances on Earth.

Unfortunately, no tourist cave impacted by severe disturbances has ever been completely restored to its former ecological state (Elliot, 2006). This is particularly the case where the wrong decisions were made in selecting a biocide to employ in Lascaux Cave, since it caused severe irreversible population shifts in the natural cave flora. For instance, in the treatment of the Lascaux Cave, benzalkonium chloride use resulted in the selection of Gram-negative bacterial species that were adapted to this biocide (e.g. *Ralstonia, Pseudomonas* and other pathogenic bacteria). The lesson to be learned from this case is that biocides that are generally considered acceptable for combating microorganisms in surface environments are not necessarily appropriate for use in sensitive ecosystems such as caves. Interestingly, recently discovered rock-art caves such as Chauvet, France, and La Garma, Spain, that have been protected against mass tourism, have so far shown no sign of deterioration. Based on this, it is evident that it is important to protect cave ecosystems from the outset following discovery and perhaps the best protection is to limit the amount of tourism (Saiz-Jimenez, 2010).

Principally, every visitor to a cave, from the professional speleologist to tourists, has the potential to exert a negative impact on the cave ecosystem, especially if the rules for basic safe caving, as outlined by McClurg (1996), are not followed: 'Don't take anything, don't leave anything; don't break or remove cave formations; don't handle with or collect cave life; or, in other words: take nothing but pictures; leave nothing but foot prints and kill nothing but time'. It goes without saying, however, that for some regions of the world, caves serve as the only viable commodity and tourism is the only source of revenue. For example, in the Mammoth Cave region, USA, over US$400 million were brought to the area by tourists in 2009, up from an estimate of

US$52 million in 1993 (Stynes, 1999). The natural beauty of caves can also serve as a mechanism for science education to the public. Local to national strategies can be, and should be, enacted to manage and protect vulnerable or at-risk cave ecosystems while also balancing the use of caves for tourism and education.

16.5 Summary and Future Visions of Cave Life Sciences

During the last decades, caves have emerged as fascinating model systems for a number of scientific disciplines. As only a minor fraction of all caves on our planet have been found and explored, much still remains to be discovered. To accomplish this, a number of further developments in caving technology, sampling technology and analytical tools are needed and these must be applied in a systematic way to allow clear correlations to be made. Fortunately, this is being accomplished via increased research in various scientific disciplines, as well as in the emergence of various professional networking organizations (e.g. websites for CRF, the Speleogenesis Network and the UIS). While each cave may pose its own specific research questions, some general examples for future fields of research include:

- Cave life sciences, in general, because our knowledge about cave biodiversity, biogeography, endemism, function and activity status, geo-ecological interactions, population dynamics, nutrient cycling, adaptation mechanisms and life cycles of various cave adapted species is still very limited.
- Biology of different types of extremophilic organisms in oligotrophic, dark life environments.
- Different applications in geobiology, such as interactions between life and minerals and subsurface sciences, as caves may provide a unique opportunity for *in situ* exploration of overall subsurface habitat biodiversity, function and its mutual interactions with the upper spheres.
- Astrobiology whereby caves may serve as interesting model systems (analogues) for hypotheses on the origin and development of life on Earth, as well as in outer space.
- Geotechnological applications, such as drilling, caving and mining.
- Environmental sciences addressing topics such as climatology, global warming, pollution and the screening of potentially novel species with interesting degradation traits (with the potential to allow water purification and/or degradation of contaminants).
- Cultural history such as archaeology and development of conservation techniques.
- Medical sciences and different types of biotechnological applications whereby caves could be explored for, e.g. useful enzymes, pathogenic organisms, screening of antibiotic-producing organisms, to name but a few.

Clearly, cave science is an exciting and ever-expanding field with enormous future potential.

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UIS International Union of Speleology: www.uis-speleo.org (accessed 2 May 2011).
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Anhang B

Linking phylogenetic and functional diversity to nutrient spiraling in microbial mats from Lower Kane Cave (USA)

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Linking phylogenetic and functional diversity to nutrient spiraling in microbial mats from Lower Kane Cave (USA)

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Microbial mats in sulfidic cave streams offer unique opportunities to study redox-based biogeochemical nutrient cycles. Previous work from Lower Kane Cave, Wyoming, USA, focused on the aerobic portion of microbial mats, dominated by putative chemolithoautotrophic, sulfur-oxidizing groups within the *Epsilonproteobacteria* and *Gammaproteobacteria*. To evaluate nutrient cycling and turnover within the whole mat system, a multidisciplinary strategy was used to characterize the anaerobic portion of the mats, including application of the full-cycle rRNA approach, the most probable number method, and geochemical and isotopic analyses. Seventeen major taxonomic bacterial groups and one archaeal group were retrieved from the anaerobic portions of the mats, dominated by *Deltaproteobacteria* and uncultured members of the *Chloroflexi* phylum. A nutrient spiraling model was applied to evaluate upstream to downstream changes in microbial diversity based on carbon and sulfur nutrient concentrations. Variability in dissolved sulfide concentrations was attributed to changes in the abundance of sulfide-oxidizing microbial groups and shifts in the occurrence and abundance of sulfate-reducing microbes. Gradients in carbon and sulfur isotopic composition indicated that released and recycled byproduct compounds from upstream microbial activities were incorporated by downstream communities. On the basis of the type of available chemical energy, the variability of nutrient species in a spiraling model may explain observed differences in microbial taxonomic affiliations and metabolic functions, thereby spatially linking microbial diversity to nutrient spiraling in the cave stream ecosystem.
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Introduction

Karst terrains extend over ~20% of the earth's surface (Ford and Williams, 2007), and caves are common features of karst terrains that allow study of subsurface habitats and ecosystems. Caves systems with surface entrances, but relative hydrological isolation from the surface, share basic physicochemical conditions, including complete darkness, year-round thermal stability, and relatively constant humidity. Caves are also generally isolated from the influence of solar UV radiation and physical weathering due to wind, rain, or freezing. Compared to our understanding of biogeochemical cycling in photosynthetic microbial mats (Ferris *et al.*, 1997; Ward *et al.*, 1998), our knowledge of nutrient cycling in aphotic systems is limited, especially in the context of how nutrient cycling and microbial diversity might be linked. But, the redox-stratified microbial mats from a hydrogen sulfide (H_2S)-bearing, anoxic to dysoxic cave stream habitat in Lower Kane Cave, Wyoming, USA (Figure 1), provide an opportunity to evaluate the relationships among habitat geochemistry, nutrient cycling, ecosystem function, and microbial diversity.

The microbial mats in Lower Kane Cave consist of filamentous and web-like morphotypes in contact with moving stream water. The filamentous surface

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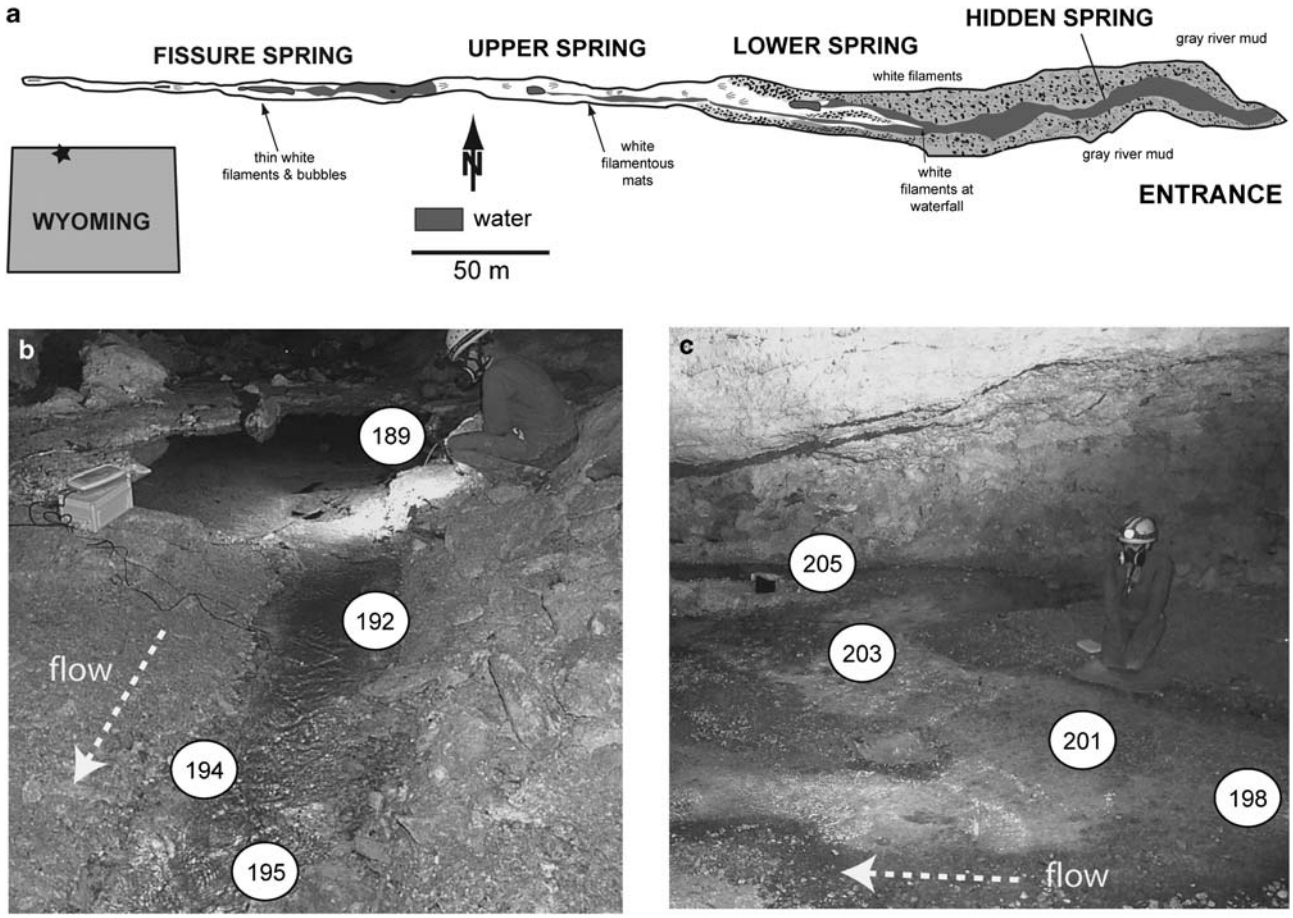

Figure 1 (**a**) General cave location in Wyoming and plan-view cave map from Engel *et al*. (2004a). (**b** and **c**) Representative photographs from the Upper Spring transect, from the spring orifice (**b**) through the microbial mats downstream (**c**). Dashed arrows indicate flow direction. Numbers in circles represent sample locations, in meters.

covers a dense, gel-like mat interior. Dissolved oxygen profiles reveal that the first 3–5 mm of the mats are oxygenated, but that the mat interior is anoxic ($PO_2 < 10$ Pa) (Engel *et al.*, 2004a). Similar microbial mat and biofilm stratification has been reported recently from the Frasassi Caves in Italy (Macalady *et al.*, 2008). On the basis of the previous full-cycle rRNA investigations from Lower Kane Cave, the aerobic portions of the mats are dominated by uncultured representatives from the *Epsilonproteobacteria* class and culturable representatives from *Thiothrix* spp. from the class *Gammaproteobacteria* (Engel *et al.*, 2003, 2004a). These groups form the bulk of the mats and provide chemolithoautotrophically fixed carbon and other nutrients to the ecosystem, but the diversity and dynamics of anaerobic microbial guilds, and their physical and functional relationships to aerobic groups, remain unknown.

In this study, we focused on the diversity, spatial arrangement, and abundance of anaerobic guilds to better evaluate ecosystem function and nutrient cycling in the microbial mats. We combined the full-cycle 16S rRNA approach, including clone library construction and fluorescence *in situ* hybridization (FISH), with the most probable number (MPN) method to correlate to guild function. Stream water advection provided an opportunity to evaluate the oxidation state transformations between mobile (for example dissolved in moving stream water) and immobile (for example incorporated as relatively stationary biologic forms) dissolved solutes associated with abiotic and biotic processes. With the expanded view of microbial diversity, the nutrient spiraling concept (Webster and Patten, 1979) was evaluated using carbon and sulfur geochemical and stable isotope data. The distance of solute transport before biogeochemical transformation has been evaluated earlier in stream reaches through nutrient enrichment (Payn *et al.*, 2005) or transport-based analyses (Runkel, 2007), and has been used to describe nitrogen and phosphorus cycling and transport in surface stream ecosystems (Newbold *et al.*, 1981, 1982; Ensign and Doyle, 2006) and algal biofilms (Paul *et al.*, 1991), but to our knowledge this is the first time that nutrient spiraling has been

compared with the spatial patterns of aerobic and anaerobic microbial groups within aphotic microbial mats.

Materials and methods

16S rRNA gene diversity and analyses
The microbial diversity of the anaerobic portion of the mats was evaluated using earlier established methods to describe the aerobic portions of the mats (Engel *et al.*, 2003, 2004a; Meisinger *et al.*, 2007). Specific details regarding these methods, including sampling site information, geochemical analyses, DNA extraction, amplification, and sequencing, as well as FISH preparation and sample examination, and MPN estimations, are provided in the Supplementary material (abbreviated 'SM'). Nearly full-length (>1100 bp) 16S rRNA gene sequences from this study were proof-read and evaluated by the ARB software (Ludwig *et al.*, 2004), which consists of ~200 000 16S rRNA gene sequences that represent relevant relatives and outgroups to the retrieved clones according to BLAST searches in GenBank (http://www.ncbi.nlm.nih.gov/; Altschul *et al.*, 1990), the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/; Cole *et al.*, 2007), and the SILVA databases (http://www.arb-silva.de/; Pruesse *et al.*, 2007). Chimeric clone sequences were identified by the ARB software using a partial treeing approach with two different filter sets that split the full-length sequence into two parts (for example *Escherichia coli* positions 1–750 and 751–1500) and performed separate treeing calculations on each section. If the two parts of the clone sequences were affiliated to different groups, then the sequences were identified as chimeric; ~10% of the sequences were identified as chimeric and were not included in further analyses. Nonchimeric sequences were submitted to GenBank under the accession numbers AM490641–AM490771.

Operational taxonomic units (OTUs) at the 98% similarity level were determined using DOTUR (Schloss and Handelsman, 2005). Coverage of the clone library was determined by rarefaction curves generated by aRarefactWin (Analytic Rarefaction ver. 1.3, http://www.uga.edu/strata/software/software.html). Sequences from the cave, close relatives, and outgroups were aligned by comparing select regions of the target gene sequences with alignment positions from multiple data sets (Ludwig *et al.*, 2004) and the resulting overall alignment was visually refined. A general phylogeny was reconstructed using the neighbour-joining distance method (Figure 2). For more refined analyses, sequences for major microbial groups were separated to reconstruct phylogenies using distance, maximum parsimony, and randomized a(x)ccelerated maximum likelihood methods (Stamatakis *et al.*, 2005). For each of these groups, a consensus tree was constructed in ARB from all treeing procedures (Supplementary Figures SM1–SM10).

Distribution patterns from FISH and MPN counts
Twenty-nine rRNA-targeted fluorescently labeled oligonucleotide probes were used for FISH to screen for different microbial groups present in 30 different mat samples distributed along the cave (Supplementary Table SM1). Although detection was limited to groups with at least 10^5 cells ml^{-1} in the mats and a relatively high ribosome content (Schleifer, 2004), this approach allowed for screening major taxonomic groups from many different aerobic and anaerobic mat samples without generating clone libraries. Cultivable microbes capable of fermentation, sulfate reduction, sulfur reduction, iron reduction, nitrate-reducing sulfide oxidation, and methanogenesis were enumerated using the MPN method (Hurely and Roscoe, 1983). Details for each enrichment medium are included in the Supplementary material.

Assessment of nutrient cycling and spiraling
Applicability of a nutrient spiraling model was evaluated using previous analyses of aqueous geochemistry and stable isotopes from cave stream waters and microbial mats (Engel *et al.*, 2004a, b). We focused on the Upper Spring transect, in which cave spring water had pH values of 7.1 ± 0.1 and a nearly constant temperature of 21.5 °C. Stream velocity was previously determined from salt-dilution traces, which were approved in 2002 by the Bureau of Land Management as conservative and nondestructive to the microbial mats and cave ecosystem (which includes a federally endangered species); these restrictions currently limit use of radioactive or isotopic tracers. Engel *et al.* (2004b) measured dissolved and atmospheric gases to determine the theoretical $C_TS^=$ concentrations in the cave stream if sulfide loss was due to (1) volatilization of gas-phase H_2S from the cave stream and not considering turbulence, (2) autoxidation of $C_TS^=$ along the stream reach (Engel *et al.*, 2004b), or (3) a net first-order loss of $C_TS^=$ along the stream estimated from $C_TS^=$ at the upstream and downstream ends of the reach:

$$\frac{C}{C_0} = e^{-kt} \quad (1)$$

where C is $C_TS^=$ (shortened for clarity), C_0 is $C_TS^=$ at the upstream end of the reach, t is the transport time along the reach, and k is the first-order rate of $C_TS^=$ transformation. Observed longitudinal distributions of $C_TS^=$ were compared with the three simple models of sulfide loss listed above (volatilization, autoxidation, and net first-order loss). These comparisons were used to evaluate spatial variability in sulfide production and consumption along

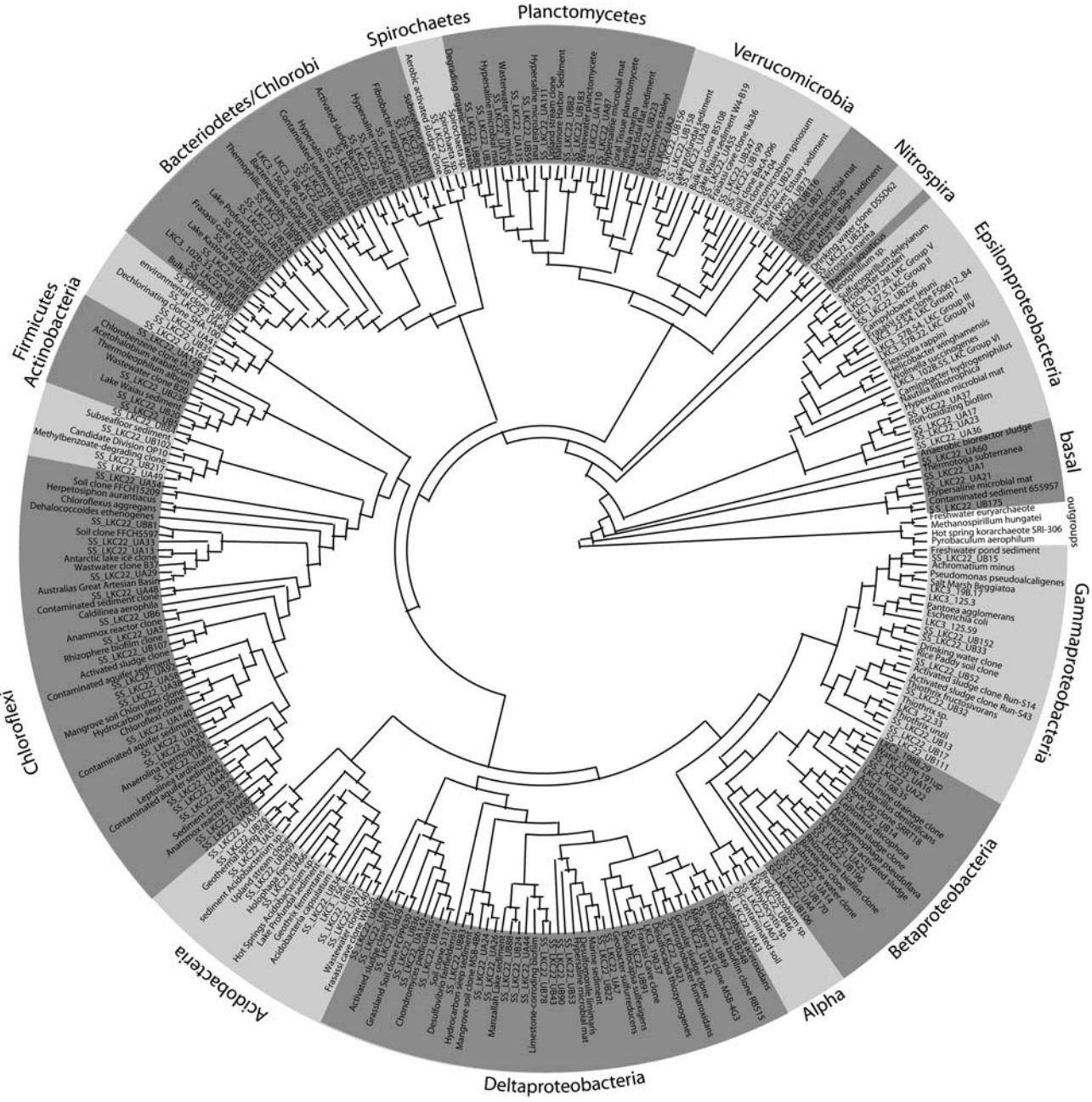

Figure 2 Neighbor-joining phylogeny of 16S rRNA gene sequences (⩾1100 base pairs) from each of the representative bacterial taxonomic diversity from Lower Kane Cave and closest relatives. Major groups are indicated, and the tree was rooted using four archaeal sequences (AY822003, M60880, L07510, and AF255604).

the stream, and to compare these patterns to the microbial community structure and function.

