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Life Strategies of Microorganisms in the Environment and in Host Organisms

Leopoldina Symposium
Bremen, April 5 to 8, 2006

Rudolf Amann, Werner Goebel, Barbara Reinhold-Hurek, Bernhard Schink and Friedrich Widdel (Eds.)

Deutsche Akademie der Naturforscher Leopoldina, Halle (Saale) 2008
Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Life Strategies of Microorganisms in the Environment and in Host Organisms

NOVA ACTA LEOPOLDINA

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Vizepräsident der Akademie

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Life Strategies of Microorganisms in the Environment and in Host Organisms

Leopoldina Symposium

Deutsche Akademie der Naturforscher Leopoldina
in Collaboration with
the Max Planck Institute for Marine Microbiology, Bremen
and the University of Bremen

Bremen
April 5 to 8, 2006

Program Committee:

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Member of the Academy

With 22 Figures and 1 Table

Deutsche Akademie der Naturforscher Leopoldina, Halle (Saale) 2008
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Cover image:
Sponges form one of the deepest radiations of the Metazoa, with a fossil record dating back 580 million years. In the 1960s and 1970s, it was recognized that many species of Demospongiae, the largest class of sponges, are associated with enormous numbers of microorganisms. All species of the Demospongiae orders Verongida, Agelasida, Chondrosida, and Lithistida contain high numbers of microorganisms. The Mediterranean sponge *Aplysina aerophoba* (Verongida, Demospongiae) can be considered as a model bacteriosponge as its microbiology has been studied in detail for more than three decades (underwater photography by T. WINTER, Würzburg; see BAYER et al. in this volume p. 71)

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Nova Acta Leopoldina NF *96*, Nr. 356, 7–8 (2008)

Preface

Friedrich WIDDEL ML (Bremen)

A symposium having in its title both "microorganisms in the environment" and "in host organisms" may at first glance appear to be an unrealistic attempt to cover the entire area of organismic microbiology within a couple of days. Any microorganism of interest is, of course, either free-living in the open environment, or symbiotic or pathogenic inside a host organism. However, the emphasis of the symposium title lies in the words "life strategies". When this term is used in biology, higher life forms will first come to mind: We are accustomed to viewing the anatomic forms, sensory accomplishments, and behaviors of animals as the life strategies employed to make optimum use of the abiotic and biotic settings in their surroundings. Such a view may not immediately come to mind when we look at the simple life forms of microorganisms. But the deeper we study their function, the more aware we become that prokaryotic capabilities can just as well be regarded as highly evolved strategies. Also these strategies, which are more on the level of molecular reactions and interactions, appear amazingly "intelligent". Such thoughts happened to become the topic of an earlier discussion inspired by Werner GOEBEL among those that have now become the program committee. We first discussed pathogenic and symbiotic microorganisms, with their astounding adaptations to their host's molecular structures and biochemistry. When we then considered that also many free-living (environmental) microorganisms "know" how to make optimal use of the physical, energetic, and chemical settings of sometimes bizarre-looking environments, the discussion soon resulted in the concept of a symposium dealing with such life strategies.

We were delighted that when inquired, several experts in the areas of environmental microbiology, symbiosis research, or medical microbiology expressed their interest in contributing to such a symposium. I wish to thank these scientists for their state-of-the-art contributions and stimulating exchange of ideas during the symposium.

Let me close these opening remarks by expressing my gratitude to those who helped with the organization of this symposium. This event would not have been possible without the input and work of Allgrid HILLMER, Heiko LÖBNER, Manfred SCHLÖSSER, and Ulrike TIETJEN.

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Welcome Greetings

Rudolf K. Thauer ML (Marburg/Lahn)

Member of the Presidium of the German Academy of Sciences Leopoldina

Dear colleagues and friends,
Ladies and gentlemen,

It is a great pleasure for me to welcome you to this Leopoldina Symposium on behalf of the presidium of the Deutsche Akademie der Naturforscher Leopoldina. The president of the Leopoldina, Volker ter Meulen, who is also a microbiologist, would have liked to be here, but unfortunately he has other pressing obligations this week. He sends his warmest regards.

The present symposium seeks to cover important developments in the study and understanding of the fascinating life strategies of free-living as well as of host-associated microorganisms and to bridge these areas from an environmental and evolutionary perspective. The presidium of the Leopoldina is pleased that the program has been so well perceived and that so many scientists from outside Bremen are participating. We are delighted that many of the invited speakers are accompanied by junior scientists and that the Leopoldina could help make this financially possible. I want to thank not only the members of the program committee for putting together such a very interesting and challenging program but also the invited speakers for accepting the invitation. I also want to thank the local organizing committee for all their efforts to make us participants feel as welcomed guests in Bremen.

Most of you are probably not too familiar with the Deutsche Akademie der Naturforscher Leopoldina, who is sponsoring this symposium. Therefore, here are just a few facts: The Leopoldina was founded 354 years ago, in 1652 in Schweinfurt, Germany. Since 1878, the academy has resided in Halle/Saale. The Leopoldina is one of the oldest academies in Europe − even a bit older than the Royal Society of the United Kingdom, which was founded in 1660 in London, and only a bit younger than the Académie Française, which was founded in 1635 in Paris. The Leopoldina, which has 1100 members both from Germany (70 %) and from abroad, is the *independent scientific Academy of Germany* dedicated to promoting excellence in science. The Academy supports developments in science, engineering, and technology in a wide range of ways. One of the many ways is to organize symposia at the cutting edge of scientific progress, such as this symposium on "Life Strategies of Microorganisms in the Environment and in Host Organisms".

This symposium was suggested by Werner Göbel from Würzburg, who as senator heads section 13 "Microbiology and Immunology" of the Leopoldina. Other members of this

section who are present are Jörg HACKER, Bernhard SCHINK, Karl STETTER, and Friedrich WIDDEL. We are all looking forward to the opening lecture of Karl STETTER, who has been a member of the academy since 1995. We are excited to hear his presentation on microorganisms living in hot and inorganic environments.

Thank you all for being here.

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Nova Acta Leopoldina NF *96*, Nr. 356, 9–10 (2008)

Microorganisms in the Environment – Unique Adaptations, Basic Metabolism, and Evolution

Nova Acta Leopoldina NF *96*, Nr. 356, 13–18 (2008)

Hyperthermophiles – Life in a Hot and Inorganic Environment

Karl O. Stetter ML (Regensburg)

With 3 Figures

Abstract

Natural hot-water-containing environments harbor complex communities of hyperthermophilic archaea and bacteria (optimal growth: 80–106 °C), representing the upper temperature limit of life (maximal growth temperature: 113 °C). Most hyperthermophiles are anaerobic chemolithoautotrophs, which obtain energy from inorganic redox reactions. In line with the scenario of a hot early Earth, hyperthermophiles cover the deepest and shortest branches within the phylogenetic tree of life. The earliest archaeal phylogenetic lineage is represented by members with extremely tiny cells belonging to the novel kingdom of Nanoarchaeota.

Zusammenfassung

Wasserhaltige heiße Gebiete beinhalten komplexe Gemeinschaften hyperthermophiler Archaeen und Bakterien (optimales Wachstum: 80–106 °C), welche die oberste Temperaturgrenze von Leben repräsentieren (maximale Wachstumstemperatur: 113 °C). Die meisten Hyperthermophilen sind anaerobe Chemolithoautotrophe, die ihre Energie aus anaeroben Redoxreaktionen gewinnen. Alle kürzesten tiefsten Äste im phylogenetischen Baum sind von Hyperthermophilen besetzt, im Einklang mit dem Bild von einer heißen frühen Erde. Die früheste phylogenetische Linie innerhalb der Archaeen wird von den extrem kleinen Mitgliedern des neuen Reiches der Nanoarchaeota repräsentiert.

On Earth, most known life forms are adapted to ambient temperatures within the range of 15–45 °C. Among microorganisms, thermophiles growing optimally between 45 and 70 °C have been known for a long time. During the past decades, novel microbes exhibiting unprecedented optimal growth temperatures in excess of 80 °C have been cultivated. To distinguish them from the usual thermophiles, I designated these newly cultivated microorganisms as hyperthermophiles (Stetter 1992). As a rule, they grow fastest between 80 and 105 °C (upper temperature limit of *Pyrolobus fumarii:* 113 °C) and are unable to propagate at 50 °C or below. For some species of hyperthermophiles, 80 °C is still too low to support growth. Here, I give an overview of the modes of life, biotopes, and phylogeny of hyperthermophiles, and discuss evidence for their probable existence since the dawn of life in the early Archaean age.

Hyperthermophiles are found in natural and artificial environments. On land, volcanic emissions from deep magma chambers heat up soils and surface waters, forming sulfur-

containing solfataric fields and neutral to slightly alkaline hot springs. The surface of solfataric soils is rich in sulfate and exhibits low pH values (pH 0.5–6). Deeper down, solfataric fields are less acidic (pH 5–7) and are anaerobic. As a rule, solfataric fields contain large amounts of elemental sulfur. Deep subterranean, non-volcanic, geothermally heated biotopes were discovered about 3,500 m below the bottom of the North Sea and below the Alaskan North Slope permafrost, where *in situ* temperatures reach approximately 100 °C (Stetter et al. 1993). These Jurassic oil-bearing sandstone and limestone formations harbor hyperthermophilic communities, as indicated by hydrogen sulfide formation ("reservoir souring") and a mixture of about 10^7 viable cells of various species of hyperthermophiles per liter of extracted fluids. These organisms are very similar to those found in submarine hydrothermal systems. Marine biotopes can be shallow (e.g. at the beach of Vulcano Island, Italy) or deep hot sediments and hydrothermal systems. Most impressive are the deep sea "smoker" vents, where mineral-laden hydrothermal fluids with temperatures of up to 400 °C build huge rock chimneys. Although these hot fluids are sterile, the surrounding rock material with a much lower temperature is teeming with hyperthermophiles (e.g. 10^8 cells of *Methanopyrus* spp. per g of rock at the Mid-Atlantic Snake Pit vent). A further type of submarine high temperature environment is provided by the active seamounts (e.g. Teahicya and Macdonald seamounts, close to Tahiti). When the Macdonald seamount erupted, about 10^4 viable cells of hyperthermophiles per liter were detected within its submarine plume (Huber et al. 1990).

Volcanic emissions usually contain large amounts of steam, carbon dioxide, and hydrogen sulfide; variable quantities of carbon monoxide, hydrogen, methane, and nitrogen; and traces of ammonia, all of which could serve as energy sources and nutrients for hyperthermophiles. Although unable to grow at the low ambient temperatures, hyperthermophiles are able to survive under these conditions for several years. This is true even for strict anaerobes in the presence of oxygen, as long as they are kept cold in the laboratory.

During the past decades, powerful molecular techniques had been developed to investigate phylogenetic relationships of living organisms. Based on the pioneering work of Carl Woese, small subunit (ss) rRNA is widely used in phylogenetic studies (Woese and Fox 1977, Woese et al. 1990). In bacteria and archaea, ss rRNA consists of about 1500 bases. ss rRNA sequence comparisons have been used to construct a universal phylogenetic tree (Fig. 1). The tree shows a tripartite division of the living world into the bacterial (formerly eubacterial), archaeal (formerly archaebacterial), and eukaryal (formerly eukaryotic) domains. Deep branches within the phylogenetic tree are evidence for early separation. The separation of the bacteria from the stem shared by archaea and eukarya represents the deepest and earliest branching point. Short phylogenetic branches indicate a rather low rate of evolution. In contrast to the eukaryal domain, the bacterial and archaeal domains within the universal phylogenetic tree exhibit some extremely short and deep branches. Surprisingly, these branches are covered exclusively by hyperthermophiles, which therefore form a cluster around the phylogenetic root. The deepest and shortest phylogenetic branches are represented by *Aquifex* and *Thermotoga* within the bacteria and *Methanopyrus*, *Pyrodictium*, and *Pyrolobus* within the archaea. On the other hand, mesophilic and moderately thermophilic bacteria and archaea, as a rule, represent long lineages within the phylogenetic tree, and, therefore, had a high rate of evolution (e.g. Gram-positive bacteria, Proteobacteria, *Halobacterium,* and *Methanosarcina*).

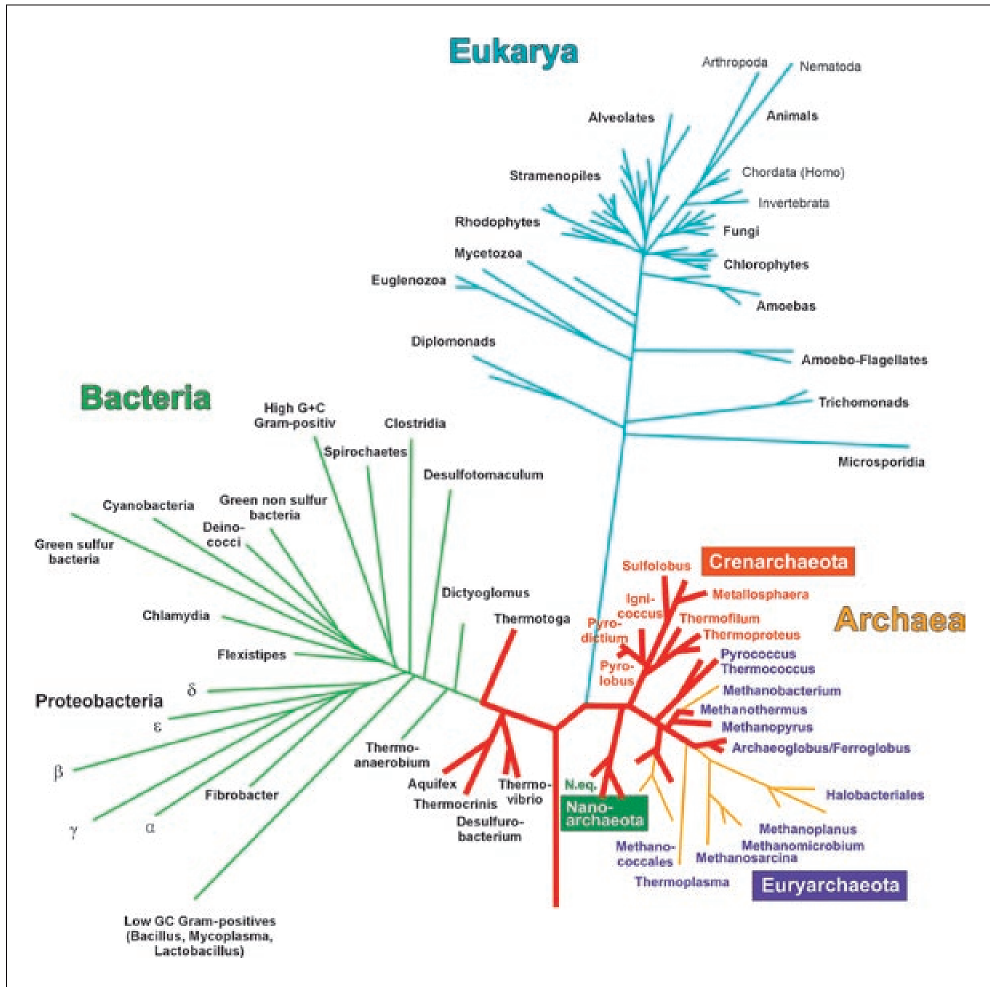

Fig. 1 Phylogenetic tree of life based on the small subunit ribosomal RNA (ss rRNA). Hyperthermophile lineages are represented by thick red lines.

To date, about 70 species of hyperthermophilic bacteria and archaea have been described. These are grouped into 29 genera in 10 taxonomic orders. Hyperthermophiles are well adapted to growing in extremes of temperature, pH, redox potential, and salinity (for a review, see Stetter 2005). The energy sources of hyperthermophiles are very simple. Most species have a chemolithoautotrophic mode of nutrition (Fig. 2). Within their biotopes, they form complex ecosystems that can consist of both primary producers and consumers of organic matter.

