Molekularbiologische Untersuchung der Diversität und Funktion methanogener Mikroorganismen im Reisfeldboden

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Die in dieser Dissertation beschriebenen Ergebnisse sind in folgenden Originalpublikationen veröffentlicht bzw. zur Veröffentlichung eingereicht:


Weiterhin waren bei der Erstellung dieser Schrift die Arbeiten zu folgendem Manuskript noch nicht vollständig abgeschlossen, die Ergebnisse werden daher in einer Rohfassung präsentiert:

Lueders T. und Friedrich M. Evaluation of PCR amplification bias by T-RFLP analysis of SSU rRNA and mcrA genes using defined DNA template mixtures of methanogenic pure cultures and soil DNA extracts. (in preparation)
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Abkürzungen

DGGE  Denaturierende-Gradienten-Gel-Elektrophorese
DSMZ  Deutsche Sammlung von Mikroorganismen und Zellkulturen
FRB   eisenreduzierende Mikroorganismen („ferric-iron-reducing bacteria“)
MCR   Methyl-Coenzym-M-Reduktase
mcrA  Gen für die $\alpha$-Untereinheit der MCR
MPN   „most probable number“
OTU   Operative taxonomische Einheit („operational taxonomic unit“)
PCA   „principal component analysis“
PVPP  Polyvinylpolypyrrolidon
RAF   Relativer Anteil der Amplifikate („relative amplicon frequency“)
RFU   Relative Fluoreszenz Einheit („relative fluorescence unit“)
RC-I  Rice Cluster I
SSCP  Einzelstrang-Konformations-Polymorphismus „single strand conformation polymorphism“
SRB   sulfatreduzierende Mikroorganismen („sulfate-reducing bacteria“)
T-RF  terminales Restriktionsfragment
T-RFLP Terminaler-Restriktionsfragment-Längen-Polymorphismus

Weitere Abkürzungen wurden entsprechend der „Information for Authors“ des European Journal of Biochemistry verwendet.
Zusammenfassung


Zusammenfassung

Acetat) direkt nach der Flutung korreliert. Bei geringen Substratkonzentrationen (nach 10 Tagen) zeigte dagegen die Analyse der 16S rRNA vor allem eine Zunahme für Methanosaeta spp.

Der Einfluss verschiedener Verfahren zur Emissionsminderung („Mitigation“) auf die methanogene Gemeinschaft wurde in der Form der Zugabe alternativer Elektronenakzeptoren untersucht. Die Zugabe von Ferrihydrit verursachte bereits am 3. Tag nach der Flutung eine starke Hemmung der Methanogenese, was auf eine sofort konkurrenzfähige Population der Eisenreduzierer hinweist. Entsprechend stark unterdrückt war die methanogene Populationsdynamik. Die Zugabe von Gips dagegen resultierte erst nach 10 Tagen in einer vollständigen Hemmung der Methanogenese und in einer nur teilweise inhibierten methanogenen Populationsdynamik, was mit einem zunächst wenig effizienten Verbrauch des Acetats durch die Sulfatreduzierer korreliert war. Es konnte gezeigt werden, dass die methanogene Gemeinschaft im italienischen Reisfeldboden nicht nur funktionell, sondern auch strukturell differenziert auf die Zugabe alternativer Elektronenakzeptoren reagiert.
A. Einleitung

A.1. Mikrobielle Ökologie gefluteter Reisfelder

Intermediate des anaeroben Abbaus, um die verschiedene respiratorische Gilden konkurrieren (Abb. 1).

Die Aktivität verschiedener respiratorischer Gilden und ihre Konkurrenz um gemeinsame Elektronendonatoren wird durch die Verfügbarkeit der jeweiligen Elektronenakzeptoren bestimmt. Dabei sind jeweils die Mikroorganismen energetisch im Vorteil, die den Elektronenakzeptor mit dem höheren Redoxpotential reduzieren können (Zehnder und Stumm, 1988). Die überlegene Gruppe ist durch eine höhere Affinität (geringerer $K_m$) und/oder eine niedrigere minimal verwertbare Schwellenkonzentration („Threshold Concentration“) bezüglich der gemeinsamen Substrate gekennzeichnet (Achnich et al., 1995; Conrad, 1999; Kristjansson et al., 1982; Lovley, 1985; Robinson und Tiedje, 1984; Schönheit et al., 1982). Diese Konkurrenz der verschiedenen respiratorischen Gilden

(Lovley, 1991) bedingt unter limitierenden Substratkonzentrationen in gefluteten Böden eine sequentielle Reduktion verfügbarer Elektronenakzeptoren in der Reihenfolge NO\textsubscript{3}\textsuperscript{-} > Fe\textsuperscript{3+} > SO\textsubscript{4}\textsuperscript{2-} > CO\textsubscript{2} (Ponnamperuma, 1972). Nur wenn gemeinsame Elektronendonatoren in nicht-limitierenden Konzentrationen vorhanden sind, können verschiedene respiratorische Gilden diese gleichzeitig veratmen.

Die in dieser Arbeit untersuchten italienischen Reisfeldböden sind durch sehr geringe natürlicher vorhandene NO\textsubscript{3}\textsuperscript{-} und SO\textsubscript{4}\textsubscript{2-}-Konzentrationen nach der Flutung gekennzeichnet, so dass die entsprechenden Reduktionsprozesse im anoxischen Teil des nicht-durchwurzelten Bodens bereits nach wenigen Tagen beendet sind. Bis auf oxisch/anoxische Übergangsbereiche, in denen „Redoxcycling“ stattfindet (Brune et al., 2000; Liesack et al., 2000), werden NO\textsubscript{3}\textsuperscript{-} und SO\textsubscript{4}\textsubscript{2-} erst mit der nächsten Trockenlegung des Feldes regeneriert. Die Phase der Fe\textsuperscript{3+}-Reduktion kann dagegen, je nach organischem Kohlenstoffgehalt des Bodens, bis zu mehrere Wochen andauern und bis zu 24% der gesamten anaeroben Mineralisationsleistung ausmachen (Jäckel und Schnell, 2000a). Spätestens wenn alle energetisch günstigeren Elektronenakzeptoren verbraucht sind, wird die Methanogenese zum dominanten terminalen Atmungsprozess. Methanproduktion wird aber bereits am ersten Tag nach der Flutung detektiert, obwohl zu diesem Zeitpunkt noch Elektronenakzeptoren wie SO\textsubscript{4}\textsuperscript{2-} oder Fe\textsuperscript{3+} vorhanden sind (Achtnich et al., 1995; Roy et al., 1997; Yao et al., 1999). Dies lässt sich durch sehr hohe Substratkonzentrationen (H\textsubscript{2}, Acetat) erklären, die eine gleichzeitige Aktivität verschiedener respiratorischer Gilden erlauben. Das produzierte Methan entweicht, wenn es nicht an den oxisch/anoxischen Übergangszenen durch methanotrophe Mikroorganismen zu CO\textsubscript{2} re-oxidiert wird, durch Diffusion, Blasenbildung oder durch das Aerenchym der Reispflanzen in die Atmosphäre (Brune et al., 2000).

A.2. Methanemission aus Reisfeldern

Neben der auf der Vielfalt der mikrobiellen Prozesse beruhenden Attraktivität des Modellsystems Reisfeld sind besonders die durch Reisfelder verursachten Methanemissionen von globaler Relevanz. Reis ist eines der wichtigsten Grundnahrungsmitte der Weltbevölkerung und wird auf einer Fläche von über 145 Mio ha angebaut (Minami und Neue, 1994). Die jährlichen Methanemissionen aus Reisfeldern werden auf ~60 Tg eingeschätzt, die bis zu ~20% der anthropogen bedingten oder ~12% der gesamten

Doch während die Hemmung methanogener *Archaea* durch die Konkurrenz anderer respiratorischer Gilden thermodynamisch und physiologisch gut verstanden ist, fehlen Daten über den Einfluss alternativer Elektronenakzeptoren auf die Struktur und Dynamik der beteiligten mikrobiellen Populationen noch vollständig. Ein solches Wissen ist aber notwendig, um die verschiedenen Möglichkeiten zur Emissionsminderung vollständig zu evaluieren.

**A.3. Methanogene *Archaea* im Reisfeldboden**


Jedoch zeigten intensivere Analysen zusätzlich die Anwesenheit mehrerer bislang nicht-kultivierter archaeeller Linien (Abb. 2), sowohl innerhalb der *Eury*- als auch der *Crenarchaeota*, die vorläufig mit „Rice Cluster I - VI“ (RC-I bis -VI) benannt wurden (Chin et al., 1999; Grosskopf et al., 1998b). Da aber nicht alle *Archaea* zwangsläufig methanogen sind, war es von großem Interesse, ob einige dieser neuen Cluster möglicherweise neuartige Methanogene darstellen. Die RC-IV und -VI gruppieren zusammen mit einer Vielzahl weiterer Umweltsequenzen (Übersicht siehe Buckley et al. (1998); DeLong (1998); Jurgens et al. (2000)) innerhalb der mesophilen *Crenarchaeota*. Bis heute ist es noch nicht gelungen, diese Organismen zu kultivieren, und ihre Physiologie ist noch völlig
unbekannt. Es gibt aber bisher keine Hinweise darauf, dass ein methanogener Stoffwechsel auch innerhalb der *Crenarchaeota* existiert.


Andere neue Entwicklungslinien aber, speziell das RC-I, sind nah mit bekannten methanogenen Familien verwandt. Da aber eine Zuordnung lediglich über 16S rRNA
Daten möglich war und ein Zusammenhang zwischen 16S rRNA-Phylogenie und Physiologie nicht per se existiert (Liesack et al., 1997), konnte ein methanogener Phänotyp des neuen Clusters lediglich vermutet werden (Grosskopf et al., 1998b). Ein weiterer Hinweis war der Nachweis von RC-I Archaea in methanogenen Anreicherungskulturen von Reiswurzeln (Lehmann-Richter et al., 1999).

A.4. Funktionelle Marker in der mikrobiellen Ökologie


Die Bandbreite der mikrobiellen Gilden, für die funktionelle Marker etabliert und eingesetzt wurden, nimmt ständig weiter zu. Bekannte Beispiele sind Methanoxidierer, die mittels der löslichen und partikulären Methanmonooxygenase nachgewiesen werden (McDonald et al., 1995; McDonald und Murrell, 1997a; McDonald und Murrell, 1997b), oder die über das Gen der Ammoniummonooxygenase analysierten Ammoniumoxidierer (Horz et al., 2000; Rotthauwe et al., 1997). Auch die Diversität anaerob atmender Gruppen wie der Nitrat- oder Sulfatreduzierer wurde bereits über ihre Schlüsselenzyme
A. Einleitung

Doch die rein auf dem Nachweis funktioneller Marker beruhende Diversitätsanalyse funktioneller Gilden erlaubt noch keine Aussage über Struktur und Dynamik der mikrobiellen Populationen. Solche Informationen sind aber notwendig, um die Reaktion funktioneller Gilden auf verschiedene Umweltfaktoren verfolgen zu können und um die tatsächlich aktiven Mikroorganismen zu identifizieren. Um diese Fragestellungen zu beantworten, werden zunehmend auch für funktionelle Marker sog. „Fingerprinting“-Methoden entwickelt, die in der 16S rDNA-Analytik bereits etabliert und weit verbreitet sind. Klassische „Fingerprinting“-Methoden sind z.B. die DGGE, T-RFLP und SSCP-Analysen, die zur Auftrennung gemischter PCR-Produkte anhand unterschiedlicher Charakteristika (Schmelzverhalten, Schnittstellen, elektrophoretische Mobilität) eingesetzt werden. Auf DGGE oder T-RFLP beruhende Methoden zur Analyse methan- und ammoniumoxidierender sowie nitratreduzierender mikrobieller Gemeinschaften wurden bereits entwickelt (Braker et al., 2001; Fjellbirkeland et al., 2001; Henckel et al., 1999; Horz et al., 2001; Oved et al., 2001).


untersucht (Braker et al., 1998; Hallin und Lindgren, 1999; Minz et al., 1999; Perez-Jimenez et al., 2001).
A.5. Ziele der Arbeit

Das Ökosystem Reisfeld stellt ein sowohl physiko-chemisch als auch mikrobiologisch äußerst vielfältiges Habitat dar, in dem methanogene Prozesse von fundamentaler Bedeutung sind. Doch während die relevanten biogeochemischen Prozesse bereits gut untersucht und verstanden sind, befindet sich die Analyse der beteiligten mikrobiellen Populationen noch an ihrem Anfang. Zentrale Fragen, die zum besseren Verständnis der methanogenen Populationen im Reisfeld beitragen können, sollen in dieser Arbeit mit Werkzeugen der molekularen mikrobiellen Ökologie behandelt werden:

- Ist die Diversität der Methanogenen im Reisfeldboden vollständig bekannt oder beinhaltet er möglicherweise noch nicht kultivierte Methanogene?
- Wie sind methanogene Populationen strukturiert und wie variabel sind sie in Reisfeldböden unterschiedlicher globaler Herkunft?
- Welche Populationsdynamik durchlaufen methanogene Mikroorganismen während ihrer Aktivierung nach der Flutung?
- Wie wirken sich verschiedene Verfahren zur Emissionsminderung auf die methanogenen Populationen aus?
B. Material und Methoden

B.1. Boden

Der in allen Experimenten verwendete Reisfeldboden wurde im Februar 1997 aus einem noch nicht gefluteten Reisfeld des „Istituto Sperimentale per la Cerealicoltura“ bei Vercelli (Po-Ebene) entnommen. Die genauen Bodenparameter wurden bereits beschrieben (Chin und Conrad, 1995). Der Boden wurde luftgetrocknet, bei Raumtemperatur gelagert und vor Versuchsbeginn in einem Backenbrecher (Typ BB1, Retsch, Haan) zerkleinert und mit einer Siebmaschine (Dietz, Motoren GmbH & CO KG, Dettingen) auf eine Korngröße \( \leq 2 \text{ mm} \) gesiebt.

B.2. Chemikalien und Gase

Alle verwendeten Chemikalien wurden, sofern im Text nicht gesondert aufgeführt, im Reinheitsgrad „zur Analyse“ oder in vergleichbaren Reinheitsgraden von den folgenden Firmen bezogen: Fluka (Buchs, Schweiz), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) und Sigma-Aldrich (Steinheim). Enzyme und molekularbiologische Reagenzien wurden von den Firmen Amersham Pharmacia Biotech (Freiburg), Applied Biosystems (Weiterstadt), Promega (Mannheim), Roche Diagnostics (Mannheim) und Sigma-Aldrich (Steinheim) geliefert. Oligonukleotide wurden bei MWG-Biotech (Ebersberg) synthetisiert. Alle verwendeten Gase wurden von Messer-Griesheim (Darmstadt) bezogen.

B.3. Versuchsansätze

Reisfeldbodenaufschlämungen wurden in 12,5- und 60-ml Serumflaschen mit 3 g trockenem, gesiebtem Boden und 3 ml sterilem, anoxischem ddH\(_2\)O (bzw. 10 g Boden + 10 ml ddH\(_2\)O) angesetzt. In einigen Experimenten wurde der trockene Boden vorher mit 1,5% Ferrihydrit (Fe(OH)\(_3\), hergestellt nach Jäckel und Schnell (2000b)) oder 0,15% Gips (Ca\(_2\)SO\(_4\) x 2 H\(_2\)O) supplementiert. Die Aufschlämungen wurden mit Butyl-Stopfen verschlossen, 5 min mit N\(_2\) begast, und statisch bei 25°C inkubiert. Für jeden Zeitpunkt einer Zeitreihe wurden drei parallele Flaschen angesetzt und am Ende der Inkubationszeit vollständig analysiert.
B. Material und Methoden

B.4. Methanogene Referenzstämmme


B.5. Chemische und physikalische Analysen

B.5.1. Gaschromatographie (Methan, Kohlendioxid, Wasserstoff)


<table>
<thead>
<tr>
<th>Trägergas:</th>
<th>Wasserstoff 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Säule:</td>
<td>2 m Edelstahlsaule, Durchmesser 1/8 Zoll, Trägermaterial Poropak QS 50/100 mesh, Temperatur 40(^\circ)C</td>
</tr>
<tr>
<td>Methanisator:</td>
<td>Eigenbau, 20 cm Edelstahlsaule, Durchmesser 1/8 Zoll, NiCr-Ni-Katalysator (Chrompack, Middelburg, Niederlande), Temperatur 350(^\circ)C</td>
</tr>
<tr>
<td>Detektor:</td>
<td>Flammenionisationsdetektor, Brenngase H(_2) und FID-Gas, Quenchgas N(_2), Temperatur 110(^\circ)C</td>
</tr>
</tbody>
</table>
B. Material und Methoden

Niedrige H₂ Konzentrationen (<100 ppmv) wurden an einem Gaschromatographen mit Reduktivgasdetektor (RGD2, Trace Analytical, Stanford, Kalifornien) gemessen. Die Einpunkteichung wurde mit 50 ppmv H₂ in N₂ durchgeführt.

Trägergas: Synthetische Luft
Säule: 1,4 m Edelstahlsäule, Durchmesser 1/2 Zoll, Trägermaterial 5 Å Molekularsieb 80-100 mesh, Temperatur 60°C
Detektor: HgO-Detektor, Temperatur 280°C

H₂ Konzentrationen über 100 ppmv wurden an einem Gaschromatographen mit Wärmeleitfähigkeitsdetektor (WLD) gemessen. Die Eichung wurde mit 1000 ppmv H₂ in N₂ durchgeführt.

Trägergas: Stickstoff 4.5
Säule: 2 m Edelstahlsäule, Durchmesser 1/8 Zoll, Trägermaterial 5 Å Molekularsieb 80-100 mesh, Temperatur 80°C
Detektor: WLD, Temperatur 80°C

B.5.2. Ionenchromatographie (Sulfat, Nitrat, Nitrit)

Zur Messung von SO₄²⁻, NO₃⁻ und NO₂⁻ wurde ein Ionenchromatograph (Sykam, Gilching) eingesetzt (Bak et al., 1991). Die Anlage bestand aus einer Pumpe (S1000), einem temperierbaren Säulenofen (S4110) mit Anionentrennsäule (LCA09), einem Leitfähigkeitsdetektor (S3110) zur Bestimmung von Sulfat, einem Suppressorsystem (S2210, S6330) und einem nachgeschalteten UV/VIS-Detektor (UVIS 204, Linear, Reno, Nevada) zur Bestimmung von Nitrit und Nitrat. Pro Messung wurden je 50 µl Probenvolumen über einen automatischen Probenaufgeber Jasco 851-AS (Jasco, Japan) injiziert. Als Eichstandard dienten 0,1 und 1 mM Referenzlösungen mit Nitrat, Nitrit und Sulfat. Die Chromatogramme wurden mit dem Programm PEAK SIMPLE (SRI Instruments, Torrence, Kalifornien) integriert und ausgewertet.

Fließmittel: 3 mM Natriumhydrogencarbonat, 6 mM Natriumcarbonat, Fluss 2 ml min⁻¹
Säule: 6 cm Edelstahlsäule, Durchmesser 4,6 mm, Trägermaterial Styrol/Divinobenzol 10 µm Korngröße, Temperatur 65°C
Detektor: Leitfähigkeitsdetektor, UV-Detektor, Wellenlänge 218 nm
B. Material und Methoden

B.5.3. HPLC (organische Säuren)


| Fließmittel: | 1 mM H₂SO₄, Fluss 0,8 ml/min |
| Säule:      | 30 cm Edelstahlsäule, Durchmesser 7,8 mm, Trägermaterial ORH-801 Ion-exclusion form, H⁺ (Interaction, Vertrieb Schambeck), Temperatur 60°C |
| Detektor:   | RI-Detektor, Temperatur 40°C, UV-Detektor, Wellenlänge 205 nm |

B.5.4. Kolorimetrische Bestimmung von Eisen(II)

HCl extrahierbares Fe²⁺ in Reisfeldbodenaufschlämmungen wurde kolorimetrisch mit der Ferrozin-Methode bestimmt (Achtnich et al., 1995). 0,5 g Bodenaufschlämmung wurden in 4,5 ml 0,5 M HCl gegeben und 24 h bei 25°C extrahiert. 100 µl des Extraktes (oder einer 1/10 Verdünnung davon) wurden anschließend mit 1 ml Ferrozin-Reagenz (0,1% Ferrozin in 200 mM HEPES Puffer, pH 7) vermischt und 2 min im Dunkeln inkubiert. Nach kurzer Zentrifugation wurde die Extinktion des Überstands (1 ml) in einem Uvikon 930 Spektrophotometer (Kontron Instruments, Neufahrn) bei 562 nm gemessen. Als Eichstandard diente eine Verdünnungsreihe einer 2 mM Fe(NH₄)₂(SO₄)₂ x 6 H₂O in 0,5 M HCl.

B.6. Molekularbiologische Analysen

B.6.1. DNA-Extraktion

DNA aus Reisfeldbodenaufschlämmungen oder aus dem Zellmaterial methanogener Reinkulturen wurde über eine direkte Lyse-Technik extrahiert. Das Verfahren basiert auf...

Bis zu 600 µl Bodenaufschlämmung (oder Zellmaterial) wurden zusammen mit 0,7 g Zirconium-Kugeln (0,1 mm; Biospec Products Inc., Bartlesville, Oklahoma), 700 µl Na-Phosphat-Puffer (120 mM, pH 8) und 230 µl Natriumdodecylsulfat (SDS)-Lösung (10 % SDS, 0,5 M Na-Phosphat-Puffer pH 8, 0,1 M NaCl) in ein 2-ml Reaktionsgefäß mit Schraubdeckel überführt. Die Lyse der Zellen erfolgte in einer Zellmühle (FastPrep 120 Bead Beater, Qbiogene, Heidelberg) für 45 s bei maximaler Beschleunigung (6,5 m s⁻¹). Zur Abtrennung der Zirkonium-Kugeln und des lysierten Zellmaterials wurde 3 min bei 20.000 g und 4°C zentrifugiert, wonach 600 µl des DNA-haltigen Überstandes in ein steriles 2-ml Gefäß überführt wurden. Das Pellet wurde ein zweites Mal mit bis zu 600 µl Na-Phosphat-Puffer re-extrahiert (30 s, 6,5 m s⁻¹). Proteine, Zellbestandteile und andere Verunreinigungen wurden für 5 min auf Eis mit 0,4 vol 7,5 M Ammoniumacetat-Lösung aus dem Bodenrohextrakt gefällt. Bei starker Verunreinigung oder hohem Proteingehalt (Reinkulturen) wurde statt dessen eine sukzessive Extraktion mit je 500 µl Phenol, Phenol/Chloroform/ Isoamylalkohol (25:24:1) und Chloroform/Isoamylalkohol (24:1) durchgeführt. Zwischen den einzelnen Schritten wurde zur Phasentrennung jeweils 3 min zentrifugiert. Eine optimale Phasentrennung (Erhöhung der Ausbeute) wurden dabei bei den letzten beiden Extraktionen durch Einsatz von 2-ml Phase Lock Gel Heavy Gefäßen (Eppendorf, Hamburg) erreicht. Diese beinhalten eine Gel-artige Matrix, die sich durch die Zentrifugation über die organische Phase legt und so ein sauberes Abgießen der wässrigen Phase erlaubt. Die DNA im wässrigen Überstand wurde durch Zugabe von 0,7 vol. Isopropanol und Zentrifugation (1 h, 25°C) ausgefällt. Anschließend wurde das DNA-Pellet vorsichtig mit 250 µl 70% Ethanol bei 4°C gewaschen, erneut abzentrifugiert, und kurz (max. 5 min) unter Vakuum getrocknet. Die DNA wurde je nach Ausbeute in 50 - 100 µl EB-Puffer (10 mM Tris HCl, pH 8,5) aufgenommen und nach Elektrophorese in einem 1% Agarosegel durch Ethidiumbromid-Färbung visualisiert.

Entfernung von Huminsäuren aus DNA-Extrakten. Huminsäuren, die aufgrund ähnlicher chemischer Eigenschaften zusammen mit der DNA aus Böden extrahiert werden, wirken als Inhibitoren der PCR und müssen deshalb entfernt werden (Wilson, 1997). Eine geeignete Reinigungsmethode ist die durch Henckel et al. (2000) in unserem Labor etablierte Adsorption der Huminstoffe an Polyvinylpolypyrrolidon (PVPP) nach Holben et al. (1988). 7,5 g PVPP (Sigma) wurden in 100 ml 3 M HCl über Nacht durch Rühren...
suspendiert. Danach wurde das PVPP für 5 min sedimentiert, der Überstand dekantiert, und durch 10 mM TE-Puffer pH 8,0 ersetzt. Dieser Vorgang wurde mindestens fünfmal wiederholt, um das PVPP auf pH 8,0 zu äquilibrieren. Der pH-Wert der Suspension wurde vor jeder Verwendung im dekantierten Überstand kontrolliert. Zur DNA-Reinigung wurden 0,5 ml PVPP-Suspension in Mikro Bio-Spin Zentrifugen-Kartuschen (Bio-Rad, München) gefüllt, 1 min bei 380 g zentrifugiert, und zweimal mit 100 µl EB-Puffer äquilibriert (1 min, 380 g). Das DNA-Extrakt wurde vorsichtig auf das gepackte Säulenbett aufgetragen und 1 min bei 380 g zentrifugiert. Das klare Eluat wurde zur weiteren Verwendung aufgefangen, die Huminsäuren blieben sichtbar als braune Bande auf der PVPP-Matrix adsorbiert.

B.6.2. RNA-Extraktion

Gesamt-RNA aus Bodenproben und Reinkulturen wurde mittels eines leicht modifizierten Protokolls nach Lüdemann et al. (2000) extrahiert. Bis zu 600 µl Bodenaufschlämmung (oder Zellmaterial) wurden zusammen mit 700 µl vorgekühltem TPM-Puffer (50 mM Tris-Cl [pH 7,5], 1,7% Polyvinylpyrrolidon, 10 mM MgCl₂ (Felske et al., 1996)) in 2-ml FastRNA Extraktionsgefäße mit Lyse-Matrix B (Qbiogene, Heidelberg) gegeben. Die Lyse wurde in einer Zellmühle (FastPrep 120 Bead Beater, Qbiogene) für 40 s bei maximaler Beschleunigung (6,5 m s⁻¹) durchgeführt. Das Lysat wurde 4 min bei 20000 g und 4°C zentrifugiert, wonach 600 µl des Überstandes in ein steriles 2-ml Gefäß überführt wurden. Das Pellet wurde mit 700 µl eines phenohlaltigen Lyse Puffers (50 mM Tris-Cl [pH 7,5], 10 mM EDTA, 1% SDS, 6% H₂O-gesättigtes Phenol) ein zweites Mal extrahiert (30 s, 6,5 m s⁻¹) und zentrifugiert, erneut wurden 600 µl Überstand abgenommen. Der vereinigte Überstand wurde sukzessive mit je 500 µl Phenol, Phenol/Chloroform/Isoamylalkohol (25:24:1) und Chloroform/Isoamylalkohol (24:1) extrahiert. Zwischen den einzelnen Schritten wurde zur Phasentrennung jeweils 3 min zentrifugiert. Die beiden letzten Extraktionen wurden in 2-ml Phase Lock Gel Heavy Gefäßen (Eppendorf, Hamburg) durchgeführt. Nach dem letzten Reinigungsschritt wurde die RNA im wässrigen Überstand durch Zugabe von 3 vol. Ethanol und 0,1 vol 3 M Na-Acetat ausgefällt (-80°C, über Nacht). Das RNA wurde abzentrifugiert (1 h, 20.000 g, 4°C), mit 250 µl 70% Ethanol bei 4°C gewaschen, erneut abzentrifugiert, kurz (max. 5 min) unter Vakuum getrocknet und 50 µl EB-Puffer resuspendiert. Die Qualität der extrahierten rRNA wurde durch Standard-Gelelektrophorese mit *E. coli* 16S und 23S
rRNA (Roche) als Referenz überprüft. Alle RNA-Lösungen wurden bei –80°C aufbewahrt.

**RNA-Reinigung.** Auch aus RNA-Extrakten mussten co-extrahierte Huminsäuren entfernt werden; hier wurde eine Gelfiltration über Sephadex G-75 eingesetzt (Moran et al., 1993). Eine PVPP-Reinigung wurde nicht durchgeführt, da diese in Bezug auf die quantitative rRNA-Hybridisierung mit einem zu großen Ausbeuteverlust verbunden ist. 1 ml in DEPC-H₂O vorgequollenes Sephadex G75 (Fluka) wurde 1 min bei 2000 g in Mikro Bio-Spin Zentrifugen-Kartuschen (Bio-Rad) gepackt und 2 x mit je 100 µl EB-Puffer gespült (1 min, 2000 g). Das RNA-Extrakt über die Säule filtriert (2 min, 2000 g) und zur weiteren Verwendung aufgefangen.

**DNAse Verdau.** Co-extrahierte DNA wurde bei Bedarf durch Behandlung mit RNase-freier DNAse I (Roche) aus den RNA-Extrakten entfernt. Dieser Schritt war besonders vor der RT-PCR, aber auch vor RiboGreen-Messungen notwendig (s.u.). 5 µl RNA-Extrakt, 5 µl 10x DNAse-Puffer (200 mM Tris-HCl pH 7,5, 100 mM MgCl₂, 20 mM CaCl₂) und 10 U DNAse I wurden mit DEPC-H₂O auf ein Volumen von 50 µl aufgefüllt und 1 h bei 37°C inkubiert. Danach wurde die DNAse durch Erhitzen (10 min, 80°C) inaktiviert und der vollständige Verdau der DNA gegebenenfalls durch PCR überprüft (s.u.).

**Vorsichtsmaßnahmen im Umgang mit RNA.** Bei der Extraktion und Arbeit mit RNA ist ständig die Gefahr der Kontamination durch RNA-degradierende Enzyme (RNAsen) gegeben. Um eine Kontamination zu vermeiden, wurden alle verwendeten Glasgeräte 6 h bei 180°C sterilisiert. Wasser zur Herstellung von Puffern wurde zur Inaktivierung von RNAsen mit 0,1% DEPC (vol/vol) vorbehandelt (Sambrook et al., 1989).

**B.6.3. Quantifizierung von Nukleinsäuren**

Die Konzentration von DNA- und RNA-Lösungen wurde durch photometrische Messung der Absorption bei 260 nm ($A_{260nm}$) einer 5 + 65 (vol+vol) Verdünnung in Wasser bestimmt (BioPhotometer, Eppendorf). Hierbei wurde die Konzentration doppelsträngiger DNA nach $C \ [\text{ng} \, \mu\text{l}^{-1}] = 50 \times$ Verdünnungsfaktor $\times A_{260nm}$ und einzelsträngiger RNA nach $C \ [\text{ng} \, \mu\text{l}^{-1}] = 40 \times$ Verdünnungsfaktor $\times A_{260nm}$ berechnet (Sambrook et al., 1989).
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**RiboGreen-Messung von RNA.** Analog zur genauen Messung von dsDNA mit PicoGreen, wurden RNA-Lösungen bei Bedarf mit dem RiboGreen RNA Quantifizierungs-Kit bestimmt (Molecular Probes). Als Eichstandard diente *E. coli* rRNA in Mengen von 1 – 50 ng [100 µl]⁻¹. Bei der RNA-Messung war jedoch zu beachten, dass RiboGreen auch an DNA bindet, deshalb musste eventuell coextrahierte DNA zuvor durch DNase Verdau entfernt werden (s.o.).

**B.6.4. PCR-Amplifikation**

**PCR-Amplifikation archaeeller 16S rDNA.** Verwendete Primerpaare für die Amplifikation von Matrizen-DNA aus Umweltproben und Reinkulturen sind in Tab. 1 aufgeführt. Alle PCR-Ansätze wurden in einem Gesamtvolumen von 50 µl durchgeführt. Als Negativkontrolle wurde ein Reaktionsansatz mit Wasser anstelle von Matrizen-DNA mitgeführt. Das Reaktionsgemisch enthielt 1 x PCR-Puffer II (Applied Biosystems), 1,5 mM MgCl₂, 50 µM jedes Desoxynukleotidtriphosphats (Amersham), 0,5 µM beider Primer, 1 U AmpliTaq DNA Polymerase (Applied Biosystems), und 1 µl Matrize-DNA. Die Reaktionen wurden mit 25 – 30 Amplifikationszyklen (3 min 94°C, 25 – 30 x [30 s 94°C, 45 s 52°C, 90 s 72°C], 5 min 72°C) in einem GeneAmp 9700 Thermocycler (Applied Biosystems) durchgeführt. Die Amplifikate wurden durch Gelelektrophorese überprüft und bei erfolgreicher Reaktion mit dem MinElute PCR Purification Kit (Qiagen, Hilden) nach Anweisungen des Herstellers aufgereinigt, um nicht inkorporierte Nukleotide und Primer zu entfernen.
### B. Material und Methoden

<p>| Tab. 1. Verwendete PCR-Primer für unterschiedliche phylogenetische und funktionelle Amplifikationsmatrizen der <em>Archaea</em>. |</p>
<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENZ (5' – 3')</th>
<th>ZIELSEQUENZ</th>
<th>REFERENZ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Archaea 16S rDNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ar21f</td>
<td>TTC CGG TTG ATC CYG CCG GA</td>
<td>Archaea 16S rDNA</td>
<td>(DeLong, 1992)</td>
</tr>
<tr>
<td>Ar1383r</td>
<td>CGG TGT GTG CAA GGA GCA</td>
<td>Archaea 16S rDNA</td>
<td>(Weber et al., 2001)</td>
</tr>
<tr>
<td>Ar109f</td>
<td>ACK GCT CAG TAA CAC GT</td>
<td>Archaea 16S rDNA</td>
<td>(Grosskopf et al., 1998a)</td>
</tr>
<tr>
<td>Ar912r *</td>
<td>CTC CCC CGC CAA TTC CTT TA</td>
<td>Archaea 16S rDNA</td>
<td>(Lueders und Friedrich, 2000)</td>
</tr>
<tr>
<td>Ar109f</td>
<td>s.o.</td>
<td>Archaea 16S rDNA</td>
<td></td>
</tr>
<tr>
<td>Ar915r *</td>
<td>GTG CTC CCC CGC CAA TTC CT</td>
<td>Archaea 16S rDNA</td>
<td>(Stahl und Amann, 1991)</td>
</tr>
</tbody>
</table>

| **Archaea 16S rRNA** | | | |
| Ar109f | s.o. | Archaea 16S rRNA | |
| Ar912rt * | GTG CTC CCC CGC CAA TTC CTT TA | Archaea 16S rRNA | Diese Arbeit |

**Methyl-Coenzym-M-Reduktase**

| MCRf * | TAY GAY CAR ATH TGG YT | mcrA | (Springer et al., 1995) |
| MCRr | ACR TTC ATN GCR TAR TT | (–500 bp) | |
| ME1 | GCM ATG CAR ATH GGW ATG TC | mcrA | (Hales et al., 1996) |
| ME2 | TCA TKG CRT AGT TDG GRT AGT | (–760 bp) | |

* Gekennzeichnete Primer wurden fluoreszenzmarkiert für die T-RFLP-Analyse eingesetzt.

**RT-PCR-Amplifikation archaeeller 16S rRNA.** Reverse Transkription der rRNA und Amplifikation der cDNA wurden mit dem Access One-Tube RT-PCR System (Promega) in einem Gesamtvolumen von 50 µl durchgeführt. Als Negativkontrolle wurde sowohl ein Ansatz mit Wasser anstelle von Matrizen-DNA als auch ein Ansatz mit rRNA aber ohne Reverse Transkriptase mitgeführt ( Kontrolle des DNAse-Verdaus). Das Reaktionsgemisch enthielt 1 x AMV/Tfl Reaktions-Puffer, 1 mM MgSO₄, 200 µM jedes Desoxynukleotidtriphosphats, 0,5 µM beider Primer, 5 U AMV Reverse Transkriptase, 5 U Tfl DNA Polymerase, und 1 µl DNA-freie RNA-Matrice. Die reverse Transkription wurde 45 min bei 48°C durchgeführt und ging direkt in die Amplifikation über (2 min 94°C, 25 – 27 x [30 s 94°C, 45 s 52°C, 90 s 68°C], 5 min 72°C).

**PCR-Amplifikation von mcrA-Genen.** Die Amplifikation partieller mcrA-Sequenzen aus Umwelt- und Reinkultur-DNA wurde ebenfalls in 50 µl-Ansätzen durchgeführt. Das Reaktionsgemisch enthielt 1 x MasterAmp PCR PreMix B (Epicentre Technologies, Madison, WI), 0,5 µM beider Primer, 1,25 U AmpliTaq DNA Polymerase
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(Applied Biosystems), und 1 µl DNA-Matrize. Die Reaktionen wurden mit 30 – 35 Amplifikationszyklen (3 min 94°C, 30 – 35 x [30 s 94°C, 45 s 50°C, 90 s 72°C], 5 min 72°C) in einem Mastercycler Gradient Thermocycler (Eppendorf) durchgeführt.

B.6.5. T-RFLP-Analyse

Archaea 16S T-RFLP. Die T-RFLP-Analyse archaellarer 16S rDNA Amplifikate wurde mit FAM (6-Carboxyfluorescein) markierten PCR-Produkten durchgeführt, die mit einem 5’-FAM-markierten revers-Primer generiert wurden (Tab. 1). Die gereinigten Amplifikate wurden anschließend mit dem Restriktionsenzym TaqI (Schnittstelle T’CGA) verdaut. Ein Restriktionsverdau enthielt in einem Gesamtvolumen von 10 µl ≈100 ng fluoreszenzmarkierte Amplifikate, 3 U TaqI (Promega), 1x 4-CORE Puffer E (Promega) und 1 µg BSA, und wurde 2 h bei 65°C inkubiert. 1,25 µl des Verdaus wurden dann mit 0,95 µl Formamid Loading-Dye (Amersham) und 0,3 µl des internen GeneScan-1000 (ROX) Längenstandards (Applied Biosystems) vermischt, 3 min bei 95°C denaturiert, und sofort auf Eis gekühlt. Die T-RFLP-Analyse wurde auf einem ABI Prism 377 DNA Sequenzierer (Applied Biosystems) im GeneScan Modus durchgeführt. 1,9 µl des Fragment-Gemisches wurden auf ein 36 cm 5% Polyacrylamid-Gel mit 6 M Harnstoff in 1x TBE-Puffer (89 mM Tris-Borat, 2 mM EDTA) geladen und 14 h elektrophoretisch getrennt (2500 V, 40 mA, 30 W). Die Elektropherogramme wurden mit Hilfe der GENESCAN Analyse Software 3.1 (Applied Biosystems) ausgewertet. Über den internen Größenstandard wurde die Länge der T-RFs gemessen. Gleichzeitig wurde die Intensität (Höhe und Fläche) der einzelnen Fluoreszenzsignale bestimmt. Hintergrundssignale (Peaks <100 RFU) wurden aus der Analyse ausgeschlossen. Die relative Abundanz eines T-RFs wurde als sein Anteil [in %] an der Summe der Fluoreszenzsignale des gesamten Elektropherograms bestimmt.

mcrA T-RFLP. Die T-RFLP-Analyse der mcrA Amplifikate wurde nach dem gleichen Prinzip durchgeführt, allerdings war der vorwärts-Primer 5’-FAM markiert (Tab. 1). Verdaut wurde mit dem Restriktionsenzym Sau96 I (Promega, Schnittstelle G’GNCC) in 1x 4-CORE Puffer C (Promega) 2 h bei 37°C. Als Längenstandard diente der GeneScan-500 (ROX) Standard (Applied Biosystems).
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B.6.6. Erstellung von Klonbibliotheken

Aus Umwelt-DNA amplifizierte PCR-Produkte wurden über Klonierung vereinzelt, um ihre Sequenzierung und phylogenetische Einordnung zu ermöglichen. Die gereinigten Amplifikate wurden mit Hilfe des pGEM-T Vector Systems II (Promega) nach den Angaben des Herstellers in *E. coli* JM109 hochkompetenten Zellen (Promega) kloniert. Transformierte Zellen wurden auf LB-Ampicillin-Nährböden angezogen und mittels „Blau-Weiβ-Kontrolle“ selektioniert. Die DNA aus transformierten Zellen wurde durch Aufkochen (10 min, 100°C) gewonnen, worauf die klonierten DNA-Inserts über PCR mit Vektor-gerichteten Primern (Tab. 2) auf ihre korrekte Länge überprüft wurden. Die Vektor-gerichtete PCR enthieiti in 50 µl Gesamtvolumen 1 x PCR-Puffer II (Applied Biosystems), 1,5 mM MgCl₂, 50 µM jedes Desoxynukleotidtriphosphats (Amersham), 0,5 µM beider Primer, 1 U AmpliTaq DNA Polymerase (Applied Biosystems), und 1 µl DNA-Matrice. Die Reaktionen wurden mit 25 Zyklen (3 min 94°C, 25 x [30 s 94°C, 30 s 55°C, 60 s 72°C], 5 min 72°C) in einem GeneAmp 9700 Thermocycler (Applied Biosystems) durchgeführt und durch Gelelektrophorese evaluiert.