Results

Microbial phylogenetic diversity

At least 17 major taxonomic bacterial groups and one archaeal group could be identified based on RFLP screening and partial (<600 bp) 16S rRNA screening of all retrieved (346) clones from the 203 g mat sample (Figure 2; detailed results in Supplementary Table SM2). Rarefaction estimates indicate that coverage barely approached saturation (Figure 3) and that the anaerobic mat community described by this work was more diverse than the aerobic mat samples reported earlier (Engel *et al*., 2004a). Of the OTUs identified, only ~7% were ⩾98% similar to previously identified groups in GenBank, corresponding to species-level relationships, and 122 novel OTUs were delineated based on 98% sequence identity. More than 50% of the clones were affiliated with three taxonomic groups: the *Deltaproteobacteria* (δ-*Proteobacteria*), *Chloroflexi*, and

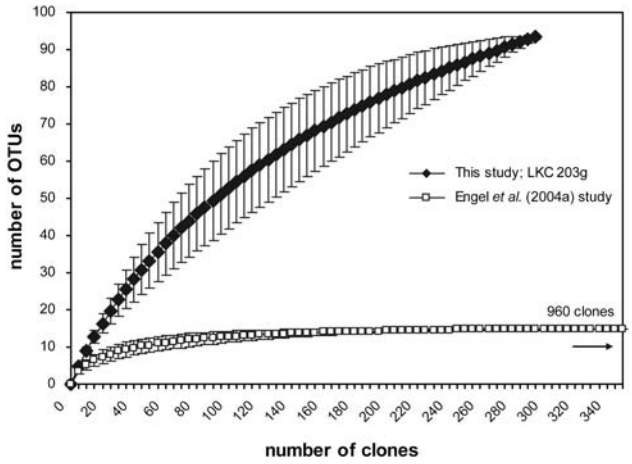

Figure 3 Rarefaction curves based on operational taxonomic unit designation showing the diversity of the 203 g 16S rRNA gene sequence bacterial clone library from this study and of the clones from 10 libraries from the aerobic mat samples described in Engel *et al.* (2004a).

Gammaproteobacteria (γ-*Proteobacteria*). Nearly full-length sequences (>1100 bp) were retrieved for 135 representative clones for more detailed phylogenetic evaluation (Figure 2).

Distribution and ecophysiology within the microbial mats
As clone libraries do not provide distribution information (Juretschko *et al.*, 2002), FISH offered a more realistic view of the distribution and abundance of the dominant and tentatively active (that is with relatively high ribosome contents) groups identified by the clone library approach. Different levels of prokaryotic diversity were targeted with 29 oligonucleotide probes (Supplementary Table SM1), and all but 7 of the 29 probes yielded positive probe signals in at least one of the samples. No signals were observed using the probes targeting *Archaea* (Arch344), *Chloroflexi* (CFX1223 and GNSB941), nitrifying bacteria (Ntspa662 and Ntspa712), and *Verrucomicrobia* (EUBIII) (Table 1). Depending on the sample type, and of the 30 samples examined, *Bacteria* were detectable by FISH using the general EUB338mix probe for only 18 mat samples. Many of the samples were not suitable for FISH due to prevalent suspended or entrained inorganic material. Higher percentages of detectable cells could be identified by FISH in aerobic samples versus the anaerobic samples (Supplementary Figure SM11). Most of the aerobic and anaerobic mat samples analyzed had high abundances of six probe-targeted groups: the γ-*Proteobacteria*, *Epsilonproteobacteria* (ε-*Proteobacteria*), *Acidobacteria*, α-*Proteobacteria*, δ-*Proteobacteria*, and the *Firmicutes* (Table 1; Supplementary Figure SM11). Filaments were the most targeted cell type, whereby positive signals were observed with probes for ε-*Proteobacteria*, δ-*Proteobacteria*, γ-*Proteobacteria*, α-*Proteobacteria*, and Bacteroidetes. The remaining probe-targeted groups were found in <30% of the investigated mat samples. As functional gene primers are not established for many possible metabolic lifestyles, construction of functional gene clone libraries would have been too time-consuming for the purposes of this study. Therefore, as a way to understand guild distribution in the microbial mats, MPN was used to screen and evaluate potentially lower abundance groups representing different metabolic life styles. Compared to the overall cell estimates of $\sim 10^{10}$ cells ml^{-1} from the aerobic portions of the mats based on cell carbon content (Engel *et al.*, 2004a), the MPN results enumerated five to six orders of magnitude less cells for each of the various anaerobic guilds (Figure 4).

Screening of the mat samples by FISH indicated that at least members affiliated to the δ-*Proteobacteria* and *Firmicutes* were present (Table 1; Supplementary Figure SM11), and although the MPN results did not provide information regarding which sulfate-reducing microbial groups (abbreviated as SRP) were enriched, cell estimates for formatotrophic and acetotrophic SRP were equally high (up to 10^6 cells ml^{-1}) from almost all samples (Figure 4). Autotrophic SRP were not detected in all samples and generally had up to four orders of magnitude fewer cells. SRP cell abundance increased from the Upper Spring orifice downstream through the microbial mats. SRP were considerably less abundant in the cave stream (Figure 4). The aerobic and anaerobic mat samples had nearly equivalent SRP MPN estimates. Sulfate reduction is a polyphyletic metabolic process (Loy *et al.*, 2002), and although several lineages within the δ-*Proteobacteria* class, the phyla *Thermodesulfobacteria*, *Nitrospirae* (for example *Thermodesulfovibrio*), *Firmicutes* (for example *Desulfotomaculum*), and some Archaea (for example *Archaeoglobus*) gain energy from sulfate reduction, FISH results indicated positive hybridization signals with the δ-proteobacterial and *Firmicutes* probes in 70% of the mat samples (Table 1; Supplementary Figure SM11). *Firmicutes* represented ~7% of the 16S rRNA gene clones, and none were related to known sulfate-reducers (for example, *Desulfotomaculum*) (Supplementary Table SM2). The majority of clones in the library was affiliated with the δ-*Proteobacteria* related to environmental sequences retrieved from soil, sediments, the Frasassi Cave sulfidic stream microbial mats, and to cultured SRP like the filamentous *Desulfonema* spp., *Desulfomonile* spp., and *Chondromyces* spp. (Figure 2). S^0RP taxonomic diversity is poorly known (Schauder and Kroger, 1993; Kletzin *et al.*, 2004; Kodama *et al.*, 2007). Several species are capable of disproportionating sulfur to sulfide and sulfate, including *Desulfocapsa thiozymogenes* (Finster *et al.*, 1998), which was retrieved in the clone library (Figure 2). Up to 10^2 S^0RP cells ml^{-1} were enumerated from MPN (Figure 4).

Table 1 Summary of fluorescence *in situ* hybridization observations from microbial mat samples from Lower Kane Cave that were distinguished as aerobic (ae) and anaerobic (ana) mat types based on geochemistry

Probe-targeted group	*Probes*	*Quality of probe signal*	*Cell morphologies observed*	*Number samples ae: ana*	*Distribution*
Bacteria	EUB338	Weak to strong positive	Long+short rods; filaments; cocci	12:6	n.q.
Acidobacteria	Hol1400, 23S subd 7/8	Strong positive	Long+short rods	9:4	60%
Actinobacteria	HGC69a	Weak positive	Short rods	1:0	10%
Bacteroidetes/Chlorobi	CF319a,b,c	Strong positive	Long thin filaments; short rods	5:1	30%
Firmicutes	LGC354A,B,C	Weak positive	Long filaments; short rods	8:1	40%
Planctomycetes	EUB338-II/PLA46	Weak positive	Thin filaments	3:0	10%
Alphaproteobacteria	Alf 1b+968	Strong positive	Long filaments; short+long rods	10:2	40%
Betaproteobacteria	Bet42a+Gam42a comp	Weak positive	Long filaments	5:1	30%
Deltaproteobacteria	DELTA495a,b,c	Strong positive	Long filaments; short+long rods	9:3	40%
Epsilonproteobacteria	LKC1006; LKC59; EPSY549	Strong positive	Mainly long filaments	11:2	60%
Gammaproteobacteria	Gam42a+Bet42a comp	Strong positive	Long, wide filaments; short+long rods; cocci	11:7	70%
Archaea	Arch915, Arch344	Negative	n.d.	—	n.d.
Chloroflexi	CFX1223, GNSB941	Negative	n.d.	—	n.d.
Nitrospirae	Ntspa662/712	Negative	n.d.	—	n.d.
Verrucomicrobia	EUB338-III	Negative	n.d.	—	n.d.

The distribution of each probe-targeted group was estimated visually from all 30 microbial mat samples analyzed, and uncertainty is $\sim \pm 10\%$. Results for each sample are described on Supplementary Figure SM11. n.q., not quantifiable for all samples due to auto-fluorescence; n.d., no signal detected.

MPN revealed that fermenting microbes were present in 80% of the mat samples, at abundances up to 10^6 cells ml^{-1} in the downstream samples (Figure 4). As fermentation is not limited to select taxonomic groups, different mixed-acid fermentation reactions were assessed (see Supplementary material). Of the 28 mixed-acid fermentation cultures, eight distinct types of heterotrophic growth were identified, predominately from downstream gray filament samples in which ~70% of the mixed-acid fermentation enrichments produced acidity and CO_2 gas. Twenty-eight percent of the mixed-acid fermentation enrichments generated H_2S. Ten percent of the enrichments grew on the TSI agar, but did not decrease medium pH or produce gas, suggesting one of four possibilities: (1) no fermentation; (2) fermentation but no decrease in pH or CO_2 production because of 2,3-butanediol or ethanol production; (3) only lactose fermentation; or (4) peptone catabolism. Hence, in addition to identifying different organic acid utilization pathways, we could also distinguish between butanediol and ethanol production that is common among Gram-positive bacteria like the *Firmicutes* (Talarico *et al.*, 2005), which represented 7% of the clone library. This group has not been found in high abundances from sulfidic cave systems previously, but it has been identified from cave-wall microbial communities and cave sediments (Laiz *et al.*, 2003; Schabereiter-Gurtner *et al.*, 2003; Cacchio *et al.*, 2004; Chelius and Moore, 2004; Ikner *et al.*, 2007).

Fourteen percent of the 16S rRNA gene sequence clones were related to uncultured *Chloroflexi* species (Figure 2; Supplementary Table SM2). Despite *Chloroflexi* being retrieved from the Frasassi Caves (Engel *et al.*, 2007) and the H_2S-bearing Zodletone Spring, Oklahoma (Elshahed *et al.*, 2003), the cave clones were more related to clones retrieved from soil, river sediments, or sludge (90–98% sequence similarity), and distantly related to cultured, anaerobic, filamentous strains (88–90% sequence similarity). FISH revealed that none of the microbial mats showed unambiguous positive hybridization signals to standard *Chloroflexi*-targeting fluorescently labeled probes (Table 1). However, preliminary studies using horse-radish peroxidase labeled *Chloroflexi* targeting probes (CFX 1223) and subsequent catalyzed reporter deposition-FISH (Pernthaler *et al.*, 2002) have recently revealed that a significant number of filamentous cells in the mats hybridized with this probe (Meisinger *et al.*, unpublished data). Because of their distant relationship to a large number of uncultured specimens found in anaerobic environments and to culturable nonobligate anaerobic species, like the novel candidate genera *Leptolinea* and *Caldilinea* in subclass I and VI of *Chloroflexi* (Kohno *et al.*, 2002; Yamada *et al.*, 2005) (Supplementary Table SM2), it is plausible that the novel *Chloroflexi* groups have an anaerobic lifestyle.

The importance of sulfur-oxidizing bacterial groups to sulfuric acid speleogenesis has been made apparent (Engel *et al.*, 2004b; Macalady *et al.*, 2006). The γ-*Proteobacteria* are predominately affiliated with the filamentous and sulfur-oxidizing genera *Thiothrix* spp. and *Beggiatoa* spp., and these groups were retrieved from the clone libraries (Figure 2), and FISH revealed that 70% of all mats had positive hybridization signals with the γ-proteobacterial

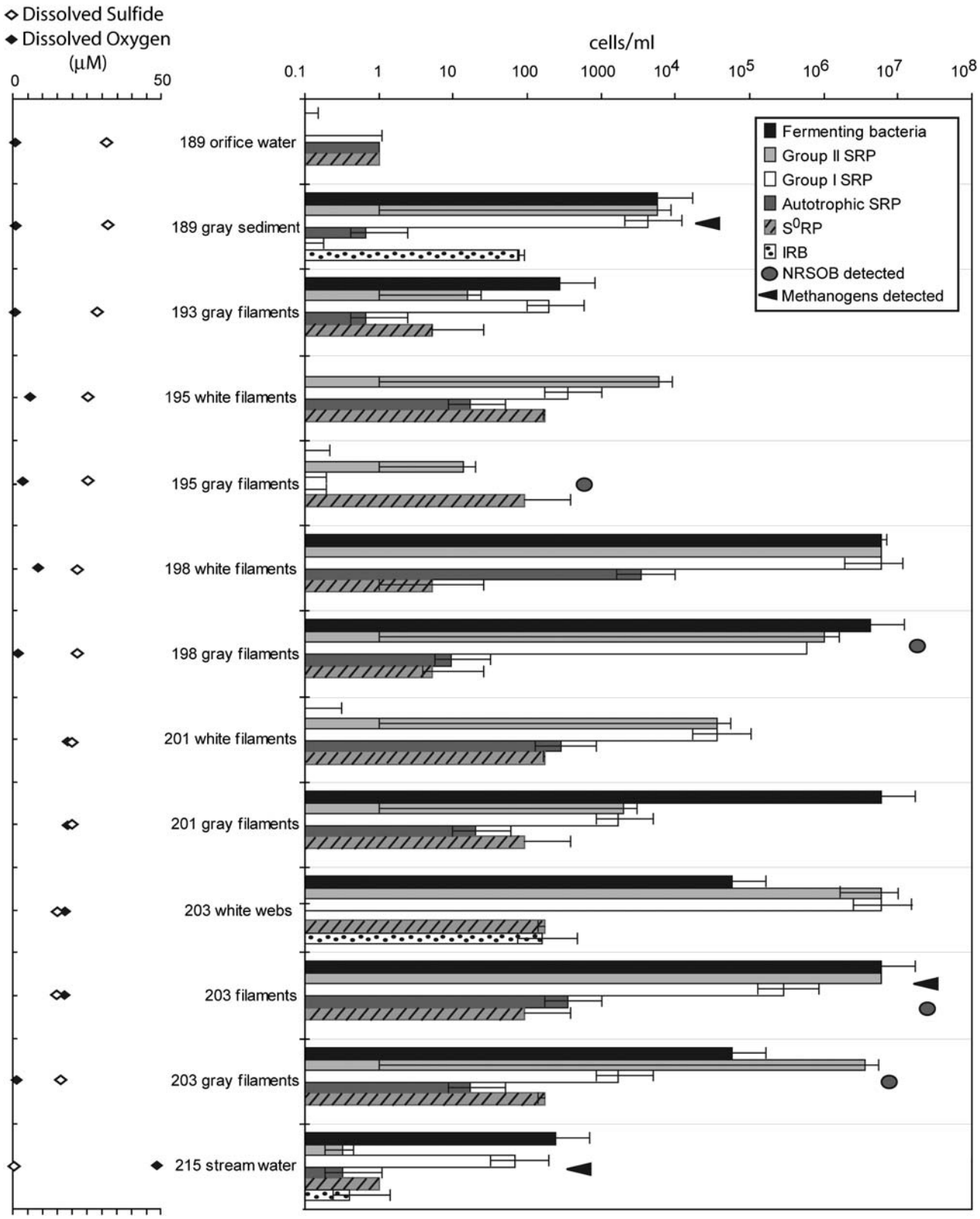

Figure 4 Most probable number (MPN) estimates for anaerobic microbial guilds from Lower Kane Cave water, sediment, and microbial mat samples compared with dissolved oxygen and sulfide concentrations for each of those samples. Samples are along a transect of the Upper Spring (from 189 to 215 m). Number at left represents site location, in meters, from the back of the cave forward (the spring orifice is the lowest number). White mats are generally considered to be aerobic, while gray mats are anaerobic. SRP, sulfate-reducing prokaryotes (groups I and II, and autotrophic); S^0RP, sulfur-reducing prokaryotes; DIRB, dissimilatory iron-reducing bacteria. Error bars are 95% confidence intervals calculated by the MPN program (Hurely and Roscoe, 1983). For nitrate-reducing sulfur-oxidizing bacteria (NRSOB) and methanogens, biomass estimates were not made and therefore only the presence of these groups is indicated; see text for methodological details.

probes, mostly to filaments, as well as to short and long rods and cocci (Table 1; Supplementary Figure SM11). The anaerobic metabolism of nitrate reduction linked to sulfide oxidation was evaluated by MPN. Half of the nitrate-reducing sulfide-oxidizing bacterial (NRSOB) enrichments had positive growth, predominately from the gray mat samples (Figure 4). As there are currently no FISH probes for this guild because the metabolism is polyphyletic, it was not possible to use FISH to identify NRSOB distribution patterns. But, 60% of the screened mat samples had positive signals for long filaments associated with the ε-*Proteobacteria*, which could indicate the presence of NRSOB (Macalady *et al.*, 2006).

The remaining 16S rRNA clones represented a range of taxonomic and metabolic groups (Figure 2). None of the clones were closely related (>99% sequence identity) to known cultured organisms (Supplementary Table SM2). Nearly 10% of the clones were affiliated with Bacteroidetes/Chlorobi members that were related (89–98% similarity) to clones retrieved from lake, sludge, and tar-oil contaminated aquifers (Supplementary Table SM2). The Bacteriodetes/Chlorobi were only detected in downstream samples by FISH (Supplementary Figure SM11). Despite members of this large group being common in caves, including nonsulfidic examples, their functional role is generally poorly understood and attributed to fermentation or metal cycling (Angert *et al.*, 1998; Schabereiter-Gurtner *et al.*, 2003; Chelius and Moore, 2004; Macalady *et al.*, 2006; Ikner *et al.*, 2007). Up to 10^3 cells ml^{-1} of dissimilatory iron-reducing bacteria were identified from anaerobic sediment in the Upper Spring from MPN estimates, but were also detected from the aerobic mat samples downstream (Figure 4). None were detected from spring or stream water. Although Meisinger *et al.* (2007) hypothesized that some *Acidobacteria* may be capable of iron reduction, such as those distantly related to *Geothrix fermentans*, very few clones were retrieved that were closely related to known dissimilatory iron-reducing bacteria (Supplementary Table SM2). Cells hybridized with *Acidobacteria*-specific probes were detected by FISH in 60% of the samples, and in some instances, this was the most abundant group identified by FISH (Supplementary Figure SM11). Several of the remaining OTUs could not be assigned to a particular division, and given the low sequence identities (86–95%) it is possible that novel groups, even at the familial level, may exist due to affiliations with basal or unlabeled groups (Figure 2).

Diverse methanogens have been identified from ε-*Proteobacteria*- and *Thiothrix*-dominated microbial mats from photic sulfidic springs (Moissl *et al.*, 2002; Elshahed *et al.*, 2004). However, there are strong indicators that methanogenic guilds have low population sizes in Lower Kane Cave, including trace detectable CH_4 in the stream water (Engel *et al.*, 2004a), low cell yields from MPN enrichments (Figure 4), low numbers of archaeal clones using three different primer pairs, and dubious fluorescent signals when using Archaea-specific FISH probes. All of the retrieved clones belonged to the *Euryarchaeota*, and were most closely related to a methanogenic freshwater sediment clone (Supplementary Table SM2). Interestingly, the rest of the clones amplified by PCR for *Archaea* were closely related to *Thiothrix* spp. FISH results were inconclusive for the *Archaea* because cell numbers may be too low to be detected by FISH, cell inactivity, or low ribosomal content, or because the *Archaea* probes, ARC344 and in particular Arch915, had unspecific probe signals to certain filaments. When Arch915 was combined with the γ-*Proteobacteria* probe Gam42a and *Thiothrix* spp.-specific probes, the filaments had overlapping signals. These results support several previous studies that indicate the present probe (Arch 915) and primer sets (A112f-A934b) used to target *Archaea* may yield erroneous (nonarchaeal) results (Amann and Fuchs, 2008).

Sulfide dynamics and nutrient spiraling

Spiraling metrics are typically based on travel distance along the stream (as defined by Newbold *et al.*, 1981), assuming an average influence of flow velocity on solute concentrations along a stream reach. Stream velocity was relatively constant from ~190 to 204 m at 0.5 m s^{-1}, but decreased to 0.2 m s^{-1} downstream of the microbial mat terminus (from ~204 to 219 m). We considered changes in $C_TS^=$ relative to transport time to account for the distinct change in velocity, yielding a more traditional time-based kinetic model. We returned the time-based kinetic analyses to a variably scaled spatial spiraling context (Figure 5) to directly compare locations of $C_TS^=$ processing and specific microbial guilds along the stream (Doyle, 2005). Although the concentrations of dissolved bicarbonate and sulfate did not vary appreciably along the cave stream, dissolved oxygen increased while $C_TS^=$ values decreased (Figures 5b and c). The mats abruptly terminate between 203 and 204 m where dissolved oxygen exceeded 45 μmol $l^{-1}$and $C_TS^=$ was half the initial concentration (~28.7 s). From Equation (1), k was estimated as 0.02 s^{-1} from the difference between upstream and downstream $C_TS^=$. From 0 to 5 s, and beyond 30 s travel time, the observed $C_TS^=$ values generally follow a first-order $C_TS^=$ loss curve, although the observed values are slightly less than the expected values from 0 to 5 s (Figure 5c, black curve). Between 5 and 30 s, however, observed $C_TS^=$ differed from the first-order loss curve. At any given dissolved oxygen concentration, the observed $C_TS^=$ values were lower than would be expected for $C_TS^=$ if loss was due to either autoxidation or volatilization processes (Figure 5c, gray curves) (Engel *et al.*, 2004b).

