Isolierung und kultivierungsunabhängige Untersuchungen von magnetotaktischen Bakterien aus marinen und limnischen Sedimenten

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Abkürzungen

A	Adenin
Abb.	Abbildung
AMB-1	*Magnetospirillum magneticum*
ARB	Softwareprogramm für die Berechnung phylogenetischer Stammbäume
ARB-1	separates phylogenetisches Cluster innerhalb der Gruppe magnetotaktischer Kokken
ARDRA	amplified ribosomal DNA restriction analysis
b	Basen
BAC	bacterial artificial chromosomes
BSA	Rinderserumalbumin
bzw.	beziehungsweise
C	Cytosin
°C	Grad Celsius
ca.	circa
cAMP	Zyklisches AMP
cm^3	Kubikzentimeter
DAPI	4,6-Diamino-2-phenylindol
DGGE	denaturierende Gradienten Gelelektrophorese
DNA	Desoxyribonucleinsäure (engl. acid)
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dsrAB	Gene der dissimilatorischen Sulfitreduktase, Alpha- und Beta-Untereinheit
et al.	und andere
EUB338	Oligonukleotidsonde, spezifisch für Bakterien
FISH	Fluoreszenz *in situ* Hybridisierung
g	Gramm
G	Guanin
h	Stunde
HaeIII	Restriktionsenzym, Schnittstelle GG´CC
J	Joule
K	Kilo

l	Liter
m	Meter, milli
M	magnetisch angereichert
µ	mikro
mamA, *B*, *C* und *D*	an der Magnetosomenbiomineralisation beteiligte Gene
MamA, B, C und D	an der Magnetosomenbiomineralisation beteiligte Proteine
MC-1	Isolat eines magnetotaktischen Kokkus
MHB-1	magnetotaktisches Stäbchen
min	Minute
MMP	magnetotaktisches multizelluläres Aggregat (magnetotactic, many-celled prokaryote)
MS-1	*Magnetospirillum magnetotacticum*
MSM-1, MSM-3, MSM-4 MSM-6 bis MSM-9	Isolate der Gattung *Magnetospirillum*
MSR-1	*Magnetospirillum gryphiswaldense*
MTB	magnetotaktische Bakterien
MV-1, MV-2, MV-4	isolierte magnetotaktische Vibrionen
OATZ	Übergangsbereich zwischen oxischer und anoxischer Zone (oxic anoxic transition zone)
p	Seite (engl. Page)
PBS	Phosphat-gepufferte Saline (phosphate buffered saline)
PCR	Polymerase Kettenreaktion (polymerase chain reaction)
pH	negativer dekadischer Logarithmus der Protonenkonzentration
rDNA	Gen, das die rRNA kodiert
rRNA	ribosomale Ribonukleinsäure
RS-1	*Desulfovibrio magneticus*
RsaI	Restriktionsenzym, Schnittstelle GT´AC
RT	Raumtemperatur; „Race-track“
S	Svedberg-Einheit, Seite
SRB	Sulfat-reduzierende Bakterien
SRR	Sulfatreduktionsraten
T	Thymin, Tesla
Tab.	Tabelle
Taq	*Thermus aquaticus*

TEM	Transmissionselektronenmikroskopie
TOPO TA	Kit zur Klonierung von PCR-Produkten
z. B.	zum Beispiel

Zusammenfassung

In den letzten Jahren wurden über verschiedene biochemische und molekularbiologische Untersuchungen von Reinkulturen viele neue Erkenntnisse zur Genetik der Magnetitbiomineralisation von magnetotaktischen Bakterien (MTB) gewonnen. Allerdings blieben dabei sowohl die Physiologie und Ökologie als auch die Verbreitung und Diversität dieser Bakterien und der an der Biomineralisation beteiligten Gene weitestgehend ungeklärt. In der vorliegenden Arbeit wurde deshalb über eine Kombination verschiedener kultivierungsunabhängiger Untersuchungsmethoden die Ökophysiologie von MTB in Süßwassersedimenten untersucht. In einem zweiten Ansatz wurde die Diversität magnetotaktischer Bakterien über verschiedene Isolierungsversuche und kultivierungsunabhängige Methoden bestimmt und die Voraussetzungen für eine molekularbiologische Untersuchung der an der Biomineralisation beteiligten Gene von bisher unkultivierbaren MTB geschaffen.

Die Verbreitung magnetotaktischer Bakterien wurde in verschiedenen limnischen und marinen Habitaten Norddeutschlands untersucht. Abgesehen von einigen stark eutrophierten Standorten konnten in fast allen Proben verschiedene magnetotaktische Kokken, Spirillen, Vibrionen und Stäbchen gefunden werden. Im Rahmen dieser Arbeit ließen sich erstmals magnetotaktische multizelluläre Aggregate in Sedimenten der deutschen Bucht und der Kieler Förde nachweisen.

Als eine Folge der Inkubation von MTB in Mikrokosmen konnte generell eine Zunahme der MTB Zellzahlen und eine deutliche Abnahme der Diversität beobachtet werden. Die meisten Proben wurden von magnetotaktischen Kokken dominiert, die aufgrund von 16S rDNA Analysen einer separaten Linie innerhalb der „Alphaproteobakterien“ zu zuordnen waren. Zwischen den verschiedenen in dieser Arbeit identifizierten magnetotaktischen Kokken konnten Sequenzunterschiede zwischen 0,1 und 11% festgestellt werden, wobei die Untersuchung verschiedener Zeitpunkte eines von magnetotaktischen Kokken dominierten Mikrokosmos zeigte, dass große Unterschiede auch innerhalb der MTB Population eines Mikrokosmos vorkommen können. Eine Besonderheit stellte die Massenentwicklung eines magnetotaktischen Stäbchens (MHB-1) in einem Mikrokosmos eines Badesees in Bremen-Walle dar. Mit dem Nachweis dieses nahe mit „Magnetobacterium bavaricum“ verwandten Bakteriums als zweiten magnetotaktischen Vertreters des *Nitrospira* Phylums konnte erstmals eine größere geographische Verbreitung und Diversität dieser Gruppe von MTB bewiesen

werden. Eine Korrelation zwischen der Entwicklung einer bestimmter MTB Spezies innerhalb eines Mikrokosmos und der geographischen Lage des ursprünglichen Habitats konnte in dieser Arbeit nicht beobachten werden.

Obwohl keine klare Korrelation zwischen der vertikalen Verteilung von MTB und verschiedenen physiko-chemischen Parametern in den untersuchten Mikrokosmen beobachtet werden konnte, deutet die über die Untersuchung der Lebendzellzahlen bestimmte heterogene Verteilung von MTB auf eine Anpassung an spezielle Gradienten hin. So wurde in allen untersuchten Mikrokosmen die Mehrheit (63 bis 98%) der untersuchten MTB im anoxischen Sediment gefunden. Darüber hinaus besitzen magnetotaktische Bakterien vermutlich innerhalb bestimmter Sedimenthorizonten aufgrund ihres hohen intrazellulären Eisengehalts, ihrer hohen Zellzahlen von bis zu $1{,}5 \times 10^7$ MTB/cm^3 und einer Abundanz von bis zu 1% der Gesamtzellzahl einen wesentlichen Einfluß auf den mikrobiellen Eisenkreislauf.

Über eine Kombination verschiedener kultivierungsunabhängiger Versuche konnte die selektive Anreicherung von MTB sowohl über die magnetischen Anreicherung von MTB in der Wassersäule der Mikrokosmen als auch über eine als “Race-track“ (RT) bezeichneten Methode belegt werden. Die Spezifität und Selektivität verschiedener magnetischer Anreicherungstechniken wurde dabei erstmals über molekularbiologische Methoden wie der denaturierenden Gradienten Gelelektrophorese (DGGE) und der Untersuchung des Restriktionspolymorphismus (ARDRA) von 16S rRNA Genen mit anschließenden Sequenzvergleichen bestimmt. In den untersuchten Proben konnten keine Unterschiede zwischen den verwendeten Anreicherungsmethoden beobachtet werden.

Im Rahmen dieser Arbeit wurden zahlreiche Kultivierungsexperimente mit verschiedenen organischen und anorganischen Substraten zur Isolierung neuer MTB Spezies durchgeführt. Trotz der geringen Anzahl nicht-magnetotaktischer Kontaminanten und der Dominanz magnetotaktischer Kokken oder im Fall von MHB-1 eines magnetotaktischen Stäbchens in den verwendeten Inokula konnten keine MTB dieser beiden Morphotypen isoliert werden. Dieses Ergebnis legt nahe, dass konventionelle Kultivierungsmethoden zur Isolierung magnetotaktischer Bakterien nicht ausreichend sind und durch neue Isolierungsstrategien ergänzt werden müssen. Dennoch konnten 10 heterotrophe magnetotaktische Spirillen von drei verschiedenen Süßwasserstandorten isoliert werden. Diese gehörten allerdings weder in den Mikrokosmen noch in den eingesetzten Inokula zu den abundanten MTB Spezies. Alle

Stämme waren mikroaerophil und konnten auf 16S rDNA Basis als Vertreter des Genus *Magnetospirillum* identifiziert werden.

Zur Untersuchung der Verbreitung und Diversität der an der Magnetitbiomineralisation beteiligten Gene (*mam* Gene) wurden erste Versuche zur Entwicklung von universellen *mam* Primern unternommen, die jedoch aufgrund der geringen Größe des vorhandenen Datensatzes nicht erfolgreich waren. Allerdings konnte die Existenz von *mam*A in 9 von 15 kultivierten *Magnetospirillum*-Stämmen mittels eines für *Magnetospirillum gryphiswaldense* entwickelten nicht degenerierten Primerpaares nachgewiesen werden. Alle Sequenzen waren nahezu identisch zu *mamA* von *Magnetospirillum magnetotacticum* oder *M. gryphiswaldense*, wobei die Zuordnung dieser Sequenzen zu den genannten Arten nicht ihren auf 16S rDNA Basis bestimmten phylogenetischen Verwandschaftsverhältnissen entsprach.

Mit der selektiven Anreicherung von MTB mittels magnetischer Feldlinien wurde die Voraussetzung für die Erstellung einer Genbank aus subgenomischen Fragmenten von bisher nicht kultivierbaren MTB geschaffen. Aus der erhaltenen Genbank konnte ein Klon identifiziert werden, der vermutlich große Teile des *mamAB* Clusters eines unkultivierten MTB enthält.

A Einleitung

Bedingt durch die vielfältigen potenziellen Anwendungsmöglichkeiten magnetischer Nanopartikel wie z. B. bei der Krebsdiagnostik (Baeuerlein et al. 1998), gewinnt die kontrollierte Mineralisation von Magnetit zunehmend an Bedeutung. Biogene, intrazellulär gebildete Magnetitpartikel sind dabei aufgrund ihrer einheitlichen Größe, ihrer nahezu perfekten kristallinen Struktur und ihrer hohen Reinheit synthetisch hergestellten Magnetitkristallen weit überlegen. Um solche Nanopartikel mit definierten Eigenschaften entweder über biotechnologische Verfahren oder im Idealfall *in vitro* im großtechnischen Maßstab herstellen zu können, ist jedoch ein tiefergehendes Verständnis der an der Biomineralisation beteiligten Prozesse nötig. Eine Möglichkeit diese Prozesse besser untersuchen und verstehen zu können, besteht in der Isolierung intrazellulär magnetitbildender Mikroorganismen und der Bestimmung der Diversität, Funktion und Regulation der an der (Magnetit-)Biomineralisation beteiligten Gene. Darüber hinaus wird diesen Bakterien aufgrund ihrer ubiquitären Verbreitung in aquatischen Habitaten, ihrer Abundanz und ihrer hohen intrazellulären Eisenkonzentration auch eine wichtige Rolle im mikrobiellen Eisenkreislauf zugeschrieben. Bedingt durch die geringe Anzahl verfügbarer Reinkulturen, gilt es a) über die Untersuchung der Parameter, die die Verbreitung dieser Organismen beeinflussen, erste Einblicke in ihre Physiologie zu erhalten um neue Strategien zu ihrer Isolierung zu entwickeln und b) verschiedene molekularbiologische Methoden zur kultivierungsunabhängigen Analyse dieser Organismen zu etablieren.

Im Folgenden sollen deshalb vor allem die bisher bekannten Informationen zur Ökologie, Verbreitung, Diversität und Physiologie dieser Organismen kurz zusammengefaßt werden. Darüber hinaus sollen verschiedene Methoden zur Isolierung und molekularbiologischen Charakterisierung bisher unkultivierbarer Mikroorganismen vorgestellt werden.

1. Magnetotaxis

1975 beobachtete der Mikrobiologe Richard Blakemore erstmals Bakterien, die sich mittels intrazellulär gebildeter Magnetit- (Fe_3O_4) oder Greigitpartikeln (Fe_3S_4) am Magnetfeld ausrichteten und entlang der Feldlinien bewegen konnten (Blakemore 1975). Aufgrund dieses als Magnetotaxis bezeichneten Verhaltens bezeichnete er diese Organismen als

magnetotaktische Bakterien (MTB). Da es sich im Gegensatz zur Chemotaxis bei der Ausrichtung der Zellen im Magnetfeld um einen passiven Prozess handelt, der auch bei abgetöteten Zellen zu beobachten ist, wurde später auch der Begriff „magnetische Bakterien“ verwendet (Blakemore und Blakemore 1990), der sich aber in der Literatur nicht durchsetzen konnte. Der Vorteil der Magnetotaxis gegenüber der Chemotaxis besteht in der Reduzierung eines dreidimensionalen (Orientierungs-)Problems auf ein zweidimensionales, so dass MTB die für sie optimalen Mikrohabitate wesentlich schneller auffinden können als rein chemotaktische Bakterien (Blakemore und Blakemore 1990). Die vertikale Orientierung von MTB beruht dabei auf der Inklination der magnetischen Feldlinien, die in der nördlichen Hemisphäre nach unten und in der südlichen nach oben gerichtet sind. In Abhängigkeit von der Hemisphäre schwimmen MTB deshalb entweder parallel (Nordhalbkugel) oder antiparallel (Südhalbkugel) zu den Feldlinien um in tiefere Wasser- bzw. Sedimentschichten zu gelangen, wobei die Lokalisierung bzw. der Verbleib der Zellen in der für sie optimalen Position im Habitat vermutlich über eine rein chemotaktische Reizantwort gesteuert wird (Blakemore 1982; Mann et al. 1990a). Ihrer Schwimmrichtung entsprechend werden MTB als nord- bzw. südsuchend bezeichnet (Blakemore et al. 1980; Kirschvink 1980; Frankel und Blakemore 1989). Allerdings weist immer ein kleiner Teil (<0,5%) der Population neben der ökologisch „richtigen“ Polarität eine entgegengesetzte Orientierung auf (Blakemore 1982). Unklar ist, wie weit die Orientierung der Zelle genetisch determiniert ist. Interessanterweise sind am Äquator, an dem die Feldlinien parallel zur Erdoberfläche verlaufen, beide Zellpolaritäten paritätisch vorhanden (Blakemore et al. 1980; Frankel und Blakemore 1980; Frankel et al. 1981). Die Orientierung dieser Zellen erfolgt vermutlich über eine rein chemotaktische Reizantwort, wobei die Chemotaxis über die Magnetotaxis dominiert (Frankel 1982). Nach Erreichen der bevorzugten Position sorgt die magnetische Ausrichtung der Zellen für einen Verbleib in der entsprechenden Wasser- bzw. Sedimentschicht (Spring 1993b).

Obwohl alle mikroaerophilen MTB die Fähigkeit besitzen, sich im magnetischen Feld zu orientieren, gehen Frankel und Mitarbeiter (Frankel et al. 1997) davon aus, dass die Magnetotaxis mikroaerophiler MTB auf zwei unterschiedlichen Systemen basiert: Als axiale Magnetoaerotaxis wird die Ausrichtung magnetischer Spirillen entlang der Feldlinien verstanden, wobei die Schwimmrichtung durch eine temporäre aerotaktische Reizantwort bestimmt wird. Bei der polaren Magnetoaerotaxis, wie sie magnetotaktischen Kokken zugesprochen wird, beeinflußt die Orientierung der Feldlinien die Schwimmrichtung der Zellen, wobei die Überschreitung bzw. Unterschreitung einer kritischen Sauer-

stoffkonzentration zu einer parallelen bzw. antiparallelen Schwimmbewegung führt. Neuere Untersuchungen von Schüler und Mitarbeitern (1999) belegen jedoch, dass auch magnetotaktische Spirillen eine polare Orientierung im Magnetfeld aufweisen können.

2. Charakterisierung der Magnetosomen

In allen bisher untersuchten MTB konnten die magnetischen Eisenmineralien Magnetit und/oder Greigit nachgewiesen werden. Elektronenmikroskopische und biochemische Untersuchungen zeigen, dass es sich bei diesen Partikeln um Monokristalle handelt, die von einer Membran umgeben sind (Balkwill et al. 1980; Gorby et al. 1988; Grünberg 2000). Diese als Magnetosomen bezeichneten Partikel sind in der Zelle entweder irregulär oder in linearen Ketten entlang der Zellachse angeordnet, wobei sich die Kristalle zum Ende hin verjüngen (Meldrum et al. 1993a). Die für die magnetische Ausrichtung der Zelle optimale Partikelgröße wird im wesentlichen durch die magnetischen Eigenschaften der Eisenmineralien bestimmt. Während kleinere Magnetitpartikel (<35 nm) bei Raumtemperatur supermagnetisch sind und daher kein richtungsstabiles magnetisches Moment besitzen, führt die Ausbildung mehrerer Domänen bei Kristallen über 120 nm zu einer antiparallelen Spinorientierungen und damit zu einer Erniedrigung der magnetostatischen Energie, (Butler und Banerjee 1975; Denham et al. 1980; Stolz 1993; Moskowitz 1995; Fabian et al. 1996). Die Partikelgröße der meisten Magnetosomen liegt mit 35 bis 120 nm im “single-domain”-Bereich, der sich durch ein stabiles Dipolmoment auszeichnet. Eine Ausnahme stellt ein MTB mit bis zu 200 nm großen Kristallen dar, das in einem Gebiet mit ungewöhnlich niedrigem Erdmagnetfeld (25 µT) gefunden wurde (Farina et al. 1994). Neben ihrer einheitlichen Größe zeichnen sich Magnetosomen durch ihre nahezu perfekte kristalline Struktur und hohe Reinheit aus, wobei die Anordnung und Form (Abb. 1) der Magnetosomen kultivierungsunabhängig und art- oder stammspezifisch zu sein scheint (Meldrum et al. 1993a; Meldrum et al. 1993b; Bazylinski et al. 1994; Bazylinski et al. 1995). Während der Kristallisationsprozess einer strengen genetischen Kontrolle unterliegt, ist die Partikelbildung und Größe von den Kultivierungsbedingungen abhängig (Blakemore et al. 1985; Bazylinski et al. 1995; Schüler und Baeuerlein 1998; Heyen und Schüler 2003). So können verschiedene im Fermenter angezogene *Magnetospirillen*-Arten Magnetosomen nur bei Sauerstoffkonzentrationen unterhalb 24 µmol/l bilden (Heyen und Schüler 2003). Durchschnittlich enthalten magnetotaktische Bakterien 22 Partikel pro Zelle. Ausgehend

von 50 nm Durchmesser, einer linearen Anordnung und einem Erdmagnetfeld von 50 µT reicht diese Menge aus, um sich im Habitat orientieren zu können (Blakemore 1982). Aufgrund des hohen Eisengehalts insbesondere einiger magnetotaktischen Bakterien, die mit bis zu 1000 Magnetosomen deutlich mehr Partikel besitzen als für die Orientierung notwendig wäre, werden den Magnetosomen alternativ zur Zellorientierung verschiedene Bedeutungen bei der Energiegewinnung, der Energiespeicherung und der Eisenspeicherung zugesprochen (Short und Blakemore 1986; Mann et al. 1990a; Guerin und Blakemore 1992; Spring et al. 1993; Spring 1993b). Eine Remineralisation des gebildeten Magnetits bzw. Greigits durch MTB konnte bisher nicht beobachtet werden. Ebenso wird die Bildung von Eisenmineralien als Folge der Detoxifizierung schädlicher Sauerstoffperoxide und Metallkonzentrationen diskutiert (Blakemore 1982; Schübbe et al. 2003).

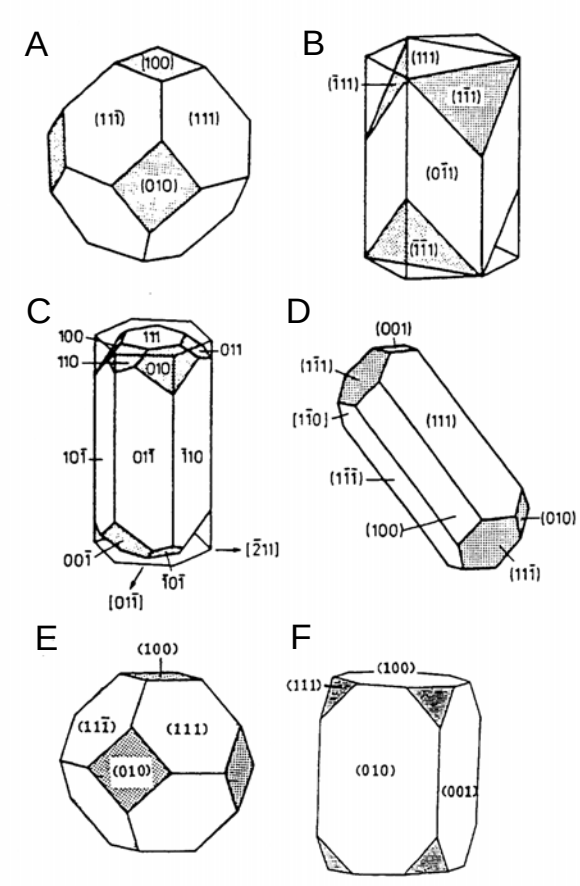

Abb. 1: Idealisierte Magnetit- (a - d) und Greigitkristallformen (e - f) magnetotaktischer Bakterien. a) und e) Kubo-Oktaeder; b), c) und f) hexagonale Prismen; d) elongierte Kubo-Oktaeder (Bazylinski und Moskowitz 1997).

Aufgrund der Identifizierung mehrerer magnetosomenspezifischer Proteine und der Untersuchung verschiedener *Magnetospirillum*-Spezies auf genetische Ebene werden bestimmte chromosomale Gene für die Magnetitbiomineralisation in MTB verantwortlich gemacht (Matsunaga et al. 1992; Nakamura et al. 1995; Dubbels et al. 1998; Grünberg 2000; Wahyudi et al. 2001; Schultheiss und Schüler 2003; Grünberg et al. 2004). Bisher konnte jedoch nicht geklärt werden, ob die Fähigkeit sich an magnetischen Feldlinien orientieren zu können, mehrfach voneinander unabhängig entstanden ist oder ob es sich dabei um einen horizontalen Gentransfer handelt. Eine erste vergleichende Analyse wurde mit der Totalsequenzierung zweier MTB durch das Doe Joint Genome Institute (www.jgi.doe.gov/JGI_microbial/html) und die Identifizierung verschiedener magnetosomenspezifischen Gene (*mam* Gene) in *Magnetospirillum gryphiswaldense* möglich. Alle von Grünberg und Mitarbeitern untersuchten *mam* Gene konnten sowohl in *M. gryphiswaldense*, als auch in *Magnetospirillum magnetotacticum* und dem magnetotaktischen Kokkus MC-1 gefunden werden, jedoch nicht in einer spontanen Mutante von *M. gryphiswaldense* oder anderen nicht-magnetotaktischen Bakterien (Grünberg 2000; Schübbe et al. 2003; Schüler 2004). Die hohe Konserviertheit und die Anordnung innerhalb eines Clusters deuten auf einen horizontalen Gentransfer innerhalb der „Alphaproteobakterien“ hin. Aufgrund dieser und anderer gemeinsamer Merkmale zu bekannten Pathogenitätsinseln wurde von Schübbe und Mitarbeitern (2003) die Existenz einer Magnetosomeninsel postuliert.

3. Vorkommen und Diversität magnetotaktischer Bakterien

MTB sind morphologisch divers (Kokken, Spirillen, Vibrios, Stäbchen und multizelluläre Aggregate) und kommen ubiquitär sowohl in verschiedenen limnischen und marinen Sedimenten als auch in der stratifizierten Wassersäule einiger sauerstoffarmer, aber schwefelreicher Brackwasserhabitate und in feuchteten Böden vor (Blakemore 1975; Moench und Konetzka 1978; Blakemore et al. 1980; Blakemore und Frankel 1981; Frankel et al. 1981; Sparks et al. 1986; Stolz et al. 1986; Oberhack et al. 1987; Matitashvili und Matojan 1989; Petersen et al. 1989; Sparks et al. 1989; Fassbinder et al. 1990; Adamkiewicz et al. 1991; Stolz 1992; Petermann und Bleil 1993; Stolz 1993; Bazylinski et al. 1995; Bazylinski und Moskowitz 1997; Riemann et al. 1999; Cox et al. 2002). MTB können sowohl in nährstoffarmen Habitaten, als auch in Bereichen mit hohen organischen

Nährstoffgehalten gefunden werden, wo sie Zellzahlen zwischen 10^3 - 10^4 MTB/cm^3 erreichen können (Blakemore et al. 1979; Blakemore 1982). Lediglich an extremen Standorten wie heißen Quellen, Kalksteinhöhlen, sauren Minengewässern oder Öl-, Chlor- oder Kupfersulfat-kontaminierten Habitaten ließen sich bisher keine MTB nachweisen (Mann et al. 1990a; Stolz 1993). Im Gegensatz zu magnetitbildenden MTB konnten bei greigitbildenden MTB signifikanter Unterschiede in ihrem Verbreitungsmuster festgestellt werden: Während magnetitbildende MTB nicht auf ein spezielles Habitat beschränkt zu sein scheinen, wurden greigitbildende MTB bisher nur in marinen Habitaten beobachtet, in denen Sulfid in Form von Schwefelwasserstoff verfügbar ist (Mann et al. 1990b; Pósfai et al. 1998). Eine Besonderheit stellt dabei ein multizelluläres Aggregat dar, in dem neben Greigit auch Pyrit (FeS_2) als unmagnetisches Eisenmineral nachgewiesen wurde (Mann et al. 1990b). Bei diesen Partikeln handelt es sich vermutlich um modifiziertes Greigit.

16S rRNA Genanalysen zeigen, dass MTB sowohl innerhalb der „Alpha-„ und „Deltaproteobakterien“ als auch innerhalb des *Nitrospira* Phylums vorkommen, wobei die Fähigkeit Magnetit zu bilden nicht auf eine phylogenetische Linie beschränkt ist. Die meisten phylogenetisch klassifizierbaren magnetitbildenden MTB gehören dabei zur Gruppe der „Alphaproteobakterien“ (Eden 1991; Schleifer et al. 1991; Burgess et al. 1993; Spring et al. 1994; Spring und Schleifer 1995; Spring et al. 1998; Amann et al. 2000b). Allerdings scheinen magnetotaktische Kokken und Spirillen zwei eigene phylogenetische Linien zu bilden (Spring et al. 1992; DeLong et al. 1993; Schüler 1999), wobei einige magnetotaktische Stämme näher verwandt mit nicht-magnetotaktischen schwefelfreien Purpurbakterien sind als mit anderen MTB. Im Verhältnis zu ihrer relativ einheitlichen Morphologie weisen insbesondere magnetotaktische Kokken eine deutlich größere phylogenetische Diversität auf (Spring et al. 1992). Innerhalb der „Deltaproteobakterien“ konnten bisher nur zwei MTB gefunden werden: ein unkultiviertes, greigitbildendes multizelluläres Aggregat (MMP, DeLong et al. 1993) und *Desulfovibrio magneticus*, bei dem bisher jedoch keine Ausrichtung der Zellen im magnetischen Feld beobachtet werden konnte, dass jedoch aufgrund seiner Fähigkeit intrazelluläres Magnetit bilden zu können ebenfalls zu den magnetotaktischen Bakterien gezählt wird (Farina et al. 1983; Rodgers et al. 1990; Kawaguchi et al. 1995). Als einziger magnetotaktischer Vertreter des *Nitrospira* Phylums wurde ein als „Magnetobacterium bavaricum“ beschriebenes, bisher unkultiviertes, magnetitbildendes Stäbchen identifiziert (Spring et al. 1993). Weitere molekularbiologische Untersuchungen versprechen jedoch zusätzliche Informationen zur phylogenetischen Diversität von MTB über die bisher bekannten Spezies hinaus.

4. Vertikale Verteilung und Ökologie magnetotaktischer Bakterien

Die Entdeckung der Magnetotaxis als vertikale Orientierungshilfe führte zu verschiedenen Diskussionen über die bevorzugte Position von MTB im Habitat, ihre Physiologie und ökologische Bedeutung (Blakemore 1975; Blakemore und Frankel 1981; Blakemore 1982; Guerin und Blakemore 1992; Frankel und Bazylinski 1994; Amann et al. 1995; Frankel et al. 1997). Im Allgemeinen wurde angenommen, dass die meisten magnetotaktischen Bakterien einen mikroaeroben Stoffwechsel betreiben und Sedimentschichten mit niedrigen Sauerstoffkonzentrationen bevorzugen (Blakemore 1975; Blakemore und Frankel 1981).

1992 veröffentlichte Stolz eine erste Untersuchung zur vertikalen Verteilung magnetotaktischer Bakterien in der Wassersäule eines Brackwasserhabitats (Pettaquamscutt River Estuary, USA). Während in der oxischen Zone mit Sauerstoffkonzentrationen zwischen 80 und 100 µmol/l nur Kokken nachweisbar waren, konnten im mikrooxisch-sulfidischen Bereich (ca. 10 µmol/l O_2 und S^{2-}) neben Kokken und Stäbchen vor allem Vibrionen gefunden werden. In der anoxischer Zone mit mehr als 100 µmol/l Sulfid konnten neben Stäbchen und Vibrionen auch eine geringe Anzahl multizellulärer Aggregate beobachtet werden, die große morphologische Ähnlichkeiten zu bekannten greigitbildenden MTB aufwiesen. Die mit $>2 \times 10^5$ Zellen/cm^3 höchste Zellzahl magnetotaktischer Stäbchen wurde bei einer Sulfidkonzentration von 2 mmol/l erreicht. Stolz folgerte daraus, dass a) Sauerstoff und Sulfid die (vertikale) Verteilung der verschiedenen Morphotypen determinieren b) das Vorkommen magnetotaktischer Bakterien generell von der Verfügbarkeit von Eisen abhängt und c) MTB aufgrund der Präzipitation von Eisenmineralien eine Rolle in der Verteilung partikulären Eisens und der Magnetisierung der Sedimente spielen (Stolz 1992). Auf einen Beitrag von MTB bei der Magnetisierung der Sedimente deutet dabei auch die große Ähnlichkeit der bakteriellen Magnetitkristalle mit den in den Sedimenten gefundenen Partikel hin (Kirschvink und Lowenstam 1979; Kirschvink und Chang 1984; Petersen et al. 1986; Stolz et al. 1986; Chang et al. 1987; Karlin et al. 1987; Vali et al. 1987; von Dobeneck et al. 1987; Chang und Kirschvink 1989; Stolz 1990; Oldfield 1991).

In einer anderen Studie von Bazylinski und Mitarbeitern wurde die vertikale Verteilung magnetit- und greigitbildender MTB innerhalb dieses Habitats untersucht. Generell konnten mehr magnetitbildende MTB innerhalb und oberhalb der Übergangszone zwischen oxischer und anoxischer Zone (OATZ) gefunden werden, während die meisten greigitbildenden MTB im anoxisch-sulfidischen Bereich vorkamen. Mit zunehmender Wasser-

tiefe und steigender Sulfidkonzentration wurde in einem sowohl magnetit- als auch greigitbildendem Stäbchen eine Zunahme des Greigitanteils pro Zelle beobachtet, der auf einen direkten Einfluß der Umweltbedingungen auf die nichtmetallische Mineralphase der Magnetosomen hindeutet (Bazylinski et al. 1995).

Die vertikale Verteilung von MTB in Tiefseesedimenten aus dem Südatlantik wurde 1993 von Petermann und Bleil untersucht. MTB konnten dabei in den oberen Sedimentschichten bis zu <10 cm Tiefe gefunden werden, jedoch nicht in der Wassersäule. Die meisten MTB kamen in der anoxischen Zone vor, wo Nitrat verfügbar war. Petermann und Bleil gehen daher von einer Nitrat- oder Stickoxidreduktion in MTB aus, wobei in nitratlimitierten Mikrohabitaten möglicherweise alternativ Eisen als terminaler Elektronenakzeptor genutzt wird (Petermann und Bleil 1993).

In einem Review-Artikel von 1997 wurde von Bazylinski und Moskowitz die vertikale Verteilung magnetotaktischer Bakterien in der Wassersäule eines Brackwassersees (Salt Pond, USA) beschrieben. Im Gegensatz zum Pettaquamscutt River Estuary wurde im Salt Pond keine Überlappung der Sauerstoff- und Sulfidgradienten beobachtet. Innerhalb der OATZ und am oberen Ende des anoxisch-sulfidischen Hypolimnions konnten von Bazylinski und Moskowitz fünf verschiedene Morphotypen gefunden werden. Ähnlich wie in den Untersuchungen vom Salt Pond (Bazylinski et al. 1995) wurden die meisten magnetitbildenden MTB in der OATZ nachgewiesen, während die meisten greigitbildenden MTB in der sulfidischen, aber auch in der sulfidfreien anoxischen Zone des Hypolimnions vorkamen. Die Präsenz größerer Mengen magnetotaktischer Bakterien korrelierte sowohl mit der maximalen Menge gelösten Eisens als auch der Menge partikulären Eisens und bekräftigte die Bedeutung magnetotaktischer Bakterien im marinen Eisenzyklus (Bazylinski und Moskowitz 1997).

Die vertikale Verteilung von MTB eines limnischen Habitats wurde erstmals 1993 von Spring und Mitarbeitern untersucht. Dabei wurde gezielt die vertikale Verteilung eines aus dem Chiemsee stammenden und als “M. bavaricum“ bezeichneten MTB bestimmt. Dieses bisher unkultivierbare Bakterium unterscheidet sich von allen anderen bekannten MTB sowohl durch seine ungewöhnliche Zellgröße (bis zu 10 µm Länge und 2 µm Breite), Magnetosomenform und Magnetosomenanzahl (bis zu 1000 projektilförmige Magnetitpartikel pro Zelle) als auch durch seine phylogenetische Position innerhalb des *Nitrospira* Phylums. Zur Zellzahlbestimmung wurde “M. bavaricum“ aus verschiedenen Horizonten magnetisch angereichert und mit einer spezifischen Oligonukleotidsonde hybridisiert (Fluoreszenz *in situ* Hybridisierung - FISH). Unter Laborbedingungen konnte

„M. bavaricum“ weder im Oberflächenwasser noch innerhalb der obersten oxischen Sedimentschicht (<4 mm) gefunden werden. Obwohl „M. bavaricum“ in allen untersuchten anoxischen Sedimentschichten (bis 15 mm Tiefe) nachweisbar war, konnten mit 7×10^5 Zellen/cm^3 die meisten Zellen im Übergangsbereichs zwischen oxischer und anoxischer Zone (<20 µmol/l O_2) gefunden werden (Abb. 2).

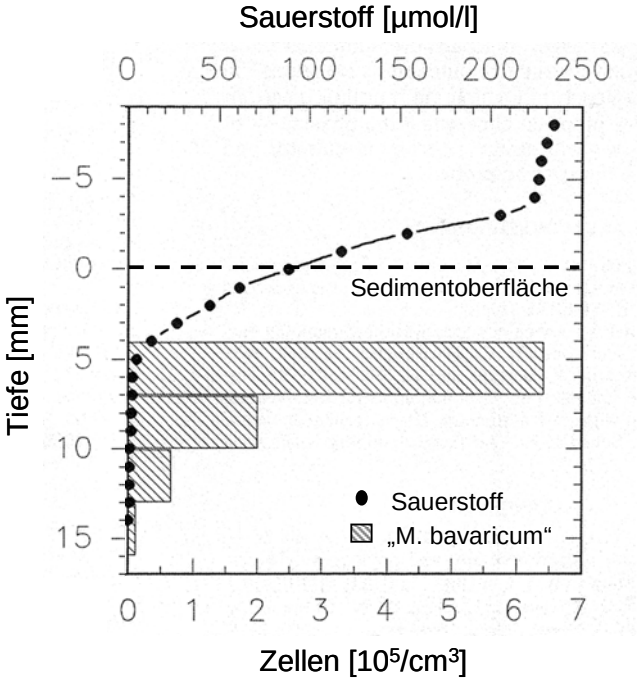

Abb. 2: Vertikale Verteilung von „M. bavaricum“ in Korrelation zur Sauerstoffkonzentration (Spring et al. 1993, modifiziert).

Aufgrund des großen Biovolumenanteils (ca. 30%), der sich aus der Abundanz (0,64 ± 0,17%) und dem Zellvolumen (durchschnittlich 25,8 ± 4,1 µm^3) zusammensetzt, wird “M. bavaricum“ eine dominante Rolle in der mikrobiellen Ökologie dieser Sedimentschicht zugesprochen. Obwohl in den untersuchten Sedimentschichten keine meßbaren Sulfidkonzentrationen vorhanden waren, schlugen Spring und Mitarbeiter aufgrund der intrazellulären Schwefeleinschlüsse, der hohen Magnetosomenzahl und der Position von “M. bavaricum“ innerhalb des Sauerstoffgradientens eine eisenabhängige Sulfidoxidation mit Sauerstoff als terminalen Elektronenakzeptor vor, wobei Magnetit als Nebenprodukt entsteht (Spring et al. 1993). Folglich würde die Bildung von Magnetit primär der Energiegewinnung dienen und nur sekundär der räumlichen Orientierung im Habitat. Allerdings konnte ein solcher Metabolismus bisher noch in keinem MTB nachgewiesen werden.

Darüber hinaus scheint eine Verbreitung dieses Stoffwechseltyps in anderen MTB aufgrund der geringen Magnetosomenanzahl von durchschnittlich 22 Partikeln pro Zelle und dem damit verbundenen geringen Energiegewinn nicht wahrscheinlich zu sein.

Anhand der in diesem Kapitel vorgestellten Ergebnisse lassen sich keine generellen Zusammenhänge zwischen der vertikalen Verteilung von MTB und einzelnen physiko-chemischen Parametern erkennen, so dass keine generellen Rückschlüsse auf die Ökologie und Physiologie von MTB gezogen werden können. Darüber hinaus deutet die unterschiedliche Positionierung diverser Morphotypen auf eine Anpassung verschiedener MTB Spezies an unterschiedliche Gradienten hin. Um mehr über den Einfluß verschiedener physiko-chemischer Parameter auf die Verteilung diverser MTB Spezies insbesondere in limnischen Systemen zu erfahren, bietet sich deshalb neben der Analyse weiterer Habitate vor allem eine detailliertere Untersuchung der vertikalen Verteilung verschiedener magnetotaktischer Morpho- bzw. Phylotypen an. Darüber hinaus verspricht die Kombination verschiedener Analysemethoden zur Bestimmung diverser physiko-chemischer Gradienten und mikrobieller Aktivitäten über die bisher durchgeführten Untersuchung hinaus neue Erkenntnisse zur Ökologie und Physiologie von bisher unkultivierbaren MTB, die zur Isolierung neuer MTB Spezies führen könnten.

5. Physiologie kultivierbarer magnetotaktischer Bakterien

Von verschiedenen marinen und limnischen Habitaten konnte bisher mehrere MTB Spezies isoliert werden (s. Tab. 1). Eine Gemeinsamkeit zwischen allen bisher kultivierten Stämmen besteht in ihrer Fähigkeit mittels einer aeroben oder anaeroben Atmung Energie zu gewinnen. Ein fermentatives Wachstum konnte hingegen bisher in keinem der untersuchten Stämme nachgewiesen werden (Bazylinski und Frankel 2004). Des weiteren können alle bisher isolierten MTB verschiedene organische Säuren als einzige Energie- und Kohlenstoffquelle nutzen (Blakemore et al. 1979; Blakemore et al. 1985; Matsunaga et al. 1991; Schleifer et al. 1991; Schüler et al. 1999; Heyen und Schüler 2003). Allerdings ließen sich zwischen den verschiedenen bisher isolierten MTB Spezies folgende Unterschiede feststellen.

Tab. 1 Verschiedene magnetotaktische Isolate von marinen und limnischen Habitaten.