Tab. 2. PCR-Primer für die Amplifikation klonierter Inserts aus dem p-GEM Vektor.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENZ (5' – 3')</th>
<th>ZIELSEQUENZ</th>
<th>REFERENZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13f(-40)</td>
<td>TTT TTC CCA GTC ACG AC</td>
<td>p-GEM Vektor</td>
<td>Promega</td>
</tr>
<tr>
<td>M13r(-48)</td>
<td>AGC GGA TAA CAA TTT CAC ACA GGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.6.7. Sequenzanalyse


Die Sequenzreaktion wurde mit dem ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit mit AmpliTaq Polymerase FS (Applied Biosystems)
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durchgeführt. Das Reaktionsgemisch enthielt in einem Volumen von 20 µl ca. 75 ng Matrizen Amplikon, 5 pmol eines geeigneten Sequenzierprimers (Tab. 3) und 4 µl BigDye Kit. Die Reaktionen wurden mit 25 Zyklen (10 s 96°C, 5 s 50°C, 4 min 60°C) in einem GeneAmp 9600 Thermocycler (Applied Biosystems) durchgeführt. Anschließend wurde das Reaktionsgemisch über Microspin G-50 Gelfiltrations-Säulen (Pharmacia, Upsala, Schweden) gereinigt, unter Vakuum getrocknet, in 1,8 µl Formamid Loading-Dye (Amersham) re-eluiert, 3 min bei 90°C denaturiert, und sofort auf Eis gekühlt. Die Sequenzanalyse wurde auf einem ABI Prism 377 DNA Sequenzierer (Applied Biosystems) durchgeführt. 1,5 µl des DNA-Gemisches wurden auf ein 48 cm 4.25% PAGE-Plus Gel (nach Angaben des Herstellers) geladen und 18 h elektrophoretisch separiert (3000 V, 60 mA, 40 W).

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENZ (5’ – 3’)</th>
<th>ZIELSEQUENZ</th>
<th>REFERENZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13uni</td>
<td>CGA CGT TGT AAA ACG ACG GCC AGT</td>
<td>p-GEM Vektor</td>
<td>Promega</td>
</tr>
<tr>
<td>M13rev</td>
<td>CAG GAA ACA GCT ATG AC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.6.8. Quantitative rRNA-Hybridisierung mit Oligonukleotidsonden

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Denhardts-Lösung (Sambrook et al., 1989), 1% Blocking Reagenz (Roche), 0,1% N-Lauroylsarcosin, 0,1% SDS) prähybridisiert und über Nacht in 3 ml Hybridisierungspuffer mit 50 pmol DIG-markierter Sonde hybridisiert. In je 50 ml Waschpuffer (1x SSC, 1% SDS, pH 7) wurde 2 x je 30 min bei 40°C unspezifisch und 1 x 30 min bei der sondenspezifischen stringenten Waschtemperatur (T\textsubscript{d}) gewaschen. Alle verwendeten Oligonukleotidsonden wurden mit einer 5'-DIG-Markierung bei MWG (Ebersberg) bezogen und sind in Tab. 4 zusammen mit dem jeweiligen Reinkultur-Standard und den T\textsubscript{d}-Werten dargestellt.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
SONDE\textsuperscript{a} & SEQUENZ (5' – 3') & ZIEL-rRNA RNA-STANDARD & T\textsubscript{d} \\
\hline
S-*-Univ-1392-a-A-15 & ACG GGC GGT GTG TRC & Alle Mikroorganismen \textit{E. coli} & 40°C \\
\hline
S-D-Arch-0915-a-A-20 & GTG CTC CCC CGC CAA TTC CT & Alle \textit{Archaea} \textit{M. barkeri} & 56°C \\
\hline
S-F-Mbac-0310-a-A-22 & CTT GTC TCA GGT TCC ATC TCC G & \textit{Methanobacteriaceae} \textit{M. bryantii} & 57°C \\
\hline
S-F-Msae-0825-a-A-23 & TCG CAC CGT GGC CAC CTA GC & \textit{Methanosacetaceae} \textit{M. concilii} & 59°C \\
\hline
S-G-Msar-0821-a-A-24 & CGC CAT GCC TGA CAC CTA GGC AGC & \textit{Methanosarcina} spp. \textit{M. barkeri} & 60°C \\
\hline
S-*-RCI-0645-a-A-23 & CCT CTC CCA GTC CCA AGC AAT GT & \textit{Rice Cluster I} \textit{In vitro} Transkript Klon AS04-16 & 58°C \\
\hline
\end{tabular}
\caption{Verwendete Oligonukleotidsonden für die quantitative rRNA-Hybridisierung, deren Ziel- und Referenzorganismen sowie stringenten Waschtemperaturen T\textsubscript{d}.}
\end{table}

\textsuperscript{a} Nomenklatur der Sonden nach Alm et al. (1996), \textit{E. coli} Nummerierung nach Brosius et al. (1978)

Die auf der Membran verbliebenen DIG-Sonden wurden über an Alkalische Phosphatase gekoppelte Anti-DIG Antikörper (Roche) nach Angaben des Herstellers nachgewiesen. Die Blots wurden mit dem ECF Fluoreszenz-Substrat (Amersham) auf einem Storm 860 Gel and Blot Imaging System (Molecular Dynamics, Sunnyvale, Kalifornien) visualisiert (Stubner und Meuser, 2000) und mit der IMAGEQUANT 5.0 Software (Molecular Dynamics) quantitativ ausgewertet.

\textit{In vitro} Transkription von \textit{Rice cluster I} rRNA. Um die auch die rRNA der noch nicht-kultivierten Methanogenen des RC-I in Umweltproben quantifizieren zu können, wurde eine für diese Linie spezifische Sonde entworfen (Tab. 4, siehe auch Abschnitt...
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Die stringente Waschtemperatur der neuen Sonde wurde über eine modifizierte Elutions-Methode (Zheng et al., 1996) bestimmt. Ca. 100 ng der RC-I RNA wurden pro Slot geblottet, hybridisiert (s.o.), und mit zunehmender Temperatur gewaschen. Die Menge der auf der Membran verbleibenden Sonde bei jeder Waschtemperatur wurde visualisiert und die Temperatur, bei der 50% der Sonde eluiert waren, als T_d bestimmt.

B.6.9. Real-Time PCR


Die Messungen wurden auf einem iCycler iQ Real-Time Detection System (Bio-Rad, München) über den Nachweis der SybrGreen-Fluoreszenz der gebildeten Amplifikate durchgeführt. Die Reaktionen wurden in MicroAmp 8-Tube-Streifen angesetzt und mit optischen MicroAmp 8-Cap-Streifen verschlossen (Applied Biosystems). Jede 50 µl-Reaktion enthielt 1x SybrGreen PCR Master Mix (Applied Biosystems), je 0,3 µM der Primer Ar109f und Ar915r, sowie 2 µl der Matrizen-DNA. Das Temperaturprofil der
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Die Eichung der Real-Time PCR-Messungen erfolgte über eine 10-fache Verdünnungsreihe der DNA von *M. jannaschii*. Das Genom dieses Organismus (1664970 bp, (Bult et al., 1996)) enthält zwei *rrn*-Operons. Bei bekannter DNA-Menge [ng µl⁻¹] ergibt sich daher ein Verhältnis von 1,196 x 10⁶ 16S rRNA-Genkopien ng⁻¹ DNA. Eichgeraden wurden mindestens über Doppelbestimmung erstellt. Unter Annahme einer konstanten Amplifikationseffizienz (\(\varepsilon_c\)) wurde diese aus der Steigung s der Eichgeraden wie folgt berechnet: \(\varepsilon_c = 10^{-\frac{1}{s}} - 1\) (Becker et al., 2000).

B.7. Bioinformatische Analysen

B.7.1. Sequenzverarbeitung

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(Benson et al., 2000). Nah verwandte Sequenzen wurden heruntergeladen und in die eigene ARB-Datenbank importiert.

B.7.2. Phylogenetische Analyse von 16S rRNA-Genen


Zunächst wurde mit Hilfe einer möglichst großen Anzahl möglichst vollständiger (>1400 bp) Referenzsequenzen ein Basenfrequenz-Filter für die jeweils bearbeitete phylogenetische Gruppierung (z.B. Archaea) erstellt. Ein solcher Filter ermöglicht es, in nachfolgende Berechnungen nur diejenigen Nukleotidpositionen einzubeziehen, die bei mindestens einem definierten Prozentsatz (z.B. 50%) der betrachteten Organismen konserviert ist. Somit werden hochvariable Regionen der 16S rRNA, deren phylogenetischer Informationsgehalt aufgrund hoher Mutationsraten fraglich ist, aus den Berechnungen ausgeschlossen.

Dann wurde ebenfalls mit einer geeigneten Auswahl möglichst vollständiger Referenzsequenzen ein präziser phylogenetischer „Basisbaum“ rekonstruiert. Diese Berechnung wurde mit verschiedenen Algorithmen durchgeführt, um die Topologie der jeweiligen Bäume miteinander zu vergleichen. Im Idealfall sollte sich ein übereinstimmendes Bild der wahren phylogenetischen Beziehung ergeben (Ludwig et al., 1998). Drei prinzipiell unterschiedliche Berechnungs-Methoden sollen an dieser Stelle kurz dargestellt werden:

- **Distanz-Matrix Verfahren.** Sequenzdifferenzen werden aus dem Alignment paarweise in eine Distanz-Matrix umgerechnet, die dann nach verschiedenen Evolutionsmodellen (Jukes und Cantor, 1969; Kimura, 1983) in phylogenetische
Distanzen umgerechnet wird. Die Distanzwerte werden dann z.B. mit der Neighbor-Joining Methode (Saitou und Nei, 1987) in Dendrogrammen dargestellt.

- **Maximum Parsimony.** Im Gegensatz zu Distanz-Verfahren werden bei dieser Methode die eigentlichen Sequenzdaten zur Berechnung der Bäume verwendet. Es wird von allen möglichen Baumtopologien diejenige mit der geringsten Gesamtdistanz gesucht (Fitch, 1971), d.h. es wird versucht, die beobachteten Sequenzunterschiede mit einem Minimum an Mutationen zu erklären („sparsamster Baum“).

- **Maximum Likelihood.** Auf der Basis verschiedener evolutionärer Modelle wird von allen möglichen Bäumen der wahrscheinlichstes rekonstruiert (Felsenstein, 1981). Diese Methode gilt derzeit als die exakteste Methode zur Rekonstruktion phylogenetischer Beziehungen und verwendet ein Maximum an Informationsgehalt der Originalsequenzen. Allerdings sind Maximum Likelihood-Algorithmen sehr rechenintensiv, wodurch die Anzahl der zu verrechnenden Sequenzen eingeschränkt wird (Ludwig et al., 1998).

In den so rekonstruierten „Basisbaum“ wurden nun kürzere Sequenzen (z.B. von Umwelt-Klonen) nachträglich eingefügt. Dies ist in ARB mit einem Maximum-Parsimony-Tool möglich, ohne die Gesamt-Topologie des Baumes zu verändern.

**B.7.3. Phylogenetische Analyse von mcrA-Genen**

B.7.4. Entwicklung von Oligonukleotidsonden

Oligonukleotidsonden wurden mit Hilfe des PROBE-DESIGN Programms (ARB Software-Paket) entwickelt. Dieses Programm ermöglicht die Suche nach konservierten Gensequenzen, die eine definierte phylogenetische Gruppe von anderen Organismen unterscheidet.

B.7.5. Statistische Auswertung von T-RFLP-Daten


Um die in großen T-RFLP-Datensätzen enthaltene Information reduziert und komprimiert darzustellen, wurde Faktorenanalyse (principal component analysis, PCA) eingesetzt. Die Faktorenanalyse zur Auswertung umfangreicher T-RFLP- (Clement et al., 1998; Dollhopf et al., 2001), DGGE- (McCaig et al., 2001), oder RFLP-Datensätze (Baleiras Couto et al., 1995; Poly et al., 2001) wird zunehmend in der molekularen mikrobiellen Ökologie eingesetzt, um die aus komplexen Mustern ableitbaren Zusammenhänge vereinfacht darzustellen. Dabei wird die Beziehungen aller Variablen untereinander simultan dargestellt. Wechselseitig korrelierte Variablen werden durch hypothetische Faktoren ersetzt, die einen gegebenen prozentualen Anteil der Gesamt-Varianz erklären. In dieser Arbeit wurde zum Vergleich der T-RFLP-Profile verschiedener Messreihen (Boden, Zeitpunkt, Nukleinsäure) eine Kovarianz-Matrix über paarweise Deletion und Varimax Faktor-Rotation extrahiert. Die Datenreduktion ergab eine zweifaktorielle Anordnung der Varianz der T-RFLP-Profile, die in zweidimensionaler Ordination dargestellt wurde.
C. Ergebnisse

C.1. Nachweis des methanogenen Phänotyps einer neuen Entwicklungslinie der Archaea über die molekulare Analyse von Methyl-Coenzym-M-Reduktase-Genen (mcrA) im Reisfeldboden und in Anreicherungskulturen

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Molecular analyses of methyl-coenzyme M reductase \( \alpha \)-subunit (mcrA) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage

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Summary
The diversity of methanogen-specific methyl-coenzyme M reductase \( \alpha \)-subunit (mcrA/mrtA) genes in Italian rice field soil was analysed using a combination of molecular techniques and enrichment cultures. From 75 mcrA/mrtA clones retrieved from rice field soil, 52 were related to members of the Methanosarcinaceae, Methanosaetaceae and Methanobacteriaceae. However, 19 and four clones formed two novel clusters of deeply branching mcrA sequences, respectively, which could not be affiliated to known methanogens. A new methanogen-specific fingerprinting assay based on terminal restriction fragment length polymorphism (T-RFLP) analysis of fluorescently labelled polymerase chain reaction (PCR) products allowed us to distinguish all environmental mcrA/mrtA sequences via group-specific Sau96I restriction sites. Even genes for the isoenzyme methyl-coenzyme M reductase two (mrtA) of Methanobacteriaceae present in rice field soil were represented by a unique 470 bp terminal restriction fragment (T-RF). Both cloning and T-RFLP analysis indicated a significant representation of novel environmental mcrA sequences in rice field soil (238 bp T-RF). To identify these mcrA sequences, methanogenic enrichment cultures with rice field soil as inoculum were established with \( \mathrm{H}_2/\mathrm{CO}_2 \) as substrates at a temperature of 50°C, and these were monitored using molecular tools. In subsequent transfers of these enrichment cultures, cloning and T-RFLP analysis detected predominantly SSU rRNA genes of rice cluster I (RC-I), an uncultivated euryarchaeotal lineage discovered previously in anoxic rice field soil. In parallel, both mcrA cloning and T-RFLP analyses of the enrichment culture identified the more frequent cluster of novel environmental mcrA sequences as belonging to members of RC-I. Thus, we could demonstrate the genotype and phenotype of RC-I Archaea by the presence of a catabolic gene in a methanogenic enrichment culture before the isolation of pure cultures.

Introduction
Methanogens are of fundamental importance in anaerobic habitats, as they contribute to the degradation of organic matter by reducing a variety of carbon compounds to methane. They inhabit typical anaerobic environments, such as wetlands, sediments, geothermal springs and the digestive tracts of animals (Garcia et al., 2000). Methane released from these habitats into the atmosphere significantly affects global warming (Lelieveld et al., 1998), which underscores the importance of understanding methanogenic community structure and function. But most polymerase chain reaction (PCR)-based culture-independent investigations of methanogenic populations have been restrained to SSU rRNA genes as genetic markers, thus representing the total archaeal community rather than methanogens in particular. Thus, a recently developed SSU rDNA-based terminal restriction fragment length polymorphism (T-RFLP) assay allowed for the rapid fingerprinting of archaeal communities but did not fully resolve methanogenic and non-methanogenic lineages (Chin et al., 1999; Lueders and Friedrich, 2000).

Well-defined guilds of microorganisms, such as the methanogenic Archaea, may be studied more specifically using a characteristic ‘functional’ marker gene. A characteristic of methanogens is their unique ability to form methane, their limited substrate range and specialized biochemistry. A specific marker for methanogens is the methyl coenzyme-M reductase (MCR), the key enzyme of methanogenesis (Ermier et al., 1997). Unlike other enzymes in methanogenic metabolism (Ghiosserova et al., 1998), MCR appears to be unique to methanogens (Thauer, 1998). MCR catalyses the reduction of
methyl-coenzyme M, which leads to the release of methane (Ellermann et al., 1988). Although the operon encoding MCR-I (mcrBCDGA) is present in all methanogens (Reeve et al., 1997), members of the Methanobacteriales and Methanococcales additionally contain an isoenzyme, the methyl coenzyme-M reductase two (MCR-II, mrrBDGA (Lehmacher and Klenk, 1994; Pihl et al., 1994) or mrrBGA (Butt et al., 1996)). The presence of an additional MCR operon in other methanogens, however, cannot presently be excluded. The mcrA and mrrA genes are highly conserved (Lehmacher and Klenk, 1994; Springer et al., 1995; Nölling et al., 1996), which renders them suitable for use in molecular ecology studies, but the phylogeny of several important taxa (i.e., the Methanosetaeaceae and Methanospirillaceae; taxonomy according to Boone et al., 1993) has not been studied so far.

Using degenerate primers has facilitated the detection of a wide range of mcrA genes in environmental samples. Environmental, PCR-amplified mcrA sequences have been retrieved from a variety of different habitats, such as termite guts (Ohkuma et al., 1995), peat bogs (Hales et al., 1996; Lloyd et al., 1998; Nercessian et al., 1999) and marine sediments (Bidle et al., 1999), specifically to detect methanogens and study their diversity. However, degenerate primer pairs for the amplification of mcrA genes may have limited diversity coverage (Edwards et al., 1998) and therefore fail to detect all methanogens present in a given habitat.

Flooded Italian rice fields are typical methane-producing wetlands and contain a high diversity of methanogens (Grosskopf et al., 1998a; Chin et al., 1999; Lueders and Friedrich, 2000). Besides SSU rDNA sequences related to those of cultivated methanogens within the orders Methanobacteriales, Methanomicrobiales and Methanosarcinales, several novel euryarchaeotal lineages of unknown physiology were detected using molecular ecology tools (Grosskopf et al., 1998a). Two of these novel clusters, rice clusters I and II (RC-I, -II), group within the phylogenetic radiation of the Methanosarcinaceae and Methanomicrobiales (Grosskopf et al., 1998a), and the detection of RC-I in a microcosm experiment culture inoculated with samples from rice roots (Lehnmann-Richter et al., 1999) suggested that RC-I might have a methanogenic phenotype.

In this study, we retrieved mcrA and mrrA sequences from rice field soil to analyse the diversity of the methanogenic community. By a combination of molecular and enrichment culture techniques, we were able to show that a clade of deeply branching novel environmental mcrA sequences represents Archaea of RC-I, which have a methanogenic phenotype. In parallel, a rapid mcrA- and mrrA-targeted T-RFLP fingerprinting method for methanogenic communities was developed.

Results

Diversity of mcrA genes in rice field soil

Methanogen-specific mcrA fragments were amplified with MCR (Springer et al., 1995) and ME (Hales et al., 1996) primer pairs. Both degenerate primer sets allow the amplification of an overlapping region of ~503 bp (corresponding to Methanothermobacter marburgensis nucleotide positions 945–1448; Bokranz et al., 1998) and ~760 bp (M. marburgensis nucleotide positions 684–1444) of the mcrA gene, respectively, which is equivalent to ~160 inferred amino acids available for phylogenetic comparison. We amplified mcrA fragments from community DNA extracted from methanogenic rice field soil and constructed two clone libraries: RS-MCR (rice field soil mcrA amplicons) and RS-ME (rice field soil ME amplicons). Fifty clones from each library were randomly selected and checked for the expected insert size. Although only two clones of the RS-MCR library displayed an incorrect insert size, 19 RS-ME clones were significantly too small and were probably primer–dimer artifacts. Both strands of the remaining 48 RS-MCR and 31 RS-ME clones were sequenced, and deduced amino acid sequences were fitted into an alignment with mcrA gene products (McrA) from reference strains.

Phylogenetic analysis of mcrA clones revealed the presence of six distinct clusters of McrA sequences in Italian rice field soil (Fig. 1), two of which could not be affiliated to any reference sequences available. Furthermore, four chimeric or truncated mcrA clones were detected, which were excluded from further analyses. The first two clusters of clones were related to McrA and Mra sequences of members of the Methanobacteriaceae (20 and eight clones respectively). The mcrA clones were most closely related to Methanobacterium bryantii. A third cluster of environmental clones grouped with McrA sequences of Methanosarcina sp. (n = 14). We were able to affiliate clones falling into the fourth cluster (n = 10) with the Methanosetaeaceae after analysing the Mra sequences of Methanoseta concilii and M. concilii VeAc9. McrA fragments from the methanomicrobial Methanospirillum hungatei and Methanoculleus thermophilus were repeatedly amplified from pure cultures, but no related rice field soil mcrA sequences were found in our clone libraries. Instead, we detected two clusters of novel, deeply branching McrA sequences within the phylogenetic radiation of the Methanosarcinales, which could not be affiliated to any reference mcrA gene products. One of these clusters was represented by four RS-MCR clones that were clearly distinct from other clone groups with a maximum identity of only ~74% (DNA) and ~80% (amino acids) to other mcrAMcrA sequences detected in rice field soil. As the affiliation of this novel McrA cluster is as yet unclear, it was tentatively named ‘unidentified
Enrichment culture of rice cluster I

Using rice field soil slurries as inoculum, RC-I Archaea were selectively enriched at 50°C with H₂/CO₂ as energy sources and acetate (2 mM) for assimilation after repeated transfers. We followed the enrichments by measuring methane production and by T-RFLP analysis of archaeal SSU rRNA genes (Chin et al., 1999), which allowed us to monitor the increasing relative gene frequencies of the 392 bp T-RF characteristic of RC-I (Lueders and Friedrich, 2000) (see below). Eleven out of 12 archaeal SSU rDNA clones retrieved from a highly enriched culture could be assigned to RC-I, and only one clone was related to the Methanobacteriaceae (GenBank accession numbers AF313892 to AF313903). RC-I enrichment cultures produced methane, but the methanogen-specific cofactor F₄₃0₄ fluorescence was not detected. Cells were small cocci of up to 0.5 µm in diameter. We specifically amplified mcrA fragments from the enrichment culture using both MCR and ME primers and constructed two clone libraries: MRE-MCR (methanogenic rice field soil enrichment MCR amplicons) and MRE-ME (methanogenic rice field soil enrichment ME amplicons). Sequence analysis and phylogenetic placement of six MRE-MCR and four MRE-ME clones revealed that all mcrA clones retrieved from the enrichment culture grouped with the more frequent cluster of novel mcrA genes obtained directly from rice field soil (Fig. 1). Thus, with the help of the enrichment culture, we were able to affiliate this novel
cluster of mcrA genes with members of the euryarchaeotal RC-I lineage before the establishment of a pure culture.

**T-RFLP fingerprinting of rice field soil mcrA genes**

The analysis of microbial communities by a cloning/sequencing approach is time intensive and may also be subject to cloning-inherent biases (von Wintzingerode et al., 1997; Lueders and Friedrich, 2000). Therefore, we developed a T-RFLP fingerprinting method for environmental mcrA sequences to allow the rapid and cloning-independent investigation of methanogenic communities in environmental samples. A comparison of the composition of the RS-ME and RS-MCR clone libraries revealed a higher diversity coverage for the MCR primer set. The ME primer set recovered only mcrA sequences within the Methanosarcinaceae, Methanobacteriaceae and the novel RC-I type (Fig. 2), of which the methanobacterial mcrA clones were clearly most frequent (47%). In contrast, the MCR primer pair additionally detected Methanosaetaeacea mcrA, Methanobacteriaceae mtrA and the unidentified rice field soil mcrA cluster. In the MCR library, relative clone frequencies of methanogenic lineages detected were between 9% and 22%, and most frequent were mcrA clones related to the Methanosaetaeacea (22%; n = 10). Because of the reduced diversity coverage of the ME primer set, we subsequently conducted T-RFLP analyses using the MCR primer set.

We checked our aligned mcrA sequences for conserved, group-specific restriction sites of the different methanogenic lineages. Sau96I digests in combination with a fluorescently labeled forward primer resulted in distinct and highly conserved group-specific terminal restriction fragments (T-RFs) for all the different groups of rice field soil mcrA/mtrA sequences (Table 1). Some groups displayed a single, 100% conserved specific T-RF, i.e. RC-I mcrA (238 bp) and Methanobacteriaceae mtrA (470 bp), whereas other lineages separated into two (Methanosaetaeacea, Methanosarcina spp. and unidentified rice field soil mcrA) or three characteristic T-RFs (Methanobacteriaceae mcrA). Most importantly, none of the distinct T-RFs was shared by different groups of methanogens, which allowed a clear separation of all methanogenic lineages detectable in Italian rice field soil with this mcrA-targeted T-RFLP analysis.

The predicted mcrA T-RFs of different pure cultures and clones were verified by T-RFLP analysis (data not shown). T-RFLP analysis of mcrAmtrA fragments from *M. bryantii* revealed, as expected, the presence of both mcrA and mtrA genes, i.e. 470 bp and 503 bp T-RFs. Moreover, both characteristic T-RFs were represented at similar signal intensities, indicating equal amplification

![Fig. 2. Relative composition of rice field soil mcrA and mtrA clone libraries generated with ME and MCR primer pairs, as analysed by the affiliation of clone sequences to major methanogenic lineages. Numbers of clones of each library are indicated in brackets.](image-url)

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**Table 1. Affiliation of distinct terminal restriction fragments (T-RFs) predicted for mcrA and mtrA clone sequences retrieved from Italian rice field soil with detected methanogenic lineages.**

<table>
<thead>
<tr>
<th>Phylogenetic affiliation of clones</th>
<th>T-RF (bp)</th>
<th>No. of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanosarcinaceae mcrA</td>
<td>147, 419</td>
<td>7, 3</td>
</tr>
<tr>
<td>Rice cluster I mcrA</td>
<td>238</td>
<td>19, (10)</td>
</tr>
<tr>
<td>Methanosarcina spp. mcrA</td>
<td>394, 427</td>
<td>12, 2</td>
</tr>
<tr>
<td>Methanobacteriaceae mcrA</td>
<td>403, 406; 503</td>
<td>1, 10</td>
</tr>
<tr>
<td>Methanobacteriaceae mtrA</td>
<td>470</td>
<td>8</td>
</tr>
<tr>
<td>Unidentified rice field soil mcrA</td>
<td>409, 506</td>
<td>1, 3</td>
</tr>
</tbody>
</table>

T-RFs were predicted *in silico* from aligned sequence data assuming amplification of partial mcrA and mtrA sequences with MCR primers (Springer et al., 1995), a fluorescently labeled forward primer and restriction of PCR amplicons with Sau96I. Numbers of rice field soil clones producing the different characteristic T-RFs are indicated; RC-I enrichment culture mcrA clones are specified in brackets.
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rRNA genes of members of RC-I (Lueders and Friedrich, 2000) was detectable in the methanogenic enrichment culture (Fig. 3D), which was established with Italian rice field soil as inoculum (Fig. 3C). A minor T-RF (90 bp) characteristic of Methanobacteriaceae SSU rRNA genes was detectable but below the quantification threshold. Thus, both independent T-RFLP analyses indicated the presence of predominantly RC-I Archaea in the enrichment culture.

Comparison of mcRA and SSU rRNA phylogeny

With the enlarged mcRA and mrtA gene database, which now includes representatives of the Methanothepumaceae, rice cluster I and additional representatives of the Methanomicrobiales, we evaluated the phylogeny of methanogens based on deduced amino acid sequences of mcRA and mrtA genes in comparison with SSU rRNA-based phylogeny. The McrA/MrtA-derived phylogenetic tree was rooted with the McrA sequence of Methanopyrus kandleri (Fig. 4A), which is the deepest branching methanogen, as inferred from rRNA gene phylogeny (Burggraf et al., 1991). The SSU rDNA-derived tree of Euryarchaeota also includes non-methanogenic lineages and crenarchaeotal reference outgroups (Fig. 4B). Sequence similarities between M. kandleri and members of the Methanosarcinaceae were ~67% at both the DNA and the amino acid level for mcRA fragments and ~71% for SSU rRNA genes. Overall, the tree topologies of SSU rRNA and mcRA/mrtA gene products were highly similar (Fig. 4; Lehmann and Klein, 1994; Ohkuma et al., 1995). The relative branching order of RC-I varied with different treeing methods in MorA analysis, resulting in a closer association with either the Methanosacetaceae (neighbour-joining and maximum parsimony analysis) or the Methanosarcinaceae (Fitch and maximum likelihood analysis). But RC-I always grouped clearly with the Methanosarcinaceae, as supported by a bootstrap value of 76% in neighbour-joining analysis. This fuzziness in the relative branching order of RC-I, the Methanosacetaceae and the remaining Methanosarcinales is reflected in an adjusted consensus McrA/MrtA dendrogram, which indicates the lack of phylogenetic resolution for this part of the McrA/MrtA tree by a manually introduced truncation (Ludwig et al., 1998). This is the best reconstruction possible at present, as only one complete mcRA sequence (Methanosarcina barkeri, Bokranz and Klein, 1987) is available within the phylogenetic radiation of the Methanosarcinales and Methanomicrobiales. In contrast to the McrA/MrtA-based analysis, RC-I clearly branched off between the Methanosarcinales and Methanomicrobiales based on SSU rDNA analysis. This position was
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Fig. 4. Phylogenetic trees indicating the relationship of methanogenic Archaea as reconstructed from McrA and MrtA sequences (A) and SSU rRNA genes (B). Tree (A) was generated as described in the legend to Fig. 1 and contains 84 partial McrA and MrtA sequences. A full circle indicates a manually adjusted consensus trifurcation. Tree (B) contains a total of 437 partial or near-complete archaean SSU rRNA gene sequences. Bootstrap values were calculated by neighbour-joining analysis of 100 replicate data sets using a ‘core’ data set of 45 SSU rRNA gene sequences (> 1300 bp). Non-methanogenic lineages are shown in brackets. Bars represent 10% sequence divergence. Accession numbers of species and clones included in both trees are available as supplementary information under http://www.uni-marburg.de/mpi/conrad/mcr.html

Consistently recovered by distance matrix and maximum likelihood treeing methods, but was only supported by a low bootstrap value (42%; Fig. 4B), which might result from the fact that only two almost complete (> 1300 bp) SSU rRNA sequences of this lineage (clone WCHD3-34; Dojka et al., 1998, and a clone retrieved from Italian rice field soil; S. Weber et al., in preparation) are available for treeing.

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Discussion

Recent molecular SSU rDNA-based surveys of Italian rice field soils revealed a high diversity of Archaea (Grosskopf et al., 1998a; Chin et al., 1999; Lueders and Friedrich, 2000), including several uncultivated eury- and crenarchaeotal lineages (rice clusters I to VI). This prompted us to speculate that at least the RC-I and -II lineages, both within the phylogenetic radiation of the Methanosarcinales and Methanomicrobiales, represent novel, as yet uncultivated methanogens (Grosskopf et al., 1998a; Lehmann-Richter et al., 1999). Therefore, we studied the diversity of rice field soil methanogens using the specific functional marker gene mcrA to detect novel methanogens. Using a combination of molecular and enrichment culture techniques, we were able to identify Archaea of RC-I as methanogens.

Prerequisites for the study of environmental gene sequence diversity are a PCR assay, which detects a wide range of organisms, and the establishment of a phylogenetic framework, which allows the identification of microorganisms. We amplified mcrA fragments from rice field soil samples using two different degenerate primer sets. The ME primer set allowed the retrieval of a longer mcrA fragment (~760 bp), which contains more phylogenetic information, but the trade-off was reduced diversity coverage (Fig. 2) (Edwards et al., 1998). Notably, the Methanosetaeae and the methanobacterial mrtA genes could not be amplified with the ME primers. Nevertheless, the mcrA sequence types detected with both MCR and ME primers were phylogenetically similar within each cluster.

So far, the presence of the paralogous mrtA gene of the Methanococcaceae and Methanobacteriaceae has not been considered in environmental studies. Whereas the mrtA sequence of Methanococcus jannaschii appears to be a true parologue, the mrtA sequence of the Methanobacteriaceae was most probably acquired laterally from a methanococcal donor species (Reeve et al., 1997), as indicated by its phylogenetic position well within the McrA/MrtA branch of the Methanococcaceae (Lohmacher and Klenk, 1994; Pith et al., 1994; Nölling et al., 1996). This has consequences for the origin of environmental mrtA sequences, as these may represent mrtA genes of either the Methanococcaceae or the Methano- bacteriaceae. In rice field soil, it is likely that the methanobacterial mcrA and mrtA sequences originated from the same Methanobacteriaceae, as T-RFLP analysis of M. bryantii pure culture and rice field soil DNA clearly showed the presence of both paralogues (470 bp and 503 bp; Fig. 3). Thus, inferences of methanogenic species composition based on relative mcrA/mrtA gene frequencies can be biased by the presence of both paralogous MCR genes originating from the same species.

The phylogenetic positions of all other methanogenic lineages within the McrA/MrtA tree were highly similar to their SSU rRNA gene phylogeny, as indicated by a similar relative branching order (Fig. 4), which reflects the conserved nature of the MCR (Springer et al., 1995). Several functionally important amino acids near the active site of the MCR that are co- or post-translationally modified in M. marburgensis (e.g. His₃257, Arg₄271, Gln₃400, Gly₄445 and Cys₅₄52; Ernster et al., 1987) have been reported to be 100% conserved in a variety of different methanogenes (Selmer et al., 2000). This site conservation was also found in all rice field soil sequences, including RC-I methanogens, suggesting that these amino acids may be post-translationally modified as well.

The high diversity of methanogens in Italian rice field soil was adequately reflected by the mcrA approach, as we detected mcrA sequences related to Methanosarcina spp., the Methanosetaeae and Methanobacteriaceae (Grosskopf et al., 1998a; Chin et al., 1999; Lueders and Friedrich, 2000). Surprisingly, we failed to detect mcrA sequences related to those of the Methanomicrobiales. This was particularly intriguing, as SSU rDNA sequences grouping with the methanomicrobial endosymbiont of the anaerobic ciliate Plagiopyla nasuta have been retrieved from Italian rice field soil (Grosskopf et al., 1998a), and we were able to amplify mcrA fragments from pure cultures of M. hungatei and M. thermophillus. The mcrA fragments of Methanomicrobiales present in the rice field soil were possibly not amplified because of a suboptimal match of the primer pairs used to their target sites. The design of improved primer sets and the investigation of a possible amplification bias may solve the lack of detection of methanomicrobial mcrA genes.

In addition to mcrA and mrtA sequences of known rice field soil methanogens, we detected two novel environmental mcrA sequence types, representing separate, deeply branching clades within the phylogenetic radiation of the Methanosarcinales (Figs 1 and 4); however, with unknown phylogenetic identification. Traditionally, the enrichment and isolation of microorganisms has been used to determine their phenotype; however, our inability to mimic growth conditions of microorganisms adequately may leave a large proportion of prokaryotic diversity uncultivated and, consequently, uncharacterized with respect to their ecological function. Several strategies can be pursued to link the phenotypic information of catalobic genes to the phylogenetic information contained in SSU rRNA genes of uncultivated organisms. Such a correlation may be achieved in an undirected way by screening environmental metagenome clones (Béja et al., 2000; Rondon et al., 2000) with the goal of linking phylogenetic and functional information potentially present on large fragments of community DNA. In this study,
we have used a combination of molecular analyses and enrichment culture techniques. The preferential enrichment of members of RC-I over other Archaea was monitored under different enrichment conditions by following methane formation, T-RFLP analysis and cloning of archaeal SSU rRNA genes. We succeeded in enriching members of RC-I using Italian rice field soil as inoculum and H₂/CO₂ as growth substrates at a temperature of 50°C (A. Fey et al., in preparation). With the aid of this highly enriched culture of RC-I Archaea, we were able to identify the more frequent cluster of novel rice field soil McrA sequences as RC-I. Cloning and T-RFLP analysis of both McrA and SSU rRNA genes revealed that predominantly RC-I genes were detectable in the enrichment culture, whereas the genes of members of the Methanobacteriaceae, as found in rice field soil, were detected only at a very low frequency (Fig. 3). The congruent phylogenetic position of the novel McrA sequences and RC-I SSU rRNA genes (Fig. 4) further supports the identity of RC-I mcrA genes. A PCR bias affecting both independent marker genes, mcrA and SSU rDNA, appears to be highly unlikely. Only a preferential amplification of RC-I SSU rRNA genes over all other methanogens in the enrichment culture in parallel with a preferential amplification of mcrA genes of a further unknown methanogen over all other mcrA genes would explain such a double PCR bias. However, T-RFLP analyses of rice field soil community DNA shows clearly that neither RC-I SSU rRNA genes nor the novel mcrA genes are preferentially amplified to such an extent that the genes of other methanogens become undetectable (Fig. 3A and C). Additional PCR-independent evidence for the identity of the novel mcrA cluster as RC-I is based on fluorescent in situ hybridization (FISH) probing with an RC-I-specific SSU rRNA probe, which indicated the presence of RC-I cells in the enrichment culture (Chin et al., in preparation).

The second, less frequently detected ‘unidentified rice field soil McrA’ cluster clearly grouped within the phylogenetic radiation of the Methanosarcinales (Figs 1 and 4). Currently, we can only speculate whether these mcrA sequences originated from RC-II. SSU rDNA phylogeny recovered RC-II within the phylogenetic radiation of the Methanomicrobiales, albeit with low bootstrap support (30%; Grosskopf et al., 1998a). On the other hand, our own analyses indicated a loose affiliation of RC-II with the Halobacteriaceae (Lueders and Friedrich, 2000) but, again, with low bootstrap support (data not shown). Thus, the phylogenetic placement of the ‘unidentified rice field soil McrA’ makes an affiliation with RC-II unlikely.

The mcrA/mrtA-targeted T-RFLP analysis represents a novel approach for the rapid fingerprinting of methanogenic communities. All methanogens detectable were clearly discriminated by distinct T-RFs (Table 1), and non-methanogenic Archaea were excluded. So far, a specific fingerprinting assay for methanogens has not been available. SSU rRNA gene-based fingerprinting methods, i.e. T-RFLP, albeit used successfully to characterize Archaea in rice field soils, have their shortcomings. For instance, different archaeal lineages share the same TaqI restriction sites (Chin et al., 1999; Lueders and Friedrich, 2000), e.g. methanogenic members of the Methanosarcinaceae and the (probably) non-methanogenic crenarchaeotal members of RC-VI (terrestrial mesophilic Crenarchaeota), which complicates the identification of individual phylogenetic groups considerably.

In this study, we could demonstrate that Archaea from RC-I were the predominant methanogens detectable in a methane-producing enrichment culture via both mcrA and SSU rRNA gene analysis, which proves their methanogenic genotype and phenotype. However, little is known about the ecology of this novel clade of methanogens. Moderately thermophilic temperatures (50°C) and H₂/CO₂ as substrates were an important prerequisite for the enrichment of RC-I methanogens. In addition, transfers of these enrichments to 30°C failed to continue growth, which further underscores the requirement for relatively high temperatures at least for the RC-I enrichment culture. Currently, these observations are puzzling, as the typical temperature range of rice field soils is between 15°C and 30°C (Schusatz et al., 1990). On the other hand, methanogenic consortia grown in enrichment cultures at 25°C, which had been inoculated with the terminal positive dilutions of the most probable number (MPN) counts on H₂ from rice roots, were characterized by a predominance of SSU rDNA clones related to RC-I Archaea (Lehmann-Richter et al., 1999). At this point, we cannot exclude the possibility that RC-I may contain both mesophilic and thermophilic methanogens; however, other methanogenic families such as the Methanosarcinaceae are also characterized by the presence of mesophilic and thermophilic species (i.e. Methanosarcina thermophila versus Methanosarcina mazei and M. barkeri). Nevertheless, high relative frequencies of RC-I SSU rRNA genes in Italian rice field soils (Chin et al., 1999; Lueders and Friedrich, 2000) and their predominance in MPN counts from rice roots (Lehmann-Richter et al., 1999) imply that these Archaea must be functionally important in rice field soil. Consequently, these novel methanogens may contribute significantly to the emission of the greenhouse gas methane from rice fields into the atmosphere. The role of RC-I in global methane production is emphasized further by the detection of RC-I SSU rRNA genes in rice fields of diverse geographical origin (B. Ramakrishnan et al., in preparation) and the methanogenic zone of a north American aquifer (Dojka et al., 1998). Furthermore, SSU rRNA genes related to RC-I have been detected recently in the anoxic hypolimnion of a boreal forest lake in Finland.
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(Jurgens et al., 2000). However, only the isolation of pure cultures of RC-1 Archaeca will provide further insight into the function and ecological niche of these novel methanogens in anaerobic environments.

Experimental procedures

**Pure cultures of methanogens**

*Methanobacterium bryanti DSM 863*³, *M. thermophilus DSM 2624*², *M. concilii DSM 3671*² and *M. hungatei JF1 DSM 864*² were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Strains were grown in standard media as specified by the DSMZ before DNA extraction. *Methanoseta concilii VeAc9* (Grosskopf et al., 1998b) was from our own culture collection.