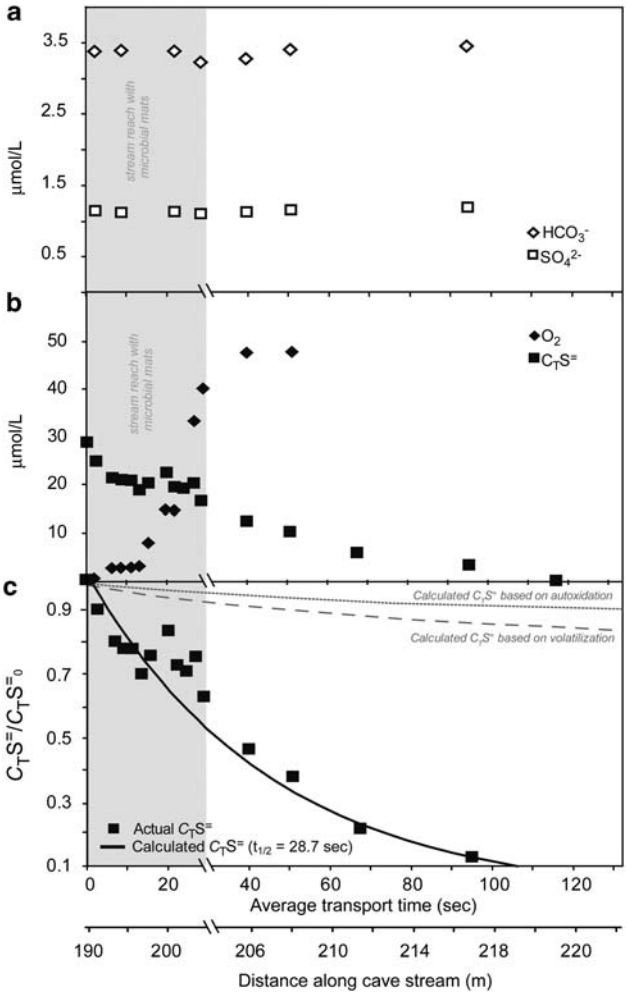

Figure 5 (**a**) Dissolved bicarbonate and sulfate concentrations. (**b**) Dissolved oxygen and total dissolve sulfide ($C_TS^=$) concentrations for the Upper Spring transect. (**c**) Observed $C_TS^=$ versus modeled $C_TS^=/C_TS_0^=$ based on a first-order loss from $t_{1/2}$ of 28.7 s (black line), and on autoxidation and volatilization loss (gray lines) according to Engel *et al.* (2004b). The broken *x* axis indicates where there is a change in velocity. The shaded box in all panels denotes the stream reach with microbial mats (from 0 to ~30 s, or 190–204 m).

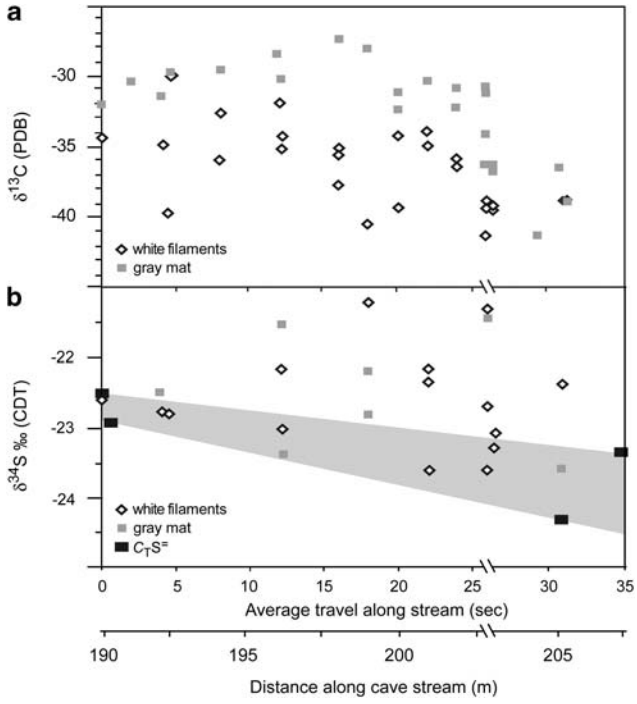

Figure 6 (**a**) Carbon isotope compositions summarized from Engel *et al.* (2004a) for white (aerobic) and gray (anaerobic) filamentous microbial mat samples. (**b**) Sulfur isotope compositions for microbial biomass and the dissolved sulfide from the cave stream (Engel *et al.*, 2007). The broken *x* axis indicates a change in velocity.

Discussion

In the Lower Kane Cave stream system, the microbial mats are likely to alter the hydrodynamics of a system, increase the contact time of water and solutes with both aerobic and anaerobic portions of the mat, and thereby compress nutrient consumption and production spirals along the cave stream (Battin *et al.*, 2003). So, the susceptibility of a molecule to metabolic transformation, such as from a mobile to immobile phase, increases as solute transport into mats from the water column becomes limiting. Gas-phase nutrients, such as H_2S, O_2, and CO_2, have the greatest potential to be limiting, and we hypothesized that the resulting variations in the uptake (consumption) and release (production) rates of these nutrients downstream should influence the longitudinal distribution of specific metabolic groups. We combined the full-cycle 16S rRNA approach with the MPN method to realize the physical and functional distribution of anaerobic microbial guilds and communities in the microbial mats. Our phylogenetic investigations uncovered the potential for undescribed biodiversity, and some groups have not been identified previously in subsurface systems. These results made evaluating the mechanisms that control changes in diversity difficult (Doebeli and Dieckmann, 2003; Mizera and Meszena, 2003; Hansen *et al.*, 2007). But, the spatial distribution of microbial diversity was placed into a nutrient spiraling framework by interpreting hydraulic, geochemical, and biological processes that balance nutrient and energy flow along the cave stream (Webster and Patten, 1979; Newbold *et al.*, 1981, 1982; Runkel, 2007).

Consideration of stable carbon and sulfur isotope ratio analyses from the microbial mats and cave waters illustrate that nutrient spiraling influences changes in microbial diversity, and that a single net fractionation cannot be applied to the system due to the diversity of biological processes that offset the isotopic composition along stream (Figure 6). For Lower Kane Cave, chemolithoautotrophically fixed carbon serves as the trophic basis of this microbial ecosystem because photosynthetically produced carbon is unlikely to provide any significant energy to the system due to the cave's isolation from the

surface (Engel *et al.*, 2004a; Porter *et al.*, 2009). The $\delta^{13}C$ values for microbial biomass are typical of autotroph-dominated biomass because they reflect the large, irreversible enzymatic discrimination against ^{13}C from a dissolved inorganic carbon reservoir (Figure 6a). The dissolved inorganic carbon $\delta^{13}C$ values decrease by ~2‰ from the spring orifice (190 m) to the end of the mats at ~204 m (Engel *et al.*, 2004a). Porter *et al.* (2009) indicate that at least 30% of the autotrophically fixed carbon in Lower Kane Cave microbial mats is cycled through a microbial detrital loop. From 0 to 10 s (~190–195 m), where measured $C_TS^=$ values are only slightly lower than expected (given experimental error) from the first-order loss curve (Figure 5c), the $\delta^{13}C$ values for upstream samples, which consist predominately of ε-*Proteobacteria* and few other groups (Supplementary Figure SM11), differ by <5‰ (Figure 6a). By ~16–17 s (~197 m), however, the differences between $\delta^{13}C$ values from the white and gray mat samples increases to >7‰. As excretion, respiration, and heterotrophic cycling of carbon should have negligible effects on carbon isotope values, the differences in $\delta^{13}C$ values could be linked to changes in the abundances of different autotrophic ε- and γ-proteobacterial groups and variations in the rates and expression of ^{13}C discrimination during autotrophy (Engel *et al.*, 2004a). From 20 to 30 s (~200–205 m), the white and gray $\delta^{13}C$ biomass compositions are less variable, suggesting that the proximity of heterotrophs to autotrophically produced organic carbon may cause immediate sequestration of carbon rather than its downstream transport (Battin *et al.*, 2003). In other words, the biomass $\delta^{13}C$ decreases with each carbon spiral along the stream because of a feedback effect between autotrophic fractionation (negative for ^{13}C) and the continual respiration of autotrophically fixed organic carbon from upstream. Carbon spiraling should homogenize the $\delta^{13}C$ in the organic and inorganic carbon pools with increasing distance downstream, as well as cause the ensuing decrease in the $\delta^{13}C$ dissolved inorganic carbon downstream, because CO_2 respired upstream is used by downstream autotrophs.

The $\delta^{34}S$ values for $C_TS^=$ in the cave stream decrease by ~1.6‰ along the cave stream, and white and gray biomass $\delta^{34}S$ values are variable (Figure 6b). The gray mat samples from the middle of the stream have the highest abundance of sulfate-reducing and heterotrophic groups (Figure 4), and these samples also correspond to a reach where measured $C_TS^=$ exceeds the first-order $C_TS^=$ decay loss (Figure 5c). The increase in $C_TS^=$ could not be due to abiotic processes, so the large and diverse community of SRP and fermenters are responsible for producing autochthonous sulfide. As autochthonous H_2S would have very low $\delta^{34}S$ values compared with allochthonous dissolved sulfide in the cave stream, consumption of this autochthonous sulfide by sulfur-oxidizing community would shift the average $\delta^{34}S$ values for microbial biomass downstream by more than ±1‰ (Fry *et al.*, 1988; Mandernack *et al.*, 2003). Differences in autochthonous H_2S uptake rates downstream, as well as differences in retention of sulfur-containing compounds within the mats downstream, may result in compositional variability. The mean sulfur content of white filaments is ~30% w/w, whereas gray filaments only have ~2% sulfur (Engel *et al.*, 2004a). In contrast, most cells have values approximately equal to 1% in the absence of stored sulfur (Fagerbakke *et al.*, 1996). Engel *et al.* (2007) confirmed that sulfur in white filaments is cyclooctasulfur (S_8), or elemental sulfur, which accumulates as a byproduct of sulfur oxidation, but that sulfur in the gray filaments is mostly as monosulfane from the accumulation of amino acids.

These correlations among microbial abundance and diversity, $C_TS^=$ dynamics, and the isotopic composition of water and mats provide a basic understanding that nutrient spiral dimensions lengthen or shorten (that is loosen or tighten; Webster and Patten, 1979) in different segments of the stream, even if we do not yet fully understand uptake and turnover dynamics between the aerobic and anaerobic portions of the mats. For instance, it is possible that some groups may facultatively switch from autotrophy to mixotrophy as autochthonous organic carbon availability increases. Therefore, given the complexity of this natural system, future work will require a more detailed characterization of the hydrology (Doyle, 2005; Runkel, 2007), nutrient addition experiments, and the introduction of conservative hydrologic tracers to quantify how spiraling dimensions change with shifts in community composition and metabolism, or how biological or hydraulic retention influence spiral lengths due to reactive transport and nutrient retention and storage within the microbial mats. More detailed modeling of the system will also require characterizing gas (H_2S) transfer across the stream–air interface, which is not implemented in the more commonly used active solute transport models (for example OTIS; Runkel, 1998). Lastly, microbial functional processes should also be explored and quantified, such as from functional genomics and expression studies.

In conclusion, this study expands the known diversity of some microbial groups to caves and the subsurface, even though the microbial diversity of various sulfidic caves and aquifers has been investigated for more than a decade (Sarbu *et al.*, 1996; Vlasceanu *et al.*, 1997, 2000; Hose *et al.*, 2000; Barton and Luiszer, 2005; Macalady *et al.*, 2006). In Lower Kane Cave, anaerobic mat communities were more taxonomically and metabolically diverse relative to the aerobic mat communities. Although groups like SRP and fermenters present a fraction of the whole cell abundance of the system, their metabolic impact is reflected and recorded in the geochemistry (that is isotopically) of the system.

Changes in the forms and abundances of nutrients influence the overall functional diversity with distance downstream, and this may result in the emergence of potentially specialized groups of differing genetic and functional compositions downstream (Battin *et al.*, 2003; Mizera and Meszena, 2003; Parnell *et al.*, 2009). Evaluation of carbon and sulfur nutrient spiraling in Lower Kane Cave improves our understanding of ecosystem function in aphotic habitats and will help us to understand microbial processes occurring in other modern, redox-stratified microbial mat systems.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)

Ergänzendes Material

Material und Methoden

Tabellen SM1 und SM2

Literaturliste

Abbildungen SM1-SM11

Supplemental Materials

Linking Phylogenetic and Functional Diversity to Nutrient Spiraling in Microbial Mats from Lower Kane Cave (USA)

Annette Summers Engel, Daniela B. Meisinger, Megan L. Porter, Robert A. Payn, Michael Schmid, Libby A. Stern, K.-H. Schleifer, Natuschka M. Lee

Sample acquisition

Sampling strategies and geochemical analyses were preformed previously by Engel et al. (2004). Each sample number refers to the location in distance (meters) from the back of the cave (i.e. 0 m), with water flow towards the cave entrance (i.e. 325 m). Conservative quantities of microbial mats were aseptically collected at the Lower Spring, the Upper Spring, and the Fissure Spring. One anaerobic sample ("203g"), from gray mats collected 2 cm below white filaments at 203 m along the Upper Spring stream, was used for the 16S rRNA clone libraries.

16S rRNA gene diversity and sequencing

Community diversity was assessed using the previously described full-cycle 16S rRNA approach. Total environmental DNA was extracted using the protocol of Griffiths *et al.* (2000), with modifications described in Meisinger *et al.* (2007). Near-full length 16S rRNA bacterial gene fragments were amplified using two universal different primer pairs, as previously explained (Meisinger *et al.*, 2007). A clone library was also constructed using the archaeal primers A112f (5´-GCTCAGTAACACGTGG-3´) and A934b (5´-GTGCTCCCCCGCCAATTCCT-3´) (Großkopf *et al.*, 1998). PCR products were purified (Quickstep PCR Purification Kit V.5, Edge Biosystems, USA) and cloned into the vector pCR.2, using a TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany). Inserts from clone plasmids

were screened using the M13F and M13R PCR primers (Invitrogen). Plasmids with the correct inserts were purified, and unique 16S rRNA gene fragments from 346 clones were identified by screening restriction fragment length polymorphism (RFLP) patterns, as described in Meisinger *et al.* (2007). Nearly full (>1100 bp) gene sequences for each unique pattern were obtained for at least one and up to three clones, where appropriate and if partially sequenced clones were similar to each other by 98% sequence similarity.

FISH sample fixation, microscopy, and screening

Samples were shipped on dry ice within 48 hr of collection. Samples were fixed with 4% (wt/vol) paraformaldehyde, as described by Manz *et al.* (1992) for optimal fixation of Gram-negative bacteria, and with 50% ice-cold ethanol for optimal fixation of Gram-positive bacteria, according to Roller *et al.* (1994). Mat samples, oligonucleotide probes, and hybridization buffers were prepared as described in Engel *et al.* (2003) and Meisinger *et al.* (2007). Table SM1 contains details regarding the 29 rRNA-targeted fluorescently labeled oligonucleotide probes used for detecting different groups present. For control of unspecific adsorption of fluorescent dyes to the fixed samples, the NonEub probe was used for comparison (Table 1). Total cell numbers detectable by FISH were determined after hybridization by post-staining with SYBR Green I (FMC Bioproducts, USA) (Engel *et al.*, 2003). FISH results were visualized using a LSM510 scanning confocal microscope (Zeiss, Oberkochen, Germany) equipped with an Argon laser (450 to 514 nm) and two HeNe lasers (543 and 633 nm). All images were recorded using a Plan-Apochromat 63x (1.4; oil immersion) objective. Image processing was performed with the LSM510 software package (ver. 1.6). Due to confounding background material in some samples

(e.g., naturally autofluorescing minerals), relative estimations of cell abundances for different probe-targeted groups were made visually.

Most probable number counts

Various media were prepared using the pre-reduced and sterile (PRAS) technique of Holdman (1972). Briefly, three replicates were done per dilution series, and each series consisted of 10 bottles each. In the cave, ~10 ml of microbial mat samples were aseptically collected, placed into 15-ml sterile Falcon tubes with 5 ml of cold-filtered cave water (to 0.2 μm), and shaken vigorously for 1 to 5 min to disrupt the mat structure. All of the inoculated bottles were removed from the cave and dilutions were initiated within 3-7 hr, depending on transport time from cave. Inoculated bottles were incubated in the dark in a Coy anaerobic chamber at ~21-23 ^{o}C with N_2:H_2 mixed gas. The program MPN Calculator (Build 2.0; http://www.i2workout.com/mcuriale/mpn/index.html) was used to calculate MPN estimates, with 95% confidence intervals. Specific details for all media can be found in Engel (2004).

Fermenters were enumerated using 25% Schaedler's Broth (Difco Laboratories, Detroit, MI) and then screened using triple-sugar iron (TSI) agar slants (Difco). Although TSI agar is traditionally used to screen for the physiological activities of some *Enterobacteriacea*, we used this method to assess specific metabolic pathways, including acid production, gas generation, and medium blackening (due to H_2S production). Sulfate-reducing prokaryotes (SRP) were enumerated using media designed specifically for this work, from modified Baar's and Postgate's C media (MacFarlane and Gibson, 1991). Non-acetate oxidizers (Group I SRP) were enriched with a mixture of lactate and formate. Group II SRP were enriched with acetate. SRP capable of autotrophic growth were not provided organic carbon. To improve the scoring method

(e.g., Bekins *et al.*, 1999), and to assess additional information on metabolites, bottles were scored for positive growth after one week by measuring evolved headspace H_2S by direct-injection into a SRI 310 gas chromatograph (GC) (SRI Instruments, Torrance, CA) using a 60 m, 0.53mm MXT-1 column with flame ionization and flame photometric detectors (FID and FPD). This was found to be a much more sensitive and accurate method for determining activity and growth of SRP (Engel, 2004). Sulfur-reducing prokaryotes (S^0RP) were enriched on an elemental sulfur slurry designed by Engel (2004). The medium used to enumerate dissimilatory iron-reducing bacteria (DIRB) was described by Lovley and Phillips (1986) and Bekins *et al.* (1999). Acetotrophic, formatotrophic, and hydrogenotrophic methanogens were enriched using PRAS-prepared dilute mineral salts media, and screened for evolved headspace methane by GC analyses, as previously described in Bekins *et al.* (1999). Although the MPN method was not used to estimate nitrate-reducing sulfur-oxidizing bacteria (NRSOB), we attempted to enrich this group from the microbial mats using the strategy of Nemati *et al.* (2001). Medium color was noted (yellowing represented S^0 accumulation compared to uninoculated controls) and cell growth was visualized and verified using phase-contrast microscopy (Engel, 2004).

Table SM 1. Probe sequences and conditions used in this study. Probe names are based on Alm *et al.* (1996).

Probe	Probe name	Target group	Probe sequence (5'→ 3')	Target Site[a]	FA[b] (%)	Reference
			DOMAIN specific probes and negative controls			
Arch344	S-D-Arch-0344-a-A-20	*Archaea*	TCG CGC CTG CTG CIC CCC GT	16S (344)	0	Raskin *et al.* (1994)
Arch915	S-D-Arch-0915-a-A-20	*Archaea*	GTG CTC CCC CGC CAA TTC CT	16S (915)	0	Stahl and Amann (1991)
EUB338	S-D-Bact-0338-a-A-18	*Bacteria*	GCT GCC TCC CGT AGG AGT	16S (338)	0-40	Daims *et al.* (1999)
EUK516	(also EUK502)	*Eucarya*	ACC AGA CTT GCC CTC C	18S (502)	n.d.	Amann *et al.* (1990)
NonEUB		Negative control	ACT CCT ACG GGA GGC AGC	16S (338)	0	Wallner *et al.* (1993)
			Specific probes within *Bacteria* (probe names in alphabetical order)			
ALF1b	S-Sc-aProt-0019-a-A-17	*Alphaproteobacteria*	CGT TCG (C/T)TC TGA GCC AG	16S (19)	20	Manz *et al.* (1992)
ALF968	S-Sc-aProt-0968-a-A-18	*Alphaproteobacteria*	GGT AAG GTT CTG CGC GTT	16S (968)	20	Neef *et al.* (1998)
BET42a	L-P-Bet-1027-a-A-17	*Betaproteobacteria*	GCC TTC CCA CTT CGT TT	23S (1027)	35	Manz *et al*. (1992)
CF319b	S-P-Bact-0319-b-A-18	Some "*Flavobacteria"*	TGG TCC GTA TCT CAG TAC	16S (319)	35	Manz *et al.* (1996)
CFX1223	S-P-GNS-1223-a-A-20	Most *Chloroflexi*	CCA TTG TAG CGT GTG TGT MG	16S (1223)	35	Björnsson *et al.* (2002)
DELTA495a	S-C-dProt-0495-a-A-18	most *δ-Proteobacteria*; *Gemmatimonadetes*	AGT TAG CCG GTG CTT CCT	16S (495)	35	Loy *et al*. (2002)
DELTA495b	S-*-dProt-0495-b-A-18	some*δ-Proteobacteria*	AGT TAG CCG GCG CTT CCT	16S (495)	35	Loy *et al.* (2002)
DELTA495c	S-*-dProt-0495-c-A-18	some *δ-Proteobacteria*	AAT TAG CCG GTG CTT CCT	16S (495)	35	Loy *et al.* (2002)
EPS710	S-Sc-eProt-0710-a-A-18	some ε-*Proteobacteria*	CAG TAT CAT CCC AGC AGA	16S (710)	30[d]	Watanabe *et al.* (2000)
EPSY549[c]	S-C-eProt-0549-a-A-18	some ε-*Proteobacteria*	CAG TGA TTC CGA GTA ACG	16S (549)	n.d.	Lin *et al.* (2006)
EUB338-II	S-*-BactP-0338-a-A-18	*Planctomycetes*	GCA GCC ACC CGT AGG TGT	16S (338)	0-40	Daims *et al.* (1999)
EUB338-III	S-*-BactV-0338-a-A-18	*Verrucomicrobia* (and others)	GCT GCC ACC CGT AGG TGT	16S (338)	0-40	Daims *et al.* (1999)
GAM42a	L-P-Gam-1027-a-A-18	*Gammaproteobacteria*	GCC TTC CCA CAT CGT TT	23S (1027)	35	Manz *et al.* (1992)
GNSB941	S-P-GNS-0941-a-A-17	Most *Chloroflexi*	AAA CCA CAC GCT CCG CT	16S (941)	35	Gich *et al.* (2001)
G123T[c]	S-G-Thioth-0697-a-A-18	*Thiothrix*	CCT TCC GAT CTC TAT GCA	16S (697)	40	Kanagawa *et al.* (2000)
LGC354A	S-P-Firm-0354-a-A-18	*Firmicutes*	TGG AAG ATT CCC TAC TGC	16S (354)	20	Meier *et al.* (1999)
LGC354B	S-P-Firm-0354-a-A-18	Same as LGC344A	CGG AAG ATT CCC TAC TGC	16S (354)	20	Meier *et al.* (1999)
LGC354C	S-P-Firm-0354-a-A-18	Same as LGC344A	CGG CGT CGC TGC GTC AGG	16S (354)	20	Meier *et al.* (1999)
LKC59	S-*-eProt-0059-a-A-18	ε-proteobacterial Group I from LKC[e]	TCC TCT CAT CGT TCG ACT	16S (59)	30	Engel *et al.* (2003)
LKC1006	S-*-eProt-1006-a-A-18	ε-proteobacterial Group II from LKC[e]	CTC CAA TGT TTC CAT CGG	16S (1006)	30	Engel *et al.* (2003)
Ntspa662[c]	S-G-Ntspa-0662-a-A-18	genus *Nitrospira*	GGA ATT CCG CGC TCC TCT	16S (662)	35	Daims *et al.* (2001)
Ntspa712[c]	S-*-Ntspa-0712-a-A-21	most *Nitrospirae*	CGC CTT CGC CAC CGG CCT TCC	16S (712)	50	Daims *et al.* (2001)
PLA46	S-P-Planc-0046-a-A-18	*Planctomycetes*, *Chlamydiaceae*	GAC TTG CAT GCC TAA TCC	16S (46)	25-35	Neef *et al*. (1998)

[a] *E. coli* 16S or 23S rRNA position in brackets, according to Brosius *et al.* (1981).