Members of the extremely acidophilic genera *Sulfolobus*, *Metallosphaera*, *Acidianus*, and *Stygiolobus* are lobed cocci and are found in acidic, hot, solfataric fields and coal refuse piles. They are aerobic, facultatively aerobic and anaerobic chemolithoautotrophs that gain energy through the use of H_2, H_2S and S^0 as electron donors and S^0 and O_2 as electron acceptors.

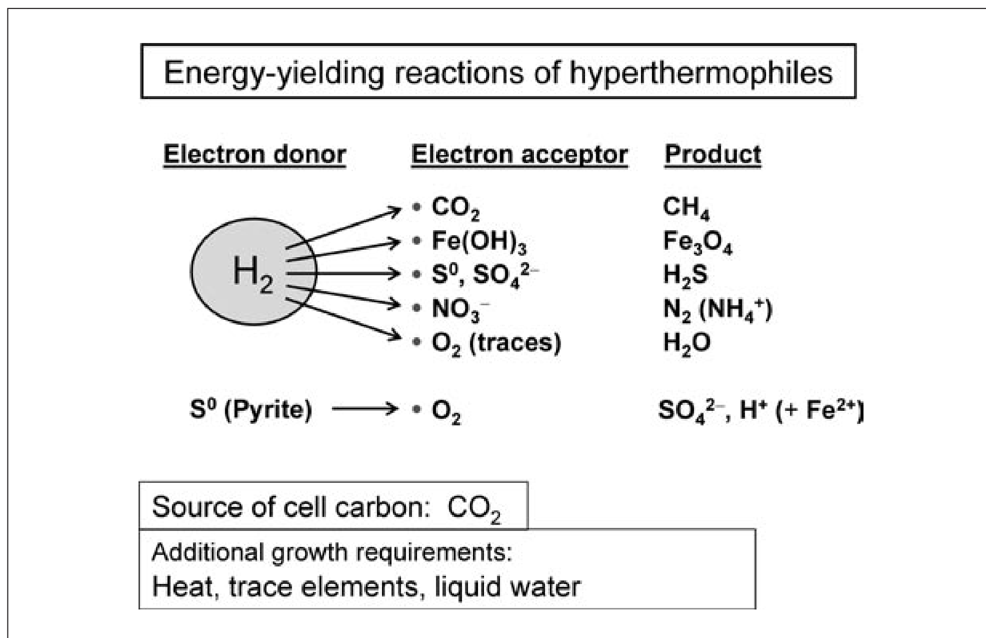

Fig. 2 Main energy-yielding reactions in chemolithoautotrophic hyperthermophiles.

Slightly acidic to alkaline terrestrial hydrothermal systems contain members of the rod-shaped archaeal genera *Thermoproteus*, *Thermofilum*, and *Pyrobaculum* and the coccoid-shaped archaeal genus *Desulfurococcus*; the species of these genera are either chemolithoautotrophs or facultative and obligate heterotrophs. Most of them are anaerobes and usually grow by sulfur respiration.

The highest growth temperatures observed occur among communities of marine hyperthermophiles consisting of members of the bacterial genera *Aquifex* and *Thermotoga* and the archaeal genera *Pyrobaculum*, *Staphylothermus*, *Pyrodictium*, *Thermodiscus*, *Thermococcus*, *Pyrococcus*, *Archaeoglobus*, *Methanopyrus*, and *Methanococcus*. They are found within shallow and deep submarine hydrothermal systems. Species of *Aquifex* are rod-shaped, obligate chemolithoautotrophs and grow at the highest growth temperature known among the bacteria (95 °C). They gain energy by oxidizing H_2 or S^0 under microoxic conditions. In the absence of O_2, members of *Aquifex* can grow by nitrate reduction.

Within archaea, the organisms with the highest growth temperatures (110–113° C) are of the genera *Pyrolobus*, *Pyrodictium*, and *Methanopyrus*. *Pyrodictium* is an anaerobic, facultatively chemolithoautotrophic sulfur respirer. It forms disk-shaped cells that connect to one another through unique networks of hollow tubules about 30 nm in diameter. Although resting forms, such as spores, have never been observed, cultures of *Pyrodictium occultum* grown at 110 °C are extraordinarily heat resistant and even survive autoclaving for 1 h at 121 °C. The temperature record of growth is held by *Pyrolobus fumarii*, a deep sea hyperthermophile that grows up to 113 °C (Blöchl et al. 1997). *Methanopyrus* is a rod-shaped methanogen that grows optimally at 100 °C, with a population doubling time of 50 min. It contains a primitive geranyl-ether lipid in its membrane; this lipid serves as a lipid precursor in other archaea.

Archaeoglobus spp. are coccoid, facultatively lithoautotrophic, archaeal sulfate reducers. They share several coenzymes with methanogens. In its genome, *Archaeoglobus* has acquired whole sets of genes from bacteria, e.g. those for fatty acid degradation.

A novel group of hyperthermophilic archaea had been completely overlooked until recently. From a submarine hydrothermal system at the Kolbeinsey Ridge, north of Iceland, we were able to isolate *Nanoarchaeum equitans*, which represents a novel kingdom of archaea (Huber et al. 2002). In ecological studies based on the polymerase chain reaction, its ss rRNA gene was not detected using the commonly used primers that were thought to be universal. With a cell diameter of only 400 nm, *Nanoarchaeum equitans* is the smallest living organism known. Cells grow attached to the surface of a specific crenarchaeal host, a new member of the genus *Ignicoccus* (Fig. 3).

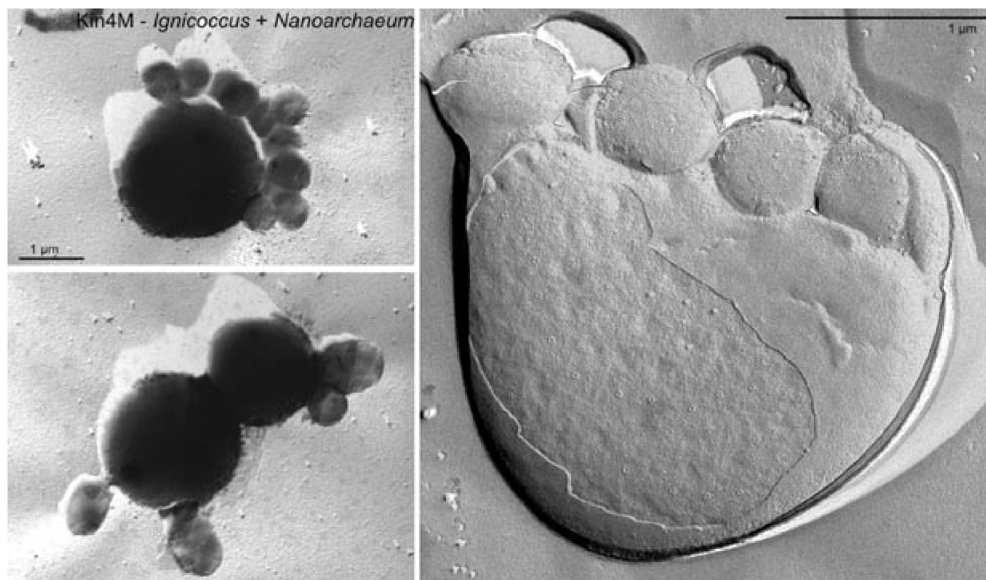

Fig. 3 Transmission electron micrographs of cells of *Nanoarchaeum equitans* (small cells) attached to *Ignicoccus* strain Kin 4M (large). Left: platinum shadowing. Right: freeze etching.

With only 490,885 base pairs, *N. equitans* harbours the smallest microbial genome known to date (Waters et al. 2003). This genome encodes the complete machinery for information management and repair, but lacks genes for amino acid, nucleotide, lipid, and cofactor biosynthesis. The limited biosynthetic and catabolic capacity of *N. equitans* suggests that its symbiotic relationship to its host may be parasitic. However, unlike the small genomes of bacterial parasites that are undergoing reductive evolution, the small genome of *N. equitans* has a well-equipped DNA recombination system and very few pseudogenes. On the molecular level, *N. equitans* harbors further unexpected, most likely primitive features, such as separately encoded enzyme modules and tRNA gene fragments (Waters et al. 2003, Randau et al. 2005). At present, we are still far from a deeper understanding of the *Nanoarchaeum–Ignicoccus* relationship and further investigations are required. The discovery of the *Nanoarchaeota* suggests that other groups of microbes with unusual ss rRNA genes that cannot be recognized by “universal” primers might still be out there, waiting to be discovered.

Acknowledgements

I wish to thank Reinhard Rachel for electron microscopy and Harald Huber for redrawing the phylogenetic tree. The work presented from my laboratory was supported by the DFG, the BMBF, the EEC, and the Fonds der Chemischen Industrie.

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Archaea, Methane, and Oases of the Deep

Victoria J. Orphan (Pasadena), Christopher H. House (College Park), and Shana K. Goffredi (Pasadena)

With 1 Figure

Abstract

The deep sea, fed by a slow trickling input of photosynthetically derived carbon, has historically been considered a low energy, oligotrophic environment. In localized areas, however, oases of elevated microbial biomass and activity within the deep sea do exist. Perhaps the most famous are hydrothermal vents, emerging along spreading centers and subduction zones, fueled by hot reduced fluids re-circulated within the Earth's crust. Equally rich, although less well known, areas of stimulated biomass production and activity also occur in the psychrophilic depths of the seafloor, fueled by large organic accumulations (i.e. food falls) and subsurface reservoirs of methane. The microbial ecology within these locally active deep-sea habitats is unique, supporting novel microbial associations and diverse pathways for carbon remineralization.

Zusammenfassung

Die Tiefsee, welche durch langsam herabrieselnden organischen Kohlenstoff photosynthetischen Ursprungs ernährt wird, wurde in der Vergangenheit oft als eine oligotrophe Welt mit geringen Energieressourcen für Lebewesen angesehen. An bestimmten Stellen in der Tiefsee gibt es jedoch Oasen mit stark erhöhter bakterieller Biomasse und Aktivität. Die wohl bekanntesten dieser Oasen sind Hydrothermalquellen an den Spreizungs- und Subduktionszonen, unterhalten durch chemisch reduzierte heiße Fluide, welche durch die Erdkruste zirkulieren. Um biologisch nicht minder reichhaltige, wenn auch weniger bekannte Tiefseeoasen handelt es sich bei den kalten Systemen, die durch große Ansammlungen organischen Materials (d. h. abgesunkene Nahrungshaufen) oder Methanvorkommen unter der Oberfläche des Meeresbodens unterhalten werden. Die mikrobielle Ökologie an solchen Oasen hoher biologischer Aktivität ist einzigartig und umfasst zuvor unbekannte Vergesellschaftungen und diverse Stoffwechselwege für die Reoxidation organischen Kohlenstoffs.

Investigations examining the succession of microbial communities and remineralization processes within organically enriched whale fall habitats in the Monterey Canyon, California, have revealed a somewhat unexpected role for methanogenic archaea within these deep sea habitats (Goffredi et al. 2008). Although prior studies have focused primarily on sulfidogenic-based carbon remineralization, whale falls also promote the establishment of methanogenic assemblages within shallow (0–3 cm bsf) seafloor sediments. We detected evidence of active methane production as well as the enrichment of putative hydrogenotrophic methanogens affiliated with the Methanomicrobiales (by both 16S rRNA diversity surveys and FISH) in seafloor sediments immediately underlying whale carcasses.

The presence of H_2-utilizing methanogens within the sulfate replete upper 0–3 cm sediments is surprising given the standard redox gradients in marine sediments, where sulfate-reducing microorganisms frequently out compete methanogens for common substrates such as H_2. Although largely uncharacterized, a handful of studies have promoted the dominance of sulfidogenic degradation pathways within the whale fall habitat (Smith and Baco 2003), presumably with bacterially generated sulfide supporting the colonization of sulfide oxidizing chemosynthetic megafauna. Our work studying the microbial ecology of whale falls in the Monterey Canyon indicate that cycling of carbon is significantly more complex with the co-existence of both sulfate reduction and methanogenic processes within the organically enriched sediments. Sulfide concentrations between whale fall habitats were variable, from undetectable levels at the deepest site (2900 m in depth) to 3 mM at the 1017 m whale fall, but methane concentrations were consistently elevated in both. In addition to geochemical evidence, the persistence of members of the putative hydrogenotrophic Methanomicrobiales within both whale fall locations from multiple samplings over a 39-month period, as well as the lack of methanogenic assemblages detected in adjacent background 'control' deep-sea sediments suggest atypical anaerobic remineralization pathways within these organically enriched environments, possibly supported by enhanced rates of fermentation and associated H_2 production (Goffredi et al. 2008). Future work building on these initial observations which incorporate microbial rate measurements and additional biogeochemical analyses will assist in further constraining the local physico-chemical environment supporting enhanced microbial activity and methane production in this unusual seafloor environment.

As observed in the whale fall habitat, unusual microbial interactions and direct linkages between the carbon and sulfur cycle are also sustained within marine methane seep habitats through the process of anaerobic oxidation of methane coupled to sulfate reduction. The discovery of extremely $\delta^{13}C$ depleted archaeal isoprenoids in tandem with the documentation of tightly coupled physical associations between Euryarchaeota, affiliated with the Methanosarcinales, and sulfate-reducing deltaproteobacteria in methane seeps lent strong evidence for the hypothesis of anaerobic oxidation of methane catalyzed by a syntrophic consortium comprised of a methanogen and sulfate-reducing microorganism (Hinrichs et al. 1999, Boetius et al. 2000 and Orphan et al. 2001a,b). Since these early observations, we now know that direct physical associations between methane oxidizing ANME archaea and sulfate reducing bacteria are not always observed *in situ*, introducing the potential that methane oxidation may be catalyzed solely by the ANME archaea, independent of the sulfate-reducing partner. Using a microanalytical method known as FISH-SIMS that enables the stable isotopic characterization of phylogenetically identified single cells, we have been investigating the dynamics of interspecies interactions and isotopic heterogeneity within individual uncultured methane-oxidizing assemblages recovered from methane seep environments. In agreement with our initial observations (Orphan et al. 2002), single cells belonging to the ANME-1 lineage recovered from 4 discrete sediment intervals (2 to 16 cm) exhibited extremely depleted stable isotope values of their cellular carbon indicative of growth on methane, with values down to −89 ‰ (n = 19). Similarly, monospecific aggregations of microorganisms related to the ANME-2 also displayed $\delta^{13}C_{biomass}$ suggestive of methane assimilation ($\delta^{13}C$ −75 ‰; n = 6). The depleted $\delta^{13}C$ measured for ANME-2 clusters are on the same order as the values obtained for members of the ANME-2 physically associated with sulfate-reducing bacteria and again, may be indica-