**Enrichment cultures**

Slurries of Italian rice field soil collected in 1998 (Italian Rice Research Institute, Vercelli, Italy) were preincubated at 50°C for 3 weeks under anoxic conditions, and then 5 ml of slurry was used for the inoculation of enrichment cultures. These were established in 120 ml serum bottles with a total volume of 50 ml of mineral medium [2 mM acetate, 0.1 ml rice field soil extract (Cote and Gherma, 1994), 10 mg of yeast extract, 10% inoculum] and a headspace of H₂/CO₂ (80:20 v/v). For the primary enrichment cultures, lysozyme (5 mg ml⁻¹) was added to eliminate Bacteria. The anoxic mineral medium was prepared as described earlier (Chin et al., 1998) with slight modifications. In addition, selenite/fungate solution (Tschech and Pfenning, 1984) was added, as well as riboflavin (0.05 mg ml⁻¹) and a lower concentration of mineral salts (0.1 x). Enrichments were incubated at 50°C, and methane formation was followed by gas chromatography (Conrad et al., 1987). The selective enrichment of novel methanogens was achieved by subsequent transfers and monitored by molecular analyses.

**DNA extraction and PCR amplification**

Rice field soil slurries were incubated and analysed as described previously (Lueders and Friedrich, 2000). Soil enrichment and pure culture DNAs were extracted and purified as previously described in detail (Henckel et al., 1999). *McrA* fragments were amplified using the primer combinations MCR1 (5'-TAY GAY CAR ATH TGG YT-3') and MCR2 (5'-ACR TTC ATN GCR TAR TT-3') (Springer et al., 1995) or ME1 (5'-GOM ATG CAR ATH GGW ATG TC-3') and ME2 (5'-TCA TKG CRT AGT TGD GRT AGT-3') (Hales et al., 1996). In a total volume of 50 µl, PCR reactions contained 1 x MasterAmp PCR PrepMix B (Epicentre Technologies), 0.5 µM each of one primer pair (MWG Biotech) and 1.25 U of Amplitaq DNA polymerase (Applied Biosystems). Diluted pure culture or soil community DNA (1 µl) was added as template. PCR amplification was performed in a Gene Amp 9700 thermocycler (Applied Biosystems) with an initial denaturation step (3 min, 94°C) followed by 30–35 cycles of denaturation (45 s, 94°C), annealing (45 s, 50°C) and extension (90 s, 72°C) and a terminal extension step (5 min, 72°C). Archaeal SSU rRNA genes were amplified as described previously (Chin et al., 1999). After amplification, 5 µl aliquots of the PCR products were analysed by standard agarose gel electrophoresis. Amplicons were purified using the QIAquick PCR purification kit (Qiagen).

**Cloning and sequencing**

McrA fragments amplified from pure culture DNA were sequenced directly using the respective amplification primer sets. Environmental and enrichment culture PCR products were cloned using the pGEM-T Vector System II cloning kit (Promega). Clones were selected randomly and checked for correct insert size via standard vector-targeted PCR and agarose gel electrophoresis. Sequencing was performed on an ABI Prism 377 DNA sequencer using BigDye terminator chemistry as specified by the manufacturer (Applied Biosystems).

**Sequence data and phylogenetic analysis**

Nucleotide and inferred amino acid sequences of all partial or complete *mcrA* genes available in public databases (Benson et al., 2000) were aligned using the *ares* software package (version 2.5b, O. Strunk and W. Ludwig, Technische Universität München, Munich, Germany, http://www.biol.chemie.tu-muenchen.de/putz/ARBi/). Partial *mcrA* sequences obtained in this study were assembled and checked using the LASERGENE software package (DNASTAR) and fitted into the *mcrA*-derived amino acid sequence alignment. Phylogenetic analyses were performed using Fitch (version 3.573c; J. Felsenstein, University of Washington, http://evolution.genetics.washington.edu/phylip.html). An amino acid frequency filter was generated from a data set of 74 sequences to include only McrA/MtA amino acid positions into phylogenetic analyses with more than 25% invariance (152 positions). Distance matrices were calculated using PRODIST with the Dayhoff PAM 001 matrix as amino acid replacement model. Phylogenetic dendrograms of partial *McrA/MtA* sequences were reconstructed from distance matrices using Fitch analysis with branch swapping (global rearrangement) and randomization of the species input order (random number seed 7, jumble seven times) as implemented in *ares* and verified by neighbour-joining, maximum parsimony and maximum likelihood methods. Bootstrap values were calculated by neighbour-joining analysis (*protdist*) of 100 replicate data sets. For phylogenetic analyses of SSU rDNA, the sequence alignment of the *ares* SSU rDNA database was used. A phylogenetic “core” tree of archaeal SSU rDNA sequences was reconstructed from a distance matrix (Kimura 2 parameter model as evolutionary distance correction) of almost complete SSU rRNA genes (> 1300 bp; n = 45) using Fitch (with global rearrangement of branches and randomized species input order: random number seed 7, jumble seven times) and validated by neighbour-joining and maximum likelihood methods. The latter method especially is very slow, which constrains the number of taxa that can be analysed. To avoid treeing artifacts caused by highly variable nucleotide positions or those that do not align unambiguously, only positions that were present in at least 50% of all sequences

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analysed were included for treeing analyses (Grosskopf et al., 1998a). The base frequency was determined with the 'cors' SSU rDNA data set (n = 45) using the appropriate urn tool. Additional nearly full-length (> 1300 bp, n = 125) and shorter SSU rDNA sequences (< 800 bp, n = 267) were inserted using the urn parsimony tool, which allows the addition of partial sequences to an optimized and validated core tree without altering the global tree topology. In a recent review, Ludwig et al. (1998) have given a detailed description of this approach.

T-RFLP analysis

Fluorescently labelled PCR products were generated according to the above PCR protocol using the MCR primer pair (Springer et al., 1995) with a 5'-6-carboxyfluorescein (FAM)-labelled forward primer. DNA concentrations of purified PCR products were determined by standard UV photometry (GeneQuant; Amersham Pharmacia Biotech). Restriction digests were performed in a total volume of 10 μl containing ≈ 100 ng of PCR amplicons, 3 U of Sau96I (Promega), 1 x 4-CORE buffer C (Promega) and 1 μg of bovine serum albumin (Promega) and incubated for 2 h at 37°C. T-RFLP analysis of archaical SSU rDNA amplicons was performed as described earlier (Chin et al., 1999). Digested amplicons (1.25 μl) were mixed with 1 μl of formamide loading dye (Amersham Pharmacia Biotech) and 0.25 μl of the GeneScan-1000 (ROX) size standard (Applied Biosystems) and analysed on an ABI Prism 377 DNA sequencer in GeneScan mode. Fragments were separated on a 36 cm 6% (w/v) polyacryl-amide gel containing 6.3 M urea and 1 x TBE buffer (89 mM Tris-borate, 2 mM EDTA) for 14 h with the following settings: 2500 V, 40 mA and 30 W. Electropherogram analysis was performed with the GENESCAN analysis software (version 3.1, Applied Biosystems) using third-order least squares size calling. T-RFs were quantified by peak area integration using a minimum peak height analysis threshold of 100 relative fluorescence units.

Nucleotide sequence accession numbers

All sequences generated in this study were deposited in the GenBank, EMBL and DDBJ databases under the following accession numbers: pure culture mcrA sequences, AF313802 to AF313806; environmental mcrA and mtrA clones RS-MCR, AF313807 to AF313851; environmental mcrA clones RS-ME, AF313852 to AF313881; methanogenic rice field soil enrichment culture mcrA clones MRE-MCR, AF313882 to AF313887; methanogenic rice field soil enrichment culture mcrA clones MRE-ME, AF313888 to AF313891; methanogenic rice field soil enrichment culture SSU rDNA clones, AF313892 to AF313903.

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References


Ermier, U., Graparbe, W., Shima, S., Goubeaud, M., and Thauer,
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C.2. Populationsdynamik der *Archaea* während der sequentiellen Reduktion im Reisfeldboden

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Archaeal Population Dynamics during Sequential Reduction Processes in Rice Field Soil

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The population dynamics of *Archaea* after flooding of an Italian rice field soil were studied over 17 days. Anoxically incubated rice field soil slurries exhibited a typical sequence of reduction processes characterized by reduction of nitrate, Fe(III), and sulfate prior to the initiation of methane production. Archaeal population dynamics were followed using a dual approach involving molecular sequence retrieval and fingerprinting of small-subunit (SSU) rRNA genes. We retrieved archaeal sequences from four clone libraries (30 each) constructed for different time points (days 0, 1, 8, and 17) after flooding of the soil. The clones could be assigned to known methanogens (i.e., *Methanosarcinaeae*, *Methanosetaeae*, *Methanomicrobiaeae*, and *Methanobacteriaceae*) and to novel euryarchaeotal (rice clusters I, II, and III) and crenarchaeotal (rice clusters IV and VI) lineages previously detected in anoxic rice field soil and on rice roots (R. Grosskopf, S. Stuhrn, and W. Liesack, Appl. Environ. Microbiol. 64:4983–4989, 1998). During the initiation of methanogenesis (days 0 to 17), we detected significant changes in the frequency of individual clones, especially of those affiliated with the *Methanosetaeae* and *Methanomicrobiaeae*. However, these findings could not be confirmed by terminal restriction fragment length polymorphism (T-RFLP) analysis of SSU rDNA amplicons. Most likely, the fluctuations in sequence composition of clone libraries resulted from cloning bias. Clonal SSU rDNA gene sequences were used to define operational taxonomic units (OTUs) for T-RFLP analysis, which were distinguished by group-specific TaqI restriction sites. Sequence analysis showed a high degree of conservation of TaqI restriction sites within the different archaeal lineages present in Italian rice field soil. Direct T-RFLP analysis of archaeal populations in rice field soil slurries revealed the presence of all archaeal lineages detected by cloning with a predominance of terminal restriction fragments characteristic of rice cluster I (389 bp), *Methanosetaeae* (280 bp), and *Methanosarcinaeae* (rice cluster VI (182 bp). In general, the relative gene frequency of most detected OTUs remained rather constant over time during the first 17 days after flooding of the soil. Most minor OTUs (e.g., *Methanomicrobiaeae* and rice cluster III) and *Methanosetaeae* did not change in relative frequency. Rice cluster I (37 to 30%) and to a lesser extent rice cluster IV as well as *Methanobacteriaceae* decreased over time. Only the relative abundance of *Methanosarcinaeae* (182 bp) increased, roughly doubling from 15 to 29% of total archaeal gene frequency within the first 11 days, which was positively correlated to the dynamics of acetate and formate concentrations. Our results indicate that a functionally dynamic ecosystem, a rice field soil after flooding, was linked to a relatively stable archaeal community structure.

In most anoxic ecosystems, methanogenic *Archaea* significantly affect carbon cycling, since fermentable substrates are degraded completely to CO₂ and CH₄ via the anaerobic food chain, with methanogenesis as the predominant terminal respiratory process (41). Important habitats for methane-forming *Archaea* are wetlands, including rice field soils. The latter are estimated to contribute about 25% to the budget of global methane emissions (36) and therefore have a significant impact on global warming (25).

In periodically submerged wetlands such as rice field soils, CH₄ production is initiated shortly after flooding, only after inorganic electron acceptors such as oxygen, nitrate, manganese(IV), iron(III), and sulfate have been reduced sequentially. Although biogeochemical processes after the flooding of rice field soil have been studied in detail, the factors controlling the initiation of methanogenesis are not fully understood (10). The sequence of reduction processes is best described by the thermodynamic theory, which predicts preferential reduction of available electron acceptors with the most positive redox potential (55, 50). For a variety of soils, this sequence of reduction processes has been demonstrated after flooding (1, 32, 33). However, in most soils, initiation of methane production deviates from the predicted concept of sequential reduction. Methane formation, albeit at trace amounts, could be observed directly after flooding of rice field soils despite the presence of oxidants such as nitrate, iron(III), and sulfate (40, 48, 49). Similarly, methane formation was observed at redox potentials of 0 to 70 mV after flooding of rice soils (33) and in pure cultures of *Methanosarcina barkeri* (14), although it is generally assumed that methanogens become active only at redox potentials below –150 mV (50). Apparently, methanogenic *Archaea* in soils are activated much faster than was previously thought (40).

Understanding the function of ecosystems requires comprehension of both biogeochemical processes and microbial community structures (10, 44). With respect to the archaeal populations involved in the initiation of methanogenesis after the flooding of soils, the central questions are which factors control the diversity and abundance of methanogens and how, if at all, populations alter in response to changing environmental conditions.

Few data are available about the population dynamics of methanogens after the flooding of soils. Using most-probable-number counts, it has been shown for geographically diverse rice field soils that the total population size of methanogens...
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remains rather constant even after periods of drainage and aeration (30) and when monitored over 2 years (3). However, the methanogenic activity may undergo a shift from hydrogenotrophy to acetotrophy during the initiation of methan production, as suggested by methyl fluoride inhibition experiments (40). Currently, we can only speculate whether this shift in activity is also reflected in methanogenic population dynamics. The known limitations of cultivation-dependent approaches (26) have hampered the retrieval of numerically abundant and functionally relevant microorganisms from complex environmental communities (2). The slow-growing or difficult-to-cultivate methanogenic Archaea especially may require extremely long incubation times (>2 year) to obtain most-probable-number cell counts (16). The application of molecular methods (i.e., the analysis of small-subunit [SSU] rDNA genes as universal markers [17, 47]) for community structure analysis facilitates detection and identification of microorganisms that are difficult to cultivate or may be regarded as unculturable from our current knowledge of the growth requirements of these microorganisms. With these methods at hand, the study of population structures becomes feasible for methanogenic ecosystems.

Molecular surveys of SSU rDNA sequences in anoxic rice field soil (9, 16) and on rice roots (15, 24) revealed a high archaean diversity, including known methanogenic families (Methanosaetaeae, Methanobacteriaceae, and Methanococciaceae) as well as phylogenetically novel eury- and crenarchaeal lineages, termed rice clusters I to VI (RC-I to RC-VI). The phylogenetic positions of only two of these clusters (RC-I and II) and their presence in methanogenic enrichment cultures (24) suggest an affiliation with methanogenic taxa. The ecophysiology of members of all other clusters (RC-III to RC-VI) is still unknown.

In this investigation, we studied the dynamics of archaean populations after the flooding of rice field soil to establish a link between structure (which species) and function (metabolic activities) of the archaean populations involved in the initiation of methanogenesis. Soil slurries were incubated anaerobically over 17 days and sampled for chemical and molecular analyses. We followed changes in the archaean community structure by analyzing SSU ribosomal DNA (rDNA) clone libraries derived from soil DNA extracts. In parallel, we determined relative gene frequencies as analyzed by terminal restriction fragment length polymorphism (T-RFLP) (27) analysis of archaean SSU rDNA PCR fragments (9). Our results indicate a surprisingly stable archaean community structure as analyzed by T-RFLP, considering the changes associated with the flooding of the soil. Only Methanosarcina-like populations were found to increase significantly in relative gene frequency.

MATERIALS AND METHODS

Soil samples and slurry experiments. Soil was sampled in February 1997 from a drained rice field of the Italian Rice Research Institute near Verzelli (Po River valley, Italy). Soil parameters were described previously (8). The soil was air dried and stored at room temperature. Preparation of the soil and sieving (mesh size, 2 mm) were done as previously described (8). Soil slurries (3 g of dry soil and 3 g of sterile double-distilled water) were prepared in triplicate for each time point in 125-mL serum vials which were sealed with butyl rubber septa. Anoxic incubations of soil slurries for each time point were started at daily intervals by the addition of anoxic water with a syringe. The headspace of vials was flushed with N₂ for 10 min, and vials were statically incubated at 25°C. At the end of the experiment, the vials were analyzed for gases (CH₄, H₂, and CO₂). Slurry samples were taken, frozen, and stored (~7°C) for analyses of biochemical parameters (Fe²⁺, NO₂⁻, NO₃⁻, SO₄²⁻, and fatty acids) and molecular analyses. Chemical analyses. Pore water samples were analyzed by ion chromatography for the determination of nitrate and sulfate (5). Acetate and formate were analyzed by high-pressure liquid chromatography (23). Fe²⁺ was analyzed in slurry samples using the ferrozine reaction method (1). Gas samples (CH₄, H₂, and CO₂) were taken from the headspaces of the flasks and measured by gas chromatography (40). Low H₂ concentrations were determined by using a reducing gas detector (RGD2; Trace Analytical, Menlo Park, Calif.) (40).

Extraction of soil DNA and PCR amplification. Total DNA was extracted from one soil sample for each time point using a direct lysis technique for cell disruption modified from Morel et al. (31) as described previously (19). Briefly, cells in soil samples (300 mL of slurry) were fixed by heat-shocking (45 s at 85 °C in the presence of sodium dodecyl sulfate solution). DNA was purified from the supernatant with ammonium acetate, isopropanol, and ethanol precipitation steps. Finally, the DNA extract was further purified using a silica matrix-based purification protocol (Prep-a-gene; Bio-Rad, Munich, Germany). Aliquots of DNA extracts were analyzed for standard gel electrophoresis. DNA concentrations were quantified fluorometrically (PicoGreen dsDNA quantitation kit; Molecular Probes, Leiden, The Netherlands).

Archaeal SSU rRNA genes were specifically amplified from total soil DNA extracts using the primer combination Ar190F (5'-AAGTCGTCCGACGACTCACTATAGGG-3') and Ar918R (5'-CTCCGGCTCAGCAGGAAACC-3') as previously described in detail (15). Briefly, new sequences were fitted into an alignment of SSU rDNA sequences containing 758 partial (5'-700 nucleotides) or complete archaean sequences retrieved from public databases (6) using the automated alignment tools of the ARB software package. When necessary, alignments were corrected manually. Chimera formation has been avoided by using an amplification protocol performed by using the ARB software package (version 2.50; G. Strunk and W. Ludwig, Technische Universität München, Munich, Germany; http://www.ibis.bcm.tmc.edu/chimera/teblastn.html) as described previously (19). The terminal 240 sequence positions of the 5'-9 and 3'-9 ends of the archaean SSU rDNA sequences were used for fractional treeing to identify possible chimeras based on mismatches of the secondary SSU rDNA structure (28). For treeing, almost full-length SSU rDNA sequences (>1,400 bases) were selected to construct an archaean base frequency filter (50 to 100% similarity), which was subsequently used to generate an initial neighbor-joining tree using selected eury- and crenarchaeal SSU rDNA reference sequences (1,440 bases). Sequences from rice field soil clone libraries (635 to 770 nucleotides in length) were then added to this tree using the ARB parsimony tool, which allows the addition of short sequences to phylogenetic trees without changing global tree topologies (29). In addition, the tree topology was evaluated using fastDNAML as implemented in ARB. Public databases were screened for close relatives of rice field soil SSU rDNA clones as described previously (15). Rabbitation analysis of SSU rDNA clone libraries was performed using the Analytical Rabbitation software (version 1.2, S. M. Holland, University of Georgia, Athens, http://www.uga.edu/~strata/Software.html).

T-RFLP analysis. T-RFLP analysis was performed as described by Chum et al. (9) with minor modifications. PCR assays (100 μL) were used for the amplification of archaean SSU rDNA genes following the above PCR protocol. From 400 to 800 ng of template DNA was used. PCR products were labeled with 6-carboxyfluorescein (FAM) at the 5'-end using the AmpliTaq polymerase. Alternatively, the 5' FAM-labeled Ar190F primer was used. Prior to digestion, PCR product concentrations were determined photometrically. DNA (50 ng), 3 μL of the appropriate 10X incubation buffer (Promega), and 1 μg of bovine serum albumin were combined in a total volume of 10 μL and digested for 2 h at 60°C. Fluorescently labeled terminal restriction fragments (T-RFs) were size-separated on an ABI 377A automated DNA sequencer (PE Applied Biosystems). T-RFLP electropherograms were analyzed by peak area integration of the different T-RFs (GeneScan 2.1 software; PE Applied Biosystems). The percent fluorescence intensity represented by single T-RFs was calculated relative...
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**RESULTS**

**Sequential reduction in rice field soil slurries.** Reduction processes in the rice field soil started immediately after the onset of anoxia. Nitrate was present at a low concentration (116 μM) initially and fell below the detection limit within 24 h (Fig. 1A). Fe(III) increased rapidly after the start of incubation and approached a maximum amount of 80 μM Fe(II) (per gram dry weight [dw] of soil) after 7 days. Sulfate in the pore water increased from 390 to 490 μM during the first day, probably due to HCO₃⁻ or Fe(II) reduction-mediated desorption from ferric iron minerals (49), but was rapidly reduced subsequently and could not be detected after 4 days. After only 1 day of incubation, methane formation was detectable, albeit at low methane mixing ratios (40). Within the first 4 days, methane increased exponentially, as revealed by logarithmic plotting (Fig. 1B), although electron acceptors other than CO₂ were still present (Fig. 1A). After 6 days of incubation, methane was produced at a constant rate of 0.5 μmol day⁻¹ (g dw)⁻¹.

The predominant methanogenic substrates, H₂ and acetate, accumulated to high concentrations during the first day (511 ± 10.6 Pa and 1,108 ± 144 μM, respectively) (Fig. 1B). Both were then rapidly consumed, but while H₂ remained detectable only in low mixing ratios (<6 Pa), acetate accumulated again, with a second concentration maximum on day 6 (521 ± 73 μM). Formate accumulated to concentrations of about 70 μM between days 9 and 11. CO₂ mixing ratios in-
creased logarithmically during the experiment, with final amounts reaching 9 kPa (data not shown).

**Diversity of Archaea in rice field soil slurries.** We constructed four clone libraries (days 0, 1, 8, and 17) of archaeal SSU rDNA fragments, which were PCR amplified from soil DNA, to assess the archaeal diversity present, identify dominant populations, and follow population dynamics during sequential reduction processes. More than 30 clones of each library were randomly selected and analyzed by comparative SSU rDNA sequencing. Within a total of 125 clones analyzed, 5 chimeric sequences were detected and excluded from further analyses. Phylogenetic placement of the nonchimeric clones revealed that they all fell within known eury- and crenarchaeotal lineages (Fig. 2 and 3), i.e., major methanogenic groups such as the *Methanosarcinaceae*, *Methanosaetaceae*, *Methanobiaceae*, and *Methanobacteriaceae*, as well as the as yet uncharacterized *Archaeo* assigned to RC-I, II, III, IV, and VI (9, 15, 16). Most clone sequences were closely related to archaeal rice field clones (>97% sequence similarity) reported earlier, but some phylogenetically more distant sequences were also detected. For example, the *Methanomicrobiaceae* differed by more than 5% in sequence from its closest relative, *S.15-24* (9). Clone AS 08-10, grouping with the *Methanomicrobiaceae*, differed 6% from its closest relative *S.15-30*, and clone AS 01-23 (*Methanobacteriaceae*), exhibited 6.5% sequence difference from ARRI1 (15), an SSU rDNA clone from rice roots.

The four clone libraries showed significant differences in relative archaeal population structures over time (Fig. 4). On day 0, immediately after flooding, populations related to *Methanosarcina* spp. (47%) and RC-I (30%) appeared to be predominant. Other groups were represented by only one or two clones, while no RC-III clone was found at all. On day 1, *Methanostraeta* spp. accounted for the largest number of clones (37%), *Methanosarcina* spp., *Methanobacteriaceae*, and RC-I represented 17% each, and the crenarchaeotal RC-IV represented 10%. One clone could be affiliated with RC-VI, while the remaining groups were not detected. On day 2 *Methanosarcina* spp. dominated (50%), whereas no other group exceeded a proportion of 10%. Interestingly, two clones of RC-III appeared, while no *Methanobacteriaceae* clone could be found. Finally, on day 17, a more balanced distribution of the major groups reappeared, 37% RC-I and 20% each *Methanostraeta* spp. and *Methanosaeta* spp. clones.

**Phylogenetic richness.** The analysis of total community diversity by clone approaches may be biased by undersampling (43). Therefore, we determined the diversity coverage of our archaeal SSU rDNA clone libraries by rarefaction analysis (10). This calculation allows the determination of the number of clones necessary to cover the expected species diversity. The calculated species richness $\hat{S}(n)$ in a sample of $n$ individuals selected at random from a community containing $N$ individuals and $S$ species. Since SSU rDNA clones in this study originated from the same soil, we combined all clones sampled at different time points with the rationale of estimating the total archaeal diversity in the soil independent of community structure shifts over time. The expected number of different SSU rDNA sequences was plotted against the number of clones retrieved (Fig. 5). We defined sequences with >99% sequence similarity (<8 different nucleotides per ~800 bp of SSU rDNA fragment) as belonging to the same species. However, even SSU rDNA sequences differing by >3% may represent a single species. Therefore, rarefaction calculations were performed at both the 99% and 97% sequence similarity levels. Species rarefaction calculations for 120 clones reached saturation at neither 99% (56 different species) nor 97% (25 different species) sequence similarity levels, indicating undetected archaeal diversity. The SSU rDNA sequences detected in our clone libraries fell into nine major clone groups (Fig. 4). By plotting the expected number of groups against the number of SSU rDNA clones retrieved, rarefaction calculations were shown to approach saturation at 120 clones, indicating sufficient diversity coverage at this level of resolution. According to these calculations, a random sample of 30 clones may contain seven to eight of the different major lineages, and in fact we detected six, seven, seven, and eight groups in the four clone libraries.

**T-RFLP analysis.** It is well known that analysis of the microbial community structure by cloning may be biased (43, 45). Thus, the significant changes in archaeological community structure as detected by clone frequencies during sequential reduction processes may have been subject to cloning biases as well. Therefore, we analyzed PCR-amplified SSU rDNA fragments directly by T-RFLP to monitor archaeological community structure changes.

Analysis of community structures by T-RFLP results in a pattern of T-RFs that may be unrelated to the phylogeny of the SSU rDNA sequences. In general, most archaeal group affiliations can be represented in the same operational taxonomic unit (OTU) (27). Therefore, we analyzed 267 archaeological clones obtained from Vercelli rice field samples in this study and in independent previous studies (9, 15, 16, 24) for group-specific TaqI restriction sites by sequence data comparison. The combined data set revealed a high degree of conservation of different group-specific restriction sites within the abundant and also within some of the less abundant clone groups (Table 1). For example, 95% of 77 *Methanosarcina*-like clones exhibited a 182-bp T-RF, 95% of 35 *Methanosaeta*-like clones showed a common T-RF of 280 bp, and 89% of 62 RC-I clones exhibited a 389-bp T-RF. Unfortunately, each of the three most frequent clone groups exhibited a common T-RF with one of the less frequent groups. *Methanosarcina*-like and RC-VI clones mainly shared a T-RF of 182 bp (9), *Methanosaeta*-like and RC-V clones exhibited a common 280-bp T-RF, and the majority of the RC-I and RC-II clones contributed to the 389-bp OTU. However, of the 120 clones analyzed in this study, only 1 (AS 00-12) fell within RC-II and none fell within RC-V. Thus, the 280-bp and 389-bp OTUs predominantly represented *Methanosaeta*-like and RC-I SSU rDNA sequences, respectively, and only to a negligible extent RC-II and RC-V sequences. In contrast, although *Methanosarcina*-like clones were clearly most abundant (40 of 120 clones), small numbers of the crenarchaeotal RC-VI clones were present in all clone libraries ($n = 6$), and therefore this population was likely to be represented in the 182-bp OTU. A typical archaeological community fingerprint obtained by T-RFLP analysis of SSU rDNA ampli
cons of slurry DNA is shown in Fig. 6A. All OTUs predicted by sequence data analysis (Table 1) could be detected in the T-RFLP electropherogram and assigned to the corresponding archaeological lineages.

**Population dynamics of Archaea.** Direct T-RFLP analysis was performed with PCR amplicons from soil DNA extracts for all time points of our slurry experiment. By integrating the fluorescence intensity of the different OTUs, relative archaeal SSU rDNA gene frequencies were quantified (Fig. 7). The major archaeal groups detected via T-RFLP analysis were identical to the most abundant lineages found in clone libraries, e.g., *Methanosarcinaceae*, RC-I, and *Methanosaetaeaceae*. But while the clone libraries displayed a large shift in relative composition over time (see above, Fig. 4), gene frequencies as determined by T-RFLP were rather constant, indicating a potential bias inherent in the procedure of cloning. Most prominent was the 389-bp OTU assigned to RC-I, but its frequency
FIG. 2. Phylegetic tree showing the placement of selected SSU rDNA clone sequences recovered from slurry samples after flooding of rice field soil (AS clones, days 0, 1, 8, and 17 [boldface]) within the Euryarchaeota. Selected sequences of cultivated representatives (nearly full-length rDNA) from curviculturals lineages as well as environmental sequences (partial sequences) from Verucilli rice field soil (ARB, ARK, EIOH, H2, ST1, ST5, and ST8 clones) (5, 15, 16, 24 and other environments as indicated were used as references to construct an evolutionary-distance dendrogram. SSU rDNA sequences of *A. pyrophilus* and *T. maritima* as well as members of the Cren- and Korarchaeota were used as outgroup references. The scale bar represents 1% sequence difference. Genbank accession numbers of sequences are indicated.
remained relatively constant over time, varying only between 30 and 35% of total archaeal gene frequency. The 182-bp OTU representing Methanosarcina-like and RC-VI populations was the only OTU which increased significantly in intensity during sequential reduction processes. A steady increase in the 182-bp OTU from 15% on day 0 to 29% of total archaeal gene frequency on day 13 was accompanied by an apparent relative decrease in especially the 88-bp, 389-bp, and 754-bp OTUs.

Using a novel T-RFLP assay with the fluorescently labeled forward primer Ar109F, we succeeded in differentiating Meth-
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![Graph showing the rarefaction analysis of the 120 archaean SSU rDNA clones from rice field soil slurry samples (days 0, 1, 8, and 17). Calculations were performed at the species (99 and 97% sequence similarity) and group level. The archaean groups were identical to those indicated in Fig. 4. Error bars indicate the variance of the expected number of species, E(S).](image)

anoarcina-like and RC-VI populations. This approach resulted in a different T-RF pattern for the same archaean community obtained in slurry soil samples (Fig. 6B). Most of the *Methanoarcina*-like and RC-I clones exhibited a common T-RF of 297 bp and therefore could not be separated, but highly conserved group-specific restriction sites were found within the *Methanoarcina*-like (611 bp) and RC-VI (532 and 604 bp) clones. Based on this novel T-RFLP assay, we observed that RC-VI gene frequencies equaled 1/3 of the *Methanoarcinaeae* gene frequencies on day 0, e.g., 25% of all amplicons represented in the 182-bp OTU. Over time, a doubling of the *Methanoarcinaeae* relative gene frequencies could be detected, while RC-VI frequencies decreased slightly. On day 17, RC-VI gene frequencies were only 1/6 of the *Methanoarcinaeae* gene frequencies, or 14% of the amplicons represented in the 182-bp OTU (using the Ar912r assay). Therefore, we concluded that the increase in the 182-bp OTU signal intensity was due to increasing *Methanoarcinaeae* gene frequencies within the first 13 days of incubation.

**DISCUSSION**

After flooding of rice field soils, methane production is typically initiated shortly after the reduction of electron acceptors such as nitrate, Fe(III), and sulfate. The sequence of these biogeochemical processes (metabolic activities) and the initiation of methane production observed in this study were in good agreement with previous reports (1, 39, 48, 49). Here, we focused on establishing a link between the observed changing biogeochemical processes (which ecosystem function) upon soil flooding to changes in archaean populations, especially the methanogens (which species).

The different molecular approaches used resulted in pronounced differences in community composition determined during the initiation of methanogenesis. Analysis of SSU rDNA clone libraries (Fig. 4) suggested significant shifts in archaean community compositions, whereas T-RFLP fingerprints exhibited a rather constant population structure when followed closely at daily intervals (Fig. 7). For example, after only 1 day of incubation, *Methanoarcina*-like and *Methanobacteriaeae* clones increased 10- and 5-fold in abundance, respectively. Subsequently, the same populations either decreased fourfold or disappeared completely after 7 days (Fig. 4).

Several lines of evidence suggest that most likely the cloning step was biased, resulting in large apparent fluctuations in the population structure. First, *Methanoarcina* spp. are characterized by slow growth (20). In fact, enumeration of acetotrophic methanogens in rice field soil required more than 40 weeks of incubation for the detection of these fastidious microorganisms (16). Although the in situ growth rates of these *Archaeae* in soils are largely unknown, it is unlikely that the *Methanoarcina*-like populations increased 10-fold within 1 day in soil slurry experiments. Moreover, high concentrations of methanogenic substrates, such as acetate, present during the first 8 days of incubation (Fig. 1B) are known to be more favorable for development of the faster-growing *Methanoarcina* spp., which

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*Sequence data are from this study (120 clones obtained on days 0, 1, 8, and 17; Fig. 4) and from previous studies (9, 15, 16, 24). The lengths of restriction fragments were predicted from aligned sequence data, assuming a labeled Ar912r primer. The numbers of clones within the different phylotypic lineages exhibiting a certain T-RF are indicated. Conserved group-specific T-RFs are in bold.*
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are adapted to higher acetate concentrations than are *Methanoanaella* spp. (20).

The strongest argument for a cloning bias, however, results from the comparison of the two methods employed in this study, cloning and T-RFLP analysis of SSU rDNA PCR products. We observed large shifts in community structure only with the cloning approach. Likewise, large fluctuations in archaean SSU rDNA sequence abundances in clone libraries were observed in a recent study of archaean communities before and after a temperature shift in methanogenic rice field soil (9). Again, T-RFLP analysis revealed less pronounced differences in community structure than were suggested by cloning analysis. On the other hand, in a study of a bioreactor sample, a combination of cloning and subsequent analysis of clones by

![Diagram](https://via.placeholder.com/150)

**FIG. 6.** Typical T-RFLP electropherograms of archaean SSU rDNA amplicons from a rice field slurry sample on day 1 after flooding. Fingerprints were generated using FAM-labeled primers Ar912r (A) and Ar109f (B). The archaean lineages represented in different OTUs are *Methanobacteria* (MB), *Methanomicrobiales* (MM), *Methanomarcinales* (MS), and RC-1 to RC-VI. T-RF lengths of selected peaks (in base pairs) are indicated.

![Diagram](https://via.placeholder.com/150)

**FIG. 7.** Archaean population dynamics in rice field soil slurries as determined by T-RFLP analysis. The figure shows the integrated fluorescence of each individual OTU. T-RFLP fingerprints were generated with the Ar912r FAM-labeled primer. Archaean lineages represented by the different OTUs are abbreviated as in Fig. 6. Data are means of replicate PCR analysis (n = 3). The average SD of single OTUs was below 1%.
ARDRA performed in three replications suggested that cloning appeared to be rather reproducible (12).

Several factors possibly influencing the abundance of sequences in clone libraries have been pinpointed, especially the PCR and the cloning procedure itself (for a review, see reference 45).

We cannot exclude an underlying PCR bias possibly affecting both methods, cloning and T-RFLP, because both methods rely virtually on the same PCR protocol (with the exception that primer Ar912r was fluorescently labeled for T-RFLP).

Since the observed large fluctuations in community composition over time were restricted to cloning analysis, we were prompted to conclude that the cloning step in particular was subject to bias.

Possible bias related only to the cloning procedure may be attributed to random error resulting from undersampling of diversity (45), toxicity of vector inserts to the transformed host, or the choice of cloning kit. Rainey et al. (unfortunately, no original data were provided in this short communication [37]) reported differences in SSU rDNA clone library composition depending on the cloning system used, i.e., blunt-end versus sticky-end cloning. In our study we used TA cloning for separating SSU rDNA PCR products amplified from environmental samples. Although widely used (54), the influence of bias related to this cloning protocol has not yet been analyzed with respect to the sequence distribution in SSU rDNA clone libraries.

Despite the principal caveat that clone abundance in libraries does not necessarily reflect population abundance in environmental samples (17), our SSU rDNA clone libraries contained valuable information about the sequence diversity of Archaea in rice field soil at different time points (i.e., days 0, 1, 8, and 17) after flooding. We detected most of the archaean groups (Methanococciaceae, Methanosphaeraceae, Methanomicrobiales, Methanobacteriales, RC-I to -IV, and RC-VI) detected previously in studies of rice field soils from Vercelli focusing either on the diversity (15, 16, 24) or on community structure changes after a temperature shift (9). We failed to detect sequences related to the novel RC-V previously shown to be present in bulk soil of 90-day-old microcosms (15). However, it is still unknown whether RC-V as well as the other as yet uncultured Archaea assigned to RC-I to -VI (15, 16) are methanogenic. Their relevance during the initiation of methanogenesis in rice field soil remains to be elucidated. It is striking that although the soil was sampled in different years and certainly not at exactly the same site near the Vercelli Rice Research Institute, the majority of our SSU rDNA clones were closely related or even identical to previously reported archaean sequences. Only a few clones were more distantly related, with up to 7% sequence differences from their closest previously reported relatives.

As indicated by rarefraction analysis, our clone libraries (n = 120) did not fully cover the phylotype richness of Archaea in Vercelli rice field soil (Fig. 5). However, rarefraction analysis focusing on the level of lineages detected revealed that it was unlikely to detect sequences representing additional or novel archaean lineages (Fig. 5). Moreover, the high similarities of our SSU rDNA sequences to those from previous studies on Vercelli rice field soil also suggested that we would not detect novel phylotypes by analyzing additional clones.

By using a large data set comprising 267 clones from our study, we obtained a similar PCR assay in the previous studies mentioned above, we were able to confirm previously detected restriction sites for the major phylogenetic lineages present in Vercelli rice field soil (Table 1) (9). Moreover, we could show a high degree of conservation of group-specific T-RFs for most of the lineages present (Table 1). For example, 95% of frequently found clones related to Methanosaetae spp. had a T-RF of 182 bp and 89% of all RC-I clones had a T-RF of 308 bp. On the other hand, clones related to Methanosaetae spp. shared the 182-bp T-RF with RC-VI clones. These RC-VI clones were also a frequent group and thus had to be considered for the analysis of relative gene frequency of this OTU.

We emphasize that the group specificity of restriction sites described here is valid only in the context of sequences retrieved from the same environment. Other sequences not detected in Vercelli rice field soil by PCR or cloning and belonging to other taxonomic groups may share common restriction sites with sequences retrieved from rice field soil samples (e.g., Methanospirillum sp. and RC-I) due to the conserved nature of the SSU rRNA gene. Knowing these limitations but equipped with the detected degree of conservation of TaqI restriction sites, we were able to expand the utility of the T-RFLP analysis considerably.

All predicted OTUs were actually found in direct T-RFLP analysis of slurry from arable land samples (unpublished data). To further analyze the temporal dynamics, we extended the T-RFLP analysis from a qualitative assessment of OTUs to a quantitative assessment of relative gene frequencies by integrating peak areas of individual OTUs. T-RFLP analysis was highly reproducible, as indicated by the relatively constant gene frequencies of most OTUs measured at daily intervals over 17 days (Fig. 7). PCR replications (n = 3) were also highly reproducible, as indicated by a low average standard deviation (SD) (<1%) for all T-RFs. A similar approach was used successfully by Suzuki et al. (43) to determine relative gene frequencies of fluorescently labeled SSU rDNA PCR fragments of different lengths.

In general, the relative gene frequency of most detected OTUs (Fig. 7) remained rather constant over time during the first 17 days after flooding of the soil. Most minor populations (as, Methanocoribacteriales and RC-III) and Methanospirillales apparently did not change at all in frequency. Notably RC-I (37 to 30%) and to a lesser extent RC-IV as well as Methanobacteriales gene frequencies decreased over time. The remarkable stability of the archaean populations after the flooding of the soil is in contrast to the changes in environmental parameters such as redox potential (40), availability of electron acceptors (Fig. 1A), and availability of electron donors such as acetate and hydrogen (Fig. 1B). Denitrification intermediates were probably too low in concentration in our slurry experiments to affect methanogenesis by nitric oxide inhibition (21), as indicated by the low initial nitrate concentration (<116 μM).

The most striking point was the significant increase in relative gene frequency of the 182-bp OTU comprising Methanocoribacteriales and RC-VI populations from 15 to 29% over 13 days (Fig. 7). By using a novel T-RFLP assay (i.e., with the fluorescently labeled primer Ar1096) (Fig. 6b), which allowed the differentiation of these two groups, we could demonstrate that in fact only the relative gene frequencies of the Methanocoribacteriales increased. Theoretically, we have to consider that an increase in relative gene frequency of the Methanocoribacteriales could be linked to a decrease in the absolute gene frequencies of the other populations, caused, for instance, by predatory protozoa (11) and phages (4). However, it is more likely that growth of Methanocoribacteriales-like populations occurred because of the energetically permissible concentration of acetate and hydrogen during the initiation of methanogenesis (Fig. 1B). We detected a similar temporal pattern of community structure change in a study analyzing the effect of soil aggregate size on the initiation of methanogenesis (37a). By
T-RFLP analysis, Chin et al. (9) also detected an increase in the OTU comprising Methanosarcinae neces and RC-VI upon anoxic incubation of methanogenic rice field soil and an even stronger increase in the RC-I-related OTU. The different incubation temperature (30°C) used in their study as well as other factors (i.e., soil sample, acetate accumulation to higher values, and lower methane production rate) may be responsible for differences in community structure changes from what was found in our study.