Stamm	**Morphotyp**	**Habitat**	**Quelle**
Desulfovibrio magneticus (RS-1)	Vibrio	marin	Sakaguchi et al. 1993
Magnetospirillum gryphiswaldense (MSR-1)	Spirillum	limnisch	Schüler 1990
Magnetospirillum magneticum (AMB-1)	Spirillum	limnisch	Kawaguchi et al. 1992
Magnetospirillum magnetotacticum (MS-1)*	Spirillum	limnisch	Blakemore et al. 1979
MC-1	Kokkus	marin	Meldrum et al. 1993a
MSM-1, MSM-3, MSM-4, MSM-6, MSM-7, MSM-8 und MSM-9	Spirillum	limnisch	Schüler et al. 1999
MV-1	Vibrio	marin	Bazylinski et al. 1988
MV-2, MV-4	Vibrio	marin	Meldrum et al. 1993b

*früher *Aquaspirillum magnetotacticum* Umbenennung durch Schleifer et al. (1991)

Im Gegensatz zu allen anderen bisher isolierten MTB ist *D. magneticus* weder in der Lage Sauerstoff noch Nitrat als terminalen Elektronenakzeptor zu nutzen. Darüber hinaus stellt *D. magneticus* das einzige isolierte MTB dar, das Sulfat reduzieren und verschiedene Alkohole verwerten kann. *M. magneticum* unterscheidet sich von *M. magnetotacticum*, *M. gryphiswaldense* und allen marinen Isolaten durch das Unvermögen Distickstoffoxid (N_2O) als terminalen Elektronenakzeptor nutzen zu können (Mencke 2003). Eine Fixierung molekularen Stickstoff konnte bisher nur in *M. magnetotacticum*, *M. gryphiswaldense* und *M. magneticum* festgestellt werden (Bazylinski und Blakemore 1983; Bazylinski et al. 2000). Ein schwaches Wachstum mit Fe(III) unter anoxischen Bedingungen ließ sich lediglich bei *M. magnetotacticum* beobachten (Guerin und Blakemore 1992). Im Gegensatz zu allen anderen bisher isolierten MTB können MV-1, MV-2 und MC-1 alternativ mit Thiosulfat oder Sulfid als Elektronendonor wachsen, wobei intrazelluläre Schwefeleinschlüsse gebildet werden (Bazylinski et al. 1988; Meldrum et al. 1993a; Kimble und Bazylinski 1996; Frankel et al. 1997; Dean und Bazylinski 1999). MV-4 kann hingegen nur Thiosulfat verwerten (Kimble und Bazylinski 1996). Von MV-1 und MV-2 ist darüber hinaus bekannt, dass sie verschiedene Aminosäuren zur Energiegewinnung nutzen können. Insgesamt unterscheiden sich alle marinen Isolate von MTB aus limnischen Habitaten

durch ihre Fähigkeit autotroph mit Kohlendioxid zu wachsen. Die Fähigkeit von Polyhydroxybuttersäure (PHB) als intrazellulären Speicherstoff bilden zu können, ließ sich hingegen bisher nur in *M. magnetotacticum*, *M. gryphiswaldense* und *M. magneticum* nachweisen (Guerin und Blakemore 1992; Grünberg 2000; Wahyudi et al. 2003). In verschiedenen Kultivierungsexperimenten konnte auch eine unterschiedliche Toleranz gegenüber Sauerstoff beobachtet werden. So vermag das Katalase-positiv aber Oxidase-negative MTB *D. magneticus* als einziges Isolat nur unter strikt anaeroben Bedingungen zu wachsen, während beispielsweise *M. gryphiswaldense* (Katalase- und Oxidase-positiv) im Vergleich zu *M. magnetotacticum* (Katalase-negativ) höhere Sauerstoffkonzentrationen tolerieren kann (Maratea und Blakemore 1981; Schleifer et al. 1991). Durch die Zugabe von Katalase kann jedoch die Sauerstofftoleranz von *M. magnetotacticum* deutlich erhöht werden (Blakemore et al. 1979).

Gemessen an der großen morphologischen und phylogenetischen Diversität von MTB ist die Anzahl der bisher isolierten Arten jedoch äußerst gering. Darüber hinaus gehören alle bisher kultivierten MTB Spezies nicht zu den im Habitat abundanten Spezies (Blakemore et al. 1979; Bazylinski et al. 1988; Kawaguchi et al. 1992; Meldrum et al. 1993a; Meldrum et al. 1993b; Sakaguchi et al. 1993; Spring et al. 1993; Spring und Schleifer 1995). Aus diesem Grund und zur weiteren Untersuchung der Magnetitbiomineralisation in MTB sind weitere Isolierungsversuche zur Kultivierung neuer MTB zwingend erforderlich.

6. Eisenaufnahme

In Mikroorganismen spielt Eisen, neben seiner Funktion als potenzieller Elektronendonor bzw. Akzeptor (Lovley 1991b; Das et al. 1992; Widdel et al. 1993; Edwards et al. 2003), eine wichtige Rolle als anorganischer Bestandteil zahlreicher Häm- und Ferroproteine wie z. B. Cytochromen, Eisen-Schwefel-Proteinen, Oxygenasen, Peroxidasen und Katalasen (Brock und Madigan 1997) und kann beispielsweise in *Escherichia coli* 0,014% des Trockengewichts ausmachen (Moench und Konetzka 1978). Die Verfügbarkeit von Eisen wird entscheidend durch dessen Oxidationsstufe bestimmt (Hughes und Poole 1991). Während Fe(II) sehr gut löslich ist (bis zu 100 mmol/l bei neutralem pH-Wert, Neilands 1984), löst sich Fe(III) nur schlecht in Wasser ($<10^{-8}$ mol/l, Thamdrup 2000). Fe(II) entsteht entweder biologisch durch Fe(III)-reduzierende Bakterien oder chemisch durch die

reduzierende Wirkung verschiedener organische Verbindungen oder Sulfid und kann im Porenwasser Konzentrationen von bis zu 1 mmol/l erreichen (Aller et al. 1986; Ghiorse 1988; Lovley 1991a; Luther et al. 1992; Canfield et al. 1993; Lovley 1993; Thamdrup et al. 1994). Aufgrund der schnellen Reoxidation beträgt die Halbwertzeit von Fe(II) in aeroben, aquatischen Habitaten jedoch nur wenige Minuten, so dass freies, gelöstes Fe(II) in diesen Bereichen für Mikroorganismen limitiert ist (Millero et al. 1987). Zur Abdeckung ihres Eisenbedarfs exkretieren daher viele Mikroorganismen verschiedene Siderophore, die Fe(III) komplexieren können und damit für eine verbesserte Löslichkeit des Eisens sorgen (Neilands 1981; Martinez et al. 2000).

In MTB kann der intrazelluläre Eisengehalt bis zu 4% des Trockengewichts betragen, wobei über 90% des Eisens auf die Magnetosomen entfallen (Moench und Konetzka 1978; Towe und Moench 1981; Schüler und Baeuerlein 1998; Grünberg 2000; Heyen und Schüler 2003; Schüler 2004). Im Vergleich zum extrazellulären Milieu stellt dies eine 20000 bis 40000fache Anreicherung von Eisen dar (Blakemore 1982). Folglich müssen MTB über ein effizientes Aufnahmesystem verfügen. Darüber hinaus ist aufgrund der Toxizität hoher Eisenkonzentrationen eine strenge Regulation der Eisenaufnahme nötig (Guerinot 1994). MTB können Eisen sowohl in Form von Fe(II) als auch Fe(III) aufnehmen (s. Abb. 3). Im Gegensatz zu *M. magnetotacticum* und MV-1 wurde in *M. gryphiswaldense* bisher jedoch nur von einer Eisenaufnahme in Form von Fe(III) über einen energieabhängigen Prozess ausgegangen (Schüler 2002). Allerdings konnten in *M. gryphiswaldense* bisher keine Siderophore gefunden werden. Verschiedene Experimente mit Kulturüberständen lassen jedoch auf die Existenz eines effizienten Eisenaufnahmesystems schließen (Schüler und Baeuerlein 1996). Neuere Untersuchungen deuten darüber hinaus auch auf eine Fe(II)-Aufnahme in diesem MTB hin (Udo Heyen, persönliche Mitteilung). In *M. magnetotacticum* konnten Paoletti und Blakemore (Paoletti und Blakemore 1986) die Bildung eines Siderophors des Hydoxamattyps nachweisen. Die Exkretion eines Hydroxamat- und eines Catecholsiderophors wurde 2003 von Calugay und Mitarbeitern (2003) in *M. magneticum* beobachtet. In letzter Zeit konnte die Existenz eines Siderophors auch in MV-1 nachgewiesen werden, wobei es sich wahrscheinlich ebenfalls um ein Siderophor des Hydroxamattyps handelt (Bazylinski und Frankel 2004). Eine Sättigung der Eisenaufnahme tritt in *M. gryphiswaldense* bei maximaler Magnetitbiomineralisation im Bereich von 15 bis 20 µmol/l Eisen auf (Schüler und Baeuerlein 1996).

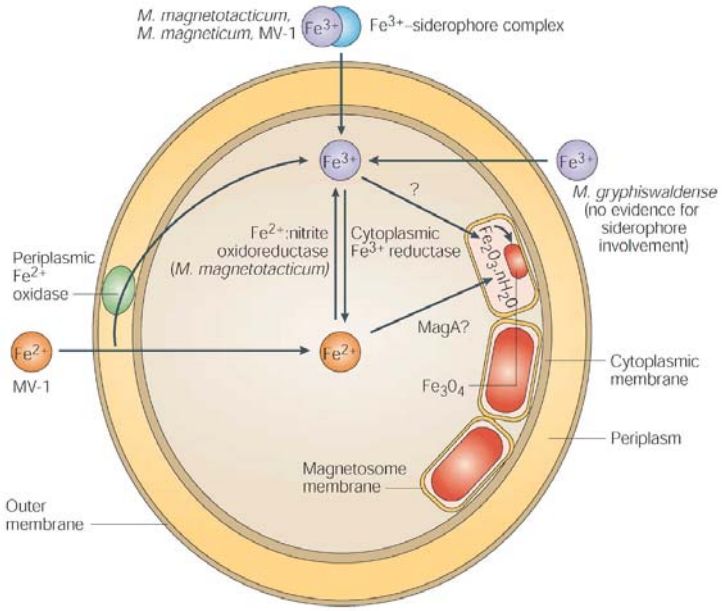

Abb. 3: Schematische Darstellung der Eisenaufnahme und Magnetitbiomineralisation in MTB (Bazylinski und Frankel 2004). Eisen wird dabei entweder in Form von Fe(III) über einen energieabhängigen Schritt (*M. gryphiswaldense*) bzw. verschiedene Siderophore (*M. magnetotacticum*, *M. magneticum* MV-1) oder als Fe(II) in die Zelle aufgenommen. Mittels verschiedener Proteine wird Eisen in die Magnetosomen transportiert, wo - vermutlich über Ferrihydrit als Zwischenprodukt - Magnetit gebildet wird.

7. Methoden zur Anreicherung und Isolierung von Mikroorganismen

7.1. Konventionelle Methoden zur Anreicherung und Isolierung von Mikroorganismen

In konventionellen Anreicherungs- und Kultivierungsansätzen werden Mikroorganismen durch die Zugabe verschiedener Substrate zu einer Umweltprobe (substratinduzierte Anreicherung) und/oder durch die Verwendung von Verdünnungsreihen isoliert. Während es bei einer substratinduzierten Anreicherung vor allem zur Isolierung schnellwachsender Mikroorganismen kommt, zielt die Verwendung von Verdünnungsreihen auf die Isolierung der am häufigsten vorkommenden, möglicherweise aber langsam wachsenden Spezies ab. Wie verschiedene mikroskopische und molekularbiologische Untersuchungen diverser Habitate belegen, konnten mit konventionellen Kultivierungsmethoden bisher jedoch nur ein geringer Anteil (0,001 bis 1%) aller in der Natur vorkommenden Mikroorganismen kultiviert werden (Jannasch und Jones 1959; Jones 1977; Kogure et al. 1980; Amann et al.

1995; Torsvik und Ovreas 2002). Darüber hinaus gehören die meisten der bisher isolierten Stämme nicht zu den im Habitat abundanten Mikroorganismen (Giovannoni et al. 1990; Ward et al. 1990; Schmidt et al. 1991; Pinhassi et al. 1997; Wieringa et al. 2000).

Mögliche Gründe für die geringe Kultivierungseffizienz können sowohl in der Zusammensetzung des Mediums als auch in der Höhe der eingesetzten Substratkonzentrationen bestehen. So können z. B. zu hohe Nährstoffkonzentrationen zum Absterben von Mikroorganismen führen (Postgate und Hunter 1964). Des weiteren können Zellen im Ruhezustand oder in einem als „viable but nonculturable“ bezeichneten Status vorliegen, die ein Anwachsen der Zellen in konventionellen Medien nicht erlaubt (Roszak und Colwell 1987b; Roszak und Colwell 1987a; Bruns et al. 2002). Außerdem berücksichtigen konventionelle Kultivierungsmethoden nur selten die unter natürlichen Bedingungen vorhandenen diversen chemischen Gradienten und komplexen Wechselwirkungen zwischen verschiedenen Organismen wie z. B. Zellkommunikation und Synthrophien (Mann et al. 1990a; Caldwell et al. 1997; Bruns et al. 2002; Bruns et al. 2003). Die Kultivierung von MTB wird darüber hinaus noch durch ihre physiologische Diversität erschwert, da sie eine gezielte Anreicherung bzw. Isolierung dieser Organismen mit selektiven Medien beeinträchtigt. Lediglich im Fall von *D. magneticus* konnte bisher über eine konventionelle (substratinduzierte) Anreicherung die Isolierung eines MTB erreicht werden (Sakaguchi et al. 1993). Darüber hinaus sind MTB in allen bisher untersuchten Habitaten nicht abundant genug um über die alleinige Verwendung von Verdünnungsreihen isoliert werden zu können. Eine wesentliche Voraussetzung für die Kultivierung von MTB besteht deshalb in der Verwendung alternativer Anreicherungs- und Isolierungsmethoden.

7.2. Innovative Methoden zur Anreicherung und Isolierung von Mikroorganismen

Eine Möglichkeit verschiedene Mikroorganismen voneinander zu separieren, besteht in der Durchflußzytometrie. Bei dieser Methode werden die Zellen einem Laserstrahl ausgesetzt und anhand ihrer Streuungseigenschaften sortiert (Amann et al. 1990; Spring et al. 1993). Die so voneinander getrennten Zellen können anschließend in verschiedenen Kultivierungsansätzen isoliert werden. Zellen mit sehr ähnlicher Größe und Morphologie können allerdings häufig nur über eine zuvor durchgeführte Fluoreszenzmarkierung unterschieden werden, der jedoch eine Abtötung der Zellen vorausgeht. Deshalb eignet sich diese Variante der Durchflußzytometrie zwar für diverse molekularbiologische Studien

jedoch nicht für die Isolierung von Mikroorganismen. Aufgrund ihrer gerichteten Bewegung in Magnetfeldern bietet sich bei MTB allerdings auch eine mechanisch-physikalische Anreicherung der Zellen an (Moench und Konetzka 1978). Dabei werden die Zellen durch Anlegen eines Magnetfeldes so manipuliert, dass sie sich entweder an einem Punkt in der Wassersäule ansammeln oder dass sie mittels einer mechanischen Barriere von nicht-magnetotaktischen Bakterien getrennt werden (Moench und Konetzka 1978; Wolfe et al. 1987; Spring et al. 1992; Spring 1993b; Schüler et al. 1999). Ähnlich wie bei der Durchflußzytometrie können die so gewonnenen MTB als Inokulum in verschiedenen Kultivierungsexperimenten eingesetzt werden, wobei etwaige nicht-magnetotaktische Kontaminanten durch die Verwendung von Verdünnungsreihen eliminiert werden.

Eine vergleichsweise neue Methode stellt die Kultivierung verschiedener Mikroorganismen in „Microdroplets“ dar. Dabei werden einzelne Zellen in Geltropfen immobilisiert. Diese werden anschließend in einem Fermenter inkubiert und kontinuierlich mit einer gleichbleibend konzentrierten Nährlösung versorgt, wobei etwaige freilebende Mikroorganismen ausgewaschen werden. Über die Durchflußzytometrie werden anschließend die Geltropfen nach den Streuungseigenschaften der in ihnen herangewachsenen Mikroorganismen getrennt und stehen so weiteren Kultivierungsexperimenten oder molekularbiologischen Untersuchung zu Verfügung (Zengler et al. 2002).

Zur Simulierung natürlicher Umweltbedingungen bietet sich alternativ eine andere Isolierungstechnik an, die auf der Verwendung einer speziellen Diffusionskammern basiert. Dabei werden die Zellen mittels einer dünnen Agarschicht immobilisiert und zwischen zwei Polykarbonatmembranen fixiert. Durch Inkubation auf der Sedimentoberfläche bleiben der Substrataustausch und die Zellkommunikation zwischen den Zellen und dem Habitat weiter bestehen (Kaeberlein et al. 2002).

8. Kultivierungsunabhängige Methoden zur Untersuchung von Mikroorganismen

8.1. Methoden zur Bestimmung der mikrobiellen Diversität

Seit den Arbeiten von Woese und Fox werden vergleichende Sequenzanalysen von rRNA Genen (16S und 23S rRNA) zur Bestimmung von phylogenetischen Verwandschaftsbeziehungen von Prokaryonten genutzt (Woese und Fox 1977). Diese Gene zeichnen sich durch ihre universelle Verbreitung in allen Mikroorganismen und ihre

Unterteilung in konservierte und variable Bereiche aus, die sowohl die Bestimmung der Verwandtschaftsverhältnisse von entfernt als auch näher verwandten Organismen erlauben. Aufgrund des geringeren Aufwands und der geringeren Kosten hat sich die Analyse der 16S rDNA als phylogenetischer Marker durchgesetzt, obwohl die 23S rDNA aufgrund ihrer Länge (etwa 2900 statt ca. 1500 Nukleotiden) und der größeren Variabilität mehr Sequenzinformationen bietet und sich damit prinzipiell besser für eine detailliertere Auflösung insbesondere bei besonders nah verwandten Arten eignet (Ludwig et al. 1992; Ludwig und Schleifer 1994; Ludwig et al. 1995). Da Arten mit sehr ähnlichen oder identischen 16S rRNA Sequenzen deutliche geno- und phänotypische Unterschiede aufweisen können (Perna et al. 2001), werden bei der Bestimmung der Verwandschaftsbeziehungen mitunter auch verschiedene andere molekularbiologische Techniken eingesetzt (z. B. enterobacterial repetitive intergenic consensus – ERIC-PCR) (Jaspers et al. 2001).

Die Einführung verschiedener molekularbiologischer Methoden in die mikrobielle Ökologie ermöglichte neben der Bestimmung der phylogenetischen Stellung von Reinkulturen erstmals auch die phylogenetische Einordnung bisher unkultivierbarer Organismen (Olsen et al. 1986; Giovannoni et al. 1988; Eden 1991; Burgess et al. 1993; DeLong et al. 1993; Muyzer et al. 1993; Amann et al. 1995). Zur Strukturanalyse und Bestimmung der mikrobiellen Diversität von Umweltproben wird meist die auf dem 16S rRNA Ansatz basierende quantitative Fluoreszenz *in situ* Hybridisierung **(**FISH) eingesetzt (Stahl et al. 1985; Olsen et al. 1986; Amann et al. 1995). Dabei werden verschiedene fluoreszenz-markierte Oligonukleotidsonden verwendet, die z. B. gattungs-, art oder unterartspezifisch an bestimmte 16S rRNA Abschnitte binden und so eine Identifizierung der Zielorganismen ermöglichen (Göbel et al. 1987; Giovannoni et al. 1988; Stahl und Amann 1991).

Andere auch als „Fingerprinting“-Technik bezeichnete Methoden, die auf der Analyse PCR-amplifizierter DNA-Fragmente beruhen (denaturierende Gradienten Gelelektrophorese – DGGE, amplified ribosomal DNA restriction analysis - ARDRA), eignen sich besonders gut für die Untersuchung räumlicher oder zeitlicher Populationsverschiebungen, da sie vor allem einen schnellen Einblick in die Komplexität einer Umweltprobe liefern (Muyzer et al. 1993; Martinez et al. 1995). Bei der DGGE werden PCR amplifizierte DNA-Fragmente direkt anhand ihrer Sequenzunterschiede in einem denaturierenden Gel aufgetrennt. Durch das Ausschneiden der Banden und der anschließenden Reamplifikation der einzelnen DNA-Fragmente ist eine Sequenzierung und damit eine phylogenetische Einordnung der detektierten Mikroorganismen möglich. Im Gegensatz zur DGGE werden bei ARDRA die PCR-Produkte mittels Restriktionsenzymen geschnitten

und in einem Agarosegel nach ihrer Länge aufgetrennt. Die erhaltenen DNA-Fragmente lassen sich aufgrund fehlender definierter Primerbindungsstellen jedoch nicht direkt sequenzieren. Um dieses Problem zu umgehen, können die PCR-Produkte zunächst kloniert und erst nach einem weiteren Amplifikationsschritt geschnitten und aufgetrennt werden, so dass anhand der Fragmentmuster einzelne Klone ausgewählt und sequenziert werden können. Bei beiden Methoden kann es jedoch aufgrund der Existenz mehrerer sich unterscheidender rRNA Operone innerhalb eines Organismus zu einer Überschätzung der phylogenetischen Diversität kommen (Nübel et al. 1996; Rainey et al. 1996; Amann et al. 2000a; van Berkum et al. 2003). Ein anderes Problem stellt die Bildung von Mischsequenzen dar, die aus zwei verschiedenen Sequenzen zusammengesetzt sind und so ebenfalls zu einer Fehleinschätzung der Diversität führen können. Diese als Chimären bezeichneten Sequenzen können während der PCR durch einen vorzeitigen Abbruch der Polymerisation entstehen, wobei das unvollständige PCR-Produkt mit dem Gegenstrang eines nicht identischen Amplifikats hybridisiert (Wang und Wang 1996; Hugenholtz und Huber 2003). Des weiteren eignen sich beide Methoden nicht für eine detaillierte Diversitätsanalyse, da ARDRA nur Unterschiede in den Restriktionsschnittstellen erkennt und die DGGE nur die Auftrennung kleiner DNA-Fragmente (<700 Basenpaaren) erlaubt. Darüber hinaus können beide Methoden aufgrund ihrer Selektivität nicht für quantitative Analysen genutzt werden. Die DNA-Isolierung, die PCR-Amplifikation und im Fall von ARDRA auch die Klonierung kann die Präsenz bestimmter Sequenzen so stark beeinflussen, dass von der Häufigkeit bestimmter Sequenzen z. B. in einer Genbibliothek keine Rückschlüsse auf die Abundanz dieser Organismen im Habitat gezogen werden können (Reyensbach et al. 1992; Muyzer et al. 1993; Farrelly et al. 1995; Suzuki und Giovannoni 1996).

Ein generelles Problem des 16S rDNA Ansatzes besteht jedoch bei der Untersuchung phylogenetisch sehr diverser Organismengruppen, die eine Entwicklung gruppenspezifischer Primer und Sonden nicht zulassen. So konnte zwar in verschiedenen Experimenten die Verbreitung und Abundanz einzelner bzw. mehrerer eng verwandter MTB Spezies über spezifische 16S rDNA-Primer und Oligonukleotidsonden untersucht werden (Spring et al. 1992; Spring et al. 1994; Thornhill et al. 1995; Spring et al. 1998), jedoch ließen sich aufgrund ihrer 16S rDNA Divergenz keine alle magnetotaktischen Bakterien erfassenden universellen Primer oder Oligonukleotidsonden konstruieren. Im Gegensatz zu FISH bieten PCR-basierende Techniken jedoch eine Möglichkeit alternativ zum 16S bzw. 23S rRNA Ansatz die Verbreitung funktioneller Organismengruppen, wie sie

z. B. auch MTB darstellen, in verschiedenen Habitaten mittels funktioneller Gene (Markergene) zu untersuchen. Die Untersuchung von Markergenen liefert darüber hinaus Informationen zur Diversität der untersuchten Gene. Darüber hinaus liefert der rRNA Ansatz zumeist keine Informationen über den Stoffwechsel und die Aktivität der Organismen (Fry 1990; Karner und Fuhrman 1997; Spring et al. 2000). Die Zuordnung bestimmter Stoffwechselaktivitäten zu einzelnen Mikroorganismen ist durch die Kombination von Mikroautoradiographie und FISH (MAR-FISH) möglich. Dabei können innerhalb eines Ansatzes sowohl die physiologisch aktiven Zellen über die Aufnahme radioaktiver Substrate markiert werden als auch ihre phylogenetische Identität mittels FISH bestimmt werden (Karner und Fuhrman 1997; Lee et al. 1999; Ouverney und Fuhrman 1999; Gray et al. 2000; Ito et al. 2002; Adamczyk et al. 2003; Kindaichi et al. 2004).

8.2 Genomische Untersuchungen unkultivierter Mikroorganismen

Bei der Untersuchung der genetische Ausstattung von bisher unkultivierbaren Mikroorganismen gewinnt die Genomforschung gegenüber herkömmlichen Klonierungstechniken zunehmend an Bedeutung. Der Vorteil des genomischen Ansatzes besteht dabei in der direkten Klonierung verschiedener DNA-Fragmente ohne vorherige Amplifikation und ermöglicht darüber hinaus die Analyse ganzer Operonstrukturen. Obwohl der Nachweis eines Gens keinerlei Aussagen über dessen Expression und die Aktivität des entsprechenden Enzyms erlaubt, stellt die genomische Analyse aufgrund der Identifizierung potenzieller Stoffwechselwege einen interessanten Ansatz zur Entwicklung neuer Strategien zur Isolierung bisher nicht kultivierbarer Mikroorganismen dar (Torsvik und Ovreas 2002). Darüber hinaus bietet der genomische Ansatz aber auch eine Möglichkeit die Diversität verschiedener funktioneller Gene zu analysieren und über weiterführende Untersuchungen wie z. B. verschiedenen Expressionsstudien oder Komplementierungsversuche mehr über die Funktion dieser Gene zu erfahren.

Bei der metagenomischen Analyse eines Habitats werden große Genomfragmente (>20 Kb) erzeugt, welche theoretisch der Gesamtheit aller im Habitat vorhanden Genome entsprechen, und in Cosmid- oder Fosmid-Vektoren oder „bacterial artificial chromosomes“ (BAC) kloniert. Diese Vektoren werden anschließend in einen leicht zu kultivierenden Mikroorganismus wie *E. coli* transformiert bzw. transduziert (Wahl et al. 1987;

Kim et al. 1992; Shizuya et al. 1992; Handelsman et al. 1998). Die Wahl des am besten geeigneten Vektors hängt von der Qualität und Quantität des Ausgangsmaterials und vom Untersuchungsschwerpunkt ab und stellt immer einen Kompromiss zwischen der Effizienz der Klonierung, der Stabilität der rekombinanten DNA und der Fragmentgröße dar. Aufgrund der heterogenen Zusammensetzung der meisten Umweltproben ist im Gegensatz zur Genomanalyse von Reinkulturen eine Zuordnung der erhaltenen Sequenzinformationen und den daraus abzuleitenden potenziellen Eigenschaften zu bestimmten Mikroorganismen meist nur über die Nachbarschaft zu phylogenetischen Markern möglich, wie sie beispielsweise die 16S rDNA darstellt (Stein et al. 1996; Béjà et al. 2000a; Béjà et al. 2000b). Alternativ ist eine solche Zuordnung aber auch über die Identifikation verschiedener anderer Markergene möglich. Allerdings können die über die Untersuchung von Markergenen bestimmten Verwandschaftsverhältnisse von der auf 16S rDNA Analysen basierenden Phylogenie stark abweichen. Vermutlich hängt dies mit dem horizontalem Transfer funktioneller Gene zusammen, wie es beispielsweise für die Gene der dissimilatorischen Sulfitreduktase (*dsrAB)* diskutiert wird (Klein et al. 2001).

9. Zielsetzung

Magnetotaktische Bakterien sind eine ubiquitär in aquatischen Habitaten verbreitete physiologisch und phylogenetisch diverse Gruppe von Bakterien, welche die Fähigkeit verbindet, sich im magnetischen Feld orientieren zu können. In der vorliegenden Arbeit sollten über eine Kombination verschiedener Isolierungsversuche und kultivierungsunabhängiger Experimente sowohl die Verbreitung und Diversität von magnetotaktischen Bakterien und der an der Magnetitbiomineralisation beteiligten Gene untersucht werden, als auch neue Erkenntnisse zur Ökologie dieser Organismen gewonnen werden. Deshalb sollte in dieser Arbeit zunächst die Verbreitung von MTB in verschiedenen limnischen und marinen Habitaten in Norddeutschland untersucht werden.

Um den Einfluß verschiedener Umweltbedingungen auf die Verteilung von MTB zu untersuchen und verschiedene Rückschlüsse auf die Physiologie und Ökologie von MTB ziehen zu können, sollte im Rahmen dieser Arbeit in einem Modellsystem (Süßwassermikrokosmen) die vertikale Verteilung von MTB mit verschiedenen physiko-chemischen Parametern korreliert werden. Dazu sollten über kultivierungsunabhängige Versuche die Zellzahlen und Abundanzen von MTB und mittels verschiedener Mikrosensormessungen und geochemischer Analysen das pH-Profil und die Verteilung von Sauerstoff, Nitrat, Sulfat Sulfid, Eisen, Sulfat- und Eisen-Reduktionsraten zeitnah bestimmt werden.

Im Hinblick auf eine anschließende Isolierung und molekularbiologische Charakterisierung von bisher unkultivierbaren MTB sollte in einem weiteren Versuchsansatz vor allem über kultivierungsunabhängige Methoden die Selektivität verschiedener magnetischer Anreicherungstechniken analysiert werden. Zusätzlich sollte die Dynamik der MTB Population innerhalb verschiedener Mikrokosmen über kultivierungsunabhängige Analysen langfristig untersucht werden.

Aufgrund der geringen Anzahl von Reinkulturen magnetotaktischer Bakterien sollten im Rahmen dieser Arbeit systematische Versuche zur Isolierung neuer MTB Spezies unternommen werden.

Neben der Isolierung neuer MTB sollte in dieser Arbeit über einen kultivierungsunabhängigen Ansatz wie der 16S rDNA Analyse die Diversität von bisher nicht kultivierbaren MTB be-

stimmt werden. Zusätzlich sollten zur Untersuchung der Verbreitung und Diversität der an der Magnetitbiomineralisation beteiligten Gene die Voraussetzungen für die Analyse einzelner Gene und die Klonierung subgenomischer Fragmente von bisher unkultivierbaren MTB geschaffen werden. Die Analyse einer solchen Genbank könnte neben zusätzlichen Informationen zur Verbreitung, Diversität und genomischen Organisation der *mam* Gene vor allem eine Möglichkeit bieten, die metabolischen Fähigkeiten dieser Bakterien zu rekonstruieren und auf diese Weise neue Strategien zur Isolierung bisher unkultivierbarer MTB zu entwickeln.

B Ergebnisse und Diskussion

1. Untersuchungen zur Verbreitung und Diversität magnetotaktischer Bakterien

In Rahmen dieser Arbeit sollte die Verbreitung und Diversität magnetotaktischer Bakterien (MTB) in verschiedenen Gewässern Norddeutschlands untersucht werden. Anhand ihrer Fähigkeit sich im magnetischen Feld orientieren zu können, lassen sich MTB leicht nachweisen (s. Manuskript 1 und 2). Der Vorteil des magnetischen Nachweises von MTB im „hängenden Tropfen“, bei der sich von wenigen Ausnahmen (<1%) abgesehen alle MTB an der dem magnetischen Südpol zugewandten Seite des Tropfens ansammeln, besteht gegenüber konventionellen Methoden darin, dass nur vitale Zellen erfaßt werden. Allerdings beschränkt sich diese Technik auf schnell schwimmende MTB, so dass schwach bewegliche (Amann et al. 2000b), gleitende (Blakemore 1982), durch Pili oder Fimbrien an Partikel anhaftende (Frankel et al. 1997; Cox et al. 2002) oder sich im magnetosomenfreien Zustand befindende MTB nicht oder nur in geringerem Maße erfaßt werden können.

Wie in Manuskript 1 dargestellt, konnten in fast allen in dieser Arbeit untersuchten Proben verschiedene MTB (Kokken, Spirillen, Vibrionen, Stäbchen und multizelluläre Aggregate) nachgewiesen werden. Lediglich in einigen stark eutrophierten Habitaten ließen sich keine MTB beobachten. Die Präsenz magnetotaktischer Bakterien sowohl in geographisch getrennten Gebieten als auch in verschiedenen Habitaten, die sich zum Teil durch ihren pH-Wert, Salzgehalt, Korngröße oder Anteil organischer Bestandteile wie z. B. Blätter und Zweige deutlich voneinander unterschieden, bestätigt das von anderen Autoren beschriebene ubiquitäre Vorkommen von MTB (Blakemore 1975; Moench und Konetzka 1978; Blakemore et al. 1980; Blakemore und Frankel 1981; Frankel et al. 1981; Sparks et al. 1986; Stolz et al. 1986; Oberhack et al. 1987; Matitashvili und Matojan 1989; Petersen et al. 1989; Sparks et al. 1989; Adamkiewicz et al. 1991; Stolz 1992; Stolz 1993; Bazylinski et al. 1995; Bazylinski und Moskowitz 1997; Riemann et al. 1999; Cox et al. 2002). In allen untersuchten MTB konnte eine cluster- oder kettenförmige Anordnung der Magnetosomen gefunden werden. Analog zu früheren Untersuchungen (Balkwill et al. 1980; Towe und Moench 1981; Torres de Araujo et al. 1986; Bazylinski et al. 1988; Blakemore und Frankel 1989; Spring et al. 1998; Schüler 1999) besaßen die meisten Magnetosomen eine Partikelgröße von 30 bis 120 nm. Eine Ausnahme stellte ein MTB mit 155 nm großen Magnetosomen dar (s. Manuskript 3). Partikel dieser Größe befinden sich normalerweise oberhalb des zur Zellorientierung optimalen „single-domain“-Bereichs. Neuere Untersuchungen belegen jedoch,

dass sich auch größere Magnetosomen aufgrund der magnetischen Interaktion zwischen kettenförmig angeordneten Kristallen wie „single-domain“-Partikel verhalten können (McCartney et al. 2001). Diese Ergebnisse unterstützen die Hypothese, dass auch diese Magnetosomen der räumlichen Orientierung im Habitat dienen.

Obwohl die magnetische Anreicherungen im „hängenden Tropfen“ eine schnelle und effiziente Möglichkeit darstellt, die Verbreitung und morphologische Diversität von MTB zu untersuchen, erlaubt sie keine Aussagen über die phylogenetische Stellung dieser Organismen. Aufgrund ihrer häufig sehr geringen Abundanz im natürlichen Habitat werden für die Untersuchung von MTB meist Anreicherungen in Mikrokosmen ohne Substratzugabe verwendet. Die Ergebnisse der in Manuskript 1 und von anderen Autoren durchgeführten Versuche (Moench und Konetzka 1978; Vali et al. 1987; Sparks et al. 1989) zeigten jedoch, dass diese Anreicherung selektiv ist, da es dabei nur zu einer Massenentwicklung einzelner MTB Spezies kommt bei einer gleichzeitigen Abnahme der Diversität. Abgesehen von sehr sandigen Süßwassermikrokosmen, die sich durch eine größere morphologische Vielfalt der MTB Population von anderen Mikrokosmen unterschieden, wurden die meisten der in dieser und in anderen Arbeiten untersuchten Mikrokosmen von magnetotaktischen Kokken dominiert (Moench und Konetzka 1978; Sparks et al. 1986; Sparks et al. 1989). Die Dominanz magnetotaktischer Kokken in den Anreicherungen erklärt, warum bisher nur wenige Sequenzen anderer Morphotypen bekannt sind. Wie verschiedene 16S rDNA Analysen gezeigt haben, gehören alle bisher phylogenetisch untersuchten magnetotaktischen Kokken einem einzigen Cluster innerhalb der „Alphaproteobakterien“ an. (Spring et al. 1992; Spring et al. 1994). Auch alle in dieser Arbeit identifizierten magnetotaktischen Kokken konnten diesem Cluster zugeordnet werden, wobei sich zwischen den einzelnen Spezies Sequenzunterschiede von bis zu 11% feststellen ließen. Überraschenderweise wurden selbst zwischen Kokken verschiedener Entwicklungsstadien eines Mikrokosmos Sequenzunterschiede von bis zu 7% beobachtet. (s. 16S rDNA Stammbaum Manuskript 1). Eine Besonderheit stellte die zwischenzeitliche Massenentwicklung eines nah mit „M. bavaricum“ verwandten magnetotaktischen Stäbchens (MHB-1) in einem Mikrokosmos mit Sedimenten eines Badesees in Bremen-Walle dar. Die Abundanz dieses Bakteriums in magnetisch angereicherten Proben konnte mit einer ursprünglich spezifisch für „Magnetobacterium bavaricum“ entwickelten Oligonukleotidsonde belegt werden. Darüber hinaus deutet die sowohl in MHB-1 als auch in „M. bavaricum“ beobachtete Präsenz projektilförmiger Magnetosomen auf eine generelle Verbreitung dieses Kristalltyps in MTB des *Nitrospira* Phylums hin. Eine gezielte Anreicherung dieser oder anderer MTB Spezies ließ sich jedoch bisher in keinem der unter-

suchten Mikrokosmen erreichen. Vermutlich bestimmen kleine und örtlich begrenzte Unterschiede in der (chemisch-physikalischen) Zusammensetzung der Habitate die Entwicklung bestimmter MTB Spezies (Mann et al. 1990a).

Der Nachweis von MHB-1 als zweiten magnetotaktischen Vertreter des *Nitrospira* Phylums und die Vielzahl morphologisch unterscheidbarer, molekularbiologisch meist aber noch nicht identifizierter MTB Spezies deuten auf eine weit größere als in dieser und anderen Arbeiten (Spring et al. 1992; Spring et al. 1994; Riemann et al. 1999; Cox et al. 2002) bestimmten phylogenetischen Diversität von MTB hin. Da die Inkubation von MTB in Mikrokosmen generell zu einer Abnahme der Diversität führte und die anschließende molekularbiologische Untersuchung der verbliebenen MTB Spezies folglich nur einen kleinen Ausschnitt der natürlichen Diversität von MTB wiedergeben kann, sollte in zukünftigen Experimenten vor allem die phylogenetische Klassifizierung von MTB aus Umweltproben weiter vorangetrieben werden. Eine Möglichkeit MTB ohne vorherige Anreicherung zu separieren, besteht dabei in der Durchflußzytometrie bei der die verschiedenen Mikroorganismen anhand ihrer Streuungseigenschaften voneinander getrennt werden (Amann et al. 1990; Spring et al. 1993). Die anschließende phylogenetische Klassifizierung dieser Organismen könnte möglicherweise zur Identifizierung neuer bisher unbekannter phylogenetischer Linien von MTB führen und so neue Erkenntnisse über die Verbreitung und den Ursprung der Magnetotaxis liefern. Wie weit es sich bei der Magnetotaxis wie von DeLong und Mitarbeitern (1993) diskutiert um eine polyphyletische Entwicklung handelt oder um horizontalen Gentransfer, könnte jedoch nur über verschiedene Experimente geklärt werden, die sowohl eine Identifizierung verschiedener magnetosomenspezifischer Gene erlauben würde als auch ihre Zuordnung zu einzelnen MTB Spezies.

2. Evaluation verschiedener magnetischer Anreicherungsmethoden

Da MTB trotz ihrer Anreicherung in Mikrokosmen nur maximal 1% der Gesamtzellzahl ausmachen und dies auch nur in eng begrenzten Sedimentschichten (Spring et al. 1993; Manuskript 2), ist für die Isolierung und kultivierungsunabhängige Charakterisierung von MTB eine zusätzliche Anreicherung nötig. Aus diesem Grund wurden verschiedene magnetische Methoden zur Separierung von MTB eingesetzt. In den bisherigen Arbeiten wurde die Reinheit der einzelnen magnetischen Anreicherungsmethoden meist nur über Mikroskopie und Kultivierungsversuche und in seltenen Fällen über Fluoreszenz *in situ* Hybridisierung (FISH) untersucht (Moench und Konetzka 1978; Moench 1988; Spring et al. 1992; Schüler et

al. 1999). Ein Ziel dieser Arbeit war es, die Selektivität und Effizienz diverser magnetischer Methoden zur Anreicherung von MTB über verschiedene Isolierungsexperimente und kultivierungsunabhängige Methoden zu bestimmen und miteinander zu vergleichen, um so die jeweils optimalen Anreicherungsbedingungen für beispielsweise die Isolierung oder die Erstellung einer Genbank von bisher unkultivierbaren MTB zu ermitteln. Die in Manuskript 1 ausführlicher diskutierten Ergebnisse dieser Untersuchungen sollen hier noch einmal kurz dargestellt werden.

Die Kombination verschiedener mikroskopischer Untersuchungen, Kultivierungsexperimente und molekularbiologischer Analysen (Restriktionspolymorphismus - ARDRA und denaturierende Gradienten Gelelektrophorese - DGGE von 16S rRNA Genen) belegen, dass die hier verwendeten magnetischen Anreicherungsmethoden - die magnetische Sammlung und die „Race-track“-Aufreinigung - hochselektiv für MTB sind. Unter den untersuchten Bedingungen ließen sich mittels ARDRA und DGGE zwischen diesen beiden Anreicherungsmethoden keine Unterschiede in ihrer Selektivität feststellen. Des weiteren konnten auch nach der wiederholten Beprobung der Mikrokosmen innerhalb weniger Tage mittels DGGE und Sequenzanalysen keine Änderung in der Zusammensetzung der magnetisch anreicherbaren MTB Population beobachtet werden. Darüber hinaus deuteten die mittels ARDRA und DGGE Analysen erzielten Ergebnisse auf die Präsenz nur einer oder weniger eng verwandter MTB Spezies in den Anreicherungen an. Eine Aussage über die tatsächliche Abundanz einzelner Spezies ist allein über eine Untersuchung mittels DGGE und ARDRA allerdings nicht möglich, da sowohl die PCR-Amplifikation als auch die Klonierung selektiv sein können und so zur überproportionalen Repräsentanz einzelner Sequenzen führen können (Reyensbach et al. 1992; Muyzer et al. 1993; Clayton et al. 1995; Farrelly et al. 1995; Cilia et al. 1996; Suzuki und Giovannoni 1996). Aufgrund der Existenz verschiedener 16S rRNA Operone innerhalb eines Organismus, wie sie z. B. von *Clostridium paradoxum* und anderen Mikroorganismen bekannt sind, kann es jedoch auch zu einer Überschätzung der Diversität kommen (Nübel et al. 1996; Rainey et al. 1996; Amann et al. 2000a; van Berkum et al. 2003). Zur Bestimmung der Abundanzen einzelner MTB Spezies sollten deshalb in weiteren Untersuchungen verschiedene spezifische Oligonukleotidsonden entwickelt und eingesetzt werden.