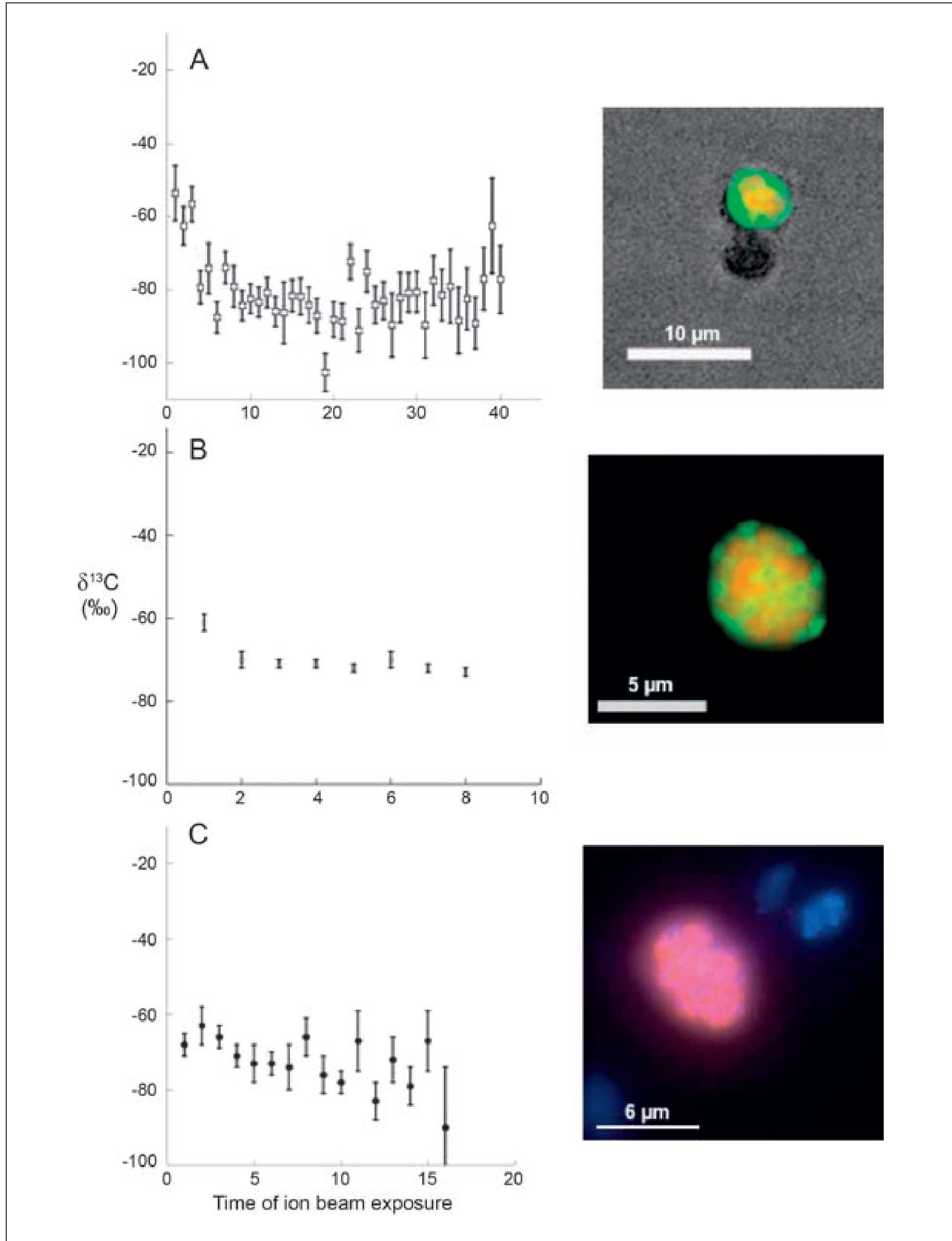

Fig. 1 Ion microprobe measurement of $\delta^{13}C$ depth profiles of morphologically diverse cell aggregates belonging to the ANME-2 lineage and members of the *Desulfosarcina*/*Desulfococcus* group. FISH images of the methane oxidizing cell aggregates prior to SIMS analysis are depicted on the right. Panel *A*: ANME-2/*Desulfosarcina*, layered shell type aggregate. Panel *B*: ANME-2/ *Desulfosarcina* mixed aggregate morphology. Panel *C*: mono-species aggregation of ANME-2 cells.

tive of methanotrophic activity by the archaea independent of a tightly-coupled bacterial partner. Metagenomic investigations of uncultured ANME assemblages have shed light on potential genes involved in the methane oxidation pathway, but have not yet uncovered evidence of dissimilatory sulfate reduction capabilities localized to the genomes of methanotrophic Archaea (Hallam et al. 2004, Meyerdierks et al. 2005). More in depth research is needed to distinguish between the capability of methanotrophic ANME archaea to oxidize methane independently of the associated sulfate reducing populations, or whether the observed mono-specific ANME clusters and single cells recovered from the environment represent inactive microorganisms which originated from disbanded syntrophic consortia previously growing on methane.

In addition to the phylogenetic diversity represented by the uncultured methanotrophic archaea, members of the three ANME clades also exhibit diversity in the spatial associations with sulfate-reducing deltaproteobacteria. Two major morphotypes are commonly observed within the methane seep environment; a layered architecture consisting of a central core of ANME-2 archaea surrounded by a shell of sulfate reducing bacteria (Fig. 1*A*, inset), or alternatively, in a mixed arrangement, with a more randomized distribution of single ANME-2 cells interspersed with sulfate reducing microorganisms (Fig. 1*B*, inset). Comparison of $\delta^{13}C$ FISH-SIMS profiles of individual ANME/DSS aggregations representing differences in spatial arrangement between the two microbial partners show evidence of methane in their biomass (Fig. 1). Examination of individual $\delta^{13}C$ SIMS depth profiles collected from mixed and shell aggregates also appear to correlate with the spatial distribution of the bacterial and archaeal partners. Where the majority of shell type aggregates displayed a progressive depletion in ^{13}C during the analysis, ranging from −58 to −90 ‰, as previously described in Orphan et al. (2001a; Figure 1*A*). Analyses of the more homogeneously mixed aggregates as well as ANME-2 only aggregates yielded a more constant ^{13}C profile averaging ~ −70 ‰ for the entire mixed aggregate and −75 ‰ for the ANME-2 (Fig. 1*B*, 1*C*). Investigations by Knittel et al. (2005) as well as observations by our group have shown these two morphologies represent two phylogenetically distinct ANME-2 ecotypes, belonging to the ANME-2c and ANME-2ab clades (Orphan et al. 2001b).

Assuming a limited free energy yield from sulfate-coupled methane oxidation and a reliance on minimal diffusion distances between the syntrophic partners, the geometry and variable diffusion distances represented by these two structurally distinct ANME/DSS associations is likely to impact the overall efficiency of the reaction. Microcosm incubations with methane seep sediment amended with ^{15}N labeled ammonium enabled an initial assessment of potential differences in assimilatory activity associated with architecturally diverse aggregates. Using ^{15}N-ammonium incorporation into biomass as a proxy for metabolically activity, $^{15}N_{biomass}$ values in shell and mixed ANME-deltaproteobacterial consortia as well as aggregations of ANME archaea was assessed. Preliminary findings suggest the highest levels of ^{15}N assimilation occurred within layered shell aggregates. In comparison, ANME-SRB aggregates with a mixed architecture were variable in ^{15}N incorporation between individual aggregates, whereas little to no ^{15}N incorporation was measured within ANME-only archaea. These findings suggest that members of the methanotrophic ANME archaea obtain some direct benefit from the physical association with the sulfate reducing bacteria and in turn influences the ecology of these globally important microorganisms.

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Natur und Migration

Vorträge anlässlich der Jahresversammlung vom 5. bis 7. Oktober 2007 zu Halle (Saale)

Nova Acta Leopoldina N. F., Bd. *97*, Nr. 358
Herausgegeben von Harald zur Hausen (Heidelberg)
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„Natur und Migration“ – assoziiert sehr verschiedenartige Phänomene, die sich durch Wanderungsprozesse auszeichnen. In diesem Band wurden besonders interessante Gebiete ausgewählt, u. a. Migration und Seuchen, Reisen und Epidemien in einer globalisierten Welt, der Vogelzug, aber auch die Migration geologischer Fluide, die Elektronenmigration in Halbleitern, die Migration als treibende Kraft in der Organogenese, die Biophysik der Zellbewegungen, die Migration von Tumorzellen, Migration als Phänomen in der Neurobiologie oder die Migration wissenschaftlicher Ideen. Besondere Akzente setzen die Themen „Diversität als neues Paradigma für Integration?“ und „Vorspiel der Globalisierung. Zur Emigration deutscher Wissenschaftler 1933 bis 1945“.
Die Beiträge sind von herausragenden Experten der jeweiligen Gebiete, u. a. durch die Leopoldina-Mitglieder Markus Affolter, Lorraine Daston, Wolfgang Frühwald, Michael Frotscher, Jörg Hacker, Hans Keppler und Otmar Wiestler, in anspruchsvoller, aber durchaus gut verständlicher Form verfasst.

Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Nova Acta Leopoldina NF *96*, Nr. 356, 25–32 (2008)

Microbes in the Deep Biosphere – Who Feeds Them?

R. John Parkes (Cardiff)

With 3 Figures

Abstract

The early data and concepts which considered deep, sub-seafloor sediments to be devoid of life below a few 10's of meters is described. More recent research is then presented which shows that a surprisingly large prokaryotic biomass is present in these sediments. Finally, the energy sources for this biomass is described and discussed.

Zusammenfassung

Zunächst werden ältere Ergebnisse und Konzepte vorgestellt, gemäß denen Sedimentbereiche in Tiefen von mehr als einigen zehn Metern unter dem Meeresboden kein Leben beherbergen. Danach werden neuere Forschungsergebnisse erläutert, welche hingegen eine erstaunlich hohe prokaryotische Biomasse in solchen Sedimentbereichen aufzeigen. Schließlich werden Energiequellen für diese Biomasse beschrieben und diskutiert.

1. Introduction

Although, microbial populations dominate the degradation of organic matter in near surface sediments and, hence, drive diagenesis and biogeochemical cycles (Jørgensen 2000), their activity is so efficient that little biodegradable organic matter is buried in deeper sediments to allow continued bacterial activity. For example, in the marine environment less than <0.5% of productivity is buried in sediments (Hedges and Keil 1995). In addition, this organic matter only survives because of its recalcitrance and or restricted bioavailability (Hedges and Keil 1995), hence, it seemed reasonable that bacterial activity would be extremely restricted or absent in deep marine sediments, which can be over 10 km thick (Fowler 1990). Early evidence supported this contention, for example, Morita and Zobell (1955) found that bacteria could not be cultured below 7.47 m depth in Pacific Ocean sediments and considered that they had reached the end of the marine biosphere. Similarly, Oremland et al. (1982) could only measure active methanogenesis down to 12 m in Gulf of California sediments but even at these depths activity was patchy (only 6 out of 12 samples positive), and there was no activity at 21 m. Therefore, despite marine sediments containing, globally, the largest store of organic carbon, they were considered to be devoid of active bacteria below a few meters to 10's of meters.

In contrast, there was some geochemical evidence indicating continuing bacterial activity in deeper sediments, for example, concentration and isotopic data on CH_4 from Walvis Ridge suggested that biogenic CH_4 may occur down to several 100's of meters (Meyers 1984), and methanogen biomarkers were present in Cretaceous age marine sediments (Brassell et al. 1981). However, in the absence of direct evidence for the presence of active bacteria this data could equally be interpreted as being fossil products from past microbial populations and activity. However, the situation of microbial activity ceasing at depths of a few 10's of meters, and then nothing happening until thermogenic processes starting at several kilometers depth and temperatures in excess of 100 °C (Quigley and Mackenzie 1988), was still a surprising and curious situation, and hence, we began to study deep sediment bacteria in 1986. Since then we have investigated the microbiology of 20 ODP and IODP Legs (Ocean Drilling and Integrated Ocean Drilling Programs, http://iodp.tamu.edu/). Consistent results from a range of complementary approaches (direct cells counts, numbers of different types of viable prokaryotes, physiology of deep sediment bacteria, activity measurements, geochemistry, molecular genetic and biomarker analysis) have demonstrated that bacteria are ubiquitous in deep sub-seafloor sediments and have a globally significant biomass, at least 10 % of all living carbon (Horsfield et al. 2006, Parkes et al. 2005, Schippers et al. 2005, D'Hondt et al. 2004, Newberry et al. 2004, Wellsbury et al. 2002, Parkes et al. 2002, Wellsbury et al. 1997, Bale et al. 1997, Cragg et al. 1996, Parkes et al. 1994).

The large biomass, great depth (at least 840 m, deepest so far analyzed, Wellsbury et al. 2002) and age (~15 Myr) is surprising considering the recalcitrant nature of the organic matter energy supply and geochemical modeling has suggested that "most microorganisms in sub-seafloor sediments are either inactive or adapted for extraordinarily low metabolic activity" (D'Hondt et al. 2002). However, these results may reflect more the limitations of the geochemical modeling approach to determine low activities in deep sediments rather than deep biosphere microbial cells being dormant or dead. A growing number of studies are providing evidence for the viability and range of energy sources for deep biosphere prokaryotes and some of these are summarized below.

2. Lithological and Geochemical Interfaces

ODP Leg 201 to the Eastern Equatorial Pacific and the Peru Margin was the first dedicated deep biosphere drilling leg and provided a range of new insights regarding prokaryotic metabolism and diversity in sub-seafloor sediments, including diverse metabolism (D'Hondt et al. 2004) which was not predicted by geochemical modeling. The shallow, high productivity Peru Margin sites have a brine incursion at depth which supplies sulfate into deeper methane rich layers (Fig. 1, Site 1229), and hence, there are two sulfate-methane interfaces. Bacterial numbers and activity are stimulated at these interfaces, presumably due to sulfate reduction (SR) coupled to anaerobic oxidation of methane (AOM). There are also changes in prokaryotic diversity with increasing depth and particularly at the two interfaces (shown in marked changes in Principal Components analysis profiles of bacterial diversity, Fig. 1*E*, also Parkes et al. 2005). These results clearly show that deep geochemical interfaces can provide energy for subsurface sediment bacteria and that these populations are active and dynamic and not just dormant, dieing slowly or fossil DNA (Dell'Anno

and Danovaro 2005). The viability of the majority of deep sediment prokaryotes from the Peru Margin was directly confirmed by ribosomal RNA detection in cells (CARD-FISH, Schippers et al. 2005). Stimulation of subsurface bacteria also occurred at a deep water, Equatorial Pacific site (1226, Parkes et al. 2005), but here it was within zones of high diatom content. Presumably diatom associated organic matter is resistant to degradation and elevated concentrations can continue to stimulate bacterial populations to great depth and age (deepest diatom zone at this site was ~250 to 320 m, between 7 and 11 Myr old). A similar stimulation occurs in the high organic matter sapropel layers in Mediterranean sediments (Coolen et al. 2002), and these are dominated by previously unknown members of the green non-sulfur bacteria (*Chloroflexi*) which were also important *Bacteria* in Peru Margin sediments (Parkes et al. 2005). Sapropel prokaryotes, however, are growing incredibly slowly (division every ~105 kyr, Parkes et al. 2000), hence degradation of sapropel organic matter must also be very slow, which explains its persistence over long timescales. How deep sediment prokaryotes survive on such little energy is remarkable.