After the complete reduction of sulfate (day 4), acetate concentrations accumulated to a second maximum of about 600 μM around day 6, whereas hydrogen concentrations remained below 6 μM. Apparently, the ac trogenic activity of methanogens was the limiting factor during the initiation of methanogenesis and led to the observed accumulation of acetate. This is in agreement with results from methyl fluoride inhibition experiments in anoxic rice field soil, which suggested that hydrogenotrophic methanogenesis dominates the initial phase of methane production (40). In addition, Chidhasaiga and Conrad (7) also reported that acetate utilization by methanogens was slower than acetate production from glucose in rice field soil during the reduction phase (i.e., in the presence of sulfate and FeC3). The second increase in acetate concentration was well correlated with the steady increase in the Methanosarcinae OTU (Fig. 7). From day 6 on, acetate concentrations decreased consistent with acetotrophic methanogenesis, e.g., by Methanosarcina spp.

Another important observation with respect to a link between metabolic activity and population dynamics was that the Methanosarcinae still increased (day 13; Fig. 7) when pore water concentrations of acetate (Fig. 1B) were well below the known threshold (>200 to 300 μM) for growth of Methano sarinae spp. Methanothrixaceae populations apparently could not benefit from their lower threshold for acetate and grow, as indicated by constant gene frequencies of the 28S-rp T-RF. Most likely, the versatile Methanosarcinae spp. switched to other electron donors, such as hydrogen or methanol (not measured). Interestingly, formate was present in a concentration of up to 120 μM between days 9 and 13 (Fig. 1B), and Methano sarinaeaceae did not increase further after formate was depleted below the detection limit. Although formate cannot be utilized by the known Methanosarcinae spp., formate is probable in equilibrium with H2/CO2 as catalyzed by fermentative bacteria. Typically, only small amounts of methane are formed, probably from H2/CO2 during the initiation of methanogenesis (40, 41). The small amounts of methane (1-156 μM; Fig. 1B) that we detected during the first 3 days after flooding of the soil are contradicted by a significant increase in gene frequency (4-14%) of Methanosarcinae-like populations. The small amounts of methane formed were probably not sufficient to explain the observed increase in Methanosarcinae-like populations by de novo growth. Further investigations are necessary to find out which methanogens actually become active during the important phase of initiation of methanogenesis. In a forthcoming study, we will therefore address this question by targeting directly the rRNA of Archaea. The rRNA content of cells is generally accepted as an indicator of the metabolic activity of microbial populations.

In summary, we observed that the population structure of Archaea remained remarkably constant over time after flooding of rice field soil. Only Methanosarcinae-like populations increased significantly in gene frequency relative to other populations. Probably due to a high organic matter content of the soil utilized, fermentation processes were vigorous. This was indicated by the initially high levels of acetate and hydrogen (day 1, Fig. 1). These conditions were obviously favorable for Methanosarcinae-like populations. Even the switch from hydrogenotrophic to acetotrophic methanogenesis was apparently not accompanied by a population shift but instead by a shift in activity of the Methanosarcinae-like populations, indicating that the same population could manage different ecosystem functions. We conclude that a functionally extremely dynamic ecosystem, a flooded rice field soil, was linked to a relatively stable archaeal community structure. This is in agreement with the temporal stability of the population structure observed in other environments such as cyanobacterial hot spring mats (13) as well as oxic (19) and anoxic rice field soils (9, 37a). On the other hand, ecosystems such as an apparently functionally stable methanogenic hot spring mat (12) or a closed rice field system may exhibit an extremely dynamic community structure (12), indicating that a general principle for microbial community dynamics relative to ecosystem function is not yet feasible.

ACKNOWLEDGMENTS

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REFERENCES


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C.3. Ergebnisse

C.3. Auswirkungen der Zugabe von Ferrihydrit und Gips auf die Struktur und Aktivität methanogener Populationen im Reisfeldboden

Tillmann Lüders und Michael W. Friedrich

Effect of ferrihydrite and gypsum amendment on structure and activity of methanogenic populations in rice field soil

Tillmann Lüders and Michael W. Friedrich

Abstract

The effects of alternative electron acceptor supplementation on methanogenesis and methanogen population dynamics after the flooding of Italian rice field soil were studied. Methanogenic population dynamics were followed by archaeal SSU rDNA- and rRNA-based terminal restriction fragment length polymorphism (T-RFLP) analysis, and by quantitative SSU rRNA hybridization for major methanogenic lineages, including the novel (presumably hydrogenotrophic) methanogens of rice cluster I (RC-I). T-RFLP patterns were evaluated using relative signal intensities of terminal restriction fragments (T-RFs) and principal component analysis (PCA) to compare temporal community changes. Our study revealed considerable effects of ferrihydrite and gypsum amendment on methanogenic population dynamics. The amendment of ferrihydrite and gypsum stimulated the activity of iron-reducers and sulfate-reducers, respectively, and inhibited methanogenesis. Kinetics and extent of inhibition varied for the different treatments. In natural rice field soil, a strong increase of *Methanosarcina* spp. populations within the first 10 days after flooding was detected for both SSU rDNA and rRNA abundance, which correlated with high substrate availability and indicated population growth. For *Methanosaeta* spp., increasing SSU rRNA amounts were detected after 10 days, when pore water acetate concentrations decreased below 100 µM, while SSU rDNA ratios remained constant. This indicates an activation of these acetotrophic methanogens without significant changes in population size. Under ferrihydrite amendment, acetate was consumed very efficiently and a rapid inhibition of methanogenesis after 3 days was observed. Thus, a high iron-reduction potential capable of immediate successful competition with methanogens was naturally present. However, inhibition was incomplete and low amounts of methane were constantly produced. Acetate was always present in low concentrations (~60 µM), and, while methanosarcinal population dynamics were largely suppressed, an even stronger increase of *Methanosaeta* spp. SSU rRNA was
observed compared to the unamended soil. Unexpectedly, the rRNA of RC-I methanogens was transiently stimulated under ferrihydrite amendment, while only little methane was produced. We speculate that energy conservation of these methanogens might have been transiently coupled to iron(III)-reduction. The addition of gypsum resulted in a rapid and efficient consumption of hydrogen, but acetate depletion was nearly unchanged compared to the control. Inhibition of methane production only became apparent after 7 days, and an absolute inhibition was observed after 10 days. This coincided with a depletion of all major substrates, which indicates that only H$_2$-dependant sulfate-reduction potential was intrinsically present, while acetate-utilizing sulfate-reducers first had to be activated. Consistently, the suppression of acetotrophic methanogenic population dynamics was less pronounced as under ferrihydrite amendment. SSU rRNA quantities of the novel RC-I methanogens were lowest under gypsum amendment, which underscores the role of sulfate-reducers as key competitors for H$_2$. In this study we could show, that different mitigation strategies based on the supplementation of alternative electron acceptors induce distinct functional and structural reactions within the methanogenic microbial community in Italian rice field soil.

Introduction

Over 50% of the global annual methane emissions are of anthropogenic origin and the cultivation of irrigated rice may account for up to 20% of this efflux (22). Methane is an important greenhouse gas, since it efficiently absorbs infrared radiation (11). Its atmospheric mixing ratio is 1.745 ppm at present and has increased by 7 ppb a$^{-1}$ in average over the last 10 years (22). In the context of planetary warming, the reduction of global methane production is of great importance and several mitigation strategies to reduce rice paddy emissions have been proposed (for recent reviews see (26,66)). Directly after flooding of rice field soils common electron donors (i.e. acetate, H$_2$) for anaerobically respiring microorganisms are present in excess due to the vigorous fermentation of organic matter and methanogenesis occurs in parallel to sulfate- and iron-reduction (1,41,50). Once electron acceptors other than CO$_2$ have been reduced in the bulk volume of the soil methanogenesis is the dominating terminal respiratory process (7). When electron donors for respiratory processes become limiting, methanogenesis can be suppressed by the supplementation of alternative electron acceptors such as iron(III) or sulfate (1). This mitigation strategy is based on the thermodynamic theory, which predicts that the
energetically more favorable electron acceptor will be utilized first under substrate limiting conditions (67). Microorganisms, which can reduce the energetically more favorable electron acceptor (e.g. nitrate, iron(III), sulfate) will out-compete those using the less favorable electron acceptor (e.g. CO₂) (30). The thermodynamically superior functional group is believed to have either a higher affinity (e.g. lower Kᵣₚ) (25,49), and/or a higher Vₘₐₓ (63), and/or a lower threshold for common substrates (1,8,29,52).

The mitigative potential of electron acceptor supplementation in the form of amorphous iron(III) oxides (i.e. ferrihydrite, (1,4,23)), sulfate containing fertilizers (i.e. ammonium sulfate, (10,27,53)), or other sulfate containing amendments (i.e. gypsum or phosphogypsum, (14,28)) has been demonstrated in field and laboratory studies and reductions in CH₄ emission rates of >70% have been reported (15,23). The out-competition of methanogens for common substrates like H₂ or acetate by ferric-iron-reducing bacteria (FRB) or sulfate-reducing bacteria (SRB) is well understood on thermodynamic and process-based level. Conversely, little is known about the effects of alternative electron acceptor supplementation on the community structure of methanogens in rice field soil. How rapidly can inhibitory effects be observed, and are they reflected in structure and dynamics of the methanogenic population or in rRNA expression levels? This knowledge is important for the better understanding of mitigation mechanisms, and hence also for possible implementations for rice agriculture.

The aim of this study was to monitor the effects of different mitigation strategies on the methanogenic community in rice field soil using tools of molecular microbial ecology. Slurries of rice field soil were amended with ferrihydrite and gypsum, and, together with an unamended control series, incubated anoxically for 30 days. SSU rDNA and rRNA dynamics of major methanogenic lineages, including the novel methanogens of rice cluster I (RC-I) (19,32), were followed by terminal restriction fragment length polymorphism (T-RFLP) profiling. Furthermore, we determined SSU rRNA expression levels by PCR-independent quantitative rRNA hybridization. Our results revealed pronounced differences in methanogenic community structure and activity, which were well correlated to biogeochemical measurements and dependent on soil treatment. We could show that the supplementation of iron(III) and sulfate as alternative electron acceptors induces different functional and structural reactions within the methanogenic microbial community in Italian rice field soil.
Materials and Methods

Soil slurry experiments and chemical analyses. Rice field soil was sampled from a field of the Italian Rice Research Institute near Vercelli (Italy) in 1997 and stored as described previously (33). Soil slurry experiments were set up by adding 10 ml of sterile, anoxic, double-distilled water to 10 g of dry, sieved rice field soil in 60-ml serum vials. Supplementary electron acceptors were amended by adding 1.5% (wt:wt, 140 µmol g⁻¹) ferrihydrite (Fe(OH)₃), prepared as described by Jäckel and Schnell (23)) or 0.15% (8.7 µmol g⁻¹) gypsum (Ca₃SO₄ x 2 H₂O, Fluka, Buchs, Switzerland) to the dry soil, respectively. Triplicate vials for each time point (0 – 30 days) were sealed with butyl rubber septa, flushed with N₂, and incubated at 25°C. At the end of incubation, each vial was analyzed for gases (9) and slurry aliquots were taken and stored at -20°C for chemical analysis as previously described (33) and at -80°C for nucleic acid extraction.

Extraction of nucleic acids. DNA from soil samples was extracted as described previously (20) and purified using polyvinyl-polypyrrolidone (PVPP) spin columns (21). Total RNA was extracted using the protocol of Lüdemann et al. (35) with minor modifications. 600 µl slurry aliquots were placed into 2-ml FastRNA tubes with lysing matrix B (Qbiogene, Heidelberg, Germany) containing 700 µl pre-cooled TPM buffer (50 mM Tris-HCl [pH 7.5], 1.7% [wt/vol] polyvinylpyrrolidone, 10 mM MgCl₂ (17)). The mixture was shaken for 40 s at maximum speed in a FastPrep 120 bead beater (Qbiogene) and cell debris and soil particles were sedimented by centrifugation (4 min, 20,000 g, 4°C). The supernatant was saved, while the pellet was re-extracted with 700 µl of a phenol-based lysis buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1% [wt/vol] sodium dodecyl sulfate, 6% [vol/vol] water-saturated phenol) by a second round of bead beating. After centrifugation, the combined supernatants were extracted with 500 µl each of water-saturated phenol, phenol-chloroform-isoamyl alcohol (25:24:1 [vol:vol:vol]), and finally chloroform-isoamyl alcohol (24:1 [vol:vol]) using 2-ml Phase Lock Gel Heavy cups (Eppendorf, Hamburg, Germany) for the last two steps to facilitate the clean separation of aqueous and organic phases and thereby maximize RNA yield. Total nucleic acids were precipitated from the aqueous phase with 3 volumes of ethanol and 0.1 volume of 3 M sodium-acetate by freezing (-80°C, overnight) and centrifugation (1 h, 20,000 g, 4°C). The precipitate was washed once with 250 µl of cooled 70% ethanol, dried and re-suspended in 50 µl of EB buffer (10 mM Tris HCl, pH 8.5, Qiagen, Hilden, Germany). RNA extracts were further purified by gel filtration with Sephadex G-75 spin columns (37) to remove residual humic acids. Extracted nucleic acids were checked by standard gel electrophoresis on a 1% agarose gel using DNA SmartLadder (Eurogentec, Seraing, Belgium) or E. coli 16S and 23S rRNA (Roche Diagnostics, Mannheim, Germany) as reference and ethidium bromide staining. Furthermore, nucleic acids extracts were quantified by standard UV photometry (BioPhotometer, Eppendorf).

PCR and RT-PCR amplification. PCR amplification of archaeal SSU rRNA genes for T-RFLP was done with slurry DNA extracts as described before (33) using the primer combination Ar109f (5'-ACK GCT CAG TAA CAC GT-3') (18) and a 5' 6-carboxyfluorescein (FAM)-labeled Ar912rt primer (5'-GTG CTC CCC CAA TTC CTT TA-3') (MWG Biotech, Ebersberg, Germany). This modification of the original Ar915r primer (57) carries a 3 bp elongated 3'-end and was designed to minimize non-specific primer binding during reverse transcription, especially as the selectivity of the original oligonucleotide has been shown to be questionable recently (56). The elongation of the primer has little effects on the absolute numbers of archaeal target sequences with 0 – 4 mismatches in our ARB database (data not shown), but
drastically reduces the numbers of non-target bacterial matches with 3 and 4 mismatches (from $n = 186$ to 8 and from 4213 to 195, respectively). Consequently, it allows a more efficient discrimination between archaeal target- and bacterial non-target-templates. This is especially important during reverse transcription, since RNA–DNA duplexes are more stable than DNA–DNA structures (51), and a low stringency temperature of 48°C had to be used. The specificity of archaeal rRNA-targeted T-RFLP fingerprints was verified by comparison of rRNA and rDNA derived profiles generated with both the original Ar915r and the modified Ar912rt oligonucleotides. While only defined terminal restriction fragments (T-RFs) were detected for rDNA profiles with both primers (profiles were practically identical), the new primer eliminated putative non-target amplicons (e.g. undefined T-RFs) below detection threshold for rRNA profiles (data not shown).

For reverse transcription, co-extracted DNA was removed from the slurry RNA extracts by DNase digestion of 1:10 diluted extracts. We added 5 µl of soil RNA to 5 µl of 10x DNase digestion buffer (200 mM Tris-HCl [pH 7.5], 100 mM MgCl₂, 20 mM CaCl₂), 10 U of RNase-free DNase I (Roche Diagnostics) and DEPC treated H₂O to a total volume of 50 µl. Incubation was for 1 h at 37°C, and heating to 80°C for 10 min stopped the reaction. Complete removal of DNA from the RNA extracts was verified by PCR (see above). RT-PCR was performed using the Access one-tube RT-PCR system (Promega, Hilden, Germany). The reaction mixture contained, in a total volume of 50 µl, 1x AMV/Tfl reaction buffer, 1 mM MgSO₄, 200 µM dNTPs, 0.5 µM of each primer (see above), 5 U of AMV reverse transcriptase, and 5 U of Tfl DNA polymerase. 1 µl of DNase treated, 1:10 diluted soil RNA extract was added as template. Reverse transcription was carried out for 45 min at 48°C, followed immediately by 28 amplification cycles (30 s, 94°C; 45 s, 52°C; 90 s, 68°C) and a terminal extension step (5 min, 68°C). After amplification, PCR aliquots (5 µl) were visualized by standard agarose gel electrophoresis. Amplicons were purified with the MinElute PCR purification kit (Qiagen), re-eluted in 25 µl of EB buffer and quantified by UV photometry.

**T-RFLP analysis.** Since rDNA-targeted T-RFLP analysis has been shown earlier to be highly reproducible with replicate DNA extractions (34,38), DNA was extracted only once from each time point and analyzed by triplicate T-RFLP profiling. T-RFLP analyses of rRNA were from triplicate RNA extractions for each time point. Restriction digests (TaqI, Promega) and T-RFLP analysis were performed as previously described (32). 1.25 µl of the digest were mixed with 0.95 µl of formamide loading dye (Amersham Pharmacia Biotech, Freiburg, Germany) and 0.3 µl of GeneScan-1000 (ROX) size standard (Applied Biosystems, Weiterstadt, Germany), denatured for 3 min at 95°C, and placed immediately on ice. T-RFLP analysis was performed on an ABI Prism 377 DNA sequencer (Applied Biosystems) in GeneScan mode. 1.9 µl of the fragment mixture were loaded into the wells of 36-well shark tooth comb on a 36 cm 5% (wt/vol) polyacrylamide gel containing 6 M urea and 1x TBE buffer (89 mM Tris-borate, 2 mM EDTA).

**Statistical analysis of T-RFLP data.** For statistics, T-RFLP data was evaluated as recently described (34,46) using SYSTAT 10 software (SPSS Inc., Chicago, Il.). Principal component analysis (PCA) was performed on T-RFLP profiles with each sample (soil treatment, time point, and targeted nucleic acids) as rows and the relative fluorescence intensities of T-RFs as columns. A covariance data matrix was extracted with pairwise deletion and varimax factor rotation. Data reduction provided a two-factorial ordering of the variance of T-RFLP profiles, which was plotted on a map.

**Quantitative rRNA hybridization.** Non-radioactive slot-blot quantification of environmental RNA extracts was performed using the digoxigenin (DIG) system (Roche Diagnostics) as recommended by the
manufacturer and described by Manz et al. (36) with minor modifications. In order to analyze all 18 different rRNA extracts (3 treatments, 6 time points) together with an appropriate standard dilution series on one membrane for each probe, the RNAs originally extracted in triplicate from each sample were blotted in duplicate using a Hoefer PR 648 slot blot manifold (48 slots, Amersham Pharmacia Biotech). All hybridization, washing and detection steps were done in 15-cm hybridization tubes (Ochs, Göttingen, Germany) in an OV4 hybridization oven (Whatman Biometra, Göttingen, Germany). After pre-hybridization, 50 pmol of DIG-labeled probe (MWG Biotech) were hybridized to the membrane-bound rRNA overnight at 40°C in 3 ml hybridization buffer (5x SSC, 5x Denhardt's solution, 1% blocking reagent (Roche), 0.1% N-lauroylsarcosine, 0.1% SDS). Washing steps were carried out for 30 min in 1x SSC, 1% SDS (pH 7) twice at 40°C and once at the probe-specific stringent washing temperatures $T_d$ (48,57,62). DIG-labeled probes were then detected with Fab fragments of anti-DIG antibodies conjugated to alkaline phosphatase (Roche Diagnostics) and the ECF fluorescence substrate (Amersham Pharmacia Biotech) on a Storm 860 gel and blot imaging system (Molecular Dynamics, Sunnyvale, Calif.) (59). Blot images were quantitatively evaluated using ImageQuant 5.0 (Molecular Dynamics).

**Oligonucleotide probes and reference RNAs.** The following oligonucleotide probes were used in this study: S-*-Univ-1392-a-A-15 (39), S-D-Arch-0915-a-A-20 (57), S-F-Mbac-0310-a-A-22, S-F-Msae-0825-a-A-23, S-G-Msar-0821-a-A-24 (48), and S-*-RCI-0645-a-A-23 (62). *E. coli* 16S and 23S rRNA (Roche) was used as universal standard. *Methanobacterium bryantii* DSM 863T, *Methanoseta concilii* DSM 3671T and *Methanosarcina barkeri* DSM 800T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and grown in standard media as recommended by the DSMZ. RNA was extracted from harvested cell pellets following the above protocol as archaeal and family-specific standards. For the quantification of RC-I Archaea, 16S rRNA of a nearly full-length RC-I clone (AS04-16, accession number AJ308972) was in vitro transcribed (43) as previously described (62).

**Results**

**Biogeochemistry of reduction processes.** Reduction processes and transient accumulation of H$_2$, acetate, propionate, isovalerate and caproate in all three parallel experiments (unamended, ferrrihydrite- and gypsum amended) were followed over 30 days (Fig. 1). The duration and rates of reduction processes are summarized in Table 1. Fe$^{3+}$-reduction in the unamended soil was terminated within 8 days (Fig. 1A). The addition of ferrrihydrite (140 µmol g$^{-1}$ added) elongated the initial phase of rapid Fe$^{3+}$-reduction (Fig. 1D) and steady reduction rates were 4.8 µmol d$^{-1}$ g$^{-1}$ in average (Table 1). Porewater SO$_4^{2-}$-concentrations in the unamended soil were depleted within 5 days (Fig. 1A). The addition of ferrrihydrite drastically reduced maximum SO$_4^{2-}$-concentrations to ~120 µM (Fig. 1D).
C.3. Ergebnisse
C.3. Ergebnisse

FIG. 1. Time course of reduction processes in unamended (A, B, C), ferrihydrite-amended (D, E, F), and gypsum-amended (G, H, I) rice field soil slurries. (A, D, G) Concentrations of SO\(_4^{2-}\) and Fe\(^{2+}\), and CH\(_4\) partial pressure. (B, E, H) H\(_2\) partial pressure and concentration of acetate. (C, F, I) Concentrations of propionate, isovalerate, and caproate (mean ± SD, n = 3).

TABLE 1. Rates of CH\(_4\)-production, Fe\(^{3+}\)-reduction, and SO\(_4^{2-}\)-reduction in unamended, ferrihydrite-, and gypsum-amended rice field soil slurries within 30 days of anoxic incubation; termination and inhibition time points of different processes; and contribution of different respiratory processes to total reducing equivalents formed.

<table>
<thead>
<tr>
<th>Soil Treatment</th>
<th>Process</th>
<th>Product formation [µmol d(^{-1}) g(^{-1})] (between days)</th>
<th>CH(_4) formation [µmol d(^{-1}) g(^{-1})] (between days)</th>
<th>Reductant formed [µmol g(^{-1})]</th>
<th>Total reducing equivalents formed [µmol g(^{-1})] (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Maximum</td>
<td>Initial</td>
<td>Steady-state</td>
<td>Inhibited</td>
</tr>
<tr>
<td>No amendment</td>
<td>CH(_4)-production</td>
<td>1.25</td>
<td>0.83</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d09)</td>
<td>(d04-d10)</td>
<td>(d11-d30)</td>
<td>-</td>
</tr>
<tr>
<td>Ferrihydrite amendment</td>
<td>CH(_4)-production</td>
<td>0.38</td>
<td>0.15</td>
<td>-</td>
<td>0.08 (d03-d30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d28)</td>
<td>(d03)</td>
<td>-</td>
<td>(d03-d30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d03)</td>
<td>(d01-d07)</td>
<td>4.8</td>
<td>(d07-d20)</td>
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<tr>
<td></td>
<td></td>
<td>(d04)</td>
<td>(d01-d05)</td>
<td>-</td>
<td>(d01-d05)</td>
</tr>
<tr>
<td></td>
<td>SO(_4^{2-})-reduction</td>
<td>0.10</td>
<td>0.05</td>
<td>-</td>
<td>(d02-d06)</td>
</tr>
<tr>
<td>Gypsum amendment</td>
<td>CH(_4)-production</td>
<td>0.84</td>
<td>0.53</td>
<td>-</td>
<td>&lt;0.01 (d11-d22)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d06)</td>
<td>(d04-d10)</td>
<td>-</td>
<td>(d11-d22)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d02)</td>
<td>(d01-d05)</td>
<td>-</td>
<td>(d01-d05)</td>
</tr>
<tr>
<td></td>
<td>SO(_4^{2-})-reduction</td>
<td>0.69</td>
<td>0.27</td>
<td>0.5</td>
<td>(d04-d12)</td>
</tr>
</tbody>
</table>

\(a\) Amount of either CH\(_4\), or Fe\(^{2+}\), or SO\(_4^{2-}\) reduced.

\(b\) The amount of product formed by each process was normalized to reducing equivalents [H]: CH\(_4\)-production (8 [H]), dissimilatory Fe\(^{3+}\)-reduction (1 [H]), or SO\(_4^{2-}\)-reduction (8 [H]) to S\(_2^\text{2-}\); SO\(_4^{2-}\)-reduction was measured by following disappearance of SO\(_4^{2-}\); the contribution of NO\(_3^-\)-reduction was neglected (<20 µM present initially).

Amendment of gypsum elevated SO\(_4^{2-}\) concentrations up to 6.5 mM (Fig. 1G). High SO\(_4^{2-}\)-reduction rates were observed only after day 8 and were 0.5 µmol d\(^{-1}\) g\(^{-1}\) in average (Table 1). After day 26, the amended SO\(_4^{2-}\) was depleted. Methane formation started directly after flooding (50) in all three experiments and increased to similar levels of ~140 Pa on day 3 (Fig. 2). In the unamended soil, methane mixing-ratios continued to increase, production rates were constant 0.5 µmol d\(^{-1}\) g\(^{-1}\) in average after day 10. Under ferrihydrite
amendment, CH₄-production was strongly suppressed after day 3 (Figs. 1D, 2), but mixing ratios still increased with low constant production rates of 80 nmol d⁻¹ g⁻¹ thereafter. In the gypsum-amended soil, an inhibition of CH₄-production only became apparent after day 6, but was absolute between day 11 and 22 (Figs. 1G, 2, Table 1). Only after the end of sulfate reduction (day 26), methane mixing ratios started to increase again in low amounts. Total methane emissions during the experiment were reduced by 85% under ferrihydrite-, and by 69% under gypsum-amendment.

H₂, produced by vigorous fermentation processes directly after flooding, accumulated up to ~92 Pa on day 1 in all parallels (Fig. 1). Hydrogen was depleted to ~1 Pa on day 4 in all three experiments, but remained below 1 Pa (0.3 Pa in average between days 13 and 20) only under gypsum-amendment (Fig. 1H). In the other two series, H₂ partial pressures increased again to a second transient maximum of ~10 Pa around day 9, and decreased to low levels of ~0.5 Pa towards the end of incubation (Figs. 1B, E). Maximum acetate concentrations (~2 µM) also accumulated on day 1 after flooding (Fig. 1). Acetate consumption was most efficient in the ferrihydrite series, concentrations rapidly decreased to constant ~60 µM from day 4 onward (Fig. 1E). In the other two series, depletion was much slower and reached concentrations below 100 µM only after 8 - 10 days. But while acetate slowly decreased from 60 to 25 µM after day 11 in the unamended slurries (Fig. 1B), it was depleted below detection limit (~5µM) between day 12 and 26 in the gypsum series (Fig. 1H). Other volatile fatty acids, like propionate,
isovalerate and caproate, transiently accumulated to substantial concentrations in the
unamended experiment (Fig. 1C). Under ferrihydrite supplementation, depletion of all
three compounds was accomplished on day 4 (Fig. 1F), while in the gypsum series only the
consumption of propionate was similarly efficient (Fig. 1I).

**T-RFLP fingerprinting of archaeal SSU rDNA and rRNA dynamics.** Nucleic
acids of all three flooding experiments were extracted from days 1, 5, 10, 16, 22, and 30
after flooding and analyzed by T-RFLP. As an example, T-RFLP electropherograms of
amplicons generated with archaeal rDNA and rRNA templates, which were extracted from
unamended slurries on day 16 after flooding, are shown in Fig. 3.

![T-RFLP electropherograms](image)

FIG. 3. Example of T-RFLP electropherograms generated with archaeal SSU rDNA (A) and SSU rRNA (B)
amplicons from unamended slurry samples 16 days after flooding. T-RFs (lengths in bp) affiliated with
major methanogenic lineages are *Methanobacteriaceae* (MB), *Methanosarcinaceae* (MS), *Methanosaetaceae*
(MX), and rice cluster I (RC-I) are indicated and have been described previously (6,33,46). RFU, relative
fluorescence units.

Relative population dynamics within the archaeal communities of the three flooding
experiments as revealed by combined rDNA- and rRNA-targeted T-RFLP analysis are
shown in Fig. 4. Structure and compositional shifts of the natural archaeal community
(Fig. 4A) were similar to those found recently (33,45). Most prominent were the T-RFs
characteristic for RC-I (392 bp) and *Methanosarcina* spp. (185 bp), both contributing
~30% relative amplicon frequency (RAF) in average.
FIG. 4. Archaeal population dynamics in unamended (A, B), ferrihydrite-amended (C, D), and gypsum-amended (E, F) rice field soil slurries as determined by SSU rDNA (A, C, E) and SSU rRNA (B, D, F) targeted T-RFLP analysis. Shown is the percentage of individual T-RFs relative to the total integrated fluorescence (mean ± SD, n = 3 T-RFLP replicates for rDNA and 3 extraction replicates for rRNA). Major methanogenic lineages represented by selected T-RFs are abbreviated as in Fig. 3. RAF, relative amplicon frequencies.
While the methanosarcinal T-RF more than doubled from 17 to 39% between day 1 and day 10, the RC-I T-RF decreased from 37 to 25% concomitantly. Also abundant were T-RFs with 283 bp (Methanosaetaceae, ~11%) and 91 bp length (Methanobacteriaceae, ~9%). The T-RFLP profiling of SSU rRNA revealed an even more pronounced dynamic of Methanosarcina spp., since their T-RF increased to 47% RAF on day 10 (Fig. 4B). Also the methanosaetal T-RF was always more abundant in rRNA profiles and doubled its RAF from 13% on day 5 to 26% on day 30. The RC-I T-RF remained stable (~21%), but was always less abundant in rRNA profiles. rRNA-based RAFs of the Methanobacteriaceae were only initially comparable to rDNA ratios, but decreased to ~3% from day 10 onward.

Under ferrihydrite amendment, SSU rDNA-based population dynamics of Archaea as observed in the unamended rice field soil were largely suppressed (Fig. 4C). Only methanosarcinal RAFs increased temporarily from 16 to 24% between days 1 and 5. All other major T-RFs were relatively stable during incubation. On rRNA level, similarly reduced methanosarcinal dynamics were observed. Towards the end of the experiment the rRNA-based RAF of Methanosaeta spp. increased (Fig. 4D) even more pronounced than in unamended soil and reached ~27% already after 22 days. Differences were also detected for RC-I SSU rRNA RAFs, they increased from 19 to 27% within the first 5 days after flooding and remained constant (~23%) thereafter. Addition of gypsum, on the other hand, resulted in archaeal rDNA dynamics quite similar to unamended soil. Again, a strong, but less marked increase of Methanosarcina spp. amplicon ratios was observed with time, while all other T-RFs remained relatively stable (Fig. 4E). Differences to the unamended soil became evident only by rRNA profiling (Fig. 4F). Methanosarcinal frequencies reached a maximum after a strong initial increase already on day 5, decreased thereafter but increased again slightly on day 30. The frequencies of RC-I rRNA, however, were only ~17% in average and always reduced compared to both unamended and ferrihydrite-amended rice field soil.

**Statistical analysis.** PCA is a preferred method for reduction of complex data sets and has been frequently used to facilitate the comparison of microbial communities with data derived from signature fatty acid or amplified gene restriction patterns (16,42,44,47). PCA of T-RFLP profiles was done to visualize patterns of time-dependent community shifts within the three differentially treated soils by generalizing variations in archaeal SSU rDNA and rRNA pools. To enhance visual clarity, PCA scores of averaged triplicate T-
RFLP results were plotted. The total variance of T-RFLP fingerprints was reduced to two hypothetical principal components, which explained 62 and 28% of variance, respectively. The factorial map (Fig. 5) shows the position, in ordination space, of archaeal SSU rDNA and rRNA pools in the three soils at different time points.

FIG. 5. Principal component ordination of the overall variance between averaged triplicate T-RFLP results generated with archaeal SSU rDNA and rRNA amplicons of natural, ferrihydrite-, and gypsum-amended rice field soil. Successive time points [days] for each treatment and nucleic acid are connected by lines and indicated by numbers to illustrate time-dependent population shifts.

PCA allowed for a clear separation of rDNA and rRNA derived profiles in ordination space. Furthermore, data points of day 1 after flooding were grouped for both rDNA and rRNA, and thus indicate closely related communities with similar T-RFLP patterns. Chronological rDNA dynamics within the natural and gypsum-amended slurries were then quite similar, e.g. shifts in principal component ordination ran in parallel and end points were closely grouped. Furthermore, PCA clearly showed that the ferrihydrite rDNA time series performed different and strongly reduced dynamics. For rRNA samples, consecutive shifts of principal component scores were more pronounced than for rDNA fingerprints (i.e. more dynamic). Here, the unamended and gypsum-amended time series again followed similar dynamics, but were better separated than for rDNA and clearly different from the ferrihydrite series.
Quantitative SSU rRNA dynamics. We performed quantitative hybridization probing of environmental rRNA extracts for the PCR independent quantification of methanogen-specific rRNAs. In addition to universal and Archaea specific probes, we used probes targeting the rRNA of *Methanosarcina spp*, members of the *Methanosaetaceae*, and *Methanobacteriaceae*, as well as the novel methanogens of RC-I (19,32).

Total amounts of extractable rRNA as detected by the universal probe decreased from ~6.6 µg g\(^{-1}\) to ~3.7 µg g\(^{-1}\) within the 30 days of incubation in all three flooding experiments (Fig. 6A). Total amounts of Archaea specific rRNA (Fig. 6B) remained relatively constant (~1.45 µg g\(^{-1}\)) in unamended rice field soil over time, while they decreased in both ferrihydrite and gypsum amended slurries to ~1 µg g\(^{-1}\) (day 30). Relative to the total extractable rRNA (universal probe), the percentage of archaeal rRNA was ~22% on day 1 in all slurries and 36, 30, and 27% on day 30 in the unamended, ferrihydrite- and gypsum-amended slurries, respectively.

Among the different methanogen-specific probes, the one targeting the rRNA of *Methanosarcina spp.* revealed the most significant quantitative dynamics (Fig. 6C). Between days 1 and 5, levels and increases were similarly in all three series. Obvious differences between the three treatments became evident on day 10 after flooding, when methanosarcinal rRNA quantities increased further to above 540 ng g\(^{-1}\) in the unamended slurries, to 405 ng g\(^{-1}\) under gypsum addition, but remained below 300 ng g\(^{-1}\) in the ferrihydrite series. On day 30 after flooding, methanosarcinal rRNA quantities again approached similar amounts of 350 - 400 ng g\(^{-1}\) in all three experiments. The rRNA dynamics of members of the *Methanosaetaceae* were different and less pronounced, quantities increased steadily with incubation time in all three flooding series (Fig. 6D). Final amounts were lowest (115 ng g\(^{-1}\)) under gypsum amendment, and, surprisingly, highest under ferrihydrite addition (223 ng g\(^{-1}\)). rRNA quantities of the *Methanobacteriaceae*, on the other hand, remained very stable with time in all slurries (~126 ng g\(^{-1}\) in average), with the exceptions of slightly elevated levels (168 ng g\(^{-1}\)) on day 5 under ferrihydrite amendment (Fig. 6E). Finally, we followed the rRNA dynamics of the yet uncultured methanogens of RC-I (32), using an oligonucleotide probe designed to specifically target this novel lineage (62). rRNA amounts of this lineage in the unamended soil were very stable, although they increased slowly from 361 to 409 ng g\(^{-1}\) during the experiment (Fig. 6F).
C.3. Ergebnisse

FIG. 6. Methanogenic population dynamics in unamended and electron acceptor-amended rice field soil slurries as determined by quantitative slot blot hybridization of environmental rRNA extracts (mean ± SD, n = 2 soil samples per timepoint). (A) Total rRNA, (B) archaeal rRNA, (C) *Methanosarcina* spp., (D) *Methanosetaeaceae*, (E) *Methanobacteriaceae*, (F) rice cluster I.

Dynamics were similar in the gypsum-amended slurries, however absolute levels were constantly ~100 ng g⁻¹ below those in natural soil. Surprisingly, under ferrihydrite addition, RC-I rRNA levels increased drastically from 280 to 462 ng g⁻¹ between day 1 and day 5 after flooding, and decreased again to ~330 ng g⁻¹ towards the end of the experiment.
Discussion

Recently, we found that the archaeal community structure in freshly flooded Italian rice field soil slurries was rather stable in a functionally highly dynamic ecosystem (33). Only *Methanosarcina* spp. were significantly stimulated by the availability of excess acetate and H$_2$ present during the first 13 days after flooding as detected by T-RFLP analyses of SSU rDNA. In this study, we induced major functional shifts on rice field soil microbial populations by amending either ferrihydrite or gypsum as electron acceptors to stimulate FRB and SRB as competitors of the methanogenic population for common electron donors (i.e. acetate, H$_2$). These treatments had significant effects on both, the methane production and the dynamics of methanogenic populations.

The dynamics of methanogens were followed by PCR/T-RFLP analysis of archaeal SSU rDNA and SSU rRNA, as well as by quantitative rRNA hybridization probing. This approach facilitates the comparative assessment of population dynamics (SSU rDNA abundance) and activity dynamics of populations as indicated by changes in rRNA content. It is generally assumed that the cellular rRNA content reflects the activity of microorganisms (3), however for most of the populations detected in molecular ecology studies the ribosome content under (varying) natural growth conditions is unknown. Nevertheless, we found that rRNA expression patterns may reveal population activity much more sensitively than this is possible by following the SSU rDNA abundance. For example, the increase of *Methanosarcina* spp. rRNA (Fig. 4, 6) was much more pronounced than the increase detectable for SSU rDNA (Fig. 4), which suggests both activation and growth of this population. On the other hand, *Methanosaeta* spp. exhibited doubling of rRNA content as detected by both T-RFLP (Fig. 4) and hybridization probing (Fig. 6), but no increase in rDNA ratio (Fig. 4), which suggests that this population was activated, but did not grow. In fact, these findings suggest an important principle for the population dynamics of methanogenic populations in rice field soil: Only fast growing populations such as *Methanosarcina* spp. increase their biomass if sufficient substrate is available. However, most other populations, i.e. *Methanosaeta* spp., RC-I and *Methanobacterium* spp. only regulate their rRNA content, while their population size remains rather constant. Similarly, the SSU rDNA derived composition of the methanogenic community in an anaerobic digester remained constant (prevalence of *Methanobacteriaceae*), while large shifts were detected via rRNA towards a prevalence of *Methanosaeta* spp. during 28 days an acetate crisis (13).
Addition of 1.5% ferrihydrite extended the phase of iron reduction to over 30 days compared to ~8 days in unamended soil. This was accompanied by a rapid, but incomplete inhibition of methanogenesis after day 3 and significant effects on the population dynamics of the methanogens, indicating that a potent population of FRB capable of successful competition with methanogens was indigenously present in the soil. However, the mechanism of inhibition of methanogenesis by ferrihydrite addition in rice field soil is not totally clear. Previous studies have shown that ferric iron reducers can out-compete methanogens for H\(_2\) in rice field soil (1) by lowering the H\(_2\) partial pressure to thresholds (>0.4 Pa) thermodynamically not permissive for methanogenesis (31). In principle, competition for acetate should be similar to H\(_2\) (4), but the experimental data available are contradictory with respect to the underlying mechanism of inhibition. FRB were shown to either maintain a lower threshold for acetate in Texas rice field soil than methanogens (55) or have a similar threshold as found in Italian rice field soil (2).

Different from these previous observations, in our experiments neither the H\(_2\) partial pressures nor the acetate thresholds were lower in ferrihydrite-amended slurries compared to unamended soil, albeit methanogenesis was strongly (but not completely) inhibited (Table 1). Especially the second transient accumulation of H\(_2\) to up to ~10 Pa on day 9, when iron was reduced at a rate of ~7.5 µmol d\(^{-1}\) g\(^{-1}\), indicates that H\(_2\) was a less competitive substrate for FRB than fatty acids. In contrast, acetate and other short chain fatty acids were consumed more rapidly in ferrihydrite amended slurries than in controls, but threshold concentrations for acetate were similar and did not decrease below 60 µM. This concentration is still permissive for acetotrophic methanogenesis by Methanosaeta spp. (24,68). The lack of full inhibition of methanogenesis may be attributable to a decreasing bioavailability of Fe\(^{3+}\) as indicated by the progressively slowing of the iron reduction rate from day 7 onward (Fig. 4D). Only ~200 µmol Fe\(^{3+}\) g\(^{-1}\) of available 240 µmol g\(^{-1}\) (~100 µmol g\(^{-1}\) indigenous plus 140 µmol g\(^{-1}\) added) had been reduced after 30 days. It cannot be ruled out that the synthetic ferrihydrite utilized for amendment was less bioavailable for FRB due to a higher degree of crystallinity compared to the indigenous ferrihydrite (54).