Wie die mikroskopische Analyse der gesammelten Zellen und verschiedene Kultivierungsexperimente zeigten, bestehen allerdings einige für spätere Untersuchungen relevante Unterschiede zwischen den beiden magnetischen Anreicherungsmethoden. So konnte über die „Race-track“-Aufreinigung im Vergleich zur magnetischen Zellsammlung eine selektivere Anreicherung von MTB erzielt werden, während sich letztere vor allem durch eine höhere

Ausbeute von bis zu 10^8 MTB pro Sammlung und einem geringerem experimentellen Aufwand auszeichnete. Für die Erstellung einer Genbank von bisher unkultivierbaren MTB eignet sich folglich aufgrund der höheren Zellzahlen die magnetische Anreicherung von MTB besser als die „Race-track"-Aufreinigung.

3. Untersuchungen zur vertikalen Verteilung und Ökologie von magnetotaktischen Bakterien in Süßwassermikrokosmen

Um erste Rückschlüsse auf die Physiologie und Ökologie insbesondere von bisher unkultivierbaren MTB ziehen zu können, wurde im Rahmen dieser Arbeit der Einfluß verschiedener Umweltbedingungen auf die Verteilung von MTB untersucht. Dazu wurden in vier verschiedenen Süßwassermikrokosmen, die sich vor allem durch eine relative hohe Anzahl von MTB auszeichneten, die vertikale Verteilung von MTB mit verschiedenen physiko-chemischen Parametern verglichen. Die Ergebnisse dieser Untersuchungen sind in Manuskript 2 ausführlicher dargestellt und sollen hier noch einmal kurz zusammengefaßt werden.

Analog zu früheren Untersuchungen von „M. bavaricum" (Spring et al. 1993) konnte in allen untersuchten Mikrokosmen eine heterogene Vertikalverteilung von MTB beobachtet werden. Dieses Ergebnis deutet auf eine Anpassung von MTB an verschiedene physiko-chemische Gradienten hin. Interessanterweise kamen in allen vier Mikrokosmen die meisten MTB - zwischen 63 und 98% - innerhalb des anoxischen Sediments vor. Dabei wurden die maximalen Zellzahlen von $9{,}7 \times 10^5$ bis $1{,}5 \times 10^7$ MTB/cm^3 in Bereichen knapp unterhalb der oxischen Zone gefunden, in denen kein Nitrat nachweisbar war. Folglich ließen sich im Gegensatz zu den Untersuchungen von Petermann und Mitarbeitern (1993) in dieser Arbeit keine direkten Zusammenhänge zwischen der Verteilung von MTB und der Verfügbarkeit von Nitrat als Elektronenakzeptor erkennen. Obwohl für die meisten MTB ebenfalls keine positive Korrelation zwischen ihrer vertikalen Verteilung und der Präsenz von molekularem Sauerstoff gefunden werden konnte, deutet die unterschiedliche vertikale Verteilung der verschiedenen Morphotypen in Mikrokosmos B an, dass einige magnetotaktische Spirillen speziell an mikrooxische Sedimentzonen angepasst sind. Auf eine Anpassung an bestimmte Sauerstoffkonzentrationen deutet auch die für mikroaerophile Bakterien typische Bandenbildung (Fenchel 1994; Zhulin et al. 1996; Frankel et al. 1997) verschiedener kultivierter *Magnetospirillen* Arten in aeroben Weichagarkulturen hin (Abb. 1A und B). Dass die vertikale Position magnetotaktischer Bakterien ebenfalls durch die Präsenz verschiedener Nitrat-

konzentration beeinflußt werden kann, belegen die von Henk Jonkers durchgeführten Mikrosensoruntersuchungen an Gradientenkulturen von *M. gryphiswaldense*.

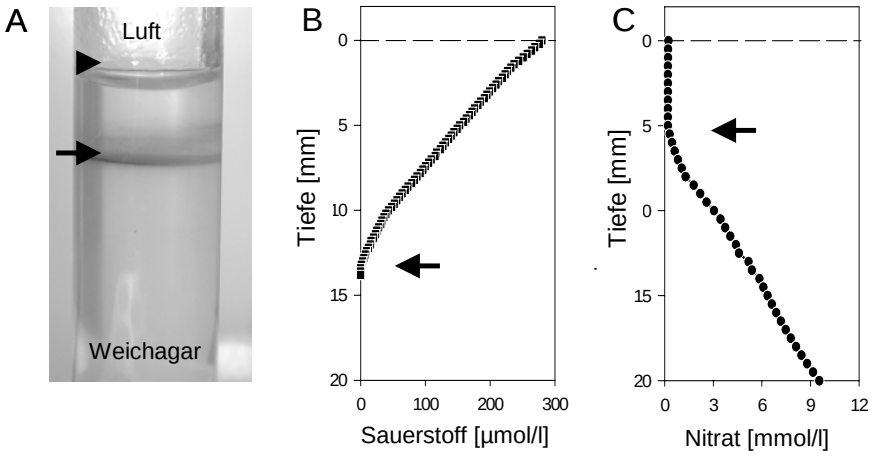

Abb. 1 A) Fotografie einer aeroben Weichagarkultur von *M. gryphiswaldense* mit der für mikroaerophile Bakterien typischen Bandenbildung; B) Verteilung von *M. gryphiswaldense* und vertikales Profil der Sauerstoffkonzentration; C) Verteilung von *M. gryphiswaldense* und vertikales Profil von Nitrat in einer Weichagarkultur mit Nitratgradienten. Der Pfeilkopf (A) bzw. die gestrichelten Linien (B und C) markieren die Agaroberfläche, die Pfeile die als scharfe Bande erkennbare Position der MTB innerhalb der Gradienten.

Neben der Verteilung von Sauerstoff und Nitrat wurde in den in dieser Arbeit untersuchten Mikrokosmen auch die vertikale Verteilung verschiedener anderer potenzieller Elektronendonatoren und Akzeptoren wie Sulfid, Sulfat und Eisen bestimmt. Eine klare Korrelation zwischen der Verteilung dieser Substrate und der Abundanz magnetotaktischer Bakterien war jedoch nicht erkennbar, so dass sich keine Rückschlüsse auf die physiologischen Eigenschaften von MTB ziehen ließen. In zukünftigen Arbeiten sollte deshalb das Spektrum der zu untersuchenden Elektronendonatoren und Akzeptoren weiter erhöht werden. Da alle bisher isolierten MTB ihre Energie ausschließlich durch eine aerobe oder anaerobe Atmung gewinnen und die meisten der in dieser Arbeit untersuchten MTB im anoxischen Bereich gefunden wurden, bietet sich daher die Untersuchung verschiedener organischer Verbindungen an, die eine anaerobe Atmung ermöglichen. Ein räumlicher Wechsel zwischen verschiedenen Horizonten, wie es von einigen *Beggiatoa* und *Thioploca* Arten bekannt ist (Jørgensen und Gallardo 1999; Zopfi et al. 2001; Mußmann et al. 2003) und für „M. bavaricum“ diskutiert wird (Spring et al. 1993), wäre ebenfalls denkbar. Bedingt durch die geringe Speicherkapazität der nur etwa 1 µm großen Zellen und der scharfen Begrenzung der MTB auf einen nur wenige Millimeter breiten Sedimenthorizont scheint trotz der in verschiedenen MTB be-

obachteten Schwefeleinschlüsse (Moench 1988; Spring et al. 1993; Frankel und Bazylinski 1994; Iida und Akai 1996; Cox et al. 2002) ein solcher Ortswechsel nicht wahrscheinlich zu sein.

Eine wichtige Rolle bei der Positionierung von MTB könnte aufgrund des hohen Eisenbedarfs für die Bildung der Magnetosomen auch die Verfügbarkeit von Eisen spielen (assimilative Eisenaufnahme, Stolz 1993). Verschiedene Untersuchungen lassen dabei vermuten, dass die meisten MTB Eisen in Form von Fe(II) und Fe(III) aufnehmen (Bazylinski und Frankel 2004; Udo Heyen, persönliche Mitteilung). In den in dieser Arbeit untersuchten Sedimentschichten, in denen MTB zu den abundanten Mikroorganismen gehörten, wurden Fe(II)konzentrationen zwischen 6 und 60 µmol/l gemessen, die denen in verschiedenen Kultivierungsexperimenten mit *M. gryphiswaldense* ermittelten optimalen Eisenkonzentrationen für das Zellwachstum und die Bildung der Magnetosomen nahe kamen (Schüler und Baeuerlein 1996). Die genaue Positionierung der Zellen scheint jedoch noch von weiteren Faktoren wie z. B. der begrenzten Verfügbarkeit von Elektronendonatoren bzw. Akzeptoren abzuhängen, da gelöstes Eisen auch in tieferen Sedimentschichten nachweisbar war, wo keine oder nur noch wenige MTB zu finden waren. Da die Verfügbarkeit von gelöstem Eisen und verschiedenen Elektronendonatoren und Akzeptoren aber auch die Präsenz potenziell toxischer Substanzen wie z. B. Sulfid neben abiotischen Faktoren auch durch verschiedene mikrobielle Prozesse wie z. B. der Reduktion von Sulfat bestimmt wird, sollte in zukünftigen Arbeiten der Einfluß dieser Stoffwechselwege auf die Entwicklung und vertikale Verteilung von MTB genauer untersucht werden. Eine Möglichkeit bestimmte Stoffwechselwege zu unterbinden - und damit auch die Etablierung bestimmter Gradienten zu verhindern - besteht im Einsatz spezifischer Inhibitoren wie sie z. B. Molybdat und Selenat für die Reduktion von Sulfat (Postgate 1949; Sørensen et al. 1981) und Allylthioharnstoff für die Nitrifikation (Oremland und Capone 1988) darstellen. Allerdings lassen sich unabhängig von der Zugabe bestimmter Substrate oder Inhibitoren die im Labor beobachteten Ergebnisse nicht automatisch auf die Verhältnisse im ursprünglichen Habitat übertragen (Mann et al. 1990a). In zukünftigen Untersuchungen sollte deshalb auch die vertikale Verteilung von MTB im natürlichen Habitat untersucht werden.

Aufgrund der hohen intrazellulären Eisenkonzentrationen von MTB (bis zu 4% Trockengewicht, (Moench und Konetzka 1978; Towe und Moench 1981; Schüler und Baeuerlein 1998; Grünberg 2000; Heyen und Schüler 2003; Schüler 2004), ihrer Abundanz von bis zu 1% der Gesamtzellzahl (s. Manuskript 2; Spring et al. 1993) und im Fall von „M. bavaricum“ ihres Biovolumenanteils von 30% (Spring et al. 1993) haben MTB mög-

licherweise einen starken Einfluß auf den mikrobiellen Eisenkreislauf innerhalb bestimmter Sedimentzonen. Welchen Einfluß magnetotaktische Bakterien auf die Stoffkreisläufe in Umweltproben wirklich haben, kann allerdings nicht allein durch die Bestimmung ihrer Abundanz geklärt werden. Untersuchungen von Luna und Mitarbeitern (2002) zeigten, dass 70 bis 74% aller Bakterien eines Sediments tot waren und sich nur 4% der Bakterien im Wachstum befanden. Durch die auf einer aktiven Magnetotaxis basierenden Zellzahlbestimmung kann jedoch sichergestellt werden, dass es sich bei den in dieser Arbeit erfaßten MTB um lebende, grundsätzlich stoffwechselaktive Organismen handelt. Wie weit diese MTB allerdings an den Umsätzen verschiedener Substrate beteiligten sind, bleibt unklar. Eine Möglichkeit die Aktivität verschiedener Mikroorganismen kultivierungsunabhängig zu bestimmen, besteht in der Verwendung radioaktiv-markierter Substrate (Roszak und Colwell 1987a). In Kombination mit der hoch selektiven Anreicherung von MTB mittels magnetischer Feldlinien ließen sich so direkte Rückschlüsse sowohl auf die Art der Eisenaufnahme als auch auf die Physiologie von bisher unkultivierbaren MTB ziehen. Bedingt durch ihre Position im Gradienten, den vielfach in MTB beobachteten Schwefeleinschlüssen und dem Wachstum einiger isolierter MTB auf Sulfid und anderen reduzierten Schwefelverbindungen (Bazylinski et al. 1988; Meldrum et al. 1993a; Kimble und Bazylinski 1996; Frankel et al. 1997; Dean und Bazylinski 1999) bietet sich dabei vor allem der Einsatz verschiedener radioaktiv-markierter reduzierter Schwefelverbindungen an. Da alle bisher isolierten MTB organische Säuren nutzen können (Blakemore et al. 1979; Blakemore et al. 1985; Matsunaga et al. 1991; Schleifer et al. 1991; Meldrum et al. 1993a; Meldrum et al. 1993b; Schüler et al. 1999; Sakaguchi et al. 2002; Heyen und Schüler 2003), sollte darüber hinaus in weiteren Experimenten die Umsetzung verschiedener organischer Säuren untersucht werden. Eine sichere Zuordnung der erhaltenen Ergebnisse zu einzelnen MTB Spezies könnte über die Hybridisierung der beteiligten Organismen mit spezifischen Oligonukleotidsonden erfolgen (MAR-FISH; Karner und Fuhrman 1997; Lee et al. 1999; Ouverney und Fuhrman 1999; Gray et al. 2000; Ito et al. 2002; Adamczyk et al. 2003; Kindaichi et al. 2004). Die Kombination dieser Ergebnisse könnten zur Entwicklung neuer Kultivierungsstrategien genutzt werden und zur Isolierung neuer MTB Spezies führen.

4. Kultivierung magnetotaktischer Bakterien

MTB sind physiologisch divers und können nicht gezielt über selektive Medien angereichert und isoliert werden. Deshalb sind bisher nur wenige Reinkulturen von MTB verfügbar, die

analog zu Umweltstudien von anderen Mikroorganismen (Giovannoni et al. 1990; Ward et al. 1990; Schmidt et al. 1991; Wagner et al. 1993; Pinhassi et al. 1997; Suzuki et al. 1997; Felske et al. 1999; Wieringa et al. 2000), jedoch nicht zu den im Habitat abundanten Spezies gehören (Spring et al. 1993; Spring und Schleifer 1995). Daher war ein Teilziel dieser Arbeit, neue Reinkulturen magnetotaktischer Bakterien zu isolieren.

4.1. Anreicherung und Isolierung magnetotaktischer Bakterien

Wie in Manuskript 1 dargestellt, wurden im Rahmen dieser Arbeit 10 Stämme der Gattung *Magnetospirillum* von Weichagarkulturen mit verschiedenen organischen Säuren isoliert. Darüber hinaus wurden verschiedene Experimente zur substratinduzierten Anreicherung von MTB durchgeführt, obwohl bisherige Anreicherungsversuche mit verschiedenen organischen Säuren und anderen Substraten meist nicht erfolgreich waren (Schüler 1994). Allerdings zeigt die Isolierung von *D. magneticus*, eines Magnetosomen bildenden aber nicht im klassischen Sinne magnetotaktischen Bakteriums (Sakaguchi et al. 1996), dass auch eine substratinduzierte Anreicherung von MTB ohne eine vorherige magnetische Anreicherung möglich ist. In dieser Arbeit wurden Wasser- und Sedimentproben eines Süßwasserhabitats homogenisiert und mit verschiedenen potenziellen meist aus Kultivierungsexperimenten mit bereits isolierten MTB bekannten Elektronenendonatoren und Akzeptoren versetzt und ungeschüttelt bei Raumtemperatur im Dunkeln inkubiert (s. Manuskript 1). Ausgehend von der von Mann diskutierten Anpassung von MTB an spezielle Habitate mit periodisch auftretenden Störungen (der Gradienten) (Mann et al. 1990a), wurden darüber hinaus einige Ansätze ohne Substratzugabe untersucht, die a) nicht, b) alle 12 Stunden für 15 Minuten oder c) permanent geschüttelt wurden. Abgesehen von den ungestörten Kontrollansätzen ohne Substrat kam es jedoch in allen untersuchten Anreicherungen innerhalb von zwei Wochen zu einem völligen Zusammenbruch der MTB Population. Entgegen den Ergebnissen der in Kapitel 1 beschriebenen Mikrokosmosexperimente wurde allerdings auch in den nicht geschüttelten Kontrollansätzen ohne Substrat nur eine kurzfristige Zunahme der MTB Zellzahlen beobachtet bevor es zu einer drastischen Abnahme der MTB Zellzahlen kam, die sich auch nach längerer Inkubation nicht wieder erhöhten. Alle Anreicherungsversuche wurden deshalb mit Proben eines anderen Süßwasserstandorts wiederholt, führten jedoch zu ähnlichen Ergebnissen. Eine mögliche Erklärung sowohl für den Zusammenbruch der MTB Population in den Anreicherungskulturen als auch für die sprunghaften Änderungen der MTB Zellzahlen und taxonomischen Zusammensetzungen in einigen marinen oder nährstoffarmen Süßwassermikro-

kosmen könnte in der Abwesenheit stabiler Gradienten liegen. In zukünftigen Arbeiten sollten deshalb wie bereits in Kapitel 3 erwähnt, weitere Untersuchungen zum Einfluß verschiedener physiko-chemischer Gradienten auf die vertikalen Verteilung von MTB durchgeführt werden. Darüber hinaus gilt es in weiteren Kultivierungsversuchen unter anderem verschiedene Gradientensysteme nachzustellen um so neue MTB Spezies isolieren zu können.

4.2. Zukünftige Isolierungsstrategien

Wie die Ergebnisse dieser und anderer Arbeiten gezeigt haben, scheinen die bisherigen Ansätze zur Anreicherung und Isolierung nicht ausreichend zu sein, um eine größere Anzahl physiologisch diverser MTB isolieren zu können. Neben der Verwendung weiterer Substrate sollten in zukünftigen Kultivierungsexperimenten vor allem verschiedene Faktoren einbezogen werden, die das Wachstum von MTB direkt oder indirekt beeinflussen können. Im Folgenden werden deshalb verschiedene teilweise kombinierbare Kultivierungsansätze dargestellt, die möglicherweise zur Isolierung neuer MTB Spezies führen könnten.

Wie in Manuskript 2 und im vorherigen Kapitel bereits beschrieben, scheinen MTB besonders an Mikrostandorte angepasst zu sein, die sich durch das Vorhandensein verschiedener Stoffgradienten auszeichnen. Allerdings lassen sich die komplexen Gradientensysteme, wie sie im natürlichen Habitat normalerweise vorkommen, in Kultivierungsversuchen nur schwer nachahmen (Mann et al. 1990a). Eine interessante Alternative stellt deshalb der Einsatz von Diffusionskammern dar, bei der die in einer Agarschicht zwischen zwei Membranen eingebetteten Zellen unter Umweltbedingungen kultiviert werden (Kaeberlein et al. 2002). Im Gegensatz zum herkömmlichen Verfahren sollten die Diffusionskammer jedoch nicht horizontal auf der Sedimentoberfläche inkubiert werden, sondern vertikal im Sediment, um die Überlappung verschiedener (vertikaler) Gradienten gewährleisten zu können.

Eine andere Möglichkeit die Kultivierungseffizienz mikroaerophiler MTB zu erhöhen, besteht im systematischen Entzug der bei der (mikro-)aeroben Kultivierung entstehenden toxischen Sauerstoffradikale. Dieses Ziel kann beispielsweise durch den Einsatz von Katalase- (Blakemore et al. 1979) oder aktivkohlehaltiger Medien (Schultheiss und Schüler 2003) erreicht werden. Aufgrund der Toxizität unnatürlich hoher Nährstoffkonzentrationen (Postgate und Hunter 1964) sollten in zukünftigen Isolierungs- und Anreicherungsexperimenten jedoch auch die Konzentrationen alle übrigen Substrate weiter erniedrigt werden. Auf diese Weise konnten in den letzten Jahren eine Vielzahl neuer Arten isoliert werden (Eilers et al. 2001; Connon und Giovannoni 2002; Rappe et al. 2002; Cho und Giovannoni 2004). Neben kon-

ventionellen Methoden bietet sich dabei die „Microdroplet“-Methode an (Zengler et al. 2002). Der Vorteil dieser Technik besteht in der Fixierung und Separierung der Zellen durch die Einbettung in einzelne Geltropfen und ihrer Kultivierung im Chemostaten, die eine gleichbleibende Versorgung der Zellen mit besonders niedrigen Substratkonzentrationen ermöglicht.

Die Schwierigkeiten bei der Isolierung von MTB könnten jedoch auch auf der fehlenden intraspezifischen Kommunikation zwischen den Zellen in konventionellen Kultivierungsansätzen zurückzuführen sein (Kaiser und Losick 1993; Bruns et al. 2002; Bruns et al. 2003). Die Kultivierungseffizienz heterotropher Bakterien konnte beispielsweise durch die Zugabe von zyklischem AMP (cAMP) deutlich gesteigert werden (Bruns et al. 2002). Eine Alternative besteht in der Erhöhung der Zelldichte in den Kultivierungsansätzen. Da aufgrund der für die Isolierung von MTB nötigen magnetischen Anreicherung eine Erhöhung der Zellzahlen in den eingesetzten Inokula nur begrenzt möglich ist, kann die Zelldichte nur über eine Erniedrigung der eingesetzten Kultivierungsvolumina erhöht werden. Um mögliche synthrophe Wechselwirkungen zwischen verschiedenen Mikroorganismen und MTB einzubeziehen, wäre auch der Einsatz von Co-Kulturen denkbar. Aufgrund der in Kapitel 3 diskutierten Bedeutung reduzierter Schwefelverbindungen als potenzielle Elektronendonoren für MTB bietet sich dabei eine Co-Kultivierung mit Schwefel- oder Sulfat-reduzierenden Bakterien an. Ein bekanntes Beispiel für eine bidirektionale Substratübertragung stellt die Assoziation eines Sulfid-oxidierenden Grünen Schwefelbakteriums (*Chlorobium* sp.) mit dem Schwefel-reduzierenden Bakterium *Desulfuromonas acetoxidans* dar (Warthmann et al. 1992).

Wie die Isolierung von *D. magneticus* gezeigt hat (Sakaguchi et al. 1996), kann eine Reduktion von Sulfat in magnetotaktischen Bakterien nicht vollkommen ausgeschlossen werden. Aus diesem Grund sollten in zukünftigen Arbeiten auch verschiedene Kultivierungsexperimente zur Isolierung Sulfat-reduzierender Bakterien durchgeführt werden. Eine andere Möglichkeit magnetotaktische Bakterien zu isolieren, könnte in der Disproportionierung von Schwefel bestehen. Eine Energiegewinnung ist dabei jedoch nur bei gleichzeitigem Entzug des entstehenden Schwefelwasserstoffs möglich (Thauer et al. 1977). Ein Wachstum von *Desulfocapsa sulfoexigens* mit Schwefel als einziger Energiequelle konnte beispielsweise nur in Kulturen beobachtet werden, bei denen Eisen zur Eliminierung des entstehenden Schwefelwasserstoff zugesetzt wurde (Finster et al. 1998).

5. Molekularbiologische Charakterisierung magnetotaktischer Bakterien

Aufgrund der beschränkten Anzahl bisher isolierter Mikroorganismen stellt die molekularbiologische Analyse von Umweltproben eine gute Alternative zur Erfassung der mikrobiellen Diversität dar (Stahl et al. 1985; Olsen et al. 1986; Amann et al. 1995). Eine Möglichkeit die Diversität, das Vorkommen und die vertikale Verteilung insbesondere von bisher nicht kultivierbaren Mikroorganismen zu bestimmen, besteht in der Untersuchung funktioneller Gene. Um zusätzliche Informationen zur genetischen Ausstattung und der Organisation verschiedener Gene zu erhalten, bietet sich vor allem die Analyse subgenomischer Fragmente an.

5.1. Entwicklung universeller Primer zur Detektion und Amplifikation magnetosomenspezifischer Gene

Wie frühere Untersuchungen und die in Kapitel 1 dargestellten Ergebnisse belegen, sind magnetotaktische Bakterien phylogenetisch zu divers um über spezifische aber für MTB universelle 16S rDNA Primer und Sonden erfaßt zu werden. Allerdings legen erste Sequenzanalysen nahe, dass zumindestens die Entwicklung einer einzigen für magnetotaktische Kokken spezifischen Sonde möglich sein könnte, ohne jedoch eine vollständige Gruppenabdeckung erzielen zu können.

Alternativ zum rRNA Ansatz besteht jedoch bei funktionellen Organismengruppen die Möglichkeit, ihre Diversität und Verbreitung über die Untersuchung verschiedener Markergene zu bestimmen, wie sie bereits erfolgreich bei verschiedenen Ammoniumoxidierern (*amoA*), Nitratreduzierern (*narH*), Denitrifizierern (*nosZ*), Stickstofffixierern (*nifH*), Schwefelreduzierern (*aprA*), Sulfidoxidierern (*soxB*), und Anoxischen Phototrophen (*pufM*) durchgeführt werden konnte (Rotthauwe et al. 1997; Scala und Kerkhof 1998; Petri und Imhoff 2000; Achenbach et al. 2001; Lovell et al. 2001; Petri et al. 2001; Friedrich 2002). Aufgrund ihrer Fähigkeit sich im magnetischen Feld orientieren zu können, stellen magnetotaktische Bakterien ebenfalls eine funktionelle Gruppe von Mikroorganismen dar. Für magnetotaktische Bakterien bietet sich deshalb eine Untersuchung der an der Bildung der Magnetosomen beteiligten *mam* Gene an. Für die Analyse von MTB über verschiedene Markergene müssen diese Gene jedoch a) innerhalb der Zielgruppe universell verbreitet sein und b) nicht in anderen Organismen vorhanden sein.

Zu Beginn dieser Arbeit waren die Sequenzen von *mamA, B, C* und *D* aus *M. gryphiswaldense, M. magnetotacticum* und MC-1 bekannt (Grünberg et al. 2001);

www.jgi.doe.gov/JGI_microbial/html). Inzwischen konnte *mamA* auch in *M. magneticum* (Komeili et al. 2004) nachgewiesen und weitere vermutlich an der Magnetosomenbiomineralisation beteiligte Gene in *M. gryphiswaldense, M. magnetotacticum* und MC-1 identifiziert werden (*mamE* bis *U*) (Schübbe et al. 2003). Sequenzvergleiche von MamC und D ergaben keine Ähnlichkeiten zu bekannten Proteinen nicht magnetotaktischer Organismen. Allerdings zeigte die Analyse von MamA und MamB signifikante Ähnlichkeiten zu verschiedenen Tetratricopeptid repeat (TPR) Proteinen bzw. Kationentransportern (Grünberg et al. 2001). Obwohl *mamA* und *B* folglich keine optimalen Voraussetzungen für die Entwicklung MTB-spezifischer Primer bieten, besteht dennoch die Möglichkeit, über eine Identifizierung von nur in MTB vorkommenden Sequenzbereichen spezifische Amplifikate zu erhalten. In jedem Fall müssen jedoch für die Entwicklung spezifischer Primer ausreichend konservierte DNA Sequenzabschnitte für die Bindung der Primer zur Verfügung stehen. Außerdem ist für eine vergleichende Sequenzanalyse die Existenz genügend variabler Positionen innerhalb der zu untersuchenden Gene erforderlich.

Zur Identifizierung konservierter Bereiche für die Entwicklung geeigneter Primer wurde zunächst ein Alignment von MamA, B, C und D von *M. gryphiswaldense, M. magnetotacticum* und MC-1 erstellt (s. Anhang). Zwischen den untersuchten Proteinen ließen sich Sequenzidentitäten von bis zu 50% beobachtet (37% für MamA, 44% für MamB, 50% für MamC und 31% für MamD). Trotz der vergleichsweise hohen Sequenzidentitäten eignet sich MamC aufgrund der relativ geringen Länge von 125 bis 134 Aminosäuren jedoch nur bedingt für vergleichende Analysen. Darüber hinaus wurden zwar bei allen vier Proteinen mehrere hochkonservierte Bereiche gefunden, jedoch ließen sich aufgrund des degenerierten genetischen Kodes auf DNA-Ebene keine hochkonservierten Primerbindungsstellen identifizieren. Aus diesem Grund wurden verschiedene degenerierte Primer mit einer durchschnittlichen Länge von 18 Nukleotiden und maximal vier variablen Positionen entwickelt. Alle getesteten Primer führten jedoch bereits in Experimenten mit *M. gryphiswaldense* nicht zum Erhalt spezifischer Amplifikate.

Bedingt durch die Schwierigkeit mit degenerierten Primern spezifische Amplifikate zu erhalten, sollte der Datensatz alternativ über die Untersuchung verschiedener *Magnetospirillum* Spezies unter Verwendung bereits für *M. gryphiswaldense* entwickelter spezifischer Primer vergrößert werden. Obwohl sich *mamA* und *B* aufgrund der genannten Einschränkungen nur bedingt für die Entwicklung universeller Primer und Sonden zur Detektion von MTB eignen, wurden die weiteren Untersuchungen auf diese Gene beschränkt, da zu Beginn dieser Arbeit eine clusterförmige Anordnung von *mam* Genen sowohl in

M. gryphiswaldense als auch in *M. magnetotacticum* und MC-1 nur bei *mamA* und *B* beobachtetet werden konnte (Grünberg et al. 2001). Ein Vorteil bei der Untersuchung von geclusterten Genen wie *mamA* oder *B* besteht darin, dass über den Einsatz universeller Primer und Sonden verschiedene Klone einer Genbank identifiziert werden könnten, deren Sequenzierung nicht nur die Analyse einzelner *mam* Gene erlauben würde, sondern zusätzlich auch Informationen zur Diversität und Operonstruktur anderer vermutlich an der Magnetitbiomineralisation beteiligter Gene wie z. B. *mamQ* und *R* liefern könnte.

Zu Versuchsbeginn standen zwei von Cathrin Wawer und Dirk Schüler für *M. gryphiswaldense* entwickelte nicht degenerierten Primerpaare zur Verfügung (s. Tab. 1).

Tab. 1 Sequenzen der zu Beginn dieser Arbeit verfügbaren Primer für *mamA* und *B* von *M. gryphiswaldense*

	Vorwärtsprimer (5´->3´)	**Rückwärtsprimer (5´->3´)**
mamA	DS24NF ATGTCTAGCAAGCCGTCG	CW4-1R CCAATGAACTCGATGAACG
mamB	CW7_2F AAACTGCCGGGAAAAGG	CW7_1R CGAACAGGCGGATATCTC

Wie erste Versuche mit verschiedenen *Magnetospirillum* Spezies zeigten, waren jedoch die für *mamB* entwickelten Primer aufgrund einer Vielzahl unspezifischer Bindungen für vergleichende Untersuchungen nicht geeignet. Im Gegensatz dazu konnte *mamA* unter Verwendung der Primer DS24NF und CW4-1R in 9 der 15 untersuchten *Magnetospirillum* Spezies amplifiziert und anschließend über eine Sequenzanalyse eindeutig identifiziert werden. Alle untersuchten Sequenzen waren dabei entweder zu mehr als 98% identisch zu *mamA* von *M. gryphiswaldense* oder *M. magnetotacticum*, die sich untereinander durch eine Sequenzdivergenz von 14% unterscheiden. In sechs der untersuchten Stämme konnte *mamA* aufgrund von unspezifischen Bindungen der Primer nicht nachgewiesen werden. Folglich sind die ursprünglich nur für *M. gryphiswaldense* entwickelten Primer DS24NF und CW4-1R für vergleichende Untersuchungen nur eingeschränkt nutzbar. Dennoch unterstützen die in dieser Arbeit erzielten Ergebnisse die Vermutung einer in allen magnetotaktischen Spirillen universellen Verbreitung von *mamA*. Interessanterweise entsprach die Zuordnung der analysierten Stämme anhand der untersuchten *mamA* Sequenzen zu *M. gryphiswaldense* bzw. *M. magnetotacticum* nicht den auf 16S rDNA Basis bestimmten Verwandschaftsverhält-

nissen dieser Stämme. Dies könnte auf einen horizontalen Gentransfer hindeuten und sollte in zukünftigen Versuchen genauer untersucht werden.

5.2. Genomische Analyse bisher unkultivierbarer magnetotaktischer Bakterien

Aufgrund der in Kapitel 5.1. dargestellten Schwierigkeiten bei der Entwicklung universeller Primer sollte in einem zweiten Ansatz ein Primer-unabhängiges System zur Untersuchung der *mam* Gene bisher nicht kultivierbarer MTB etabliert werden. Eine gängige Methode zur genomischen Analyse bisher unkultivierbarer Organismen aus Umweltproben besteht in der ungerichteten Klonierung größerer DNA-Fragmente. Wie bereits im vorherigen Kapitel beschriebenen, bietet die Analyse einer solchen Genbank die Möglichkeit, die Diversität verschiedener Gene und ihrer Organisation im Genom zu untersuchen. Des weiteren liefert die Klonierung kompletter Gene und Operone die nötige Voraussetzung für die funktionelle Untersuchung dieser Gene. Darüber hinaus stellt die Genomanalyse einen der wichtigsten Ansätze zur Untersuchung der metabolischen Fähigkeiten bisher unkultivierbarer Mikroorganismen dar. In Kombination mit einer effizienten Anreicherung mittels magnetischer Feldlinien eignet sich der genomische Ansatz deshalb besonders zur Untersuchung der Physiologie und Magnetitbiomineralisation von bisher unkultivierbaren MTB.

Zur Erstellung einer Genbank von bisher unkultivierbaren MTB wurden in dieser Arbeit Zellen über mehrere Tage mittels Magnetsammlungen aus einem Mikrokosmos eines Dorfteichs in Staßfurt angereichert. Aus der mikroskopisch einheitlich aus magnetotaktischen Kokken bestehenden Zellsuspension wurden ca. 400 ng DNA isoliert. Mittels DGGE und Sequenzierung konnten drei fast identische 16S rDNA Sequenzen gefunden werden, die eine hohe Ähnlichkeit zu einer bereits bekannten Sequenz eines unkultivierbaren MTB (Accession Nummer X61607) aufwiesen. Da die DNA-Menge und Qualität für die Herstellung von „bacterial artificial chromosomes“ (BAC) mit Insertgrößen von bis zu 300 Kb nicht ausreichend war, wurde von Anke Meyerdierks die Methode der Fosmid-Klonierung angewendet, bei der die in die Klonierung eingesetzten DNA-Fragmente nur eine Größe von ca. 33 – 50 Kb aufweisen müssen. Die erhaltene Genbank wurde anschließend von ihr mittels eines Gemischs verschiedener, ursprünglich für *M. gryphiswaldense* entwickelter Oligonukleotidsonden für *mam*A, *B*, *E* und *U* gescreent. Ein auf diese Weise identifizierter Klon wurde anschließend ansequenziert. Über einen anschließenden Sequenzvergleich des erhaltenen Fragments ließ sich, auf eine Länge von 104 bzw. bei MC-1 111 Aminosäuren bezogen, eine Homologie von 49% zu *mamS* aus *M. magnetotacticum*, 45% zu

M. gryphiswaldense und 36% zu MC-1 feststellen. Interessanterweise scheint dieses Gen also trotz der Dominanz magnetotaktischer Kokken im Ausgangsmaterial näher mit *mamS* aus *M. magnetotacticum* als mit dem magnetotaktischen Kokkus MC-1 verwandt zu sein. Eine eindeutige Zuordnung dieses oder anderer Gene zu einer bestimmten MTB Spezies wäre jedoch nur über die Nachbarschaft zu einem phylogenetischen Markergen wie der 16S rDNA möglich. Ein bekanntes Beispiel für eine solche Zuordnung stellt die Entdeckung des Proteorhodopsins in Bakterien der SAR86-Gruppe bzw. die Identifikation einer RNA-Helicase in Archaeen dar (Stein et al. 1996; Béjà et al. 2000a; Béjà et al. 2000b). Bedingt durch die beschränkte Größe der klonierten DNA Fragmente kann allerdings nur ein kleiner Teil des Genoms auf diese Weise erfaßt werden. Aufgrund der relativ geringen Insertgröße der eingesetzten Vektoren, der Clusterung der *mam* Gene innerhalb einer Magnetosomeninsel und der normalerweise geringen Kopienzahl der 16S rRNA Gene ist die Wahrscheinlichkeit beide Gene innerhalb eines Klons zu finden jedoch als gering anzusehen.

Auch ohne die eindeutige Zuordnung der erhaltenen Sequenzinformationen zu einer bestimmten MTB Spezies stellt die Klonierung und Sequenzierung von *mam*S aus einer Umweltprobe den ersten Nachweis eines vermutlich an der Biomineralisation beteiligten Gens aus einem bisher unkultivierbaren Mikroorganismus dar. Im Zuge der geplanten vollständigen Sequenzierung ließe sich die Existenz weiterer *mam* Gene innerhalb dieses Klons untersuchen. Die daraus entwickelten homologen Primer und Sonden könnten für ein sensitiveres Screening der in dieser Arbeit erhaltenen Klone genutzt werden und möglicherweise zur Identifizierung weiterer *mam* Gene tragender Klone führen. Über die Assemblierung überlappender Klonsequenzen könnten so möglicherweise größere Teile der von Schübbe und Mitarbeitern (2003) postulierten Magnetosomeninsel rekonstruiert und weitere Informationen zur Organisation dieser Gene gewonnen werden. Darüber hinaus könnten über einen Vergleich der erhaltenen Daten mit den bereits bekannten Sequenzen möglicherweise verschiedene universelle Primer und Sonden zur weiteren Untersuchung der Diversität von *mam* Genen entwickelt werden.

Insbesondere der Transfer kompletter Gencluster bietet darüber hinaus eine Möglichkeit, mehr über die Funktion und Regulation der beteiligten Gene zu erfahren. So konnte beispielsweise die Fähigkeit zur anaeroben Nitratreduktion von *Thermus thermophilus* auf eine aerobe Unterart dieses Organismus übertragen werden (Ramirez-Arcos et al. 1998). Allerdings ließ sich bisher in keinem der in dieser oder anderen Arbeiten untersuchten Klone die Fähigkeit Magnetosomen bilden zu können nachweisen. Wahrscheinlich hängt dies mit der unvollständigen Übertragung der von Schübbe und Mitarbeitern (2003) postulierten

Magnetosomeninsel und der damit verbundenen Abwesenheit eines oder mehrerer für die Magnetitbiomineralisation essentieller Gene zusammen. Eine möglicherweise im Verhältnis zu den anderen *mam* Genen periphere Lokalisierung dieser essentiellen Gene, die eine Übertragung der gesamten für die Bildung der Magnetosomen nötigen Sequenzinformationen innerhalb eines Klons erschweren würde, kann jedoch nicht ausgeschlossen werden. Darüber hinaus könnte auch ein Problem in der Bildung für den Empfängerstamm toxischer Produkte bei der Expression dieser Gene bestehen. Diese könnten zum Zelltod der Klone führen und so die Biomineralisation der Magnetosomen verhindern. Möglicherweise fehlen in den verwendeten *E. coli* Stämmen aber auch die genetischen Voraussetzungen z. B. für die Erkennung der Promotersequenzen und somit der Expression dieser Gene. Eine Alternative um die Funktion einzelner *mam* Gene zu untersuchen, besteht deshalb in der Durchführung von Komplementationsversuchen mit verschiedenen Mutanten bereits isolierter MTB Spezies. Die so gewonnenen Ergebnisse ließen sich in zukünftigen Experimenten möglicherweise zur Herstellung diverser Magnetitkristalle mit definierten Eigenschaften oder zur Herstellung größerer industriell relevanter Magnetosomenmengen nutzen.

5.3. Perspektiven der Genomanalyse bisher unkultivierbarer magnetotaktischer Bakterien

Die Genomanalyse stellt einen der wichtigsten Ansätze dar, um Informationen über die genetische Ausstattung eines Organismus zu erhalten und neue Strategien zur Isolierung bisher unkultivierbarer Mikroorganismen zu entwickeln (Torsvik und Ovreas 2002). Mit der im Zuge dieser Arbeit erfolgten Erstellung einer genomischen Datenbank aus magnetisch angereicherten Zellen besteht nun erstmals die Möglichkeit, mehr über die genetische Ausstattung bisher unkultivierbarer magnetotaktischer Bakterien zu erfahren. Ausgehend von einer Reinkultur, einer angenommenen Genomgröße von 5 Mb, einer Insertgröße von 33 Kb und der Existenz von 820 Klonen sollte diese Genbank theoretisch einer fünffachen Abdeckung und statistisch gesehen einer Erfassung von 99,3% des Genoms eines Mikroorganismus entsprechen (Wolff und Gemmill 1997). Da es sich bei der verwendeten Zellsuspension, jedoch nicht um eine Reinkultur, sondern um Material aus einer Umweltprobe handelte, kann allerdings trotz der hocheffizienten Anreicherung, nicht zwangsläufig auf die ausschließliche Repräsentanz von DNA-Fragmenten magnetotaktischer Bakterien oder einzelner MTB Spezies geschlossen werden. Über die Assemblierung verschiedener Contigs mit überlappenden Sequenzbereichen (Shigenobu et al. 2000) wäre jedoch die Rekonstruktion einzelner Teile des

Genoms/der Genome von bisher unkultivierbaren MTB denkbar. Bei einer Mischpopulation verschiedener phylogenetisch eng verwandter Organismen mit großen Sequenzähnlichkeiten innerhalb bestimmter Genombereiche, könnte es jedoch zu einer fehlerhaften Assemblierung der Contigs kommen. Dass eine solche Strategie dennoch erfolgreich sein kann, belegt die in diesem Jahr über eine Shotgun-Sequenzierung und anschließende Assemblierung erzielte nahezu komplette Rekonstruktion zweier Genome aus einem relativ homogenen Biofilm (Tyson et al. 2004). Allerdings liefert der Nachweis eines Gens innerhalb eines Genoms keinerlei Aussagen über die Expression und die Aktivität des ensprechenden Enzyms. So konnte zwar im Genom von *Desulfocapsa sulfoexigens* die Existenz eines Gens für die Adenosin-5-Phosphosulfat-Reduktase (*aprA*) festgestellt werden, jedoch ließ sich bisher in keiner Kultur eine Reduktion von Sulfat nachweisen (Finster et al. 1998; Friedrich 2002). Dennoch könnte die Sequenzierung der in dieser Arbeit erhaltenen Klone zu verschiedenen Rückschlüssen auf die Physiologie bisher unkultivierbarer MTB führen und so zur Entwicklung neuer Isolierungsstrategien beitragen. In zukünftigen Arbeiten sollten deshalb vor allem die Genome von MHB-1, „M. bavaricum“ oder MMP kloniert und analysiert werden, da eine Isolierung dieser MTB in Hinblick auf ihre ungewöhnliche phylogenetische Position und Magnetosomenform bzw. Zusammensetzung besonders interessant ist und möglicherweise zu einem besseren Verständnis der Magnetosomenbiomineralisation führen könnte.