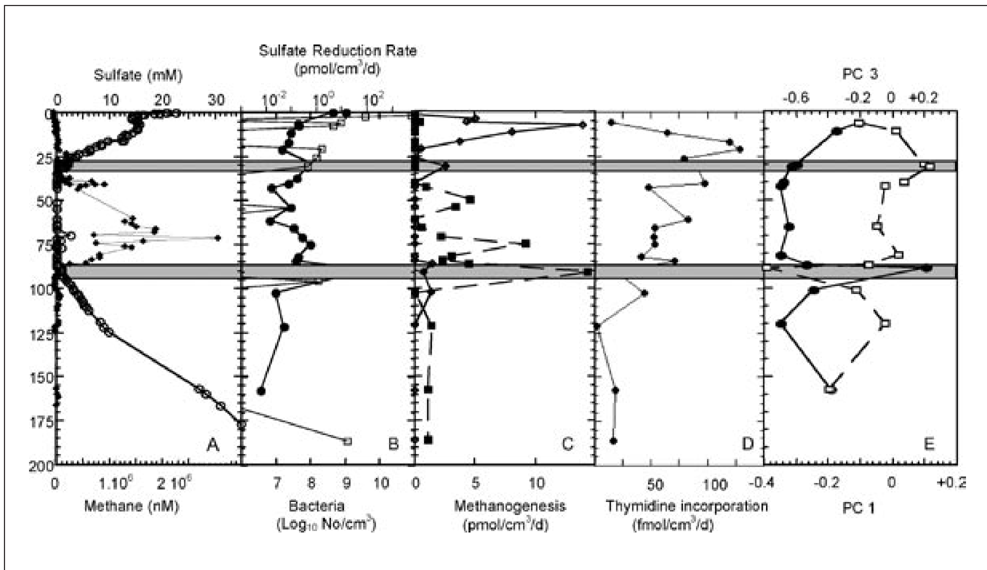

Fig. 1 Biogeochemical process and prokaryotic biodiversity profiles at the Peru Margin Site (ODP 1229). (*A*) geochemistry: ○ pore water sulfate (mM), ♦ CH_4 (nM), (*B*) □ sulfate reduction rates (pmol/cm^3/d), ● total prokaryotic population (Log_{10} numbers/cm^3), (*C*) rates of methanogenesis (pmol/cm^3/d) ◊ H_2/CO_2, acetate ■, (*D*) rates of prokaryotic growth ● thymidine incorporation (fmol/cm^3/d), (*E*) Principal Components depth profile of diversity of *Bacteria* based on DGGE analysis of 16S rRNA gene sequences of extracted DNA: ● Component 1 (56 % of variation), □ Component 3 (9 % of variation); Component 2 (24 % of variation) shows a similar profile to Component 1. Shaded boxes highlight elevated prokaryotic processes and sulphate:methane interfaces. From Parkes et al. 2005.

3. Gas Hydrate Containing Sediments

Gas hydrates form under the low temperature and high-pressure conditions which exist in most marine sediments and particularly in ocean margin sediments where there are sufficient methane gas concentrations. Hydrates contain large concentrations of methane which

globally may represent twice the amount of carbon in "normal" fossil fuels (Kvenvolden 1988). In Blake Ridge (North Atlantic) gas hydrate sediments prokaryotic activity is stimulated at depth around the base of the gas hydrate stability zone (Fig. 2), where increasing temperatures cause the hydrate ice to melt and release methane gas. Stimulation includes AOM, presumably as a response to increased methane concentrations, methanogenesis (acetate and H_2/CO_2), acetate oxidation, bacterial growth and cell numbers (Wellsbury et al. 2000). Some of this general stimulation may reflect large increases in pore water volatile fatty acid concentrations, particularly acetate (to mM rather than the normal few µM concentrations). Similar high acetate concentrations have been detected in subsurface gas hydrate deposits from Southern Hydrate Ridge (Pacific Ocean, Lorenson et al. 2006), and stimulation of prokaryotic activity in deep gas hydrate deposits has also been shown in Cascadia Margin sediments (Pacific Ocean, Cragg et al. 1996). General stimulation of deep prokaryotic activity may be enhanced by fluid flow into the base of the hydrate zone (Egeberg and Barth 1998). Stimulated deep methanogenesis may contribute to the methane in hydrate deposits (Fig. 2).

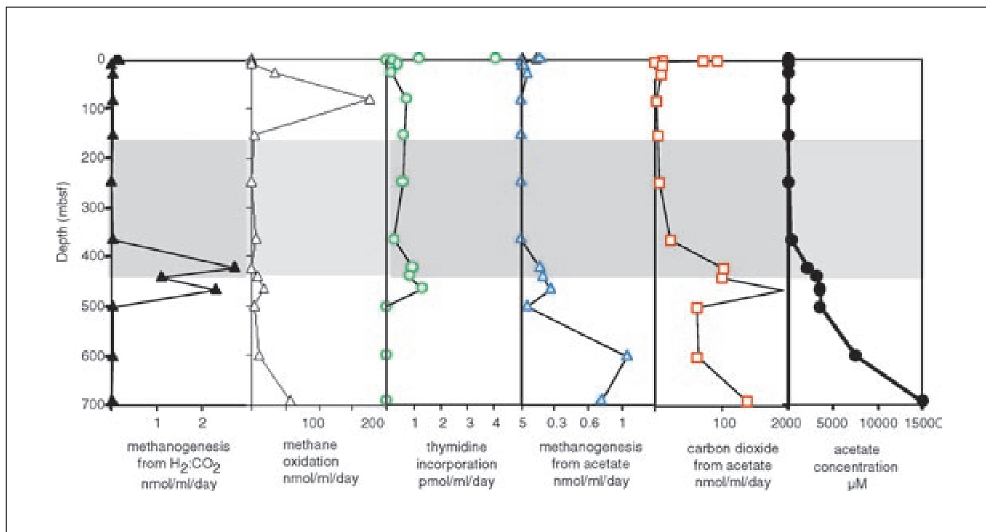

Fig. 2 Depth profiles of rates of prokaryotic activity and pore water acetate concentrations in gas hydrate sediments from Blake Ridge (ODP Leg 164). Shaded area is the gas hydrate zone. From Parkes et al. 2000.

4. Activation of Organic Matter during Sediment Burial and Heating May Provide a Continuing Deep Biosphere Energy Supply

Formation of high acetate concentrations can be simulated in the laboratory by heating sediments to stimulate microbially-driven diagenetic reactions (Wellsbury et al. 1997), and this together with the high organic matter burial rates at Blake Ridge may help to explain the high volatile fatty acid concentrations. Longer term heating experiments (to 90 °C) show that this warming can produce a sequence of reactions that mimic those occurring in deep, hot sediments, such as in the Nankai Trough (sulfate removal, CH_4 and acetate production, H_2 and sulfate formation, and "deep" methanogenesis, Parkes et al.

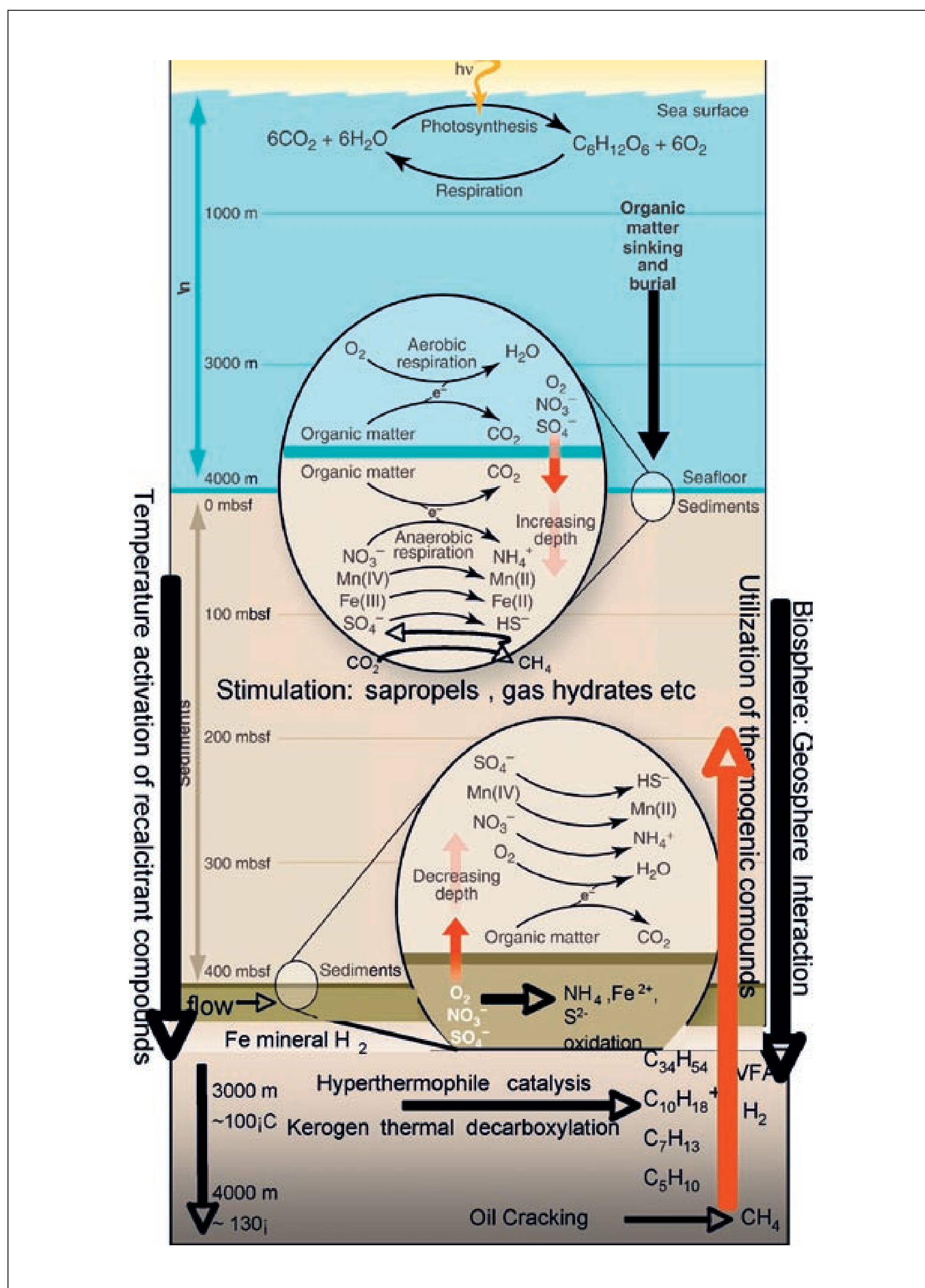

Fig. 3 Schematic of prokaryotic metabolisms, energy sources and interactions in deep, sub-seafloor sediments. Modified from DeLong 2004.

2007). At higher temperatures such reactions have traditionally been considered to be thermogenic (Cooles et al. 1987), but in these experiments they were bacterially-driven as sterile controls produced few products. Hence, slow temperature increases during burial may enable recalcitrant organic compounds to be slowly "activated", and hence, sustain a deep biosphere over great depths and sediment ages. This includes deep, high-temperature sediments, where "thermogenically" produced substrates may stimulate bacterial processes (Horsfield et al. 2006). However, some of these "thermogenic" processes may, in fact, be bacterially catalyzed such as the aromatization of organic matter to produce H_2 (Parkes et al. 2007). Therefore, given that the upper temperature limit for prokaryotes is at least 113 °C (Blochl et al. 1997), bacteria are likely to be present to several kilometers depth in marine sediments (~4 km at the average temperature gradient of ~30 °C/km) and at higher temperatures there can be upward diffusion of thermogenically generated compounds which feeds the base of the deep biosphere (Fig. 3, and Parkes et al. 1994).

5. Inorganic H_2 Generation as a "Dark Energy" Supply

In deep rock environments basalt weathering to produce H_2 has been suggested to be a "Dark Energy" energy supply for a deep biosphere which is independent of surface photosynthetic activity (Stevens and McKinley 1995). Although this has been criticized (Anderson et al. 1998) and it has been shown that not all iron minerals produce H_2 (Stevens and McKinley 2000) under abiotic conditions, we have found that in the presence of bacteria many of these iron minerals do produce H_2 and for extended periods of time (Parkes et al. 2007, and unpublished results). This indicates that prokaryotes are intimately involved in the H_2 generation reaction. The H_2 produced is utilized by other prokaryotes for sulfate reduction and to produce CH_4 and volatile fatty acids, which in turn are substrates for further metabolism. Although fine particle size and fresh surfaces are required for maximum mineral activity which would restrict H_2 generation *in situ*, this would also slow the processes down so that iron mineral H_2 generation could last for long periods of time. These reactions are also stimulated by increased temperatures, potentially providing continuing slow H_2 generation with increasing depth.

6. Conclusions

A range of sedimentary conditions can provide energy for deep biosphere prokaryotes over great depths and geological time scales (Fig. 3). These include organic and inorganic ("Dark Energy") sources both of which are stimulated by temperature increases with depth and may result in deep sediment bacterial activity driving previously considered thermogenic processes. In addition, there is clear interplay between deep biosphere and abiological processes in deep sediments, with thermogenic processes feeding the base of the biosphere. A remarkable aspect is that the rate of energy supply must be very slow to enable energy resources to last for millions of years and how deep prokaryotes cope with this situation is currently a major scientific puzzle.

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From Inorganic to Organic: Prokaryotic CO_2 Fixation

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With 1 Figure

Abstract

The ability to build organic compounds from inorganic precursor molecules marks the beginning of chemoevolution. Extant life depends on this process, which is dominated by carbon fixation in chloroplasts of plants. These organelles are derived from a cyanobacterial endosymbiont that used the Calvin cycle. However, in bacteria and archaea, at least three other pathways of autotrophic CO_2 fixation are known. These pathways are compared to shed light on their distinguishing features, distribution, and possible evolutionary roots.

Zusammenfassung

Die Befähigung zur Synthese von organischen Molekülen aus anorganischen Vorläufermolekülen markiert den Beginn der Chemoevolution. Das Leben hängt von diesem Prozess ab, der von der CO_2-Fixierung in Chloroplasten der Pflanzen beherrscht wird. Diese Organellen stammen von cyanobakteriellen Endosymbionten ab, die den Calvin-Zyklus verwendeten. In Bakterien und Archaeen kennt man jedoch wenigstens drei weitere Wege der autotrophen CO_2-Fixierung. Dieser Beitrag stellt diese Wege gegenüber, um ihre Charakteristika, Verbreitung und geschichtlichen Wurzeln zu beleuchten.

1. Introduction

Autotrophic carbon fixation is the most important synthetic process in biology. It enables organisms to synthesize a central cell carbon precursor exclusively from inorganic carbon. This capability is a characteristic feature of prokaryotes, and only through endosymbiosis was it conferred on eukaryotic cells (MARTIN and SCHNARRENBERGER 1997). Chloroplasts, as well as many autotrophic bacteria, use the Calvin cycle for CO_2 fixation. However, there are at least three other fundamentally different processes in prokaryotes that allow cellular building blocks to be synthesized from CO_2: the reductive citric acid cycle, the reductive acetyl-CoA pathway, and the 3-hydroxypropionate cycle.