The low acetate concentrations observed (see above) corroborate both, the increase of methanosaelal rRNA (Fig. 4D, Fig. 6D), which was strongest under ferrihydrite amendment, and the suppressed dynamics of Methanosarcina spp. (Fig. 4D), as indicated by rDNA and rRNA analysis. Most likely, the acetotrophic Methanosaeta spp. contributed
substantially to the low amounts of methane produced in spite of ferrihydrite mediated inhibition, whereas Methanosarcina spp. were apparently out-competed for both, hydrogen and acetate by FRB after day 4. The minor initial increase of Methanosarcina spp. was likely to be related to excess substrate availability until day 4 (Fig. 1). Unexpectedly, RC-I methanogens were significantly stimulated temporarily (up to day 5) only under ferrihydrite amendment as indicated by rRNA analysis (Fig. 4D, 6F) as were members of the Methanobacteriaceae albeit to a lesser extent (Fig. 6E). Enrichment cultures of the novel RC-I methanogens have been shown to grow on H\textsubscript{2}/CO\textsubscript{2} (32), and also the members of the Methanobacteriaceae are hydrogenotrophic (68). The transient stimulation of these hydrogenotrophic methanogens was paralleled by an increase of the total archaeal rRNA content between day 1 and 5, which was strongest under ferrihydrite amendment (~160 ng g\textsuperscript{-1}). In the same samples, no rRNA-stimulation of the acetotrophic Methanosarcina and Methanosaeta spp. was observed. Furthermore, in the ferrihydrite-amended slurries, initial sulfate concentrations were much lower than in the control, probably due to sulfate-anion adsorption to ferric iron minerals (5,40,58). Hence the activity of SRB, major competitors for H\textsubscript{2} (8), was also reduced. Thus, under ferrihydrite amendment, it is likely that a much larger fraction of the initially produced H\textsubscript{2} was available for hydrogenotrophic methanogens than in unamended soil. However, the strong transient increase of especially RC-I rRNA cannot be explained considering the energy available from the small amount of methane actually produced within the first 5 days under ferrihydrite amendment. Rather, we speculate that under these conditions energy conservation of hydrogenotrophic methanogens was coupled to iron(III)-reduction, a process, which has been shown to occur in hyperthermophilic methanogens such as Methanopyrus kandleri and Methanococcus thermolithotrophicus (61).

Addition of 0.15% gypsum to the slurries increased pore water sulfate concentrations substantially, however without exceeding the maximum gypsum solubility of ~2 g l\textsuperscript{-1} (i.e. 11.6 mM). The phase of sulfate reduction was extended from 5 days after flooding in unamended soil to 26 days under gypsum amendment. However, the immediate metabolic potential of the intrinsic SRB population in Italian rice field soil was not sufficient to inhibit methanogens by substrate competition as indicated by the delayed inhibition of methanogenesis from day 6 on only. SRB populations required more than twice as much time than FRBs to induce similar inhibitory effects on the methanogens. In contrast to this longer "lag-phase", SRBs were capable of completely inhibiting methanogenesis after day
10, most likely after a sufficient population size was established, until sulfate finally was depleted. Interestingly, the activation of SRB populations and the gradual inhibition of methanogens were paralleled by two phases of substrate depletion. $H_2$ was depleted to mixing ratios as low as 0.3 Pa already after day 4 under gypsum amendment (Fig. 1H) and a second transient accumulation to up to 10 Pa as in the ferrihydrite- and un-amended slurries was not observed. Thus, a competitive population of $H_2$-utilizing SRB was indigenously present in Italian rice field soil and, upon addition of sulfate, capable of outcompeting methanogens for $H_2$ directly, which has been observed earlier (1,2).

Conversely, the consumption of acetate in gypsum-amended slurries was almost identical to the unamended soil initially and depleted below 100 µM only on day 9. Subsequently however, acetate was depleted below detection (<5 µM) limit between days 12 and 26 under gypsum supplementation. This was also the phase of constant sulfate reduction rates and complete inhibition of methanogenesis (Fig. 1G). Apparently, a second population of SRB using acetate as electron donor was intrinsically present only with low potential and capable of completely out-competing acetotrophic methanogens only after ~11 days of incubation. Most likely, acetate-utilizing SRB were either present at lower numbers than $H_2$-utilizers, or were present as spores, i.e. Desulfotomaculum spp. (65), which became active only after germination. In fact, in dry Italian rice field soil MPN counts of $H_2$-utilizing SRB ($9.2 \times 10^4$ cells x g dry soil$^{-1}$) were significantly lower compared to acetate-utilizing SRB ($0.5 \times 10^4$ cells x g dry soil$^{-1}$), which however, became more abundant upon incubation for 13-weeks in rice field soil microcosms (64).

T-RFLP, PCA, and quantitative rRNA hybridization showed, that methanogenic population dynamics under gypsum amendment were not as different to the unamended soil as under ferrihydrite addition. We relate this to the delayed inhibition of namely the acetotrophic methanogens, resulting in "intermediate" methanosarcinal rRNA dynamics, and nearly unchanged rDNA dynamics. Interestingly, RC-I rRNA levels as revealed by T-RFLP and quantitative rRNA analysis were significantly lower under gypsum amendment, which is basically a reversion of the effect observed on day 5 under ferrihydrite addition and therefore underscores the potential of $H_2$-utilizing SRB for out-competing hydrogenotrophic methanogens.

In this study, we induced major functional shifts within the rice field soil microbial community by supplementing alternative electron acceptors in the form of ferrihydrite and gypsum, and thereby promoting respiratory processes other than methanogenesis. The
enhanced activity of FRB and SRB resulted in a more or less complete inhibition of methanogenesis under conditions of limiting substrate- and non-limiting electron acceptor availability. Steady-state methane production rates in unamended slurries after day 10 (0.5 µmol d\(^{-1}\) g\(^{-1}\)) corresponded to sulfate-reduction rates in gypsum-amended slurries after day 8 (0.5 µmol d\(^{-1}\) g\(^{-1}\)) and to iron-reduction rates in ferrihydrite-amended slurries after day 7 (4.8 µmol d\(^{-1}\) g\(^{-1}\)). Considering the electron-uptake potential of 8 electrons per CO\(_2\) and SO\(_4^{2-}\) and 1 electron per Fe\(^{3+}\), the amounts of sulfate reduced perfectly matched the quantity of methane, which was not produced under inhibition. The budget of ferrihydrite reduction was not as efficient, since more electron equivalents were consumed indicating that electron donors other than acetate and H\(_2\) were utilized by FRB, and inhibition of methanogenesis was incomplete. The total amount of gypsum added was only 1/10 of the ferrihydrite amendment, but still the mitigative effect was comparable (69 and 85% methane reduction, respectively). Gypsum and other sulfate containing amendments like phosphogypsum are inexpensive and readily available (15), while ferrihydrite is not commercially available. Both reduced sulfur- and iron(II)-compounds can be reoxidized in the rhizosphere or upon drainage (12,60), but especially sulfate will be more rapidly lost from the system due to washout than iron-compounds. More research on the long-term mitigation potential of alternative electron acceptor supplementation is needed to better understand the different relevant processes. Moreover, touching on the effects of amendments on population dynamics of not only methanogens, as presented in this study, but also SRB and FRB, would greatly expand our knowledge of the microbial ecology involved, and should be object of future studies.

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References


C.4. Einfluss der Größe von Bodenaggregaten auf Methanogenese und Struktur der archaeellen Gemeinschaft in anoxischem Reisfeldboden

Balsubramanian Ramakrishnan, Tillmann Lüders, Ralf Conrad und Michael Friedrich

Effect of soil aggregate size on methanogenesis and archaeal community structure in anoxic rice field soil

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Abstract

In anoxically incubated slurries of Italian rice field soil, CH₄ production is initiated after a lag phase during which ferric iron and sulfate are reduced. The production of CH₄ was affected by the size of soil aggregates used for the preparation of the soil slurry. Rates of CH₄ production were lowest with small aggregates (< 50 and 50–100 μm), were highest with aggregates of 200–2000 μm size and were intermediate with aggregates of 2000–15,000 μm size. The different amounts of CH₄ accumulated were positively correlated to the concentrations of acetate, propionate and caproate that transiently accumulated in the slurries prepared from different aggregate sizes and also to the organic carbon content. The addition of organic debris that was collected from large-size aggregates to the aggregate size fractions < 200 and < 50 μm resulted in an increase of CH₄ production to amounts that were comparable to those measured in unamended aggregates of 200–2000 μm size, indicating that CH₄ production in the different aggregate size fractions was limited by substrate. The distribution of archaeal small-subunit rRNA genes in the different soil aggregate fractions was analyzed by terminal restriction fragment length polymorphism which allowed seven different archaeal ribotypes to be distinguished. Ribotype-182 (consisting of members of the Methanosarcinaceae and rice cluster VI), ribotype-389 (rice cluster I and II) and ribotype-820 (undigested DNA, rice cluster IV and members of the Methanosarcinaceae) accounted for > 20%, > 30 and > 10% of the total, respectively. The other ribotypes accounted for < 10% of the total. The relative quantity of the individual ribotypes changed only slightly with incubation time and was almost the same among the different soil aggregate fractions. Ribotype-389, for example, slightly decreased with time, whereas ribotype-182 slightly increased. At the end of incubation, the relative quantity of ribotype-182 seemed to be slightly higher in soil fractions with larger than with smaller aggregates, whereas it was the opposite with ribotype-80 (Methanomicrobiaceae) and ribotype-88 (Methanobacteriaceae). Ribotype-280 (Methanosetaeaceae and rice cluster VI), ribotype-375 (rice cluster III), ribotype-389 and ribotype-820, on the other hand, were not much different among the different soil aggregate size fractions. However, the differences were not significant relative to the errors encountered during the extraction of polymerase chain reaction (PCR)-amplifiable DNA from soil. In conclusion, soil aggregate size and incubation time showed a strong effect on the function but only a small effect on the structure of the methanogenic microbial community.

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Keywords: CH₄ production; Fermentation; Ribosomal RNA; Terminal restriction fragment

1. Introduction

Rice fields annually contribute about 100 ± 50 Tg of the greenhouse gas CH₄ to the atmospheric budget [1]. Methane is produced when flooded rice field soils become anoxic and reduced. After cropping, the fields are drained and the soils become dry and oxic until the next cultivation. The chemical changes after flooding of anoxic paddy soils and the microbiological processes leading to CH₄ production have been investigated in detail [2,3]. Vigorous CH₄ production is usually not observed before oxidants such as ferric iron and sulfate are largely reduced. However, trace amounts of CH₄ are usually produced immediately upon flooding in the presence of these oxidants [4,5].

Soils contain diverse groups of microorganisms, including the methanogenic archaea, which all contribute to the decomposition of organic matter under anoxic conditions [6,7]. Environmental factors such as soil type, rice variety, field management or season are possibly important determinants of the composition of the methanogenic microbial community in rice field soils. Mayer and Conrad [8] reported that the methanogenic population size in Italian rice field soil was almost constant, even during dry fallow
periods. Asakawa and Hayano [9] also reported that changes in the populations of \( \text{H}_2\text{CO}_2\text{H} \), methanol- and acetate-utilizing methanogens, determined by the most probable number (MPN) cultivation method, were negligible, when investigating Japanese paddy soils under different moisture regime (flooded or non-flooded), crop rotation (rice or wheat), fertilizer treatment and soil depth (0–1, 1–10 and 10–20 cm). Recent studies of archaean small-subunit (SSU) rDNA retrieved from anoxic Italian rice field soil demonstrated a larger archaean diversity than suggested by MPN analysis [10–13]. Together with analyses of the archaean communities on rice roots [10,11,14,15] these authors demonstrated a relatively large diversity of Archaea in rice fields, including members of the families Methanosarcinaceae, Methanosaetaceae, Methanomicrobiaceae and Methanobacteriaceae (taxonomy according to [16]). In addition, members of the kingdom Euryarchaeota have been detected and grouped into novel phylogenetic clusters termed rice clusters I, H, III and V [10,11]. Finally, members of the kingdom Crenarchaeota have been detected and grouped into phylogenetically defined clusters termed rice clusters IV and VI [10–12].

Soil microbial communities are known to respond to the agricultural management practices and environmental perturbations [17–19]. In soils, bacteria tend to occur immobilized through adsorption to the soil particles [20]. These organisms also play an important role in the formation and stabilization of soil aggregates (groups of primary particles that adhere to each other more strongly than to surrounding soil particles). Tsidall and Oades [21] suggested that soil aggregation is a complex hierarchical process. They classified the organic binding agents, depending on the effectiveness of these agents, at different stages in the structural organization of aggregates into: (a) transient, mainly polysaccharides; (b) temporary, such as root and fungal hyphae, and (c) persistent, such as humic and phenolic compounds associated with polyvalent metal cations and strongly-sorbed polymers. Disintegration of the bulk soil into aggregates can occur by disruptive forces which include cultivation (tillage/plowing), flooding and erosion (wind and water). The resulting aggregate size distribution in soils is often considered as a function of soil properties and also of the cultivation method [22].

Here, we have studied the initiation of \( \text{CH}_4 \) production upon flooding of dry soil aggregates of different size ranges, together with the archaean community structure. Different specified aggregate size fractions of dry Italian rice field soil were obtained by rotary sieving. These samples were incubated as anoxic slurries at 25°C and the change of concentrations of \( \text{CH}_4 \), \( \text{H}_2 \), \( \text{CO}_2 \) and volatile fatty acids was analyzed. The archaean community structure in different aggregate size fractions was studied by terminal restriction fragment length polymorphism (T-RFLP), a molecular genetic fingerprinting technique [12,13,23–25].

2. Materials and methods

2.1. Soil samples and slurry incubation

Soil samples were collected in 1993 and 1997 from the experimental fields of the Italian Rice Research Institute, Veredelli, and were stored as dry lumps at room temperature. The site descriptions and soil characteristics are presented in previous reports [26,27]. The larger dry lumps were broken manually by slight impounding before passing the samples through stainless steel sieves of 15000, 2000, 500, 400, 200, 100 and 50-μm mesh size on a mechanical rotary shaker (K. Retsch, Haan, Germany) at a shaking speed of 50 rpm for 5 min. The different-sized fractions of soil aggregates (the fractions passing through the 15000 μm sieve and collected on the 2000 μm sieve are designated as aggregates 2000–15000 μm and analogously for the other fractions) were collected and stored in large plastic containers. Total carbon and nitrogen contents of these soil aggregates (Table 1) were determined with a CHN analyzer (Analytical Chemical Laboratory of the Phillips University, Marburg, Germany). For determination of the available iron content, soil samples (0.5 g) were extracted with 4.5 ml of 0.5 M HCl at room temperature for 24 h. After centrifugation at 10000 rpm for 5 min, the supernatant was mixed with 4-(2-pyridylidiazyl) resorcinol and concentrations of Fe(II) and Fe(III) were measured by ion chromatography using a high performance polymer-coated silica-based cation exchange column [28]. The sulfate content was determined after 2 h extraction at room temperature using 1 ml of sodium carbonate (100 mM) or by centrifuging the aliquots of the soil slurries and filtration through a 0.2-μm membrane filter (Minisart...

<table>
<thead>
<tr>
<th>Soil aggregate size (μm)</th>
<th>Carbon (%)</th>
<th>Nitrogen (%)</th>
<th>Iron (μmol g⁻¹)</th>
<th>Sulfate (μmol g⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>2000–15000</td>
<td>1.46 ± 0.16</td>
<td>0.17 ± 0.04</td>
<td>99.66 ± 11.62</td>
<td>1.86 ± 0.02</td>
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<tr>
<td>500–2000</td>
<td>1.36 ± 0.37</td>
<td>0.12 ± 0.03</td>
<td>97.88 ± 6.19</td>
<td>2.07 ± 0.13</td>
</tr>
<tr>
<td>200–500</td>
<td>1.87 ± 0.59</td>
<td>0.20 ± 0.02</td>
<td>105.16 ± 2.02</td>
<td>2.73 ± 0.38</td>
</tr>
<tr>
<td>&lt; 200</td>
<td>1.50 ± 0.34</td>
<td>0.14 ± 0.06</td>
<td>113.27 ± 0.86</td>
<td>2.24 ± 0.13</td>
</tr>
<tr>
<td>50–100</td>
<td>1.31 ± 0.14</td>
<td>0.12 ± 0.04</td>
<td>71.22 ± 17.75</td>
<td>1.94 ± 0.08</td>
</tr>
<tr>
<td>&lt; 50</td>
<td>1.26 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>120.57 ± 6.81</td>
<td>2.58 ± 0.03</td>
</tr>
</tbody>
</table>
C.4. Ergebnisse

RC15, Sartorius, Göttingen, Germany). The sulfate concentrations were then analysed in a Sykam ion chromatographic system [29].

For the preparation and incubation of slurries, 10 g dry weight (d.w.) of different-sized soil aggregates were filled into 120-ml serum bottles and suspended at a weight ratio of 1:1 in anoxic and sterile distilled water. The serum bottles were closed with sterile butyl rubber stoppers, crimped with aluminum caps, and flushed with N2. Serum bottles were incubated in the dark at 25°C without shaking to avoid damage of the methanogenic community [30].

In another experiment, the soil aggregate fractions of 500–2000 and 200–500 μm were mixed with water at a 1:1 soil and water ratio, stirred briefly and then centrifuged at 10000 rpm for 5 min on a centrifuge (Universal 30F; Hettich, Tuttlingen, Germany). Pieces of rice roots and straw were collected from the supernatant and then dried at 60°C for 2 days. These native organic substances were added as substrate amendment (150 mg) to the soil slurries prepared from aggregates <50 and <200 μm. The preparation and incubation of the substrate-amended soil aggregate slurries were carried out as described above. The experiments were carried out by preparing in parallel numerous bottles which were subsequently sacrificed in triplicate at each time of sampling.

2.2. Analysis of gases

At given time intervals, gas samples were withdrawn from the headspace after vigorously shaking the bottles by hand to allow equilibration between the liquid and gas phases by using a gas-tight pressure-lock syringe. The concentrations of H2, CO2 and CH4 in the gas samples were measured by gas chromatography as previously described [31]; H2 by using a molecular sieve 5 Å column (80–100 mesh, 70 cm length) with HgO-Hg vapor conversion detector (RQDG2, Trace Analytical, Stanford, CA, USA); CO2 after conversion to CH4 by a methanizer (Ni-catalyst at 330°C, Chrompack-Middleburg, The Netherlands) and CH4 by using an 80-cm-long Propak Q 60–80 mesh column at 50°C on a Shimadzu GC 8A with flame ionization detector (Shimadzu, Japan).

2.3. Analysis of volatile fatty acids

Volatile fatty acids were measured at regular time intervals. To collect the liquid samples, the soil slurry in serum bottles was mixed briefly on a vortex mixer, transferred into 2.0-ml microcentrifuge tubes and then centrifuged at 14000 × g for 5 min at 4°C. The supernatant was collected and filtered through 0.2-μm membrane filters (Minisart SRP-15, Sartorius). The filtered samples were stored frozen at −20°C until analysis. The concentrations of the volatile fatty acids were analyzed in a high pressure-liquid chromatograph (Sykam, Gauting, Germany) with refractive index and UV detectors [32].

2.4. DNA extraction from soil slurry

The sampling for molecular analysis was carried out with incubated soil slurries and dry soil samples. Slurry samples of 0.5 ml were collected at different intervals after mixing of the serum bottles thoroughly, and then stored in 2.0-ml microcentrifuge tubes at −20°C until analysis. For extraction of total community DNA from dry soil samples, 0.5 g of different soil aggregate size fractions were filled into microcentrifuge tubes, mixed with 0.5 ml of degassed distilled water and incubated for 1 h.

The direct DNA extraction procedure of Moré et al. [33] was adapted with modifications as described by Henckel et al. [34]. The cells in slurry samples were lysed on a beadbeater (BIO 101 Fast Prep, Savant Instruments, Farmingdale, NY, USA) for 45 s at a setting of 6.5 m s−1 in the presence of an SDS solution. Total soil community DNA was extracted, washed, pelleted and resuspended in 100 μl of Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, pH 8). Then, the DNA extracts were further purified by centrifugation (2100 × g, 2 min) through spin columns (Bio-Rad, Munich, Germany) filled with acid-washed polyvinylpolypyrrolidone (Sigma-Aldrich, Steinheim, Germany) in 30 mM potassium phosphate buffer (pH 8.0) as described by Berthelet et al. [35]. The purified extracts were clear and colorless. The nucleic acid concentration of these purified extracts, representing total soil community DNA, was determined either spectrophotometrically (GeneQuant, Pharmacia Biotech, Uppsala, Sweden) or fluorometrically by using the PicoGreen dsDNA quantitation kit (Molecular Probes Europe BV, Leiden, The Netherlands).

2.5. PCR amplification of archaeal SSU rDNA

Archaeal SSU rRNA genes were specifically amplified from total soil community DNA extracts using the primers Ar109F (5′-ACK GCT TAA CAC GT-3′) and Ar912r (5′-CTC CCC CGC CAA TTC CTT TA-3′), modified from Großkopf et al. [10]. For PCR amplification, 20 ng of purified DNA extract was added to a final volume of 100 μl of reaction mixture containing 10 mM Tris-HCl, 1.5 mM MgCl2, 50 μM of each deoxynucleoside triphosphate, 0.3 μM of each archaeal SSU rRNA gene primer and 2.5 U of AmpliTaq DNA polymerase (PE Applied Biosystems, Weiterstadt, Germany). The reverse primer, Ar912r, was labeled at the 5′ terminus with 6-carboxyfluorescein. The PCR thermal profile included the following steps using a GeneAmp PCR system 9700 (PE Applied Biosystems): an initial denaturation for 5 min at 94°C, then 27 cycles of 1 min denaturation at 94°C, 1 min annealing at 52°C and 1 min extension at 72°C, finished by a 3 min final extension at 72°C. PCR amplicons were analyzed by electrophoresis in 1% (w/v) agarose gels and then purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The DNA concentra-
2.6. T-RFLP analysis

The PCR amplicons were analyzed by T-RFLP [12,13,23,25]. PCR amplicons (75 ng), 2.5 U of TaqI restriction enzyme (Promega, Mannheim, Germany), 1.0 µl of the appropriate incubation buffer supplied by the manufacturer and 1 µg of bovine serum albumin were made up to a total volume of 10 µl and incubated at 65°C for 2 h. The digested fluorescently-labeled SSU rDNA fragments were size-separated on an automated DNA sequencer (Model 373A, PE Applied Biosystems). The RFLP pattern of the 5’-terminal SSU rDNA fragments of each sample was determined in comparison to that of the internal standard by using GeneScan analysis software (version 2.1, PE Applied Biosystems). This software package estimates the length of T-RFs and integrates the florescence emission intensity of individual T-RF peaks. Based on the principle that the relative proportion of the integrated fluorescence of each T-RF corresponds to the proportion of each amplicon in the PCR products, the relative abundance of amplicons was estimated as the ratio between the integrated fluorescence of each of the T-RFs and the total integrated fluorescence of all T-RFs [36]. Thus, the percentage distribution of different ‘ribotypes’ (i.e. T-RFs) within the archaeal community structure in a particular soil sample was determined. The major ribotypes consisted of several phylotypes, which were identified by calculating the theoretical lengths of T-RFs from 16S rRNA sequences by using the ARB software [37], from various clone libraries of archaeal SSU rDNA retrieved from Italian paddy soil (Table 2). The detailed description of the clone libraries is provided elsewhere [10–14,25].
3. Results

3.1. Ecophysiological function of different soil aggregate sizes

In the anoxically incubated slurries of different-sized aggregates from rice field soils, the production of detectable levels of CH₄ (assessed on an exponential scale) began as early as after 1–2 days of incubation (data not shown). The onset of CH₄ production only on day 2 of incubation was typical for the fractions with soil aggregates < 100 μm. CH₄ accumulation on a linear scale showed that during an incubation of 25 days, CH₄ was produced if small aggregates (< 100 μm) were used compared to other aggregate fractions (Fig. 1A). The CH₄ production potential among these aggregate fractions exhibited clear differences after about 10 days incubation and could be grouped into three levels. The highest level of accumulation was apparent in the aggregates of 200–500 and 500–2000 μm size. The lowest level was observed with aggregates of 50–100 and < 50 μm size. The other two aggregate size fractions (2000–15000 and < 200 μm) showed intermediate levels of CH₄ accumulation.

In order to relate the production potential of different

<table>
<thead>
<tr>
<th>Ribotype: length of T-RF</th>
<th>Phylotype</th>
<th>Number of positive clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>Methanomicrobiaceae</td>
<td>9</td>
</tr>
<tr>
<td>88</td>
<td>Methanobacteriaceae</td>
<td>18</td>
</tr>
<tr>
<td>182</td>
<td>Methanosarcinaceae and rice cluster VI</td>
<td>73 and 22</td>
</tr>
<tr>
<td>280</td>
<td>Methanosetaeaceae and rice cluster V</td>
<td>34 and 5</td>
</tr>
<tr>
<td>375</td>
<td>rice cluster III</td>
<td>6</td>
</tr>
<tr>
<td>389</td>
<td>rice cluster I and II</td>
<td>55 and 3</td>
</tr>
<tr>
<td>820</td>
<td>undigested DNA (clones belonging to rice cluster IV and Methanosarcinaceae)</td>
<td>12 and 4</td>
</tr>
</tbody>
</table>

The major ribotypes are classified based on the theoretical lengths of T-RFs from the aligned SSU rDNA archaeal sequences of clone libraries and also from the T-RFLP analysis using SSU rDNA clone libraries constructed from DNA retrieved from Italian paddy soil. These clone libraries comprise a total of 267 clones described by [10–14,25]. The degree of conservation in restriction site positions in the SSU rDNA was about 90% [25].
aggregates to the availability of substrates, we measured the total carbon and nitrogen contents in the different soil aggregate size fractions (Table 1) and followed the change of H₂ partial pressure (data not shown) and of dissolved volatile fatty acids (Fig. 1). Spearman rank correlation analysis showed that higher amounts of CH₄ accumulated were positively correlated with the soil carbon content ($r^2 = 0.83$). H₂ partial pressures increased to high values of 6–14 Pa within 1 day of incubation. Within 3–4 days, the H₂ partial pressures rapidly declined and stabilized at values of 0.1–0.5 Pa in all samples except those with the smaller soil aggregate sizes (<100 μm), in which they stabilized around 0.8–2.0 Pa H₂. Similar trends in the accumulation of H₂, CH₄ and CO₂ were observed when the soil aggregate size fractions were prepared from soil collected in 1993 and 1997 (data of 1997 are not shown).

Acetate, propionate, caproate, lactate and butyrate were the only volatile fatty acids detectable at concentrations >10 μM. Butyrate was detected only on day 1 (data not shown). Similarly, lactate was >20 μM only on day 1 (data not shown). Acetate (Fig. 1B), propionate (Fig. 1C) and caproate (Fig. 1D), on the other hand, transiently accumulated during the incubation and exhibited different accumulation patterns among the different aggregate size fractions. The maximum concentrations of acetate ($r^2 = 0.94$), propionate ($r^2 = 0.82$) and caproate ($r^2 = 0.93$) were correlated with the amount of CH₄ accumulated in the different soil aggregate fractions. Acetate reached concentrations of 1–5 mM (Fig. 1B). In the soil aggregate fractions <100 and 200–1500 μm, the maximum acetate concentrations that were reached after 15 days of incubation were relatively low (<2 mM) and decreased thereafter only slightly. In the soil aggregate fractions of 200–2000 μm, on the other hand, much higher acetate concentrations (5 mM) were reached after 10 days incubation, which thereafter decreased to <200 μM acetate until the end of incubation (Fig. 1B).

On a visual inspection, the slurries of the most active aggregate sizes (200–500 and 500–2000 μm) showed root pieces and other organic debris. Increased amounts of native organic substances in these soil aggregates were also evident from the soil carbon content (Table 1). Collection of these native organic substances and addition to the smaller soil aggregate size fractions (i.e. <200 and <50 μm) resulted in increased amounts of CH₄ accumulated. Accumulation of CH₄ in the amended small aggregate size fractions was then comparable to those in the unamended large aggregate size fractions of 200–500 and 500–2000 μm (data not shown).

3.2. Archaeal community structure of different soil aggregate sizes

The total community DNA extracted from slurries prepared with different soil aggregate sizes were used for T-RFLP analysis. An example of such an analysis is shown in Fig. 2A. The T-RFLP analysis identified seven major T-RFs representing seven ribotypes (Table 2). Analysis of the archael SSU rDNA sequences of 267 clones retrieved from Italian rice field soil allowed the assignment of the different ribotypes to corresponding phylogenotypes which had been identified by the construction of phylogenetic trees.
Unfortunately, the individual ribotypes were not strictly represented by only one phylotype (Table 2). For example, ribotypes -182, -280 and -389 each comprised two different phylotypes which possibly vary in proportion to each other from sample to sample. However, each ribotype was reproducibly retrieved from duplicate analysis using the same DNA, both in quality and in its relative proportion (determined as peak area) to the total ribotypes (data not shown). The relative proportion of each ribotype to the total was also independent of the number of PCR amplification cycles which were varied between 22 and 44 cycles (Fig. 2B). Only ribotype -389 and -820 exhibited slightly decreased frequencies at PCR cycles > 27. Therefore, we routinely used 27 PCR cycles for T-RFLP analysis. Under these conditions, the analysis of triplicate soil samples generally showed an accuracy of better than ± 5-10% ribotype frequency (Fig. 2C).

The seven archaeal ribotypes were also detected in dry soil samples prior to incubation as anoxic slurries (Fig. 3; incubation time = zero). Ribotypes -182, -389 and -820 were the most abundant ribotypes and made up 16.23, 43.48 and 13.18%, respectively, of the total ribotypes detected in the various dry soil aggregate sizes. The narrow range of percentages indicates that the composition of the archaeal community by the dominant ribotypes was relatively constant at the beginning of anoxic incubation. The other ribotypes were detected at proportions of < 10%.

During anoxic incubation, the proportional contribution of the individual ribotypes changed only slightly with incubation time and also was only slightly different in the different soil aggregate size fractions. These changes and differences were not statistically significant when compared to the error (± 5-10% ribotype frequency) encountered during T-RFLP analyses of replicate soil samples (Fig. 2C). However, there seemed to be tendencies. For example, the relative proportion of ribotype -80 to the total increased from about 0.5-1.5% in dry soil aggregates to 3-6% in 25-day-old slurries (Fig. 3A). The proportion of ribotype -88 on the other hand decreased from initially 10% to about 5% after 6 days incubation (Fig. 3A). Subsequently, the relative proportion of ribotype -88 tended to increase with higher values for the larger soil aggregate sizes (Fig. 3A). Noteworthy is the relatively high proportion of ribotype -182 which stayed relatively constant with incubation time at about 15-25% among the small-sized aggregates (< 100 μm), but tended to increase to > 40% among the larger-sized aggregates (Fig. 3B). Ribotype -280 contributed only little (4-9%) to the total archaeal ribotypes and stayed relatively constant with incubation time (Fig. 3B). Ribotype -375, on the other hand, also contributed little (< 6%) but tended to increase with incubation time (Fig. 3C). Ribotype -389 was the most abundant ribotype and tended to slightly decrease with time (Fig. 3A). The same was observed with ribotype -820, which was also relatively abundant (Fig. 3C).

4. Discussion

The production of CH₄ is accomplished by methanogenic archaebacteria which usually disproportionate acetate into CH₄ plus CO₂, or reduce CO₂ with H₂ to CH₄. In the present study, we demonstrated that the production of CH₄ from different-sized aggregates from Italian rice field soil was less if small aggregates (< 100 μm) were used, thus confirming earlier results [8]. Furthermore, we showed that anoxic slurries prepared from different soil aggregate sizes exhibited systematic differences in the transient accumulation of metabolic intermediates that were produced and consumed during the methanogenic degradation of soil organic matter. Thus, the amounts of CH₄ produced correlated with the maximum concentrations of acetate, propionate and caproate that transiently accumulated in the different aggregate size incubations. More than 80% of the differences in CH₄ production were explained by the differences in accumulated volatile fatty acids, which are direct or indirect methanogenic precursors [3]. Apparently, syntrophic microorganisms, which typically degrade volatile fatty acids, such as propionate, butyrate and other longer chain volatile fatty acids, did not fully couple with their syntrophic H₂-oxidizing partner organisms as indicated by different degrees of accumulation of these fatty acids in the different aggregate size fractions, whereas CH₄ production increased. On the other hand, the composition of the methanogenic archaeal community, which was determined by T-RFLP analysis, was not significantly different among the different soil aggregate sizes and was also relatively constant with time. Thus, differences in volatile fatty acid accumulation between aggregate size fractions were not affected by methanogenic population shifts.

There are suggestions that the decomposition process leads to the development of an aggregate hierarchy [38]. Much of the organic matter in soil is particulate, not evenly distributed and physically protected from microorganisms by adsorption onto inorganic clay surfaces and by entrapment in aggregates. The highest concentrations of volatile fatty acids and the highest rates of CH₄ production were observed in intermediate soil aggregate sizes (200-2000 μm), whereas lower values were found in both the largest (> 2000 μm) and the smallest (< 100 μm) soil aggregate fractions. Less production of CH₄ from small soil aggregates is likely due to less available substrates. Diffusion limitation could have been responsible for reduced microbial activities in the larger size aggregates, but it is unknown to which degree size aggregates remain physically intact after flooding of the soil. Elliott [39] showed that organic matter associated with microaggregates was more recalcitrant than organic matter associated with macroaggregates. Less production of CH₄ from large soil aggregates may also be due to less available substrate. Indeed, the intermediate soil aggregate fractions contained the highest amounts of organic debris indicating
that this material controlled the accumulation of volatile fatty acids as well as the subsequent CH₄ formation. This conclusion was confirmed by stimulated CH₄ production in the small-size fractions when native organic debris from the intermediate soil fractions was added.

The ecology of methanogenic archaea in soils is little explored and there is a need to establish the link between the community structure (speciation) and function (metabolic activity). We have used T-RFLP as a molecular fingerprinting technique for the purpose of a direct analysis of the archael community structure in the different-sized soil aggregates. This technique enabled us to compare unique T-RFs (ribotypes) generated from the digestion of PCR amplicons with those derived from SSU rRNA gene sequence database in order to make phylogenetic inference. In addition, the relative amount of the individual ribotypes in the PCR amplicons (‘gene frequencies’; [23,36]) were quantified. Our results showed that soil aggregate disruption, cell lysis for extraction of total community DNA, amplification of archael SSU rDNA by PCR, restriction and separation of the terminally-labeled fragments were all efficient and reproducible and yielded community fingerprints which were qualitatively and quantitatively consistent.

Thus, we found highly reproducible PCR yields and T-RFLP patterns from replicate aliquots of the same DNA extract. By using the SSU rDNA of *Methanosarcina barkeri* for PCR reactions and subsequently measuring the dsDNA concentration of amplicons fluorometrically using the ‘PicoGreen’ method [40], we found that the relative proportion of integrated fluorescence of the restriction fragment peak linearly increased with increasing concentrations of amplicon digests (unpublished results). Similarly, duplicate sets of PCRs using the same DNA from a single soil community gave highly reproducible community fingerprints with the same relative distribution of ribotypes. We also found that the relative distribution of the different ribotypes was not significantly affected by the number of PCR amplification cycles if varied between 24 and 32. The kinetic bias effect reported by Suzuki et al. [36] therefore did not seem to pose a problem in the PCR reaction using rice field soil community DNA. Different soil replicates, on the other hand, created a somewhat larger error. Nevertheless, the error of the total procedure using replicate soil samples was only in the order of ± 5–10% ribotype frequency, similar to that recently described for T-RFLP analysis of the community of Bacteria in rice field soil [24].

The T-RFs obtained by T-RFLP analysis of our dry rice field soil samples had sizes in the following order of dominance: 389 > 182 > 820 > 280 > 80 > 88 ≈ 375 bp. Based on the size of the T-RFs, representing individual ribotypes, all the major phylogenetic archael groups were identified that had previously been detected in Italian rice field by either isolation and cultivation or by cloning and sequencing, i.e. those belonging to either Methano-
sarcinaeae, Methanosetaeaeae, Methanomicrobiaeae, Methanobacteriaeae or one of the taxonomically undefined archael rice clusters I to VI [10–12,14]. The resolution of T-RFLP analysis is generally limited to that of high order taxa due to the variable conservation of restriction site positions in 16S rDNA. In addition, the production of T-RFs of identical size by different taxa with a given restriction enzyme [41] or multiple ribotypes due to 16S rDNA sequence heterogeneity within one taxon [42,43] can pose problems in attributing a phylogenetic position to a particular ribotype. Thus, we were unable to differentiate between members of the Methanosarcinaeae and rice cluster VI, between members of the Methanosetaeaeae and rice cluster V and between rice cluster I and rice cluster II. Since the phylogenetic differentiation of the individual ribotypes is based on the sequence information presently available, we can not exclude that ribotype 80, -88, -375 and -820 consisted of more than the one phylotype indicated in Table 2.

Our data suggest that there may be slight temporal changes in the relative quantities of ribotypes, but the dominance of individual ribotypes did not change. The euryarchaotal rice clusters I and II, which are represented by ribotype-389, dominated in the beginning of incubation. Their contribution slightly declined with incubation time with the concurrent increase of ribotype-182 representing members of the Methanosarcinaeae and eurearchaeotal rice cluster VI. These changes suggest that the populations of individual species within the archael community changed during the incubation period. Microbial isolates representing rice cluster I and II do not exist and, therefore, the phenotype of these archael populations is unknown. Indirect evidence suggests that they represent methanogenic archaela which perhaps utilize H₂ or ethanol as electron donors [14]. The genus *Methanosarcina*, on the other hand, is known for acetoclastic methanogenesis. If the temporal change in the frequency of the two dominant ribotypes (ribotype-182 and -389) is real, the results suggest that acetoclastic methanogens may have slowly replaced CO₂-reducing methanogens during the course of incubation. A change in the activity from predominantly H₂/CO₂-utilizing to acetoclastic methanogenesis has recently been concluded from inhibition studies with anoxic rice field soil [4]. Our results of the process-oriented experiments support this view. While CH₄ production began as early as 1–2 days after incubation, the accumulated acetate did not start to decrease before day 10, being consistent with the view that the acetoclastic methanogenic populations increased with time. Similar population increases of acetoclastic *Methanosarcina* or *Methanoseta* species were recently reported for cellulose-degrading anaerobic enrichment cultures inoculated with rice field soil [13] and during sequential reduction processes after flooding of rice field soil [25]. However, a statistically significant proof for the temporal change of the dominant methano-
genic populations will only be possible when the individual
C.4. Ergebnisse

ribotypes detected by T-RFLP analysis will be determined with an even better accuracy to that in the present study. Further refinement in the procedures for cell lysis, DNA extraction, DNA purification [44] and quantification of DNA from the soil environment [40,45] will possibly enhance the accuracy.

Besides the possible slight change in the populations of \( \text{H}_2/\text{CO}_2 \)-utilizing and acetotrophic methanogenic popula-
tions, the frequency of the third-most dominant ribotype[20], representing undigested DNA and clones of crenarcheotal rice cluster IV and of members of the Methanosarcinaceae, also seemed to decrease slightly with incubation time. We have presently no clue what the physiological consequences of this decrease might be. All the other ribotypes made up < 10% of the total.

In general, our assessment of relative frequency of the different ribotypes revealed a relatively stable archael community structure both in time and among the different aggregate sizes. Especially with regard to the different soil aggregate fractions, the stable archael community pat-
terns were quite surprising. The different soil aggregate fractions revealed quite different patterns of production of volatile fatty acids and \( \text{CH}_4 \); large differences in the archael community structure have been reported for rice field soil incubated at different temperatures [12]; dif-
ferent community structures were found for the flora of the bulk soil [10] and the rice rhizosphere [11]; and different structures were also observed among rice field soils sampled from different rice-growing regions (unpublished results). Other studies have shown the presence of pheno-
typically and phylogenetically distinct members of both Euryarchaeota and Crenarchaeota in different soil types [46–48]. Our results, however, clearly show that the pre-
dominant representatives of Archaea form a relatively sta-
ble part of the microbial communities in the Italian rice field soil. However, we did not estimate the total number of species (‘species richness’) and it may well be that differ-
ces occur among the different species belonging to the same ribotype.

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References

tion in different rice paddy soils from China, the Philippines and Italy. Soil Biol. Biochem. 31, 463–473.
lation of methanogenic bacteria and the initiation of methane produc-
tion upon flooding of paddy soil. FEMS Microbiol. Ecol. 73, 103–112.
ture of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl. Environ. Microbiol. 64, 966–969.
eaetal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. Appl. Environ. Microbiol. 64, 4983–4989.

sierung methanogener Populationen an Wurzeln verschiedener Rein-
ial populations and activities in reduced chemical input agroecosys-
terization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63, 4516–4522.