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Publikationen

Publikationsliste mit Erläuterung

Diese Dissertation beruht im wesentlichen auf den folgenden Publikationen. Vorangestellt sind kurze Erläuterungen über meinen Anteil an der jeweiligen Arbeit.

1 A polyphasic approach for the characterization of uncultivated magnetotactic bacteria from freshwater and marine environments

Christine Flies, Jörg Peplies, Dirk Schüler

Das Konzept dieser Arbeit wurde von Dirk Schüler und mir entwickelt. Alle Experimente wurden von mir durchgeführt. Die Rekonstruktion des phylogentischen Stammbaums erfolgte in Zusammenarbeit mit Jörg Peplies. Bei der Erstellung des Manuskripts wurde ich von Dirk Schüler unterstützt.

2 Diversity and vertical distribution of magnetotactic bacteria along chemical gradients in stratified freshwater microcosms

Christine Flies, Henk Jonkers, Dirk de Beer, Katja Bosselmann, Michael Böttcher, Dirk Schüler

Das Konzept dieser Arbeit wurde von Dirk Schüler, Michael Böttcher, Henk Jonkers und mir entwickelt. Alle mikro- und molekularbiologischen Versuche wurden von mir durchgeführt. Das Manuskript wurde in Zusammenarbeit mit Dirk Schüler, Henk Jonkers, Katja Bosselmann, Dirk de Beer und Michael Böttcher erstellt.

3 Crystal size and shape distributions of magnetite from uncultured magnetotactic bacteria and magnetite as a potential biomarker

Baláz Arató, Zoltán Szányi, Christine Flies, Dirk Schüler, Richard Frankel, Peter Buseck, Mihály Pósfai

Vier der sieben analysierten Proben wurden von mir genommen. Für die elektronenmikroskopische Untersuchung wurden die Zellen von mir vorpräpariert. An der Erstellung des Manuskripts habe ich redaktionell mitgewirkt.

4 Intracellular magnetite and extracellular hematite produced by *Desulfovibrio magneticus* strain RS-1

Baláz Arató, Richard Frankel, Dennis Bazylinski, Bruce Moskowitz, Christine Flies, Dirk Schüler, Mihály Pósfai

Alle Kulturen wurden von mir angezogen und für die elektronenmikroskopische Untersuchung präpariert. An der Erstellung des Manuskript war ich beteiligt. Der von Bruce Moskowitz bearbeitete Teil der Arbeit wird gerade in das Manuskript eingearbeitet.

5 Phylogeny and *in situ* identification of magnetotactic bacteria

Rudi Amann, Ramon Rossello-Mora, Christine Flies, Dirk Schüler

An der Überarbeitung und Editierung dieses Buchkapitels war ich beteiligt.

1.

A polyphasic approach for the characterization of uncultivated magnetotactic bacteria from freshwater and marine environments

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Abstract

Both magnetic collection and ”race-track” purification techniques were highly effective for the selective enrichment of MTB as revealed by amplified ribosomal DNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA genes. Using these methods, the diversity of magnetotactic bacteria (MTB) in microcosms from different marine and freshwater environments was assayed by a combined microscopic, molecular and cultivation approach. Most microcosms were dominated by magnetotactic cocci, which affiliated with a distinct cluster within the “Alphaproteobacteria”. Sequence divergence between 0.1 to 11% within this lineage was found between magnetotactic cocci from various microcosms as well as between MTB from different stages of succession of the same microcosms. A novel magnetotactic rod (MHB-1) was detected in a microcosm from a lake in Northern Germany by fluorescence *in situ* hybridization (FISH). MHB-1 was closely related to ”Magnetobacterium bavaricum” within the *Nitrospira* phylum. In extensive cultivation attempts, we failed to isolate MHB-1 as well as most other MTB present in our samples. However, although magnetotactic spirilla were usually not abundant in the enrichments, 10 heterotrophic strains of the genus *Magnetospirillum* were isolated in pure culture.

Introduction

Magnetotactic bacteria (MTB) are a heterogeneous group of aquatic microorganisms which share the ability to orient themselves along magnetic field lines due to the presence of magnetosomes. Magnetosomes are intracellular membrane-bounded crystals of magnetic iron mineral which consist of magnetite or greigite (Balkwill et al. 1980; Bazylinski 1995; Schüler 1999; Schüler 2004). Diverse MTB, including cocci, spirilla, rods, vibrio and multicellular aggregates, were found in different aquatic habitats (Blakemore 1975; Moench and Konetzka 1978; Bazylinski et al. 1995; Cox et al. 2001). Most cultivated and uncultivated MTB have been affiliated with the “Alphaproteobacteria”. The magnetotactic sulfate-reducing bacterium *Desulfovibrio magneticus* (Kawaguchi et al. 1995) and a magnetotactic, many-celled prokaryote (MMP, DeLong et al. 1993), belong to the “Deltaproteobacteria”, while a giant magnetotactic rod, tentatively named “Magnetobacterium bavaricum”, affiliates to the *Nitrospira* phylum (Spring et al. 1993). The phylogenetic and morphological diversity of MTB is matched by a remarkably variation in the shapes, organization, and numbers of mag-

netosome crystals found in diverse MTB (for review, see Schüler 1999). While reasonable progress was recently made in the genetic and biochemical analysis of magnetosome formation in a limited number of cultivated *Magnetospirillum* strains (Bazylinski and Frankel 2004; Schüler 2004), the intriguing diversity of magnetosome biomineralization has remained entirely unexplored at a biochemical and genetic level. This is due to the fact that most MTB were recalcitrant to isolation, and only very few strains are available in pure cultures. Therefore, there is an urgent need for innovative methods to explore the vast diversity of uncultivated MTB.

Because of their unknown growth requirements and presumptive physiologic diversity, there is no general strategy for the conventional enrichment of MTB based on metabolic selection. However, in contrast to most other uncultivated bacteria, MTB can be physically enriched by taking advantage of their directed swimming behaviour in magnetic field lines, which has been successfully used in a number of previous attempts to isolate several strains of MTB (Bazylinski et al. 1988; Schüler 1990; Kawaguchi et al. 1992; Meldrum et al. 1993a; Meldrum et al. 1993b; Schüler et al. 1999). However, it is unclear how selective these enrichment techniques were with respect to the recovered diversity of MTB. In addition, as previous detection and isolation of MTB were from laboratory enrichments, it was not addressed how prolonged incubation of environmental samples did affect the community structure and diversity of MTB.

The aim of this study was to thoroughly evaluate different magnetic enrichment methods for their selectivity and efficiency in the collection and purification of uncultivated MTB from environmental samples. This was done by molecular and microscopic characterization. In addition, we investigated the diversity and succession of MTB populations in various microcosms from different aquatic habitats by the combination of a culture independent 16S rDNA based approach with extensive cultivation experiments.

Materials and methods

Sampling and set up of microcosms

During March 2000 and April 2003 sediment samples from the upper sediment layer and surface water were taken from more than 50 different sites in Germany and Sweden. The samples were transferred to aquaria, glass or plastic bottles, covered loosely and incubated in

these microcosms under low-light conditions or in the dark for several months. The occurrence of MTB was studied in various microcosms from freshwater, brackish and marine habitats. MTB from 14 microcosms (A to N) from 9 different habitats were collected for molecular analysis. Four of these microcosms from different marine and freshwater sites from Germany were investigated in more detail because of their abundant and diverse MTB populations (A: Wadden sea near Sahlenburg, German bight; B: public swimming area from a lake in Bremen (”Waller See”); C: drainage ditch in Bremen, D: freshwater pond near Staßfurt).

Magnetic collection of MTB

The collection of MTB was performed essentially as described before (Moench 1978). Cells were enriched by attaching the south pole of a permanent magnet outside the jar 1 cm above the sediment surface. After 2 to 4.5 hours 200 to 400 µl of the water near the south pole of the magnet were collected by a pipette.

Race-track (RT)-Purification of MTB

MTB were purified by the capillary “race-track”-method (Wolfe et al. 1987) which was modified by Schüler et al. (1999). In this method the narrow tip of a Pasteur pipette was sealed in a gas flame and the capillary (1 to 9 cm in length) was filled with sterilized habitat water using a long hypodermic needle. Sample material (sediment or magnetic collected cells) was placed on top of a sterile, wetted cotton plug in the wide-mouthed end of the pipette which served as a reservoir. The filled pipette was exposed to a magnetic field produced by a permanent magnet along the capillary. MTB migrated through the cotton plug towards the end of the capillary. After 30 to 165 min the tip containing the accumulated MTB was broken off. Using a sterile hypodermic needle the MTB were removed and transferred into sterilized habitat water.

Phase-contrast and electron microscopy

The swimming behavior and cell morphology of MTB was investigated by the "hanging" drop method (Schüler 2002) using a phase-contrast microscope (Zeiss). The arrangement and morphology of the magnetosomes were analyzed by transmission electron microscopy (TEM). For TEM, cells were adsorbed onto 300-mesh formvar coated copper grids (Plano) and analyzed without staining. Alternatively, cells were negatively-stained using 4% uranyl acetate. The samples were examined with an EM 301 transmission electron microscope (Philips) at 80 kV.

DNA extraction

The DNA of uncultivated cells was extracted according to the method of Zhou et al. (1996) modified by Sievert et al. (1999). The DNA was dissolved in 100 µl PCR water.

PCR amplification

For DGGE the bacterial 16S rRNA genes were amplified with the primer pair GM5F with GC-clamp and 907R (Muyzer et al. 1995) by using the MasterTaq-system from Eppendorf or the RedTaq system from Sigma. The PCR reactions were prepared in comply with manufacturer's instructions including bovine serum albumin (BSA, Fluka, final concentration of 0.3 mg/ml) and using the MasterTaq system, 4% enhancer (v/v). Isolated DNA or magnetic enriched cells were used as template. The touchdown PCR was initiated by a heating step of 94 °C. When the RedTaq system was used, the PCR reaction was cooled down to 80 °C and the Taq was added. In the following cycles, the temperature decreased continuously at 1 °C after two cycles from 65 °C to 56 °C. The PCR was finished with 19 cycles at 55 °C. For cloning, nearly-complete 16S rRNA genes were amplified using the RedTaq PCR system with the universal bacterial primer pair GM3F and GM4R, (Muyzer et al. 1995). PCR was performed by 33 cycles at an annealing temperature of 42 °C. The inserts from clones were amplified using the MasterMix from Promega and vector-specific primers. The PCR was performed by 35 cycles at an annealing temperature of 60 °C. All PCR products were checked by electrophoresis in an agarose gel and ethidium bromide staining.

DGGE

The DGGE was performed using the D-Gene™ system (Bio-Rad Laboratories) and the DNA fragments were separated in a 1 mm thick polyacrylamide gel (6% wt/vol) with a 20 - 70% denaturant gradient and 1 x TAE electrophoresis buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) at 60 °C. After 16 h electrophoresis at a constant voltage of 100 V, the gel was stained with ethidium bromide and photographed by the Image Master (Amersham Pharmacia). DNA bands were excised with a sterile scalpel and eluated in 100 µl PCR water over night at 4 °C. An aliquot of the eluates were reamplified with GM5F and 907R and were purified using an agarose gel and the Quiaquick Gel Extraction Kit from Quiagen. The purified DNA was used for sequencing.

ARDRA

The DNA was cloned into a pCR®2.1-TOPO® or pCR®4-TOPO® vector and transformed into competent *Escherichia coli* cells (TOP10 One Shot® from Invitrogen, DH5α from GIBCO BRL or XL-10 Gold cells from Stratagene). The clones were analyzed by PCR (see above). The PCR products from more than 60 positive clones were restricted with HindIII and RsaI (final activity 0.5 U/µl) for 3 h at 37 °C and separated in a 3.5% agarose gel. Plasmids from clones with various band patterns were isolated with the Quiaprep Spin Miniprep Kit from Quiagen and sequenced.

Sequencing

The DNA was sequenced with the primer described above or vector specific primers and GM1 (5´-CCAGCAGCCGCGGTAAT-3´) by a capillary sequencer (Applied Biosystems/Hitachi 3100 Genetic Analyzer).

Phylogenetic analysis

The partial and full length 16S rDNA sequences retrieved in this study were added to an internal ARB data set containing approx. 31.500 partial and full length small subunit ribosomal RNA sequences, based on the database of the Technical University of Munich (release

June 2002). The tool ARB EDIT was used for automatic sequence alignment. The alignment was checked by eye and corrected manually. The phylogenetic tree presented was reconstructed based on maximum-likelihood analysis of selected full length sequences affiliated with known magnetotactic bacteria, the full length 16S rRNA sequences generated in this study and all sequences within the tree "tree-demo" of the public ARB data set from June 2002. The tree topology was evaluated by maximum-parsimony and neighbor-joining analysis of the full data set in combination with different filters excluding highly variable positions. A consensus tree was constructed taken into consideration the results obtained by applying the various tree reconstruction methods. Discrepancies of the different reconstruction methods are indicated by multifurcations. Partial 16S rRNA gene sequences obtained by DGGE analysis were added to the tree using the "Parsimony quick add" tool of ARB.

Fluorescence in situ hybridization (FISH)

Cells of a magnetotactic rod (MHB-1) were magnetically collected and fixed with paraformaldehyde as described by Pernthaler et al. (2001). Than the cells were embedded in agarose (final concentration 0.02%) and fixed on glass slides (Amann type, Paul Marienfelder KG). A second fixation (hydration) is performed by applying increasing concentrations of ethanol (50 - 80 - 96%). The dried samples were hybridized with a specific Cy3-labeled probe for “Magnetobacterium bavaricum” (Spring et al. 1993) for 90 min at 46 °C without any formamide (Pernthaler et al. 2001). The fluorescein-labeled probe EUB338 (Amann et al. 1990) and the non-EUB probe (Wallner et al. 1993) were used as controls. The samples were washed without EDTA for 20 min at 48 °C, rinsed with water (Millipore) and stained with 4´,6´-diamidino-2-phenylindole (DAPI, Sigma) (Pernthaler et al. 2001). The samples were washed with H_2O and 70% ethanol. After drying they were embedded in Citifluor (Citifluor Products) and investigated by a fluorescence microscope from Zeiss.

Cultivation experiments

Various minimal and complex media with 0 - 0.8% agar were used for cultivation experiments. Medium constituents used as potential electron donors included reduced sulfur compounds and a variety of organic compounds such as glucose, amino acids, lactate, malate,

succinate, Alpha-ketogluterate, citrate, pyruvate, acetate, formiate, propionate, butyrate, iso-butyrate, 2-methylbutyrate, valerianate and capronate, which were added as mixtures as sole carbon or energy sources. Different potential electron acceptors including oxygen, nitrate, N_2O and sulfate were added in different combinations and different concentrations. In addition, several media were used in combination with oxygen-sulfide-gradients (Nelson and Jannasch 1983). Ferric quinate or ferric citrate were added as iron sources. Sulfide or cysteine were used as reducing agents and resazurine was used as redox indicator in most media. Complex media were supplemented with various vitamin cocktails, yeast extracts, peptones, meat extract and sediment extract. The pH was between 6.8 and 7.2. 20 ml Hungate tubes were filled with 10 ml media and closed with screw caps. Anaerobic media were prepared by the method of Widdel and Bak (1992). For anaerobic or microaerobic growth the gas phase of the tubes was replaced by different gas mixtures of various concentrations of O_2, N_2, N_2O and CO_2 and sealed with butyl stoppers. Inocula of purified MTB were applied to the media in dilutions down to 10^{-6}. All tubes were incubated at room temperature under low light conditions. Growth was analyzed by visual screening (colony or band formation) and microscopy. For further cultivation of isolated magnetic spirilla the medium contained (per liter): 0.068 g KH_2PO_4, 0.108 g $MgSO_4$ x 7 H_2O, 0.1 g NH_4Cl, 0.166 g sodium succinate, 2 ml ferric citrate (10 mM), 5 ml mineral solution (Wolin et al. 1963) and 0.2% agar noble (Difco). The medium was adjusted to pH 7.0 and autoclaved.

Results

Magnetic collection and enrichment of MTB

In order to find optimal conditions for collection of MTB, we first evaluated two different magnetic enrichment techniques with respect to recovered cell numbers and selectivity. Magnetic collection from the water column of several microcosms yielded a visible pellet of accumulated MTB close to the south pole of the magnet, which was equivalent to $10^7 – 10^8$ magnetotactic cells. Typically, up to 100 ng genomic DNA could be obtained from this amount of cells. Small magnetotactic crustaceans (*Ostracoda*) were frequently observed among collected cells. Repeated collections (approximately 5 - 10x) within several days resulted in an increasing depletion of magnetotactic cells. In the shown example (Fig. 1), DGGE profiles of magnetically collected cells revealed only two distinctive bands (a and b),

which yielded nearly identical sequences with high similarity to uncultivated magnetic cocci from the database. Sequence and position of band b matched a weak band in the complex DGGE patterns obtained from DNA extracted from the upper sediment layer (approx. 0 - 5 mm depth) indicating that the collected MTB were among the more abundant species in this layer. DGGE and ARDRA analysis of magnetically collected MTB revealed identical results. Several magnetic morphotypes, which could be observed in samples collected directly from the sediment, were reluctant to swim out of the sediment, even if the water column was rendered anoxically by sparging with nitrogen.

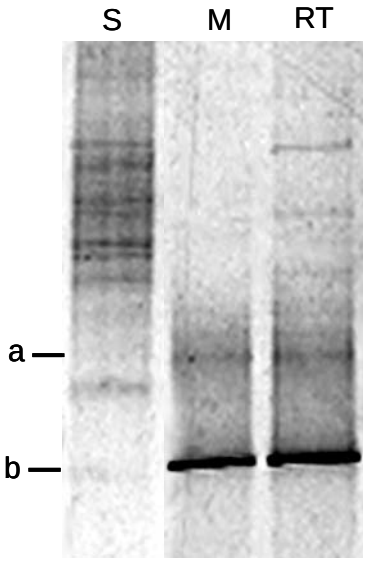

Fig. 1 DGGE profiles of amplified 16S rDNA fragments from microcosm D. Lane 1: isolated DNA from the upper sediment layer (S), lane 2: magnetically collected cells (M), lane 3: magnetically collected and RT-purified cells (RT). Bands which were excised for reamplification and sequencing are indicated (a and b).

Using the race-track (RT) method, MTB could be enriched to virtual homogeneity as evident by microscopy and were visible as a pellet at the tip of the pipette. ARDRA, DGGE and 16S rDNA sequence analysis of RT-purified MTB confirmed their identity with the abundant MTB obtained by magnetic collection (Fig. 1). Approx. $10^5 – 10^7$ cells could be obtained per single run. All attempts to scale-up this method by using larger volumes did not result in significantly increased cell numbers. Only the addition of a reductant (200 µM sodium cysteine) to the water within the capillary seemed to slightly increase the yield of MTB. With a running distance of 9 cm, visible amounts of MTB reached the tip of the capillary after 10 min. This is equivalent to a migration speed of more then 50 µm/s and in most cases the

major fraction of MTB arrived the tip after 30 min. The variation of running times, conditions and distances had no effect on the selectivity of the magnetic collection and RT-purification (Fig. 2).

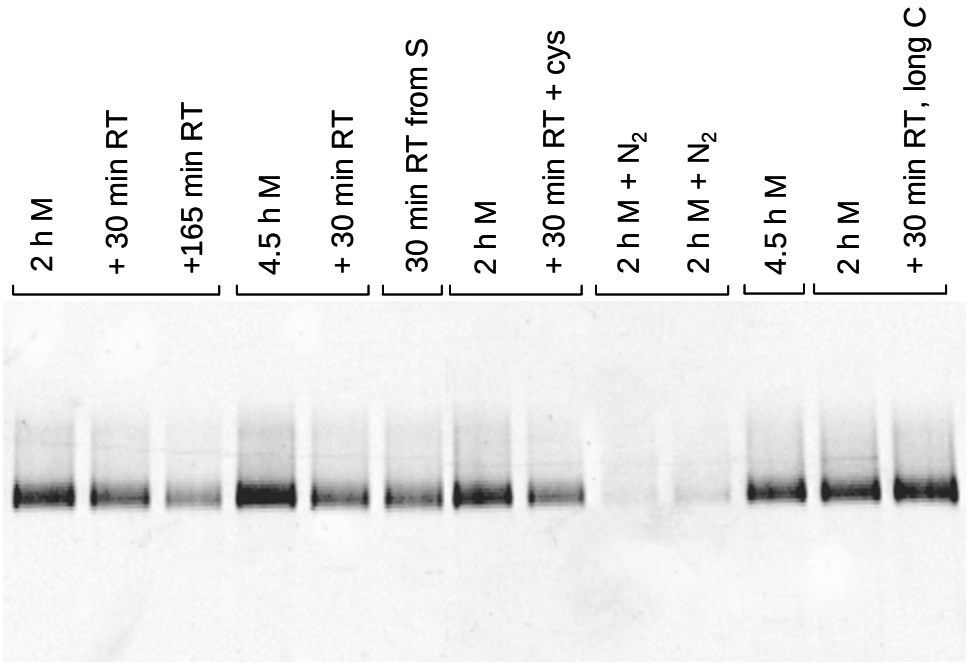

Fig. 2 DGGE band patterns from amplified 16S rDNA fragments of microcosm C obtained after repeated collection under various enrichment conditions. Brackets indicate samples taken simultaneously, (+) indicate samples, which were pre-enriched by magnetic collection. M, magnetic collected; RT, race-track purified cells; S, sediment; cys, cysteine, N_2, nitrogen; C, capillary.

Diversity and succession of MTB in microcosm experiments

In a survey of more than 150 samples from marine coastal and freshwater habitats, MTB were detected in the majority of samples. We failed to detect MTB in wet soil samples and in sediments from highly eutrophic habitats including swine waste pits and sewage sludges. Conspicuous magnetotactic multicellular aggregates were observed in several marine samples from the Wadden sea (Sahlenburg) and sediment cores from the Baltic Sea (Eckernförder bight), strongly resembling those that were previously described as MMP by Rodgers et al. (1990). However, we consistently failed to obtain 16S rDNA sequences with similarity to MMP, probably due to their low abundance in our samples. Most samples from different sites

were dominated by various morphotypes of MTB such as cocci, rods, vibrio and spirilla (Fig. 3).

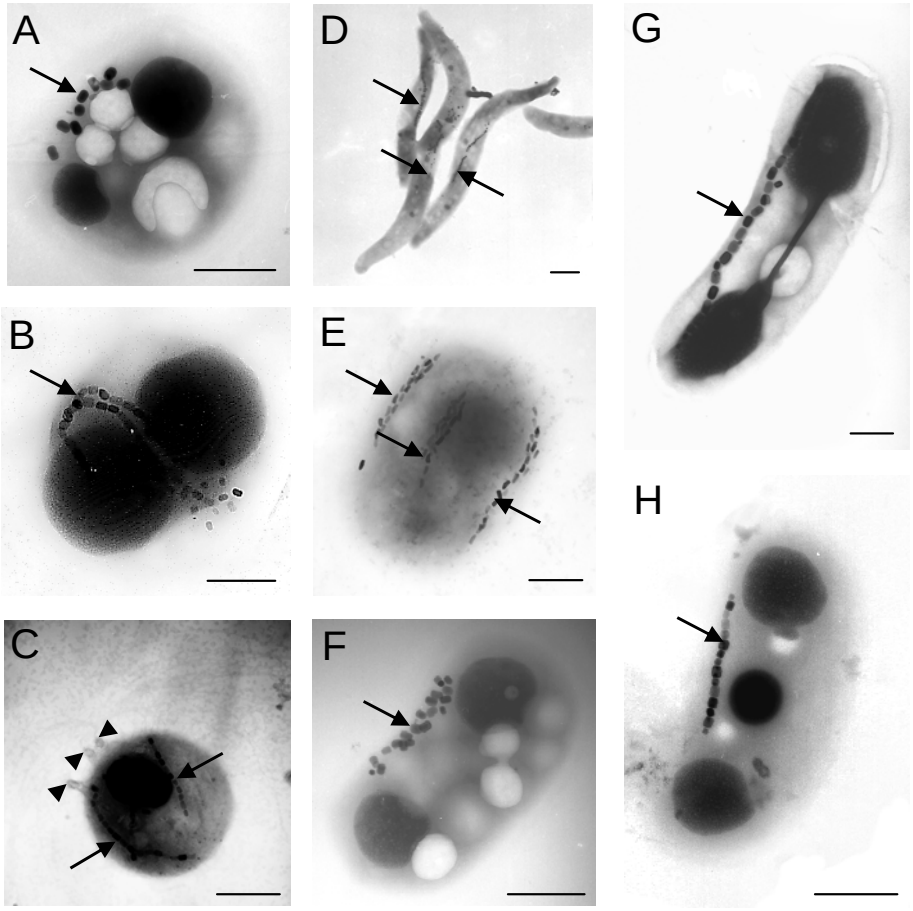

Fig. 3 Electron micrographs of different morphotypes of MTB identified in this study from different habitats. The morphological diversity include cocci (A to C), spirilla (D) and ovoid to rod shaped cells (E and H). Arrows indicate magnetosomes; arrowheads mark insertion of flagella bundles; sample C) was stained with uranyl acetate; bar = 0.5 µm.

Most of the sequences obtained from magnetically collected cells display highest similarities to previously identified uncultivated magnetotactic cocci within the ”Alphaproteobacteria” (Fig. 4 cluster I). Sequence divergence between these phylotypes was between 0.1 and 11%. Sequences unrelated to known MTB from the database were occasionally obtained from magnetic collections and corresponded often to very weak and unreproducible bands in DGGE. Interestingly, several of these sequences affiliated with the Rhodospirillaceae, but were not closely related to *Magnetospirillum* species. Although we cannot fully exclude that some of them potentially represent so far unknown phylogenetic lineages of MTB, they were attributed to non-magnetic bacteria present in these samples.

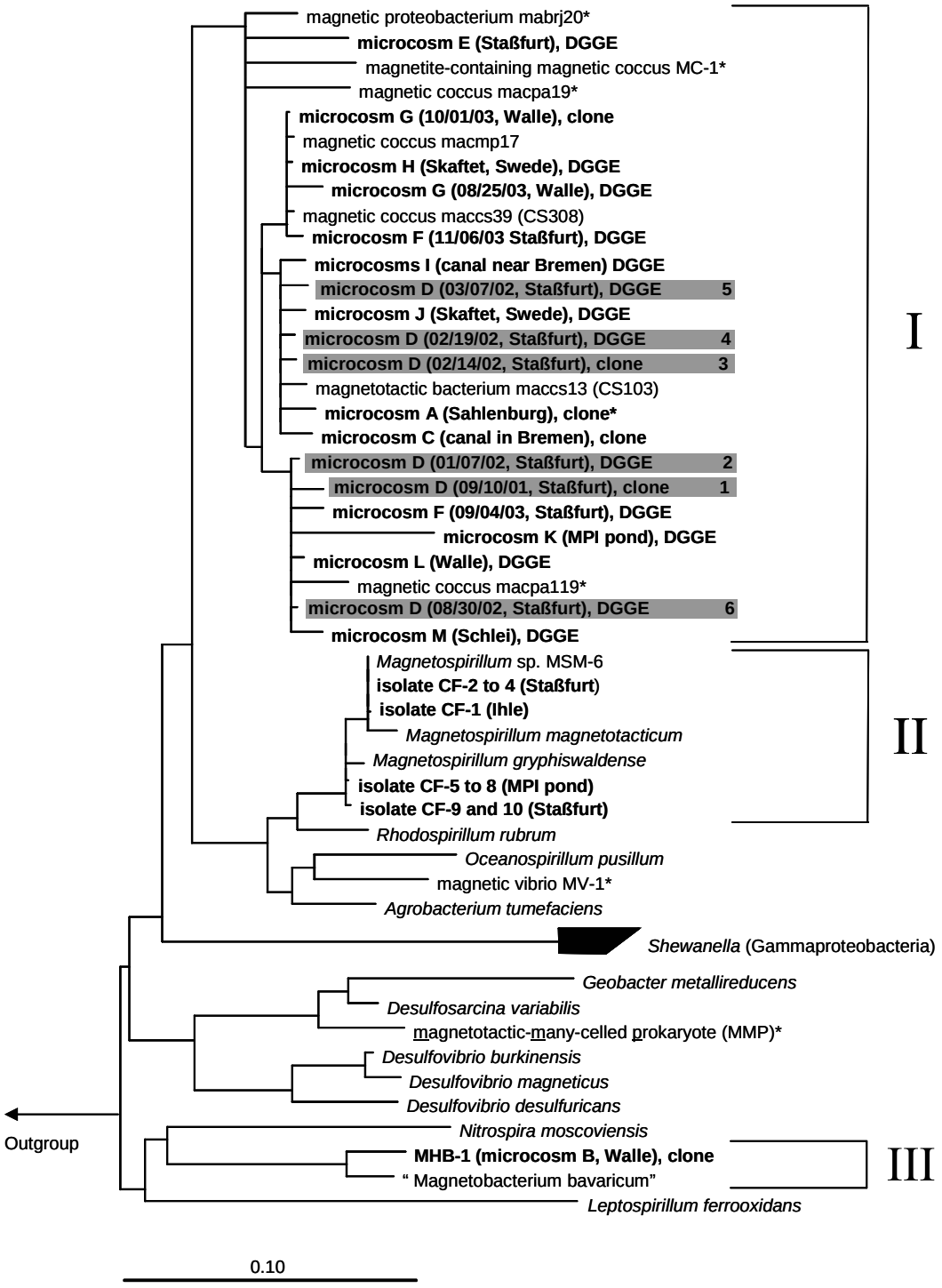

Fig. 4 Phylogenetic tree of magnetotactic bacteria based on comparative sequence analysis of 16S rRNA genes. Bar indicates 10% estimated sequence divergence. If identical 16S rDNA sequences from both DGGE and ARDRA from the same microcosms were available, only the clone sequences were shown. Sequence differences lower than 0.3% from isolates from the same habitat were not itemized. The sequences determined in this study are in bold. Shaded sequences and numbers indicate the order of collection from microcosm D. (*) indicates sequences from marine habitats.

In one microcosm (B), the MTB population was found dominated after 19 months of incubation by a small (2 - 3 µm in length, 1 µm in width), slowly moving magnetotactic rod. Sequence analysis of a 16S rDNA clone obtained from magnetically collected cells displayed high similarity (91%) to ”Magnetobacterium bavaricum”, which is affiliated with the *Nitrospira* phylum (Spring et al. 1993) (Fig. 4, cluster III). In FISH experiments, a rod-like cell designated MHB-1 was recognized both by the EUB338 (eubacteria) and the “M. bavaricum” probe, which had been previously described (Spring et al. 1993) and perfectly matched the target sequence (Fig. 5A to C). Electron micrographs of MHB-1 cells revealed magnetosome crystals, which were aligned in multiple chains and had the same bullet-shaped morphology like those from ”M. bavaricum” (Fig. 5D). However, unlike the several hundred magnetosome particles organized in 3 - 5 bundles of chains, which were present in the giant cells of ”M. bavaricum” (Hanzlik et al. 2002), MHB-1 cells only contained 30 – 60 magnetosomes within a single bundle.

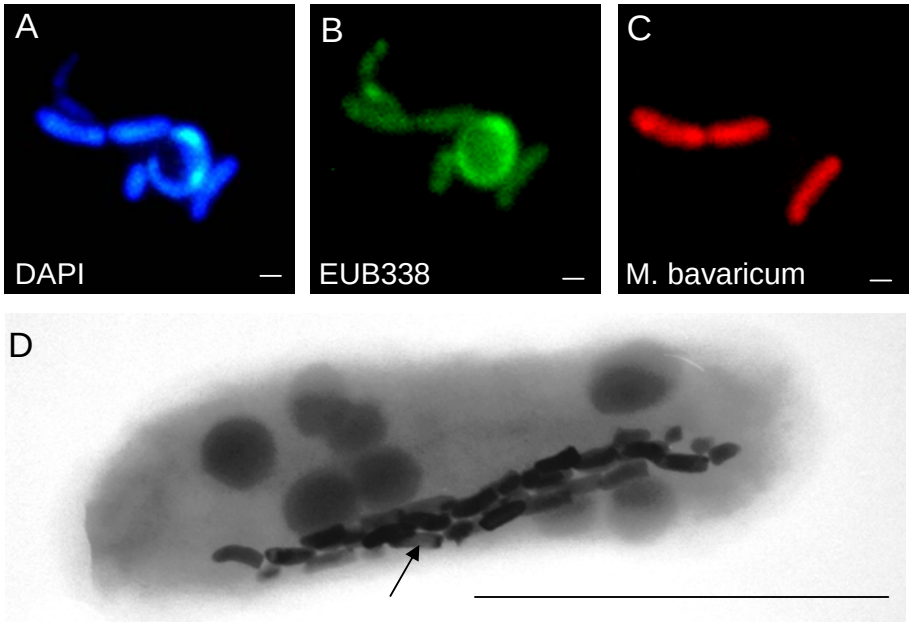

Fig. 5 Magnetically collected cells of MHB-1 from microcosm B. DAPI stained (A), cells hybridized with a bacterial probe EUB338 (B) and a probe for “M. bavaricum” (Spring et al. 1993) (C). Electron micrograph of MHB-1 (D). Bar = 1 µm; arrow indicates magnetosomes.

Generally, freshwater microcosms underwent a characteristic succession upon prolonged incubation. We often noticed an increase in total cell numbers of MTB and a change of morphological diversity as revealed by microscopy. Therefore, we were interested, if the population of MTB remained stable after the mass development of magnetotactic cocci, which occurred in most microcosms after several weeks to months. Fig. 6A and B show the DGGE profiles of magnetically collected cells from two microcosms over several months of incubation. In microcosm C, the same phylo- and morphotype (Fig. 6C) was persistently detected over 16 months. In contrast, a shift in the predominant MTB species was indicated by divergent community profiles in DGGE profiles from microcosm D, while the collected magnetotactic cells virtually displayed the same morphology (cocci) over many months by light microscopy. Nevertheless detailed analysis by electron microscopy showed the presence of two different morphotypes of magnetotactic cocci within a sample. Sequences obtained from different time points displayed up to 7% divergence.

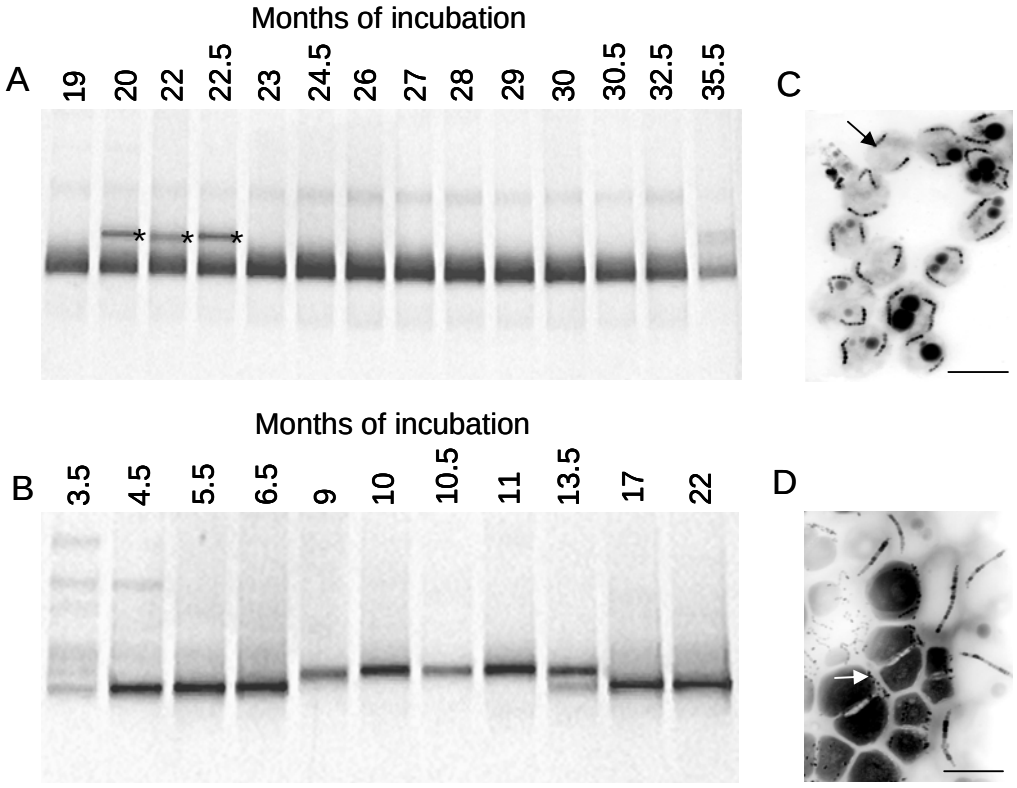

Fig. 6 A) and B) Community profiles of magnetically collected cells based on DGGE separation of amplified 16S rDNA fragments from microcosm C (A) and D (B) at different time points. All bands were excised, reamplified and sequenced; (*) bands which sequences are not related to any known MTB. C) and D) Electron micrographs of magnetotactic cocci from microcosm C (C) and D (D). Arrows indicate magnetosomes; bar = 1 µm.

We also noticed that stored samples from the same location sometimes contained different morphotypes of MTB. Therefore, four aliquots of the same sample taken from a site in Staßfurt were incubated for 22 months under nearly identical conditions in the lab. All four microcosms developed populations of abundant magnetic cocci, which displayed virtually the same morphology. However, DGGE analysis clearly revealed the presence of different MTB phylotypes in every microcosm (Fig. 7), indicating that the development of MTB population was not reproducible and apparently depends on very subtle differences in physico-chemical conditions between the microcosms.

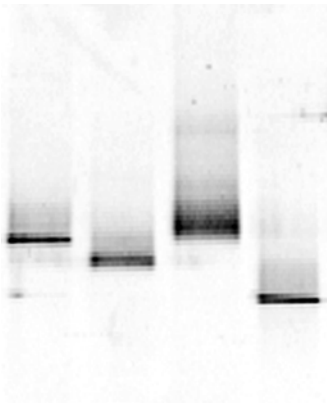

Fig. 7 DGGE profiles of amplified 16S rDNA fragments obtained from four magnetic collected samples from microcosms, which were simultaneously taken from a freshwater pond from Staßfurt.

Cultivation experiments

In extensive cultivation experiments, a multitude of different growth media were used to attempt the isolation of heterotrophic, autotrophic, or mixotrophic MTB. Typically, 10^5 - 10^7 viable (motile) MTB obtained by magnetic collection or RT-purification were used as inoculum per experiment both directly and in tenfold dilution steps. If inocula were prepared by magnetic collection, growth of non-magnetic contaminants was observed occasionally in dilutions of up to 10^{-4}. In contrast, RT-purified samples only very infrequently gave rise to contaminant growth, indicating that this purification method was strictly selective for MTB.

In numerous repeated experiments, we failed to detect growth of magnetotactic cocci, which were highly abundant in the inoculum. Neither the addition of complex supplements or redox-active compounds nor the incubation at different oxygen levels or in oxygen-sulfide-

gradients stimulated the growth of those MTB. However, in slush-agar cultures, which were inoculated with MTB from three different sampling sites (Ihle, Staßfurt and Bremen), growth of magnetotactic bacteria was repeatedly detected by the establishment of a distinct microaerophilic plate of bacteria after several days of incubation. Microscopic examinations revealed the presence of spirilla, which displayed a polar North-seeking magnetotactic reaction. While several of these cultures contained mixed populations with non-magnetic contaminants and could not be further purified, 10 isolates were obtained in pure cultures from various complex as well as minimal media containing a mixture of different organic substrates. Most strains were obtained from media with oxygen-sulfide-gradients (Schüler et al. 1999). All isolates were microaerophiles, which grew heterotrophically on minimal media on succinate and had a very similar morphology to previously isolated magnetotactic spirilla (Fig. 8). Phylogenetic analysis affiliated them all with the genus *Magnetospirillum* with closest similarity to either MSM-6 (Schüler et al. 1999) or *Magnetospirillum gryphiswaldense* (Schleifer et al. 1991) (Fig. 4, cluster II). Sequence divergences of 0 to 2.4% were found between all isolates.

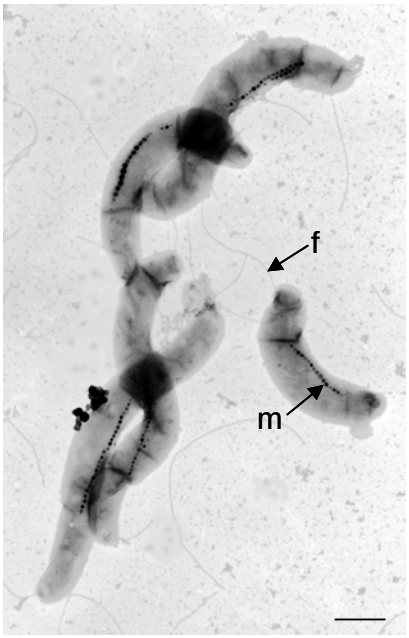

Fig. 8 Electron micrograph from a representative of the isolated *Magnetospirillum* strains. Cells were stained with uranyl acetate; m, magnetosomes; f, flagellum; bar = 0.5 µm. Other isolates obtained in this study had an identical morphology.