The four autotrophic pathways (Fig. 1) differ in numerous aspects: use of CO_2 or bicarbonate; affinity of the key carboxylase(s) for CO_2 or bicarbonate; redox potential of reduction steps; coenzymes involved; natural reducing agent; ATP requirement; concomitant assimilation of common low-molecular-mass compounds, such as fermentation products; oxygen sensitivity of essential enzymes; connection to the energy metabolism; regulation;

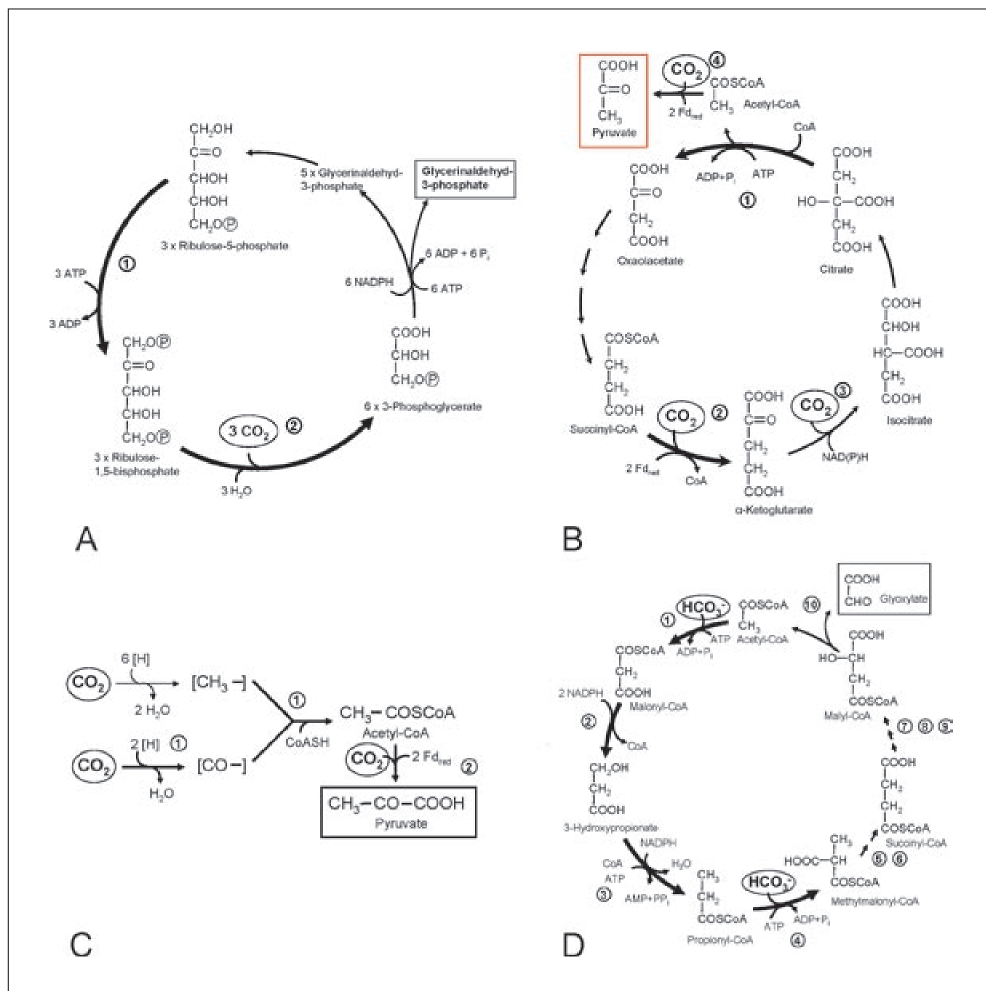

Fig. 1 (*A*)–(*D*) The four known pathways for autotropic CO_2 fixation. The reactions catalyzed by key enzymes are indicated by bold arrows. (*A*) Calvin-Bassham-Benson cycle (Calvin cycle); (1) ribulose 5-phosphate kinase (phosphoribulokinase) and (2) ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO). (*B*) Reductive citric acid cycle; (1) ATP citrate lyase, (2) α-ketoglutarate : ferredoxin oxidoreductase, (3) isocitrate dehydrogenase, and (4) Fd_{red} = reduced ferredoxin. (*C*) Reductive acetyl-CoA pathway; (1) CO dehydrogenase/acetyl-CoA synthase and (2) pyruvate : ferredoxin oxidoreductase. (*D*) 3-Hydroxypropionate cycle in *Chloroflexus aurantiacus*; (1) acetyl-CoA carboxylase, (2) malonyl-CoA reductase, (3) propionyl-CoA synthase, (4) propionyl-CoA carboxylase, (5) methylmalonyl-CoA epimerase, (6) methylmalonyl-CoA mutase, (7) succinyl-CoA:L-malate CoA transferase, (8) succinate dehydrogenase, (9) fumarate hydratase, and (10) L-malyl-CoA lyase.

use of the reverse reactions for the complete oxidation of organic compounds; and the evolutionary origin of the machinery and the distance to the presumed chemoevolutionary scenario. The pathways also differ in $^{12}C/^{13}C$ isotope discrimination, a fact that has been neglected for a long time and which has an impact on the interpretation of carbon isotope fractionation data and geological records. The distribution of the individual CO_2 fixation pathways among prokaryotes reflects their characteristic features.

2. The Calvin Cycle

This cyclic pathway was discovered by the group of Melvin Calvin and represents the common autotrophic pathway not only in chloroplasts, but also in most aerobic bacteria (Morikawa et al. 1994, Schauder et al. 1987). It is centered around carbohydrates, with ribulose-1,5-bisphosphate being the CO_2-accepting molecule. This molecule reacts in its endiol form with CO_2 (or oxygen, see below) (Spreitzer and Salvucci 2002). Basically, only two key enzymes other than common enzymes of gluconeogenesis and sugar-phosphate interconversion are required: ribulose-1,5-bisphosphate carboxylase (RubisCO), and the phosphoribulokinase that converts ribulose-5-phosphate into the starting molecule ribulose-1,5-bisphosphate.

The Calvin cycle has a high ATP demand; nine ATP are required to form one triose-phosphate molecule from three molecules of CO_2. The RubisCO enzymes are rather poor catalysts, with high K_m values especially for CO_2, low turnover numbers, and an oxygenase side activity that results in futile cleavage of the acceptor molecule to form phosphoglycolate as a side product. The enzymes of the Calvin cycle are oxygen insensitive, they are easy to control because the whole pathway is separated from most of the central metabolism of the cell, and sugar phosphates are common metabolites.

RubisCO might have been derived from a RubisCO-like enzyme that plays a role in a methionine salvage pathway in some bacteria (an enolase catalyzing the 2,3-diketo-5-methylthiopentyl-1-phosphate enolase reaction) (Ashida et al. 2003, Hanson and Tabita 2001). RubisCO enzymes form three families. The enzyme comprising the third family is exclusively found in archaea, and its role in archaeal carbon metabolism is at issue (Atomi et al. 2001, Atomi 2002, Ezaki et al. 1999, Kitano et al. 2001, Maeda et al. 1999, 2002, Morikawa et al. 1994, Watson et al. 1999). The archaeal RubisCO functions in bacterial mutants in which RubisCO is knocked out (Yoshida et al. 2006). Yet, phosphoribulose kinase has not been detected in archaea, and hence, the cycle cannot yet be closed. Notably, RubisCO is found in archaea that were not reported to be able to grow autotrophically. Conversely, RubisCO has also been found in archaea that grow autotrophically but that are considered to use the reductive acetyl-CoA pathway for CO_2 fixation. Hence, these prokaryotes contain at the same time RubisCO activity and key enzyme activities of the reductive acetyl-CoA pathway.

Despite the high energy requirement and the apparently imperfect carboxylase, the Calvin cycle dominates autotrophic carbon fixation in aerobic bacteria. Its success is not obvious if the adverse characteristics of one of its key enzymes, RubisCO, is considered. The cycle is very likely a latecomer in the metabolic scene, which was already settled before the oxygen concentration in the atmosphere increased to higher values.

3. The Reductive Citric Acid Cycle

This pathway was proposed by Arnon, Buchanan, and others as the carbon fixation pathway in *Chlorobium* sp., and it was not accepted by the scientific community for many years (Buchanan and Arnon 1990, Evans et al. 1966, Fuchs 1989, 1990, 1994). The cyclic pathway reverses the reactions of the citric acid cycle and forms acetyl-CoA from two CO_2. Three modifications of the conventional oxidative citric acid cycle are needed that

substitute irreversible enzyme steps. Succinate dehydrogenase is replaced by fumarate reductase, 2-oxoglutarate dehydrogenase by the ferredoxin-dependent 2-oxoglutarate oxidoreductase, and citrate synthase by ATP citrate lyase (Antranikian et al. 1982, Ivanovsky et al. 1980). There are variants of the ATP-driven cleavage of citrate and variants of isocitrate dehydrogenase. The pathway requires five ATP to form triosephosphates from three CO_2 and depends on oxygen-sensitive, ferredoxin-specific enzymes. Furthermore, the pathway may be used in the reversed oxidative direction for the oxidation of acetyl-CoA in one and the same organism. The use of the ATP citrate lyase reaction, i. e., acetyl-CoA + oxaloacetate + ADP + $P_i \rightarrow$ citrate + CoA + ATP, instead of the irreversible citrate synthase reaction, allows a second molecule of ATP to be generated in the cycle in addition to ATP synthesis via succinate thiokinase.

The reductive citric acid cycle pathway is found in anaerobic as well as microaerobic bacteria and archaea, such as anaerobic sulfate reducers (e. g., *Desulfobacter* sp., Deltaproteobacteria), microaerobic sulfur oxidizers (Epsilonproteobacteria), anaerobic phototrophic green sulfur bacteria (*Chlorobium*), and hydrogen-oxidizing microaerobic Aquificales (*Aquifex*, *Hydrogenobacter*). The low ATP requirement as well as the moderate oxygen sensitivity of some of its enzymes makes the reductive citric acid cycle a suitable pathway for anaerobes as well as microaerobes. The assimilation of acetyl-CoA via reductive carboxylation to pyruvate (by pyruvate : ferredoxin oxidoreductase) is common in anaerobes and applies also to the next pathway (Beh et al. 1993, Brysch et al. 1987, Fuchs et al. 1980, Schauder et al. 1987, Shiba et al. 1985).

4. The Reductive Acetyl-CoA Pathway

This is a non-cyclic pathway that also results in the fixation of two molecules of CO_2 to form acetyl-CoA. It was elucidated by Wood, Ljungdahl, Thauer, and others as a pathway used by acetogenic bacteria to synthesize acetate from CO_2 in their energy metabolism (Länge et al. 1989, Ljundahl et al. 1965, Ljundahl 1986, Ragsdale 1991, Thauer et al. 1989). One molecule of CO_2 is reduced to the level of methyltetrahydropterin. Another molecule of CO_2 is reduced to the level of carbon monoxide, catalyzed by the nickel enzyme carbon monoxide dehydrogenase. This enzyme also acts as acetyl-CoA synthase (Ragsdale 1991, Wood 1991). It accepts the methyl group from methyltetrahydropterin, which is transferred via a corrinoid protein, combines it with the carbon monoxide group to an enzyme-bound acetyl group, and releases this group thiolytically with coenzyme A to form acetyl-CoA. The key enzyme CO dehydrogenase/acetyl-CoA synthase is homologous in all prokaryotes, in contrast to the enzymes involved in the formation of methyltetrahydropterin from CO_2. There are many variants of the pathway, and they differ in the use of coenzymes, electron carriers, etc. The pathway can also be reversed and used for the oxidation of acetyl-CoA, instead of the citric acid cycle (Thauer et al. 1989).

The reductive acetyl-CoA pathway is unique in several aspects. The pathway makes extensive use of coenzymes (tetrahydropterin, cobalamin) and of metals (Mo or W, Co, Ni, Fe, Fe-S centers). It enables also CO, formaldehyde, methanol, methylamine, or methylmercaptan to be assimilated. Many of the one-carbon units react spontaneously with the cofactors of this pathway. This pathway depends on anoxic conditions since some of its

enzymes, notably CO dehydrogenase/acetyl-CoA synthase, are oxygen sensitive. It requires the above-mentioned metals, which are water soluble preferentially in the reduced oxidation state, i.e., under anoxic conditions. The process can be simulated in the laboratory to make not only acetylthioesters, but also derived products by simply incubating CO, H_2, and H_2S or methylmercaptan with Ni and Fe salts; these inorganic metals form mixed Ni-Fe sulfides that act as catalysts (Wächtershäuser 2007). Among the CO_2 fixation pathways, the reductive acetyl-CoA pathway has the lowest energetic costs, requiring only four ATP per triosephosphate.

The reductive acetyl-CoA pathway is the favored autotrophic pathway in strict anaerobes. Notably, it is found in those bacteria that encounter traces of the above-mentioned gases and one-carbon compounds in their environment. The pathway functions not only in carbon fixation; but can be also reversed to oxidize acetyl-CoA to two CO_2 in the energy metabolism. Acetyl-CoA is assimilated into cell material as described for the reductive citric acid cycle and can also serve as an energy source for ATP synthesis during its conversion to acetate. This pathway is found in acetogenic bacteria and methanogenic archaea, in sulfate-reducing bacteria (*Desulfobacterium* sp. [Deltaproteobacteria], *Desulfotomaculum* sp. [relatives of *Clostridia*], and *Archaeoglobus* [Euryarchaeota]) as well as in anaerobic ammonium-oxidizing bacteria (White 1995). It very likely represents an archaic type of metabolism that may be similar to the primordial carbon fixation pathway.

5. The 3-Hydroxypropionate Cycle

The pathway results in the fixation of three molecules of bicarbonate and forms pyruvate as the central carbon precursor molecule. The main CO_2-fixing enzyme is acetyl-CoA/propionyl-CoA carboxylase. The pathway can be divided into two metabolic cycles.

In the first cycle, acetyl-CoA is carboxylated to malonyl-CoA, which is subsequently reduced and converted to propionyl-CoA, via 3-hydroxypropionate as a free intermediate. Propionyl-CoA is carboxylated to methylmalonyl-CoA, which is subsequently converted to succinyl-CoA. Succinyl-CoA is used to activate L-malate by succinyl-CoA: L-malate coenzyme A transferase, which forms L-malyl-CoA and succinate. Succinate is oxidized to L-malate via conventional steps. L-Malyl-CoA is cleaved by L-malyl-CoA/β-methylmalyl-CoA lyase, thus regenerating the starting molecule acetyl-CoA and releasing glyoxylate as the first fixation product (Alber and Fuchs 2002, Castenholz 1969, Eggerer and Grünewälder 1964, Eisenreich et al. 1993, Friedmann et al. 2006, Gogos et al. 2004, Heider 2001, Herter et al. 2002).

Glyoxylate is an unconventional cell carbon precursor, and special enzymes are required for it to be used in biosynthesis. A second cycle was proposed to serve as the glyoxylate assimilation pathway. Glyoxylate is combined with propionyl-CoA to β-methylmalyl-CoA, catalyzed by malyl-CoA/β-methylmalyl-CoA lyase. This promiscuous enzyme not only cleaves L-malyl-CoA into acetyl-CoA and glyoxylate, but it also synthesizes β-methylmalyl-CoA from glyoxylate and propionyl-CoA. β-Methylmalyl-CoA is converted via mesaconyl-CoA to citramalyl-CoA, which is cleaved into pyruvate and acetyl-CoA; however, details of this process are not yet known. Acetyl-CoA conversion to propionyl-CoA occurs as described above.

This pathway has to date only been observed in the phototrophic bacterium *Chloroflexus aurantiacus*, a member of the green non-sulfur bacteria. A variant of this pathway appears to operate in the Sulfolobales of the Crenarchaeota (*Sulfolobus*, *Acidianus*, *Metallosphaera* sp.) (Hügler et al. 2003). This pathway allows mixotrophic growth by assimilating fermentation products such as acetate, propionate, or succinate. Its energy costs are high, ten ATP per triosephosphate. Bicarbonate rather than CO_2 is the actual inorganic carbon species used. The acetyl-CoA carboxylase reaction is virtually irreversible, and the enzyme has a high affinity for bicarbonate. This complex pathway requires various new enzymes. It is suitable for bacteria living in habitats where fermentation product may occur.

6. Autotrophic Pathways in Crenarchaea

In Crenarchaea two new autotrophic pathways have been found. The aerobic Sulfolobales and related Archaea use a 3-hydroxypropionate/4-hydroxybutyrate cycle (Berg et al. 2007). The anaerobic *Ignicoccus* sp. and probably also the Thermoproteales use a dicarboxylate/4-hydroxybutyrate cycle (Huber et al. 2008).

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Of Methanotrophic and Methanogenic Archaea

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Member of the Presidium of the German Academy of Sciences Leopoldina

Abstract

Methanogenic and methanotrophic archaea are phylogenetically relatively closely related. Both groups of anaerobic microorganisms, as far as investigated, contain high concentrations of the nickel enzyme methyl-coenzyme M reductase. In methanogenic archaea, this enzyme catalyzes the actual methane-forming step. It is therefore assumed that the nickel enzyme is also involved in the anaerobic oxidation of methane in methanotrophic archaea.