C.5. Ergebnisse

C.5. Struktur der archaeellen Gemeinschaft in Reisfeldböden unterschiedlicher geographischer Herkunft vor und nach der Initiierung der Methanogenese

Balsubramanian Ramakrishnan, Tillmann Lüders, Peter F. Dunfield, Ralf Conrad und Michael W. Friedrich

Archaeal community structures in rice soils from different geographical regions before and after initiation of methane production

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Abstract

The methane production potential of rice soils, which are situated in different geographical regions, shows inherent variations and is catalyzed by archaeal methanogens. We therefore investigated the archaeal community structure in 11 rice field soils which represent a range of climatic conditions (temperate to subtropical zones) and soil properties. Retrieval of environmental partial SSU rDNA sequences from the rice soils of Shenyang (China) and Gapan (The Philippines) showed that the communities were different from each other. However, despite the differences in soil properties and geographical region the sequences clustered in similar phylogenetic groups to those obtained earlier from rice fields of Verceili (Italy). The archaeal community structure in the other rice field soils was compared using terminal restriction fragment length polymorphism (T-RFLP) analysis targeting the SSU rRNA gene and the methyl-coenzyme M reductase \(\alpha\)-subunit gene (mrkA). The relative abundance of each terminal restriction fragment (T-RF) was determined by fluorescence peak area integration. The 182-bp SSU rDNA T-RF (representing members of \textit{Methanosarcinaeae} and rice cluster (RC) VI) was dominant (40–80% contribution) in Chinese soils (Zhenjiang, Changzhun, Jurong, Boiyuan, Shenyang) and the Philippine soil of Gapan. The other Philippine soils (Luisiana, Guangzhou, Phila) and the Italian soils (Verceili, Pavia) showed a dominant 389-bp T-RF (35-40% contribution), representing mainly the novel methanogenic RC-I. All the other T-RF (80, 88, 280, 375 and > 800 bp) contributed < 20%. Prolonged anoxic incubation (30–200 days) of the air-dried soils resulted in the production of CH\(_4\), which was in some soils preceded by a characteristic halt phase. T-RFLP analysis revealed that the soils with a methanogenic halt phase also showed dramatic archaeal population dynamics which were related to the length of the halt phase. Our results show that the archaeal communities in rice field soils of different geographical origin are highly related, but nevertheless exhibit individual patterns and dynamics, thus providing evidence for the active participation of the community members in energy and carbon flow. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Methanogen; Regional biodiversity; Methyl-coenzyme M reductase; SSU rRNA; Terminal restriction fragment length polymorphism analysis

1. Introduction

Methanogens are a highly specialized group of anaerobic microorganisms that belong phylogenetically to the domain \textit{Archaea}. Though the metabolic process of formation and emission of CH\(_4\) in rice soils worldwide has been investigated extensively, relatively few reports are available on the populations of methanogenic \textit{Archaea} present.

These reports include isolation of methanogens from rice field soils [1–7] but also molecular characterization of the archaeal SSU rRNA genes in the soil or rhizosphere [5,6,8–12]. It has been demonstrated that a relatively large diversity of \textit{Archaea}, including the members of the families \textit{Methanosarcinaeae}, \textit{Methanosaeaeae}, \textit{Methanomicrobiaeae}, and \textit{Methanohacteriaeae} (taxonomy according to [13]), are present in Italian rice soils. In addition, novel SSU rRNA sequences representing uncultivated microorganisms have been found in rice field soil. These novel sequences occur both in the kingdom \textit{Euryarchaeota}, termed rice clusters (RC) I, II, III and V, and in the kingdom \textit{Crenarchaeota}, termed RC-IV and RC-VI [6,9,11,12].

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Most recently, the methanogenic community in Italian rice field soil was also characterized by the *merA* gene encoding the α-subunit of the methyl-coenzyme M reductase, the key catalytic enzyme of methanogens [14]. This study revealed the methanogenic geno- and phenotype of the novel RC-1 lineage.

There is a need to characterize microbial communities phylogenetically in order to define the factors that regulate both their structure and function [15]. Methane production in rice soil ecosystems is considered to be accomplished by a complex community of hydrolytic, fermenting, syntrophic, homoacetogenic, and methanogenic microorganisms [16]. The high variability of the CH$_4$ production potential of rice soils is not completely accounted for by the differences in the soil chemical properties alone [17–19]. Potentially, the diversity and community structure of *Archaea* in rice soils may have important ecological significance. Therefore, our study was aimed at understanding the community structure of archael members in soils from rice fields other than the relatively well studied site at Vercelli, Italy. We used rice field soils representing a range of climatic conditions (temperate to subtropical zones) and soil organic carbon contents. These soils had been studied in detail regarding their potential to produce CH$_4$ and its control by chemical characteristics [19–21]. We used terminal restriction fragment length polymorphism (T-RFLP) analysis of SSU rRNA and methyl-coenzyme M reductase genes (*merA*) to identify archael and methanogenic phyotypes and compare their distribution and relative community composition in these soils.

2. Materials and methods

2.1. Soil sampling, analyses, and geographical regions

Soil samples were obtained from the different geographical sites, 3–4 months after rice harvest. Selected characteristics of soils and information on the geographical regions are provided in Table 1; other details are presented elsewhere [19]. Biogeochemical experiments using the same batch of soil samples have been described [19–21]. Samples were size-fractionated using stainless steel sieves to obtain soil particles between 0.1 and 1.0 mm diameter, and stored in darkness at 4°C prior to the experiments. The aerobic storage of dried soils is reported to have no significant effect on soil methane production capacity [22]. For studying methane production over time and the composition of the archaeal communities, soil samples (0.5 g) and 0.5 ml of anoxic, sterile water were placed into 2.0-ml replicate open polypropylene microcentrifuge tubes. Several tubes were then placed into replicate 120-ml serum bottles. The serum bottles were closed with butyl rubber stoppers and the head space was flushed with N$_2$ for at least 30 min. Serum bottles were incubated statically in the dark at 30°C. At given time intervals (2 h after preparation of soil slurries (day 0), and after 30 days incubation), triplicate samples were withdrawn and stored at -20°C until further analysis. Another set of bottles with soil slurries (10 g of soil and 10 ml of anoxic sterile water) were incubated under anoxic conditions at 30°C and sampled after 200 days. Slurry samples (0.5 ml) from these vessels were collected after vortexing the serum bottles, and stored in microcentrifuge tubes at -20°C.

2.2. DNA extraction, purification and quantification

DNA extraction was performed according to Moré et al. [23] with modifications as described by Henckel et al. [24]. Briefly, soil slurry samples (0.5 ml) were lysed in the presence of a sodium dodecyl sulfate solution using a bead-beating protocol as described previously [24]. Total soil community DNA was extracted, washed, pelleted, and reuspended in 100 μl of TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). For further purification, DNA extracts were centrifuged at 2100×g for 2 min and passed through spin columns (Bio-Rad, Munich, Germany) filled with acid-washed polysilin polyyprrolidone (Sigma-Al drich, Steinheim, Germany) in 30 mM potassium phosphate buffer (pH 8.0) as described by Berthelet et al. [25]. The purified extracts were clear and colorless, and
were analyzed by standard gel electrophoresis. DNA concentrations of the purified extracts were determined spectrophotometrically.

2.3. Archaeal SSU rDNA amplification

From soil community DNA, archaeal SSU rDNA was amplified by PCR using primers Ar109f (ACK GCT CAG TAA CAC GT) and Ar912r (CTC CCC CGC CAA TTC CTT TA), 20 ng of DNA as template, and 28 cycles of PCR as described previously in detail [12].

2.4. Cloning, sequencing and phylogenetic analysis of SSU rDNA clones

Clone libraries of SSU rRNA genes were generated from community DNA samples of rice field soil (day 0 after 2 h of incubation) from Shenyang (Shen-A clones) and Gapan (Gap-A clones). Archaeal SSU rDNA amplions were cloned in the Escherichia coli JEM 109 using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions (Promega, Mannheim, Germany). From each library, randomly selected clones were screened for positive inserts by PCR with the M13 primers and sequenced as described earlier [12]. Nucleotide sequences were assembled using SeqMan-II software (DNastar, Madison, WI, USA) and checked for close relatives and taxonomic assignment using BLAST searches [26]. Phylogenetic analysis of the sequences, which were more than 650 bp in length, was performed using the ARB software package [27] as described previously in detail [6,9,12]. Clones were deposited with GenBank under accession numbers AF399284-AF399315 for Shen-A clones and AF399316-AF399345 for Gap-A clones.

2.5. Archaeal SSU rDNA T-RFLP analysis

T-RFLP was performed as described previously [11,12] using primer Ar912r labeled at the 5’ end with 6-carboxy-fluorescein (FAM). Briefly, fluorescently labeled PCR amplicons (75 ng) were digested using TaqI, and subsequently analyzed using an automated sequencer (Model 373A, Applied Biosystems, Weiterstadt, Germany). T-RFLP patterns of each sample were evaluated using GeneScan analysis software (version 2.1, Applied Biosystems). Since the relative proportion of the integrated fluorescence of each terminal restriction fragment (T-RF) corresponds to the proportion of each amplicon in the PCR product [28,29], the relative abundance of amplicons was estimated and expressed as the percentage distribution of the different T-RF within each archaeal community fingerprint. By comparing the theoretical T-RF lengths of archaeal SSU rDNA clones obtained from China (Shenyang) and The Philippines (Gapan), the major T-RF were identified (Table 2) and compared to earlier analyses of Italian rice field soil [12].

2.6. Statistical analysis

The area percentage (A_p) of each T-RF was calculated as described recently [30] as

\[ A_p = \frac{n_i}{N} \times 100 \]

in which \( n_i \) represents the peak area of one distinct T-RF and \( N \) is the sum of all peak areas in individual T-RFLP electropherograms. A total of 70 different soil samples (four samples from day 0 × 10 different soils and three samples from day 30 × 10 different soils) were compared by T-RFLP. Excluded from statistical analysis were

<table>
<thead>
<tr>
<th>T-RF (bp)</th>
<th>Phyletype</th>
<th>Soils (number of clones)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Veroff¹</td>
</tr>
<tr>
<td>80</td>
<td>Methanomicrobiae</td>
<td>9</td>
</tr>
<tr>
<td>88</td>
<td>Methanobacterieae</td>
<td>18</td>
</tr>
<tr>
<td>RC-IV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RC-V1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>182</td>
<td>Methanococcaceae</td>
<td>73</td>
</tr>
<tr>
<td>RC-V1</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>280</td>
<td>Methanosetaeae</td>
<td>34</td>
</tr>
<tr>
<td>RC-V</td>
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<td>0</td>
</tr>
<tr>
<td>375</td>
<td>RC-III</td>
<td>6</td>
</tr>
<tr>
<td>389</td>
<td>RC-I</td>
<td>55</td>
</tr>
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<td>RC-II</td>
<td>3</td>
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</tr>
<tr>
<td>489</td>
<td>RC-I</td>
<td>4</td>
</tr>
<tr>
<td>667</td>
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<td>2</td>
</tr>
<tr>
<td>754</td>
<td>RC-IV</td>
<td>0</td>
</tr>
<tr>
<td>&gt; 800</td>
<td>RC-IV</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Methanococcaceae</td>
<td>4</td>
</tr>
</tbody>
</table>

¹Data taken from [12].
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Methanosarcina spp.

Rice cluster I

Methanosetaeaceae

Rice cluster II

Methanomicrobiaeae

Rice cluster III

Methanobacteriaceae

Rice cluster V

terrestrial mesophilic Crenarchaeota

/Rice cluster VI

freshwater mesophilic Crenarchaeota

/Rice cluster IV
samples from day 200, which were not analyzed in replicate, and soil Changchun, which could only be analyzed for day 0 because of PCR inhibition in soil samples from other time points. All T-RF were included as dependent variables in a single multivariate analysis of variance (MANOVA) general linear model incorporating both regression and categorical effects. Sampling time, halt phase, halt phase X sampling time, organic carbon content, nitrogen content, total free iron, and methane production rate were effects. Parametric assumptions were checked by examination of probability and residual plots, and were satisfied after transformation by logarithm (T-RF 80 and 88), or inverse sine (all other T-RF) of the T-RF relative frequencies. For effects with significant multivariate test statistics ($P < 0.01$), the univariate $F$-statistics (after Bonferroni adjustment for seven T-RF) and canonical loadings of each T-RF were examined. Statistical analyses were performed using Systat 10 (SPSS Inc., Chicago, IL, USA).

2.7. mcrA gene amplification and T-RFLP analysis

$mcrA$ fragments were amplified as described previously [14] using primer combinations $MCRf$ (5’-TAY GAY CAR ATH TGG YT-3’) and $MCRr$ (5’-ACR TTC ATN GCR TAR TT-3’) [31]. For T-RFLP analysis, PCR products were obtained using a FAM-labeled $MCRf$ primer as described previously in detail [14]. Restriction digests with SssI (Promega) were performed using $\sim 100$ ng of PCR amplicons. Digested amplicons were analyzed as described above.

3. Results

3.1. Phylogenetic analyses of SSU rDNA clones from rice field soils

Soil samples from different geographical sites exhibited a wide range of chemical characteristics and potential CH$_4$ production rates [19,20] which are summarized in Table 1. Soils were incubated anoxically for 2 h (day 0), 30 days, and 200 days (selected soils) as previously described [19,20] to compare the community structure of Archaea in the different phases of methanogenesis after flooding of the soil. The archaean community structure in the geographically different soils was analyzed by molecular methods targeting the SSU rRNA and $mcrA$ genes. Total nucleic acids were extracted from these soils and purified. Using an Archaea-specific primer set we obtained PCR amplifiers of the correct size from all soils, except from DNA extracts of a 30 days incubated soil sample from Changchun (China). For this particular soil sample, the archaeal community structure could not be analyzed, probably due to PCR-inhibiting substances still present in the DNA extract after purification.

Clone libraries of archaeal SSU rDNA were created from soil samples of Shenyang (China; Shen-A) and Gapan (The Philippines; Gap-A). The SSU rDNA insert of a total of 32 and 30 randomly selected clones from each soil, respectively, was sequenced (about 720 bp). Phylogenetic analyses showed that all clones fell within known eury- and crenarchaeotal lineages, i.e. major methanogenic groups such as the Methanosarcinaceae, Methanosaetaceae, Methanomicrobiaceae and Methanobacteriaceae, as well as the yet uncultured *Archaea* entitled RC-1, RC-2, RC-3, RC-4, and RC-VI [6,9,11]. Representative clones of SSU rDNA sequences are shown in Fig. 1 together with close database relatives and with archaeal sequences from Italian rice soils [6,9,11,12].

3.2. T-RFLP analysis of the archaean community structure

The SSU rDNA sequences retrieved from each clone library were analyzed by in silico digestion to identify restriction sites TaqI (Table 2). Ten different classes of T-RF were identified and affiliated to one or two of the 10 major phylogenetic groups of *Archaeae* (Methanomicrobiaceae, Methanobacteriaceae, Methanosarcinaceae, Methanosaetaceae, RC-1 to RC-VI). Characteristic group-specific T-RF predicted for Shenyang and Gapan SSU amplicons matched with previously defined groups for Italian rice field soil [11,12].

T-RFLP analysis was performed using the community DNA extracted from the different rice soils. Typical community fingerprints obtained by the T-RFLP analysis of SSU rRNA genes are shown in Fig. 2 (left panel) for the soils from Guangzhou (China), Gapan (The Philippines) and Pavia (Italy).

The relative contribution of the different phylotypes to the soil archaean community was quantified using peak area integration of SSU rDNA T-RF. We found that the relative peak area of a T-RF linearly increased with the increasing double-stranded DNA concentration of the amplicon digests, using the SSU rDNA of *Methanosarcina barkeri* as a standard (data not shown). Duplicate T-RFLP analyses using different DNA template concen-

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Fig. 1. Phylogenetic tree showing the placement of selected SSU rDNA clone sequences recovered from rice field soils Shenyang (Shen-A clones) and Gapan (Gap-A clones) within the *Euryarchaeota* and *Crenarchaeota*. Selected sequences of cultivated representatives (nearly full length rDNA) from archaean lineages as well as environmental sequences (partial sequences) from Verrilli rice field soil (ABS, ARR, EOH, H2, ST1, S15 and S30 clones [6,9-11]) and other environments were used as references to construct an evolutionary distance dendrogram (maximum likelihood method). SSU rDNA sequences of *Aquifex pyrophilus* and *Thermococcus maritima* as well as members of the *Korarchaeota* were used as outgroup references. The scale bar represents 10% sequence difference; GenBank accession numbers of sequences as indicated.
Fig. 2. Typical T-RFLP electropherograms of (left panel) archaeal SSU rDNA (Tsul digest) and (right panel) mcrA meraA gene (Sau96 digest) amplicons from rice field slurry samples of Guangzhou (China), Gapan (The Philippines), and Pavia (Italy), after 30 days of incubation. The archaeal lineages represented in different T-RF are Methanobacteriaceae (MB), Methanomicrobiaceae (MM), Methanomicrobiaceae (MS), Methanococcaceae (MX), and RC-I to RC-VI. In addition to mcrA gene T-RF, a 470-bp mcrA fragment specific for Methanosarcinaceae is indicated. T-RF lengths of selected peaks in bp are indicated by numbers. RFU, relative fluorescence unit.
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The results (20–60 ng) obtained from the same soil community gave reproducible fingerprints with the same relative distribution of T-RF (data not shown). The relative proportion of each T-RF to the total was independent of the number of PCR amplification cycles (24–44), as found before for Italian rice field soil [32] and other environments [33].

Certain SSU rDNA T-RF may represent more than one phylotype [12], e.g. 182 bp (RC-IV and Methanosarcinaeae), >800 bp (RC-IV and Methanosaetaeae). Therefore, the community structure of methanogens was analyzed in parallel by the more specific mcrA-targeted T-RFLP analysis (Fig. 2, right panel). In a previous study [14], the different T-RF of mcrA amplicons retrieved from Italian rice field soil were identified on the basis of a clone library of mcrA gene fragments (n=75) that had been sequenced and phylogenetically characterized. This phylogenetic classification was used to identify the major T-RF in fingerprints from the different soil samples shown in Fig. 2. In general, all major methanogenic groups (e.g. Methanosarcinaeae, Methanosaetaeae, Methanobacteriaeae, and RC-I) were detected by mcrA-targeted T-RFLP fingerprints. As in Italian rice field soil, mcrA genes characteristic of the Methanomicrobiaeae were not detected, but this lineage was present only at low SSU rDNA ratios as indicated by T-RFLP analysis (Fig. 2, left panel) and cloning of archaeal SSU rRNA genes from Shenyang and Gapan soils (Table 2).

3.3. Archaeal population dynamics

We used the seven major SSU rDNA T-RF (Table 2) for comparative analysis of the archaeal community in the different soils over time, i.e. 0, 30, and 200 days (some soils) of anoxic incubation (Fig. 3; Table 3). All seven major archaeal T-RF were detected in most soils, which had been incubated for only 2 h (day 0), indicating that these populations were either already present in the air-dried soil or that the PCR product originated from free soil DNA. Less than seven T-RF were detected in soils from Zhenjiang, Lusiana and Pila (Fig. 3). The most predominant T-RF in all soils analyzed were those of 182 bp (Methanosarcinaeae/RC-VI) and 389 bp (mainly RC-I) length. These had relative gene frequencies ranging from 22 to 54% and from 6 to 48%, respectively.

The effects of different environmental factors (hale phase, organic carbon content, nitrogen content, total free iron, and methane production rate) on the archaeal community composition were tested by MANOVA. The multivariate test statistics showed that each single factor had some significant effect on the community pattern. Many of these factors are cross correlated and of course

<table>
<thead>
<tr>
<th>Methanogenic group</th>
<th>Marker gene</th>
<th>T-RF (bp)</th>
<th>Country</th>
<th>Origin of soils (soil #)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>China</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zhenjiang (1)</td>
<td>Guangzhou (3)</td>
</tr>
<tr>
<td>Methanosarcinaeae</td>
<td>SSU rRNA</td>
<td>182</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>mcrA</td>
<td>394</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>427</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Methanosaetaeae</td>
<td>SSU rRNA</td>
<td>280</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>mcrA</td>
<td>147</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>419</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>RC-I</td>
<td>SSU rRNA</td>
<td>389</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
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<td>mcrA</td>
<td>238</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>++</td>
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<td>mcrA</td>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
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<td>88</td>
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<td>++</td>
</tr>
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<td></td>
<td>mcrA</td>
<td>406</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>503</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Table 3

Estimation of the relative gene frequencies of methanogenic Archaea in rice field soils from different geographic regions after 30 days of anoxic incubation as derived from T-RFLP analyses of SSU rDNA(TaqI digest) and mcrA genes (SacI digest).

Gene frequencies were categorized into three classes according to the percentage peak area of individual T-RF relative to the sum of all T-RF of a T-RFLP profile: 1-14%: ‘+’; 15-29%: ‘++’; >30%: ‘+++’. ‘?’ undetected mcrA gene of members of the Methanomicrobiaeae.
a causal relationship cannot be inferred from such a result. In any case, most of these effects were limited to only one or two minor T-RF. For example T-RF 80, which was present at only 0-5% total frequency (Fig. 3), was significantly affected by all factors except nitrogen content ($P < 0.01$). Other significant effects were methane production rate on T-RF 389, organic carbon on T-RF 182, nitrogen content on T-RF 88, and sampling time on T-RF 375.

On the other hand, halt phase significantly affected both dominant T-RF 182 and 389 (in each case $P < 0.01$). T-RF 80 and 280 were also significantly affected, but the effect was most evident for the dominant T-RF 182 and 389, based on canonical loadings of 0.698 and −0.495, respectively. An interaction of halt phase $\times$ sampling time was also significant on T-RF 182, 375, 389 and 800. Fig. 4 illustrates how the two major T-RF varied across soils with different halt phases. Soils with long halt phases tended initially to have a lower percentage of T-RF 182 and a correspondingly higher percentage of T-RF 389 compared to soils with short halt phases. At the later, day 30 sampling point, these differences had almost disappeared because the soils with long halt phases had developed communities more similar to those of the other soils. The statistical significance of the above conclusions was verified by dividing the soils into three groups (halt
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![Graph showing the influence of the halt phase on the relative gene frequencies of T-RF 182 and T-RF 389 on the first day of incubation (day 0) and after 30 days of incubation.](image)

Fig. 4. Influence of the halt phase on the relative gene frequencies of T-RF 182 bp and T-RF 389 bp on the first day of incubation (day 0) and after 30 days of incubation. Each point represents a separate soil and is the mean of three and four samples. Lines are least-square linear regressions.

Phase: 5 days, halt phase 9–13 days, and halt phase ≥ 23 days, performing an analysis of variance for each T-RF with sampling time and halt phase group as effects, and then doing post-hoc multiple Bonferroni contrasts for each halt phase group:sampling time pair. The pairwise comparison matrix demonstrated that T-RF 182 and 389 in soils with short halt phases did not change significantly from day 0 to 30, but did change in the soils with longer halt phases.

This observed pattern of relative SSU rRNA gene frequencies of the major archaeal groups, which appeared to be correlated to the length of the halt phase, was supported by the presence and estimated frequency of mcrA genes analyzed for soil samples after 30 days of incubation (Table 3). In general, gene frequencies of both marker genes, SSU rRNA and mcrA, were in good correlation and similar estimates were derived from both T-RFLP analyses. However, PCR/T-RFLP analyses of defined template mixtures with defined amounts of target DNA from pure cultures have revealed that methanobacterial mcrA genes (503-bp mcrA T-RF) tend to be over-represented (Lueders and Friedrich, unpublished).

The mcrA-targeted T-RFLP analysis clearly showed the presence of those methanogenic lineages which share a SSU rDNA T-RF with non-methanogenic lineages (e.g., Methanosarcinaceae/RC-VI). For example, mcrA genes of Methanosarcinaceae were abundant in soils with high levels of the 182-bp SSU rDNA T-RF (Table 3). Also the RC-I methanogens, represented by a characteristic 238-bp mcrA T-RF, were detected in all rice field soils regardless of geographical origin. Also the absence of the Methanoseta-specific SSU rDNA T-RF in the Zhenjiang soil was verified by mcrA T-RFLP analysis.

4. Discussion

Rice field soil from Italy was found to contain a diverse archaeal community comprising most of the families of known methanogens [34], but also including phylogenetic lineages within the Euryarchaeota and the Crenarchaeota for which no microbial isolate exists [6,11,12]. One of these novel lineages, RC-I, was recently identified as methanogenic [14]. Here we have shown that rice field soils from China and The Philippines exhibit a similar archaeal diversity, despite the fact that they originate from different geographical regions. Sequences of archaeal SSU rRNA genes retrieved from Chinese and Philippine soils grouped with the same major phylotypes that had been identified in Italian rice field soil, including the uncultivated RC-I to RC-VI. The same lineages were identified in a total of 11 different rice field soils from China, The Philippines, and Italy by using T-RFLP fingerprinting targeting the genes of the SSU rRNA and the methyl-coenzyme M reductase (mcrA). The mcrA gene is only found in methanogenic Archaea, whereas the SSU rRNA approach targets also non-methanogenic Archaea. The similar community patterns found in the different rice field soils analyzed by three approaches, i.e., (1) sequencing and phylogenetic analysis of SSU rRNA genes, (2) T-RFLP fingerprinting of SSU rRNA genes, and (3) T-RFLP fingerprinting of mcrA genes, suggest that rice fields all over the world contain methanogenic communities which are similarly structured with respect to the major methanogenic archaeal lineages.

The worldwide distribution of major methanogenic phylotypes in rice field soils is very interesting with regard to global dispersal and colonization of Archaea. Using classical isolation procedures, Joulian et al. [5] reported that the genera Methanobacterium and Methanospirillum were dominant among cultivable organisms and Methanobacterium bryantii, in particular, was isolated from 12 of 13 soils from France, The Philippines, and USA. The atmosphere is one of the main media for dispersal and aerosol particles help to transport microorganisms attached to the surface of droplets [35,36]. In addition, surface and subsurface waters may play a significant role in transport [37]. The question remains, however, how obligately anaerobic microorganisms such as methanogens can be dispersed so
widely, since these organisms do not possess classical resting stages (i.e., spores, cysts).

A worldwide distribution of the major methanogenic phylotypes is consistent with the observation of cosmopolitan phylogenetic clusters in other functional microbial groups, e.g., psychrophilic bacteria in ice [38] or pelagic freshwater bacteria [39]. This does not exclude, however, that the community structures with respect to taxonomic units of a level lower than that resolved by the molecular techniques applied might be different in the soils from the different geographic regions. It is worth mentioning that microbial communities were found to be composed of endemic ‘species’ provided that the genotypes were sufficiently resolved [40].

Molecular community fingerprinting by T-RFLP analysis is not only used for phylogenetic/taxonomic information but also for analysis of relative proportions of dominant phylotypes within the microbial community [28,33,41,42]. Although the rDNA-based T-RFLP analysis suffers from the lack of fine resolution at the species level, and also from biases related to cell lysis, DNA extraction and purification, and PCR amplification, it has many advantages for comprehensive sampling and rigorous comparative community analysis which can include process and biogeographical investigations [43,44]. We have used the T-RFLP fingerprinting technique for identification and quantification of the major archaenal phylotypes in different rice soils. We have quantified the relative amounts of the individual SSU rRNA gene T-RF as done before for different environments [28–30] and Italian rice field soil in particular [12,32,45,46]. These previous studies have shown that the quantification of relative amounts of individual SSU rRNA gene T-RF is highly reproducible. Our results confirm this conclusion. Since the distribution and abundance of characteristic T-RF in the different soils were not the same, the preferential amplification of certain sequences [47] or the kinetic bias effect [29] is not considered as a problem in PCR amplification and subsequent T-RFLP analysis in our studies. Osbora et al. [33] made similar observations during T-RFLP analysis of PCB-polluted and pristine soil. Biases, however, related to primer mismatches or the use of degenerate primers, especially in mcrA-targeted T-RFLP analysis, cannot be excluded and are currently being investigated (Luenders and Friedrich, in preparation). Therefore, we estimated the mcrA gene abundance by T-RFLP analysis qualitatively rather than reporting absolute quantities.

Some of the soils (Zhenjiang, Beiyan, Jurong, Shenyang, Gapan) showed a change in the relative composition of the archaenal community when they were incubated for extended periods under submerged and anoxic conditions indicating the existence of population dynamics. This dynamic may be connected to the temporal change in the activity of CH4 production. Although a change in CH4 production activity is observed in all rice field soils which generally produce CH4 in distinct phases [19], there are differences with respect to the duration of the individual phases, the reduction and methanogenic phases in particular. The reduction phase is dominated by reduction of ferric iron (and sulfate to a smaller extent) so that little CH4 is produced. The production of CH4, which is thermodynamically controlled, halts as long as the reduction of ferric iron proceeds. A pronounced halt phase is observed in many rice field soils [20], summarized in Table 1.

The archaenal population dynamics in the investigated soils appear to correlate with the duration of the halt phase, which fall into three groups: soils without or with a very short halt phase (Guangzhou, Luisiana, Pia, Pavia, Verceil), soils with a halt phase between 9 and 13 days (Gapan, Jurong, Shenyang, Beiyan) and those with an extended halt phase of up to 23 days (Changchun, Zhenjiang).

Soils without any halt phase or with only a short halt phase exhibited a very stable community structure over time, probably because the ratio of degradable organic matter to reducible iron allowed the continuous production of CH4 even during the phase of iron reduction [20]. In these soils, the archaenal population seemed to be rather stable and exhibited no dynamics, and was dominated mostly by members of RC-I and the Methanosarcinaceae. It should be noted, however, that this stability refers to the DNA of the populations and does not reflect their potential activities, which may well change with incubation time, and also does not reflect their actual activities (potentially modified by substrate availability) which definitely do change with incubation time.

With increasing duration of the halt phase, soils exhibited an increasing proportion of relative SSU rRNA gene frequencies of Methanosarcinaceae with a concomitant decrease of the RC-I populations especially at day 0. During incubation these soils developed a community structure which was comparable to soils without halt phase. In soils with the longest halt phase the initial dominance of Methanosarcinaceae was most pronounced. The observed difference in community structure of soils with a prolonged halt phase before initiation of methanogenesis may be linked to site-intrinsic factors which favor the presence of Methanosarcina spp. in certain rice field soils, but the causes are unknown.

The relatively pronounced stability of the archaenal community structure in Italian rice field soil has been noticed before [12,32]. Only the relative contribution of Methanosarcinaceae was found to increase significantly (∼50%) in the initial phases of methanogenesis (0–15 days) after flooding of the soil. We might have overlooked this temporary change of community structure since we did not analyze the initial phase of methanogenesis in this study. Enumeration by culturing methanogens also demonstrated relatively constant numbers over the season in the field and over incubation time in the laboratory [22,48]. Changes in incubation temperature, on the other hand, resulted in a pronounced change in the methanogenic community structure [11,46,49].
In summary, we found that the occurrence of major archaeal lineages was surprisingly constant among all the various rice field soils from different geographic regions. We hypothesize that differences, if they exist, are only on a taxonomic level at or below that of microbial 'species' [50]. However, we also found that the relative composition of the archaeal communities was different, though only to some degree and among some of the soils tested. Furthermore, the composition changed with time of anoxic incubation, but only in those soils that showed pronounced changes in CH₄ production during their reduction and methanogenic phases.

Acknowledgements

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References

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C.6. Untersuchungen zum Einfluss der PCR-Amplifikation auf die Wiederfindung definierter Kopienzahlen von 16S rRNA- und mcrA-Genen mittels T-RFLP

Tillmann Lüders und Michael W. Friedrich


Evaluation of PCR amplification bias by T-RFLP analysis of SSU rRNA and \textit{mcrA} genes using defined DNA template mixtures of methanogenic pure cultures and soil DNA extracts

Tillmann Lüders and Michael W. Friedrich

Abstract

Terminal restriction fragment length polymorphism (T-RFLP) analysis is an emerging fingerprinting method, which has been successfully used to monitor microbial communities in different habitats by targeting SSU rRNA and even functional marker genes. Although T-RFLP analysis is used for inferring the relative composition of microbial communities, it is not known whether gene frequencies of individual populations are adequately represented in post-PCR relative amplicon frequencies. Therefore, we generated artificial template mixtures containing genomic DNA of 4 different methanogens with defined gene content. Mixed composition PCR amplicons were generated for SSU rRNA and methyl-coenzyme M reductase (\textit{mcrA/mrtA}) genes and analyzed by T-RFLP. Relative amplicon frequencies of each methanogen were quantified by comparing fluorescence intensities of characteristic terminals restriction fragments. Defined template ratios and changes in the artificial community were adequately reflected in SSU rDNA-targeted T-RFLP analysis. Results of \textit{mcrA}-targeted T-RFLP analysis were affected by differences in operon copy number of the different methanogens and biased by the degenerate functional primers. Furthermore, we could show that PCR/T-RFLP analysis of environmental DNA extracts is highly reproducible, and independent of PCR cycle number. Our findings demonstrate that PCR/T-RFLP analysis is an accurate tool for the investigation of microbial communities, but also pinpoint limitations of the method.

Introduction

Molecular fingerprinting methods are of increasing importance in microbial ecology, as they allow to monitor diversity, structure, and dynamics of microbial populations in replicate numbers adequate to answer ecologically relevant questions. They are based on the PCR amplification of a mixture of genes representing different microorganisms from environmental nucleic acid extracts. Mixed PCR products are separated and visualized by a variety of electrophoresis techniques (denaturing gradient gel electrophoresis (DGGE),
single strand conformational polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP)), that employ different amplicon characteristics for separation (melting behavior, electrophoretic mobility, endonuclease restriction sites). Classically, this is done targeting SSU rRNA genes as universal marker, and methods to investigate a broad range of different phylogenetic lineages in natural ecosystems have been established (e.g. Bacteria (11,16,19), Fungi (2,14,31), and Archaea (5,22,25)). However, functional groups of microorganisms are often not monophyletic, and thus not easily discriminated from other, closely related, but physiologically distinct organisms by SSU rRNA genes (15). A great improvement is the utilization of functional marker genes, which allow to specifically target ecologically significant groups of microorganisms. Functional markers are genes, which encode characteristic key-enzymes of functional guilds and therefore allow the affiliation of microorganisms represented by sequences detected to their likely function in the environment. Recently, fingerprinting techniques for functional genes of methane- and ammoniumoxidizers (6,10,13,21), nitratereducers (3), and methanogens (17) have been developed.

However, these fingerprinting techniques are PCR-based, therefore they may be subject to potential PCR bias and thus may not provide a qualitative or quantitative assessment of microbial populations. Potential PCR-inherent biases like preferential amplification of certain templates (23,32,33) or template re-annealing with increasing PCR cycle numbers have been discussed (28,29).

In this study, we thoroughly evaluated the potential of fingerprinting methods for methanogenic communities using both phylogenetic and functional markers (SSU rDNA- and mcrA-targeted T-RFLP analyses). We present data on the reproducibility of environmental fingerprinting results, and also on the effects of varying amplification cycle numbers. Furthermore, we analyze defined template mixtures of methanogenic pure cultures (“artificial communities”) by T-RFLP, to evaluate, how template ratios are represented by PCR/T-RFLP fingerprinting analysis.

**Materials and Methods**

**Pure cultures, soil samples, and DNA extraction.** Methanobacterium bryantii DSM 863\textsuperscript{T}, Methanoseta concilii DSM 3671\textsuperscript{T}, and Methanospirillum hungatei JF1 DSM 864\textsuperscript{T} were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and cultivated in standard media as specified by the supplier prior to DNA extraction. Genomic DNA of Methanococcus jannaschii DSM 2661\textsuperscript{T} was provided by Dr. Gerrit Buurman (MPI Marburg, Germany). Soil
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samples were from an Italian rice field soil near Vercelli and anoxically incubated in serum bottles for 14 days as described before (18). DNA was extracted and purified from pure culture cell material and ~600 µl soil slurry aliquots as published elsewhere (Lueders and Friedrich, submitted).

DNA quantification. After extraction, DNA solutions were preliminarily quantified by standard UV photometry (BioPhotometer, Eppendorf, Hamburg, Germany). Precise quantification of diluted DNAs was performed with the PicoGreen dsDNA quantification kit (Molecular Probes, Leiden, The Netherlands) in 96-well microtitre plates on a Fluorolite 1000 fluorescence microtitre plate-reader (Dynatech Laboratories, Chantilly, VA.). Standardization of measurements was done with λ DNA (Molecular Probes) in concentration range between 1 and 50 ng [100 µl-well]⁻¹. The 96-well format permitted the simultaneous measurement of a high number of replicates for each DNA sample (three replicates each of two different dilutions).

PCR amplification. PCR amplification of archaeal SSU rRNA genes for T-RFLP analysis was done as described before (18) using the primer combination Ar109f (5’-ACK GCT CAG TAA CAC GT-3’) (8) and a 5’ 6-carboxyfluorescein (FAM)-labeled Ar915r primer (5’-GTG CTC CCC CGC CAA TAC CT-3’) (27) (MWG Biotech, Ebersberg, Germany). Partial mcrA amplicons were generated for T-RFLP analysis with the 5’ FAM-labeled MCRf (5’-TAY GAY CAR ATH TGG YT-3’) and MCRr (5’-ACR TTC ATN GCR TAR TT-3’) primer pair (26) as described earlier (17). After amplification, PCR aliquots (5 µl) were visualized by standard agarose gel electrophoresis. Amplicons were purified with the MinElute PCR purification kit (Qiagen, Hilden, Germany), re-eluted in 25 µl of EB buffer (Qiagen) and quantified by UV photometry.

Real-time PCR. The SSU rRNA gene content of pure culture DNA dilutions was quantified using a iCyler iQ real-time PCR detection system (Bio-Rad, Munich, Germany) and the amplicon detection by SybrGreen fluorescence. The reactions were set up in MicroAmp 8-tube strips (Applied Biosystems, Weiterstadt, Germany) and sealed with MicroAmp optical 8-cap strips (Applied Biosystems). Each reaction contained, in a total volume of 50 µl, 1x SybrGreen PCR Master Mix (Applied Biosystems), 0.3 µM of each primer (Ar109f / Ar915r, unlabeled), and 2 µl of standard or unknown DNA template. The thermal protocol for real-time PCR amplification and detection was 10 min of initial denaturation (94°C) followed by 40 amplification cycles (30 s, 94°C; 30 s, 52°C; 60 s, 72°C). Before each quantification run, well factors were collected with a 96-well microtitre plate containing 50 µl aliquots of a 25 nM fluorescein-5-isothiocyanate (FITC) solution. After every run, melting curves were evaluated for each PCR reaction to discriminate between specific and unspecific real-time signals. Real-time PCR collection data was analyzed using the iCylcer optical system interface software (version 2.3.1370, Bio-Rad).

T-RFLP analysis. Restriction digests of SSU rDNA amplicons were performed using TaqI, and mcrA amplicons were digested with Sau96 I, as previously described (17). Electrophoretic separation of restricted amplicons was done on an ABI Prism 377 DNA sequencer (Applied Biosystems) in GeneScan mode (Lueders and Friedrich, submitted). SSU rDNA amplicons were standardized with the GeneScan-1000 (ROX) size standard (Applied Biosystems), while the length of mcrA T-RFs was determined with the GeneScan-500 (ROX) size standard (Applied Biosystems). Relative fluorescence signals of T-RFs were measured using peak area integration. Signals with peak height below 100 relative fluorescence units (RFU) or with peak area contribution below 1% were regarded as background noise and excluded from analysis (20).
Results and Discussion

Fingerprinting analysis of microbial populations is based on two fundamental procedures prior to electrophoresis: DNA extraction and PCR amplification. To verify the reproducibility of the entire method, we extracted duplicate DNA samples from three parallel rice field soil slurries (e.g. 6 replicate DNA extractions) and analyzed these by archaeal SSU rDNA-targeted T-RFLP analysis (Fig. 1).

![Graph showing reproducibility of fingerprinting analysis of archaeal populations as shown by T-RFLP analysis of 6 replicate DNA extractions. (Fig. 1)](#)

Our data shows that DNA extraction and PCR/T-RFLP analysis were highly reproducible, 6 replicate fingerprints yielded an average SD of 0.8% RAF. The highest absolute SD was measured for the most prominent 182 bp T-RF (36.8 ± 1.7%) representing *Methanosarcina* spp. (18), highest relative SD was 2.1 ± 0.7% for the 496 bp T-RF (e.g. 32.9%). Thus, peaks of minor abundance are less accurately displayed. This is in concordance with the general practice in T-RFLP evaluation, that signals below 100 RFU peak height are excluded from further analysis, as it is impossible to discriminate their true information content from background noise (20).

To examine the effect of increasing PCR cycle numbers on T-RFLP results, we amplified archaeal SSU rRNA genes from a rice field soil DNA template with PCR.
amplification between 22 and 45 cycles. At least 26 amplification cycles were necessary to generate sufficient amplicon quantities for T-RFLP analysis (Fig. 2).

![Image](image.png)

FIG. 2. Effect of increasing PCR cycle numbers on the SSU rDNA-targeted T-RFLP analysis of a rice field soil archaeal community. (A) Ethidium bromide stained gel image of generated amplicons between 22 and 45 PCR cycles. X, no template, 45 cycles. (B) Contribution percentage of individual T-RFs to total integrated fluorescence of T-RFLP electropherograms.