[b] Formamide percentage (vol/vol) in the hybridization buffer.

[c] Used in combination with competitor probes: Eprot 549comp (5`-CAG TGA TTC CGA GCA ACG-3'); G123T-C (5'-CCTTCCGATCTCTACGCA-3') (Kanagawa *et al.*, 2000); Ntspa662comp (5'-GGA ATT CCG CTC TCC TCT -3'); Ntspa712comp (5'- CGC CTT CGC CAC CGG TGT TCC -3')

[d] This formamide concentration differs from the one suggested in the original publication (Watanabe *et al.*, 2000).

[e] LKC = Lower Kane Cave

Table SM 2. Taxonomic distribution of bacterial and archaeal clones from the 203g sample (also referred to as LKC22) based on partial 16S rRNA gene sequences. Results obtained for the *Acidobacteria* have been reported previously in Meisinger *et al.* (2007). Trees can also be downloaded from http://geol.lsu.edu/aengel/publications.htm.

Phylogenetic affiliation [a]	Representative clone sequences [Accession Numbers]	Closest relative of full clone sequences [Accession Numbers]	16S rRNA similarity (%) [b]	No. clones total [c]	Tree figure [d]
Acidobacteria (81-100 %)[d]	SS_LKC22_UA44 [AM180886]	Upland stream clone S-Rwb_47 [DQ017940]	88	2	Figure SM 1
	SS_LKC22_UB34 [AM180892]	Frasassi cave clone WM56 [DQ415770]	99	2	
	SS_LKC22_UA66 [AM180887]	Coffee Pots Hot Spring clone pC0F_65.7_E5 [EU156148]	95	1	
	SS_LKC22_UB76 [AM180890]	Geothermal spring mat clone DTB16 [EF205538]	95	1	
	SS_LKC22_UA71 [AM180888],	Wastewater clone C40 [EU234275]	99	3	
	SS_LKC22_UB55 [AM180891]		99		
	SS_LKC22_UB269 [AM180889]	Lake profundal sediment clone c5LKS46 [AM086131]	95	1	
	SS_LKC22_UA39 [AM490652]	Canyon and slope sediment clone HCM3MC78_3D_FL [EU373931]	87	1	
Actinobacteria (94-98%) [d]	SS_LKC22_UB5 [AM490645],	Lake Waiau sediment clone W4-B35 [AY345497]	98	2	Figure SM 2
	SS_LKC22_UB20 [AM490646]		98		
	SS_LKC22_UB236 [AM490647]	Wastewater clone B20 [AY426443]	98	2	
Bacteroidetes/Chlorobi (80-99%) [d]	SS_LKC22_UB12 [AM490699],	Activated sludge *Bacteroidetes* clone Skagen107 [DQ640726]	97	2	Figure SM 1
	SS_LKC22_UB231[AM490708]		99		
	SS_LKC22_UB10 [AM490700]	Freshwater pond sediment clone MVS-58 [DQ676406]	89	7	
	SS_LKC22_UB19 [AM490701]	Bulk soil reactor clone BS049 [AB240238]	97	4	
	SS_LKC22_UB85 [AM490702],	Contaminated sediment clone 655949 [DQ404821]	96	6	
	SS_LKC22_UB50 [AM490707]		96		
	SS_LKC22_UB87 [AM490703]	Lower Kane Cave clone LKC3_102b.33 [AY510256]	100	1	
	SS_LKC22_UB39 [AM490705]	Lake Kastoria, Greece, sediment clone Kas27B [EF203181]	98	1	
	SS_LKC22_UB74 [AM490706],	Thermophilic anaerobic sludge clone J1 [AY526509]	94	7	
	SS_LKC22_UB238 [AM490710]		94		
	SS_LKC22_UB203 [AM490709]	Frasassi cave clone SILK71 [EF467456]	99	1	
	SS_LKC22_UB232 [AM490711]	Lake profundal sediment clone c5LKS4 [AM086105]	95	2	
Chloroflexi (80-99%) [d]	SS_LKC22_UA20 [AM490718],	Tar-oil contaminated aquifer sediment clone D15-48 [EU266884]	98		Figure SM 3
	SS_LKC22_UA31 [AM490720]		97	11	
	SS_LKC22_UA140 [AM490733]	Mangrove soil uncultured *Chloroflexi* clone MSB-3F12 [DQ811858]	94	3	
	SS_LKC22_UA5 [AM490721]	Activated sludge clone 1641 [AB286640]	94	2	
	SS_LKC22_UB107 [AM490730]	Rhizophere biofilm clone RB390 [AB240366]	94	1	
	SS_LKC22_UA29 [AM490726]	Wastewater clone B37 [EU234165]	92	1	
	SS_LKC22_UA38 [AM490719]	Hydrocarbon seep clone BPC110 [AF154084]	93	2	
	SS_LKC22_UA13 [AM490722]	Antarctic lake ice clone LB3-100 [AF173817]	92	1	
	SS_LKC22_UA33 [AM490723]	Australias Great Artesian Basin clone B25 [AF407718]	90	1	
	SS_LKC22_UA54 [AM490724]	Soil clone FFCH15209 [EU134296]	91	3	
	SS_LKC22_UA27 [AM490725],	Granular anammox of reactor clone KIST-JJY023 [EF594055]	97		
	SS_LKC22_UA68 [AM490731]		97	11	
	SS_LKC22_UB6 [AM490729]	Granular anammox of reactor clone KIST-JJY02 [EF594058]	97	1	
	SS_LKC22_UA75 [AM490732]	*Leptolinea tardvitalis* YMTK-2 [AB109438]	98	2	
	SS_LKC22_UB79 [AM490727]	Sediment clone 35-7 [DQ833481]	96	1	
	SS_LKC22_UB81 [AM490728]	Soil clone FFCH5597 [EU133971]	97	1	

Phylogenetic affiliation [a]	Representative clone sequences [Accession Numbers]	Closest relative of full clone sequences [Accession Numbers]	16S rRNA similarity (%) [b]	No. clones total [c]	Tree figure [d]
Chloroflexi (80-99%) [d]	SS_LKC22_UA58 [AM490734]	Chloroflexi clone XME38 [EF061969]	93	2	
	SS_LKC22_UA45 [AM490735],	Tar-oil contaminated aquifer sediment clone D15-47 [EU266883]	93	4	
	SS_LKC22_UA42 [AM490736]		95		
	SS_LKC22_UA92 [AM490737]	Tar-oil contaminated aquifer sediment clone D15_4 [EU266881]	96	1	
Fibrobacter (80%) [d]	SS_LKC22_UB57 [AM490642]	Hypersaline microbial mat clone MAT-CR-MI-COB [EU245407]	91	2	Figure SM 4
	SS_LKC22_UA11 [AM490643],	Subseafloor sediment clone MD2896-B231 [EU385807]	94	3	
	SS_LKC22_UA53 [AM490644]		95		
Firmicutes (79-92%) [d]	SS_LKC22_UB84 [AM490651]	Subseafloor sediment clone MD2902-B178 [EU385957]	93	4	Figure SM 2
	SS_LKC22_UA128 [AM490650]	Chlorobenzene clone IA23 [AJ488074]	99	2	
	SS_LKC22_UA41 [AM490662],	Dechlorinating clone SHA-100 [AJ306780]	93	15	
	SS_LKC22_UB35 [AM490664],		97		
	SS_LKC22_UA46 [AM490648],		92		
	SS_LKC22_UA51 [AM490649]		83		
	SS_LKC22_UB190 [AM490666],	Anaerobic dechlorinating clone SJP-3 [AJ387900]	95	4	
	SS_LKC22_UA164 [AM490663]		92		
Nitrospira	SS_LKC22_UB224 [AM490665]	Drinking water clone DSSD62 [AY328760]	99	3	Figure SM 2
Planctomycetes (78-91%) [d]	SS_LKC22_UA2 [AM490667]	Coral tissues planctomycete clone STX_07f [EF123574]	88	1	Figure SM 4
	SS_LKC22_UB80 [AM490670]	Degrading organic solid waste clone CFB-28 [AB274517]	93	1	
	SS_LKC22_UB2 [AM490668]	Hypersaline microbial mat clone MAT-CR-P3-C03 [EU246093]	90	2	
	SS_LKC22_UA110 [AM490669],	Hypersaline microbial mat clone MAT-CR-H4-B12 [EU245214]	93	3	
	SS_LKC22_UA87 [AM490678]		93		
	SS_LKC22_UB82 [AM490671]	Baltimore Harbor sediment clone B4 [AY266450]	96	1	
	SS_LKC22_UB59 [AM490672],	Hypersaline microbial mat clone MAT-CR-P5-D08 [EU246220]	90	4	
	SS_LKC22_UA135 [AM490674]		87		
	SS_LKC22_UA111 [AM490673]	Upland stream clone S-Rwb_48 [DQ017941]	93	2	
	SS_LKC22_UA120 [AM490675]	Wastewater clone mie1-8 [AF280847]	93	1	
	SS_LKC22_UB223 [AM490676]	Naked tial flat sediment clone MSB-1E4 [EF125406]	94	1	
	SS_LKC22_UB183 [AM490677]	Wastewater treatment plant planctomycete A-2 [AM056027]	97	1	
Proteobacteria					Tree Figure SM 5
Alphaproteobacteria (83-87%) [d]	SS_LKC22_UA43 [AM490679]	Oil-contaminated soil clone LYC075 [DQ984605]	93	3	
	SS_LKC22_UB46 [AM490681]	*Bradyrhizobium* sp. clone BqE2 [DQ786797]	92	3	
	SS_LKC22_UA67 [AM490683]	*Methylocystis* sp. m261 [DQ852351]	99	2	
Betaproteobacteria (84-97%) [d]	SS_LKC22_UA14 [AM490712],	Sludge-seeded bioreactor clone DR-4 [AY945901]	98	5	Tree Figure SM 5
	SS_LKC22_UA25 [AM490686]		98		
	SS_LKC22_UA4 [AM490713],	Subsurface water clone MS149BH1062003317 [DQ354749]	93	3	
	SS_LKC22_UB170 [AM490717]		95		
	SS_LKC22_UA16 [AM490714],	Water upstream clone 191up [AY212644]	98	5	
	SS_LKC22_UA22 [AM490715]		98		
	SS_LKC22_UB14 [AM490716]	Root-tip clone SRRT18 [AB240466]	99	1	
	SS_LKC22_UB196 [AM490698]	Denitrifying industrial activated sludge clone S28 [AF072922]	96	3	
	SS_LKC22_UB54 [AM490752]	Soil clone S137 [DQ083111]	96	1	
	SS_LKC22_UB29 [AM490754]	Grassland soil clone FCPP630 [EF516042]	91	3	
	SS_LKC22_UB106 [AM490695]	Rhizosphere biofilm clone SRRT06 [AB240461]	87	1	

Phylogenetic affiliation [a]	Representative clone sequences [Accession Numbers]	Closest relative of full clone sequences [Accession Numbers]	16S rRNA similarity (%) [b]	No. clones total [c]	Tree figure [d]
Deltaproteobacteria (82-100%) [d]	SS_LKC22_UA7 [AM490740],	Marine sediment bacterium Btol [AF282178]	97	2	
	SS_LKC22_UB90 [AM490653]		95		
	SS_LKC22_UA24 [AM490738],	Mangrove soil delta proteobacterium MSB-4B6 [DQ811805]	92	34	Figure SM 6
	SS_LKC22_UA26 [AM490739]		92		
	SS_LKC22_UA12 [AM490742],	Mangrove soil delta proteobacterium MSB-4G3 [DQ811814]	90	3	
	SS_LKC22_UA32 [AM490682]		87		
	SS_LKC22_UB21[AM490743]	Granular sludge clone R1p32 [AF482435]	93	4	
	SS_LKC22_UB24 [AM490744],	Manzallah lake sediment clone Hados.Sed.Eubac.13 [AB355082]	97	6	
	SS_LKC22_UB88 [AM490746]		97		
	SS_LKC22_UB16 [AM490748]	Activated sludge clone 0112 [AB286342]	97	2	
	SS_LKC22_UB36 [AM490749],	*Chondromyces pediculatus* [AJ233940]	92	3	
	SS_LKC22_UA142 [AM490753]		94		
	SS_LKC22_UB22 [AM490741]	*Desulfomonile limimaris* [AF282177]	96	5	
	SS_LKC22_UB78 [AM490750],	Limestone-corroding biofilm clone WM28 [DQ133916]	93	10	
	SS_LKC22_UB43 [AM490755]		92		
	SS_LKC22_UB49 [AM490751]	Rhizosphere biofilm clone RB515 [AB240382]	97	1	
	SS_LKC22_UB53 [AM490680]	Hypersaline microbial mat clone GN01-0080 [DQ154835]	94	9	
	SS_LKC22_UB248 [AM490756]	*Desulfobacca acetoxidans* [AF002671]	95	2	
Gammaproteobacteria (76-97%) [d]	SS_LKC22_UB13 [AM490761],	Lower Kane Cave *Thiothrix* spp. clone LKC3_22.33 [AY510233]	96	14	Figure SM 7
	SS_LKC22_UB17 [AM490763]		98		
	SS_LKC22_UB15 [AM490762]	Suboxic freshwater-pond sediment clone MVS-58 [DQ676406]	93	6	
	SS_LKC22_UB33 [AM490764],	Drinking water clone DSSD72 [AY328770]	98	3	
	SS_LKC22_UB152 [AM490768]		96		
	SS_LKC22_UB32 [AM490765]	Activated sludge clone Run-S43 [AB247466]	97	8	
	SS_LKC22_UB52 [AM490766]	Rice paddy gamma proteobacterium clone P-41 [AM411939]	98	3	
	SS_LKC22_arch_1 [AM490769]	*Thiothrix* sp. clone KRN-B2 [AB166732]	90	3	
	SS_LKC22_UB111 [AM490767]	Activated sludge clone Run-S43 [AB247470]	89	5	
Epsilonproteobacteria (72-81%) [d]	SS_LKC22_UA48 [AM490758]	Contaminated aquifer clone 661227 [DQ404776]	88	4	Figure SM 8
	SS_LKC22_UA23 [AM490759]	Frasassi cave clone FS0612_B4 [EU101041]	86	3	
	SS_LKC22_UB256 [AM490760]	Lower Kane Cave clone LKC1.57.5 [AY191473]	95	1	
Spirochaetes	SS_LKC22_UA8 [AM490641]	Aerobic activated sludge clone XJ109 [EF648155]	95	1	Figure SM 4
Verromicrobium (79-96%) [d]	SS_LKC22_UA28 [AM490654]	Bulk soil clone BS108 [AB240261]	91	3	Figure SM 4
	SS_LKC22_UA55 [AM490655]	Lake Waiau sediment clone W4-B19 [AY345504]	93	1	
	SS_LKC22_UB23 [AM490656]	Soil clone F4-04 [EU025069]	87	3	
	SS_LKC22_UB199 [AM490657]	Soil clone BacA_096 [EU335294]	94	1	
	SS_LKC22_UA17 [AM490687]	Iron-oxidation biofilm clone 82 [AB252962]	75	1	
	SS_LKC22_UB7 [AM490689]	South Atlantic sediment clone SC3-21 [DQ289929]	92	1	
	SS_LKC22_UA60 [AM490691]	Anaerobic bioreactor sludge clone 30f03 [EF515591]	92	1	
	SS_LKC22_UB37 [AM490692],	Hypersaline microbial mat clone MAT-CR-M6-F09 [EU245808]	86	2	
	SS_LKC22_UB116 [AM490696]		85		
	SS_LKC22_UB247 [AM490658]	Frasassi cave clone Ika36 [EF467542]	98	2	
	SS_LKC22_UB73 [AM490659]	Pearl River Estuary sediment clone MidBa45 [EF999382]	87	3	

Phylogenetic affiliation [a]	Representative clone sequences [Accession Numbers]	Closest relative of full clone sequences [Accession Numbers]	16S rRNA similarity (%) [b]	No. clones total [c]	Tree figure [d]
Unclassified Candidate Divisions (70-99%) [d]	SS_LKC22_UA1 [AM490684], SS_LKC22_UA21 [AM490685]	Hypersaline microbial mat clone MAT-CR-P4-B02 [EU246135]	89 98	5	Figure SM9
	SS_LKC22_UA36 [AM490757]	Soil, rice microcosm clone PBS-III-29 [AJ390457]	84	3	
	SS_LKC22_UB156 [AM490660], SS_LKC22_UB158 [AM490661]	Lake profundal sediment clone c5LKS30 [AM086121]	93 94	2	
	SS_LKC22_UA37 [AM490688]	Hypersaline microbial mat CD OD1 clone 072DZ74 [DQ330702]	94	1	
	SS_LKC22_UA49 [AM490690], SS_LKC22_UB217 [AM490697]	4-Methylbenzoate-degrading clone UASB_TL26 [AF254404]	98 99	2	
	SS_LKC22_UB28 [AM490704]	Guerrero Negro, microbial mat clone 09DZZ45 [DQ329569]	92	2	
	SS_LKC22_UB102 [AM490693]	Candidate division OP10 bacterium [EU266864]	98	2	
	SS_LKC22_UB175 [AM490694]	Contaminated sediment clone 655957 [DQ404829]	94	1	
Archaea (100%) [e]	SS_LKC22_arch_2 [AM490770], SS_LKC22_arch_15 [AM490771]	Freshwater sediment euryarchaeote clone CH1_S2_18 [AY822003]	93 94	2 [f]	Figure SM 10
Amount of clones:	138			346	

[a] Affiliations of full 16S rRNA gene sequences of retrieved clones based on taxonomic classifications from BLAST, RDP and SILVA searches.

[b] 16S rRNA gene similiartiy between full 16S rRNA genes and closest relative (column 3) calculated with the ARB-software.

[c] Full and partial 16S rRNA gene sequences retrieved in this study. The affiliation of partial clone sequences were checked manually by addition of the partial 16S rRNA gene sequences to phylogenetic trees containing full 16S rRNA gene sequences calculated by the ARB software.

[d] 16S rRNA gene consensus trees showing all full and partial sequences of all clones retrieved in this study can be downloaded from http://geol.lsu.edu/aengel/publications.htm.

[e] 16S rRNA gene similarity between retrieved clones

[f] The number of clones listed here show the actual amount of true archaeal clones retrieved with all three primer pairs tested. The amount of clones retrieved with the archaeal-specific primers was higher (41 clones), however, the majority of the clones proved to be non-archaeal.

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Supplementary data for **Linking Phylogenetic and Functional Diversity to Nutrient Spiraling in Microbial Mats from Lower Kane Cave (USA)**

Annette Summers Engel, Daniela B. Meisinger, Megan L. Porter, Michael Schmid, Libby A. Stern, K. H. Schleifer, Natuschka M. Lee

Figure SM 1. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to the *Acidobacteria* and *Bacteroidetes/Chlorobi* 16S rRNA gene consensus tree was based on three different tree-ing procedures (neighbor joining hood, parsimony and rax maximum likelihood). All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Sequences retrieved in former Lower Kane Cave studies are depicted in bold/pink. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).

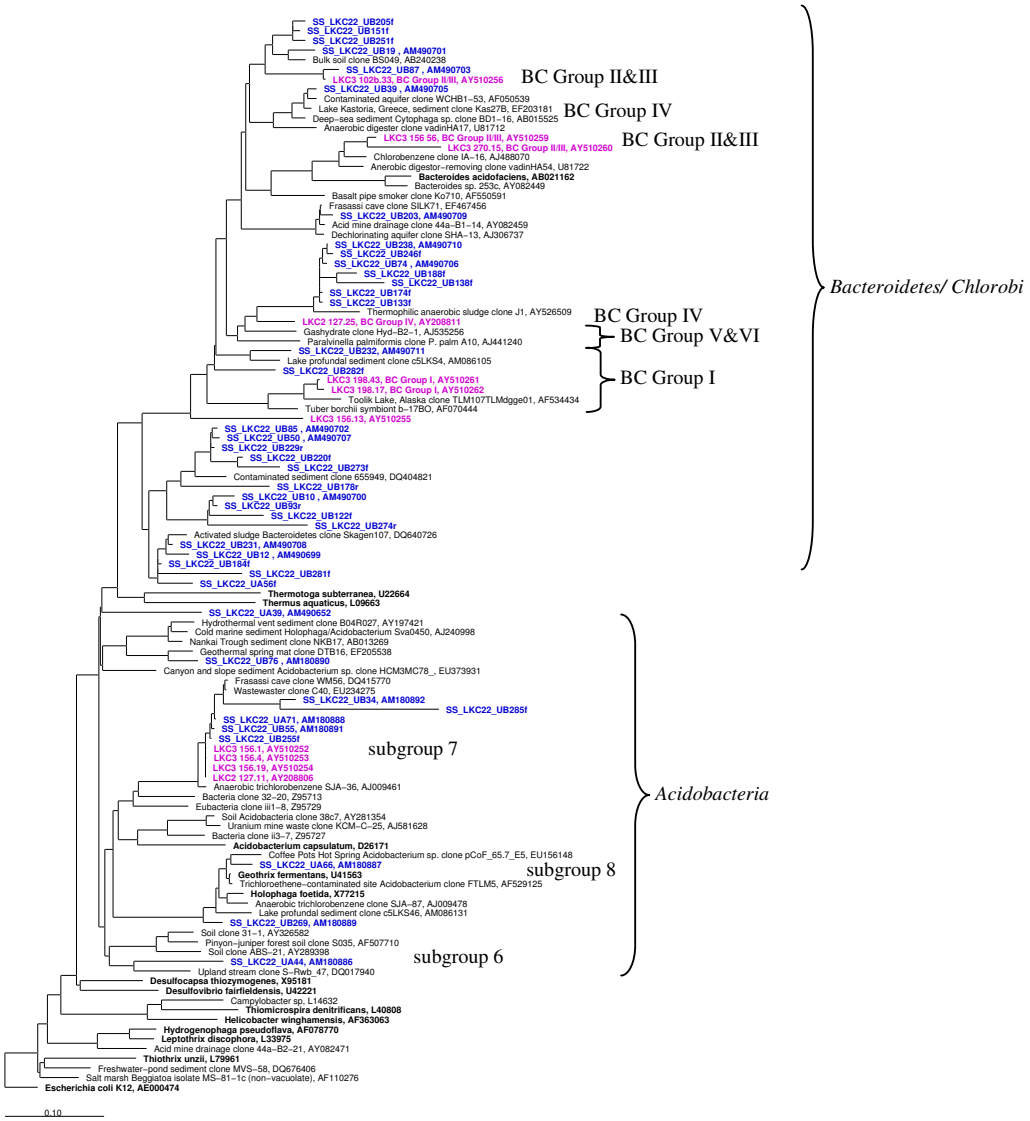


Figure SM 2. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to the *Actinobacteria, Firmicutes* and *Nitrospirae*. All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).