Discussion

The active migration of MTB along magnetic field lines provides a unique tool for their detection, manipulation and enrichment from environmental sources. Our study confirmed by molecular analysis earlier observations that uncultivated MTB can be selectively separated from sediment particles and contaminant bacteria by magnetic collection and RT-purification. While only $10^5 – 10^7$ cells could be recovered by RT-purification per single run, significantly higher amounts – we estimate up to 10^8 MTB - could be obtained by magnetic collection from the water column of several microcosms. The total MTB number could be increased by repeated collections, but hardly reached 10^9 MTB due to the increasing depletion of the microcosms. Although the collected cells still contained rare contaminants, they were sufficiently enriched for the isolation of high-molecular genomic DNA (data not shown). However, both methods were biased for fast-swimming MTB and did not fully retrieve the diversity of MTB observed by direct microscopy of sediment samples but both ARDRA and DGGE analysis revealed a low diversity within the collected MTB population. Both methods can be influenced by differential amplifications of the 16S rRNA genes during the PCR and give no reliable evidences about the abundance of the detected organism(s) in the sample (Reyensbach et al. 1992; Farrelly et al. 1995; Suzuki and Giovannoni 1996).

Similar as in previous studies (Blakemore 1975; Frankel et al. 1981; Sparks et al. 1986; Oberhack et al. 1987; Matitashvili and Matojan 1989; Petersen et al. 1989; Sparks et al. 1989; Petermann and Bleil 1993; Spring et al. 1998; Cox et al. 2002), a ubiquitous occurrence and considerable diversity of MTB was found in a survey of numerous aquatic habitats. 16S rDNA analysis has shown that all MTBs from this study affiliated with known MTB from either two phylogenetic clusters within the “Alphaproteobacteria” (cluster I and II) or the *Nitrospira* phylum (cluster III). Nevertheless, we cannot fully exclude that some of the sequences, which do not correspond to known MTB, represent so far unknown phylogenetic lineages of MTB. The general dominance of magnetotactic cocci in most of our samples may be a consequence of the selective enrichment conditions in our microcosms, which seem to favor the mass development of single or few magnetotactic species at the expense of diversity. The occurrence of closely related phylotypes of magnetotactic cocci within single samples may represent either a microdiversity of MTB or the presence of different 16S rDNA operons of a single species, which has been demonstrated for many other bacteria (Nübel et al. 1996; Rainey et al. 1996; Amann et al. 2000; van Berkum et al. 2003). Nevertheless, high sequence divergences up to 11% indicate that MTB of this morphotype seem to fall into different genera. On the other hand, samples from different sites contained nearly identical

phylotypes, while sequences retrieved from a single sample often displayed remarkable heterogeneities, indicating that the phylogenetic diversity is not correlated in an obvious way with geographical and geochemical variations between microcosms.

Only one uncultivated magnetotactic member of the *Nitrospira* phylum was previously identified and assigned ”Magnetobacterium bavaricum”. This large magnetotactic rod was detected in the sediments of various lakes in Upper Bavaria (Germany) (Spring et al. 1993). Our observation of MHB-1 in a microcosm sediment from a lake in Northern Germany indicates that the occurrence of MTB from this lineage is not restricted to oligotrophic Bavarian lakes. Although the presence of bullet-shaped magnetosomes seems to be a common trait of MTB from the *Thermodesulfovibrio*-”Magnetobacterium”-lineage of the *Nitrospira* phylum (Daims et al. 2001), there is a significant morphological and phylogenetic diversity within this magnetotactic group. Whereas isolation and cultivation-independent studies have identified *Nitrospira*-like bacteria as nitrite oxidizers (Watson et al. 1986; Ehrich et al. 1995; Wagner et al. 1996; Schramm et al. 1999; Daims et al. 2001), cultivation experiments with *Thermodesulfovibrio yellowstonii* and *Thermodesulfovibrio islandicus*, which represent the closest relatives of MHB-1, have demonstrated their ability to reduce oxidized sulfur compounds (Henry et al. 1994; Sonne-Hansen and Ahring 1999). In addition, in ”M. bavaricum“ and a closely related non-magnetic bacterium from deep-sea hydrothermal systems the oxidation of sulfide as energy source was implicated (Spring et al. 1993; Suzuki et al. 2004). Further studies will be required to analyze if these metabolic traits are present in uncultivated MTB from the *Nitrospira* phylum.

In the light of the considerable diversity observed in different environmental samples, an important and central question is, which environmental factors are controlling the occurrence and development of different MTB under natural conditions and in the microcosms stored under laboratory conditions? Consistent with earlier reports (Moench and Konetzka 1978; Schüler 1994), any of our attempts to amend the microcosms with various electron donors and acceptors such as iron (Fe^0 or Fe(III)), sulfate or nitrate under aerobic or anaerobic growth conditions did not result in increased numbers and diversity of MTB based on metabolic selection (data not shown). We found that smaller microcosms were less likely to develop stable MTB populations then larger one, probably owing to the instability of geochemical gradients within these systems. Parallel samples originating from the same site frequently developed populations of MTB which were predominated by different phylotypes. Likewise, aged microcosms, which had developed apparently stable population still underwent long-term population dynamics characterized by either sporadic collapses and

subsequent recovery of the whole MTB population or a temporary shifts towards different, yet morphological similar species. This suggests that MTB are strictly adapted to particular microniches and even subtle change in the environmental conditions may affect the community structure of MTB. Detailed geochemical analysis of microcosms and fresh undisturbed sediments cores will be required to elucidate the *in situ* physico-chemical conditions of microhabitats in which different MTB predominate.

Although magnetic cocci were highly abundant both in the sediment of microcosms and in our purified magnetic enrichments, they failed to grow under a multitude of cultivation media and conditions tried. Although magnetotactic spirilla were not abundant and could not be detected by DGGE, they were repeatedly isolated on simple media preferentially containing opposing gradients of oxygen and sulfide as described before (Schüler et al. 1999). However, the cultivated diversity of magnetotactic spirilla seems to be confined to species of the genus *Magnetospirillum*, which in physiological and morphological traits strongly resemble previously isolated species (Blakemore et al. 1979; Schleifer et al. 1991; Kawaguchi et al. 1992; Schüler et al. 1999).

In summary, our study has demonstrated that despite extensive attempts only a small fraction of MTB is amenable to cultivation by conventional methods. Beside the detailed geochemical analysis of microhabitats, which may lead to improved isolation strategies, innovative approaches are required, which should aim to reconstruct the complex chemical gradients found in stratified sediments. On the other hand, our data demonstrate that several MTB can be propagated in lab-scale microcosms, from which they can be collected in high numbers and enriched to virtually monospecific populations. In addition to the PCR-based RNA approach, genomic DNA could be isolated from those collected cells in amounts, which have shown to be sufficient for the construction of large-insert genomic libraries (Meyerdierks et al., unpublished data). Thus, the cloning and analysis of the genome of single magnetotactic species, or ultimately the ”magnetotactic metagenome” seems to be one of the most powerful approaches in order to obtain further insights into the genetic diversity of magnetosome biomineralization within uncultivated MTB.

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2.

Diversity and vertical distribution of magnetotactic bacteria along chemical gradients in stratified freshwater microcosms

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Abstract

The vertical distribution of magnetotactic bacteria (MTB) along various physico-chemical gradients in freshwater microcosms was analyzed by a combined approach of viable cell counts, 16S rDNA analysis, microsensor profiling and geochemical methods. The occurrence of MTB was restricted to a narrow sediment layer overlapping or closely below the maximum oxygen and nitrate penetration depth. Different species showed different preferences within vertical gradients, but the largest proportion (63 – 98%) of MTB was detected within the suboxic zone. In one microcosm the community of MTB was apparently dominated by a single species of a coccoid "Alphaproteobacterium", which could by detected by DGGE in all horizons from 1 to 10 mm depth. Maximum numbers of MTB were up to 1.5 x 10^7 cells/cm^3, which corresponded to 1% of the total bacterial community in the upper sediment layer. The occurrence of MTB coincided with the availability of significant amounts (6 - 60 µM) of soluble Fe(II), and in one sample with hydrogen sulfide (up to 40 µM). The putative relevance of several geochemical parameters for the distribution of MTB and potential implications for their metabolic adaptation to stratified sediments are discussed.

Introduction

Magnetotactic bacteria (MTB) are aquatic microorganisms whose swimming direction is influenced by magnetic fields (Blakemore 1975). The ability of magnetotaxis is based on magnetosomes, which are intracellular membrane-bounded crystals of a magnetic iron oxide, such as magnetite or greigite (Balkwill et al. 1980; Bazylinski 1995; Schüler 1999; Schüler 2004). The function of magnetotaxis is generally assumed to facilitate the bacteria finding and maintaining a favorable position in vertical chemical gradients in stratified environments (Blakemore 1975; Bazylinski 1995; Frankel et al. 1997). Despite the ubiquitous and abundant occurrence of diverse MTB in many marine and freshwater habitats, only a small number of magnetotactic strains could be isolated in pure culture so far (for review, see Bazylinski and Frankel 2004). Although they provide valuable models for laboratory investigation, insights into their metabolism, magnetosome biomineralization, and ecophysiology may not necessarily be generalized for the vast natural diversity of MTB, as the known cultivated species do not represent the dominant species in their natural environment (Spring et al. 1993; Spring and Schleifer 1995). Thus, further investigations of uncultivated MTB are required.

A variety of diverse MTB can be easily enriched without cultivation by taking advantage of their magnetically directed swimming behavior (Moench and Konetzka 1978; Wolfe et al. 1987; Schüler et al. 1999). This has enabled to explore the morphological and phylogenetic diversity of uncultivated magnetotactic bacteria in a number of studies (for review, see Spring and Schleifer 1995; Amann et al. in press). However, much less is known about their ecology and distribution in sediments and stratified water columns.

In an early report, the distribution of MTB in the permanently stratified water column of the Pettaquamscutt River Estuary (USA) was addressed (Stolz 1992). Whereas magnetotactic cocci could be detected only in the oxic and microoxic zone, diverse morphotypes were abundant (up to 2 x 10^5 cells/cm^3) not only in the microoxic but also in the anoxic zone in the presence of up to 2 mM sulfide. It was therefore concluded that the distribution of different MTB is determined by different optima in sulfide and oxygen gradients. Another study investigated the vertical distribution of magnetite and greigite producing MTB in the same habitat (Bazylinski et al. 1995). Generally, more magnetite producers were found at and above the oxic-anoxic transition zone (OATZ), whereas more greigite producing MTB were located in the anoxic sulfidic zone. Similar observations were reported for a stratified water column of a brackwater pond (Bazylinski and Moskowitz 1997).

MTB were only observed in the upper 10 cm of South Atlantic deep-sea sediments, while no MTB were detected in the water column (Petermann and Bleil 1993). Because the majority of MTB was found in the anoxic zone where nitrate was available, it was suggested that most MTB may reduce nitrate as the terminal electron acceptor.

The spatial distribution of an uncultivated giant MTB species ("Magnetobacterium bavaricum") from a freshwater habitat (Lake Chiemsee) was analyzed by fluorescence *in situ* hybridization (FISH) (Spring et al. 1993). In microcosm experiments the occurrence of "M. bavaricum" was restricted to deeper sediment layers. Most cells were present within the microoxic zone with a peak abundance of 7 x 10^5 cells/cm^3 equivalent to a relative abundance of 0.64 ± 0.17%. Because of its unusual large size, "M. bavaricum" accounted for approximately 30% of the biovolume within a narrow layer of the sediment, which indicates that MTB may play a dominant role in the microbial ecology of the sediment. Although these studies have indicated that MTB are major constituents of microbial communities in certain zones of aquatic habitats, the biogeochemical interactions controlling their occurrence and spatial distribution in stratified sediments have remained poorly understood.

In this study, we investigated the spatial distribution of MTB by viable counts and DGGE analysis in several freshwater microcosms. The purpose of our experiments was to

correlate the occurrence of MTB in different sediment layers to data obtained by simultaneous characterization of the chemical microenvironments using microsensor profiling, direct activity measurements of the sulfur cycle and geochemical methods. The results described here provide new insights into the physiology and ecological role of MTB and may lead to new strategies for the isolation of novel magnetotactic species in future studies.

Material and methods

Sampling and setup of microcosms

Sediment samples from the upper sediment layer and surface water were collected from several marine and freshwater habitats in Northern Germany. Microcosms were set up essentially as described earlier (Blakemore et al. 1979) in bottles (0.1 – 2 l). Alternatively, aquaria (5 l) were used for larger sample volumes. Approximately two thirds of sediment was overlaid with one third of sample water. The loosely covered containers were incubated at room temperature in dim light without agitation. Microcosms were stored in complete dark to prevent photosynthesis five days before analysis. Four microcosms were selected for comprehensive analysis, which had been previously incubated for 30 (A), 17 (B), 17 (C) or 6 (D) months, respectively and originated from the following sampling sites: (A) drainage ditch in Bremen, (B, C) eutrophic pond in Staßfurt (Sachsen-Anhalt), (D) public swimming area from a lake in Bremen (”Waller See”). Staßfurt samples (B and C) were collected from the same site, but at different times (B: April 2001, C: October 2001). Microcosm A and D were collected in March 2000 and April 2003, respectively. Cores from microcosms A and B were taken without venting and incubated for five days before analysis to reestablish physicochemical gradients, whereas cores from microcosms C and D were depressurized during sampling and could be analyzed immediately.

Viable counts

One sediment core per microcosm was sliced into 1 to 13 mm increments. The sediment samples were immediately diluted with sterile habitat water (1:2) and homogenized. For enumeration, 3 µl of sediment slurry was placed as a hanging drop onto a microscopic slide (Schüler 2002). Aliquots were diluted appropriately with sterilized habitat water, so that

50 - 300 MTB per drop could be counted. Cells with characteristic directed motility and magnetic response, which accumulated at the edge facing the magnetic south pole of a bar magnet, were considered as magnetotactic. Counting was started when virtually all MTB had reached the edge, which was typically after 10 - 12 min. Average MTB numbers were calculated from three different drops (Fig. 1). To investigate the influence of atmospheric oxygen onto the migration of MTB, a control sample was analyzed in parallel under oxygen-containing and oxygen-free atmospheres. Since both samples yielded nearly identical MTB numbers, the effect of oxygen was considered negligible and all counts were subsequently performed in the presence of air.

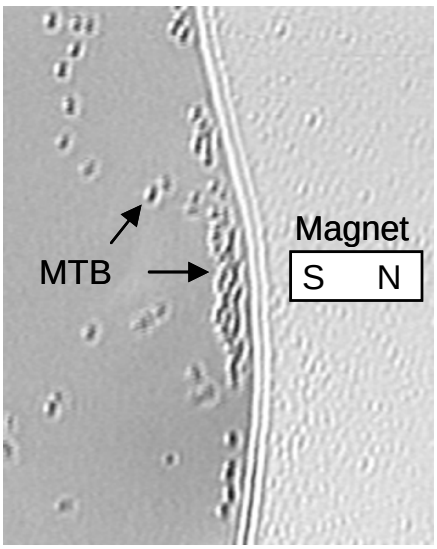

Fig. 1 Accumulation of MTB in a "hanging" drop after a magnetic field was applied for several minutes. Conspcicous bacteria appearing at the edge of the drop next to the south pole of the magnet were counted as MTB.

DAPI counts

Samples were fixed with paraformaldehyde, washed, subjected to sonification and filtered onto polycarbonate filters (pore size 0.2 µm, Millipore) as described by Pernthaler et al. (2001). The cells were stained with 4´,6´-diamidino-2-phenylindole (DAPI, Sigma) for 4 min and washed with H_2O and 70% ethanol. After the embedding in Citifluor (Citifluor Products) total cell numbers were counted using a fluorescence microscope (Zeiss). Mean cell numbers were calculated from several randomly chosen fields on each filter, corresponding to a minimum of 1000 DAPI-stained cells.

Electron microscopy

MTB from different sediment horizons were collected by a magnet as described above and adsorbed onto 300-mesh formvar coated copper grids (Plano), which were examinated without staining with an EM 301 transmission electron microscope (Philips) at 80 kV.

Magnetic collection of cells for PCR

To strictly separate MTB from sediment particles and contaminants, 50 µl of each sediment horizon were applied to a special separation chamber (Fig. 2). If necessary, the samples were diluted before with sterile habitat water (1:2). MTB were separated from the sediment slurry in a magnetic field using their active migration. Separated cells were collected from the chamber after 45 min. After concentration by centrifugation, cells were resuspended in 11 µl water.

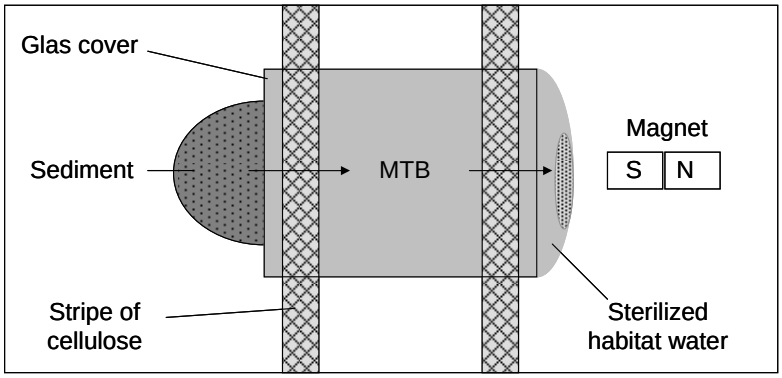

Fig. 2 Magnetic collection chamber for the separation of MTB from small sediment samples. MTB accumulate at the edge of the chamber next to the south pole of the magnet.

PCR amplification

Bacterial 16S rRNA genes from the resuspended cells were amplified with the universal primer pair GM5F with GC-clamp and 907R (Muyzer et al. 1995) and the PCR system from TaKaRa Bio Incorporation was used according to manufacturer's instructions. The touchdown PCR was initiated by one cycle at an annealing temperature of 65 °C, which was gradually decreased by 1 °C in every other cycle down to 55° C. For cloning, nearly-complete

16S rRNA genes were amplified using the same PCR system with the universal bacterial primer pair GM3F and GM4R, (Muyzer et al. 1995). PCR was performed by 33 cycles at an annealing temperature of 42 °C. The PCR product was cloned into the pCR®4-TOPO® vector and transformed into competent *Escherichia coli* cells (TOP10) from Invitrogen. The plasmid DNA from positive clones was isolated with a QIAprep Spin Miniprep Kit (Quiagen) and sequenced with GM1 (5´-CCAGCAGCCGCGGTAAT-3´) and vector-specific primers.

DGGE

DGGE was performed by using the D-Gene™ system (Bio-Rad Laboratories, Munich, Germany) as described previously (Muyzer et al. 1998). DNA fragments were separated in a 1 mm thick polyacrylamide gel (6% wt/vol) with a 20 - 70% denaturant gradient and 1 x TAE electrophoresis buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) at 60 °C. After 16 h electrophoresis at a constant voltage of 100 V, the gel was stained with ethidium bromide and documented by the Image Master from Amersham Pharmacia. DNA bands were excised and eluted in 100 µl water overnight at 4 °C. 1 to 6 µl of the eluates were reamplified with GM5F and 907R and PCR products were purified using the gel extraction kit (Eppendorf) from agarose gels and subsequently sequenced.

Sequence analysis

Purified PCR product or plasmid DNA (100 ng) were used in the sequencing reaction. DNA sequences were determined by a capillary sequencer (Applied Biosystems/Hitachi 3100 Genetic Analyzer) and compared against databases using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Microsensor measurements

Concentration profiles of oxygen, sulfide, nitrate and pH potential were measured using custom-build microsensors. Amperometric Clark type sensors were applied for oxygen and sulfide determinations, potentiometric glass sensors for pH, while biosensors were used for determination of nitrate profiles. The measuring tip of all applied sensors was typically 10 to

30 µm. See Kühl and Revsbech 1999 for detailed description and working principle of used microsensors.

Geochemical analysis

Sediment cores were sliced under nitrogen gas into 0.5 – 2 cm sections and pore water was immediately separated from the sediments by centrifugation through 0.45 µm membrane filters. Dissolved iron was measured spectrophotometrically by adding reducing Ferrozine immediately after filtration (Ferrozine reagent with 1% hydroxylamine-hydrochloride) to the pore water (Stookey 1970). To discriminate between the Fe(II) and Fe(III) ions, the reducing agent was added to the Ferrozine assay after a previous measurement in which hydroxylamine-hydrochloride was omitted. In addition, high resolution measurements of dissolved iron and manganese in the pore water were performed using the technique of diffusive equilibration in thin films (DET; Davison and Zhang 1994; Zhang and Davison 1999). In this technique solutes equilibrate with the water of the hydrogels, which are removed from the sediment after 24 h and subsequently sliced and reequilibrated for 24 h with HNO_3 (2% s. p). Dissolved iron and manganese concentrations were analyzed by optical emission spectroscopy (ICP, Perkin-Elmer Optima 3000 XL) using scandium as an internal standard.

Reactive or leachable iron is defined as the fraction of iron in sediments which is available for chemical reduction and readily reacts with sulfide (product of dissimilatory sulfate reduction) to form various iron sulfide minerals and eventually pyrite (Canfield 1989). The proportion of leachable iron was quantified by the Ferrozine method (see above) after extraction for 1 h at room temperature with either buffered Na-dithionite solution (Canfield 1989) or 0.5 M HCl (Kostka and Luther 1994). While the HCl-extraction reveals the amount of ferrihydrites and iron monosulfides, leachable iron extracted by dithionite represents both poorly and well-crystallized iron oxides, e. g. hematite, and minor iron bond to silicates (Canfield 1989; Kostka and Luther 1994). Both extraction methods showed nearly similar results therefore only data from dithionite extraction are presented in the results.

Sulfate concentrations in the pore water were determined by ion chromatography (Waters). Microbial sulfate reduction rates were measured using the whole core incubation method. The sediment core was injected with a $^{35}SO_4^{2-}$ tracer and incubated for 4 h at room temperature. Gross sulfate reduction rates (SRR) were measured for both the fraction of acid volatile sulfide (AVS, essentially FeS) and chromium reducible sulfur (CRS, essentially equivalent to elemental sulfur plus minor amounts of pyrite) by the two step distillation

method (Fossing and Jørgensen 1989). Total gross SRR is the sum of both fractions. The spatial distribution of bacterial sulfate-reducing activity was visualized by the application of the *in situ* 2D-photopaper monitoring technique, which is a modification of a method described in Lehmann and Bachofen (1999), and is based on the diffusion of hydrogen sulfide into a photographic paper with subsequent Ag_2S formation. Photographic paper was incubated for 23 – 25 h followed by the fixation in a 2 M sodium thiosulfate solution.

The water content of samples was determined gravimetrically after drying at 104 °C for 24 h. To establish the nature of mineral phases of the solid phases that precipitated at the walls of the incubation vessels, FTIR spectroscopy (Mattson 3000 type FTIR spectrophotometer) and X-ray powder diffraction (Philips X-ray powder diffractometer) were used. Pore water profiles were interpreted by the modeling program PROFILE (Berg et al. 1998) to calculate net rates of production or consumption as a function of depth.

Results

Setup and development of microcosms

Typically, various characteristic morphotypes of MTB including cocci, spirilla, rods and vibrios were present in most fresh samples. Both marine and freshwater microcosms underwent a characteristic succession within several weeks. Marine samples generally contained low numbers of MTB, which rapidly disappeared upon laboratory incubation. Generally, the total MTB numbers increased in most freshwater microcosms within several weeks. This was coincident with an apparent loss of diversity, and most microcosms were ultimately dominated by few morphotypes after prolonged incubation. In those samples, MTB populations remained apparently stable for periods of up to several years without obvious changes in cell numbers and morphologies. Therefore, four of these aged microcosms (A, B, C, D) were selected for further examination of distribution of MTBs.

Biogeochemical characteristics of microcosms

The succession in the MTB population coincided with a noticeable change in the appearance of microcosms. A characteristic stratification of the sediments became visible after several weeks of incubation, which reflected changes in the biogeochemical processes. The upper

sediment layer of microcosm C had a silty appearance, while coarse-grained sediment prevailed below about 18 mm depth. Surface sediments showed indications for bioturbation. The brownish sediment turned black below about 10 mm due to the reduction of Fe(III) minerals and the precipitation of iron monosulfides. The presence of FeS was proven by H_2S liberation from the sediment upon attack with diluted hydrochloric acid. Below about 30 mm depth the sediment turned grey due to the formation of pyrite and it contained gas bubbles (presumably methane), besides plant residues. Water contents reached about 75% at the surface that decreased to below 30% in the coarse grained bottom part. Microcosm D, containing a more sandy sediment, displayed stratification marked by color change. The light-brownish-gray oxic surface extended over 3 mm and turned into a darker layer down to 30 mm depth. The following grey-colored section of the sediment was laminated with two distinct darker layers at about 40 and 50 mm depth. Water contents ranged up to 35% and decreased with depth.

During the time of sampling, maximum oxygen penetration depth varied between the four microcosms (A: 2.5 mm; B: 1.5 mm; C: 3.0 mm; D: 2.0 mm; Fig. 3), but displayed only slight variations (less than 0.5 mm) within a single setup. In microcosms C and D additional profiles of nitrate, sulfide and pH were obtained. In both microcosms nitrate was measurable and maximum penetration depth was generally 0.5 mm deeper than oxygen (Fig. 3C and D). However, at one particular spot in microcosm C, a deeper maximum penetration depth of nitrate was observed (5.0 mm), which was possibly due to locally enhanced nitrification rates (Fig. 3C). Although photopaper-monitoring techniques indicated the occurrence of dissolved sulfide in microcosm C (data not shown), no free sulfide (H_2S) was measurable, probably due to the rapid reoxidation at the surface (Fig. 3C). In microcosm D hydrogen sulfide could be detected below a depth of 3.0 mm. Interestingly, dissolved sulfide apparently did not overlap with either oxygen or nitrate (Fig. 3D). Values of pH decreased from 8.5 at the sediment surface to 7.0 in deeper sediment layers in microcosm C, but remained stable at 6.7 in the sediment of microcosm D (data not shown).

Maximum sulfate concentrations were 1.4 mM (C) and 4 mM (D) in the surface waters and decreased further downcore (Fig. 4A and B). Modeling with PROFILE revealed a distinct zone of sulfate consumption in the upper 4 cm of microcosm C (data not shown). This result was further substantiated by the direct measurements of gross sulfate reduction rates (SRR). Gross SRR was found throughout the investigated sediment sections with maxima at about 4.5 cm depth (Fig. 4A). The surface values of sulfate reduction rates of 10 nmol/cm^3/d increased up to maximum rates of 70 nmol/cm^3/d at 4.5 cm depth with a subsequent decline. Effected by reoxidation, sulfate reduction rates showed an increase of AVS just below 1.5 cm

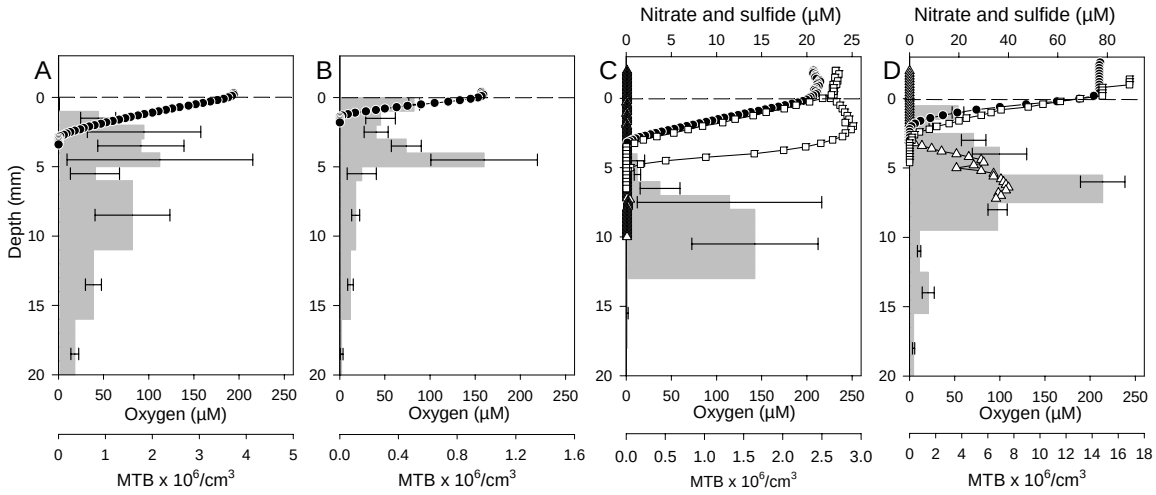

Fig. 3 Vertical distribution of MTB in comparison to the oxygen, nitrate and hydrogen sulfide profiles. A) microcosm A, B) microcosm B, C) microcosm C and D) microcosm D. Sediment surface (- -), oxygen (●), nitrate (□), sulfide (Δ), MTB numbers (vertical bars).

depth and reached maximum values at 4.5 cm depth. In microcosm D modeling with PROFILE did revealed a sulfate depletion below the maximum of SRR at approximately 2.5 cm depth (PROFILE data not shown). The rates were about 20times higher compared to microcosm C. AVS contents started to accumulate just below 1.5 cm depth, indicating effective reoxidation in the top part of the sediment (Fig. 4B).

In microcosm C an increase of Fe(II) was detected below 0.5 cm depth and reached up to 110 µM at 2 cm depth (Fig. 4C). In deeper sections, iron monosulfide was present contributing to the fraction extracted by Na-dithionite (Fig. 4E) and leading also to a decrease in dissolved Fe(II) concentrations. Corresponding to the SRR zones, net Fe(III) reduction zones near the surface were obtained from the modeling with PROFILE. For the upper sediment zone a net iron reduction rate of 17 nmol/cm^3/d was calculated (data not shown). Dissolved iron was released into the pore water below the oxygen penetration depth of about 5 mm up to 104 µM in microcosm D (Fig. 4D). The depth profile of leachable iron (Fig. 4F) indicates a depletion of Fe(III) below the first cm due to chemical reduction by sulfide in agreement with the accumulation of dissolved Fe(II). The Fe(III) reduction rate in the upper sediment layer determined by PROFILE was in the same range as described for microcosm C (data not shown). The combined results of SRR and Fe(III) reduction rates indicate that this is a zone where both chemical and microbial iron reduction takes place in both microcosms. The precipitation of ferrihydrites and manganese dioxides at the walls of microcosm D indicated that

near surface metal reduction led at least temporary to the liberation of dissolved iron and manganese to the overlying water, where reoxidation took place.

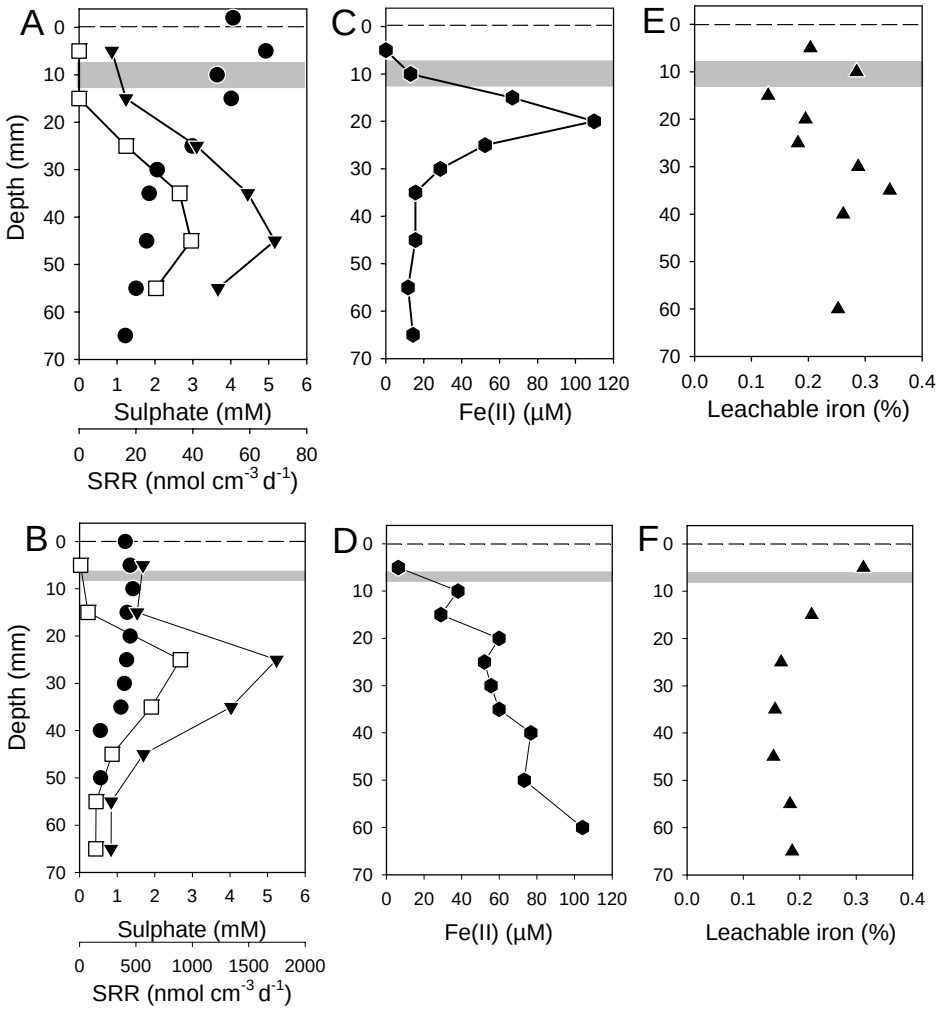

Fig. 4 Distribution of maximum MTB numbers in comparison to sulfate concentrations, sulfur reduction rates and dissolved and leachable iron. A), C) and E) microcosm C; B), D) and F) microcosm D. Sediment surface (- -), sulfate (●), sulfate reduction rate total (▼), sulfate reduction rate AVS (□), dissolved iron (⬢), leacheable iron (▲), horizons with maximum cell numbers of MTB (vertical bars).

Vertical distribution of MTB

Analysis of viable cell numbers revealed a heterogeneous vertical distribution of MTB in all microcosms. No MTB were detectable in the oxic water columns and the occurrence of MTB was restricted to a narrow layer in the sediment, which was overlapping or closely below the

OATZ. However, different microcosms displayed variations in the abundance and distributions of MTB.

In microcosm A highest numbers of MTB (2.3 x 10^6 cells/cm^3) were present between 4 and 5 mm depth (Fig. 3A). No other morphotypes than magnetotactic cocci were observed by microscopy. In contrast, two maxima of distribution were found in microcosm B (Fig. 3B). The first maximum of 5.1 x 10^5 MTB/cm^3 was found immediately below the surface in the microoxic sediment zone and consisted of different morphotypes including magnetotactic cocci and spirilla. A second peak (9.7 x 10^5 MTB/cm^3) occurred in the anoxic zone between 4 and 5 mm depth and exclusively consisted of magnetotactic cocci.

Likewise, a heterogeneous distribution of different morphotypes was observed in microcosm C. Beside the presence of cocci, this microcosm was dominated by magnetotactic spirilla. Unlike magnetotactic cocci, spirilla performed a bidirectional swimming motility (Frankel et al. 1997), but nevertheless displayed a North-seeking net polarity. Both cocci and spirilla were present in the oxic as well as in the anoxic zone of the sediment, but were most abundant between 8 and 13 mm depth (1.6 x 10^6 MTB/cm^3, Fig. 3B; proportion of cocci was 8.0%, data not shown). Interestingly, this peak did partially overlap the presence of soluble ferrous iron of 25 - 60 µM (Fig. 4B). The number of MTB steeply declined in deeper sediment layers.

In microcosm D, MTB could be detected in all horizons down to 45 mm. A single maximum of 14.8 x 10^6 MTB/cm^3 was detectable in the anoxic sediment between 6 and 8 mm depth where 6 - 40 µM Fe(II) is available (Fig. 3D and 4D). Total cell numbers in this layer were determined by the DAPI method and were found to be 10^9 cells per cm^3. Thus, MTB accounted for approximately 1% of the total microbial population of this particular horizon. In contrast to magnetotactic cocci, which were most abundant at this depth (14.6 x 10^6 cells/cm^3), magnetotactic spirilla occurred in highest numbers (0.5 x 10^6 cells/cm^3) in the upper anoxic layer between 4 and 6 mm depth, but were absent from deeper sediment layers (Fig. 5A).

Morphological and phylogenetic diversity of MTB from different horizons

Microscopic analysis indicated that the MTB distribution in our microcosms was heterogeneous not only with respect to cell numbers, but also to the composition of the magnetotactic population (Fig. 5C to E). Differences were observed between different sediment layers of a single microcosm as well as between microcosms from different

sampling sites, as indicated by microscopy and 16S rDNA analysis of MTB, which were magnetically collected without depth fractionation. While for instance a sequence from microcosm A revealed high similarity to the uncultivated magnetotactic coccus maccs13 (CS103, accession number X61605), sequences from microcosm B affiliated with a different uncultivaed MTB (accession number AJ223476).

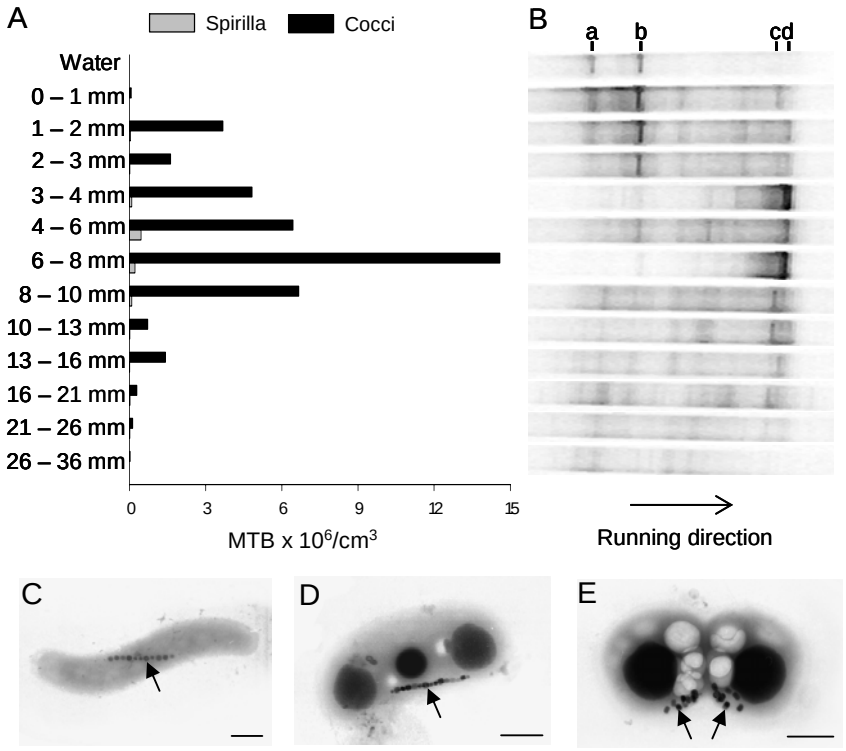

Fig. 5 A) Vertical distribution of different morphotypes in microcosm D. B) DGGE band pattern obtained with the universal bacterial 16S rDNA Primer GM5F+GC-clamp and 907R from magnetically enriched cells from different depths of microcosm D. The marked bands were excised and sequenced for phylogenetic analysis. C) to E) Electron micrographs of different MTB which were characteristic for microcosm D. Bar = 0.5 µm.

To investigate the vertical distribution of different species, MTB were collected from different sediment layers of microcosm D. Figure 5B shows the results of the DGGE of PCR-amplified 16S rDNA fragments obtained from the water column and magnetically enriched cells from different sediment layers. Two major bands (a and b) were obtained from the water column. These bands were also present in the upper zone of the sediment, but their intensity decreased in deeper sediment layers. Sequences obtained from a and b displayed highest similarity to those from the *Cytophaga-Flavobacterium-Bacteroides* cluster, while a third band (c) from the upper and deeper sediment layers yielded a sequence closely related to *Pseudomonas*

spinosa. A further distinct band (d) was abundant in DGGE patterns from 1 to 10 mm depth, which was coincident with the maximum abundance of MTB. Sequencing of this band confirmed its identity in all horizons and revealed high sequence similarity to a sequence from an uncultivated magnetic coccus in the database (accession number X80996). To obtain extended sequence information, the nearly complete 16S rRNA gene was amplified from cells collected from 6 to 8 mm depth, the horizon which had revealed only a single band in DGGE. One sequence was obtained, which matched the partial sequences from band d in the DGGE profile and displayed only four mismatches to the uncultivated magnetococcus (Spring et al. 1994). Hence, the DGGE profiles from different sediment layers confirmed the heterogeneous vertical distribution of MTB and were consistent with the distribution of total viable MTB numbers. However, molecular analysis did not fully reveal the diversity of MTB observed by microscopy during cell counts and electron microscopy. Microscopy of the magnetotactic cells collected for PCR revealed that in contrast to direct counts in the hanging drop, only magnetotactic cocci were observed in significant numbers. This might be a consequence of the more stringent collection conditions employed for PCR amplification, which had resulted in the loss of other MTB species (e. g. spirilla) below detection.

Discussion

The function of magnetotaxis is generally assumed to facilitate the bacteria finding and maintaining their favorable position in vertical chemical gradients in stratified sediments, however it has not been explored how exactly the position is correlated to geochemical parameters. Previous studies have attempted to characterize the vertical distribution by FISH (Spring et al. 1992; DeLong et al. 1993; Spring et al. 1993; Spring et al. 1994; Spring et al. 1998). Furthermore, *in situ* detection of individual cells in sediments is problematic because of a potentially low abundance of the target organisms and a high background fluorescence (Prosser and Embley 2002). In addition, no group-specific probe universal for MTB has been available because of their phylogenetic heterogeneity, confining the analysis to individual species. In this study, counts of magnetically selected cells have proven a powerful tool for the enumeration of viable MTB. However, this method probably tends to underestimate the abundance of MTB, because it is intrinsically biased for highly motile cells, while slow-moving bacteria or cells that were attached to sediment particles are possibly missed (Frankel et al. 1997; Cox et al. 2002). All of our selected samples were characterized by the development of abundant populations of MTB, however, these varied considerably in numbers and

community compositions between different microcosms. The observed downcore profiles of microbial activity and dissolved constituents confirmed the presence of steep opposing gradients of reduced and oxidized compounds in the surface sediment of the investigated microcosms. In all samples this coincided with the occurrence of MTB, which was restricted to a narrow layer in the upper sediment located closely to the OATZ. As most of the cultivated MTB strains are known to behave as typical microaerophiles, it was surprising that in all microcosms most MTB were detected in the suboxic zone immediately below the OATZ (A: 63%, B: 92%, C: 98% and D: 91%). Maximum numbers were between 9.7 x 10^5/cm^3 and 1.5 x 10^7/cm^3, thus accounting for at least 1% of the total cell numbers in this region. Interestingly, in microcosm B magnetic spirilla were more abundant in microoxic sediment layers, whereas magnetic cocci were predominantly found in the deeper suboxic zone, indicating that different species showed different preferences within the vertical gradients. The maximum MTB numbers in our study were considerably higher than MTB numbers estimated for environmental samples (10^3 – 10^4 MTB/cm^3, Blakemore et al. 1979; Blakemore 1982), but were in the same range as reported previously for other laboratory enrichments (Moench and Konetzka 1978; Blakemore et al. 1979; Petersen et al. 1989; Spring et al. 1993).