We could show, that T-RFLP profiling was independent of PCR cycle number within a range of 30 to 45 amplification cycles, as indicated by the relatively stable RAF of T-RFs detected. Minor differences were detected with 26 and 28 PCR cycles, especially the 734 and >800 bp T-RFs were more abundant than with 30 or more cycles. The 734 bp T-RF is a very long fragment and the >800 bp peak represents undigested amplicons with no TaqI restriction site. Both migrate very slowly during electrophoresis, which results in broad, less defined (“fuzzy”) peaks compared to shorter T-RFs (<500 bp). With respect to the low total amplicon quantities generated with 26 and 28 PCR cycles, and, essentially, loaded onto the gel, we assume a relative over-representation of “fuzzy” fluorescence peaks in electropherograms with a low total fluorescence intensity. Also, the 383 bp T-RF was below detection limit with 26 amplification cycles. In general, we could show, that minor peaks did not become more abundant with increasing PCR cycle numbers (once the
detection threshold was exceeded). Therefore, for this analysis we could exclude a potential bias of RAF by template re-annealing, which would impose minor T-RFs to become relatively more abundant and approach a 1:1 ratio with increasing PCR cycles (29).

While both results are very supportive of the T-RFLP method and suggest it to be both robust and reproducible, the central question of how the relative abundance of amplicons represents template gene ratios cannot be answered by the analysis of environmental DNA extracts of unknown composition. Therefore, DNA template mixtures of defined gene content have to be artificially created. Defined template mixtures for PCR analysis have been created earlier by mixing two (29) or three (28) different PCR products or pairwise mixing of pure culture genomic DNAs (12,23,30). In this study, we chose to mix the genomic DNAs of four methanogenic pure cultures, as also in environmental DNA extracts whole genomes are the actual PCR templates, and not PCR products. This also allows the comparison of results obtained for both SSU rDNA- and mcrA-targeted T-RFLP analyses of identical mixtures. The SSU rRNA gene content of pure culture DNA extracts was quantified by real-time PCR analysis. A freshly prepared 10-fold dilution series of *M. jannaschii* genomic DNA with PicoGreen-determined DNA-content was taken as standard. The genome of this organism has been sequenced (1664970 bp (4)) and it contains two *rrn*-operons and thus 1.196 x 10^6 SSU rDNA gene copies per ng of DNA. In real-time PCR, the quantity of formed dsDNA in each reaction cup is monitored continuously via fluorescence. The threshold cycle (C_T), at which a certain fluorescence threshold value is exceeded, is recorded for each cup. This threshold cycle is linearly dependent on the logarithm of the starting quantity of gene copies in each template (9). Standardization was performed with a minimum of two replicates for each measurement and was shown to be reliable between at least 10^7 and 10^9 *M. jannaschii* SSU rRNA gene copies (Fig. 3). The constant amplification efficiency (ε_c) for the ~800 bp *M. jannaschii* SSU rDNA amplicons derived from the slope of the standard curve was 0.90 and calculated as ε_c = 10^{-1/s} - 1 (1). The determined ε_c implicates a very efficient amplification, as the maximum possible ε_c = 1 imposes every template to be duplicated in every round of amplification.
FIG. 3. Real-time PCR standard curve creation with the \( C_T \) method. (A) Plots of SYBR Green fluorescence recorded for a duplicate 10-fold dilution series of \( M. jannaschii \) genomic DNA with defined SSU rRNA gene content. RFU, relative fluorescence units. (B) Standard curve shows plots of \( C_T \) versus \( \log_{10} \) of SSU rRNA gene copies. \( s \), slope. \( \epsilon_c \), amplification efficiency.

Genomic DNAs of \( M. bryantii \), \( M. concilii \), \( M. hungatei \), and \( M. jannaschii \) were freshly diluted to working solutions of \( \sim 2 \) ng \( \mu l^{-1} \) (PicoGreen measurement). The SSU rRNA gene content of working solutions was determined by triplicate real-time PCR, the average SD of measurements was below 2\% of means. These diluted genomic DNA working solutions with defined DNA and SSU rRNA gene content were then used for the preparation of defined template mixtures. For each of the four methanogens, a series with increasing template ratios between 7.1 and 78.6\% was prepared. Immediately after preparation, the standardized genomic DNA mixtures were analyzed by both SSU rDNA-
and mcrA-targeted T-RFLP analyses. The results obtained by T-RFLP analysis of mixed SSU rDNA genes in two replicate mixing experiments are shown in Fig. 4. The relative abundance of T-RFs was linearly dependent on template ratios for all tested organisms and in average in absolute correlation to the given template ratios.

FIG. 4. Relation between SSU rDNA template ratios and relative amplicon frequencies [RAF] as determined by T-RFLP analysis of defined template mixtures prepared with genomic DNA of 4 methanogens. Line indicates best linear fit for all data points.

Increasing template ratios were accurately detected for the four methanogens tested and a significant under- or over-representation of any of the utilized species was not observed. Therefore, preferential amplification (or PCR selection (33)) could be excluded for the tested genes and genomes. However, the actual values measured did fluctuate between the two replications of the mixing experiment. This might be attributed to simple pipetting errors during the preparation of defined templates, where minute volumes of DNA working solutions need to be transferred with extreme precision. The observed fluctuations could also be related to irreproducible stochastic variations in the early cycles of PCR amplification (PCR drift (33)). As mentioned above, the data presented here is only preliminary, and the authors hope to obtain clearer evidence for what might be causing the observed fluctuations by further repetitions of the entire mixing experiment.
In this study, we wanted to address not only the reliability of SSU rDNA-targeted, but also of \textit{mcrA}-targeted T-RFLP fingerprinting of methanogenic communities. This was of special interest, since differences in relative representation of certain lineages by the two different approaches had become apparent earlier (17,24). Especially, the \textit{mcrA/mrtA} genes of members of the \textit{Methanobacteriaceae} were of increased relative abundance compared to SSU-rDNA derived data, while members of the \textit{Methanomicrobiaceae} where only detected via SSU rDNA genes, and not via their \textit{mcrA} genes. To compare results for the two different markers, it is important to know the respective operon copy numbers within genomes of the four methanogens. \textit{M. jannaschii} harbors two \textit{rrn}-operons, plus one \textit{mcr}- and one \textit{mrt}-operon (4). MRT is an isoenzyme of the MCR also detected in \textit{mcrA} fingerprinting analysis (17). \textit{M. bryantii} also contains two MCR isoenzymes, but its \textit{rrn}-copy number is unknown. \textit{M. concilii} and \textit{M. hungatei} are known to contain at least one \textit{mcr}-operon, a second isoenzyme has not been detected. Also their \textit{rrn}-copy number is unknown, but in general, \textit{Archaea} have been reported to contain between 1 and 2 SSU rRNA genes (7).

We compared the measured SSU rRNA gene ratios of the defined template mixtures to their \textit{mcrA} gene ratios. Pronounced representational differences for the different species analyzed became apparent (Fig. 5). Although increasing ratios were also reflected, the \textit{mcrA} genes of \textit{M. hungatei} were under-represented, while the combined \textit{mcrA/mrtA} genes of \textit{M. bryantii} were over-represented. The \textit{mcrA/mrtA} genes of \textit{M. jannaschii} and the \textit{mcrA} genes of \textit{M. concilii} were of a more equal representation in both fingerprinting methods. These observations could be explained by the following assumptions: \textit{M. bryantii}, strongly overrepresented by its \textit{mcrA/mrtA} genes, contains only one \textit{rrn}-operon. \textit{M. hungatei}, on the other hand, contains two \textit{rrn}-operons, but only one \textit{mcr}-operon. \textit{M. jannaschii} and \textit{M. concilii} possess an equal operon distribution, e.g. one \textit{mcr}-, one \textit{mrt}-, plus two \textit{rrn}-operons for \textit{M. jannaschii}, and one \textit{mcr-} plus one \textit{rrn}-operon for \textit{M. concilii}. However, also other possible bias sources have to be taken into account for \textit{mcrA}-fingerprinting analyses. The primer set (26) for the amplification of partial \textit{mcrA/mrtA} sequences is strongly degenerate and contains a total of 9 wobbled nucleotides. Different binding energies for GC-rich permutations have to be assumed, that may lead to preferential amplification of certain templates (23). Such PCR selection bias (33) might account for the observed non-linear representation-relations of certain species (Fig. 5).
In this study we could show, that SSU rDNA-targeted T-RFLP analysis is a robust and reproducible tool for the accurate determination of archaeal gene frequencies. Different SSU rRNA gene ratios were absolutely reflected in relative amplicon frequencies, and possible bias sources like PCR selection or template re-annealing could be excluded. Increasing template ratios were also detected via mcrA-targeted T-RFLP analysis, however representation varied for the four different methanogens tested. Our results suggest that T-RFLP analyses is an appropriate tool to monitor composition and dynamics of microbial populations. Fingerprints generated with different primer sets may not be directly comparable due to differences in gene copy numbers within microorganisms, and amplicon ratios obtained with degenerate functional primers may be biased due to preferential amplification of certain templates.

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**References**


D. Zusammenfassende Diskussion

Die Methanogenese ist im globalen Kohlenstoffkreislauf von fundamentaler Bedeutung und Reisfelder stellen ein wichtiges, anthropogen beeinflusstes Habitat methanogener Mikroorganismen dar. Die Physiologie der in Reinkultur vorhandenen methanogenen *Archaea* und auch die Prozesse der Methanogenese im Reisfeld sind gut untersucht. Das Wissen aber über die methanogene Gemeinschaft im natürlichen System ist begrenzt und die generellen Prinzipien, die die Diversität und Struktur methanogener Populationen sowie die Interaktion und Konkurrenz verschiedener Spezies untereinander beeinflussen, sind weitgehend unbekannt.


D.1. Diversität methanogener *Archaea* im Reisfeldboden


Zunächst war aber noch unklar, ob die nachgewiesenen neuartigen *mcrA*-Gene tatsächlich den *Archaea* des RC-I zuzuordnen waren. Die kongruente Topologie der rekonstruierten McrA- und 16S rRNA-Dendrogramme schien dies zwar nahezulegen, der endgültige Beweis gelang aber mit Hilfe einer methanogenen Anreicherungskultur. In


D. Zusammenfassende Diskussion

D.2. Das Potential der T-RFLP-Analyse


In dieser Arbeit wurde die von Chin et al. (1999) entwickelte „Fingerprinting“-Methode für archaellle 16S rDNA sowohl zur zeitlich fein-aufgelösten Analyse von Populationsdynamiken eingesetzt (Lueders und Friedrich, 2000), als auch erstmals über RT-PCR zur Untersuchung der archaellen 16S rRNA herangezogen (Abschnitt C.3.). Ein Nachteil dieser auf der 16S rDNA basierenden Methode ist aber, dass die Gene aller anwesenden Archaea erfasst werden und eine Differenzierung zwischen methanogenen und nicht-methanogenen Archaea nicht vollständig möglich ist (Chin et al., 1999; Lueders und Friedrich, 2000). Deshalb wurde im Rahmen dieser Arbeit zusätzlich eine neue, mcrA/mrtA-gestützte T-RFLP-Analyse entwickelt und eingesetzt, die erstmals die spezifische Untersuchung von methanogenen Gemeinschaften erlaubt (Lueders et al.,

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D.3. Struktur methanogener Populationen

Die Untersuchung der Struktur der methanogenen Gemeinschaft im italienischen Reisfeldboden wirft die Frage nach der Abundanz verschiedener Populationen auf. Mit klassischen Kultivierungstechniken wie seriellen MPN-Verdünnungsreihen wurde die Zahl der hydrogenotrophen Methanogenen in 90 Tage alten Reismikrokosmen auf \(2 \times 10^6\) bis \(2 \times 10^7\) und die der acetonotrophen Methanogenen auf \(5 \times 10^5\) bis \(1 \times 10^6\) Zellen pro g trockenem Boden bestimmt (Grosskopf et al., 1998a). Gleichzeitig wurden *Methanobacterium* und *Methanoseta* spp. als dominante hydrogenotrophe bzw. acetonotrophe Methanogene identifiziert. Dieser Befund korreliert gut mit den während der Vegetationsperiode geringen Acetat-Konzentrationen im Porenwasser gefluteter Reisfelder (<100 µM), die zu niedrig für die Verwertung durch andere acetonotrophe Methanogene der Gattung *Methanosarcina* sind (Schwellenwerte von 200 – 1200 µM (Jetten et al., 1992)).


**D.3. Dynamik methanogener Populationen**


Das funktionell äußerst dynamische Ökosystem des frisch gefluteten Reisfeldes ist während der Aktivierung der methanogenen Gemeinschaft also durch eher geringe methanogene Populationsdynamik gekennzeichnet. Eine Möglichkeit zur Induktion dauerhafter funktioneller Veränderungen im Reisfeld ist die Zugabe alternativer Elektronenakzeptoren wie Fe³⁺ oder SO₄²⁻. Dadurch werden die mit den Methanogenen um gemeinsame Substrate konkurrierenden respiratorischen Gilden der FRB oder SRB stimuliert, was eine Veränderung des Kohlenstoffflusses und, unter Substratlimitierung, eine Hemmung der Methanogenese zur Folge hat (Achtinich et al., 1995; Denier van der Gon und Neu, 1994; Jäckel und Schnell, 2000b). Diese Prozesse sind aus klimatischer und mikrobiologischer Sicht von zentralem Interesse, da sie sowohl eine Strategie zur Emissionsminderung darstellen, als auch eine Möglichkeit zur Analyse der Interaktion
verschiedener funktioneller Gilden bieten. In dieser Arbeit wurde der Einfluss der Zugabe von Ferrihydrit und Gips als alternativen Elektronenakzeptoren auf die Populationsdynamik der Methanogenen nach der Flutung des Reisfeldbodens untersucht (Abschnitt C.3.).


**D. Zusammenfassende Diskussion**


**D.4. Schlussbetrachtung und Ausblick**


Fragen zu Struktur und Dynamik anderer mikrobieller Populationen im frisch gefluteten Reisfeld sind aber nach wie vor offen. Insbesondere sollten in zukünftigen Untersuchungen ebenfalls die eisen- und sulfatreduzierenden Gemeinschaften Gegenstand des Interesses sein. Nur so kann das Wissen um die äußerst vielschichtigen Interaktionen
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der verschiedenen funktionellen Gilden erweitert werden. Da es sich aber auch bei den FRB und SRB nicht um monophyletische Gruppen handelt, ist zur umfassenden Analyse dieser Gilden ebenfalls der Einsatz funktioneller Marker notwendig. Nur für die SRB sind diese in Form der Adenosin-5'-Phosphosulfat Reduktase (Deplancke et al., 2000; Friedrich, 2002) und der dissimilatorischen Sulfitreduktase (Klein et al., 2001; Wagner et al., 1998) teilweise schon vorhanden, bei den FRB besteht noch erheblicher Forschungsbedarf.

Es sollten auch weiterhin alle Anstrengungen unternommen werden, die neuartigen Methanogenen des RC-I in Reinkultur zu isolieren, denn nur mit der physiologischen Charakterisierung dieser Organismen kann eine vollständige Aufklärung ihre Rolle im Reisfeld erfolgen. Ein wichtiger Beitrag zur Aufklärung der Funktion nicht-kultivierter Mikroorganismen in natürlichen Habitaten kann aber auch über neue Techniken der molekularen mikrobiellen Ökologie geleistet werden. Über die Kombination von Mikroautoradiographie und Fluoreszenz-in-situ-Hybridisierung (FISH) kann der Umsatz von radioaktiv markierten Substraten durch phylogenetisch definierte, aber nicht in Kultur befindliche Organismen direkt in Umweltproben nachgewiesen werden (Lee et al., 1999). Des Weiteren ist über den Einbau von $^{13}$C-markierten Substraten in die DNA aktiver Populationen nach Dichtegradienten-Zentrifugation die Beteiligung vielfältiger Mikroorganismen am Umsatz eines Substrates nachweisbar (Radajewski et al., 2000). Ein ebenfalls vielversprechender Ansatz ist die direkte Klonierung und Sequenzierung großer genomischer Fragmente aus Umweltproben, die es erlaubt, Informationen über die enzymatische Ausstattung nicht-kultivierter Mikroorganismen zu erhalten (Beja et al., 2000; Rondon et al., 2000; Schleper et al., 1997).
E. Literatur


F. Anhang

F.1. Die Beteiligung methanogener Populationen am Abbau von Reisstroh in anoxischem Reisfeldboden

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Methanogenic populations involved in the degradation of rice straw in anoxic paddy soil

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Abstract

Additional of straw to anoxic rice field soil stimulates production of CH₄, an important greenhouse gas. The archaeal community colonizing rice straw was investigated by molecular methods targeting the small subunit ribosomal RNA gene. Cloning and sequencing of 60 clones detected predominantly relatives of Methanobacterium spp. (38 clones) and Methanosarcina spp. (16 clones). Terminal restriction fragment length polymorphism (T-RFLP) analysis confirmed the dominance of Methanobacteriaceae and Methanosarcinaceae, and in addition showed restriction fragments characteristic for Rice cluster I (RC-I) methanogens. A new oligonucleotide probe specific for RC-I was designed. Quantitative slot blot hybridization of extracted rRNA with this probe indicated the presence of an active population of RC-I methanogens. Other methanogenic groups (e.g. Methanomicrobiaceae, Methanosaetaeaceae), although present and active in soil, could not be conclusively detected on rice straw. The methanogenic community pattern on straw, as revealed by T-RFLP and quantitative rRNA probing, was fairly constant with incubation time (8–57 days), but the total activity of methanogenic Archaea almost doubled. Our results indicate that the methanogens colonizing rice straw are less diverse than those inhabiting the soil. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Methanogen; Small subunit ribosomal RNA gene; Terminal restriction fragment length polymorphism analysis; Rice cluster I; Slot blot hybridization; Archaea

1. Introduction

Methanogenic Archaea catalyze the last step in the anaerobic degradation of organic matter, the formation of CH₄, which is an important greenhouse gas. Methanogens are found in various anaerobic environments such as freshwater, brackish and marine sediments, hot springs, sewage digesters, intestinal tracts and flooded soils. In the anaerobic zones of flooded rice fields methanogens are common inhabitants. Flooded rice fields cover a total area of about 1.43 million km² with a total CH₄ production rate of about 60 Tg year⁻¹, and are thus one of the major sources of atmospheric CH₄ [1–3].

Rice fields are commonly fertilized with organic matter, mainly rice straw, which is ploughed under after rice harvest. The rice straw serves as substrate for a complex microbial community consisting of hydrolytic (cellulolytic), fermenting, homoacetogenic, syntrophic and methanogenic microorganisms [4,5]. Methane and CO₂ are the major end products of the degradation process. Many studies showed that fertilization of rice fields with straw increases the release of CH₄ [6–9]. In principle, the degradation pattern in soils with and without amended rice straw is similar, with acetate, propionate and H₂ as the main intermediates of anaerobic degradation and CH₄ being formed from H₂/CO₂ (11–27%) and acetate (84–89%). However, the early phase of straw degradation differs as a large variety of fatty acids accumulate transiently [6]. The bacterial community colonizing and degrading rice straw seems to consist mainly of Clostridium species belonging to the clusters I, III and XIVa [10], as found for soil that has not been treated with straw [11,12]. However, β- and γ-Proteobacteria and Acidobacteria were only found on straw and not in soil while Verrucomicrobia and Actinobacteria were detected in soil but not on straw [10–12].

The genera Methanosarcina and Methanobacterium were frequently isolated from rice field soils and were also found using molecular techniques. In addition members
of the families Methanoacetaceae, Methanomicrobiaceae and Rice cluster I (RC-I) were detected [13–15]. Members of RC-I possess the enzyme methyl-coenzyme M reductase (mcrA) gene for methane production [16] and apparently are able to form CH₄ using H₂/CO₂ as substrate [17,18]. All these studies were done with rice field soil, but decomposing rice straw has not yet been investigated. Therefore, we studied the methanogenic populations that colonize rice straw using molecular methods targeting the small subunit rRNA gene (SSU rRNA). Randomly selected SSU rDNA clones established from archaea-specific PCR amplicons were sequenced and compared to assess the diversity of methanogens in rice straw-degrading soil samples. The population dynamics over time were investigated by terminal restriction fragment length polymorphism (T-RFLP) analyses. Important methanogenic groups were quantified by slot blot analyses. A new SSU rRNA probe was designed for quantification of members of RC-I.

2. Materials and methods

2.1. Incubation of soil and straw samples

Soil was obtained from rice fields in Vercelli, Italy. The soil was a sandy loam and has been described before [19]. The soil had previously been used for growing rice in a greenhouse, and then was drained, air-dried, crushed, sieved (1-mm mesh size) and stored at room temperature. The straw originated from rice plants (Oryza sativa, var. Roma) and was air-dried and stored at room temperature. For the experiments, only stems of the straw were used, cut into pieces of approx. 2 cm length.

The setup of the experiments was described previously [6]: 40 g dry soil and 0.5 g straw were mixed with 40 ml deionized water, filled into 150-ml glass bottles (Müller and Krempel, Büllach, Switzerland) and incubated for up to 10 weeks at 25°C in the dark. The amount of straw corresponds to 37.5 t ha⁻¹, i.e. about three times higher than normal [9]. Methane formation was measured as described by Glissmann and Conrad [6].

2.2. Preparation of the soil and straw samples for DNA and RNA extraction

For DNA extraction the straw pieces were removed after different incubation times from the soil and washed twice in phosphate-buffered saline (PBS, 0.8% NaCl in 10 mM phosphate, pH 7.0). The straw was then placed in a plastic bag together with 5 ml fresh PBS and treated with a stomacher (Seward, London, UK) for 1 min at high speed [20] to release cells attached to the straw. The PBS was collected, replaced by 5 ml fresh PBS, and the procedure was repeated twice. Cells were then concentrated by centrifugation (2516×g, 15 min). The cell pellet and the treated straw were combined and stored at –20°C for later DNA extraction (see below). Straw was added back to allow the retrieval of DNA from cells which might not have been detached from the straw by the stomacher treatment. For RNA extraction (see below) both straw and soil samples were used. After removal of the straw pieces from soil, the straw was washed twice in PBS and then immediately used for RNA extraction. The residual soil was also used for RNA extraction. Straw and soil samples were from one replicate at each time point.

2.3. DNA extraction, PCR, cloning and sequencing

The procedure used for DNA extraction was as described before [10,21]. DNA extracts were purified and DNA samples amplified by PCR with the Archaea-specific primer pair 1090.915r as described elsewhere [14]. The DNA concentration was 5–25 ng for each reaction. Aliquots of PCR products were analyzed by electrophoresis on agarose gels. Four clone libraries were created with archaeal SSU rDNA amplicons from ARS1 (straw incubated in anoxic soil for 1 week), ARS2 (2 weeks), ARS3 (3 weeks) and ARS7 (7 weeks). Cloning was performed using the TOPO Cloning Kit (pCR 2.1 Vector, Escherichia coli TOP 10, Invitrogen, Groningen, The Netherlands) in accordance with the manufacturer’s instructions. For each incubation time, inserts of about 15 randomly selected clones were analyzed. Extraction of DNA from clones, amplification with primers that target vector sequences, purification of the PCR product and non-radioactive sequencing were performed as described elsewhere [22].

2.4. Sequence data analysis and phylogenetic placement

SSU rRNA sequences were added to a database consisting of about 400 complete or partial publicly available archaeal SSU rRNA sequences [23]. The resulting alignment was manually checked and corrected if necessary. Evolutionary distances between pairs of sequences were calculated using the Jukes–Cantor equation [24]. Phylogenetic trees were constructed by neighbor-joining [25] and maximum likelihood algorithms [26]. Filters for Archaea with 40 and 50% invariance were used. To exclude sequence chimeras prior to the phylogenetic analysis a separate analysis of terminal 400 nucleotide sequences was carried out [27].

The diversity index \( H \) [28] was calculated from the number \( n \) of clones and the number \( n_i \) of clones with a similarity > 97% analyzed, assuming that these represent the same species [29]:

\[
H = \ln n - 1/n \sum (n_i \ln n_i)
\]  

This index was corrected for the maximum theoretically possible \( H (H_{\text{max}}) \) giving the equitability \( J \):

\[
J = H / H_{\text{max}}
\]
2.5. T-RFLP analysis

The principle of T-RFLP analysis has been described by Liu et al. [30] and the protocol by Chin et al. [31]. T-RFLP electrophoreograms were analyzed by peak area integration of the different T-RF (GeneScan 2.1 software; Applied Biosystems). The percent fluorescence intensity represented by single T-RF was calculated relative to the total fluorescence intensity of all T-RF.

2.6. Probe design and characterization

An oligonucleotide probe specific for RC-I SSU rRNA sequences was designed using the ‘PROBE DESIGN’ tool of ARB software package and an ARB database containing a total of 1002 archaeal SSU rRNA sequences, including 90 sequences of RC-I clones. In vitro transcribed SSU rRNA [32] of RC-I was generated for probe specificity studies. In order to obtain nearly full-length 16S rRNA transcripts, archaeal 16S rRNA genes from rice field soil were amplified using the primers Ar21f (5’-TTG ATC CYG CGG GA-3’) and Ar1384r (5’-CGG TGT GTG CAA GGA GCA-3’). Amplicons were cloned with the pGEM-T vector system II (Promega, Mannheim, Germany) and the resulting clone library was screened for RC-I clones (unpublished results). A 1345-bp RC-I clone (AS04-16, accession No: AJ308972) was chosen for RNA generation with the Riboprobe in vitro transcription system (Promega) using T7 RNA polymerase according to the manufacturer’s instructions. Transcripts were purified using the RNeasy Midi Kit (Qiagen, Hilden, Germany) and quantified by slot blot hybridization using reference rRNA of methanogen pure cultures and the probe S-D-Arc-0915-a-A-20 [34]. The temperature of dissociation (T_d) for the newly designed probe was determined using a modified elution method [35]. About 100 ng of RC-I and reference organism SSU rRNA per slot were blotted as described below and then washed with increasing stringency to visualize the amount of probe remaining bound to the membrane for each washing temperature. The temperature corresponding to 50% probe elution was taken as T_d.

2.7. RNA extraction and slot blot hybridization

RNA was extracted from straw and soil samples, and from pure microbial cultures as described previously [36]. Microbial pure cultures were harvested by centrifugation (4°C, 10 min, 2500 x g) before RNA extraction and cell pellets were resuspended in 800 μl TMP buffer (50 mM Tris–HCl (pH 7.0), 1.7% (w/v) polyvinylpyrrolidone 25, 20 mM MgCl_2) (Sigma).

Reference organisms used for standard curves are listed in Table 1. E. coli RNA (16S plus 23S rRNA) was obtained from Roche Diagnostics (Mannheim, Germany) with a concentration of 4 μg µl⁻¹. Cultures of Methanobacterium bourriani (DSM 862), Methanocaeta concili (DSM 3671) and Methanospirillum hungatii (DSM 864) were provided by Dr. J.C. Scholten, the culture of Methanosarcina barkeri (DSM 800) was provided by J. Meuer (both from the Max-Planck-Institute, Marburg, Germany).

Slot blot hybridization was carried out as described by Manz et al. [37]. RNA was denatured by adding 1 vol. 20× SSC (3 M NaCl, 0.3 M Na-citrate, pH 7.0) formaldehyde (37%) at a ratio of 3 vols. 20× SSC plus 2 vols. formaldehyde. After 15 min at 65°C, samples were transferred to a positively charged nylon membrane (Amerham Pharmacia Biotech, Buckinghamshire, UK) using a Bio-Dot SF blotted (Bio-Rad Laboratories, Munich, Germany). Nucleic acids were immobilized using twice the ‘auto-cross-link’ function (120 ml) of a UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA). Membranes were prehybridized for 1 h at 40°C with 10 ml solution containing 5× SSC, 1% blocking solution (Roche Diagnostics, Mannheim, Germany). 0.1% N-lauroylsarcosine and 0.02% SDS. Hybridization was performed at 40°C for at least 14 h in 3 ml prehybridization solution with 90 pmol of one of the digoxigenin (DIG)-labeled group-specific oligonucleotide probes (Table 1). After hybridization the membranes were washed twice (30 min, 40°C) with 50

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>Sequence of probe (5’-3’)</th>
<th>Reference organisms</th>
<th>T_d-values (°C)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S*-Univ-192-a-A-15 (Univ192)</td>
<td>All life</td>
<td>ACggCGCGTTGGTGTRC</td>
<td>E. coli</td>
<td>40</td>
<td>[50]</td>
</tr>
<tr>
<td>S-D-Bact-0313-a-A-18 (Eub338)</td>
<td>Bacteria</td>
<td>GCGTGCCTCCCCGTTAGGAGT</td>
<td>E. coli</td>
<td>55</td>
<td>[51]</td>
</tr>
<tr>
<td>S-D-Arc-0915-a-A-20 (Arc915)</td>
<td>Archaea</td>
<td>GTGCGGCCCCGGCAATCTCT</td>
<td>Methanobacterium bourriani</td>
<td>56</td>
<td>[34]</td>
</tr>
<tr>
<td>S-F-Mbau-0310-a-A-22 (MB10)</td>
<td>Methanobacteriales</td>
<td>CTTGGTCCTAGGGCTTCATCCCC</td>
<td>Methanobacterium bourriani</td>
<td>57</td>
<td>[42]</td>
</tr>
<tr>
<td>S*-RC1-0653-a-A-23 (RC1)</td>
<td>Rice cluster</td>
<td>5’-CCCT CCA GTC CCA AGC</td>
<td>Clone AS04-16</td>
<td>58</td>
<td>This work</td>
</tr>
</tbody>
</table>

*aNomenclature of probes [32], E. coli numbering [40].

*bReference organisms used for standard curves.
ml washing solution (1 × SSC, 0.1% SDS) and once for 30 min at the probe-specific \( T_m \) value (Table 1). Non-specific binding was blocked by incubation of the membranes for 10 min with 10 ml 1% blocking solution in a buffer containing 0.1 M maleic acid and 0.15 M NaCl (pH 7.5). The DIG-labeled probes were detected using anti-DIG antibodies (diluted 1/1000 (v/v) in 1% blocking solution) coupled with an alkaline phosphatase (Roche Diagnostics) according to the manufacturer’s instructions. Signals were quantified after addition of the ECF substrate (Amersham Pharmacia Biotech) at 570 nm on a Storm 860 Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA, USA).

The percentage of SSU rRNA of different phylogenetic groups in relation to total soil rRNA was quantified with group-specific SSU rRNA probes. Therefore, the RNA of different reference organisms was necessary for the generation of probe-specific standard curves (Table 1). The relative content of SSU rRNA per µl RNA extract of four reference organisms was quantified by slot blot hybridization using a RNA standard (E. coli RNA, Roche Diagnostics). The E. coli standard curve was in the range of 0.3–60 ng RNA per slot. Quantification of the different methanogenic groups in the environmental samples was done with reference organisms (Table 1). The standard curves were in the range of about 0.3–150 ng per slot.

Quantification of the hybridization signal was done with the program ImageQuant 5.0 (Molecular Dynamics). The background signal was corrected using the function ‘Local Average’.

Sequences were deposited with the EMBL database under the following accession numbers: clone library ARS1 under AJ308904 to AJ308919, ARS2 under AJ308920 to AJ308936, ARS3 under AJ308937 to AJ308953, ASR7 under AJ308954 to AJ308963 and clone AS04-16 under AJ308972.

3. Results

3.1. Phylogenetic placement of SSU rDNA clone sequences

The DNA from straw samples incubated for 1, 2, 3 and 7 weeks was extracted, PCR-amplified, and the SSU rDNA was cloned. A total of 60 clones was chosen randomly from all four clone libraries. The T-RF length of each clone was determined by T-RFLP analysis and its partial SSU rDNA was sequenced. The dendrogram of Fig. 1 shows the phylogenetic affiliation of representatives

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**Fig. 1.** Phylogenetic dendrogram based on SSU rDNA sequences (about 700 bp lengths) showing the relationship of selected clones retrieved from incubated rice straw (ARS clones) to members of the domain Archaea. The tree was constructed using neighbor-joining and Jukes-Cantor correction. *Aquifex pyrophilus* was used as out-group. Scale bar = 10% estimated difference in nucleotide sequence position.
of the characterized clone sequences within the archaeal domain. Most of the sequences (38 out of 60) grouped with the genus *Methanobacterium*, and all were characterized by a T-RF of 90 base pairs (bp) length. The second most abundant group of clone sequences (16 out of 60) was related to the genus *Methanosarcina* and possessed a T-RF of 185 bp length. Four clones exhibited T-RF of 392 bp length and were closely related to clone ABS5 (similarity value 99%) previously retrieved from the same rice field soil [38]. Clone ABS5 together with other clone sequences from this rice field soil [16,31,38] belong to the new euryarchaeotal Rice cluster I. Another clone sequence (ARS1-c57) with a T-RF of 83 bp length fell into the Rice cluster VI [31,38] of the terrestrial mesophilic Crenarchaeota. The last clone (ARS1-c13) with a T-RF of 83 bp length showed close relationship to the clone ABS22 [14] which again was found in DNA extracted from the same rice field soil and is related to the Methanomicrobiaeae.

If we assume that clones with a sequence similarity of > 97% represent the same species, the archaeal community of the rice straw results in a relatively low diversity. The diversity index (equitability) was only 0.29. This does not exclude, however, that more species might occur at a low frequency that was not covered by the limited number of clones tested.

### 3.2. Population dynamics by T-RFLP analysis

Rice straw was incubated in anaerobic soil slurries for up to 57 days. Methane production started after 8 days and reached quasi steady state after 20 days of incubation (data not shown), as reported by Glassmann and Conrad [6]. Samples of rice straw for molecular investigations were taken after 8, 15, 22, 36, 43, 50 and 57 days of incubation. These samples were used for DNA extraction, followed by PCR and T-RFLP analyses. To test the reproducibility of this method, two straw samples (1 and 2) were incubated in the same way for 57 days in different bottles and the DNA was extracted in parallel. By integrating the fluorescence intensity, the relative signal intensities of the different T-RF in both samples were quantified and compared. This experiment showed that the relative contribution of single T-RF to total fluorescence was in the same range for both samples with variations between 1 and 3%.

The T-RFLP patterns of all five samples showed T-RF with 83, 90, 185, 381 and 392 bp, irrespective of incubation time. The major archaeal groups detected via T-RFLP analysis were identical to the most abundant lineages found in the clone libraries (see above), i.e. *Methanobacteriaceae*, *Methanosarcinaceae*, RC-I, RC-VI and Methanomicrobiaeae [15,31]. A T-RF with 166 bp was found only once in the straw sample after 15 day incubation. No clone sequence with a T-RF length of 166 bp could be identified. Therefore, the affiliation of this unusual peak remains unclear. In all samples, undigested amplicons (about 800 bp) appeared. It is known that clones belonging to crenarchaeotal RC-IV mostly show T-RF of 754 bp length and that amplicons with no restriction site for TaqI may represent RC-IV or Methanosarcinaceae [15].

The relative fluorescence intensities of the individual T-RF remained relatively constant with incubation time of the rice straw (Fig. 2). The T-RF with 90 bp representing *Methanobacteriaceae* was always dominant (32–49%). The fragment with 392 bp representing RC-I was relatively frequent at day 8 and after 36 and 43 day incubation (17–20%). The T-RF with 185 bp representing *Methanosarcinaceae*, on the other hand, was most frequent on day 8 (23%) and again towards the end of incubation (21–25%).

![Fig. 2. T-RFLP analyses of archaeological populations involved in the anaerobic degradation of rice straw. Straw was incubated for 57 days in soil slurries. Each time point was sampled and extracted in one replicate (see Section 3 for reproducibility test). Data show the relative contribution of each T-RF to the total signal intensity. The likely phylogroups for the individual T-RF are: Methanomicrobiaeae (MM), Methanobacteriaceae (MB), Methanosaec- naeae (MS), and Rice clusters 1 to VI (RC-I to RC-VI).](image-url)
Table 2
Target region of the newly designed rice cluster I-specific SSU rRNA probe S*-RCI-0645-a-A-23* and probe mismatches* displayed by methanogenic reference organisms

<table>
<thead>
<tr>
<th>Probe/organism</th>
<th>Probe/target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S*-RCI-0645-a-A-23</td>
<td>3’TGT AAC GAA CCC TGA CCC TCT CC 5’</td>
</tr>
<tr>
<td>Rice cluster I clone AS04-16</td>
<td>5’ACA TTG CTT GGG ACT GGG AGA GG 3’</td>
</tr>
<tr>
<td>Methanosaeta concilii</td>
<td>GGC AGA CTT GGA ACC GGG AGA GG</td>
</tr>
<tr>
<td>Methanospirillum hungatii</td>
<td>GTC TGG CTT GGA GGC GGG AGA GG</td>
</tr>
<tr>
<td>Methanobacterium bryantii</td>
<td>GTG TTC CTA GGG ACC GGG AGA GG</td>
</tr>
</tbody>
</table>

*Probe is named according to the Oligonucleotide Probe Database [52].
*Mismatches are underlined.

3.3. Design of a SSU rRNA oligonucleotide probe specific for RC-I

The probe S*-RCI-0645-a-A-23 (5’-CCT CTC CCA GTC CCA AGC AAT GT-3’) was developed to detect and quantify RC-I SSU rRNA specifically (Table 2). It binds to the positions 643-667 of the SSU rRNA (E. coli numbering according to [40]) and shows an absolute match to 86 and one mismatch to the remaining 90 RC-I clones in our ARB database, respectively. Nearest non-target sequences belonged to organisms of the ANME-1 lineage [41] and showed three (one clone) and four mismatches (seven clones), respectively. More importantly, all non-target archaeal sequences retrieved from rice field soil environments in our database [14-17,31,38] were discriminated by more than five mismatches, thus emphasizing the specificity of this new probe for the quantification of RC-I SSU rRNA in rice field soil.

As no pure culture of RC-I organisms is available, determination of the specific dissociation temperature (T_d) for this new probe was performed using in vitro transcribed SSU rRNA [32] of the nearly full-length RC-I clone AS04-16 (Fig. 3). Additionally, equal amounts of non-target pure culture SSU rRNA were blotted. The specific washing temperature, at which 50% of target bound RC-I probe was eluted, was experimentally determined as 58°C.

3.4. Quantification of methanogenic rRNA on straw and in soil

The SSU rRNA of different methanogenic groups was quantified by slot blot hybridization using SSU rRNA probes specific for four different methanogenic groups [34,42] and for RC-I. The amount of group-specific SSU rRNA was calculated in relation to the amount of total SSU rRNA detected with the universal probe Univ1392 (Fig. 4) in each sample.

For both soil and straw the percentage of all analyzed archaeal groups increased during incubation (Fig. 4). The increase was higher in the soil than on the straw, and was paralleled by an increase of the total archaeal rRNA de-
tected with the archaeal probe Arc915. The average percentage of archaeal rRNA (Arc915) to total rRNA (Univ1392) increased from 10% and 15% after 1 week to 26% and 56% after 4 weeks for straw and soil, respectively, while the percentage of the different specific SSU rRNA (individual probes) compared to total archaeal rRNA (Arc915) was constant with time (data not shown). The rRNA detected with the individual probes accounted for a relatively constant proportion (42–46%) of the total archaeal rRNA (data not shown).

4. Discussion

Methanogens can be divided into four physiological groups: (1) genera exclusively using acetate as substrate such as Methanoanaeta; (2) genera using H₂CO₂ and formate such as Methanobacterium, Methanobrevibacter and Methanogenium; (3) genera using methyl compounds (e.g., methanol) such as Methanolobus and Methanococcus; and (4) generalists forming methane from acetate, H₂CO₂, and methyl compounds such as members of the genus Methanosarcina [43]. The methanogenic community involved in the decomposition of rice straw was investigated by T-RFLP analysis, cloning and sequencing, and slot blot hybridization. Methanobacterium, Methanosarcina and RC-I methanogens were found to be the most important groups. These methanogenic groups are able to produce CH₄ from H₂CO₂ and/or acetate, which are the main products of the bacterial degradation processes of rice straw [6]. Methanol and formate were not detectable after inhibition of methanogenesis and thus are apparently not important methanogenic substrates during straw degradation [6].

The different methods used (cloning and sequencing, T-RFLP, slot blot hybridization) produced different results. In the clone libraries, Methanobacterium- and Methanosarcina-related clones were dominant. T-RFLP analysis confirmed this pattern but additionally showed high frequencies of RC-I methanogens and Methanomicrobiaeae. Compared to these PCR-dependent methods, rRNA-based slot blot hybridization indicated that RC-I methanogens were most active followed by Methanobacteriaeae, whereas Methanosarcinaeae and Methanomicrobiaeae (Methanogenium-related species) played only a minor role. It is assumed that the rRNA reflects the activity of microbial populations in a habitat [44] and thus it can be assumed that methanogens other than Methanobacteriaeae and RC-I methanogens were present on the rice straw but only at a low activity.