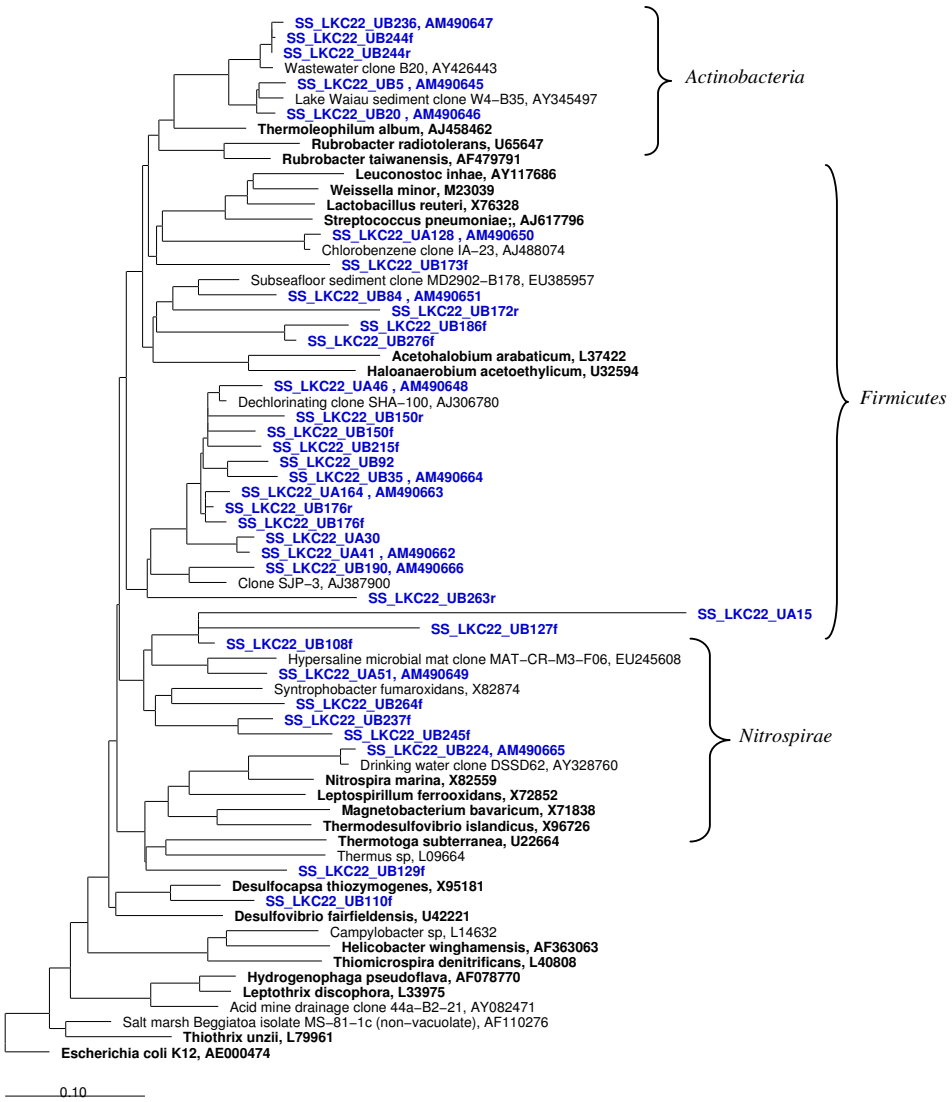


Figure SM 3. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to the *Chloroflexi*. 16S rRNA gene consensus tree was based on three different tree-ing procedures (neighbor joining hood, parsimony and rax maximum likelihood). All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).

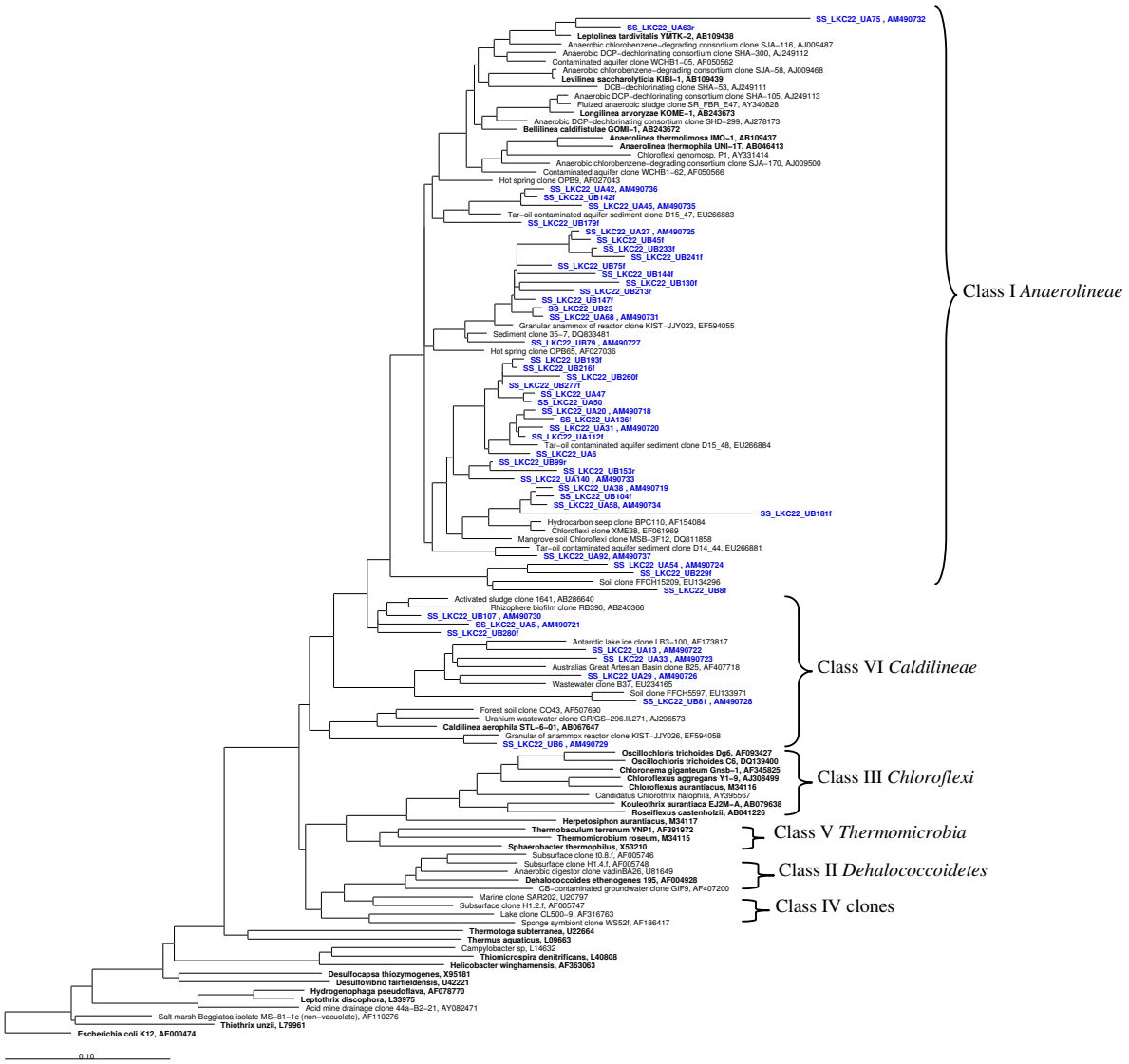


Figure SM 4. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to the *Fibrobacter, Planctomycelates, Spirochatales,* and *Verrucomicrobiales*. The consensus tree is based on three different tree-ing procedures (neighbor joining hood, parsimony and rax maximum likelihood). All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).

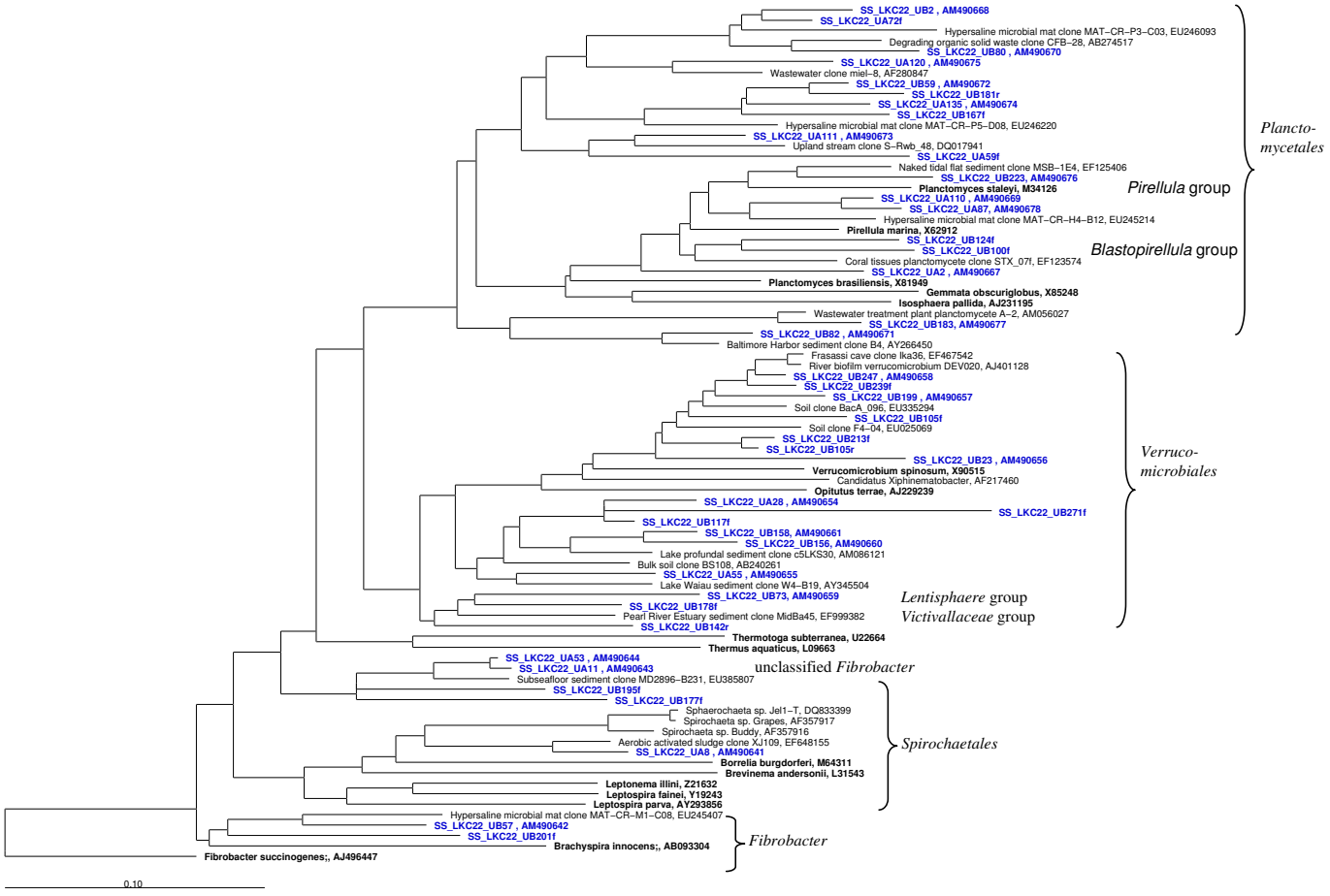


Figure SM 5. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to the *Alphaproteobacteria* and *Betaproteobacteria*. 16S rRNA gene consensus tree was based on three different tree-ing procedures (neighbor joining hood, parsimony and rax maximum likelihood). All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Sequences retrieved in former Lower Kane Cave studies are depicted in bold/pink. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).

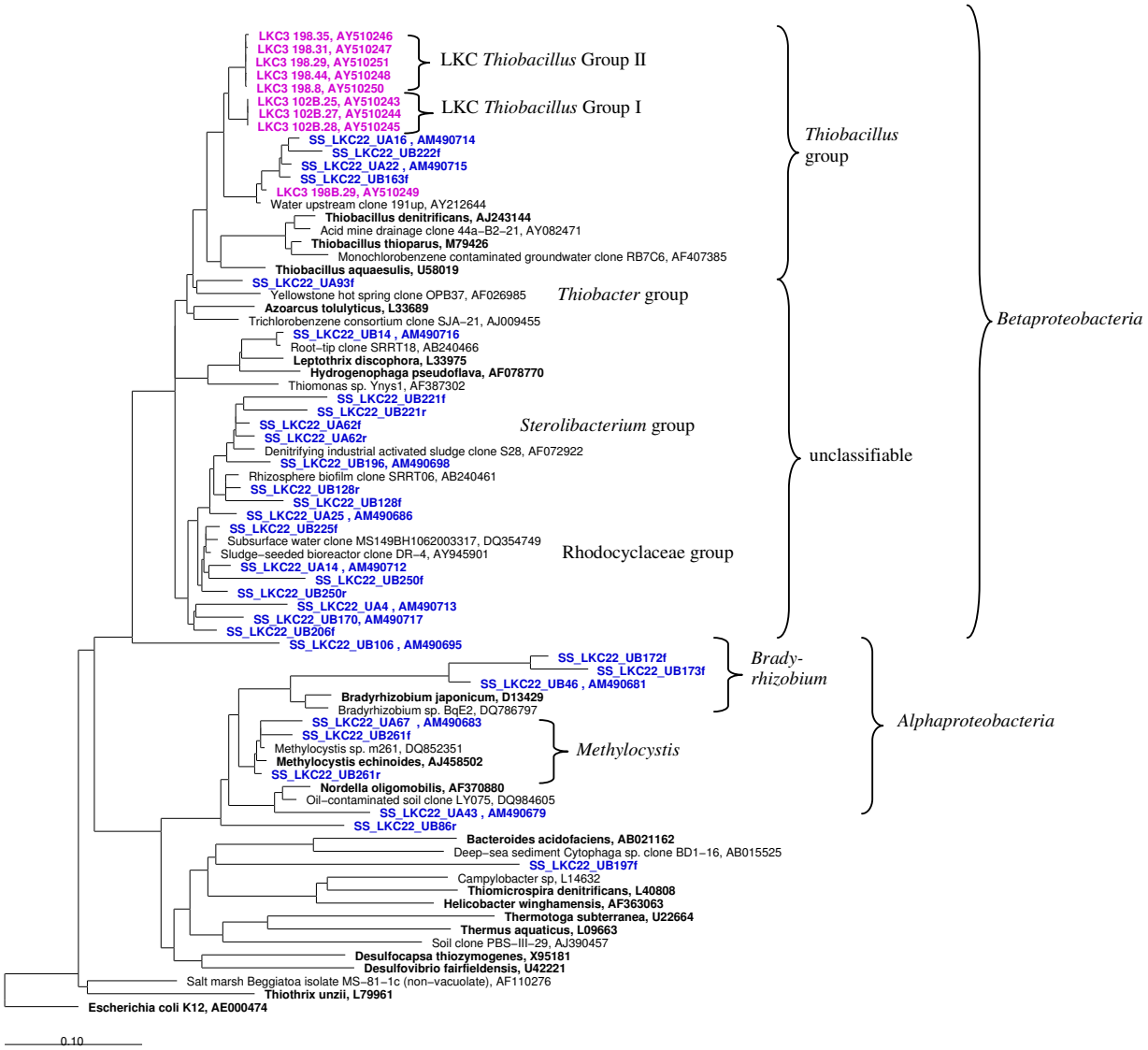
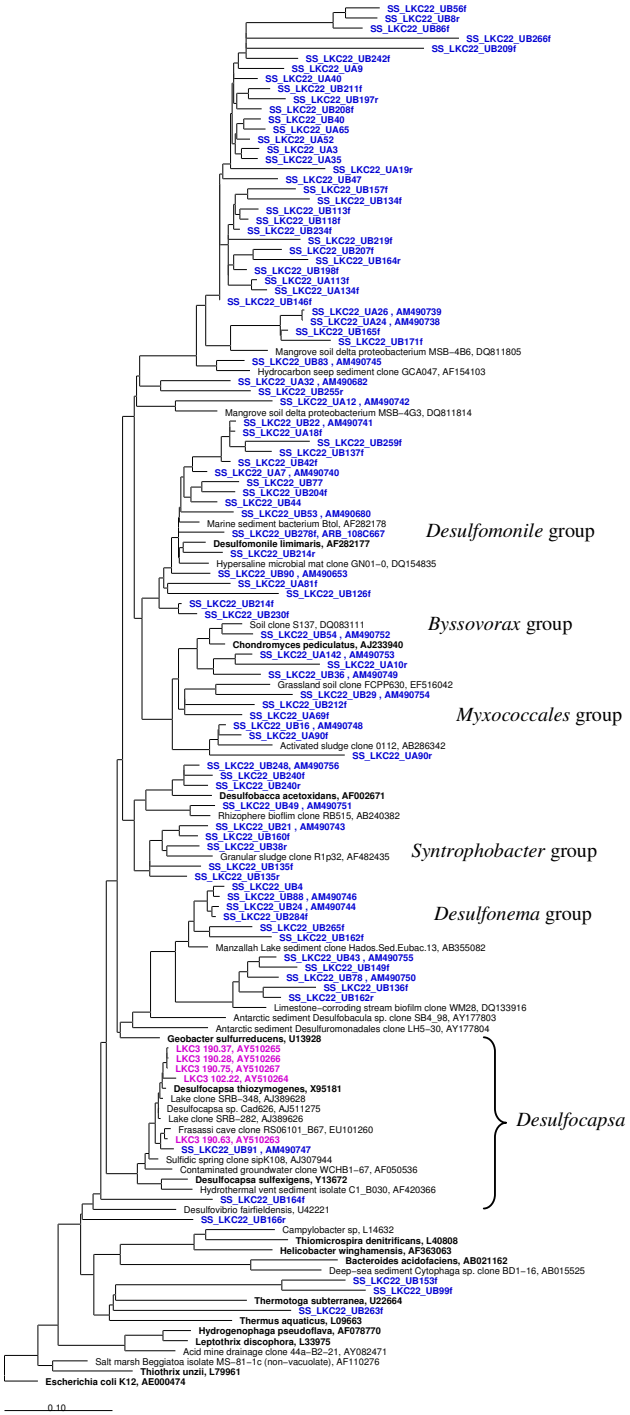


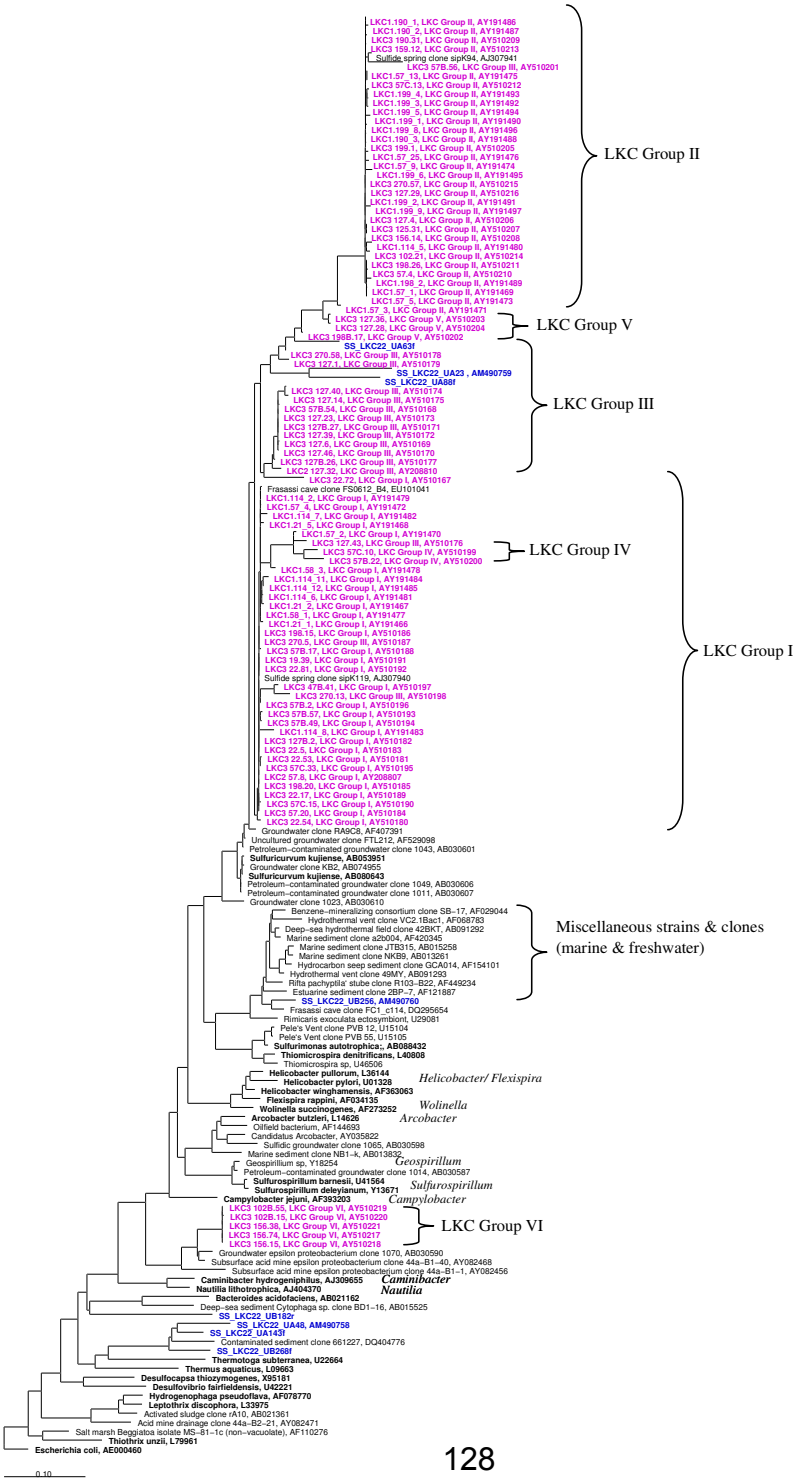
Figure SM 6. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to the *Deltaproteobacteria.* 16S rRNA gene consensus tree was based on three different tree-ing procedures (neighbor joining hood, parsimony and rax maximum likelihood). All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Sequences retrieved in former Lower Kane Cave studies are depicted in bold/pink. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).