Although all MTB displayed a very strict preference within the vertical chemical zonation, the pathway of electron transfer and their putative mode of metabolism are not immediately apparent from the distribution of potential electron donors and acceptors investigated. Cultivated MTB are metabolically versatile and all investigated species are known to perform a strictly respiratory metabolism, which involves the oxidation of organic or inorganic substrates. The distribution and identity of dissolved organic carbon was not determined in this study. However, measurements in stratified biomats indicated that the occurrence of short-chained fatty acids, which are typical fermentation products, is restricted to the upper layers slightly below the oxic zone (Jonkers et al. 2003). As these are preferred substrates for cultivated MTB strains, the peak abundance of MTB in our microcosms thus might reflect the availability of these organic carbon sources.

In addition to organic substrates, the availability of inorganic electron donors must be considered as an important factor controlling the distribution of MTB. Several isolated MTB strains including the magnetic coccus MC-1 and the magnetic vibrio MV-1 are able to grow by the oxidation of sulfide in opposing sulfide-oxygen gradients (Meldrum et al. 1993b; Kimble and Bazylinski 1996). *Sox* genes, which encode enzymes essential for the oxidation of reduced sulfur compounds in several bacteria (Wodara et al. 1994; Friedrich et al. 2000; Petri et al. 2001) have been recently detected in the genomes of *Magnetospirillum* species (DOE

Joint Genome Institute www.jgi.doe.gov/JGI_microbial/html; Flies & Schüler, unpublished), suggesting that the ability to oxidize reduced sulfur compounds is ubiquitous among MTB. In MV-1, evidence for a facultative autotrophic metabolism based on the oxidation of sulfide or thiosulfate was found (Kimble and Bazylinski 1996). The majority of the MTB found in our microcosms contained inclusions, which in light and electron micrographs were apparently identical to the sulfur globules that have been reported in numerous uncultivated MTB from freshwater and marine habitats (Moench 1988; Spring et al. 1993; Frankel and Bazylinski 1994; Iida and Akai 1996; Cox et al. 2002). Intracellular sulfur globules are commonly found in other bacteria as metabolic products of H_2S oxidation. Sulfide could be detected in microcosm D and the calculated net production of sulfate as well as sulfur intermediates (as S°) coincided with the occurrence of MTB in this microcosm. Thus, the assumption of a sulfide-oxidizing metabolism, either autotrophic or mixotrophic, would be reasonable at least for the MTB population in microcosm D.

With the exception of the sulfate-reducing strain *Desulfovibrio magneticus*, all known cultivated MTB strains can use molecular oxygen and nitrous compounds (i. e., nitrate or nitrous oxide) as terminal electron acceptors for respiration (Blakemore et al. 1979; Blakemore et al. 1985; Bazylinski et al. 1988; Matsunaga et al. 1991; Schleifer et al. 1991; Meldrum et al. 1993a; Kimble and Bazylinski 1996; Frankel et al. 1997; Dean and Bazylinski 1999; Heyen and Schüler, unpublished). However, the occurrence of MTB in our microcosms was not coincident with the presence of either nitrate and oxygen and the maximum numbers were found in horizons where these electron acceptors were not detectable. MTB thus might shuttle electrons between the zones where either electron donors and acceptors are available, respectively. One possible mode of metabolism would be that MTB migrate between zones of sulfide oxidation and oxygen reduction. Vertical displacements of several cm up to 2 m in the sediment and in the water column, respectively, have been observed in other sulfur oxidizing microorganisms from marine and freshwater environments (Huettel et al. 1996; Ramsing et al. 1996; Mußmann et al. 2003). MTB could take up H_2S in the sulfide production zone, make a partial oxidation to elemental sulfur, store the sulfur intracellularly as granules, and move up to the upper zone, where the sulfur could be oxidized to sulfate with molecular oxygen or nitrate.

Nevertheless, the predominant occurrence of MTB in zones apparently lacking an appropriate electron acceptor remains somewhat puzzling. Due to their small size, the observed MTB are unlikely to store significant amounts of an electron acceptor, such as for instance nitrate-storing sulfide-oxidizing *Thioploca* and *Beggiatoa* species (Jørgensen and Gallardo

1999; Zopfi et al. 2001; Mußmann et al. 2003). Therefore, while shuttling electrons between separate zones, the cells have to derive some energy by an unknown process to maintain their metabolic activity and motility, perhaps by some sort of fermentation or the transient use of other oxidants. Alternatively, their preferred occurrence in oxygen-free zones in our aged microcosms might reflect a metabolically inactive or resting state, which is occupied in the absence of easily accessible organic substrates. This would be consistent with the stunning observation that viable MTB can be recovered in high numbers from sediment micro- and mesocosms even after many years of incubation in the dark without any addition of a carbon or energy source (Blakemore 1975; unpublished observation).

Besides oxygen and nitrate, the use of sulfate as a further electron acceptor has been demonstrated in MTB. The "Deltaproteobacterium" *D. magneticus* is the only cultivated magnetotactic strain, which can couple the oxidation of organic acids to the reduction of either fumarate or sulfate (Sakaguchi et al. 1996). 16S rDNA analyses of other uncultivated, multicellular MTB from the marine environments affiliated them also with the "Deltaproteobacteria" with closest similarity to the known sulfate reducer *Desulfosarcina variabilis* (DeLong et al. 1993), suggesting that this type of metabolism might be more common among diverse MTB. Sulfate reduction rates in the microcosm C are comparable with rates found in oligotrophic lakes (Urban 1994; Savvichev et al. 2003), while the high sulfate reduction rates found in microcosm D are at the upper end of those commonly found in limnic sediments and compare to rates found in marine systems, where concentrations of sulfate are 2 - 3 orders of magnitude higher (Jørgensen 1990). SRB seem to be important in the shaping of gradients in the investigated sediments. However, no MTB could be detected from the depth of maximum sulfate reduction. Although some sulfate reducing activity occurred also in the upper, partially microoxic horizons, the apparently most abundant MTB detected in these layers was identified as "Alphaproteobacterium". However, so far there are no indications that members of this group might be able to reduce sulfate. Thus, sulfate reduction or sulfur disproportionation are not likely to play a role as major metabolism pathways in most MTB from freshwater habitats.

Several previous reports have implicated cultivated MTB in the reduction of iron and it has been suggested that they potentially derive energy from this process (Short and Blakemore 1986; Paoletti and Blakemore 1988; Guerin and Blakemore 1992). The depth extension of iron reduction in sediments generally coincides with the availability of Fe(III) (Thamdrup 2000). In our experiments, MTB were found in a zone where abundant solid phase Fe(III) was present and thus, potentially might serve as an electron acceptor. However, ferric iron reduc-

tion did not support growth of cultivated MTB in recent experiments (Heyen, personal communication), and all attempts to enrich and isolate MTB capable of iron respiration have failed so far (Flies and Schüler, unpublished). Thus, there are no clear indications that dissimilatory iron reduction is a potential metabolic pathway at least in cultivated MTB, and its relevance in environmental populations remains uncertain.

Besides its putative function in redox cycling, iron plays an eminent role in MTB, since large amounts of iron are required for the synthesis of magnetosome crystals that account for 2 - 4% of the dry weight (Blakemore et al. 1979; Schüler and Baeuerlein 1998). Intriguingly, the abundance of MTB in our microcosms coincided with the availability of dissolved iron in the top surface sediments. Ferrous iron concentrations in the pore water of sediment layers with highest MTB abundance were between 25 to 60 µM and 6 to 40 µM in microcosm C and D, respectively. These concentrations are in the range of those, which are saturating for both growth and magnetosome formation in cultures of *Magnetospirillum gryphiswaldense* and other MTB (Schüler and Baeuerlein 1996; Schüler and Baeuerlein 1998; Heyen and Schüler 2003). In contrast to oxic environments at neutral pH, where bacteria usually encounter conditions of iron scarcity (Millero et al. 1987), the micro- and suboxic sediment layers occupied by MTB apparently provide sufficient amounts of iron. Thus, the availability of iron for ”assimilation”, i. e. synthesis of magnetosomes appears to be an important factor determining the distribution of MTB.

The relative position of MTB within gradients of other dissolved species might thus not being strictly determined by a single factor, but rather represent a ”trade-off” between the availability of iron on the one hand and organic carbon sources and/or inorganic compounds serving as electron donors and acceptors on the other hand, which perhaps involves a shuttling or temporary displacements between different zones. How can MTB manage to navigate within these complex and steep chemical gradients? It has been suggested that behavioral responses to terminal electron acceptors may play a key role in the structure and organization of microbial communities of microbial mats and sediments (Møller et al. 1985; Krekeler et al. 1998; Alexandre et al. 2004). Interestingly, genome analysis recently revealed that known MTB are among the bacterial species with the highest number of chemotaxis transducers (e. g. *Magnetospirillum magnetotacticum*: 65, *E. coli*: 5). It therefore has been speculated that energy taxis may play a dominant role in the control of motile behaviour in these organisms (Alexandre et al. 2004). In combination with magnetic orientation, this would provide an unusually efficient mode of vertical orientation by reducing three-dimensional search to a mono-dimensional search problem (Blakemore and Blakemore 1990). Thus, MTB seem to be

perfectly adapted for the efficient navigation within steep chemical gradients in stratified environments.

Our investigated microcosms were primarily selected because of high numbers of MTB, which developed after establishment of nearly steady-state conditions after prolonged incubation. Thus, the aged microcosms do not necessarily reflect natural conditions, as indicated by the observed loss of diversity. Conditions in natural sediments are likely to be more dynamic due to the continuous influx of organic substance and the impact of photosynthesis, and the effects of vertical migration might be more pronounced. Thus, further studies, such as in *in situ* measurements of their metabolic activity (e. g. by microautoradiography and MAR-FISH) in natural sediments from marine and freshwater habitats are required. In addition, future strategies for the isolation and cultivation of abundant MTB species might benefit from the application of multiple gradient systems.

Acknowledgements

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3.

Crystal size and shape distributions of magnetite from uncultured magnetotactic bacteria and magnetite as potential biomarker

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Abstract

We studied the sizes and shapes of magnetite crystals produced by several types of uncultured magnetotactic bacteria in order to understand whether the size distributions of magnetite nanocrystals can be used for identifying their biogenic origin in geological samples. The two-dimensional projections of nanocrystals were measured on transmission electron microscope images, and features of crystal size and shape factor distributions (CSD and SFD, respectively) analyzed. In agreement with previous results, most magnetite CSD curves are asymmetric and negatively skewed; however, one magnetotactic strain produced particles that have a normal size distribution. A statistical analysis of CSDs and SFDs (from both this and previous studies) reveals similarities among magnetite from magnetotactic strains from various locations. In particular, crystals in a cultured marine strain (MC-2) were indistinguishable from magnetite from a freshwater strain. We tested whether CSDs of distinct magnetosome types can be recovered from the shape and size data of all particles in samples that contain several types of magnetosomes; such samples can be used as models for rocks that contain magnetite nanocrystals of unknown and, presumably, various origins. If the SFDs of the distinct magnetosome types occurring in the same sample differ, the CSDs of individual magnetosome types can be retrieved from bulk data. In such cases the characteristic shape of the size distribution can be used for identifying magnetite as originating from magnetotactic bacteria.

Introduction

Magnetite (Fe_3O_4) crystals produced intracellularly by magnetotactic bacteria have well-defined and often unusual morphologies, narrow size distributions, and a high degree of chemical purity (Devouard et al., 1998; Bazylinski and Frankel, 2000). The unique crystal habits and size constraints of bacterial magnetite offer the possibility of using such nanocrystals as biomarkers in geological samples. Nanoscale magnetite crystals from ocean sediments and rocks of various ages were identified as formed by biologically controlled mineralization (BCM) on the basis of crystal sizes and shapes (Chang and Kirschvink, 1989). Thomas-Keprta et al. (2000; 2002) compared magnetite extracted from the ALH84001 Martian meteorite with crystals from bacterial strain MV-1 and concluded that the MV-1 magnetite was “indistinguishable” from a subset of the meteoritical magnetite particles. The identity of the two types of magnetite was based primarily on morphological features (Clemett et al., 2002). Previously

we argued (Buseck et al., 2001) that the information obtained about the crystals is insufficient to reach a reliable conclusion on the biogenic origin of magnetite in ALH84001, and noted that not only the mean size of nanocrystals but the shape of the crystal size distribution (CSD) curve could be diagnostic for the biological control of crystal growth. In the present study we explore the possibility of using the features of the CSD and SFD of magnetite populations for the determination of the biogenic or inorganic origins of crystals.

The shapes of CSDs carry information about the growth histories of crystal populations (Eberl et al. 1998). Various inorganically formed crystals including quartz (Kile and Eberl, 1999), illite (Srodon et al., 2000), and exhibit lognormal distributions. Such CSDs can result when the crystals grow in an open system, and their growth is controlled by either their surface area or the availability of nutrients ("surface-controlled" and "supply-controlled growth," respectively; Eberl et al., 1998). In contrast to inorganically produced magnetite populations that show lognormal CSDs (Fig. 1a), magnetite particles from several cultured magnetotactic bacterial strains produce negatively skewed CSDs (Meldrum et al., 1993a,b; Devouard et al., 1998; Eberl and Frankel, 2000) (Fig. 1b). Based on the few CSDs that are available in the literature, the narrow size distributions and sharp cutoffs at large sizes may be a signature of bacterial magnetite. On the other hand, greigite (Fe_3S_4) crystals from a sulfide-producing magnetotactic microorganism ("many-celled magnetotactic prokaryote," MMP) exhibit a symmetrical, Gaussian CSD (Fig. 1c) (Pósfai et al., 2001). The specific shapes of CSDs are likely related to specific biologically controlled crystal growth mechanisms.

In principle, the distinctly biogenic CSDs could be used for identifying bacterial magnetite in geological samples such as the ALH84001 Martian meteorite. Presumably, if BCM magnetite occurs in a rock or sediment sample, the crystals were produced by several types of magnetotactic bacteria, and the magnetite grains have various sizes and shapes. In addition, if magnetite particles formed by biologically induced mineralization (BIM) or inorganic processes also occur in the sample, the unambiguous identification of BCM magnetite may be difficult or impossible. Clearly, there is a need for a method that could be used for retrieving the CSDs of magnetite populations of distinct origins from the crystal size and shape data of all magnetite particles in a sample.

In this study we describe CSDs and SFDs of magnetite from several uncultured magnetotactic species obtained from environmental samples and compare them with those previously reported for cultured species. Some samples contain several types of magnetosomes that have distinct morphologies and sizes. Assuming that magnetite particles remain unchanged after deposition, cell lysis, and diagenesis, these samples can be used as models for sediments

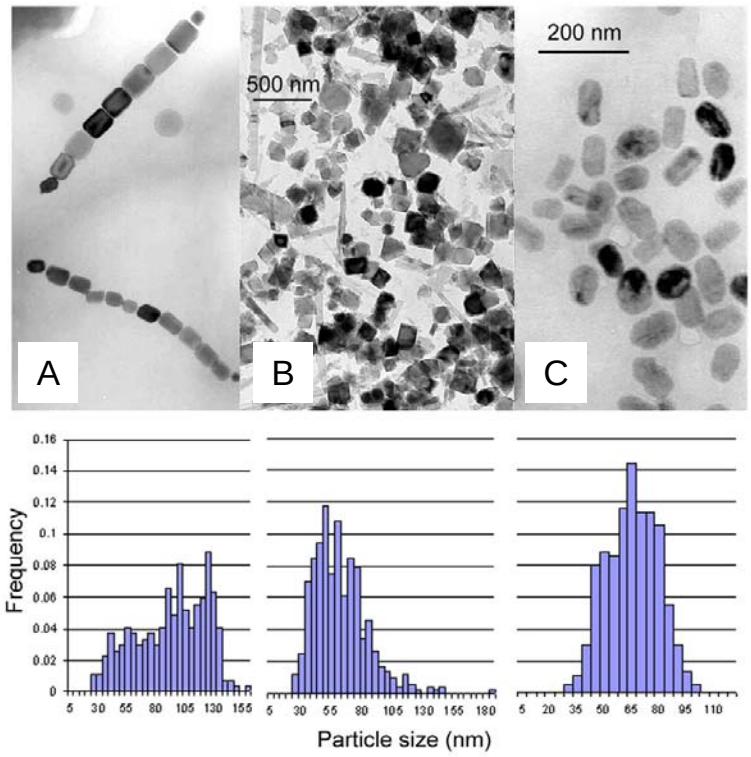

Fig. 1 Three distinctly different CSDs of magnetosomes and synthetic magnetite. (a) Magnetite from magnetotactic bacterium from Séd stream. (b) Synthetic magnetite prepared from $FeSO_4$ solution. (c) Greigite from the many-celled magnetotactic prokaryote, MMP (Pósfai et al., 2001). Upper panel represents TEM images, lower panel shows CSD of the particles.

or rocks that contain several types of biogenic magnetite. We studied whether distinct magnetosome types can be identified in such samples solely on the basis of the properties of the SFD and CSD of all magnetite particles and without knowledge of other characteristic features that would not be preserved in rocks (such as cell morphology or chain structure). Peaks in the combined SFD were deconvoluted and then size distributions plotted from the data belonging to the distinct modes of the SFD. The resulting CSD curves were compared with those obtained for individual magnetosome types using all observable features, including chain and cell structure.

We also mixed data from the bacterial crystals with those from synthetic magnetite and then tried to retrieve individual CSDs from the combined data set of crystal sizes and shapes. The aim of this experiment was to test whether an objective, relatively fast and robust method, the analysis of the CSDs and SFDs as measured in two-dimensional projections, can be used for identifying the biogenic origin of magnetite populations in samples that contain grains of both bacterial and inorganic origins.

Materials and methods

Environmental samples were collected from the sediment and water of freshwater streams and lakes in Hungary (Gyöngyös, Séd, and Tapolca), and from streams, ponds, and the sea in Northern Germany (samples S1 to S4; Table 1). Glass containers were filled with sediment and water. We enriched magnetotactic bacteria by placing a bar magnet with its magnetic south pole next to the sediment/water interface. A subsample was drawn with a pipette from next to the magnet. Further enrichment of bacteria was achieved by placing the magnet to the end of the pipette. The first drop contained a large number of magnetotactic bacteria and was placed onto Cu or Ni TEM grids covered by carbon-coated Formvar films. No further specimen treatment was applied.

Synthetic magnetite was prepared from both $FeSO_4$ and $FeCl_2$ as starting materials. Following the recipe by Sidhu (1977), 80 g $FeSO_4 \cdot 7H_2O$ was dissolved in 560 ml deionized water, with air excluded, and the solution heated to 90 ^oC in an N_2 atmosphere. Then we added a solution that was prepared by mixing 6.46 g KNO_3 and 44.9 g KOH with 240 ml deionized water. The solution was kept at 90 ^oC for 30 min while magnetite precipitated. We also synthesized magnetite, by mixing solutions of 2 M $FeCl_2$ and 1 M $FeCl_3$ in a volume ratio of 1:4 and slowly adding 0.7 M NH_4OH at room temperature. By using these two methods we obtained two magnetite populations that had different CSDs.

The morphologies of bacteria and magnetite particles were observed using a Philips CM20 TEM operated at 200 kV and a Hitachi 7100 TEM operated at 125 kV. Composition was analyzed using energy-dispersive X-ray spectrometry (EDS) in the Philips CM20 with a Noran Voyager EDS detector, and selected-area electron diffraction (SAED) was used to identify the structures of crystals.

Digitized bright-field TEM images were used for a statistical analysis of the sizes and shapes of magnetite crystals. We used Digital Micrograph software to measure the two-dimensional projections of grains; in order to consistently characterize the dimensions of crystals having various shapes, we fitted ellipses to the particles and used the major and minor axes of the best-fitting ellipse for further analysis. The particle size was defined as the average of the major and minor axes of the best-fitting ellipse, and shape factor (SF) as the ratio of the short and long axes. CSDs were obtained by plotting frequencies against particle sizes, and SFDs by plotting frequencies against the shape factor.

Table 1 Statistical parameters of crystal size and shape factor distributions (CSD and SFD, respectively) of magnetite magnetosomes from several types of uncultured magnetotactic bacteria, from three cultured strains (from previous studies), and two types of synthetic magnetite.

Sample locations and types	Sample identifiers	Magnetosome type	Number of Crystals	CSD			SFD	
				Maximum (nm)	Mean (nm)	Cut-off (nm)	Maximum	Mean
Gyöngyös stream, Szombathely	Gyöngyös	small, thick	440	60	60	95	0.85	0.841
Séd stream, Veszprém	Séd	double chain	443	100	83	120	0.925	0.897
Malom-tó (”Mill Pond”) Tapolca	Tapolca	scattered	241	85	80	105	0.625	0.655
Ihle stream, Bremen (9/10/2000)	S1 A	thick	270	125	92	150	0.875	0.837
	S1 B							
Freshwater pond at Max Planck Institute, Bremen	S2 A	thick	120	115	96	125	0.95	0.907
	S2 B	zig-zag chain	74	115	93	125	0.825	0.859
	S2 C	thin	70	75	69	95	0.625	0.680
	S2 D	double chain	30	85	75	100	0.825	0.817
Ihle stream, Bremen (3/28/2001)	S3 A	a) chain	21	120	101	130	0.725	0.748
	S3 B	b) chain	29	105	78	115	0.8	0.803
	S3 C	double chain	77	40	55	85	0.925	0.910
	S3 D	scattered	244	65	65	95	0.7	0.683
Sahlenburg (German Wadden Sea/German Bight)	S4 A	thick	100	130	115	155	0.8	0.809
	S4 B	small, thick	80	85	86	135	0.85	0.887
	S4 C	double chain	58	85	84	130	0.825	0.813
	S4 D	scattered	386	80	68	95	0.625	0.629
Cultured, marine	MV-1*	-	175	55	50	60	0.7	0.733
Cultured, freshwater	M. magneto-tacticum*	-	225	45	38	55	0.9	0.888
Cultured, marine	MC-2*	-	53	100	89	120	0.9	0.906
Synthetic	SYN1	$FeCl_2$ starting material	147	35	18	-	0.9	0.827
Synthetic	SYN2	$FeSO_4$ starting material	483	184	61	-	0.925	0.828

Results

We identified distinct types of magnetosomes on the basis of the features of both the magnetite grains and the cells that contained them. Criteria included (1) the sizes and habits of magnetite crystals, (2) the properties of magnetosome chains (intact chains or scattered crystals within cells, single or double chains, length of chains, positions of crystals within chains) and, if visible on the TEM grid, (3) the morphology of the cell. Using these criteria 16 types of magnetite magnetosomes were identified in 7 samples (Table 1). Groups that contained more than 100 particles were used for further statistical analysis only. Three samples (S2, S3, S4) contained more than one type of magnetosomes.

Magnetotactic bacteria collected from Gyöngyös stream have helical cell morphologies and contain single chains of slightly elongated magnetite crystals (Fig. 2a). Even the smallest particles at the ends of chains are euhedral and have perfect magnetite structure (Fig. 2b); thus, there is no indication of a precursor phase to magnetite, previously suggested to occur in some magnetotactic bacteria (Frankel et al., 1983; Taylor and Barry, 2004). Magnetotactic cocci in Séd stream typically contain two double chains of magnetite and large P-bearing granules. Crystals at one or both ends of the magnetosome chains are smaller than those in the middles of the chains (Fig. 2c). Magnetite particles are slightly elongated and their corners rounded. Fast-swimming cocci were collected from a pond (Malom-tó, Tapolca). They typically contain two large, high-contrast inclusions that resemble polyphosphate granules and elongated magnetite crystals that occur partly scattered within the cell (Fig. 2d). Some of the magnetosomes either do not form proper chains in this organism, or the chains partially disintegrate during or after sample preparation.

The dominant magnetotactic bacterium morphotype in sample S1 is a coccus that contains two single chains of magnetite (Fig. 3a). The crystals are fairly large, with maximum sizes of about 150 nm. Typically, one or more small (~20 nm) crystals occur at the ends of chains. Bacteria in sample S2 lysed and their cell morphologies were not preserved, although the chains of magnetite remained intact. We distinguished four types of magnetosomes, based on their sizes, shapes, and features of their chains (Fig. 3b): “thick” (A), “zigzag” (B), “narrow” (C), and “double” (D). Four magnetosome types also occur in sample S3 (Fig. 3c): two types of rod-shaped cells contain single chains of magnetosomes (A and B), a coccus contains a double chain of magnetite (C), and a large coccus contains irregularly shaped, scattered magnetite crystals and large phosphatic granules (D). All magnetotactic bacteria in sample S4 seem to be cocci, although cell morphologies are not recognizable in all cases be-

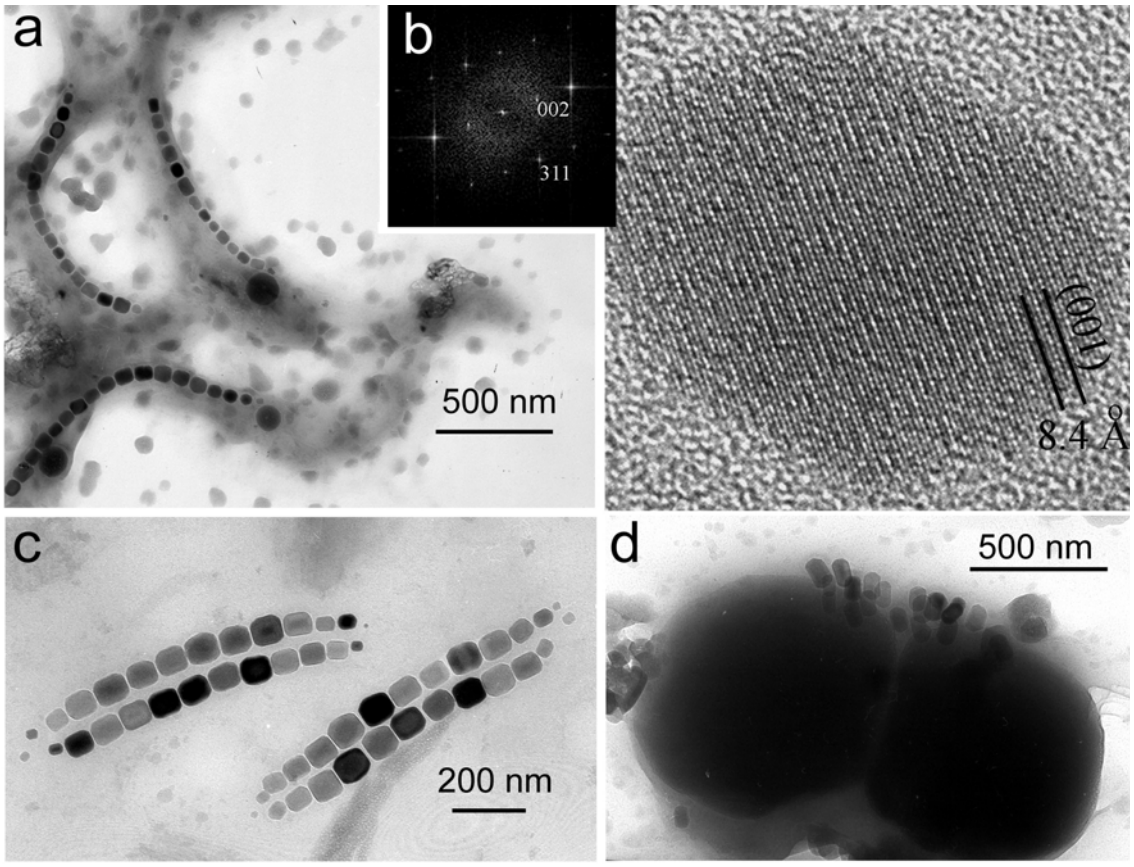

Fig. 2 Magnetite crystals in magnetotactic bacteria collected at three locations in Hungary. (a) Helical cell from Gyöngyös stream, and (b) high-resolution TEM image and Fourier transform of a small, euhedral, and structurally perfect crystal from the end of a magnetosome chain. (c) Double chain of magnetosomes in a coccus from Séd stream; (d) Scattered magnetite crystals in a coccus from Malom-tó (Mill Pond). The cell contains two large, P-bearing granules.

cause many cells form large clusters on the TEM grid. Four distinct types of magnetosomes occur (Fig. 3d): (A) and (B) form single chains of 10 to 15 magnetite crystals, but the (A) particles are significantly larger than the (B) type. A magnetosome type forming a short double chain (C) also occurs. The most abundant type in sample S4 constitutes elongated magnetite crystals, most of which are arranged in short, parallel chains at one edge of the large cells (D).

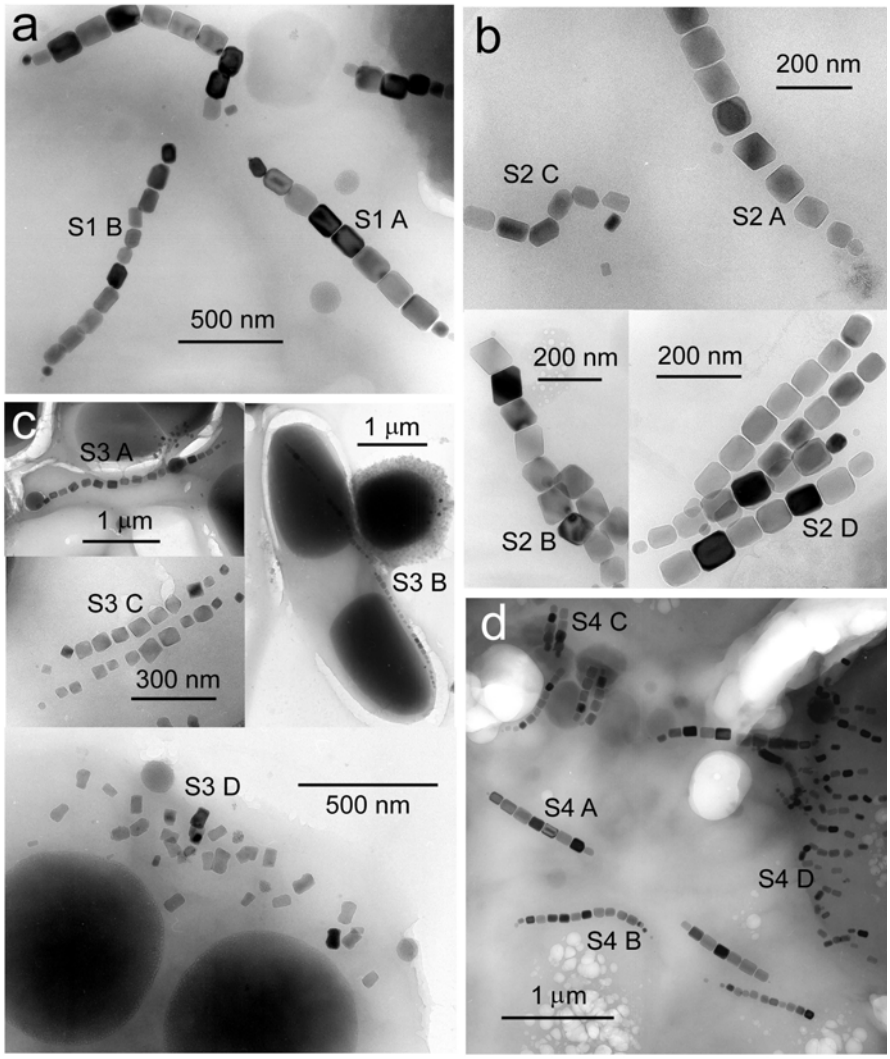

Fig. 3 Magnetite crystals from magnetotactic bacteria from four samples collected in Northern Germany. (a) S1; (b) S2; (c) S3; (d) S4 (see Table 1).

Features of SFDs and CSDs

SFDs of magnetite from the uncultured bacteria uniformly show bell-shaped distributions, with the locations of maxima ranging between 0.60 and 0.95 (Fig. 4; Table 1). Slightly elongated magnetosomes produce peaks in the SFDs between 0.8 and 0.9 (Séd, S1, S2A, S2B, S3A, S3B, S3C, S4A, S4B, S4C). Magnetite particles in samples S2C, S3D and S4D are elongated and have maxima between 0.5 and 0.7. Crystals in the Gyöngyös sample produce a significantly higher frequency maximum than the other types, reflecting the great morphological uniformity of magnetosome particles from this sample.

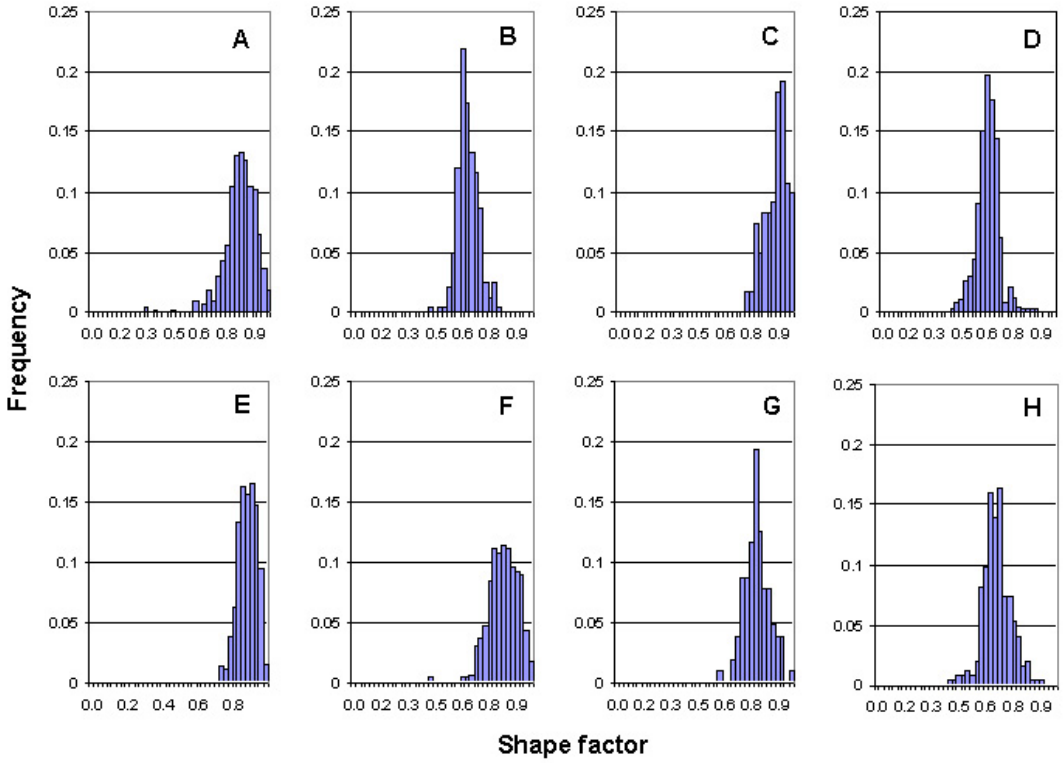

Fig. 4 SFDs of magnetite from wild-type magnetotactic bacteria.

The sizes of most magnetosomes (about 70% of all particles) range between 30 and 120 nm, and thus belong into the single magnetic domain (SD) range (Fig. 5). The largest magnetosomes are ~155 nm and occur in sample S4A. Although this size is above the SD/TD (two-domain) boundary, similar large magnetosomes from a bacterium from Itaipú Lagoon, Brazil were found to behave as SD particles because of the magnetic interactions between particles that are arranged in chains (McCartney et al., 2001). Crystals smaller than 30 nm were also shown to have a permanent magnetization, as long as they occur in chains and their magnetic fields are confined by the neighboring particles (Dunin-Borkowski et al., 1998; 2001).

The CSDs have well-defined shapes (Fig. 5). The distributions are unimodal except for S2A and S4A, which show local maxima at the smaller sizes. We distinguished two groups of CSDs depending on whether the maximum frequency value (f_{max}) is larger or smaller than 0.15 (Fig. 5); except for sample S3D, both types show asymmetry (Table 1). The negatively skewed shapes of magnetite CSDs from uncultured magnetotactic bacteria are consistent with those reported for magnetite from cultured strains (Devouard et al. 1998, Meldrum et al., 1993a, b).

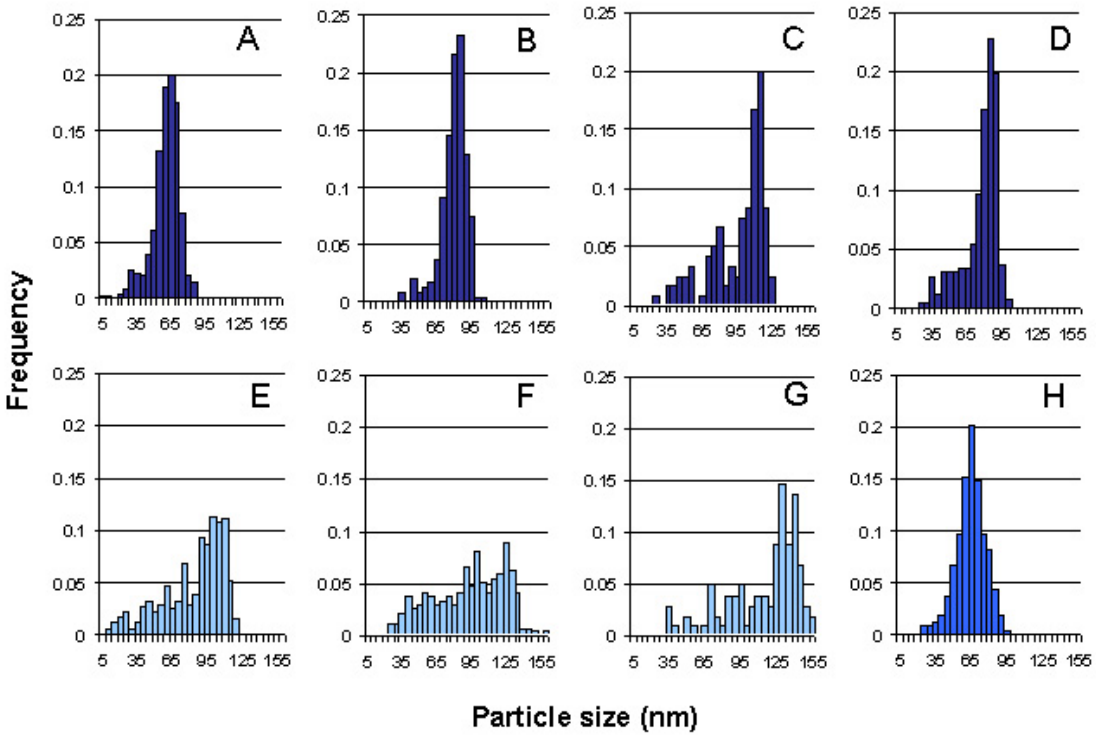

Fig. 5 Crystal size distributions (CSDs) of magnetite from wild-type magnetotactic bacteria. The three shades of the data columns refer to three different types of distributions (see text).

Magnetite particles from sample S3D show a symmetric, Gaussian-like distribution that differs from CSDs of intracellulary produced magnetite reported so far. The maximum of the CSD occurs at a particle size of 65 nm, and the SFD is broader than those of the other types. The normal CSD, broad SFD, and the scattered occurrence of crystals within the cells may indicate that in this bacterium type the biogenic control over crystal growth is not as strict as in the other magnetite-producing magnetotactic bacteria.

Comparison of CSDs and SFDs of bacterial magnetite from various samples

We wished to determine whether similar magnetosome types can be identified in distinct samples by comparing SFDs and CSDs. We used both the observations in this study and literature data, including those for bacterial magnetite (Devouard et al, 1998) and greigite (Pósfai et al., 2001). Similarities between any two curves were assessed quantitatively using chi-square and Kolmogorov-Smirnov tests. CSDs and SFDs of magnetite from distinct bacterial

morphotypes were considered similar if they matched at >1% significance level in one of the two tests (Table 2).

Table 2 Matching pairs of SFDs and CSDs of magnetite populations from several bacterial strains and synthetic samples.

Shape Factor Distributions			
Matching pairs		**^{2} Test**	**KS Test**
S1 A	S4 A	>20	>10
S2 A	MC-2	-	>10
Séd	MC-2	>20	>10
Séd	S2 A	-	1- 5
Séd	M.magnetotacticum*	10 - 20	1 - 5
M. magnetotacticum*	MC-2	-	>10

Crystal Size Distributions				
	Matching pairs		**^{2} Test**	**KS Test**
Group 1	Gyöngyös	S3 D	>20	-
	S3 D	MMP	>20	-
Group 2	Séd	MC-2	>20	>10
	Tapolca	S4 D	5 to 10	5 to 10

A group of matching SFDs result from the statistical tests, including four pairs of similar magnetosome types from Séd, S2A, *M. magnetotacticum*, and MC-2 (Fig. 6a and Table 2). SFDs of the pairs *M. magnetotacticum* – MC-2, Séd – *M. magnetotacticum*, and Séd – MC-2 match in both tests, indicating that these magnetosome types have similar elongations.