Methanosetaeae were also detectable on the rice straw by slot blot analysis albeit at a very low level. However, this methanogenic group could not be detected in the clone libraries or by T-RFLP analysis. On the other hand, slot blot analysis clearly detected Methanosemiteaeae in the soil, being consistent with previous results [14,15,45]. The acetate concentrations during rice straw degradation reached up to 24 mM [6]. This high acetate concentration is sufficient for Methanosarcina spp. that have a threshold of 0.2–1.2 mM acetate, while Methanoaeta species require < 10 μM acetate [46]. Thus, it is likely that the more versatile Methanosarcina spp. became
the dominant acetoclastic methanogens during straw degradation. The DNA-based methods showed that the community structure remained fairly constant during straw degradation, but methanogenic rRNA increased with incubation time. This increase was even more pronounced in soil than on the rice straw. Apparently, the activity of the methanogens in the soil increased more strongly than on the straw. This observation is consistent with biogeochemical measurements by Glissmann et al. [47] who found that the CH₄ produced in straw-amended soil was predominantly produced in the soil rather than on the straw. Nevertheless, the proportion of individual methanogenic rRNA relative to total archaeal rRNA stayed constant with incubation time and was also similar for both straw and soil, indicating that preferential growth of particular methanogenic taxa did not occur and that the archaeal community structures on straw and in soil were similar.

Slot blot hybridization with group-specific probes accounted for about 45% of the total archaeal SSU rRNA detected with probe Arc915. However, we do not believe that we have missed major methanogenic groups. It has been shown recently that the probe Arc915 is not absolutely specific for archaeal SSU rRNA [48]. Therefore, an overestimation of the amount of total archaeal SSU rRNA in our samples is possible. The underestimation of the SSU rRNA of RC-I and of Methanomicrobiales could be another reason for the difference between the amount of archaeal and group-specific SSU rRNA. It has been shown [49] that although nearly full-length in vitro transcribed 16S rRNA can be used as a standard for the quantification of uncultured microorganisms in environmental samples, this transcribed rRNA can also lead to an underestimation (detecting only \(~75\%\) compared to native rRNA standardization) of the real rRNA abundance. Therefore, we might have underestimated the actual amount of RC-I rRNA.

F.1. Anhang

5. United references

[39]

Acknowledgements

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References


F.2. Axiale Unterschiede in der Populationsstruktur der *Crenarchaeota* und *Euryarchaeota* im hoch-kompartimentierten Darm der bodenfressenden Termite *Cubitermes orthognathus*

Michael W. Friedrich, Dirk Schmitt-Wagner, Tillmann Lüders und Andreas Brune

**Anmerkung:** Die Untersuchung des Modellsystems „Reisfeld“ stand im zentralen Fokus dieser Dissertation. In einem Kooperationsprojekt wurde jedoch auch ein weiteres methanogenes Habitat untersucht, der Termitendarm. Die Arbeit, an der der Autor dieser Schrift vorrangig methodisch beteiligt war, erscheint deshalb an dieser Stelle lediglich im Anhang.

**Zusammenfassung:** Termitendärme stellen ein weiteres wichtiges Habitat methanogener Mikroorganismen dar. In dieser Untersuchung wurde die axiale Struktur der Besiedlung des Darms bodenfressender Termite durch *Archaea* erstmals über Klonierung und T-RFLP-Analyse untersucht. Die morphologische und physiologische Heterogenität des Darms spiegelte sich deutlich in der Besiedlungsstruktur der *Archaea* wieder. Im extrem alkalischen ersten Abschnitt des Enddarms (pH 11.9) wurden vorwiegend Mitglieder der *Methanosarcinaceae* nachgewiesen, während in den posterioren Abschnitten des Enddarms die Methanogenen der *Methanobacteriaceae* und der *Methanomicrobiales* dominierten. Darüber hinaus wurde in dieser Arbeit erstmals die Anwesenheit von *Crenarchaeota* in einem Arthropodendarm nachgewiesen.
Axial Differences in Community Structure of Crenarchaeota and Euryarchaeota in the Highly Compartmentalized Gut of the Soil-Feeding Termite Cubitermes orthognatus

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Methanogenesis represents an important electron sink reaction in the hindgut of soil-feeding termites. This is the first comprehensive analysis of the archaeal community structure within the highly compartmentalized intestinal tract of a humivorous insect, combining clonal analysis and terminal restriction fragment (T-RF) length polymorphism (T-RFLP) fingerprinting of the archaeal communities in the different gut compartments of Cubitermes orthognatus. We found that the morphological and physicochemical heterogeneity of the gut is reflected in a large phylogenetic diversity and pronounced axial differences in the composition of the archaeal gut microbiota, notably among those clones or ribotypes that could be assigned to methanogenic taxa. Comparative analysis of the relative frequencies of different archaeal lineages among the small-subunit rRNA gene (SSU rRNA) clones and their corresponding T-RF indicated that the archaeal community in the anterior, extremely alkaline hindgut compartment (P1) consists mainly of members of the Methanosarcinaceae, whereas Methanobacteriaceae and Methanomicrobiaceae predominate in the subsequent, more posterior compartments (P3/4a and P4b). The relative abundance of Thermoplasmatales increased towards the rectum (P5). SSU rRNA sequences representing Crenarchaeota, which have not yet been reported to occur in the intestinal tracts of arthropods, were detected in all gut sections. We discuss how the spatial distribution of methanogenic populations may be linked to axial heterogeneity in the physicochemical gut conditions and to functional adaptations to their respective ecological niches.

Termites are considered an important source of the climate-relevant greenhouse gas methane (for a review, see references 5 and 40), which is formed by methanogenic Archaea located in the enlarged hindguts of these insects. Methane emission rates differ strongly between termite species; these differences are closely correlated with the feeding habits of the respective taxa.

In the hindgut of most wood-feeding species, homoeocetogenesis is the major hydrogen sink reaction (8, 9). The methane emission rates of the extremely abundant and globally important soil-feeding species, however, exceed those of wood-feeding termites considerably. In the humivorous Termitinae, the rates of reductive acetogenesis measured in gut homogenates were about 10-fold lower than the rates of methanogenesis (8). Methanogenesis represents a major electron sink in the hindgut metabolism of these termites; in Cubitermes orthognatus, methane production amounts to almost 10% of the respiratory activity of the insect (46).

Initially, the factors influencing the outcome of the competition of these CO₂-reducing processes for hydrogen were quite enigmatic (9). However, several studies using microsensors and radiotracer analysis of intestinal CO₂-reduction rates have provided evidence that the radial and axial distribution of the microbial populations involved in the production and consumption of hydrogen may play a key role in controlling the fluxes of reducing equivalents in the gut (18, 41, 47).

In soil-feeding Termitinae, the hindgut is highly compartmentalized and characterized by an unusually high pH in the anterior region (4) (Fig. 1). The luminal pH increases sharply in the mixed segment, a gut region located between the neutral midgut and the first proctodeal dilation (P1), which possesses the highest alkalinity ever observed in biological systems (around pH 12; [14]). Significant amounts of hydrogen accumulate only in the mixed segment and in the P3 (41). Conversely, methane is formed mainly in the posterior gut compartments P3/4a and P4b (Fig. 1); only small amounts of methane are emitted by the anterior, alkaline compartments (41). The close contact of hydrogen-producing and hydrogen-consuming gut compartments in the abdomen and the stimulation of methanogenesis in the isolated posterior hindgut compartments by the addition of external H₂ and formate (41) indicate that a cross-epithelial transfer of reducing equivalents and the contribution of electron donors other than hydrogen also have to be considered when analyzing the functional interactions between the different microbial populations (13, 41).

Unfortunately, little is known about the microbial community involved in these processes, the numerical abundance and spatial distribution of the different hydrogen-producing and hydrogen-consuming populations, and their metabolic properties. Despite the ubiquitous presence of methanogenic activities in termites, the only methanogens isolated in pure culture from termites are three Methanobrevibacter spp. from the wood-feeding Reticulitermes flavipes (27, 28); most of the cur...
F.2. Anhang

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Section

M

P1

P3

P4

P5

Methane emission

< 0.02

0.11

2.43

0.47

0.05

nmol h⁻¹ section⁻¹

Gut segment

C

M

ms

P1

P3

P4a

P4b

P5

Average pH

6.0

7.1

11.9

10.4

7.5

4.8

1 mm

FIG. 1. Gut morphology of a C. argillaceus sp. worker termite. The gut was drawn in its unraveled state to illustrate the various segments: C, crop; M, midgut; ms, mixed segment; P1 to P5, proctodeal segments 1 to 5, respectively. Gut sections were separated at the indicated positions. Methane emission rates are for individual gut sections of C. argillaceus incubated under N₂ atmosphere with 20% H₂ (41); the average luminal pH of the major gut segments was determined with glass pH microelectrodes for C. argillaceus spectrunculus (44). (Modiﬁed after reference 41).

rent knowledge of the methanogen diversity in termite guts is based on cultivation-independent molecular studies using PCR-based 16S rRNA gene (rDNA) retrieval from total gut homogenates. All clones obtained from lower termites represent Methanobacteriaceae (33, 33b, 49) and are closely related to the Methanobrevibacter species isolated from R. ﬂavipes. Also, the archaeal 16S rDNA clones obtained by Shinzato et al. (42) from the gut of Reticulitermes speratus mainly clustered with the Methanobacteriaceae. A few clones, however, fell into the nonmethanogenic order of the Thermoplasmales (42). The archaeal clones obtained from higher termites also include members of the Methanocoraceae and Methanomicrobiales; those from the soil-feeding Pericapritermes nobelii, the only huminorous species tested to date, represent the highest methanogen diversity among all termites so far investigated (34). Interestingly, all archaeal clones so far recovered from termite guts form termite-species speciﬁc.

Nevertheless, the implications of the morphologically and physicochemically highly structured hindgut of the soil-feeding species on the diversity and distribution of archaeal populations remain to be addressed. In this study, we investigated the composition of the archaeal microbiota in the individual gut sections of the soil-feeding termite C. orthogonatus and compared it to the archaeal community structure in the nest and the surrounding soil.

MATERIALS AND METHODS

Termites. Nests of C. orthogonatus Emerson (Termitidae: Termitinae) were collected near Busia, Kenya. The termites together with nest fragments and soil from the original collection site were brought to the laboratory in polypropylene containers. Only worker caste termites were used in this study.

DNA extraction and puriﬁcation. Termites were dissected with sterile, ﬁne-tipped forceps, and guts were separated into six sections, comprising the crop, mident, and the major hindgut compartments (Fig. 1). Between 10 and 20 gut sections each were placed in sterile 2-ml tubes ﬁlled with 1 ml of buffered saline solution (46) and were stored frozen at −80°C. DNA was extracted following a direct lysis protocol modiﬁed after that of Morét et al. (33) as previously de- scribed in detail (23). Alliquots (1 g) of soil samples collected at about 3 m from the nest and of the inner nest material were extracted using the same procedure. DNA was puriﬁed from the supernatant by consecutive ammonium acetate, isopropanol, and ethanol precipitation steps. To remove humic substances, the extracts were passed through spin columns ﬁlled with polyvinylpolypyrrolidone (3) as previously described (36). Extraction efﬁciency and quality of the extracted DNA were veriﬁed by standard gel electrophoresis. DNA concentrations were determined photometrically using Hoechst dye 33258 and a DNA Quant 200 fluorometer (Amerham Pharmacia Biotech, Freiburg, Germany) as recom- mended by the manufacturer.

PCR ampliﬁcation of archael 16S rRNA genes. PCR ampliﬁcation was car- ried out as described previously (21) with modiﬁcations. Oligonucleotide primers specific for archael 16S rDNAs were Ar109F (21) and Ar192R (CTC CCC CCG CAA TTC CTT TA) (E. coll 16S rDNA numbering [11]; positions 109 to 125 and 912 to 931, respectively). PCR (30 cycles) was carried out at an annealing temperature of 52°C, and 1 µl of unclariﬁed (for P4 and P5) or 10-fldd-diluted DNA preparation (all others) was used as template. Aliquots of the 16S rDNA amplicons (5 µl) were analyzed by electrophoresis on a 1% agarose gel and visualized after staining with ethidium bromide.

Fragments of chimerataphal SSU rDNAs were speciﬁcally afﬁliated using primers Ar109F and Cen752R (5′–AGG GTG AGG GAT GAA AGC TG-3′), which were modiﬁed from those of Buckley et al. (15). All other PCR conditions were as described above.

SSU rDNA libraries. Clone libraries were created from archaeal sub-units (SSU) rDNA amplicons obtained from termite gut community DNA. PCR products (~900 bp Ar109F-Ar192R or ~750 bp ArCh21F-Cen752R long) were ligated into the pGEM-T Easy plasmid vector (Promega, Mannheim, Germany), and Es. coli JM109 (Promega) was transformed with the recombinant plasmids according to the manufacturer’s instructions. Randomly picked clones were further analyzed as described previously by Rothraue et al. (37). Clones were checked for the correct insert size by vector-targeted PCR and standard agarose gel electrophoresis. Clones were designated P1 (gut section P1; 30 clones), P3 (gut section P3; representing the P3/4a double compartment; 20 clones), P4b (gut section P4; 30 clones), P5 (gut section P5; 42 clones), or Cen (whole gut; PCR product ampliﬁed with ArCh21F-Cen752R, 30 clones).

T-REFP analysis. Terminal restriction fragment (T-REF) length polymorphism (T-REFLP) analysis was performed as described by Laeder and Friedrich (31) using Taq restriction digestion. Archaeal SSU rDNAs were ampliﬁed using primer Ar109F and 5′-carboxyﬂuorescein-labeled primer Ar192R following the PCR protocol described above, except that only 100-µl PCR mixtures and 26 cycles were used. Standards (GeneScan-1000 ROX; Applied Biosystems), sample preparation, electrophoretic separation on an ABI 373 sequencer, and data analysis using GeneScan analysis software were as previously described (16). T-REFLP electropherograms were analyzed by peak area integration of the dif- ferent T-REFs. In order to obtain a measure of the relative SSU rDNA frequency, the relative ﬂuorescence intensity of each individual band representing a single T-REF was compared to the total ﬂuorescence intensity of all T-REF bands (31).

Diversity analysis. Clones with similar (~97% similarity) SSU rDNA se- quences were assigned to the same operational taxonomic unit (OTU). Diversity coverage of SSU rDNA clone libraries was analyzed by using the analytical approximation algorithm of Harburt (24), and 95% conﬁdence intervals were estimated as described by Heck et al. (22). Rarefaction curves were produced using the Analytic Rarefaction software (version 1.2; S. M. Holland, University of Georgia, Athens [http://www.uga.edu/strata/Software.html]).

Phylogenetic analysis. Sequence data were analyzed and trees were con- structed using the ARB software package with its database (version 2.5b;
S. Strunk and W. Ludwig, Technische Universität München, Munich, Germany [http://www.biochemie.tu-muenchen.de/pub/ARB/]) as described previously in detail (20). SSU rDNA sequences were added to the database and aligned by using the Fast Aligner tool (version 1.01). Alignments were corrected manually if necessary. Sequences were phylogenetically placed by comparing them to reference sequences for the main lines of descent within the archael kingdoms Euryarchaeota and Cenarchaeota (53), as well as Korarchaeota (2). For tree construction, nearly full-length SSU rDNA sequences (~1,300 bases) from the ARB database were selected to construct an archael base frequency ﬁlter (30 to 100% similarity), and an initial tree was generated by using the neighbor-joining algorithm (37). Sequences from termite gut clone libraries were added to this
F.2. Anhang

RESULTS

DNA was successfully extracted from all gut sections. Final yields in purified extracts ranged between 50 and 100 ng of DNA per gut section. Whole-gut preparations yielded about 280 ng of DNA per gut, which corresponded to approximately 130 µg of DNA per g (dry weight); yields from purified soil and nest material were 2.0 and 2.5 µg of DNA per g (dry weight), respectively. All DNA extracts except those obtained from the crop yielded amplions with the Archaea-specific primer pair. Since SSU rDNA was successfully amplified from all extracts, including the crop, with a Bacteria-specific primer pair (27F-1492R [23, 52]) (data not shown), we concluded that the purification protocol had removed PCR-inhibiting compounds (e.g., humic substances) to such an extent that a high degree of inhibition was unlikely.

SSU rDNA clone libraries. The DNA extracts obtained from gut sections P1, P3, P4, and P5 were used to construct individual clone libraries of archaean SSU rDNA. From each clone library, 30 clones (section P5 contained 42 clones) were randomly selected and sequenced. Sequence analysis revealed that nearly all clones were affiliated with known taxa of Archaea. While most clones clustered with Euryarchaeota (Fig. 2), a small number (5%) of the clones in the libraries were more closely related to so-far-uncultured Crenarchaeota (Fig. 3). Separate phylogenetic analysis of the terminal 400 nucleotide sequence positions at the 5’ and 3’ ends of the SSU rDNA clones identified three clones as chimeras (Table 1). These four clones in the clone libraries that were not affiliated with the Archaea were excluded from further analysis (Table 1).

Archaeal SSU rDNA clones related to methanogens. The majority of the clones affiliated with the Euryarchaeota cluster within different methanogenic lineages (Fig. 2). One group of clones was related to the family Methanobacteriaceae and grouped closely with Methanobrevibacter filiformis and Methanobrevibacter curitubari (94% to 96% and 95% to 97% similarity, respectively), which have been isolated from the gut of the wood-feeding termite Reticulitermes flavipes (Kollar) (27), and to sequences of clones retrieved from the guts of other wood-, grass-, and soil-feeding termite species.

A second group of clones was related to members of the Methanomicrobiales (7) and clustered with SSU rDNA sequences retrieved from the guts of Nasatermes takasagoensis and Poreipalmarium nitobei (34) (98% to 99% similarity; 550 bp of overlapping sequence information). Clones of this cluster were 95% to 100% similar to each other, and their closest cultivated relative was Methanospirillum hungatei (86% to 89% similarity [Fig. 2]).

A third group of clones was related to members of the family Methanosarcinaceae and grouped closely with SSU rDNA sequences retrieved from the guts of Poreipalmarium nitobei and Odontotermes formosanus (34) (96% to 98% similarity; 550 bp overlapping sequence information). Clones of this cluster were 94% to 100% similar to each other, and their closest cultivated relative was Methanomicrococcus blattolae (93.5% to 97.5%, similarity, isolated from the gut of the cockroach Periplaneta americana (43).

Each of these three clone groups contained distinct subclusters within their respective clade (Fig. 2). For example, at least three distinct subclusters of Methanobrevibacter spp. (i.e., an M. cuticularis cluster, an M. filiformis cluster, and a distinct C. orthogonathiis clone group, labeled A to C in Fig. 2) were retrieved from the clone libraries that displayed intersubcluster sequence differences of 95% to 96%, whereas sequence similarities within these subclusters were between 97.5% and 100% similarity. Some of these clones even differed in their Taq restriction sites (Table 1).

Archaeal SSU rDNA clones related to members of the Thermoplasmatales. A significant proportion of clones affiliated with the Euryarchaeota, especially those recovered from the P5, clustered with members of the Thermoplasmatales (Fig. 2). The clones of this cluster were 92% to 100% similar to each other and formed two distinct subclusters. They were closely related to a clone from a fluidized-bed reactor fed with vinasses (19) (92% to 95% sequence similarity) but only distantly related to cultivated members of the Thermoplasmatales (between 75% to 78% similarity).

Crenarchaeotal SSU rDNA sequences. A smaller proportion (5%) of the clones in the clone library carried sequences affiliated with the Crenarchaeota (Fig. 3). Seven of these clones grouped closely with several clones of “rice cluster IV” (16, 20) and from agricultural soil (6); both clone groups are affiliated with the “freshwater cluster” of the kingdom Crenarchaeota. Four clones were affiliated with rice cluster VI (16, 20), a clone group within the “terrestrial cluster” of the Crenarchaeota (95% to 96%, 99%, and 98% similarity, respectively). Clones retrieved from termite gut sections within the freshwater cluster

FIG. 2. Phylogenetic tree showing the positions of SSU rDNA sequences recovered from termite hindgut gut compartments P1, P3(4a), P4(b), and P5 relative to members of the Euryarchaeota. Selected sequences (nearly full-length rDNA) of cultivated representatives from euryarchaeotal lineages were used as references to construct an evolutionary distance dendrogram. SSU rDNA sequences of members of Crenarchaeota and Korarchaeota were used as outgroup references. Partial SSU rDNA sequences (>500 bp) of clones from the guts of the termites Nasatermes takasagoensis (34), Poreipalmarium nitobei (34), Odontotermes formosanus (34), Hodotermites sp. (34), Hodotermites japonica (34, 49), Reticulitermes flavipes, and Reticulitermes speratus (34) related to archaean clones from C. orthogonathus were added to the tree using the ARB parsimony tool. Methanobacterial subclusters were designated as follows: A, M. cuticularis; B, C. orthogonathus termite cluster; C, M. filiformis. The termite hosts from which SSU rDNA sequences were retrieved are indicated. The scale bar represents 10% sequence difference. Accession numbers of reference sequences are indicated.
and the terrestrial cluster were 94 to 100% similar to each other.

To further explore the diversity of Crenarchaeota in the gut of C. orthognathus, we specifically amplified only crenarchaeotal SSU rDNA from total community DNA extracted from whole guts. A total of 24 clones from this clone library were analyzed, one of which was a potential chimera. All other clones were closely related to the crenarchaeotal clones in the archaeal clone libraries obtained from the individual gut sections, which indicated that further crenarchaeotal genotypes were not to be expected. The largest proportion of the new clones (21 clones) fell into rice cluster IV, and the remainder fell into the terrestrial cluster (Fig. 3).

Since the cloning analysis may not rectify the total community diversity due to the method-inherent undersampling bias (45), we checked the diversity coverage of the individual clone libraries by rarefaction analysis (22). We defined sequences as belonging to the same species at 97% sequence similarity, which is in agreement with current microbial species concepts (44). Results for gut sections P1 and P5 revealed a good di-
TABLE 1. Lengths of TaqI-specific T-RFs of SSU rDNA clones

<table>
<thead>
<tr>
<th>T-RF [bp]</th>
<th>P1</th>
<th>P3(4a)</th>
<th>P4(b)</th>
<th>P5</th>
<th>Phylogenetic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>88</td>
<td></td>
<td>P4b-Ar-11, -17, -23, -27</td>
<td>P5-Ar-5 to -7, -9, -14, -15; P5-Ar-2 to -4, -8, -30, -11, -15 to -19, -21 to -23, -25 to -27, -30</td>
<td>Methanobacteriaceae</td>
<td></td>
</tr>
<tr>
<td>220</td>
<td>P1-Ar-11</td>
<td>P3-Ar-5</td>
<td>P4b-Ar-26</td>
<td>P5-Ar-2</td>
<td>Carnivora</td>
</tr>
<tr>
<td>289</td>
<td></td>
<td></td>
<td>P4b-Ar-7, -19</td>
<td></td>
<td>Thermoplasmales</td>
</tr>
<tr>
<td>341</td>
<td>P3-Ar-9</td>
<td>P4b-Ar-10, -22, -25</td>
<td>P5-Ar-1 to -3, -10 to -12; P5-Ar-2 to -3, -6, -9, -13, -14 to -20, -24, -28</td>
<td>Thermoplasmales</td>
<td></td>
</tr>
<tr>
<td>388</td>
<td>P3-Ar-3, -7, -18, -19, -20, -25, -30</td>
<td>P4b-Ar-4, -29, -16</td>
<td></td>
<td>Methanomicrobiales</td>
<td></td>
</tr>
<tr>
<td>389</td>
<td>P4b-Ar-13</td>
<td></td>
<td></td>
<td></td>
<td>Carnivora</td>
</tr>
<tr>
<td>457</td>
<td>P4b-Ar-5, -8</td>
<td>P4b-Ar-1, -2, -4, -6, -10, -23, -26 to -29</td>
<td></td>
<td>Carnivora</td>
<td>Methanobacteriaceae</td>
</tr>
<tr>
<td>611</td>
<td>P3-Ar-1 to -8, -10, -12, -20 to -30</td>
<td>P4b-Ar-18</td>
<td></td>
<td>Methanoscarsinae</td>
<td></td>
</tr>
<tr>
<td>~800°F</td>
<td>P1-Ar-1 to -8, -10, -12</td>
<td>P4b-Ar-9, -12</td>
<td></td>
<td>Carnivora</td>
<td>Unidentified/chimera</td>
</tr>
<tr>
<td>ND°F</td>
<td>P1-Ar-9</td>
<td>P3-Ar-8</td>
<td>P4b-Ar-2</td>
<td>P5-Ar-12, -29</td>
<td>Methanobacteriaceae</td>
</tr>
</tbody>
</table>

T-RFs of ~800 bp represent PCR fragments without a TaqI restriction site.

Carnivora were characterized by three T-RFs of 88, 457, and 611 bp. The third of these fragments did not have a TaqI restriction site and was also characteristic for clones belonging to the Methanobacteriaceae and the Carnivora. Clones falling into the Methanomicrobiales and the Carnivora were characterized by T-RF lengths of 388 and 389 bp, which is a fragment size difference below the resolution limit of the T-RF analysis (35). However, only one carminarchaeal clone with a T-RF of 389 bp was detected in the library of the P4 section, which indicated the low frequency of these sequences. All other T-RF sizes predicted were unique for one phylogenetic group. Clones belonging to the Thermoplasmales could even be differentiated by distinct T-RF sizes of 220, 341, and 375 bp (Table 1).

Archaean community structure in different gut sections. Both T-RFLP analysis and cloning analysis revealed significant differences in the archaean community structure among the individual gut sections of C. orthogallus. In general, the relative gene frequencies of OTUs derived from T-RFLP analyses were in good agreement with the frequencies of clones falling into the respective T-RF size classes (Table 1; Fig. 6). T-RFLP analysis detected a few additional, less-frequent ri-
FIG. 5. T-RFLP analyses of archaeal SSU rDNA PCR products amplified from DNA extracts of gut sections P1 and P3. T-RFLP profiles after TaqI digestion of 6-carboxyfluorescein-labeled PCR products from P1 (A) and P3 (C) and after double digestion with TaqI and HaeIII from P1 (B) and P3 (D). The x axis shows the length (base pairs) of the terminal restriction fragments, and the y axis shows the intensity of fragments in arbitrary units. Designations of archaeal lineages are derived from in silico analysis of sequence data (see also Table 1).

FIG. 6. Relative fluorescence patterns of T-RFs from gut sections P1 and P3. (A) P1 - TaqI. (B) P1 - TaqI + HaeIII. (C) P3 - TaqI. (D) P3 - TaqI + HaeIII.

T-RF length (bp)

- **A**
  - Primer-Dimer
  - MS
  - MB 182
  - P1 - TaqI
  - MS + MB ~800

- **B**
  - Primer-Dimer
  - MS
  - MB 182
  - 366
  - P1 - TaqI + HaeIII
  - MB ~800

- **C**
  - 88
  - MB
  - 389
  - 457
  - P3 - TaqI

- **D**
  - 88
  - MB
  - 366
  - 380
  - P3 - TaqI + HaeIII
  - MM

bototypes (<4%) with T-RFs of 72, 76, and 457 bp, which were not detected by cloning analysis (Fig. 6; Table 1).

The highest diversity was found for the whole termite gut (Fig. 6B), as indicated by 10 different, mostly less-frequent ribotypes, and two frequent groups, represented by T-RFs of 389 and ~800 bp (see Table 1 and the legend of Fig. 6 for affiliation of T-RFs to phylogenetic groups). All major T-RFs, however, could be traced back to the individual gut compartments, where they were represented with a different, compartment-specific gene frequency (Fig. 6).

**Midgut section.** The midgut section, which included the increasingly alkaline mixed segment, exhibited a T-RFLP profile that resembled in the relative frequencies and size classes those observed in the following hindgut compartment (P1; see below) with a predominance of the ~800-bp OTU (72% [Fig. 6B]). Since no clone library was generated for this section, it remains open whether the ~800-bp OTU represents members of the *Methanosarcinaceae* or of the *Methanobacteriaceae* prevailing in this section.

**Hindgut section P1.** In the case of the extremely alkaline P1, we observed a high proportion of clones (90%) related to members of the *Methanosarcinaceae*, which all had a T-RF of ~800 bp (Fig. 6A), and only one crenarchaeotal clone. Direct T-RFLP analysis revealed the presence of a slightly lower relative frequency of 78% for the ~800-bp OTU, and in addition T-RFs with 88 bp (5%; OTU specific for members of the *Methanobacteriaceae*) (Table 1) and 181 bp (11%), which probably represented members of the *Crenarchaeota* (Table 1).
Hindgut section P3. In the more posterior P3/4a double compartment, the archaean community changed distinctly and was dominated by Methanobrevibacter spp. (55% of clones, 67% of T-RFs), followed by members of the Methanomicrobiales (24% of clones, 26% of T-RFs). However, only one clone each was closely related to Methanosarcinaeaceae, Thermoplasmatales, and Crenarchaeota, which is in agreement with the relative gene frequencies of <5% for the 341-, 375-, and 182-bp T-RFs, respectively (Fig. 6A). Using a modified T-RFLP assay, we were able to differentiate between Methanosarcinaeaceae-like, Methanobrevibacter-like, and crenarchaeotal amplicons, none of which had a restriction site for TaqI in the PCR fragment analyzed (~800-bp OTU [Table 1]). This approach involved a double digestion with HaeIII and TaqI, which resulted in a characteristic 34-bp T-RF (Fig. 5B) for clones falling into the Methanosarcinaeaceae and a 366-bp T-RF for Methanobrevibacter-like sequences (Fig. 5B and D). Unfortunately, the 34-bp T-RF comigrated with the primer-dimer artifact (34 bp), which rendered quantification impossible. Nevertheless, we were able to show that the Methanobrevibacter-like gene frequencies were low in the P1 section (Fig. 5B), whereas Methanomirina-like genes were virtually not present in the adjacent P3/4a section (Fig. 5D).

Gut sections P4 and P5. Towards the posterior end of the gut, the relative frequencies of the Methanobrevibacter-like sequences (88- and 611-bp T-RFs and ~800-bp OTU) decreased from 64% in the P4 section to 45% in the P5, whereas cloning analyses gave similar clone frequencies of 52 and 56% for these
sections. Sequences falling into the *Methanomicrobiales* represented 10% relative gene (389-bp T-RF) and clone frequency in both sections. The proportion of *Thermoplasmatales*-like sequences increased to 18 and 28% (341- and 375-bp T-RFs), and that of the *Crenarchaeota* (182-bp T-RF) increased to 4 and 7% in the P4 and P5 sections, respectively. Interestingly, *Methanobrevibacter*-like sequences with a T-RF of 88 bp represented a larger proportion of archaeal OTUs in the P4 and P5 sections (14 and 11%, respectively) than in the P3 section (2%). This may indicate that the phylogenetically distinct subclusters (A to C [Fig. 2]) represent *Methanobrevibacter* spp., which occupy specific niches within the respective gut segments.

Archaeal community composition in termite nest and adjacent soil. Both the T-RFLP fingerprints of the nest material and the surrounding soil were characterized by a predominant 182-bp T-RF, which represented 82 and 76% of the ribotypes in the respective samples (Fig. 6B) but which was not present in the fingerprints of any of the gut sections. Also, two other, less-frequent OTUs with T-RFs of 161 and 169 bp were observed in soil, but not in the termite gut samples. OTUs present both in soil and gut samples had relative frequencies in the fingerprints of the soil samples that were significantly lower than those of the gut samples. Only 10% of the predominant ~800-bp OTU was present in nest material, and only 5% of the 88-bp T-RF was present in the surrounding soil. The distinct differences in the community composition did not require the assignment of the OTUs to a specific archaeal group; therefore, no clone analysis was performed for the soil and nest material samples.

**DISCUSSION**

This is the first comprehensive analysis of the archaeal community structure within the highly compartmentalized intestinal tract of a soil-feeding termite. We could show that the morphological and physicochemical heterogeneity of the gut (14, 25, 41) is paralleled by a high phylogenetic diversity and pronounced axial differences in the composition of the archaeal gut microbiota.

**Phylogenetic diversity of termite gut Archaea.** Our study reveals a remarkable diversity of *Archaea* in the gut of *C. orthognathus*. Both the results of the cloning analysis and the T-RFLP profiles demonstrated considerable differences in the composition of the archaeal community in the different gut segments. It should be pointed out that many of the phylogenetic groups were detected only by a separate analysis of the individual gut sections (Fig. 6A) and by sequencing a sufficiently large sample of clones (Fig. 4 and 6A). For example, clones related to the *Methanosarcinaeae* were barely represented in the clone library of the P3 and P4 sections and were not detected at all in the P5 section, whereas they predominated in the P1 section.

Besides detecting sequences grouping with methanogenic *Archaea*, we also detected SSU RNA sequences that grouped with members of the *Thermoplasmatales* (Fig. 2). To date, only three SSU RNA clones (sequence of ~500 bp) related to the *Thermoplasmatales* have been recovered from the wood-feeding termite *R. esperatus* (42). It was not possible to determine their phylogenetic relationship with the clones retrieved from the guts of *C. orthognathus* since the overlapping region of the sequences (~20 bp) was too small for comparison. One can only speculate whether the *Thermoplasmatales*-like clones represent non-methanogenic *Archaea*.

In addition to these euryarchaeotal clones, SSU rDNAs representing *Crenarchaeota* were also detected in all hindgut sections. Phylogenetic analysis indicates a close relationship of the termite gut clones to other clones with unknown phenotypes retrieved from terrestrial or aquatic environments (Fig. 3). There is an increasing body of evidence that *Crenarchaeota*, which were originally considered to be confined to habitats characterized by high temperature, high salinity, or an extreme pH, also seem to occur ubiquitously in temperate or cold aquatic (26) and terrestrial environments (for a review, see reference 17). Our findings represent the first report on the occurrence of *Crenarchaeota* in the digestive tract of arthropods and, together with the distantly related clones recovered from the midgut of the holothurian *Onuphidus musabili* (32), also one of the first reports on the presence of *Crenarchaeota* in intestinal tracts. In the case of a crenarchaeotal clone recovered from the gut of a flounder (*Platichthys flesus*) (50), the presence of a specific intestinal population remains to be established.

Although both soil and nest material contained archaeal SSU rDNA, T-RFLP profiles of parent soil and nest material differed strongly from those of the different gut sections. It can be ruled out that the microorganisms whose DNA was amplified from the gut extracts stem from ingested soil. Rather, they seem to be specific members of the termite gut microbiota. T-RFs characteristic of *Crenarchaeota* and *Methanomicrobiales* were not even detected in the DNA amplified from the parent soil (Fig. 6B [389 bp]; Table 1), and the frequency of crenarchaeotal clones increased towards the P4b section (Fig. 6A). Interestingly, shedding of intestinal archaea seems to have little impact on the composition of the archaeal community in the nest material, despite the fact that the latter is constructed largely from feces.

**Spatial distribution and functional implications.** The environmental factors which should be most decisive for the distribution of archaeal populations in the different gut sections of *C. orthognathus* are the intestinal pH and—at least in the case of methanogenic *Archaea*—the availability of reducing equivalents. In a previous study, we found that methane is formed mainly in the P3a4a and P4b compartments (41, 46) (Fig. 1). The results of the molecular analysis performed in the present study, however, indicate that the anterior gut sections (M/Ms to P1) also harbor specific, probably autochthonous populations of methanogenic *Archaea* (Fig. 6). Based on the extreme alkalinity of the P1 compartment of all *Cubitermes* spp. (pH 11 to 12.5 [14]), one might carefully conclude that the methanogenic populations specific for this gut section are alkaliphilic. More than 60% of the T-RFs and 90% of the archaeal clones recovered from the P1 section were members of the *Methanosarcinaeae*, which were almost completely replaced by *Methanobrevibacter*-related sequences in the subsequent sections consisting of less-alkaline to neutral gut segments (Fig. 6). All *Methanosarcinaeae*-related clones from the P1 compartment clustered within a clade comprising only clones from the intestinal tracts of insects, which may reflect coevolution with their host, and were closely related to...
clones recovered from other termites with alkaline gut segments (Nasutitermes takasagoensis and Pericapritermes nitobei). However, the same clade comprised also clones or pure cultures obtained from hosts without an elevated intestinal pH (Odonotermes formosanus and Periplaneta americana) (Fig. 2), and presently it cannot be excluded that the methanogenic population in the P1 compartment is located in microhabitats characterized by a less-alkaline pH than that reported for its bulk volume. Cultivation or in situ localization of the cells within the gut will be necessary to clarify whether these methanogenic symbionts are indeed adapted to extremely alkaline microenvironments.

Hydrogen appears to be a key substrate of methanogenesis in termite guts (for reviews, see references 10 and 12). In Cubitermes spp., however, a utilization of endogenous hydrogen is feasible only in the anterior gut regions, including the P3/4a compartment (41). Based on the large methanogenic potential of the posterior hindgut and the juxtaposition of hydrogen-forming and hydrogen-consuming gut regions in the abdomen of the termite, we have proposed a cross-epithelial hydrogen transfer from hydrogen-forming gut segments (ms, P1, and P3) to hydrogen-consuming gut segments (P4a and P4b) in situ (41), which would create additional microichrons for H2-consuming populations.

Nevertheless, it is possible that different methanogenic populations use different electron donors in situ. An alternative, exogenous electron donor for methanogenesis in the posterior hindgut compartments could be formate. Formate, most probably a product of microbial fermentations in the anterior gut compartments (46), is present in the hemolymph of C. orthognathus in appreciable concentrations (2.6 mM [A. Tholen and A. Brune, unpublished results]). The stimulation of methane emission of isolated P3/4a compartments by formate is even stronger than that by exogenous H2 (41), and H2-oxidizing and formate-oxidizing methanogens seem to be present in similar numbers in Cubitermes spp. and other soil-feeding Termitidae (38, 46).

Another potential substrate for methanogenesis in the termite gut may be methanol. Methanomicroccocus blatticola, the only cultivated representative in the cluster of Methanomicrococcus-related clones recovered from insect guts, is obligately methylotrophic; i.e., it uses H2 as an electron donor only in the presence of methanol or methylamines (43). Sequence similarities between C. orthognathus clones and M. blatticola range from 93.5 to 97.5%, which may allow careful speculations regarding a methylotrophic phenotype.

While the different methanogenic community structures of P1 versus the P3 and P4 sections of C. orthognathus may be caused by axial differences in the microenvironmental conditions, the diversity among the methanogenic populations within each section may reflect a radial organization of methanogens specifically adapted to their respective microhabitats. This is supported by the apparent location of the hydrogen sinks in the posterior hindgut compartments of C. orthognathus (P3-P4b) (41) and by the observation that different morphotypes of F2g2-fluorescent cells appear to be associated with the gut wall and the cuticular spines protruding into the lumen of the P4b compartment (Tholen and Brune, unpublished results). Functional adaptations to different ecological niches may also explain why Tokura et al. (49) found that the methanogens associated with gut epithelium of the wood-feeding R. speratus and the grass-feeding Hodeotermopsis spoedestii are phylogenetically different from those harbored by the intestinal flagellates (49).

Most-probable-number enumeration yielded highest numbers of methanogens in the posterior hindgut, i.e., in P3/4a and P4b (6 once sections, but the low absolute numbers (2 × 106 cells per section [46]) are probably strongly biased by cultivation. It is important to consider that the proportions of methanogenic phylotypes among the archaean populations in different gut sections reported in this study represent only relative numbers. These are based on PCR methods, which cannot be subject to method-inherent bias (for a review, see reference 51). In order to gain more information on the absolute abundance and physiological role of methanogens and other archaea in the individual gut segments, it will be necessary to determine their absolute numbers and their spatial distribution by a PCR-independent approach, e.g., by fluorescent in situ hybridization.

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REFERENCES

27. Leadbetter, J. R., and J. A. Breznak, 1996. Physiological ecology of Meth-
anotherbauchia caldiphila sp. nov. and Methanotherbacter curvatus sp. nov., isolated from the hindgut of the termite Reticulitermes flavipes. Appl. Envi-
33. Moret, M. J., B. Herrick, M. C. Silva, W. C. Ghose, and E. L. Madison, 1994. Quantitative cell box of indigenous microorganisms and rapid extrac-
34. Okhmaka, M., and T. Kudo, 1998. Phylogenetic analysis of the symbiotic intestinal microflora of the termite Cryptotermes domesticus. FEBS Micro-
41. Schmidt-Wagner, D., and A. Brune, 1999. Hydrogen profiles and localization of methanogenic activities in the highly compartmentalized hindgut of soil-
nol- and methylamine-reducing methanogen from the hindgut of the cock-
46. Tholen, A., and A. Brune, 1999. Localization and in situ activities of hu-
mosacogenic bacteria in the highly compartmentalized hindgut of soil-
48. Tholen, A., B. Schink, and A. Brune, 1997. The gut microflora of Reticuliter-
teres flavipes, its relation to oxygen, and evidence for oxygen-dependent acetogenesis by the most abundant Enhydrobacter sp. FEMS Microbiol. Ecol. 21:177–149.
49. Tokiaru, M., M. Okhama, and T. Kudo, 2000. Molecular phylogeny of meth-
anogens associated with flagellated protists in the gut and with the gut 
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Erklärung

Ich versichere, daß ich meine Dissertation

„Molekularbiologische Untersuchung der Diversität und Funktion methanogener Mikroorganismen im Reisfeldboden“

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, 7. November 2001