Zusammenfassung

Methanogene und methanotrophe Archaeen sind phylogenetisch relativ nahe verwandt. Beide Gruppen von anaeroben Mikroorganismen enthalten, so weit untersucht, hohe Konzentrationen des Nickel-Enzyms Methyl-Coenzym-M-Reductase, die in methanogenen Archaea den eigentlich Methan-bildenden Schritt katalysiert. Es wird daher angenommen, dass das Nickel-Enzym auch an der anaeroben Oxidation von Methan beteiligt ist.

Methanotrophic and methanogenic archaea, both belong to the kingdom of euryarchaeota. Within this kingdom, the methanotrophic archaea are most closely related to the methanogenic *Methanosarcinales* and to the sulfate-reducing *Archaeoglobales* (Boetius et al. 2000, Orphan et al. 2002, Meyerdierks et al. 2005, Ciccarelli et al. 2006). Methanotrophic archaea and methanogenic archaea are both strictly anaerobic microorganisms that play key roles in the carbon cycle. About 1 % of the carbon dioxide fixed annually by photosynthesis is converted back to carbon dioxide by microorganisms via methane, which amounts to 1 billion tons of methane formed and consumed per year (Thauer 1998).

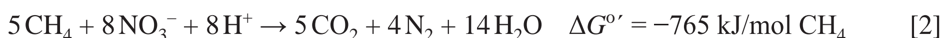
In marine anaerobic sediments, large amounts of methane are oxidized to CO_2 with sulfate as electron acceptor, which is reduced to H_2S (for a review, see Valentine and Reeburgh 2000):

$$CH_4 + SO_4^{2-} + H^+ \rightarrow CO_2 + HS^- + 2\,H_2O \quad \Delta G^{o\prime} = -21 \text{ kJ/mol } CH_4 \qquad [1]$$

Sulfate-dependent anaerobic oxidation of methane is mediated by a consortium of methanotrophic archaea (>50 %) and sulfate-reducing Proteobacteria of the genus *Desulfo-*

sarcina (>20 %) (Krüger et al. 2003). The role of the sulfate-reducing bacteria in the consortium is not yet clear. In one hypothesis, it is assumed that the methanotrophic archaea themselves are incapable of dissimilatory sulfate-reduction and therefore are obligately dependent on the sulfate-reducing bacteria for growth. In favor of this hypothesis is the formation of tightly associated aggregates of methanotrophic archaea and sulfate-reducing bacteria in microbial mats (Boetius et al. 2000). However, all attempts to find an intermediate connecting methane oxidation in the archaea with sulfate reduction in the bacteria have failed. At least in one instance, H_2, formate, methanol, and acetate were excluded as possible intermediates (Nauhaus et al. 2002, 2005). In another hypothesis, it is assumed that the methanotrophic archaea, like the related *Archaeoglobus* species, are capable of dissimilatory sulfate reduction and that the sulfate-reducing bacteria are merely present in the consortium because they feed on the extended glycocalyx of the methanotrophic archaea. Such a complex carbohydrate diet would explain why it has not yet been possible to grow the sulfate-reducing bacteria in the absence of the methanotrophic archaea. Analyses of the metagenome of the consortium have also failed to solve this problem (Hallam et al. 2004).

In freshwater anaerobic sediments, in the nitrate zone, methane is oxidized to CO_2 with nitrate (or nitrite) as electron acceptors, and the nitrogen oxides are reduced to N_2 (Raghoebarsing et al. 2006):



$$5\,CH_4 + 8\,NO_3^- + 8\,H^+ \rightarrow 5\,CO_2 + 4\,N_2 + 14\,H_2O \quad \Delta G^{o\prime} = -765 \text{ kJ/mol } CH_4 \qquad [2]$$

Anaerobic oxidation of methane (AOM) coupled to denitrification supports the growth of a consortium consisting of approximately 10 % archaea and 80 % bacteria. Sequence analysis of the bacterial clone library revealed that the dominant bacterium belongs to a novel phylum without any documented cultured species and that the archaeon is distantly related to archaea involved in AOM with sulfate (Raghoebarsing et al. 2006). The free energy change ($\Delta G^{o\prime}$ = −765 kJ/mol methane) associated with nitrate-dependent AOM is much larger than that associated with sulfate-dependent AOM ($\Delta G^{o\prime}$ = −21 kJ/mol methane). The two processes also differ in their kinetics. AOM with sulfate has been reported by Krüger et al. (2003) to proceed at a specific rate of only 0.1 nmol min^{-1} (mg protein)$^{-1}$, whereas AOM with nitrate is described by Raghoebarsing et al. (2006) as proceeding at a specific rate of 2 nmol min^{-1} (mg protein)$^{-1}$. The apparent K_m for methane is >10 mM in sulfate-dependent AOM and <1 µM in nitrate-dependent AOM (Raghoebarsing et al. 2006).

For the sulfate-dependent process, there is circumstantial evidence that a nickel enzyme, methyl-coenzyme M reductase, catalyzes the first step of methane oxidation (Krüger et al. 2003, Hallam et al. 2003). The main evidence is that the methane-consuming archaea involved, which are related to methane-producing archaea, contain high concentrations of the enzyme; this enzyme in the latter archaea catalyzes the actual methane-forming step, the reduction of methyl-coenzyme M (CH_3-S-CoM) with coenzyme B (HS-CoB) to form methane and the heterodisulfide CoM-S-S-CoB (Thauer 1998). The free energy change ($\Delta G^{o\prime}$) associated with this reaction is estimated to be –30 kJ/mol, which indicates that the enzyme will catalyze the back reaction, the oxidation of methane, with a catalytic efficiency (k_{cat}/K_m) of only approximately 0.001 % of the forward reaction (Shima and Thauer 2005). This might be just sufficient to account for the observed rates of sulfate-dependent

anaerobic oxidation of methane in microbial mats. But it is hardly consistent with the much higher specific rates and the much lower apparent K_m for methane of the nitrate-dependent process (Thauer and Shima 2007).

It is not yet known whether the archaea present in the consortium mediating AOM with nitrate contain methyl-coenzyme M reductase.

For details on various aspects of these processes, the reader is referred to recent reviews of the author (Shima and Thauer 2005, Thauer and Shima 2006, 2007, Jaun and Thauer 2007).

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On the Emergence and Early Evolution of Life

Michael J. Russell (Pasadena)

With 3 Figures and 1 Table

Abstract

Life emerged at an alkaline, formate-bearing submarine seepage. Here hydrothermal H_2 and NH_3 converged to react further with the atmospheric CO_2 dissolved in the 4 billion year old mildly acidic ocean. The "ready-made" catalysts for their interactions were greigite clusters ($NiFe_5S_8$), which also contributed to the semi-permeable compartments comprising the hydrothermal precipitates. Life then colonized ocean-floor sediments adjacent to the mound. From there it was conveyed by ocean-floor spreading to an island chain where a portion of its host sediments were thrust up into the photic zone. Here, at the Earth's surface, some bacteria became adapted to photosynthesis and a few later evolved a reaction centre to co-opt another mineral cluster, probably a tunnel manganite ($CaMn_4O_8$), to gain 4 e^- (and 4 H^+) from 2 H_2O for biosynthesis, releasing O_2 as waste product.

Zusammenfassung

Das Leben entstand an alkalischen, Formiat enthaltenden Quellen auf dem Meeresgrund. Hier kamen H_2 und NH_3 hydrothermalen Ursprungs zusammen und reagierten mit atmosphärischem CO_2, das vor vier Milliarden Jahren in einem säuerlichen Ozean gelöst war. Die „gebrauchsfertigen" Katalysatoren für die Reaktionen waren Greigit-Cluster ($NiFe_5S_8$), die auch Bestandteil der semipermeablen, aus hydrothermalen Präzipitaten gebildeten Kompartimente waren. Das entstandene Leben besiedelte dann das Meeressediment rings um die Austrittsstellen und verbreitete sich im Zuge tektonischer Ausdehnungen auf Inselketten. Aus dem besiedelten Sediment herausgelöste Teile gelangten in die photische Zone, wo einige Bakterien die anoxygene Photosynthese entwickelten und wenige davon später ein anderes Mineral-Cluster, ein Tunnel-Manganit ($CaMn_4O_8$), aufnahmen, um für die Biosynthese 4 e^- (und 4 H^+) aus 2 H_2O zu erhalten, was mit der Freisetzung von O_2 als Abfallprodukt verbunden war.

Following the formation of the Earth by planetesimal accretion, the gravitational, latent and radioactive heat so concentrated was dissipated to the cold sink of deep space by a series of convection cells that operated successively in the core, mantle, lithosphere, hydrosphere and atmosphere. While convection was successful in dissipating the physical energy from our planet, it focused strong chemical and electrochemical disequilibria wherever crustal hydrothermal convection cells met cool ocean waters (Russell and Hall 1997; Fig. 1). Indeed, life emerged as the chemical vortex through which this energy was dissipated to ocean and atmosphere, first as acetate, then, when life had found a way to obviate the higher kinetic barrier, to methane (Martin and Russell 2003, 2007). It

emerged where chemical and electro-chemical gradients were at their steepest. The acetate and methane, along with water, comprised the exhaust from energy-providing and carbon-fixing reactions, essentially between H_2 and CO_2 (as HCO_3^-) plus formate ($HCOO^-$) that were catalyzed within the hydrothermal precipitates, specifically at iron-nickel sulfides (Tab. 1, Fig. 1). The H_2 was generated from seawater as it coursed through, and oxidized, the ferrous iron-rich oceanic crust in a series of long-lived (>10^4 a) hydrothermal convection cells (RUSSELL and HALL 1997, MCCOLLOM and SEEWALD 2001). The convection cells were buffered by $Mg(OH)_2$ at pH 9–12 and perhaps rheologically thermostated at ~120 °C, though higher temperatures have been suggested at the base of such convection cells (LO-

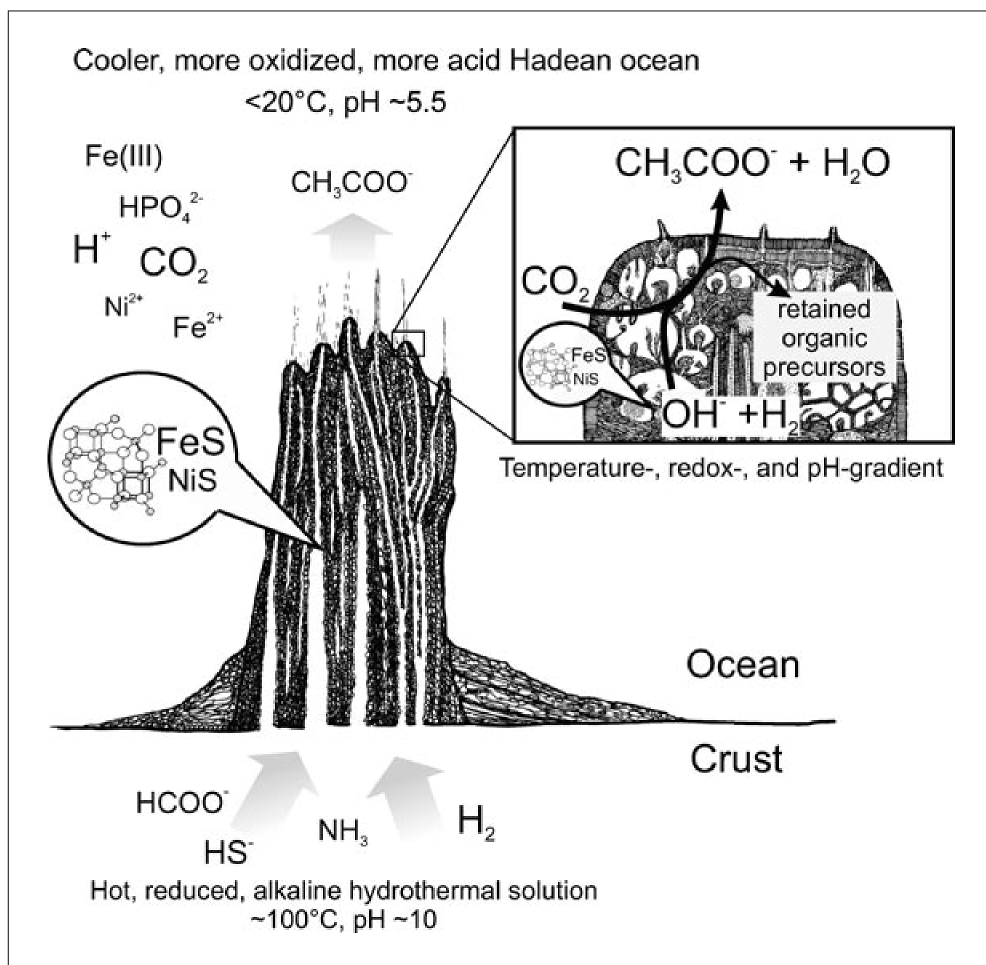

Fig. 1 The hydrothermal mound depicted as an acetate generator and hatchery for emergent life. pH ranges from ≥9 within the mound to ~6 at its periphery (from RUSSELL and MARTIN 2004, with permission). The detailed cross-section of the mound's exterior illustrates the sites where organic ions are produced, retained, react and self-organize to emerge as protolife. The main reactants and intermediaries delivered to the mound are: hydrothermal H_2 (≤15 mmol l^{-1}), oceanic CO_2 (~1 bar and hydroxylated to the bicarbonate between pH 6.6 and 10) and formate (>5 mmol l^{-1}) (MCCOLLOM and SEEWALD 2003, KELLEY et al. 2005).

WELL and RONA 2002, KELLEY et al. 2005, RUSSELL and HALL 2006). In these conditions a large fraction of the HCO_3^- entrained in the hydrothermal solution would be reduced by H_2 to $HCOO^-$ (HAGGERTY 1991, MCCOLLOM and SEEWALD 2001, 2003, SEEWALD et al. 2006). The original sources of the CO_2 dissolved in the ocean (pH ~5.5) were very high temperature volcanic exhalations (which included CO − also to be photo-oxidized to CO_2, to total at least one atmosphere) (KASTING et al. 1993; Fig. 1). The initial pathways to acetate and methane were via the two branches of an antecedent acetyl-CoA pathway, probably beginning at formate provided by the alkaline spring waters (FUCHS 1989, FUCHS and KOCKELKORN in this volume, THAUER 1998, THAUER in this volume, RAGSDALE 2004, MARTIN and RUSSELL 2007). To this day formate is provided in hot springs and metabolized by thermophilic and hyperthermophilic chemolithotrophic bacteria, e. g., *Carboxydothermus hydrogenoformans* (HENSTRA and STAMS 2004) and *Thermocrinus rubber* (JAHNKE et al. 2001).