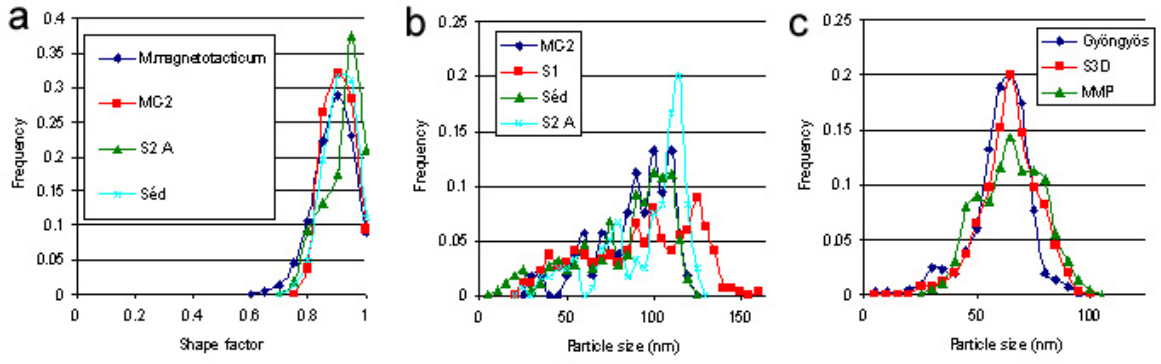

Fig. 6 Matching SFDs (a) and CSDs (b and c) of magnetite populations from various samples (also see Table 2).

According to the statistical tests, two distinct groups of similar CSDs occur. The asymmetric, negatively-skewed CSDs of magnetite from samples Séd, S1, S2A, and MC-2 (Fig. 6b and Table 2) show great similarity to one another. The other group of CSDs includes the pairs Gyöngyös and S3D, and S3D and greigite from the MMP (Pósfai et al., 2001). The CSD of magnetite from Gyöngyös is slightly asymmetric, but magnetite from S3D and greigite from MMP show nearly perfect Gaussian distributions (Fig. 6c).

Considering the features of both SFDs and CSDs, the Séd – MC-2 pair is the only one among all possible pairs of the studied crystal populations that match in both shape and size distributions. The match between magnetite particles from an uncultured freshwater magnetotactic bacterium (Séd) and a marine species (MC-2) indicates that similar magnetosomes can be produced in very different environments. Presumably, similar mechanisms of biological control over crystal growth are employed by the two bacterium types.

Even though the SFDs of S3D magnetite and MMP greigite do not match at a significance level higher than 1%, it is interesting that both crystal populations have matching Gaussian CSDs. In addition to having a normal CSD, S3D magnetite stands out in the irregular shapes of the crystals and their scattered distributions within the cells. Since similar features are typical for MMP greigite, we assume that the mechanisms controlling crystal size, shape, and position in the cell are related, and control over crystal growth is less strict in S3D and MMP than in the other magnetite-producing bacteria.

Identification of CSDs of distinct magnetosome types in samples containing only biogenic magnetite

The use of magnetite nanoparticles as biomarkers has been primarily based on the characteristic sizes and shapes of crystals from magnetotactic bacteria (Chang and Kirschvink, 1989; Thomas-Keprta et al., 2000). However, unless the magnetite crystals occur in chains, it is not straightforward to determine whether they are of inorganic or biogenic origin. With current technology, precise determination of the morphologies of a statistically significant number of crystals requires an impractically large amount of labor-intensive TEM work (Buseck et al., 2001). Since the SFDs and CSDs of large crystal populations can be measured relatively easily, the features of such distributions could serve as tools for identifying biogenic magnetite. Before attempting to distinguish between magnetite populations of biogenic and inorganic origins, we wished to determine whether the nanocrystals produced by distinct bacterial strains can be identified in samples that contain magnetite from several types of bacteria.

For the three samples that contain more than one type of magnetosomes (S2, S3, and S4), we combined the individual SFDs into one distribution, corresponding to the entire sample. Then Gaussian curves were fitted to each peak in the combined SFD. In this way, we defined relatively narrow SF intervals (Fig. 7), based on particle elongations. For the particles belonging to distinct SFD intervals we used the corresponding size data and plotted their CSDs. The resulting curves were compared with those previously determined for each magnetosome type (as in Fig. 5). In effect, we tested whether by using only "bulk" size and shape data similar CSDs can be retrieved as using all available information (size, shape, chain and cell type).

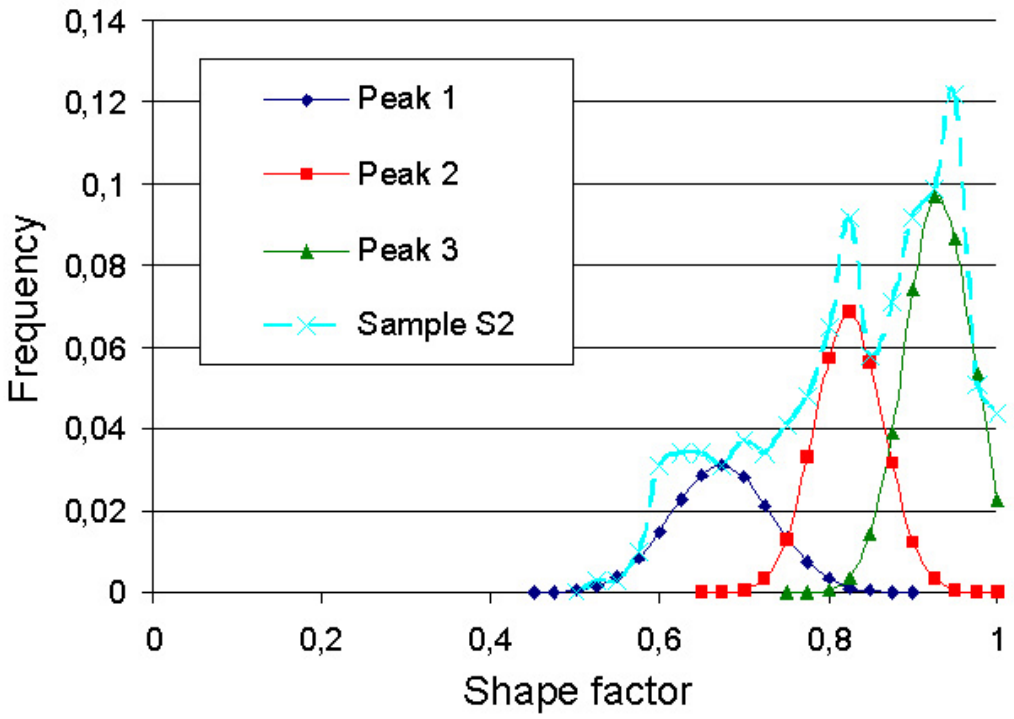

Fig. 7 An example of the deconvolution of an SFD that includes data from all magnetite crystals in a sample (S2) that contains several types of magnetosomes.

The combined CSD of all magnetite particles in sample S2 contains three distinct peaks and thus does not indicate an unambiguous bacterial origin (Fig. 8). Crystals numerically separated into three groups on the basis of SF intervals (Fig. 7) have CSDs that match those of the previously determined magnetosome types S2A, S2B, and S2C (Fig. 8).

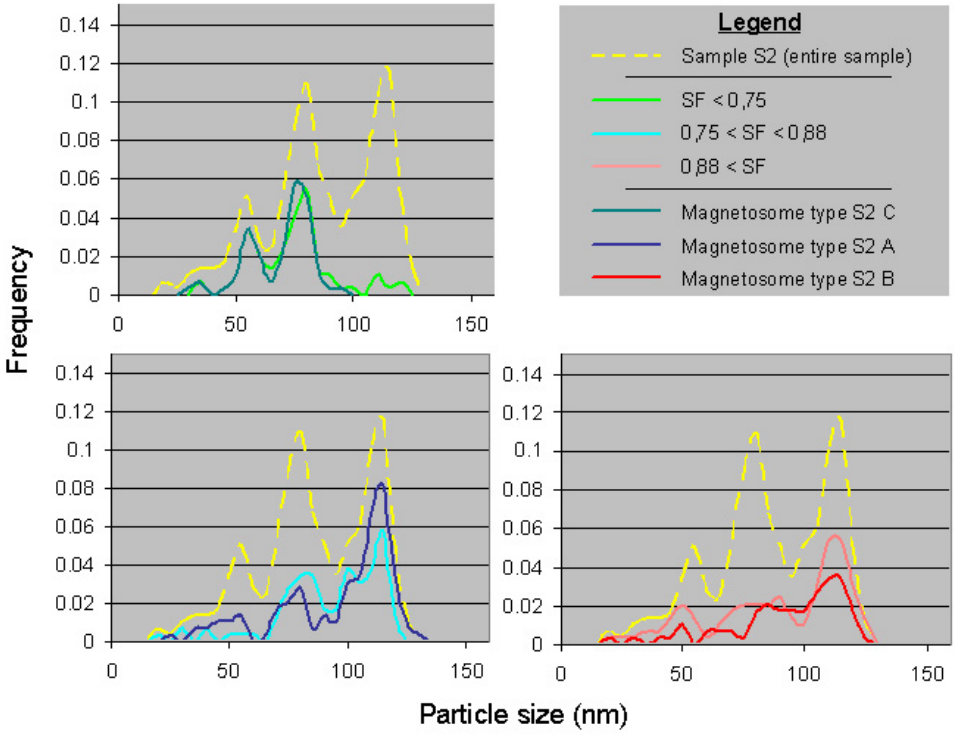

Fig. 8 Combined CSD including size data from all magnetite crystals in sample S2 (dashed line), CSDs of three distinct magnetosome types identified visually, using all available information (dark lines), and CSDs that resulted from the numerical separation of crystals from the entire sample, based on the individual peaks in the SFD of the total magnetite population (Fig. 7).

Thus, it is possible to retrieve the CSDs of distinct types from the CSD of the entire sample by separating particle groups on the basis of their SF values, if several magnetosome types in the same sample have different elongations.

In samples S3 and S4 more than 60% of all measured particles belong to one magnetosome type; consequently, the size distribution of this type dominated the combined CSD. As expected, in the case of these two samples the particles selected on the basis of their SF values (SF<0.76 and SF<0.75 in samples S3 and S4, respectively) produced CSDs that matched the size distribution curves of the major magnetosome types (S3D and S4D, respectively) identified previously (Fig. 9). The CSDs of the other, minor magnetosome types could not be retrieved from the combined size and shape data. The CSD of the S4D type has a negatively skewed CSD that is characteristic of bacterial magnetite crystals; thus, even without separating the particles on the basis of their SF values, the CSD of all particles in the sample would indicate a biogenic origin. The major magnetosome type in S3 shows a normal distribution, which makes it less likely that the bacterial origin of magnetite in this sample would be identified on the basis of the SFD and CSD of the entire sample.

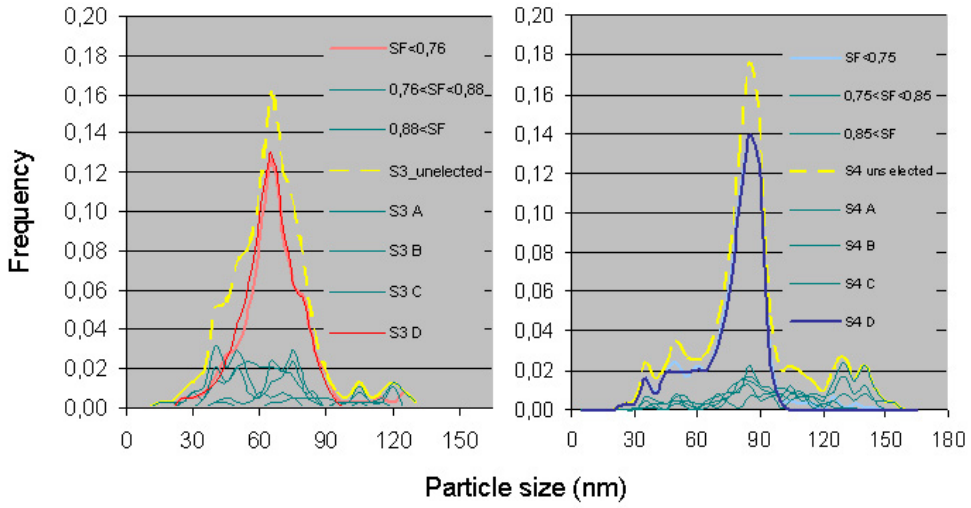

Fig. 9 Combined CSD including size data from all magnetite crystals in (a) sample S3 and (b) sample S4 (dashed lines), CSDs of three distinct magnetosome types identified visually, using all available information (dark lines), and CSDs that resulted from the numerical separation of crystals from the entire samples, based on individual peaks in the SFD of the total magnetite population.

Mixtures of bacterial and synthetic magnetite

Both types of synthetic magnetite have fairly narrow size distributions, but crystals prepared from the $FeCl_2$ + $FeCl_3$ solution are smaller (with a mean size ~18 nm) than those obtained from the $FeSO_4$ starting material (mean size ~61 nm) (Table 1). Both CSDs have lognormal shapes (Fig. 1a). The data for both types of synthetic particles were combined with those of all magnetosomes in the sample S2. The resulting combined CSD is complex, has several peaks, and does not show an apparent BCM component (Data not shown). The combined SFD (Data not shown) also exhibits several peaks; these were deconvoluted, and from the corresponding size data individual CSDs reconstructed. A comparison of the original CSDs (for the three types of magnetosomes in sample S2 and the two synthetic crystal populations) with the numerically produced ones reveals that the characteristic negatively skewed CSDs of the bacterial magnetite populations cannot be retrieved from the bulk size and shape data. Thus, in this inorganic/biogenic sample the statistical analysis of sizes and shapes cannot reveal the origin of the bacterial component.

Summary

We studied CSDs and shape SFDs of magnetite from wild strains of magnetotactic bacteria and identified 16 distinct types of magnetosomes. Our main results are summarized as follows:

1. Most but not all magnetite magnetosome CSDs are asymmetric, negatively skewed. Symmetrical, Gaussian distribution also occurs in one strain, similar to the CSD of greigite from a multicellular magnetotactic prokaryote (MMP; Pósfai et al., 2001).
2. Statistical tests are useful for identifying magnetite populations consisting of crystals that have similar morphologies and sizes and can reveal similarities between CSDs and SFDs of magnetite from wild types of magnetotactic bacteria, cultured strains (Devouard et al., 1998), and synthetic magnetite.
3. The best match among pairs of magnetite size and shape distributions was found between a wild freshwater type and a marine cultured strain, MC-2. This match may indicate that the mechanism of biochemical and genetic control of magnetite formation is very similar in these two bacterial strains.
4. “Bulk” SFD and CSD data from all magnetite particles were used for extracting information on the CSDs of distinct types of magnetosomes that occur in the same sample. If the elongations of distinct magnetosome types differ, CSDs of individual types can be retrieved from bulk data by grouping particles according to the peaks in the SFD of the entire sample. In such cases the biogenic origins of magnetite populations can be identified on the basis of crystal size and shape data. Thus, although limited to certain types of samples, the analysis of CSDs and SFDs of magnetite can serve as a robust method for determining the likely biogenic origin of magnetite in certain geological samples.
5. Size and shape data of synthetic magnetite crystals were mixed with those of bacterial crystal populations, in order to simulate a geological sample that contains magnetite of both biogenic and inorganic origins. Since both shape and size distributions of the two types of magnetite overlap, the origin of the biogenic component could not be identified on the basis of CSDs.

Acknowledgements

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4.

Intracellular magnetite and extracellular hematite produced by *Desulfovibrio magneticus* strain RS-1

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Manuscript in preparation
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Abstract

Desulfovibrio magneticus strain RS-1 is an anaerobic sulfate-reducing bacterium that also respires and grows with fumarate as a terminal electron acceptor. Cells grown with either sulfate or fumarate form intracellular nanocrystalline magnetite but are only weakly magnetotactic. In order to understand the unusual magnetic response of this strain, we studied magnetite crystals within cells grown with fumarate and sulfate. Many cells grown under either condition did not form magnetic crystals while others contained only 1 to 6 small (~40 nm) magnetite magnetosomes. Bulk magnetic measurements of whole cells showed a superparamagnetic-like behavior, indicating that the magnetite crystals are too small to have a permanent magnetic moment at ambient temperature. As a result of the small size and low number of magnetite magnetosomes, the magnetic moments of most cells are insufficient for magnetotaxis. In addition to intracellular magnetite, in some cultures another iron oxide, hematite formed on the surfaces of cells. Rounded grains of hematite range in size from 30 to 110 nm and are embedded in extracellular polymeric material, indicating that the crystals likely result from a biologically-induced mineralization process. Although the hematite particles appear to consist of many small (5 to 10 nm) grains, these grains have a consensus orientation and thus the whole particle diffracts as a single crystal. The aligned arrangement of nanoparticles within larger clusters may reflect either a templated nucleation of hematite crystallites in an extracellular organic matrix, or a self-assembling process during the crystallization of hematite from ferric gels or ferrihydrite.

Introduction

Biogenic processes are important in the formation of iron oxides in many environments. Both the biologically-controlled mineralization (BCM) of intracellular magnetite (Bazylinski and Frankel, 2000; Mann, 2001; Gajdardziska-Josifovska et al., 2001) and the biologically-induced mineralization (BIM) of magnetite, goethite, lepidocrocite, ferrihydrite, and other iron oxides (Zhang et al., 1998; McClean et al., 2001; Chatellier et al., 2001; Banfield et al., 2000) have been extensively studied. BCM magnetite recently attracted great interest as a potential biomarker (Thomas-Keprta et al., 2000; Buseck et al., 2001). An intriguing problem is how magnetotactic bacteria control the growth of magnetite crystals that have specific sizes and morphologies. Concerning BIM iron oxides, it is important to understand the nucleation processes of inorganic crystals on biological surfaces or within organic matrices (Banfield and

Zhang, 2001; Frankel and Bazylinski, 2003). We wished to obtain more insight into BCM and BIM iron oxide-forming processes by studying *Desulfovibrio magneticus* strain RS-1 that appears to produce both intracellular magnetite and extracellular hematite, as well as iron sulfides.

Desulfovibrio magneticus strain RS-1 was first isolated from freshwater sediments by Sakaguchi et al. (1993), and described as a dissimilatory sulfate-reducing obligate anaerobe that produces intracellular magnetite and extracellular iron sulfides. However, in contrast to other magnetite-bearing, magnetotactic bacteria, the response of cells of strain RS-1 to external magnetic fields was so weak that cells could not be harvested using magnetic collection methods (Sakaguchi et al., 1996). Phylogenetic analysis based on the partial 16S rDNA sequence showed that RS-1 belongs to *δ-Proteobacteria* in the genus *Desulfovibrio* (Kawaguchi et al., 1995). Based on a detailed study of the physiology and biochemical characteristics of RS-1, a novel species, *Desulfovibrio magneticus* was proposed (Sakaguchi et al., 2002).

Desulfovibrio magneticus is unique among magnetotactic bacteria both in terms of its weak magnetotactic response and phylogenetic position. The only other magnetotactic microorganism in the *δ-Proteobacteria* is the multicellular magnetotacic prokaryote (MMP; DeLong et al., 1993); however, this organism synthesizes intracellular iron sulfides, including greigite, Fe_3S_4 (Farina et al., 1990; Mann et al., 1990; Pósfai et al., 1998a, b), and mackinawite, FeS (Pósfai et al., 1998a, b), but not magnetite. Biomineralization by *D. magneticus* has significant geological implications in that this species produces magnetite in a sulfidic, reducing environment. *D. magneticus* strain RS-1 is the only magnetotactic, sulfate-reducing bacterium that is currently available in pure culture.

We studied the magnetosomes in RS-1 in order to understand why the magnetic response of RS-1 is so poor compared to that of other magnetotactic bacteria. We used bulk magnetic methods and transmission electron microscopy (TEM) imaging for characterizing the magnetosomes. While looking for intracellular iron oxide crystals, we found hematite (Fe_2O_3) grains attached to the surfaces of cells in one of the cultures, and studied them using high-resolution TEM (HRTEM) and electron diffraction.

Material and methods

Desulfovibrio magneticus RS-1 (DSM No. 13731) was obtained from the DSMZ type culture collection (Braunschweig, Germany). Two different media were used for cultivation. The sulfate-free medium (medium No. 896 from DSMZ) contained pyruvate and fumarate as electron donor and acceptor, respectively, and was prepared anaerobically according to the method of Widdel and Bak (1992). Alternatively, defined freshwater sulfate reducer medium was used containing 5 mM pyruvate as electron donor as described by Widdel and Bak (1992). Cells in both media were cultivated in 20 ml tubes containing 10 ml medium and nitrogen in the head space. The tubes were sealed by butyl rubber stoppers. Incubation was at room temperature without agitation.

Bulk magnetic measurements. This paragraph is in preparation by Bruce Moskowitz.

Samples for electron microscopy were prepared from young (one day incubation) ore stationary cultures (two weeks incubation) of *D. magneticus* RS-1 grown in both media. Cells were deposited on Cu and Ni TEM grids covered by a carbon-coated Formvar film. Electron microscopy was performed using a Philips CM20 instrument operated at 200 kV accelerating voltage. The compositions of mineral grains were determined with an attached Noran Voyager detector using energy-dispersive X-ray spectrometry (EDS). HRTEM images of magnetite and hematite were obtained using a 300-kV JEOL 3010 and a 400-kV JEOL 4000EX microscope, respectively. For image processing (filtering of HRTEM images and obtaining Fourier transforms) we used Digital Micrograph software. We obtained size distributions of magnetite and hematite grains by fitting ellipses to the outlines of crystals, and then using the average of the short and long axes of the best-fitting ellipse as the diameter of the crystal (Devouard et al., 1998; Pósfai et al., 2001).

Results

Magnetic properties of RS-1 cultures

This paragraph is in preparation by Bruce Moskowitz.

Intracellular magnetite magnetosomes by TEM

Most cells of RS-1 did not contain magnetite at all. The cells grown in either medium contained at most 6 magnetite crystals, but the majority was magnetite-free. The magnetite magnetosomes are small (~40 nm) and have elongated, irregular shapes (Fig. 1a). In contrast to most magnetotactic strains that produce magnetite crystals with asymmetric, negatively-skewed size distributions (Devouard et al., 1998; Arató et al., 2004), magnetite produced by RS-1 has a symmetric, Gaussian distribution (Fig. 1b). Similar size distributions were observed for intracellular greigite in the multicellular magnetotactic prokaryote, MMP (Pósfai et al., 2001) and in a magnetite-producing uncultured bacterium (Arató et al., 2004). In all these cases the normal crystal size distribution was associated with irregular crystal morphologies. Magnetite nanocrystals produced by RS-1 appear to be free of structural defects (Fig. 1d); in contrast to most types of bacterial magnetite, the crystals are elongated along [100], not [111]. In a SAED pattern from a magnetite crystal faint additional reflections appear that may indicate the partial oxidation of magnetite into maghemite (Fig. 1c).

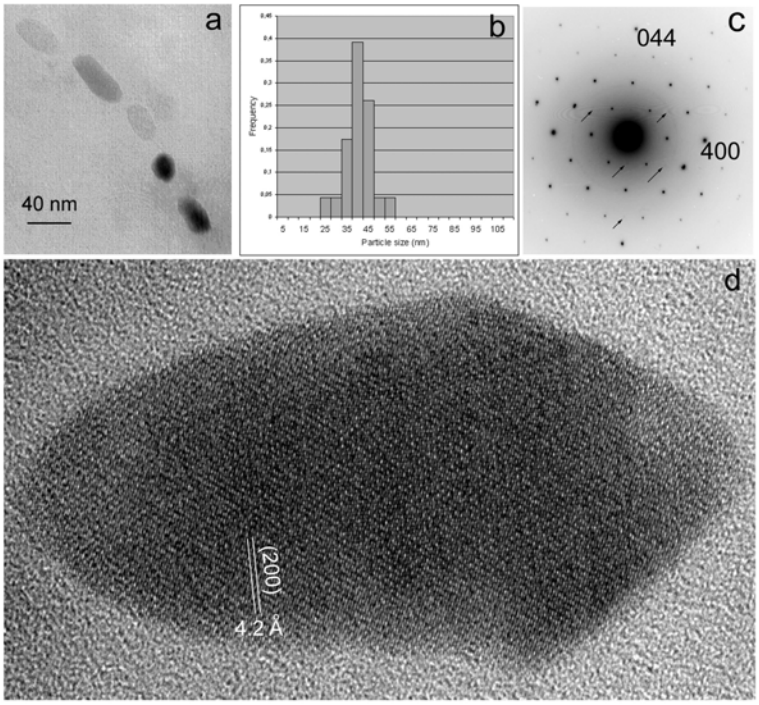

Fig. 1 (a) Magnetite magnetosomes in a cell of RS-1. (b) Crystal size distribution of intracellular magnetite crystals. (c) Selected-area electon diffraction pattern and (d) corresponding high-resolution image of a magnetite magnetosome, viewed along [110]. The weak, arrowed reflections in (c) are forbidden by the magnetite structure, and their presence indicates that the crystal is transforming into maghemite.

Extracellular hematite

Extracellular polymers are associated with the cells of RS-1. EDS analyses show that the web-like substance on the surfaces of cells consists either of carbonaceous material only (Fig. 2a), or of carbonaceous material with metal-containing deposits. In a fumarate-grown culture Fe oxide particles commonly occur on the extracellular polymers (Fig. 2b). The rounded, fairly uniformly-sized particles have a grainy appearance and seem to consist of many smaller crystallites (Fig. 2c). Nevertheless, the Fe oxide particles produce single crystal-like selected-area electron diffraction (SAED) patterns that indicate an almost identical orientation of the individual grains ("nanograins") within each larger particle (Fig. 2d). The d-spacings and angles in the SAED patterns are consistent with the structure of hematite.

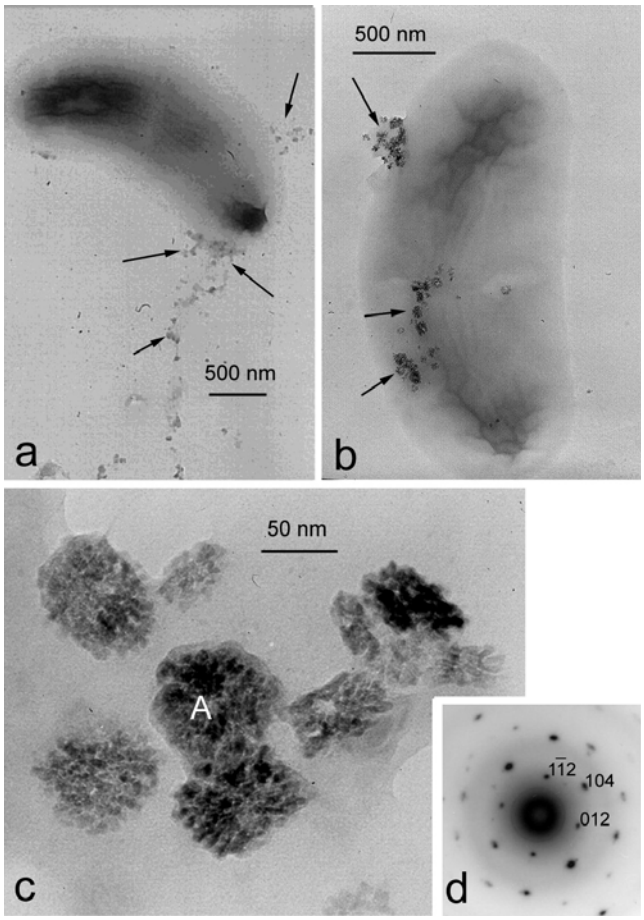

Fig. 2 (a) Extracellular polymeric material (marked by arrows) attached to a cell of RS-1. (b) Hematite particles (arrowed) embedded in extracellular material. (c) Grainy appearance of hematite particles suggests that they consist of smaller crystallites. (d) Selected-area electron diffraction pattern obtained from the particle marked A in (c); the pattern indicates that the particle diffracts as a single crystal.

HRTEM images confirm that the nanograins have a consensus orientation within each hematite particle (Fig. 3). In some nanograins in Figure 3b rows of dots along the hematite (-210) and (-120) planes are spaced at ~2.9 Å intervals and thus can be regarded as representing [FeO_6] octahedra. Similar rows of dots in the same orientation appear in other nanograins, whereas in the spaces between the grains only fringes or no structural detail can be observed. The contrast in the TEM is not uniform; some grains appear darker than others (Fig. 3a). This effect is probably caused by the slight orientational differences, although the variable size and thus thickness of nanograins may also contribute to contrast variation.

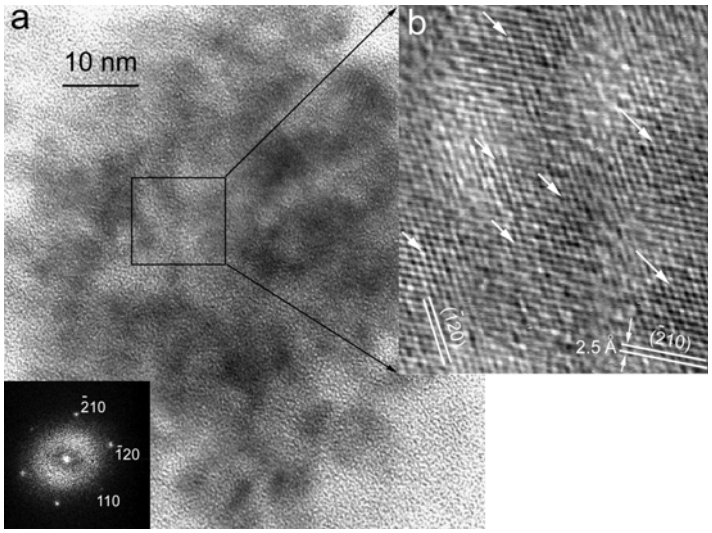

Fig. 3 (a) High-resolution TEM image of a hematite particle oriented with [001] parallel to the electron beam (as indicated by the Fourier transform in the inset), and (b) rotationally filtered image of the area marked in (a). Note the uniformly oriented, ordered islands ("nanograins," marked by arrows) in (b).

The crystal size distribution (CSD) of hematite particles is basically symmetric, with a maximum at ~70 nm (Fig. 4a). A few large particles (>110 nm) occur that may have formed by the aggregation of the original particles. The relatively broad shape factor distribution (Fig. 4b) indicates the irregular shapes of hematite particles; although most grains are only slightly elongated, particles having higher aspect ratios (lower shape factor values) also occur.

In some cultures, metal-bearing particles other than hematite are present within the extracellular polymeric material. In a sample of a fumarate-grown culture amorphous particles occur that have fairly uniform compositions and contain Na, Zn, and S. ZnS biomineralization is known to occur associated with some bacteria (Labrenz et al., 2000; Zbinden et al., 2001), but in these cases ZnS was reported to be crystalline. In the RS-1 sample the amorphous, Zn-bearing grains contain about as much Na as Zn (in terms of mol%).

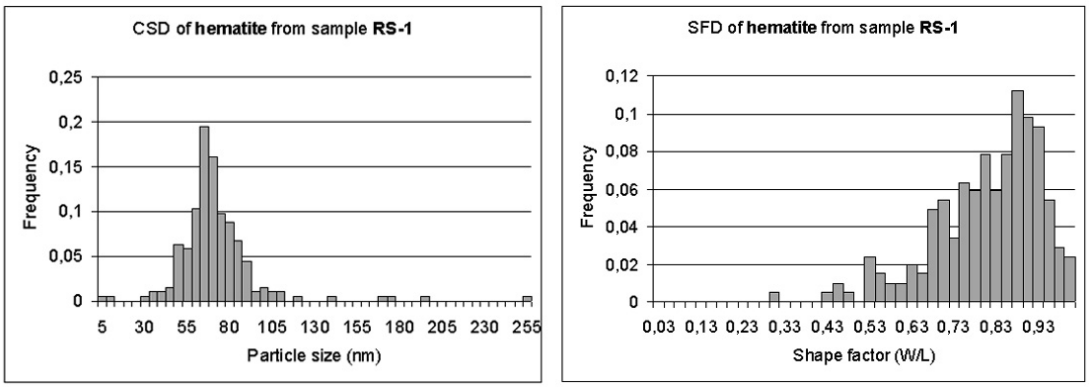

Fig. 4 (a) Crystal size distribution and (b) shape factor (width/length) distribution of extracellular hematite particles.

Discussion

The weak magnetotactic response of RS-1 cells is understandable in light of the bulk magnetic measurements and TEM observations of magnetite magnetosomes. Only a small fraction of cells contain magnetite. Frequency-dependent susceptibility and low-temperature susceptibility data indicate the presence of superparamagnetic particles. Particle sizes measured in TEM images confirm that most magnetite crystals in RS-1 are only slightly larger than 30 nm, the theoretical lower size limit of single magnetic domains in magnetite (Dunlop and Özdemir, 1997). In addition, the magnetosomes do not form proper chains; there are large gaps between magnetite crystals. In other magnetotactic strains even small (<30 nm) and larger (>120 nm) magnetite crystals are single magnetic domains because of magnetic interactions among crystals in well-organized chains (Dunin-Borkowski et al., 1998; 2001; McCartney et al., 2001); such interactions cannot occur in RS-1. Since the cells contain very few magnetite crystals that do not form ordered chains, the magnetic moments of individual cells are insufficient to orient the cells along the geomagnetic field lines.

The extracellular formation of hematite is interesting, since iron in hematite is in fully oxidized state, whereas the environment of the anaerobic RS-1 is reducing. It appears that the solution chemistry within the extracellular polymeric material differs from that of the culture medium. Although we observed hematite in one culture only, it is unlikely that its formation is an artifact; the samples were never exposed to oxidizing conditions during culturing. Further specimen handling is not expected to change the original composition and structure of iron oxides.

Whereas most of the common iron oxide and hydroxide minerals are known to be readily produced by biomineralization (Weiner and Dove, 2003; Frankel and Bazylinski, 2003), there are few reports of the biogenic formation of hematite (Konhauser, 1998). In addition to other iron minerals, aggregations of randomly oriented, 1 to 50 nm hematite nanocrystals were extracted from grass samples (McClean et al., 2001). These nanocrystals formed the inorganic cores of phytoferritin, and thus can be regarded as products of BCM processes. Poorly ordered hematite crystals were found associated with bacterial cells and their formation attributed to BIM processes (Ferris et al., 1989; Brown et al., 1994). The biogenic hematite particles described in this study are unique in that they consist of crystallographically aligned nanograins.

The extracellular polymer matrix may play a role in the oriented arrangement of hematite crystallites. Organic surfaces or matrices template the oriented nucleation and growth of nanocrystals in biomimetic systems. Examples include the oriented arrays of magnetite nanocrystals on polyvinyl alcohol surfaces (Sinha et al., 2001), assemblies of goethite nanocrystals formed on polysaccharide alginic acid fibrils (Nesterova et al., 2003), and spectacular pseudo-octahedral calcite “single” crystals composed of highly aligned nanocrystals that formed in a poly-acrylamide hydrogel network (Grassmann et al., 2003). Oriented arrays of magnetite nanocrystals occur in plants and produce single crystal-like SAED patterns (Gajdardziska-Josifovska et al., 2001). Although possible, at present we do not know whether the extracellular polymers associated with RS-1 have a templating role in hematite nucleation.

Alternatively, the ordered alignment of hematite nanograins on cells of RS-1 can be explained by a self-assembling process. Hematite is known to form by the transformation of ferric gels (Combes et al., 1990) or ferrihydrite (Schwertmann and Cornell, 2000) through dehydration and structural rearrangement. Although the complete transformation of ferrihydrite to hematite requires temperatures >80 °C (Schwertmann and Cornell, 2000), Janney et al. (2001) found hematite associated with ferrihydrite synthesized at room temperature. Ferrihydrite nanocrystals produced by iron-oxidizing bacteria were shown to aggregate and rotate so the individual crystallites shared a common orientation (Banfield et al., 2000). In general, drying can mediate self-assembly of nanoparticles (Rabani et al., 2003), and crystallographically coherent boundaries are energetically favorable over random orientations (Banfield and Zhang, 2001). In the case of the hematite formed on cells of RS-1, it is possible that the role of the extracellular organic material in the mineralization process is limited to providing nucleation sites for ferric oxides or oxyhydroxides, and the common crystallographic orienta-

tion of the nanograins is a consequence of their self-alignment during the transformation of initial precipitates into hematite.

Both intracellular magnetite and extracelllular hematite particles produced by RS-1 exhibit Gaussian CSDs (Figs. 1 and 4), suggesting a random crystal growth process. Similar CSDs are characteristic for intracellular greigite from cells of a multicellular magnetotactic prokaryote (MMP; Pósfai et al., 2001) and magnetite from a wild-type magnetotactic bacterium (Arató et al., 2004). Interestingly, greigite, magnetite, and hematite particles that exhibit symmetric CSDs also have irregular crystal morphologies and disordered or semi-ordered spatial distributions within or outside cells. In contrast, magnetite crystals from several other magnetotactic strains typically have asymmetric, negatively skewed CSDs (Devouard et al., 1998; Arató et al., 2004); these crystals have well-defined morphologies and tend to remain in ordered chains within dehydrated cells on the TEM grids. It appears that the biogenic controls over crystal size, morphology, and chain formation are related in magnetotactic bacteria.

Acknowledgements

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5.

Phylogeny and *in situ* identification of magnetotactic bacteria

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Microbial diversity and the problem of culturability

In the last decade molecular biological data have reinforced common knowledge of microbiologists: It is difficult to bring bacteria in pure culture! Some of those bacteria that are most conspicuous in the microscope have until now resisted all attempts of enrichment and cultivation. Among those are symbiotic prokaryotes like those chemolithoautotrophic bacteria found in marine invertebrates, the many bacteria and archaea dwelling in protozoa, slow growing bacteria adapted to life in oligotrophic environments but also magnetotactic bacteria [1]. The comparative analysis of bacterial 16S rRNA sequences directly retrieved from various environments by techniques pioneered by Woese [2] has proven that the about 5000 validly described bacterial species represent only a small part, likely less than 1%, of the extant bacterial diversity [1],[3]. The combination of cultivation-independent rRNA gene retrieval, comparative sequence analysis and fluorescence *in situ* hybridization has been shown to allow for phylogenetic affiliation and *in situ* identification of hitherto uncultured bacteria [4]. We will here review the application of this methodology to magnetotactic bacteria.

The rRNA approach to microbial ecology and evolution

The rRNA approach to microbial ecology and evolution was first described in its full potential by the group around Norman Pace and David Stahl in 1986 [5]. It is based on the comparative sequence analysis of ribosomal ribonucleic acid (rRNA) [2]. The different rRNA molecules, in bacteria the 5S, 16S and 23S rRNAs with approximate lengths of about 120, 1500 and 3000 nucleotides, are essential components of all ribosomes. These are the cellular protein factories present in every cell in high copy numbers. Their sequences are evolutionary quite conserved but also contain regions in which changes accumulate more rapidly. Due to their ubiquity, conserved function, and lack of lateral gene transfer, especially the longer 16S and 23S rRNA molecules are ideal chronometers for the reconstruction of bacterial evolution [2]. Furthermore, these two molecules contain highly conserved sites which allow their amplification from the rRNA genes present in environmental DNA by the polymerase chain reaction (PCR) [1],[3]. In the currently most widespread format almost full length 16S rRNA genes are amplified from conserved sites existing at the 5’ and 3’ ends of this molecule. The resulting mixed amplificates should reflect the natural bacterial community. It is subsequently ligated into a plasmid vector and cloned into *Escherichia coli* using standard techniques of molecular biology. The cloning step allows to segregate the different fragments. This is necessary for the

sequencing and in its effect comparable to the segregation of individual strains by growth on agar plates.

In addition, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments [6] was established as a less time-consuming alternative to the cloning step. The DGGE permits the cultivation-independent analysis of the composition of complex microbial communities. The method allows the separation of double-stranded DNA fragments with identical lengths but sequence heterogeneities based on the different melting properties in polyacrylamide gels with a lineary increasing gradient of the DNA denaturants urea and formamide. After excision of bands and reamplification, the DNA fragments can be sequenced. The 16S rRNA sequences thereby retrieved from environmental samples without cultivation of the original bacteria are then compared to large 16S rRNA sequence databases that contain more than 90% of the 16S rRNA sequences of the hitherto cultured, validly described bacteria.

By comparative analyses the closest known sequence can be identified and a 16S rRNA-based evolutionary tree can be reconstructed which either places the new sequence to a known phylogenetic group or on a new branch of the universal tree. The comparative analysis does, however, also allow to identify sequence idiosyncrasies that, like a fingerprint, may serve for the identification of the new sequence. This sequence can then be the target for an oligonucleotide probe which is a short, single-stranded piece of nucleic acid labeled with a marker molecule. Often a short oligonucleotide of 15 – 25 nucleotides is sufficient to discriminate by hybridization the 16S rRNA sequence retrieved from the environment from all other known sequences. The binding of a probe to a fully or partially complementary target is called hybridization. Under optimized conditions the hybridization is specific, meaning that the probe only binds to the target nucleic acid but not to other (nontarget) nucleic acids. The probes can be used to quantify the target nucleic acids in a mixture of environmental nucleic acids. In one particular technique, the fluorescence *in situ* hybridization (FISH), the nucleic acid probe is labeled with a fluorescent dye molecule and incubated with fixed, permeabilized environmental samples. During an incubation of one to several hours at defined conditions the probes diffuse into the cells and bind specifically to their complementary target sites. Due to the fact that the 16S rRNA is quite abundant in bacterial cells, e. g., a rapidly growing *Escherichia coli* cell contains about 70000 copies of the molecule, even fluorescein-monolabeled oligonucleotides are sensitive enough to visualize individual bacterial cells in epifluorescence microscopes [1]. By FISH the 16S rRNA sequence retrieved from the environment is linked to defined cells, with a certain abundance, shape, and spatial distribution.

By this so-called full cycle rRNA approach bacteria can be phylogenetically affiliated and identified without prior cultivation.