Supplementary data for Linking Phylogenetic and Functional Diversity to Nutrient Spiraling in Microbial Mats from Lower Kane Cave (USA)

Figure SM 7. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to the *Gammaproteobacteria.* The consensus tree is based on three different tree-ing procedures (neighbor joining hood, parsimony and rax maximum likelihood). All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Sequences retrieved in former Lower Kane Cave studies are depicted in bold/pink. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).

Figure SM 8. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to the *Epsilonproteobacteria.* The consensus tree is based on three different tree-ing procedures (neighbor joining hood, parsimony and rax maximum likelihood). All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Sequences retrieved in former Lower Kane Cave studies are depicted in bold/pink. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).



Supplementary data for Linking Phylogenetic and Functional Diversity to Nutrient Spiraling in Microbial Mats from Lower Kane Cave (USA)

Figure SM 9. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to unclassifiable candidate divisions 16S rRNA gene consensus tree was based on three different tree-ing procedures (neighbor joining hood, parsimony and rax maximum likelihood). All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).

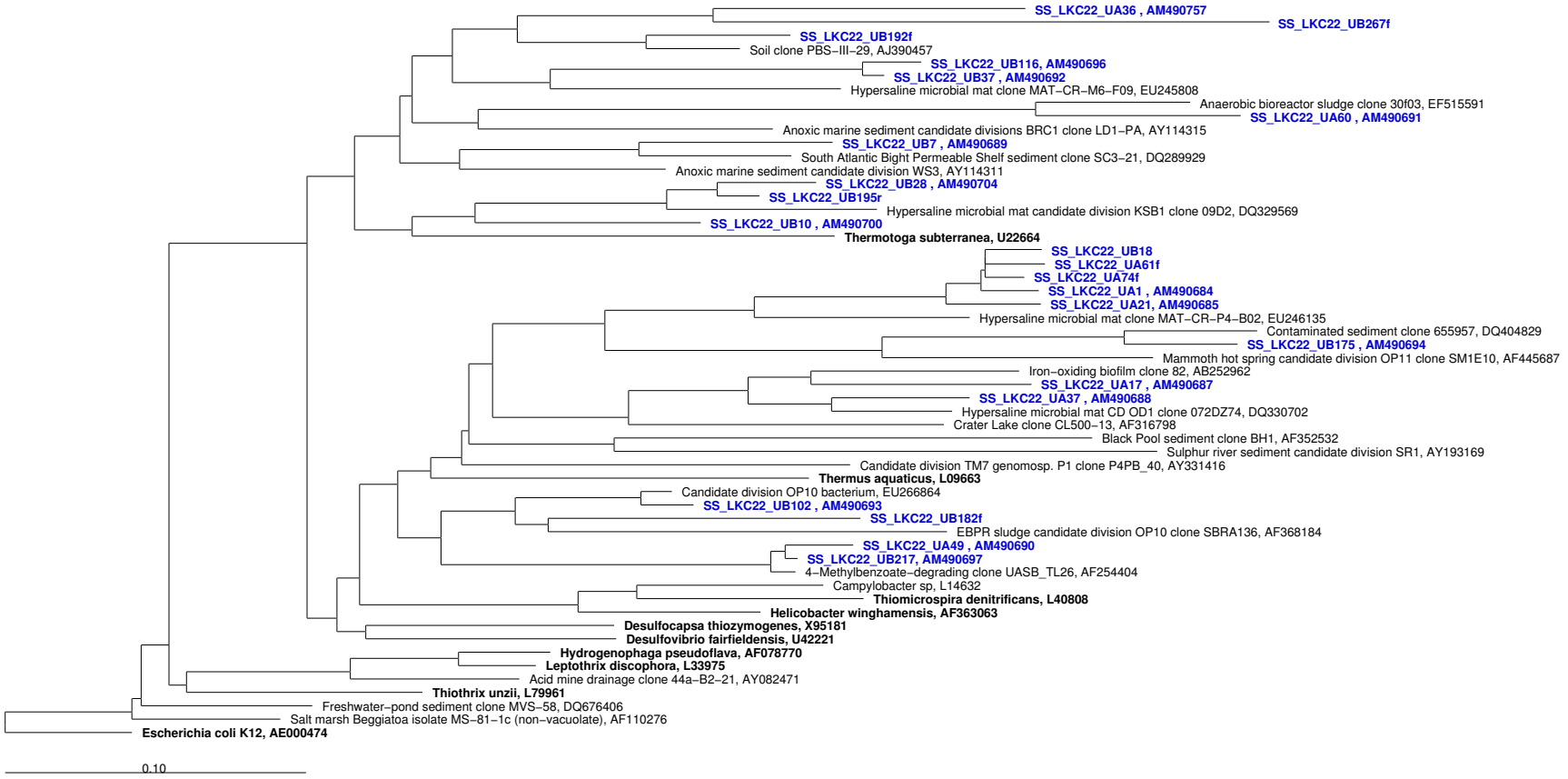
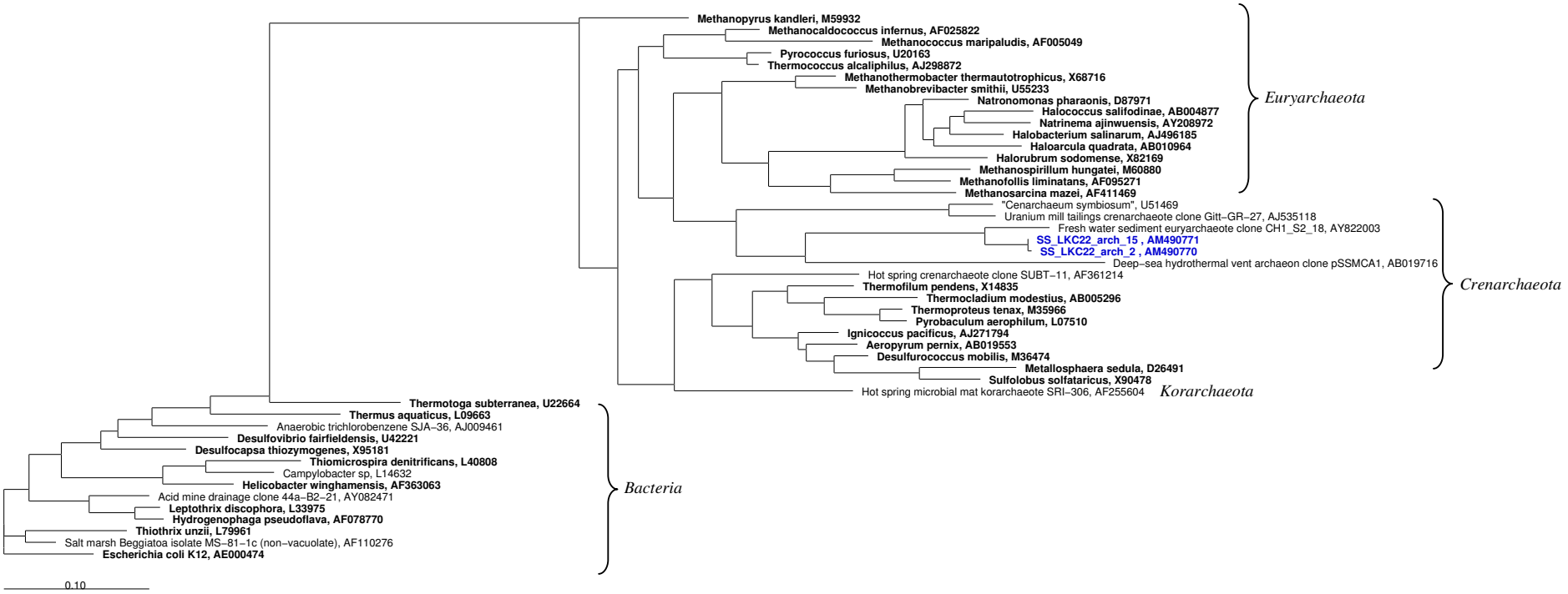


Figure SM 10. 16S rRNA gene consensus tree of clones retrieved from anaerobic microbial mats in Lower Kane Cave affiliated to the *Archaea*. 16S rRNA gene consensus tree was based on three different tree-ing procedures (neighbor joining hood, parsimony and rax maximum likelihood). All calculations are based on full 16S rRNA gene sequences ≥ 1 kb long. Partial sequences (≥ 200 kb) were added afterwards to the trees. Sequences retrieved in this study are depicted in bold/blue. Published full sequences of clones are listed with both clone name and accession number. Partial sequences are listed only with clone name, and contains the epithet f (forward) or r (reverse).



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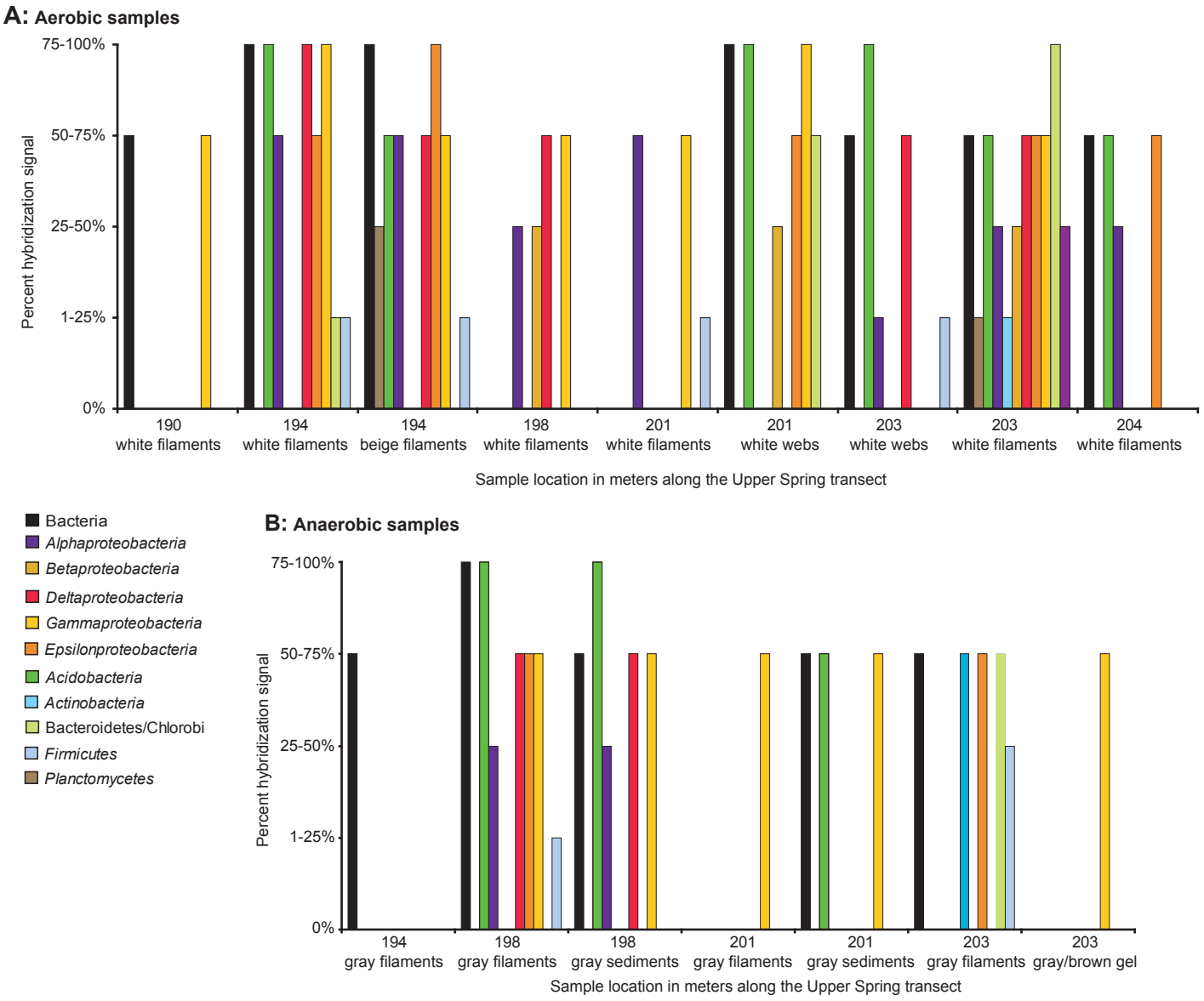

Supplemental Figure SM 11: Percentages of positive hybridization signals from microbial mats, along Upper Spring stream transect that were distinguished as aerobic (A) or anaerobic (B) mat types from geochemistry. FISH signals were obtained by using various oligonucleotide probes, as listed on Supplemental Table SM 1.

Anhang C

In situ detection of novel *Acidobacteria* in microbial mats from a chemolithoautotrophically based cave ecosystem (Lower Kane Cave, WY, USA)

Daniela B. Meisinger[*], Johannes Zimmermann[*], Wolfgang Ludwig, Karl-Heinz Schleifer, Gerhard Wanner, Michael Schmid, Philip C. Bennett, Annette S. Engel, Natuschka M. Lee

[*]DM and JZ equal contribution

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Phylogenetische Analyse (16S-rRNS): DM, NL, WL
FISH-Analyse mit 23S-rRNS Polynukleotidsonden: JZ
FISH-Analyse mit 16S- und 23S-rRNS Oligonukleotidsonden: DM, NL
Physikalisch-chemische Analyse: AE
Text: AE, NL mit unterstützender Hilfe der Co-Autoren

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In situ detection of novel *Acidobacteria* in microbial mats from a chemolithoautotrophically based cave ecosystem (Lower Kane Cave, WY, USA)

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Summary

Lower Kane Cave, Wyoming (USA), has hydrogen sulfide-bearing springs that discharge into the cave passage. The springs and cave stream harbour white filamentous microbial mats dominated by *Epsilonproteobacteria*. Recently, novel 16S rRNA gene sequences from the phylum *Acidobacteria*, subgroup 7, were found in these cave mats. Although *Acidobacteria* are ubiquitously distributed in many terrestrial and marine habitats, little is known about their ecophysiology. To investigate this group in Lower Kane Cave in more detail, a full-cycle rRNA approach was applied based on 16S and 23S rRNA gene clone libraries and the application of novel probes for fluorescence *in situ* hybridization. The 16S and 23S rRNA gene clone libraries yielded seven and six novel acidobacterial operational taxonomic units (OTUs) respectively. The majority of the OTUs were affiliated with subgroups 7 and 8. One OTU was affiliated with subgroup 6, and one OTU could not be assigned to any of the present acidobacterial subgroups. Fluorescence *in situ* hybridization distinguished two morphologically distinct, rod-shaped cells of the acidobacterial subgroups 7 and 8. Although the ecophysiology of *Acidobacteria* from Lower Kane Cave will not be fully resolved until cultures are obtained, acidobacterial cells were always associated with the potentially chemolithoautotrophic epsilon- or gammaproteobacterial filaments, suggesting perhaps a lifestyle based on heterotrophy or chemoorganotrophy.

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Introduction

The *Acidobacteria* were first recognized as a major bacterial phylum-level lineage in 1997 based on 16S rRNA gene sequences retrieved from agricultural soils (Ludwig *et al*., 1997). Subsequent 16S rRNA gene sequence-based phylogenetic analyses have included an increasing number of gene sequences retrieved from various environments, including soil, ocean water, hot springs and acid mine drainage (e.g. Hugenholtz *et al*., 1998a; Barns *et al*., 1999; Sait *et al*., 2002; Baker and Banfield, 2003; Sogin *et al*., 2006). The *Acidobacteria* were previously divided into eight deeply branching subgroups (Hugenholtz *et al*., 1998b), but have recently been expanded to 11 subgroups (Zimmermann *et al*., 2005a). Despite the widespread distribution of *Acidobacteria*, only three validly described species with apparent metabolic versatility have so far been described: *Acidobacterium capsulatum* (subgroup 1), a chemoorganotroph isolated from an acidic habitat (Kishimoto *et al*., 1991); *Holophaga foetida* (subgroup 8), a homoacetogenic, methoxylated aromatic compound degrader (Liesack *et al*., 1994); and *Geothrix fermentans* (subgroup 8), an iron-reducing chemoorganotroph (Coates *et al*., 1999). Although recent culture-based studies yielded the first culturable representatives for subgroups 2, 3 and 4 (Janssen *et al*., 2002; Sait *et al*., 2002; 2006; Joseph *et al*., 2003; Stevenson *et al*., 2004), the vast majority of *Acidobacteria* for which 16S rRNA gene sequences have been recovered, still remain uncultured, and their role in the environment is thus poorly understood.

Numerous acidobacterial 16S rRNA gene sequences have also been detected in different active and ancient

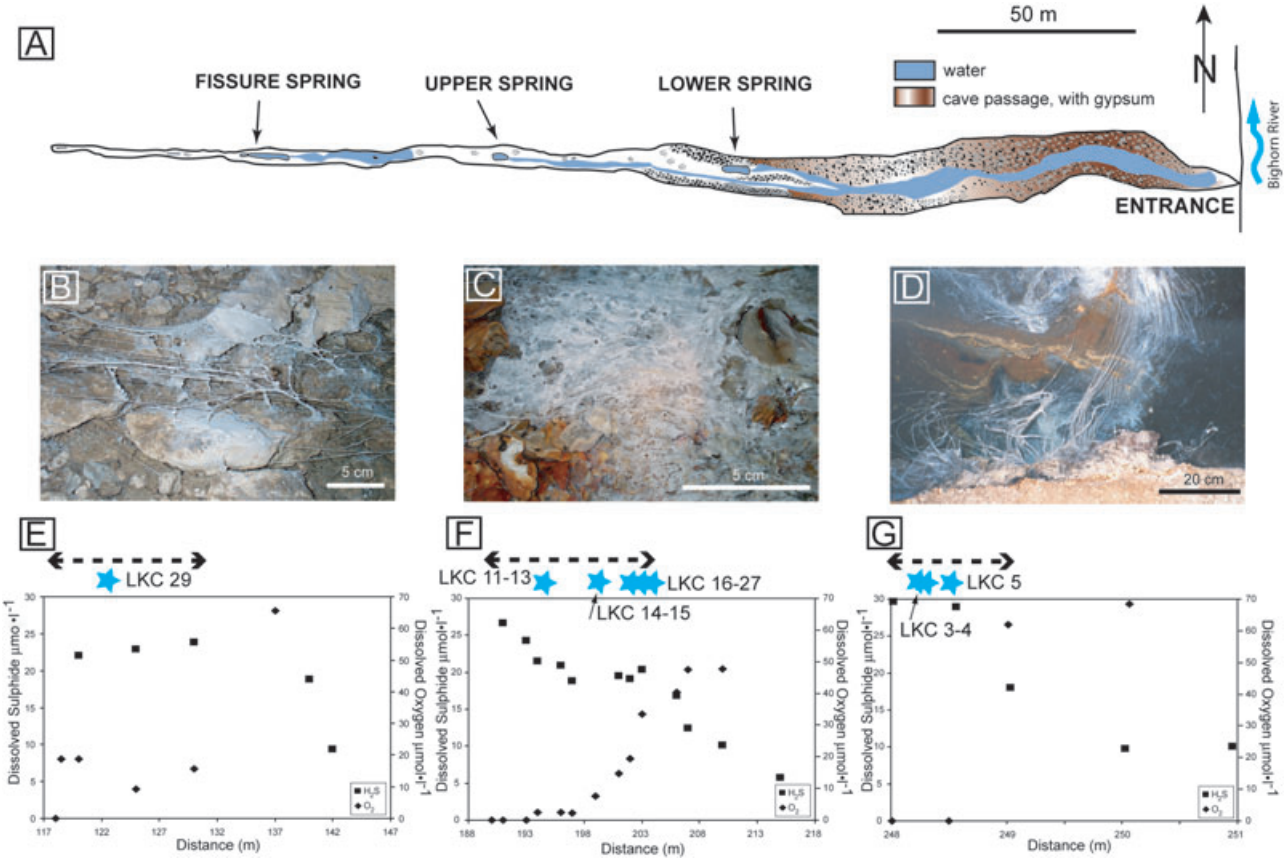

Fig. 1. Summary of physicochemical information for Lower Kane Cave, Wyoming.
A. Generalized plan-view map showing location of three main sulfidic springs, modified from Engel and colleagues (2004a) and the sample designations investigated in this study.
B. White filament bundles in Fissure Spring orifice, 118 m.
C. Microbial mat, ~5 cm thick, from Upper Spring stream channel, 201 m.
D. Microbial filaments in Lower Spring orifice, 248 m.
E–G. Distances at each corresponding spring reach versus dissolved sulfide (left *y*-axes) and dissolve oxygen (right *y*-axes) concentrations. Stars correspond to location of microbial mat sample used in this study. Dotted lines correspond to length of microbial mats at each spring.

cave systems all over the world, including the Nullarbor Caves in Australia (Holmes *et al*., 2001), the Altamira, Tito Bustillo, La Garma and Llonín caves in Spain (Schabereiter-Gurtner *et al*., 2002a,b; 2003; 2004; Zimmermann *et al*., 2005a), the Movile Cave in Romania (Hutchens *et al*., 2004), the Wind and Lower Kane Cave in the USA (Chelius and Moore, 2004; Engel *et al*., 2004a) and other subterranean systems such as catacombs (Zimmermann *et al*., 2005b). Although substantially more research has been done on the diversity and abundance of *Acidobacteria* in subaerial cave-wall settings, the ecological role of *Acidobacteria* in subsurface habitats remains unclear. Due to the unique geochemistry of Lower Kane Cave, it is an excellent environment to investigate potentially novel *Acidobacteria* that inhabit subterranean ecosystems.