Tab. 1 "Ready-made" inorganic redox catalysts and comparisons with the active centres of enzymes

Cluster	Mineral Equivalent	cf., Enzyme	Function
$[Fe_2S_2]$	Mackinawite (Fe >> Ni, Co)S	(Rieske protein) hydrogenase	e^- transfer and hydrogenase activity
2 $[Fe_2S_2]$ → $[Fe_4S_4]$	to cuboidal moiety in greigite (Fe_6S_8)	Ferredoxins $[Fe_4S_4]^{0/+}$, $[Fe_3S_4]^{+/2+}$	e^- capacitor, e^- transfer and hydrogenase activity $H_2 + \rightarrow 2\,e^- + 2\,H^+$
$NiFe_5S_8$	in greigite as $NiS_2[Fe_4S_4]S_2Fe$ $[Fe_4S_4]$cys-Ni-cys$_2$-Ni	CODH/ACS $NiFe_4S_5$ &	CH_3-corrin + CO + HSCoA → $CH_3CO(SCoA)$ + corrin
$[Fe_4S_4]$ + $[MoFe_3S_4]$	Greigite cubane twin as $[Fe_4S_3]S_4[S_3Fe_4]$	Nitrogenase $[Fe_4S_3]XS_3[S_3Fe_3Mo]$	$N_2 + 8e^- + 16\,MgATP^{2-} + 8\,H^+ \rightarrow$ $2\,NH_3 + H_2 + 16\,MgADP^- + 16\,P_i$
(MnO_4^{4-})	As tunnel manganite $CaMn_4O_8$	OEC $CaMn_4O_x \pm 2H_2O$	$2\,H_2O$ + light $\rightarrow 4\,H^+ + 4\,e^- + O_2$

That the pathways probably began at formate may explain why, to date, reactions to produce acetate and methane have defied abiotic experimental reproduction directly from CO_2 in the laboratory. However, CO_2 (as electron acceptor) has been reduced to methyl sulfide with H_2S while FeS was oxidized to pyrite, though in acidic rather than the alkaline conditions we favour (HEINEN and LAUWERS 1996). And using methyl sulfide, HUBER and WÄCHTERSHÄUSER (1997) have demonstrated the synthesis, catalyzed by NiS, of an activated thioester (CH_3–CO–S–CH_3) from CO (presumably hydrated to $HCOO^-$ at the optimal pH of 8−10). Whether the CO_2 (or HCO_3^-) in the ocean-water permeating the mound would have been reduced is not certain. In the presence of FeS it would certainly have been rapidly converted to bicarbonate in a carbonic-anhydrase-like reaction ($CO_2 + OH^- \rightarrow HCO_3^-$; LUTHER 2004). High-pressure reactor experiments are called for to assess the likely relative contributions of HCO_3^- and $HCOO^-$ to the synthesis of the methyl, carbonyl as well as other carboxyl groups.

The mineral precipitates to be expected at a warm spring that might have fabricated life's hatchery are more predictable and have been reproduced in the laboratory (RUSSELL and HALL 1997, 2006, RICKARD et al. 2001). The metals Fe, Mn, Zn, Co, and Ni (along

with phosphate) comprising these minerals had been conveyed to the mildly acidic Hadean ocean in high temperature (~400 °C) "black smoker" springs operating at ocean floor spreading centers, above mantle plumes and at subduction zones. The marine metals reacted with sulfide (as HS^-) and hydroxide at the cooler submarine alkaline springs and seepages (also bearing traces of $MoO_xS_{4-x}{}^{n-}$ and $WO_xS_{4-x}{}^{n-}$) to precipitate a porous mound of oxyhydroxides, sulfides and ephemeral carbonates. Of the sulfides, mackinawite (as $[Fe_2S_2]_n$) and greigite (as $\{[SFeS][Fe_4S_4][SNiS]\}_n$) comprised semi-permeable compartments within the mound (Fig. 1, 2*A*). Greigite consists of Ni and Fe atoms tetrahedrally coordinated to sulfur as well as $[Fe_4S_4]$ cubanes − a complex structure with strong affinities to the active centers of the bifunctional enzyme, carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH—ACS) (Tab. 1, Fig. 2 *C*−*E*). Electrons are delocalized in these clusters, and may be lost or gained with minor flexure but with no change of conformation, both factors that encourage redox catalysis (Fig. 2*B*) (Hall et al. 1974). Energy for biosynthesis was provided through acetyl phosphate, synthesized via phosphorolysis of acetyl thioesters (de Duve 1991, de Zwart et al. 2004). Reductive aminations of α-ketocarboxylic acids, as well as the peptide bonding of the residues, are optimized at the second pK (formation of $\alpha{-}NH_3$) of amino acids obtaining at pH ~9–9.7, as are phosphorylations (Huber and Wächtershäuser 2003, Huber et al. 2003, Russell and Hall 1997). The alkaline conditions at the off-ridge hydrothermal mound are appropriate for such chemistries. Short heterochiral pep-

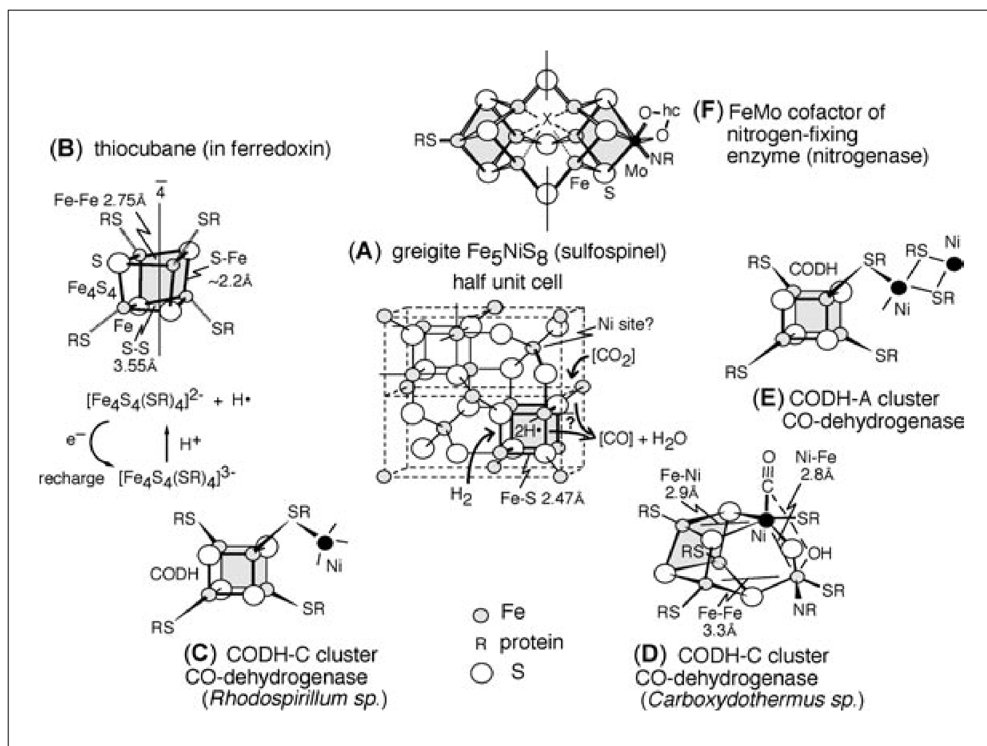

Fig. 2 Structural relatedness of: (*A*) greigite Fe_5NiS_8; (*B*) the thiocubane $[Fe_4S_4]$ unit in protoferredoxins and ferredoxins; (*C*), (*D*) and (*E*) the $[Fe_4NiS_5]$ open cuboidal complexes in CO-dehydrogenases; (*F*) the twinned center to nitrogenase (after Russell and Hall 2006).

tides condensed from simple amino acids were, by dint of their positively charged backbone, liable to sequester acetyl phosphate and the metal sulfide clusters in nests, so preventing their dissolution or, in the case of the sulfides, their crystallization. By stabilizing the reduced, somewhat more than the oxidized, forms of iron(nickel)-sulfur centers, nests are expected to increase their reduction potential (i.e., lower their effective E_h) (Milner-White and Russell 2005). Longer peptides could have contributed to a proteinaceous membrane that replaced its mineral precursor while still controlling the anions, and thereby the distribution of electrons and protons through the membrane, so enhancing metabolism.

Amino acids also contributed to the synthesis of a limited number of conjugated stable cyclic and heterocyclic organic molecules, rich in highly delocalizable π-electrons, which acted as coenzymes and catalyzed a wide variety of reactions through electron and group transfer (Pullman 1972). Martin and Russell (2007) argue that pterins were the first of such functional molecules and that they displaced sulfide in the methyl-generating branch of the Wood-Ljungdahl pathway. RNA-cofactors became involved as acetogens and methanogens eventually managed to reduce bicarbonate in this branch, energized by ATP (first formed by substrate level phosphorylation, though this was replaced by chemiosmosis at an early stage of evolution) or directly by the *pmf* in the case of the methanogens. Such energies are a feature of our scenario where an alkaline hydrothermal fluid interfaces a mildly acidic ocean, and could be exploited as soon as an ATPase was invented (Martin and Russell 2007).

The heterocyclic catalysts eventually developed to a degree where polymers comprising a variety of sequences of the energetically stable AMP and GMP, and the rather less stable CMP and UMP, emerged. Based on these associations, populations of virus-like RNA molecules and combinations of self-replicating molecular cooperatives could range through the complex of mineral compartments comprising the mound (Koonin and Martin 2005). These RNAs could replicate and eventually synthesize and encode short proteins and thereby improve the functions of proteinaceous membranes.

While still in the mound the last universal common ancestor differentiated on the basis of metabolism, the acetogens to become the bacteria and the methanogens the archaea (Fig. 3). The two prokaryotic domains expanded into the contiguous ocean-floor sediments and crust to constitute a deep biosphere protected from ocean-vaporizing giant meteorite impacts (Fig. 3; Parkes in this volume). Nitrogen, supplied first as hydrothermal NH_3, now required a nitrogenase with active centers developed along the lines of a "siamese twin" of a greigite cubane moiety, though with one distal Mo atom (Fig. 2*F*; Tab. 1).

The deep biosphere was conveyed by ocean floor spreading toward an island chain where a portion of its host sediments was thrust up into the photic zone (Fig. 3). Though most bacteria would have succumbed to high-energy photons, some happened to invade littoral precipitates at sulfurous springs. Shielded from UV radiation by a mineral cover, these bacteria evolved and assembled light-harvesting antennae from radio- and photo-resistant macrocyclic pyrrole rings (easily raised to excited states) that were first developed for group and electron transfer, perhaps by exchanging Mg for a transition metal. Photochemical reaction centers (RCs) coevolved − exploiting low energy solar photons, oxidizing H_2S to sulfur, and gaining electrons for biosynthesis. As the H_2S supply waxed and waned an alternative reaction complex (RC2) evolved from the ancestral complex (RC1) that could oxidize organic detritus ('$CH_2O_{detritus}$') from dead cells at a higher redox potential. According to Allen (2005) such a photosynthetic bacterium could switch between using RC1 and RC2, depending upon prevailing redox conditions. A manganiferous UV shield to these bacteria,

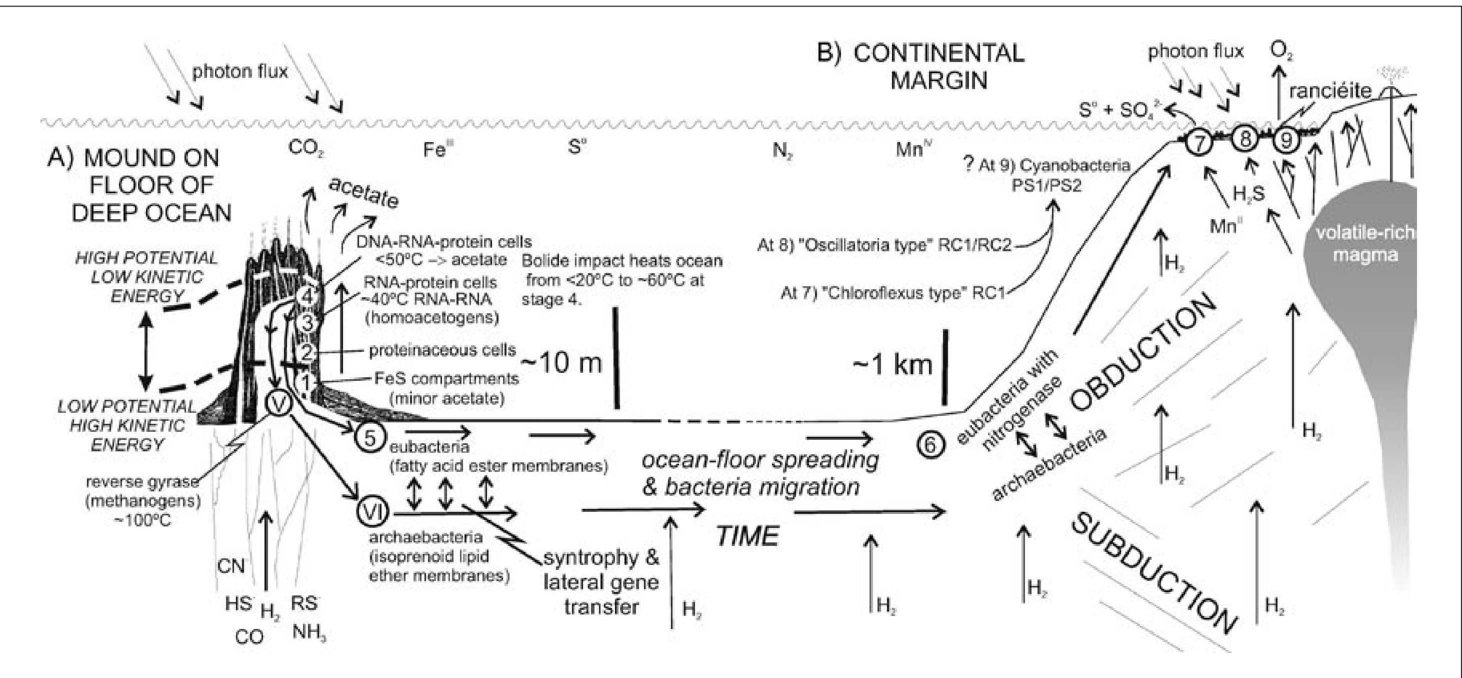

Fig. 3 Chemosynthetic life emerges at a warm alkaline seepage and at (*A*), differentiates into the precursors of the bacteria and archaea (Martin and Russell 2003), and colonizes the surrounding sediments and crust. Prokaryotes are conveyed, by ocean floor spreading, toward a constructive margin (*B*) produced by obduction. Once thrust up at the margin some of the cells happen to invade sediments in the photic zone where, at a sulfurous spring, some evolve to exploit solar photons. Numbers 1–3 show life's emergence, 4 marks the point of differentiation of the archaea from the bacteria. Roman numerals V–VI mark evolutionary stages of the archaea, and 5 and 6 indicate stages of evolution of the bacteria in the deep biosphere. Photon energy was mastered first by the green bacteria (cf. *Chloroflexus*) (7), perhaps followed at some remove by a precursor to *Oscillatoria* (Allen 2005) (8). These photosynthesizing bacteria had probably appeared by the early Archaean. Oxygenic photosynthesis (9) is a further development which may have evolved at a manganiferous hot spring, perhaps by 3.75 Ga (after Russell and Arndt 2005). Figure includes various scales.

comprised, in part, of a tunnel manganite ($CaMn_4O_8$) (cf. BARRIER et al. 2005), may have oxidized a small proportion of the ambient water to O_2 (Tab. 1) (RUSSELL and ARNDT 2005). Cooption (and eventual invagination) of such a "ready-made" $CaMn_4O_8$ cluster by RC2 produced a photosystem (PS2) that gained 4 electrons (and 4 protons) from two water molecules (2 H_2O in place of '$CH_2O_{detritus}$'). At the same time, a simple mutation disabled the redox switch and allowed RC1 (as PS1) to accept electrons directly from PS2 instead of H_2S (Fig. 3; ALLEN 2005). The two newly co-existing photosystems, by sharing electron carriers, thus became connected. These allowed the generation of electrochemical potential high enough to diurnally oxidize H_2O for biosynthesis (ALLEN 2005).

In sum, convection currents in various guises not only pumped materials and chemical energy to emergent and burgeoning life, but also were to convey life itself to further nutrients and chemical and photochemical energies. Yet for chemosynthesis and oxygenic photosynthesis to emerge at all, minerals and mineral sulfide and oxide clusters were co-opted to act as host, hatchery, catalyst, shelter and also as sources for the active centers of the precursors to the metalloenzymes.