Application of the rRNA approach to magnetotactic bacteria

The potential of the rRNA approach for analysis of hitherto uncultured bacteria has in the meanwhile been demonstrated on various conspicuous bacteria, among those the magnetotactic bacteria. Although these have shapes and sizes typical for bacteria, they, if present, become conspicuous when live mounts of marine or freshwater surface sediments are exposed to changing magnetic fields. A fraction of cells decisively swims along the lines of the magnetic field and immediately follows any change in its orientation. After their discovery in 1975 by Blakemore [7] methods for visualization and enrichment have been developed. When working with magnetotactic bacteria one has the unique advantage that they can be readily separated from sediment particles and other bacteria based on their magnetotaxis. However, of the many morphotypes detected, including spirilla, cocci, vibrios, ovoid, rod-shaped and even multicellular bacteria, only few bacteria could so far be brought into pure culture (for review see [8]). Some members of this interesting group of bacteria with its ferromagnetic crystalline inclusions were therefore investigated by the cultivation-independent rRNA approach. The main questions of interest were the following: (1) Is the morphological diversity reflected in a diversity on the level of 16S rRNA? Here, the two alternative answers are that there exist actually only few species of magnetotactic bacteria that have variable morphology (pleomorphism), or that several species are hidden behind one common morphotype. (2) Are the magnetotactic bacteria forming a monophyletic group or are ferromagnetic crystalline inclusions found in different phylogenetic groups? (3) Has this specific trait developed once or independently several times during bacterial evolution?

The genus *Magnetospirillum* encompassing culturable magnetotactic bacteria

It was the obvious starting point to determine the 16S rRNA sequences of the pure cultures available. Schleifer, Schüler and coworkers in 1991 [9] studied the two pure cultures available at that time and created the genus *Magnetospirillum* with the two species *Magnetospirillum magnetotacticum* (formerly *Aquaspirillum magnetotacticum* [10]) and *Magnetospirillum gryphiswaldense* [9]. In parallel the sequence of *A. magnetotacticum* was determined by Eden and coworkers [11]. The 16S rRNA sequences of the two species affiliated them with the

Alphaproteobacteria whereas the type species of the genus *Aquaspirillum* falls in the Betaproteobacteria. *M. magnetotacticum* and *M. gryphiswaldense* strain MSR-1 share a similarity of 94.1% while the corresponding similarity values to the other proteobacterial sequences available at that time where between 84 and 89%. The two culturable magnetospirilla have a very similar cell size (0.2 - 0.7 µm by 1 - 3 µm) and ultrastructure with respect to the arrangement (single chain of up to 60 magnetosomes), size (diameter approximately 40 – 45 nm) and cubo-octahedral crystal structure of magnetosomes as well as flagellation (single flagella at each pole). However, there are also differences such as oxidase and catalase activities that are found only in strain MSR-1 which has an increased oxygen-tolerance. The mol% G+C content of MSR-1, originally reported to be with 71% considerably higher than that of *M. magnetotacticum* (64.5%) [9], was recently reexamined by a HPLC-based technique and found with 62.7% to be close to a new value for *M. magnetotacticum* of 63% [12].

In 1993 the group of Matsunaga [13] published the evolutionary relationships between the two facultatively anaerobic strains of magnetic spirilla (AMB-1 and MGT-1) and the genus *Magnetospirillum*. The 16S rRNAs of AMB-1 and MGT-1 share 98 - 99% similarity with that of *M. magnetotacticum* but only 95 - 96% to that of *M. gryphiswaldense*. They clearly fall in the genus *Magnetospirillum* and their proximity to *M. magnetotacticum* on the 16S rRNA level does not exclude the placement of the strains AMB-1 and MGT-1 in this species. The authors also note that there are clearly two groups of magnetospirilla: the one around *M. magnetotacticum* including MGT-1 and AMB-1 and the one with *M. gryphiswaldense* that are about as distant from each other as they are from some nonmagnetotactic photoorganotrophic spirilla, e. g., *Phaeospirillum* (formerly *Rhodospirillum*) *fulvum* and *P. molischianum*.

Further diversity of magnetospirilla was recently revealed by a study of Schüler, Spring and Bazylinski [14] in which a new two-layer isolation medium with opposing oxygen and sulfide gradients was used for cultivation. With this technique seven strains of microaerophilic magnetotactic spirilla could be isolated from one freshwater pond in Iowa, USA. While the 16S rRNA sequences of five of the isolates (MSM-1, -6, -7, -8, -9) were very similar to either *M. gryphiswaldense* or *M. magnetotacticum* (>99.7%), two (MSM-3, MSM-4) are likely to represent a third phylogenetic cluster and at least one additional species. There seems to exist considerable diversity within this genus of culturable magnetic bacteria. In a recent study, a number of novel magnetotactic spirilla strains were isolated from various freshwater habitats including a ditch and several ponds in Northern Germany [15]. Again, 16S rRNA analysis affiliated them all with the genus *Magnetospirillum* with highest similarity to strain MSM-6. Interestingly, several recent reports described the isolation of bacteria, which can be

clearly identified as *Magnetospirillum* species by morphological, physiological and 16S rRNA sequence analysis, however, which lack the capability to form magnetosomes ([16],[17], and others). It will be interesting to see if these non-magnetic "Magnetospirilla" are distinguished from their magnetic relatives by the absence of biomineralization genes, i. e. the magnetosome island [18]. Nevertheless, all isolates seem to represent only a minority of the magnetotactic population and are not abundant in the environment [19].

Phylogenetic diversity and *in situ* identification of uncultured magnetotactic cocci from Lake Chiemsee

The sequences of the two cultivated *Magnetospirillum* strains were subsequently compared to sequences originating from the upper sediment layers of Lake Chiemsee, a large, mesotrophic freshwater lake in Upper Bavaria, Germany [20]. The sediment was stored on a laboratory shelf protected from direct light for several weeks in a 30 l-aquarium. At that time, high numbers of magnetotactic bacteria could be detected in wet mounts of subsamples taken right beneath the water-sediment interface. An enrichment was obtained based on magnetotactic swimming into sterile water or diluted agarose. It contained four distinct morphotypes: cocci, two big rods of distinct morphology (one slightly bent and therefore originally referred to as “big vibrio” [20]) and small vibrios. 5’ end 16S rRNA gene fragments of about 800 nucleotides were PCR-amplified directly from the enriched cells without further DNA isolation and segregated by cloning. Within the 54 clones analyzed, 21 different sequence types could be discriminated. Most of them grouped with 16S rRNA-sequences of Alphaproteobacteria, several with other proteobacteria and one sequence, later shown not to originate from a magnetotactic bacterium, was found to be identical to the 16S rRNA of *Mycobacterium chitae*.

Three probes constructed complementary to signature regions of the most frequent alphaproteobacterial sequences all bound to discrete subpopulations of the cocci which were accounting for about 50% of all cells in the magnetotactic enrichment investigated. Simultaneous applications of two differentially labeled (red, green) probes for these magnetotactic cocci indicated differences in abundance and tactic behavior of the different populations. Genotype CS308 accounted for approximately 80% of all magnetotactic cocci and was therefore more frequent than the genotypes CS103 and CS310. Under the influence of a magnetic field, cells of genotype CS103 were predominantly entrapped nearest to the agarose solution/air interface.

By comparative analysis the partial 16S rRNA sequences of the three types of magnetotactic cocci were shown to be not closely related to any known sequence. The similarities were highest among each other but even there only moderate (89 - 93%). The three newly retrieved sequences form a separate lineage of descent within the Alphaproteobacteria. Surprisingly, even though the genus *Magnetospirillum* also falls into this group the magnetococci have more sequence similarity with other nonmagnetic representatives of this Alphaproteobacteria than with the culturable magnetospirilla.

The study by Spring and coworkers [20] is interesting for several reasons. From a methodological point of view it was one of the first studies in which problems of the rRNA approach became aware. Even though three additional morphotypes were present in the enrichment, together accounting for about 50% of all magnetotactic bacteria, their sequences were obviously not among those retrieved. This might have been caused by preferential PCR amplification of the partial 16S rRNA gene fragment of the magnetotactic cocci. Alternatively, since also several non-magnetotactic bacteria were readily amplified in the experiment, the other magnetotactic bacteria might have been discriminated in any one of the following steps, cell lysis, DNA release, amplification and cloning. With regard to the diversity of magnetotactic bacteria, the discrimination of three genotypes within the magnetotactic cocci and the lack of binding of oligonucleotide probes for the cultivated magnetospirilla and the magnetotactic cocci to the other morphotypes indicated that the genotypic diversity of this bacterial group is higher than the morphological diversity. Furthermore, first hints for a polyphyletic origin of the magnetotactic bacteria were obtained since the next known relatives of both the cultivated magnetospirilla and the Chiemsee magnetococci show no magnetotaxis. Interestingly, even though the magnetotactic cocci are quite abundant in Lake Chiemsee and can be readily enriched from its sediment they have until now resisted all attempts to bring them into pure culture (Stefan Spring, personal communication). This underlines the importance of the cultivation-independent rRNA approach in the study of magnetotactic bacteria.

The magnetotactic bacteria are polyphyletic with respect to their 16S rRNA

The magnetosomes of most magnetotactic bacteria contain only iron oxide particles, but some magnetotactic bacteria collected from sulfidic, brackish-to-marine aquatic habitats contain iron sulfide or both. DeLong and coworkers analyzed three magnetotactic bacteria of the magnetite or greigite type by the rRNA approach [21], they found the two isolates with the iron oxide magnetosomes, a magnetotactic coccus and a magnetotactic vibrio, to be affiliated

with the Alphaproteobacteria. The coccus actually fell in the group of Chiemsee magnetococci, whereas the vibrio was closer to the magnetospirilla even though based on different tree reconstructions it could not be finally shown whether it was closer to *Rhodospirillum rubrum* or to the genus *Magnetospirillum*. These findings were in line with those of Spring and coworkers [20]. The 16S rRNA sequence retrieved from an uncultured many-celled, magnetotactic prokaryote (MMP) with iron sulfide magnetosomes collected at various coastal sites in New England, however, was specifically related to the dissimilatory sulfate-reducing bacteria within the Deltaproteobacteria. The closest relative is *Desulfosarcina variabilis* with a 16S rRNA similarity of 91% [21]. This indicated a polyphyletic origin for magnetotactic bacteria. The authors also argue that their findings suggest that magnetotaxis based on iron oxide and iron sulfide magnetosomes evolved independently. They state that the biochemical basis for biomineralization and magnetosome formation for iron oxide-type and iron sulfide type bacteria are likely fundamentally different and speculate that in two independent phylogenetic groups of bacteria analogous solutions for the problem of effective cell positioning along physico-chemical gradients were found based on intracellular particles with permanent magnetic dipole moments [21].

“Magnetobacterium bavaricum”

The polyphyletic distribution of magnetotaxis in bacteria was further corroborated by the phylogenetic affiliation and *in situ* identification of the large rod-shaped magnetic bacterium from Lake Chiemsee sediment which was found to belong to a third independent lineage [22]. This bacterium was conspicuous because of its large size (5 – 10 µm long; approximately 1.5 µm in diameter) and high number of magnetosomes. Up to 1000 hook-shaped magnetosomes with a length of 110 - 150 nm can be found in several chains. The large cells are gram-negative and often contain sulfur globules. The cells are mobile by one polar tuft of flagella. This morphotype, tentatively named “Magnetobacterium bavaricum”, has so far only been enriched from the calcareous sediments of a few freshwater lakes in Upper Bavaria [23]. As is the case for many other magnetotactic bacteria, microbiologists were unable to grow this bacterium in pure culture until now. This morphotype was abundant in the magnetotactic enrichment investigated by Spring and coworkers before [20] but its 16S rRNA sequence could not be retrieved in the presence of the magnetotactic cocci. “M. bavaricum” cells were therefore sorted from this enrichment by flow cytometry based on the high forward and sideward light scattering caused by the large cell size and the high amounts of magnetosomes. From the

sorted cells an almost full length 16S rRNA sequence could be retrieved that was proven by FISH to originate from “M. bavaricum” (Fig. 1). Unlike the magnetotactic cocci, this conspicuous morphotype consisted of only one genotype which was affiliating with neither the Alpha- nor the Deltaproteobacteria but with a different line of descent, tentatively referred to as the *Nitrospira* phylum since it encompasses the cultured *Nitrospira moscovensis*. The 16S rRNA of “M. bavaricum” has similarities of less than 80% with any other known sequence of magnetotactic bacteria. The magnetosomes were shown to consist of the iron oxide magnetite (N. Petersen, personal communication), suggesting that there were also multiple phylogenetic origins for the iron oxide/magnetite-based magnetotaxis.

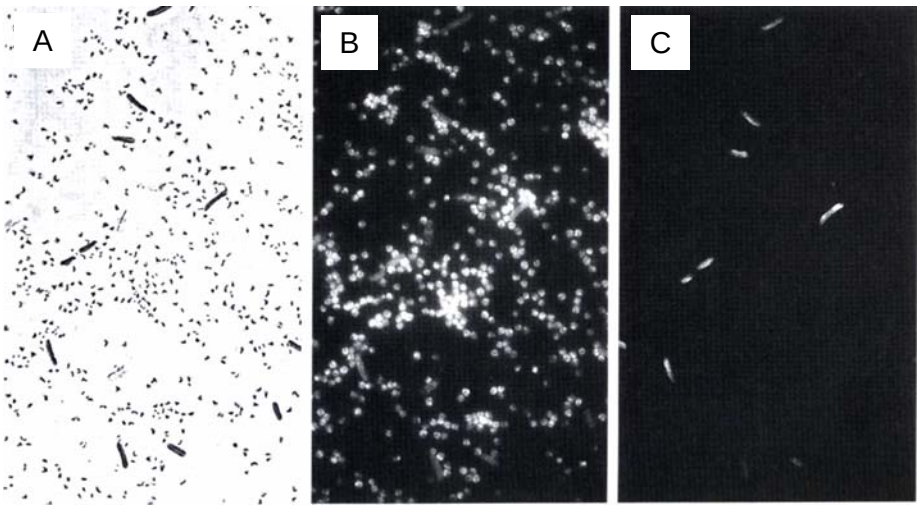

Fig. 1: *In situ* identification of the hitherto uncultured “Magnetobacterium bavaricum” by FISH with a specific, 16S rRNA-targeted oligonucleotide probe [22]. Panel A Phase contrast micrograph. Panel B Visualization of hybridization of bacterial probe EUB338-Fluorescein. Panel C Selective visualization of “M. bavaricum” by a specific tetramethylrhodamine-labeled oligonucleotide probe. Identical microscopic fields are shown in panels A-C.

Recently, it was shown that the occurrence of magnetotactic bacteria from the *Nitrospira* phylum is apparently not restricted to Bavaria. A conspicuous magnetotactic rod (MHB-1) was magnetically collected from sediment of a lake nearby Bremen [15]. The magnetosomes from MHB-1 display the same bullet-shaped crystal morphology like those from ”M. bavaricum” (Fig. 2) and are aligned in multiple chains. However, unlike the latter organism, MHB-1 has less magnetosomes, which form a single bundle. 16S rRNA analysis revealed 94% sequence similarity to ”M. bavaricum” and cells hybridized with the FISH probe originally used for the identification of “M. bavaricum” [22], indicating that there exists morphological and phylogenetic diversity within this magnetotactic lineage.

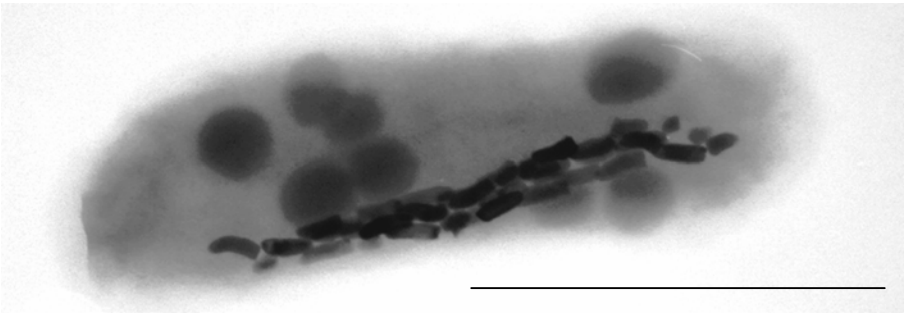

Fig. 2: Transmission electron micrograph image of a novel magnetotactic rod of the *Nitrospira* phylum, which is closely related to "Magnetobacterium bavaricum". Bar = 0.7 µm.

"M. bavaricum" could be best enriched from a reddish brown layer at a depth of 5 to 8 mm below the sediment surface. By FISH its abundance in the sediment was quantified and correlated to physico-chemical gradients determined with needle electrodes. Up to 7×10^5 mobile cells per cm^3 were present in the reddish brown zone. This layer coincided with the microaerobic zone. Using sulfide electrodes no free sulfide above the detection limit of 10 µM could be detected. However, sulfate reducing bacteria were present in the microaerobic zone and the authors argue that low levels of sulfide might be continuously produced. They suggested that "M. bavaricum" has an iron-dependent way of energy conservation which depends on balanced gradients of oxygen and sulfide [22]. Based on its relative abundance of 0.64 ± 0.17% and a large average cell volume of 25.8 ± 4.1 μm^3 it was estimated that "M. bavaricum" made up approximately 30% of the bacterial biovolume in the reddish brown zone. This demonstrates how hypotheses on the physiology and ecology of hitherto uncultured bacteria can be built based on the joint application of microscopic techniques, the rRNA approach and the *in situ* characterization of the microhabitat of the bacterium of interest.

Further diversity of magnetotactic bacteria

In the 1990s it became standard to infer evolutionary relationships of bacteria by the phylogenetic analysis. In the following we will just quickly review further publications reporting 16S rRNA sequences from both cultured strains of magnetotactic bacteria and magnetic enrichments.

In 1994 Spring and coworkers used the cultivation-independent approach to retrieve another three partial and seven almost full length 16S rRNA gene sequences from freshwater sediments of various sites in Germany [24]. By FISH all sequences were assigned to magneto-

tactic bacteria, nine to magnetotactic cocci and one to the second rod-shaped magnetotactic morphotype (“large vibrio”) originally described in Lake Chiemsee [20]. The magnetotactic rod shared a 16S rRNA similarity of 90 - 92% with the magnetotactic cocci, which among themselves mostly had similarity values below 97%. All sequences grouped with those earlier retrieved from the uncultured Chiemsee magnetotactic cocci [20]. The authors point out that the finding that most magnetotactic cocci have 16S rRNA similarities below 97% has important taxonomic implications. In several studies on culturable bacteria it has been shown that a significant DNA-DNA relatedness that would justify assignment to one species exists only above 97%. Therefore, upon isolation the different magnetococci could be placed in different species. This work of Spring and coworkers [22] did not only corroborate that the diversity of magnetotactic cocci is fairly large, but it also showed that the “Lake Chiemsee magnetococci” branch does not exclusively consist of cocci. This once again demonstrates the limited value of cell morphology in bacterial systematics.

In 1995 the group around Matsunaga published two reports related to the diversity and distribution of magnetotactic bacteria. In one, a PCR primer set specific for the 16S rRNA gene of the Lake Chiemsee magnetotactic cocci [20] was used to amplify DNA from magnetically isolated cocci. Comparative sequence analysis of the amplified 16S rDNA fragments proved their affiliation to the Lake Chiemsee magnetotactic cocci [25]. This demonstrated that this group of magnetotactic bacteria is not only occurring in German sites but also in Japan. The authors used the primer set to investigate the distribution of magnetotactic cocci in laboratory enrichments. 16S rRNA gene fragments of magnetotactic cocci were readily amplified from a water column above the sediment kept in an anoxic environment, but little was amplified from a water column kept in an oxic environment. The results suggest that the magnetotactic cocci found in the anoxic water column had migrated there from the sediment as a response to the microoxic or anoxic conditions or having been present previously in a nonmagnetic form and having become magnetic due to the change in conditions. For instance, *M. gryphiswaldense*, can grow aerobically but produces magnetosomes only under microoxic or anoxic conditions (<20 mbar O_2, [26]). Studies of their vertical distribution in freshwater sediments have indicated that most magnetotactic bacteria occurred in a narrow layer in the suboxic zone, where dissolved iron was available [27].

In their second report the group of Matsunaga [28] described the phylogenetic analysis of a novel sulfate-reducing magnetic bacterium, RS-1. The almost full 16S rRNA gene of the pure culture was amplified and partially sequenced. The comparative sequence analysis placed it with the sulfate-reducing bacteria of the Deltaproteobacteria within the genus

Desulfovibrio. Interestingly, *Desulfovibrio magneticus* RS-1 was the first bacterium reported outside the Alphaproteobacteria that contains magnetite inclusions [29], [30]. It therefore disrupts the correlation between the alpha- and deltaproteobacterial magnetotactic bacteria and iron oxide (magnetite) and iron sulfide (greigite) magnetosomes, respectively, suggested by DeLong and coworkers [21].

This list of applications of the rRNA approach to the phylogeny and *in situ* identification of magnetotactic bacteria extends to a publication of Spring and coworkers [31] in 1998. In this study, natural enrichments of magnetic bacteria from the Itaipu lagoon near Rio de Janeiro in Brazil were analyzed. These were dominated by coccoid-to-ovoid morphotypes. Some of the cells produced unusually large magnetosomes that with a length of 200 nm and a width of 160 nm are almost twice as big as those found in other magnetotactic bacteria [32]. Partial sequencing of 16S rRNA genes revealed two clusters (Itaipu I and II) of closely related sequences within the lineage of magnetotactic cocci [20],[24],[25]. For a detailed phylogenetic analysis several almost full length 16S rRNA gene sequences were determined. In order to link at high resolution the ultrastructure of the enriched cells with their 16S rRNA sequence a new methodology was applied. Instead of light microscopic FISH with fluorescent oligonucleotide probes *in situ* hybridizations with polynucleotide probes on ultra-thin section of embedded magnetotactic bacteria were examined by electron microscopy. For that one representative clone of each of the two closely related 16S rRNA clusters was used as a template for in vitro transcription of a 230 nucleotide long variable region at the 5' end of the 16S rRNA. The resulting RNA probe was during in vitro transcription labeled with digoxigenin- and fluorescein-labeled UTP. Bound polynucleotide probe was detected by incubation of the sections with gold-labeled antibodies specific for fluorescein or digoxigenin. The gold labels could then be detected in the electron microscope (Fig. 3). This enabled for the first time a detailed description of the morphological variety and ultrastructure of *in situ* identified, uncultured magnetic bacteria. Using this technique it was possible to link presence of the unusually large magnetosomes in ovoid magnetotactic bacteria to the Itaipu I 16S RNA type.

Cox and coworkers investigated the diversity of magnetotactic cocci in Baldwin Lake (Los Angeles) by restriction fragment length patterns (RFLP) analysis [33]. They found several 16S rRNA sequences, which had high similarities to known magnetotactic cocci from the database. In addition, they identified six sequences, which formed a monophyletic cluster (ARB-1 cluster) related to, but distinct from other magnetotactic bacteria (89% similarity to the magnetotactic coccus CS92).

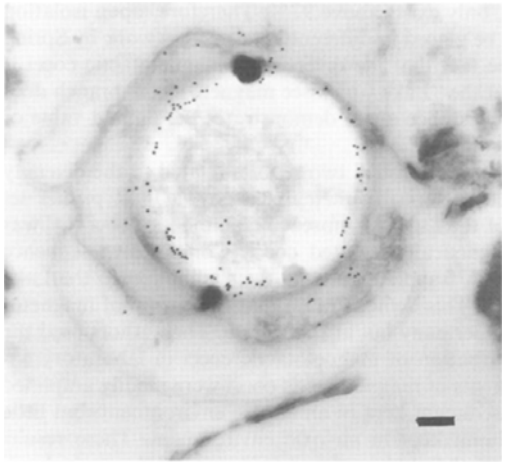

Fig 3: Electron micrograph of a hybridized thin section of magnetically enriched bacteria form the Itaipu lagoon (Rio de Janeiro, Brazil). Magnetosomes are visible as black inclusions. The digoxigenin-labeled polynucleotide probe mabrj58 specific for the morphotype Itaipu I was detected with anti-DIG-antibodies conjugated with 15 nm gold particles. Bar = 0.5 µm.

Recently, Flies and coworkers [15] have investigated the diversity of magnetotactic bacteria in various microcosms with freshwater and marine sediments from Germany and Sweden by DGGE and Amplified Ribosomal DNA Restriction Analysis (ARDRA) of the 16S rRNA genes. Initially, the sediments contained a highly diverse population of magnetotactic bacteria displaying a variety of different morphotypes. However, the magnetotactic population in the microcosms underwent a rapid succession, which usually resulted in the dominance of a magnetotactic coccus from the Alphaproteobacteria after several weeks of incubation.

While most 16S rRNA sequences from magnetotactic bacteria were identified after magnetic enrichment, two sequences putatively originating from marine magnetotactic bacteria were found without magnetic manipulation by Riemann and coworkers, who investigated the bacterial community composition in the Arabian Sea by DGGE analysis [34]. Both sequences were nearly identical to each other and were closely related to an uncultivated magnetotactic coccus from a freshwater habitat (95% similarity). This indicates that magnetotactic bacteria may also occur in the water column. Alternatively, these sequences may represent closely related non-magnetotactic species.

A current view of the phylogeny of magnetotactic bacteria

Our current view of the phylogeny of magnetotactic bacteria is shown in a 16S rRNA-based tree reconstruction in Fig. 4. Magnetotactic bacteria can be found in the *Nitrospira* phylum and within the Alpha- and Deltaproteobacteria.

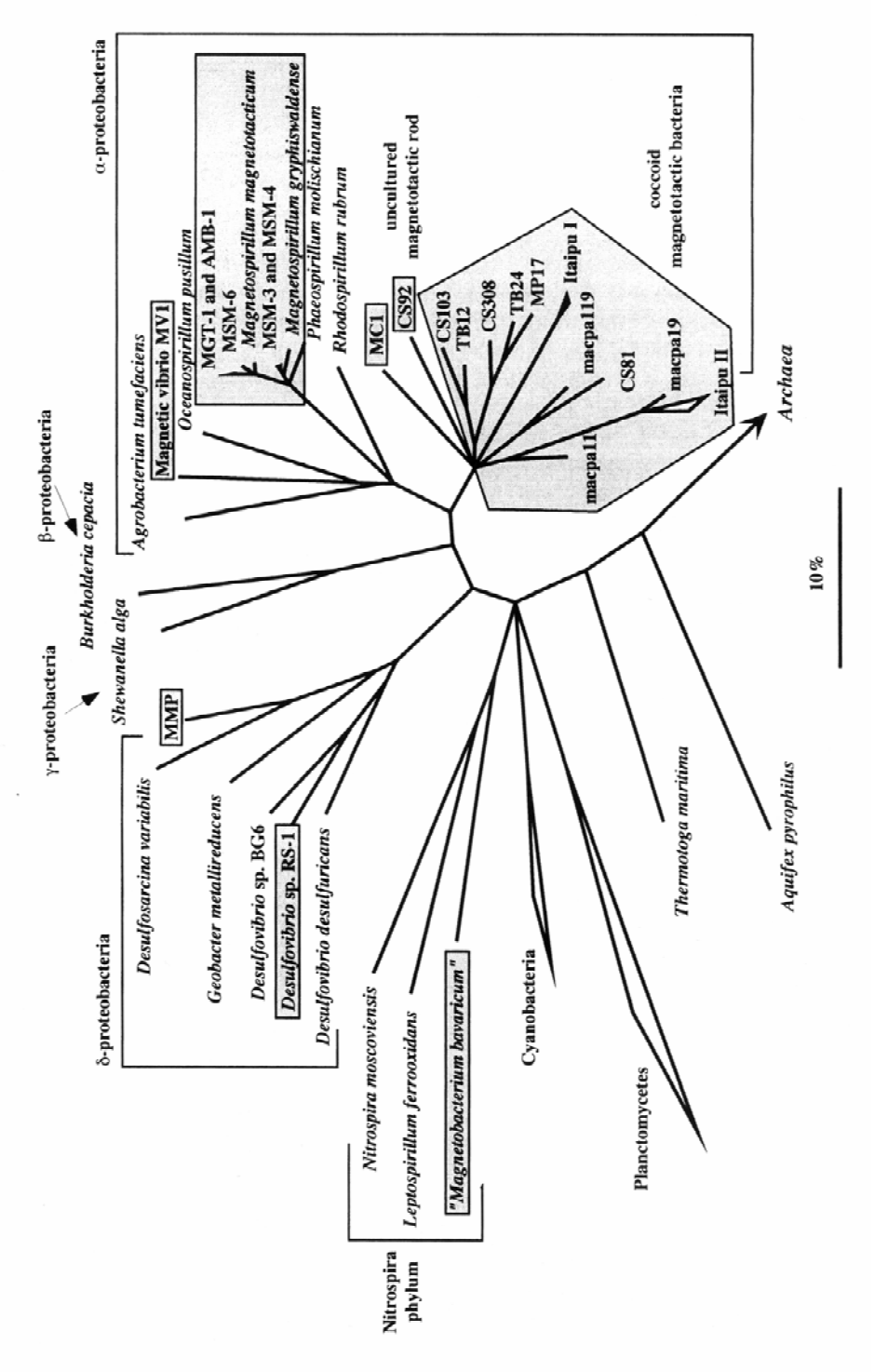

Fig. 4: 16S rRNA-based tree reconstruction showing the phylogeny of magnetotactic bacteria (grey). The tree is based on parsimony analyses and was corrected according to the results of maximum likelihood and neighbor joining methods using the ARB program package [36]. Multifurcations were drawn whenever branching orders were not stable [35]. The tree topo-logy is based on almost full-length 16S rRNA sequences. The partial sequences of strains CS103, MC-1, MV-1, MMP and RS-1 have been added with the parsimony tool without allowing changes of the overall tree topology [36].

Whereas the diversity in the *Nitrospira*-branch of the magnetotactic bacteria consists only of the still uncultured "Magnetobacterium bavaricum" [22] the two proteobacterial branches are based on rRNA sequences of pure cultures and of still uncultured magnetotactic bacteria. Within the Deltaproteobacteria there is the sequence of the pure culture *Desulfovibrio magneticus* RS-1 [25] and the sequence MMP [21] that by the rRNA approach was assigned to the hitherto uncultured multicellular magnetotactic prokaryote. The sequence of MMP is also the only sequence that is available of a magnetotactic bacterium with iron sulfide/greigite magnetosomes. The vast majority of currently known sequences of magnetotactic bacteria (>90% of those we found in the publicly available data bases) falls within the Alphaproteobacteria. Of this large diversity only several members of the genus *Magnetospirillum*, the magnetic coccus MC-1 and the magnetic vibrio MV-1 have been cultured. In the last decade cultivation stagnated and only few additional strains of magnetospirilla have been described [13],[14]. What was found in terms of new diversity over this period was mostly from uncultured magnetotactic bacteria. However, the new information obtained by the cultivation-independent approach since the phylogeny of magnetotactic bacteria was last reviewed by Spring & Schleifer in 1995 [19] is also limited. There were only few new sequences affiliated with the already described branch of Lake Chiemsee magnetotactic cocci for which the reports from Germany [20],[24], Japan [25] and Brazil [31] now suggest global distribution and considerable intragroup-diversity.

What is the reason for this stagnation? One possibility is that the extant diversity of magnetotactic bacteria is by now fully described. The other possibility is that our methods are selective. We know that for the cultivation methods but it has to be realized that this is also true for the rRNA approach, especially, if it starts from standard laboratory enrichments which themselves are selective. The methodology currently applied is biased towards motile, aero-tolerant bacteria, although the presence of atmospheric oxygen apparently did not affect the number of bacteria magnetically collected from anoxic sediment horizons [27]. New diversity might be detected if from various habitats magnetotactic bacteria are directly retrieved without prior storage of the sediments in the lab. Are there strictly anaerobic, non-motile bacteria which form intracellular magnetosomes? Also primer sets other than the standard "bacterial" ones should be tested for 16S rRNA retrieval from magnetic enrichments. It is known that every primer set has preferences and the example of the discrimination of the "Magnetobacterium bavaricum" sequence against the magnetotactic cocci has been described before. In this case, it was only the large size and the extraordinary high magnetosome content that allowed further purification of the initial magnetotactic enrichment by flow cytometric

sorting. This is not possible for less conspicuous magnetotactic bacteria. Magnetotactic bacteria might occur in other bacterial lineages. It would also be interesting to check whether archaebacterial sequences can be sequenced from magnetotactic enrichments.

Future attempts to identify and characterize new magnetotactic bacteria should be undertaken. These should make use of the potential synergistic effects of cultivation-independent *in situ* and traditional cultivation approaches. If, a cultured close relative can be identified in the 16S rRNA tree, then the affiliation of a "new magnetic sequence" may give important hints for its enrichment and cultivation. Also data on the *in situ* microhabitat of magnetotactic bacteria should be obtained and used for the formulation of suitable media.

In the last decade two of the three questions raised above have been answered. There exists a large diversity of magnetotactic bacteria that goes beyond that already indicated by the many morphotypes detected in the 70s and 80s, and, the magnetotactic bacteria are polyphyletic. The third question, however, whether the biomineralization of magnetosomes, or at least e. g. the intracellular formation of magnetite is monophyletic, is still open. It would be highly interesting to investigate by comparative analysis of genes involved in the magnetosome formation whether lateral gene transfer e.g. from the alphaproteobacterial magnetotactic bacteria to "M. bavaricum" contributed to the spreading of magnetite-based magnetotaxis or whether the mechanisms of magnetosome formation have independently developed in the different phylogenetic groups. Studies of this type will not necessarily rely on cultured strains since there is a rapidly increasing potential to directly retrieve form the environment and analyze large DNA fragments. If these fragments contain 16S rRNA genes or can be linked by overlaps to such fragments environmental genomics allows for the comparative genome analysis of identified, unculturable bacteria [35]. Recently, a substantial number of genes for magnetosome formation were identified (*mam*-genes), which apparently are ubiquitously present in the genomes of all magnetotactic bacteria from the Alphaproteobacteria that have been analyzed so far [18]. Therefore, the cultivation-independent retrieval of genetic information directly from the environment should not be restricted to phylogenetic marker genes, but should be extended to those metabolic key genes. Magnetotactic bacteria can be easily collected by magnetic enrichment directly from environmental samples in high numbers and virtually free of contaminants. In addition, most, if not all genes essential for magnetosome biomineralization apparently are clustered within a relatively small section of the chromosome as a genomic "magnetosome island". Thus, the retrieval and analysis of large continuous sequences harboring these islands, or even the analysis of the whole "magnetotactic metagenome" in the future will be an extremely powerful approach to gain further insights

in the genetic diversity of magnetosome biomineralization as well as a better understanding of the ecological interactions of these organisms. This in the future might also lead to improved strategies for their isolation and cultivation.

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E Anhang

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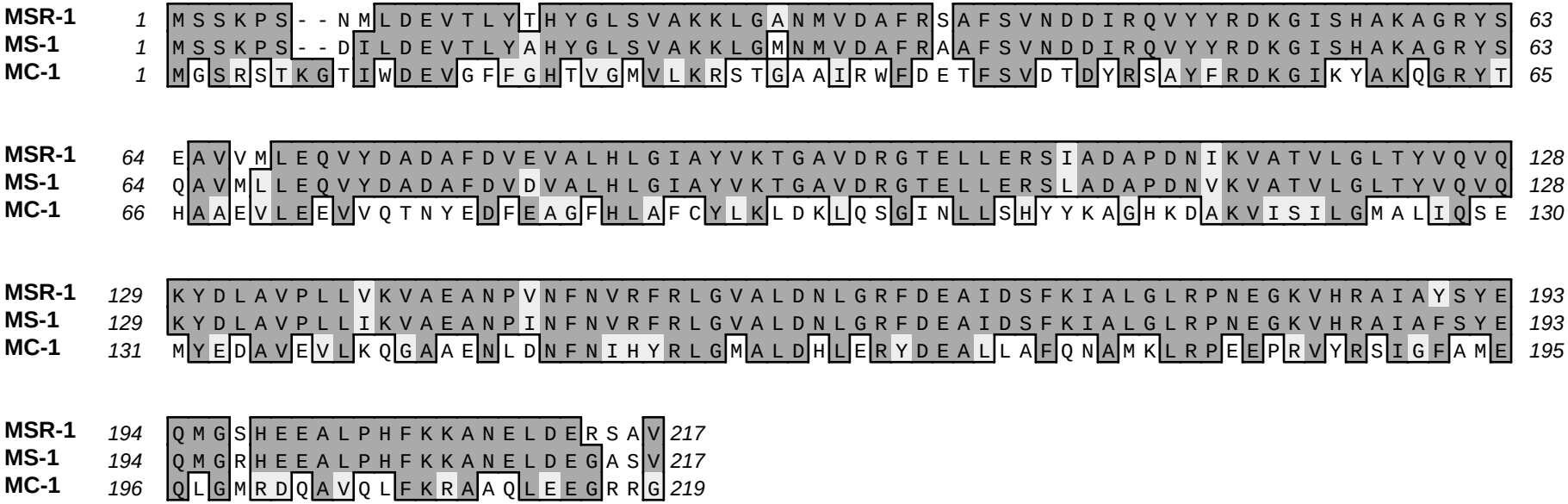
Aligment MamA



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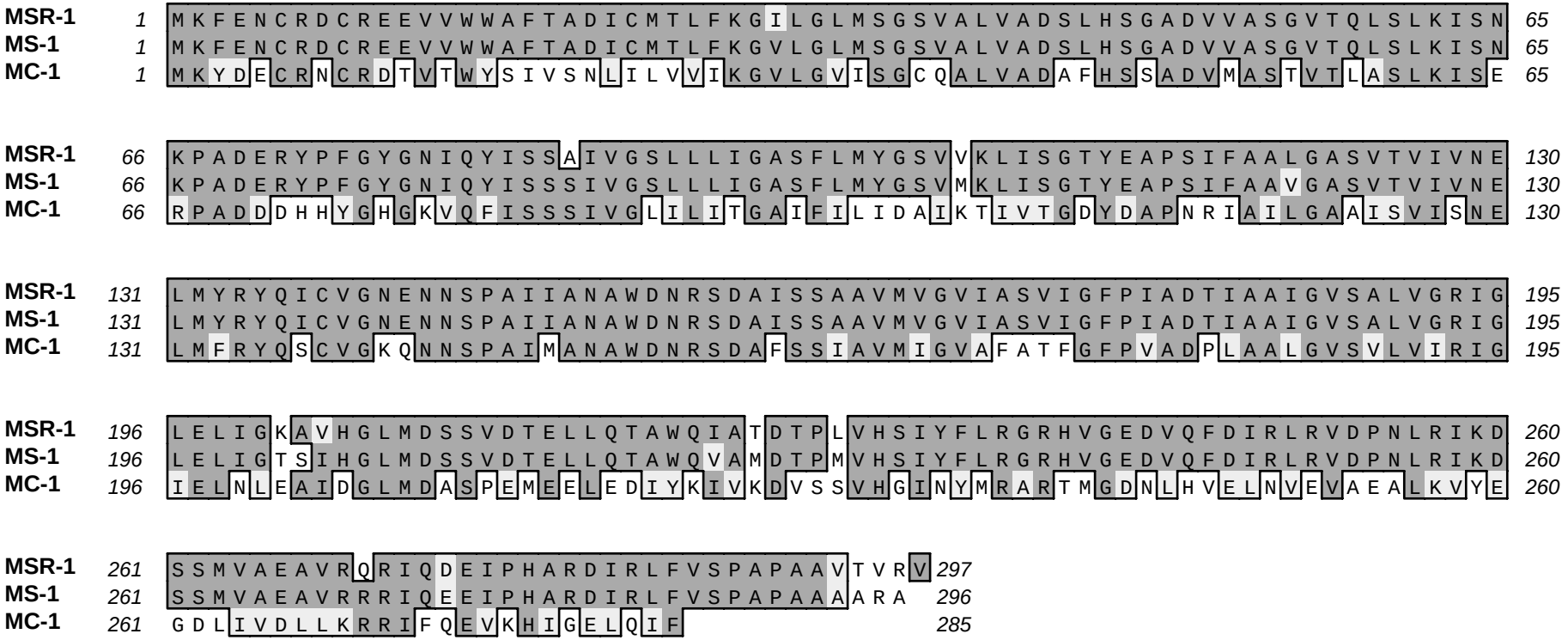

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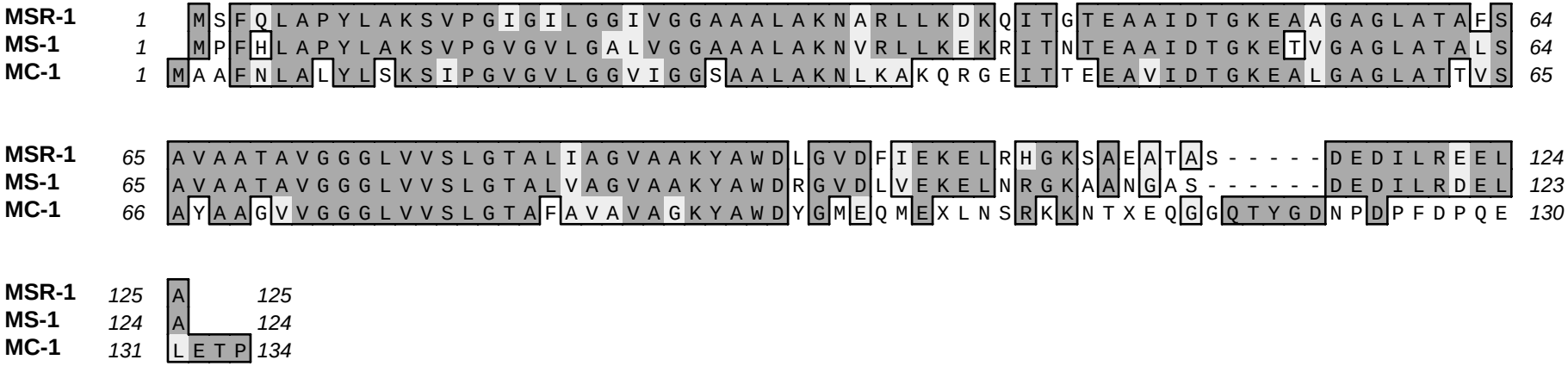

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