Lower Kane Cave, located in the northern Bighorn Basin of Wyoming, has three main thermal (~21°C), hydrogen sulfide-bearing springs that discharge into the cave (Fig. 1A) (Engel *et al*., 2004a). White filamentous microbial mats have formed in the sulfidic water emanating from each of the spring orifices (Fig. 1B–D). We sampled the mats with a range of geochemical conditions, dissolved sulfide concentration, and dissolved oxygen tension (Fig. 1E–G). The microbial mats are dominated by filamentous bacteria belonging predominately to distinct phylogenetic groups of uncultivated *Epsilonproteobacteria* (Engel *et al*., 2003; 2004a), but recently, other prokaryotic groups such as *Bacteroidetes/Chlorobi*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria* and some acidobacterial 16S rRNA genes affiliated to the subgroup 7 have been retrieved (Engel *et al*., 2004a). Here we describe a full-cycle rRNA approach to retrieve both 16S rRNA and 23S rRNA gene sequence information to design specific fluorescence *in situ* hybridization (FISH) probes, according to a multiple probe concept (e.g. Behr *et al*., 2000). The applications of novel 16S and 23S rRNA-targeted oligonucleotide probes, as

well as polynucleotide probes, were successfully applied for the *in situ* detection of acidobacterial target cells from Lower Kane Cave microbial mat samples. Ecophysiological information for the *Acidobacteria* was evaluated by comparing the associations with other microbial groups, habitat geochemistry and mat elemental content.

Results and discussion

Acidobacterial 16S rRNA phylogeny and clonal distribution

The screening of ~800 clones from four 16S rRNA clone libraries using universal prokaryotic PCR primers yielded eight almost complete acidobacterial 16S rRNA gene sequences (> 1500 nucleotides), representing seven distinct acidobacterial operational taxonomic units (OTUs) (at > 99.5% sequence similarity). Most of these OTUs could be assigned to the subgroups 7 (clone designations: SS_LKC22_UA71; SS_LKC22_UB34; SS_LKC22_UB55) and 8 (clone designations: SS_LKC22_UA66; SS_LKC22_UB269; SS_LKC29_UB26). The acidobacterial subgroup 7 16S rRNA gene sequences shared 92%–98% identity to the environmental clone sequence SJA-36 (AJ009461) recovered from an anaerobic, trichlorobenzene transforming microbial consortium in a fluidized bed reactor (von Wintzingerode *et al*., 1999). Three of the clones (SS_LKC22_UA66; SS_LKC22_UB269; SS_LKC29_UB26) grouped with acidobacterial subgroup 8 and shared 91%–94% identity to *G. fermentans*.

The OTU represented by clone SS_LKC22_UA44 was found within the radiation of subgroup 6. In addition to clone sequences retrieved from soil (Schabereiter-Gurtner *et al*., 2002a; 2004; Chelius and Moore, 2004), subgroup 6 also comprises a number of OTUs recovered from the subaerial, non-sulfidic Altamira Cave, Spain (Zimmermann *et al*., 2005a). However, overall 16S rRNA gene sequence similarities of 82.2%–82.6% indicate only a moderate relationship between the *Acidobacteria* in both cave systems. Clone SS_LKC22_UB76 shared 83.5% identity to *G. fermentans*, and was most closely related to an uncultured bacterium retrieved from a deep marine sediment (AB013269, 89% identity). Together with sequences retrieved from other marine sediment samples (Ravenschlag *et al*., 1999; Dhillon *et al*., 2003; Knittel *et al*., 2003), this clone represents either a distantly related branch to acidobacterial subgroup 10 (Zimmermann *et al*., 2005a) or a new subgroup (Fig. 2).

Acidobacterial 23S rRNA phylogeny and clonal distribution

The screening of ~50 clones from four 23S rRNA gene clone libraries based on PCR primers targeting conserved regions of *Acidobacteria* yielded 22 partial 23S rRNA gene sequences, each comprising > 1800 nucleotides. The 22 sequences could be divided into six OTUs, all of which belonged to subgroups 7 (LS_LKC21_kan1) and 8 (LS_LKC11_kan2–4, 6; LS_LKC21_kan5) (Fig. 3). Because the subgroup definition by Hugenholtz and colleagues (1998a) and Zimmermann and colleagues (2005a) is based mainly on 16S rRNA sequence data, the expansion of the subgroup assignment to the 23S rRNA gene data obtained in this study was achieved by determining both 16S and 23S rRNA sequences from the same organism or clone. For subgroup 8, the respective 23S rRNA gene sequences from cultured *H. foetida* and *G. fermentans* could be used for subgroup assignment. For a better assignment of the OTUs assumed to be affiliated to subgroup 7, the clones containing partial 16S and 23S rRNA gene sequences were generated by PCR using the universal eubacterial 16S rDNA-targeted primer 27f in combination with the 23S rDNA-targeted primer ALP544r targeting exclusively OTU LS_LKC_21_kan1 (Table 1). Sequence comparison revealed that LS_LKC21_kan1 and other 16S rRNA-based clones affiliated to subgroup 7, such as SS_LKC_22_UB55, represented the same OTU. The 23S rRNA gene sequence of OTU LS_LKC21_kan1 from subgroup 7 indicate a close relationship to novel OTUs designated as 'Doñana cluster' retrieved from dune ponds E-W at Doñana Biological Reserve in South-west Spain (J. Gonzalez, pers. comm.). The LS_LKC21_kan1 23S rRNA gene sequence shares 98.3%, 93.4% and 94.5% identity with the homologous sequences of the Doñana OTUs DON29, DON30 and DON31 respectively.

The novel OTUs from subgroup 8, with 23S rRNA gene sequence similarities between 92.9% and 97.2%, are the first representatives found in a subsurface cave habitat. All of the subgroup 8 OTUs 23S rRNA gene similarities to the cultured members, *G. fermentans* and *H. foetida*, ranged from 92.9% to 97.2% and 93.4% to 94.5% respectively. The LS_LKC11_kan2 sequence retrieved from Upper Spring sample LKC11 has 97.2% sequence similarity to *G. fermentans*, which, at the 16S rRNA gene level, would implicate a species–level relationship (Rosselló-Mora and Amann, 2001). However, given the greater variability of the 23S rRNA gene sequence, this level of nucleotide similarity can be regarded as evidence for a higher degree of genetic relatedness (de Rijk *et al*., 1995).

The 23S rRNA approach did not retrieve sequences belonging to acidobacterial subgroup 6 and from the phylogenetic vicinity of sequence SS_LKC22_UB76, but this may simply be attributed to the 15-fold lower number of clones screened in the 23S rRNA gene libraries.

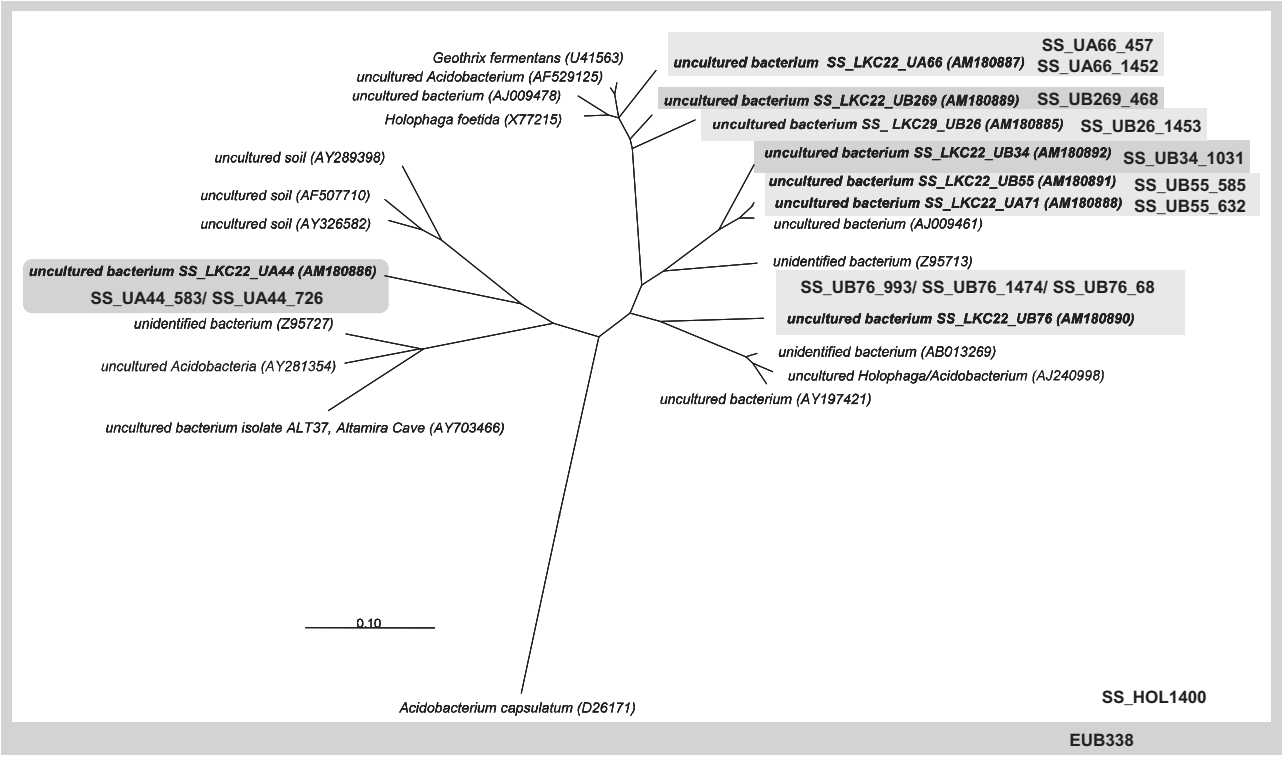

Fig. 2. Consensus tree based on a maximum parsimony analysis of 60 000 almost complete SSU rRNA sequences. Homologous primary structure positions were included at which at least 50% of the acidobacterial sequences share the same nucleotide. The topology was evaluated applying alternative treeing methods (neighbour-joining and maximum likelihood) on subsets of the data. Multifurcations indicate that a common branching order was not significantly supported by the various analyses. The shaded areas indicate the specificities of FISH 16S rRNA-targeted oligonucleotide probes designed in this study (see Table 1). Sequences AY289398, AF507710, AY326582 and SS_LKC29_UB26 have no perfect match with the probe sequence SS_HOL1400.

Fluorescence in situ *hybridization with acidobacterial 16S and 23S rRNA-targeted oligonucleotide probes*

Microbial mat samples were screened for *Acidobacteria* using FISH with 21 novel 16S and 23S rRNA-targeted oligonucleotide probes specific for the phylum *Acidobacteria* or the different clone sequences retrieved from the cave habitat (Table 1). A mixture of phylum-targeted probes LS_HOL189, SS_HOL1400, and the bacterial probe EUB338 mix (each probe labelled with a different fluorochrome) were applied to 23 mat samples for a general screening of the presence of *Acidobacteria*; however, three mat samples (LKC14, 16 and 22) were unsuitable for FISH due to strong background fluorescence caused by the high abundance of solid, mineralized material surrounding the cellular biomass. The specificity of the novel probes was evaluated using aliquots from six white filament samples (LKC11, 13, 15, 20, 21 and 29) because the biomass had low background fluorescence and contained sufficient acidobacterial cells. The specificity of the probe signals from the acidobacterial targeting probes was confirmed by the observation that fluorescent signals from probe EUB mix and the probes targeting the 16S rRNA as well as the 23S rRNA gene of the phylum *Acidobacteria* consistently overlapped with each other from 0% to 20% formamide concentration in the hybridization buffer (no signals could be observed at higher formamide concentrations). A similar hybridization pattern was observed with the pure culture strain of *Acidobacterium capsulatum* (strain DSMZ 11244).

Cells identified by probes SS_HOL1400 and LS_HOL189 were thin, and sometimes curved, rods that could be classified into two morphological groups; morphotype group A and B. Morphotype A was ~8 μm in length and had a diameter of ~0.3 μm (Fig. 4A). Morphotype B had a similar diameter, with a length of ~2.5 μm (Fig. 4B). Both morphotypes occurred in clusters of single cells, and cells did not form chains, as did representatives of acidobacterial subgroup 3, identified by the same probes in activated sludge (Zimmermann, 2002). Further identification of morphotypes A and B was done with five novel 23S rRNA and 10 novel 16S rRNA oligonucleotide probes targeting the acidobacterial different OTUs (Table 1). Morphotype A rods could be detected using the 23S rRNA-targeted probes LS_ALP544 and LS_ALP761,

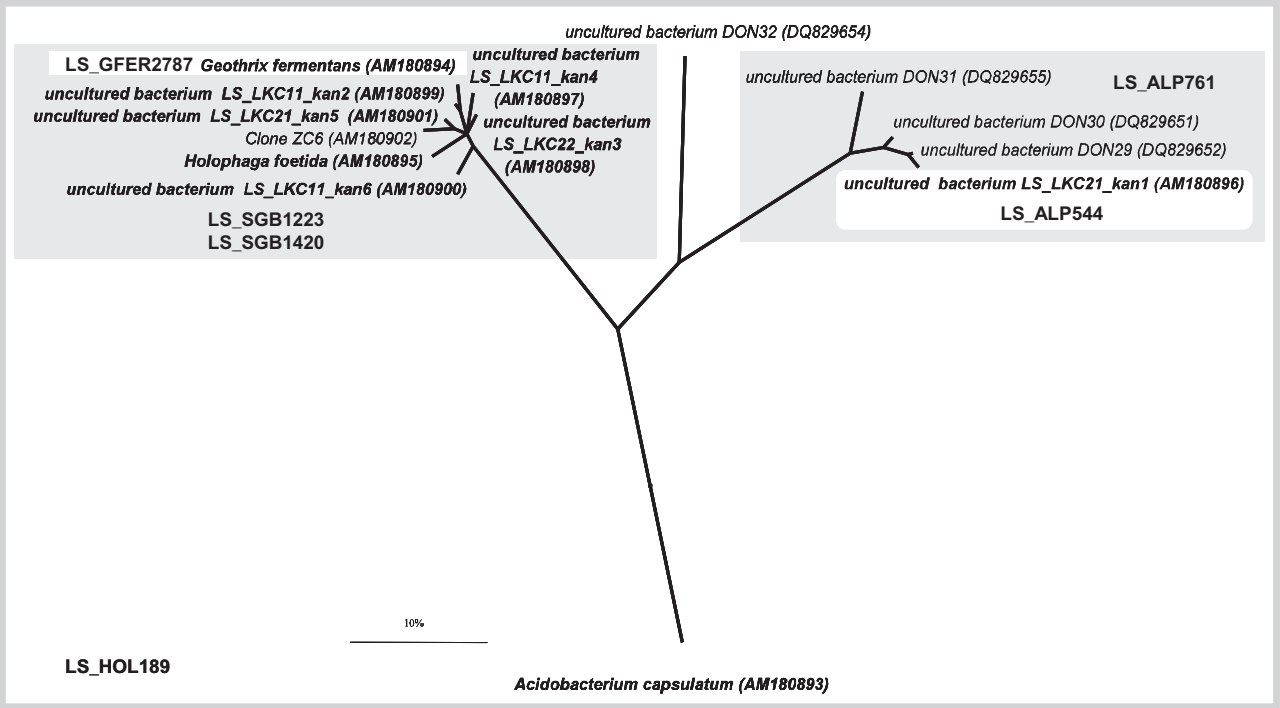

Fig. 3. Consensus tree based on a maximum parsimony analysis of 5000 almost complete LSU rRNA sequences. Homologous primary structure positions were included at which at least 50% of the acidobacterial sequences share the same nucleotide. The topology was evaluated applying alternative treeing methods (neighbour-joining and maximum likelihood) on subsets of the data. Multifurcations indicate that a common branching order was not significantly supported by the various analyses. The shaded areas indicate the specificities of FISH 16S rRNA-targeted oligonucleotide probes designed in this study (see Table 1).

as well as the 16S rRNA-targeted probe SS_subd7_UB55 specific for subgroup 7 clone sequences (Table 1).

Morphotype B cells could be detected using the 23S rRNA-targeted probes LS_SGB1223, LS_SGB1420. Three 16S rRNA-targeted probes, SS_subd8_UA66, SS_subd8_UB26 and SS_subd8_UB76, specific for subgroup 8 clone gene sequences, were also applied; however, these probes always produced questionable hybridization signals in comparison with the signals observed with the phylum targeting 16S and 23S rRNA probes as well as the 23S rRNA targeting probes against subgroup 8 (results not shown). These results suggest that the 23S rRNA gene provided better probe target sites than the 16S rRNA gene of these organisms.

Because some of the clones in subgroup 8 shared up to 97% gene sequence identity with *G. fermentans*, further identification of morphotype B cells was performed by a multiple probe set targeting exclusively organisms from the close phylogenetic vicinity of *G. fermentans* (i.e. probe LS_GFER2787) in combination with the different probes targeting the subgroup 8 clones mentioned above. Probe LS_GFER2787 detected significantly fewer morphotype B rods than the probe hybridizing the clones affiliated to subgroup 8, but the LS_GFER2787-targeted cells did exhibit a morphology closely resembling *G. fermentans* cells (Coates *et al*., 1999). Therefore, the novel acidobacterial OTUs, and not *G. fermentans* or other closely related organisms, comprise a significant amount of the acidobacterial communities in Lower Kane Cave.

Detection of cells with the several specific FISH probes failed for subgroup 6 (represented by the clone SS_LKC22_UA44) and the clone SS_LKC22_UB76 which could not be clearly assigned to any of the present subgroups. This may be due to inaccessible regions of the probes applied to target this group, or simply due to too low cell numbers.

No overlapping of hybridization signals was observed when combining 16S or 23S rRNA-targeted probes specific for subgroups 7 and 8. As all hybridization signals obtained with the clone-specific probes always yielded a signal when combined with phylum-specific probes with another fluorochrome, these observations do support the finding that at least two novel acidobacterial subgroups coexist in Lower Kane Cave. However, when we combined the clone-specific 23S rRNA probes with the clone-specific 16S rRNA-targeted probes for each subgroup we did observe some discrepancies. The 23S rRNA-targeted probes often hybridized to more cells than the 16S rRNA-

Table 1. Oligonucleotide probes used in this study.

Probe	Probe sequence (5′–3′)	Target, *E. coli* position[b]	Target group	FA (%)[c]	Reference
LS_HOL189 (L-P-Acido-0189-a-A-18)[a]	CTG AGA TGG TTC ACT TCC	23S rRNA, 189	*Acidobacteria*	20	This study
SS_HOL1400 (S-P-Acido-1400-a-A-17)[a]	TTC GTG ATG TGA CGG GC	16S rRNA, 1400	*Acidobacteria*	20	This study
LS_ALP544 (L-*-kan1–0544-a-A-18)[a]	CCA CCG GAT TGC TCC ATG	23S rRNA, 544	LS_LKC21_kan1	35	This study
LS_ALP761 (L-*-Sub7–0761-a-A-18)[a]	CCC CTA GCC TGA GCT CAT	23S rRNA, 761	Parts of Acidobacterial subgroup 7 (including LS_LKC21_kan1, DON29, DON30, DON31)	35	This study
SS_subd7_UB55–585 (S-*-Sub7–0585-a-A-18)[a]	CTC TGA CGC ACG AAA CCG	16S rRNA, 585	Clone SS_LKC22_UB55, subgroup 7 SS_LKC22_UA71, SS_LKC21_kan1	0–50	This study
SS_subd7_UB55–632 (S-*-Sub7–0632-a-A-18)[a]	TCT CGC CGT ATC AGG GGC	16S rRNA, 632	Clone SS_LKC22_UB55, subgroup 7 SS_LKC22_UA71, SS_LKC21_kan1	0–50	This study
LS_subd8_SGB1223 (L-*-Sub8–1223-a-A-18)[a]	GTC TCC AGC TGT CCT TAC	23S rRNA, 1223	Acidobacterial subgroup 8 LS_LKC11_kan2, LS_LKC22_kan3, LS_LKC11_kan4, LS_LKC21_kan5 (LS_LKC11_kan6), clone ZC6, G. *fermentans*, *H. foetida*	35	This study
LS_subd8_SGB1420 (L-*-Sub8-1420-a-A-18)[a]	CCT TCC TGC GTC CCT GCA	23S rRNA, 1420	Acidobacterial subgroup 8 LS_LKC11_kan2, LS_LKC22_kan3, LS_LKC11_kan4, LS_LKC21_kan5, LS_LKC11_kan6, clone ZC6, *G. fermentans*, *H. foetida*,	35	This study
LS_GFER2787 (L-*-Gfer-2787-a-A-18)[a]	ACC ACG GGT CTC ATA GGG	23S rRNA, 2787	*G. fermentans* and relatives (no LKC clones)	35	This study
LKC1006 (S-*-eProt-1006-a-A-18)[a]	CTC CAA TGT TTC CAT CGG	16S rRNA, 1006	Unculturable epsilonproteobacterial group II from LKC	30	Engel *et al*. (2003)
Gam42a (L-P-Gam-1027-a-A-17)[a]	GCC TTC CCA CAT CGT TT	23S rRNA, 1027	*Betaproteobacteria*	35	Manz *et al*. (1992)
G123T (S-G-Thioth-697-a-A-18)[a]	CCT TCC GAT CTC TAT GCA	16S rRNA, 697	*Thiothrix eikelboomii*, *T. nivea*, *T. unzii*, *T. fructosivorans*, *T. defluvii*, Eikelboom type 021N group I, ii, iii	40	Kanagawa *et al*. (2000)
G123T comp (S-G-Thioth-697-a-A-18)[a]	CCT TCC GAT CTC TAC GCA	16S rRNA, 697	*Thiothrix eikelboomii*, *T. nivea*, *T. unzii*, *T. fructosivorans*, *T. defluvii*, Eikelboom type 021N group I, ii, iii	40	Kanagawa *et al*. (2000)
Bet42a (L-P-Bet-1027-a-A-17)[a]	GCC TTC CCA CTT CGT TT	23S rRNA, 1027	*Betaproteobacteria*	35	Manz *et al*. (1992)
EUB338 (S-D-Bact-0338-a-A-18)[a]	GCT GCC TCC CGT AGG AGT	16S rRNA, 338	Many but not all *Bacteria*	0–60	Amann *et al*. (1990)

More detailed information about the probes, including probe mismatch files, can be found at http://www.microbial-systems-ecology.net/Meisinger_et_al_2007.html

a. Probe designations according to Alm and colleagues (1996) are given in brackets.

b. *E. coli* position according to Brosius and colleagues (1981).

c. Percentage of formamide (FA) in hybridization buffer.

The following oligonucleotide probes were also tested in this study, but produced no or questionable FISH signals.
SS_subd6_UA44–583 (5′-CGA CCT AAA AAG CCG CCT-3′), SS_subd6_UA44–726 (5′-CCG GGA AAA CCG CTT TCG-3′)
SS_subd7_UB34–1031 (5′-ACC TAA AGG ACA GCT CCT-3′), SS_subd8_UA66–457 (5′-ACA GGA CTA TTT ACC CCG-3′)
SS_subd8_UA66–1452 (5′-CAT TGA AAG CTG GCC CCT-3′), SS_subd8_UB76–68 (5′-CGG TTG CCC GCG GCC TCG-3′)
SS_subd8_UB76–993 (5′-CGG CTG ACT CCA GCA GTT-3′), SS_subd8_UB76–1474 (5′-GCC CTT GGT ACC CTT GTT-3′)
SS_subd8_UB26–1453 (5′-CCG TTG GCC GGT ACC TCC-3′) and SS_subdnew_UB269–468 (5′-ACC GTC AAG CAA CTC AGT-3′).

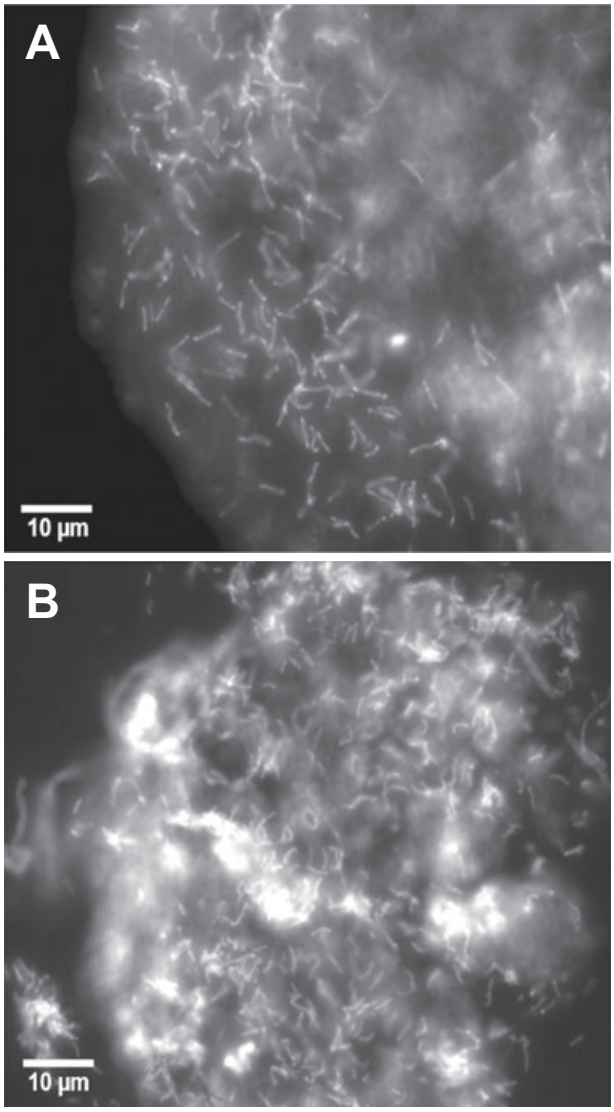

Fig. 4. Colonies of *Acidobacteria* from sample LKC29 (Fissure Spring, Fig. 1) within white microbial mats in Lower Kane Cave.
A. Morphotype A, representing subgroup 7, with Cy3-labelled oligonucleotide probe LS_subd7_ALP761.
B. Morphotype B, representing subgroup 8, with Fluorescein-labelled oligonucleotide probe LS_subd8_SGB1223.

targeted probes or yielded brighter signals (results not shown). Because all 23S rRNA probes targeting the different subgroups were always covered by the phylum-specific probe, this finding could indicate that the acidobacterial diversity in Lower Kane Cave may be even higher than so far discovered.

Fluorescence in situ *hybridization using 23S rRNA-targeted polynucleotide probes*

The assignment of morphotype A cells to subgroup 7 and morphotype B cells to subgroup 8 was further confirmed by FISH using DNA polynucleotide probes directly generated from cloned 23S rRNA gene fragments. This approach allowed the specific *in situ* detection of each 23S rRNA-based OTU, in contrast to the 16S rRNA-targeting probes that targeted all OTUs within a specific subgroup. This approach gave us also additional proof for the existence of the organisms whose sequences were retrieved by analyses of clone libraries – especially in those cases where the clone-specific 16S rRNA probes only yielded questionable FISH signals in the mat samples.

Targeting cells of subgroups 7 and 8 with the polynucleotide probes did not result in halo formation (results not shown, see further Zimmermann, 2002), as has been commonly observed in polynucleotide hybridizations using polyribonucleotide probes or deoxyribopolynucleotide probes generated by PCR amplification (Zimmermann *et al*., 2001; Zwirglmaier, 2005). Frequently, hybridization signals not restricted to the cell periphery were obtained; fluorescence signals were unevenly distributed over the cells and were concentrated near the cell poles (Zimmermann, 2002). With the exception of the OTU LS_LKC11_kan4, all other 23S rRNA gene OTUs could be detected by polynucleotide FISH, although LS_LKC11_kan4 was represented by seven clones in the 23S rRNA clone libraries from sites LKC11 and LKC29 (Zimmermann, 2002), thus indicating that the combined application of FISH and rRNA gene clone libraries avoids false negative data interpretation.

Distribution of Acidobacteria *and metabolic hypotheses*

Our culture-independent results provide new insight into the acidobacterial diversity and distribution in subterranean and sulfidic ecosystems. Because phylogenetic associations do not imply ecosystem function, we are only able to speculate about their metabolic capabilities of the acidobacterial subgroups until they are isolated in pure culture. However, based on ecological associations with other microbial groups and habitat geochemistry, we can hypothesize about the metabolic function(s) of *Acidobacteria* in Lower Kane Cave. These hypotheses will help to guide culturing efforts.

Acidobacteria were detected in 20 examined microbial mat samples from all cave springs and stream channels, and from both high and low dissolved sulfide and oxygen conditions. Detailed quantification of acidobacterial cells with FISH was not possible either due to high background sample fluorescence, patchy distribution of target cells, or due to substantial amount of strongly fluorescing filamentous epsilon- or gammaproteobacterial cells (Engel *et al*., 2003). However, *Acidobacteria* constituted ~5%–10% of all *Bacteria* in the samples that were suitable for FISH (data not shown). Both subgroups 7 and 8

were detected in seven different samples, but cells from subgroup 7 were always more abundant than cells from subgroup 8. Samples where only subgroup 7 organisms were detected originated from the more oxygenated, distal reaches of the microbial mats compared with where subgroup 8 organisms were identified or where there was a mix of subgroups 7 and 8 organisms. A rough visual assessment of the FISH results with probes specific for subgroup 7 and 8 organisms respectively, showed that, with growing distance from the Fissure Spring (Fig. 1), the relative abundance of subgroup 7 target cells in the samples increased, while the abundance of subgroup 8 organisms decreased. This shift was found both in the FISH biovolume estimates and in the clone libraries. Additionally, subgroup 7 cells alone were observed in five samples (LKC14, 15, 19, 24 and 27), and solely one sample, LKC13, contained only subgroup 8 cells.

Subgroup 7 and 8 cells were always found embedded in a matrix of epsilon- or gammaproteobacterial filaments in all of the samples (Fig. 5). Engel and colleagues (2003; 2004a) found that the microbial mats dominated by *Epsilon-* and/or *Gammaproteobacteria* (with more than 70% of the biovolume being as *Epsilonproteobacteria* for some mats, and up to 90% of the biovolume for other samples being as *Gammaproteobacteria*) had carbon isotope values consistent with chemolithoautotrophic metabolism. The *Acidobacteria* may be living as chemoorganotrophs in association with the autotrophically fixed carbon in the poorly oxygenated regions of the cave stream. Although previous environmental studies demonstrated that some *Acidobacteria* from subgroup 6 could be enriched only in the presence of *Alphaproteobacteria* (Spring *et al*., 2000), the true functional association between the cave *Acidobacteria* and *Epsilon-* and/or *Gammaproteobacteria*, if there is one, will require more investigations.

X-ray microanalysis of the microbial mats revealed that the samples contained an abundance of inorganic crystalline structures, including elemental sulfur and other minerals like calcite and iron sulfides (results not shown, but refer to Zimmermann, 2002). However, the Lower Kane Cave acidobacterial subgroups 7 and 8 had low similarity in 23S rRNA gene sequence to *G. fermentans* (< 97%), an iron-reducing acidobacterial group. These issues raise the question as to whether or not the *Acidobacteria* in Lower Kane Cave may be involved in iron cycling, or if they are potentially associated with sulfur cycling, similar to *Geobacter sulfurreducens* which can reduce sulfur under microaerophilic conditions (Caccavo *et al*., 1994).

In conclusion, our multifaceted approach, including the construction and screening of both 16S and 23S rRNA gene clone libraries, the application of the multiple probe concept for FISH analysis, evaluation of ecological associations, and the consideration of habitat physicochemistry, has unveiled novel *Acidobacteria* from a subsurface aquatic habitat. The distribution and diversity of *Acidobacteria* in the sulfidic waters of Lower Kane Cave provide clues as to the significance of *Acidobacteria* in other microbial ecosystems and yield new information regarding the metabolic function and roles of the different *Acidobacteria* subgroups for future pure culture studies.

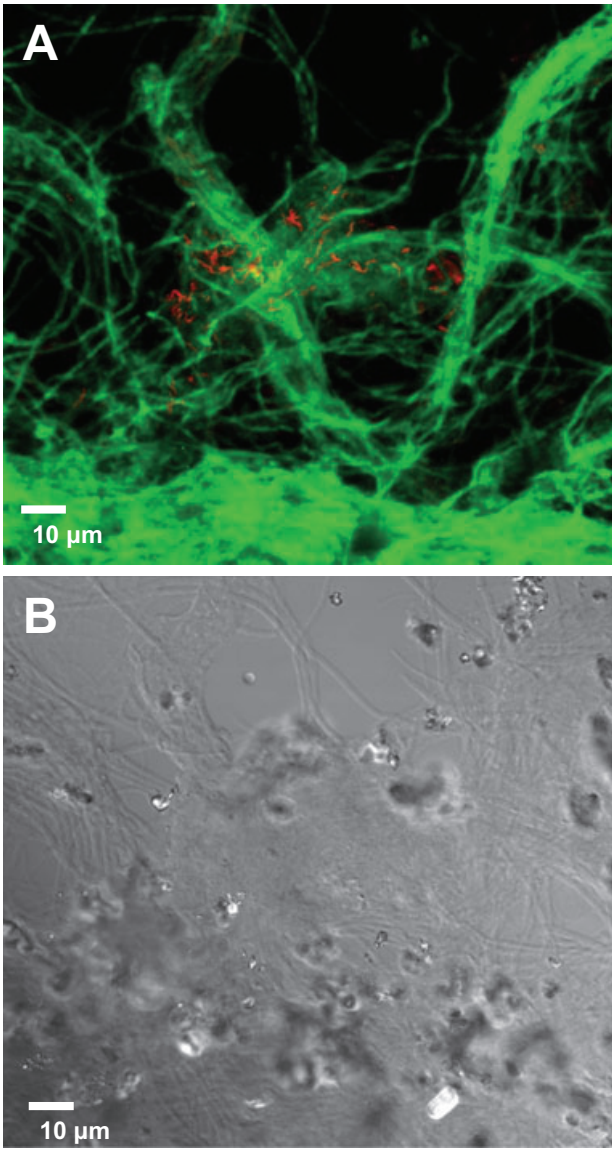

Fig. 5. A. Hybridizations with sample LKC20 using 16S rRNA-targeted FLUOSPRIME-labelled, epsilonproteobacterial LKC group II-specific oligonucleotide probe LKC1006 (green) in combination with Cy3-labelled probe LS_subd7_ALP761 (red), specific for the acidobacterial subgroup 7.
B. Phase contrast micrograph of the image (A).

Experimental procedures

Description of geochemistry of Lower Kane Cave

The major ion geochemistry from the aquatic sampling sites was similar, with all waters being calcium–bicarbonate–sulfate water type (i.e. dominated by Ca^{2+}, HCO_3^- and SO_4^{2-} ions, as described in Egemeier, 1981; Engel *et al*., 2003; 2004a,b). Although the cave is forming from sulfuric acid dissolution of limestone, the spring and stream waters are buffered to circum-neutral (pH of ~7.3) by ongoing carbonate dissolution. The dissolved sulfide concentration was > 35 μmol l^{-1} in the incoming spring water and contained no dissolved oxygen (Fig. 1E–G). At all springs, the end of the microbial mats was marked by a decrease in the concentration of dissolved sulfide to non-detectable levels and an increase in the concentration of dissolved oxygen to 40 μmol l^{-1}. Trace dissolved iron was measured in the incoming spring water, but not in downstream water (Engel *et al*., 2004a).

Sample collection

Samples for clone library studies were collected aseptically from the cave microbial mats, immediately frozen, and transported to the laboratory on dry ice. Samples originated from the three main springs: Fissure Spring, Upper Spring and Lower Spring (distance in meters from the back of the cave, 0 m, to the cave entrance, Fig. 1A). Sample sites in this study correlate to sites used for the previous bacterial diversity study by Engel and colleagues (2004a). For clone library construction, samples were used from the Fissure (123 m, sample LKC29) and Upper Spring (194 m and 203 m, samples LKC11, 21 and 22 respectively). Based on previously conducted oxygen profiles using a fluorescence-quenching microelectrode method (Ocean Optics), only the top 3–5 mm of the microbial mats were oxygenated, with the mat interior being anaerobic (pO_2 < 10 Pa) (Engel *et al*., 2004a). Three of the mat samples were obtained from the aerobic layer of the mats (LKC29, 11 and 21), whereas the fourth sample (LKC22) was obtained from the anaerobic layer of the mat sample LKC21.

For screening with FISH using different probes, 23 different mat samples, representing mats with different redox conditions (aerobic vs. anaerobic), were collected (Fig. 1E–G). The samples were fixed in two different ways within 48 h after sampling: (i) fixation with 4% paraformaldehyde; and (ii) fixation with 96% EtOH, as described by Amann (1995).

DNA extraction

Genomic DNA was extracted from samples LKC11, 21, 22 and 29 by using the following modifications to the protocol of Griffiths and colleagues (2000): the enzyme pretreatment of the samples was omitted and the precipitation of nucleic acids in the aqueous phase was performed with 0.1 vol. of 3 M sodium acetate (pH 5.2) and 0.6 vol. of isopropanol for 3 h at room temperature. DNA was collected by centrifugation at 10 000 *g* for 10 min at room temperature, washed with 70% ethanol, air dried, and suspended in 50 μl sterile water.

PCR amplification and cloning of rRNA genes

16S rRNA gene fragments were amplified using the general primer pair 616f-630r spanning *E. coli* positions (Brosius *et al*., 1981) 8–1542 (for clone library UA, Juretschko *et al*., 2002) and primer pair 27f-1492r spanning *E. coli* positions 27–1510 (for clone library UB, Lane, 1991). 23S rRNA gene sequences ranging from *E. coli* base pair position 189 to 1948 were amplified using the *Acidobacteria*-specific primer HOL189f (5′-GGAAGTGAACCATCTCAG-3′) (Zimmermann *et al*., 2005a) in combination with the bacterial primer 1037r (5′-CGACAAGGAATTTCGCTAC-3′) (Ludwig *et al*., 1992). The binding site of HOL189f targets the sequence stretch coding for the helix 11 region of the 23S rRNA molecule, which is a signature region for the *Acidobacteria* phylum. In some cases, amplification of large parts of the rRNA operon (targeting both 16S and 23S rRNA genes) was performed using conserved bacterial primers 27f and 985r (5′-CCGGTCCTCTCGTACT-3′) spanning the rRNA operon from 16S rRNA *E. coli* position 8 to 23S rRNA *E. coli* position 2669. TaKaRa Ex Taq Polymerase (TaKaRa Shuzo, Otsu, Japan) was used for *in vitro* amplification of 16S rRNA and 23S rRNA gene fragments. An initial denaturation step at 94°C for 4 min was followed by 25–35 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s, and chain extension for 90 s at 72°C. Annealing temperatures were 52°C for the 16S rRNA primers, and 58°C for the primer combination HOL189f-1037r. Purified PCR products (Quickstep PCR Purification Kit V.5, Edge Biosystems, Gaithersburg, MD, USA) were cloned into the vector pCR.2 using a TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany), following manufacturer's instructions. Approximately 800 16S rRNA and ~50 23S rRNA clones were obtained for sequencing.

Sequencing and phylogenetic analyses of the 16S and 23S rRNA gene fragments

Clone inserts were screened using the M13F and M13R PCR primers (Invitrogen) and plasmids with correct inserts were purified using the QIAquick Plasmid Purification Kit (Qiagen, Hilden, Germany). Unique 16S and 23S rRNA gene fragments were sequenced in polyacrylamide gels using the SequiTherm ExcelTM II DNA Sequencing Kit-LC (Epicentre, Madison, WI, USA) and a LI-COR Global IR2 DNA Sequencer (LI-COR Biosciences, Bad Homburg, D). Oligonucleotides carried fluorescent labels IRDye700 and IRDye800 (LI-COR Biosciences, Bad Homburg, Germany). In addition to the plasmid-targeted standard sequencing primers, oligonucleotides 1020rmod (5′-KCTGGGYTSTT YCCCT-3′) and 1019fmod (5′-TAGCTGGTTCTCYBSGAA-3′) were used for sequencing internal regions of 23S rRNA gene fragments. The sequencing gels were analysed using e-Seq DNA Sequencing and Analysis Software (LI-COR Biosciences, Bad Homburg, Germany).

Newly acquired sequences were added to an alignment of ~50 000 16S and 5000 23S rRNA gene sequences using the ARB program alignment tool (Ludwig *et al*., 2004). Alignments were visually refined and phylogenetic analyses were performed by using distance matrix, maximum parsimony and maximum likelihood (AXML in the ARB software) methods. The analysis compared select regions of the target gene

sequences and alignment positions from multiple data sets (Ludwig *et al*., 1998). Unique gene sequences or OTUs identified in the clone libraries (represented by single clones or clones carrying inserts with > 99.5% sequence similarity) were designated with the either SS (small subunit) or LS (large subunit) if containing 16S or 23S rRNA gene information respectively. The clone designation provides information on the sampling site (e.g. LKC22). All 16S and 23S rRNA gene sequences determined in this study have been submitted to Gene Bank (http://www.ncbi.nlm.nih.gov/) under the accession numbers AM180885–AM180902.

Fluorescence in situ *hybridization with 16S and 23S rRNA-targeted oligonucleotide probes*

For identification of *Acidobacteria* and other bacterial groups, such as the not-yet-cultured filamentous *Epsilonproteobacteria*, 16S and 23S rRNA-targeted oligonucleotide probes with varying specificities were used (Table 1). Design of novel probes targeting the novel clone sequences was accomplished using the PROBE_DESIGN tool from ARB (Ludwig *et al*., 2004). More detailed information about the probes, including probe mismatch files, can be found at http://www.microbial-systems-ecology.net/Meisinger_et_al_2007.html

Hybridization was performed as described by Amann (1995) with oligonucleotides labelled with the monofunctional, hydrophilic, sulfoindocyanine dye Cy3 or the fluorescein derivative FLUOS-PRIME (MWG, Ebersberg, Germany). Probe specificity and functionality were evaluated with paraformaldehyde-fixed samples from Lower Kane Cave, as no cultured target cells from this habitat were available (Engel *et al*., 2003). *Acidobacterium capsulatum* (strain DSMZ 11244) was used as a reference strain for evaluation of the probes targeting the phylum in general. Formamide concentrations were increased in incremental steps of 5% to determine the optimal hybridization stringencies that yielded FISH signals exclusively from morphotypes that could be simultaneously detected with corresponding FISH probes (Table 1).

Fluorescence in situ *hybridization using 23S rRNA-targeted polynucleotide probes*

23S rRNA-targeted polynucleotide probes were prepared by PCR using the primer pair 1900VN (5′-AAAAGCGGCCGCMADGCGTAGNCGAWG-3′) and 317RT3 and 5′-ATAGGTATTAACCCTCACTAAAGGGACCWGTGTCSGTTTHBGTAC-3′) (where M is A or C, D is A or G or T, N is any of the four bases, W is A or T, H is A or C or T, and B is C or G or T), targeting a hypervariable stretch in domain III of the 23S rRNA gene under conditions, as described by Zimmermann and colleagues (2001). The fluorescently labelled dNTPs, FluoroLink Cy3-dCTP and -dUTP (Amersham Pharmacia, Little Chalfont, UK), were incorporated during the amplification. The DNA polynucleotide probes were purified and applied for FISH, as described by Zimmermann and colleagues (2001), at a formamide concentration of 46% in the hybridization solution.

Fluorescence microscopy

Samples subjected to FISH were mounted with the antibleaching agent Vectashield H-1000 (Vector, Burlingame, CA, USA) and viewed using an Axioplan microscope equipped with an epifluorescence device and a model LSM 510 scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a UV laser (351 and 364 nm), an Ar ion laser (450–514 nm), and two HeNe lasers (543 and 633 nm). Image processing and recording were performed with the standard software package included with the instrument.

X-ray microanalysis

For analysis of the immediate surroundings of the acidobacterial cells identified by FISH with probe SS_HOL1400, paraformaldehyde-fixed samples were coated with platinum using a Magnetron SCD 050 Sputter Coater (BAL-TEC, Walluf, Germany). Samples were examined using an S-4100 Hitachi field emission scanning electron microscope with elemental analysis at 5 kV for imaging and 20 kV for elemental analysis. Elemental spectra were generated using Thermo NORAN software (Thermo NORAN, Middleton, WI, USA).

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