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On the Ancestral State of Microbial Physiology

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Abstract

Most thinking on early evolution and the origin of life is devoted to aspects of chemical synthesis, organization, information, replication, and the like. But life is a redox reaction. In all living systems, there is a main chemical reaction at the core of biochemistry, the redox potential of which is harnessed as chemical energy that allows a myriad of little side reactions that assemble the substance of life to go forward. At the origin of life there also must have been a main chemical reaction and some smaller side reactions. In the absence of evidence to the contrary, it seems reasonable to assume that life has not forgotten how it arose, that is, the trace of biochemical origins should probably be preserved somewhere in the metabolism of modern organisms – whatever worked then, should still work now. The reduction of CO_2 by H_2 to yield compounds such as acetate and methane seems to be a reasonable candidate for such a main initial reaction. If so, acetogenesis and methanogenesis might be the ancestral state of microbial physiology among the eubacteria and archaebacteria, respectively. But both acetogenesis and methanogenesis operate today only with the help of chemiosmosis, indicating the antiquity of chemiosmotic energy harnessing mechanisms. Prior to the advent of proteinaceous chemoisomotic coupling mechanisms, phosphorolysis of acetyl thioesters could have generated acetyl phosphate as the ancestral energy currency. Life might have grown up on acetyl phosphate and been weaned on compounds like CO or methyl sulfide present in hydrothermal fluid by virtue of serpentinization processes in the Earth's crust, before it learned to walk all by itself on H_2 and CO_2 alone.

Zusammenfassung

Wer über frühe Evolution und über den Ursprung des Lebens nachdenkt, hantiert in der Regel mit Begriffen wie chemische Synthese, Organization, Information, Replikation und dergleichen mehr. Aber das Leben ist eine Redox-Reaktion. In allen Lebensformen gibt es im zentralen Stoffwechsel eine Hauptreaktion, deren Redoxpotential die Synthese von Speicherformen chemischer Energie gewährleistet; diese wiederum begünstigt eine Schar kleiner Nebenreaktionen, die in ihrer Gesamtheit die Substanz des Lebens ausmachen. Am Ursprung des Lebens muss es auch eine Hauptreaktion mit einigen kleineren Nebenreaktionen gegeben haben. Es ist unwahrscheinlich, dass das Leben alle Spuren seines Ursprungs verloren hat, d. h., die Spur der biochemischen Genese sollte irgendwo im Stoffwechsel moderner Organismen zu finden sein – was damals chemisch möglich war, sollte heute kaum unmöglich sein. Die Reduktion von CO_2 mit H_2 zu kleinen organischen Substanzen wie Acetat oder Methan scheint ein gut geeigneter Kandidat für solch eine biochemische Urreaktion zu sein. Demnach spiegelten Acetogenese und Methanogenese Urzustände der Mikrobenphysiologie unter den Eubakterien beziehungsweise den Archeabakterien wider. Sowohl die Acetogenese als auch die Methanogenese funktionienen heute jedoch nur mittels der Chemiosmose, was auf eine essentielle Rolle chemiosmotischer Kopplungsmechanismen beim Urprung der ersten freilebenden Zellen hinweist. Den ersten Mechanismen proteinbasierter chemiosmotischer Kopplung ging unter Umständen die Phosphorolyse von Acetylthioestern voraus; so entstandenem Ace-

tylphosphat käme somit eine Rolle als die Urwährung biochemischer Energie zu. Möglicherweise ist das Leben mit Acetylphosphat groß geworden und hat seine Milchzähne an einer Hydrothermalquelle mit nützlichen Substanzen wie CO und Methylsulfid aus der Serpentinisierung geschliffen, bevor es schließlich das Laufen mit H_2 und CO_2 allein gelernt hat.

There are currently three main approaches to improving our grasp of how early evolution and the origin of life might have been possible. One is founded in the chemical the tradition of the Miller-Urey experiment (Miller 1953) and involves organic syntheses with the goal of demonstrating how biochemical compounds might have been prebiotically synthesized on the early Earth (Oró et al. 1962, Huber and Wächtershäuser 1997). Such endeavors provide important information about various kinds of chemical reactions that might have been plausible at life's origin, but the information obtained strongly depends upon the assumed initial conditions. Another approach involves exploration of the catalytic and replicatory functions of RNA molecules with the aim of explicating the possible properties of self-replicating systems that are thought to have existed in the RNA world (Joyce 2002, Penny 2005, Eschenmoser 2004). A third approach is founded in molecular phylogenetics. It involves making trees from different kinds of protein- or RNA-sequences and then pondering the position of the tree's root, from which properties of the most deeply branching lineages and their suspected ancestors can be inferred (Forterre 2006, Cavalier-Smith 2006, Lake et al. 2007). That approach provides food for thought as to how various genes (and by inference, cell lineages) might be related to one another (assuming that the tree is correct), while falling short of being able to explain how the starting material of such investigations – biochemicals, genes, and cells – might have arisen.

Many, but not all, concepts germane to prebiotic chemistry, the RNA world, and deep phylogeny are mutually compatible and hence render the transition from the elements to *bona fide* life forms on early Earth vaguely imaginable, but with the caveat that one best not delve too deeply into the exact details, because life is a complicated phenomenon. Some might protest that attempts to make the origin of life *imaginable* constitutes an unscientific objective, and might condemn such wording (for example, Wächtershäuser in Borgeson et al. 2002). But I find such wording not only appropriate, but also accurate. For even if we were able to create life exactly as we know it from the elements in some laboratory apparatus, we still would be unable to demonstrate that *we*, our ancestors, arose that way; we would just have a narrative that is more readily imaginable for scientists. Accordingly, all research that aims to uncover how life *actually* arose is an unfalsifiable conjecture, which is fine. Having said that, concepts common to prebiotic chemistry, the RNA world, and deep phylogeny taken together provide a common backbone for much current thinking (or speculation, if one prefers) about early evolution. Accordingly, much of the literature about early evolution deals with aspects of synthesis, organization, information, replication and the like.

But all of evolution, including early evolution, is about life. Life, in turn, is about redox chemistry (Mitchell 1967). A famous and influential chemist in the origin of life field once looked me in the eye and, witnessed by another chemist who is even more famous and more influential in the same field, said: "Redox chemistry is a product of the human imagination." That statement can be readily falsified by putting a plastic bag tightly over one's head for about 53 seconds; the ensuing physiological reaction unequivocally demonstrates that life is about redox chemistry. The mitochondria of human tissues require

oxygen, delivered by the lungs and circulatory system, as a terminal acceptor for the electron transport chain that couples a redox reaction – the oxidation of glucose with oxygen to produce carbon dioxide and water – to the storage of energy so released into a chemical carrier (ATP in modern biochemistry) so that the processes of life (biochemical reactions) may occur. Without a main redox reaction, no chemical energy can be harnessed that would promote side reactions of the main reaction to occur, and no life as we know it would be possible. That is true not only for humans, it is true for all organisms. Life is a chemical reaction, a main redox reaction that delivers chemical energy so that lots of little side reactions, driven by the main reaction, can deliver the substance of life. The principle that main redox reactions reside at the core of all life processes is universal, more universal than the genetic code, because there are no deviations and no exceptions to the rule. One might interject that photophosphorylation in *Halobacterium* is not a redox reaction, which is true, but photophosphorylation is not the same as life, and without redox reactions, *Halobacterium* cannot grow, for reasons of the plastic bag above. Life is a redox reaction. At the very beginning of life, it cannot have been otherwise. Redox chemistry is not a product of the human imagination, and some origin of life research is not really about the origin of life.

So if one ponders early evolution or the origin of life from the standpoint of energy metabolism or bioenergetics, the question ensues: What was the main exergonic (energy-releasing) chemical reaction that drove the very first organic-synthetic chemical reactions from which life eventually arose? Another way to state that question is: What was the ancestral state of microbial physiology?

There are quite a few possibilities, because different kinds of organisms are able to use hundreds of different kinds of main redox reactions to generate their chemically harnessable energy (Thauer et al. 1977, Amend and Shock 2001). But among the myriad of core chemical reactions that modern cells use to generate ATP, acetogenesis and methanogenesis stand out as particularly good candidates for very primitive and possibly the most ancient forms of core bioenergetic reactions, because acetogens and methanogens *synthesize* ATP from the process of CO_2 fixation (reduction). That sets methanogens and acetogens apart from all other autotrophs (organisms that satisfy their carbon needs from CO_2 alone), which *consume* ATP during CO_2 fixation.

One can also ponder the ancestral state of microbial physiology from a slightly different perspective. If one assumes (as I will here) that biochemistry and life started off from CO_2 (Mereschkowsky 1910, Maden 1995), rather than from some kind of preformed organic soup, and if one furthermore assumes that there is a trace of life's beginnings preserved in modern metabolism, there are only four main places to look for it: the Calvin cycle, the reductive citric acid cycle, the 3-hydroxypropionate pathway, and the acetyl-CoA pathway, because those are the only known pathways of CO_2 fixation known among autotrophs (see addendum). Although much attention has been given to the reverse citric acid cycle as a candidate for the primordial form of carbon fixation (Hartman 1975, Wächtershäuser 1992), it does not generate ATP, it consumes ATP. Of those four pathways, the acetyl-CoA pathway would seem to have the most primitive sorts of features, based on bioenergetic considerations and the simplicity of its chemistry (Fuchs and Stupperich 1986, Fuchs 1989). Acetogens and methanogens both use the acetyl-CoA pathway in their core energy-conserving reaction. The pathway is replete with FeS and FeNiS proteins, consistent with the idea that FeS centers are relics of primordial biochemistry (Hall et al. 1971).

How does the acetyl-CoA pathway work? In a nutshell, the acetyl-CoA pathway involves the synthesis of a methyl group from CO_2 and H_2, donation of the methyl group to an enzyme called bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS), the reduction of a molecule CO_2 to CO (using electrons from H_2) by CODH/ACS, the condensation of CO and the methyl group to form an enzyme-bound acetyl-group, and thiolysis of the acetyl group by CoASH to yield the energy rich thioester acetyl-CoA that is the end product of the pathway and the starting point of carbon metabolism. In their main energy-conserving reaction, the details of which are not yet fully clarified (Müller 2003, Svetlitchnaia et al. 2006), acetogens generate an ion gradient in the process of synthesizing acetyl-CoA and harness that ion gradient with an ATPase for net ATP synthesis. In the main energy-conserving reaction of methanogens, the majority of the methyl groups are further reduced to methane in a series of reactions in which an ion gradient is generated that is harnessed by an ATPase (Thauer 1998).

If either methanogenesis or acetogenesis is the ancestral state of microbial physiology, one might assume that one evolved from the other. But I will not be suggesting here that methanogens (belonging to the archaebacteria) evolved from acetogens (members of the eubacteria) or *vice versa*. Neither will I suggest that one physiology is ancestral to the other. Both modern physiologies involve the storage of chemiosmotic potential through generation of an ion gradient and harnessing of that potential in the chemical form of ATP. That in turn, requires proteins, membranes, cofactors, and a particular topological orientation of the redox reaction in order for that energy to be harnessed. That represents a very advanced state of physiology, a fully modern state for acetogens and methanogens. Is a simpler ancestral state imaginable? Arguably so, and submarine hydrothermal vents might provide important clues as to what the ancestral state of life's initial chemical reaction might have been.

A simpler ancestral state would, for example, involve the abiotic synthesis of acetate and methane from H_2 and CO_2 but without any side reactions or chemiosmotic coupling. This author does not know that modern hydrothermal systems of the kind that Mike Russell (warm, alkaline, highly reduced; see chapter by Russell in this volume) and John Baross (Baross and Hoffmann 1985) have been talking about for a couple of decades have actually been shown to produce *abiotic* methane and acetate. But because (*i*) all the ingredients are there to do it – H_2 generated from serpentinization (Bach et al. 2006) and CO_2 in ocean water –, and because (*ii*) the acetate- and methane-producing reactions are exergonic (Thauer et al. 1977), and because (*iii*) the main catalysis in CODH/ACS is afforded by transition metal sulfides as might occur in such geological settings (Russell and Hall 1997), one might would suppose that compounds like methane, acetate, and formate (simple reduced forms of carbon) ought to be formed geologically and that they should therefore be found in the effluent of systems such as Lost City (Kelley et al. 2005) or similar systems. Evidence indicating that to be the case would be of interest.

Could the abiotic synthesis of acetate and methane from H_2 and CO_2 in the absence of chemiosmotic coupling provide any form of harnessable chemical energy? In other words, are there *simple* side reactions from the main acetate- and methane-producing reactions that are imaginable and might make sense in terms of modern biochemistry? In the modern acetyl-CoA pathway, a thioester, acetyl-CoA, is the end product. The thioester bond is an energy-rich bond that can do so some additional chemical work. It has been proposed that prebiotic phosphorolysis of an acetyl thioester might produce acetyl phosphate (de Duve 1991), a reaction that occurs in many microbes today. The mixed anhydride bond in acetyl

phosphate has greater phosphorylating potential than the phosphoanhydride bonds in ATP (Stryer 1975), so acetyl phosphate might have been an important energy currency in early evolution (Ferry and House 2006, Martin and Russell 2007). That suggestion would be consistent with findings by Huber and Wächtershäuser (1997) but differs in the details from the earlier proposal that the source of thioesters in the main bioenergetic reaction at the origin of life was the Fe(III)-dependent oxidation of abiotically formed sugars existing in some form of organic soup (de Duve 1991), and that the main function of acetyl phosphate was to produce pyrophosphate as the initial energy currency. Pyrophosphate has only about 2/3 the phosporylating potential of ATP and only about half that of acetyl phosphate (Stryer 1975), so as a main carrier of chemical energy in early evolution, acetyl phosphate would seem to be very attractive. If it were to phosphorylate a substrate, acetate would be the waste product, in line with the notion that a main chemical reaction might give rise to simple side reactions at first that then could grow more complicated with time.

What sorts of substrates would acetyl phosphate be likely to phosphorylate? Obvious candidates would be simple compounds. The simplest is perhaps bicarbonate, phosphorylation of which would yield carboxyphosphate, an important chemical intermediate of most biotin-dependent carboxylation reactions today and an important intermediate in pyrimidine biosynthesis. Another simple substrate would be carbamate, the simplest form of organic nitrogen, whose phosphorylated form, carbamoyl phosphate, is also an important intermediate in pyrimidine biosynthesis. Another simple candidate phosphorylation substrate would be formate, to yield formyl phosphate, an important intermediate in purine biosynthesis. Such simple phosphorylations and their possible significance are discussed in more detail elsewhere (Martin and Russell 2007).

But there is a small bioenergetic problem with the notion that H_2, CO_2, and phosphate could react to provide net surplus abiotic synthesis of acetyl phosphate for the "purpose" of promoting side reactions en route to synthesis of a simple main initial waste product such as acetate. It has to do with the efficiency of (bio)chemical energy conservation and the circumstance that acetogens and methanogens are both dependent upon chemiosmosis. Namely, there is apparently not enough free energy to be gleaned from either acetogenesis or methanogenesis from H_2 and CO_2 to allow modern microbial growth via substrate level phosphorylation alone. That means that, under these premises, some kind of chemical "booster" at the origin of biochemistry might have been required. It is not inconceivable that such an energetic booster might have involved substantial concentrations of CO (anhydrous formate) or methyl sulfide, provided by serpentinization in the Earth's crust, in a hydrothermal exhalate (Ferry and House 2006, Martin and Russell 2007). For given either starting compound, or both (Huber and Wächtershäuser 1997), the sustained synthesis of acetyl thioesters and, given phosphate, net synthesis of acetyl phosphate for biochemical work, would seem to be thermodynamically feasible (Ferry and House 2006, Martin and Russell 2007).

If so, that would physically link the origin of biochemistry via the acetyl-CoA pathway to a geochemical setting such as a hydrothermal vent. From my point of view that is hardly a fundamental problem, and geochemists like Mike Russell might find it uniquely satisfactory, but there are a couple of details that would need to be addressed. One is how microbes could have attained the free-living state under such premises. The bioenergetic constraint that methanogens and acetogens require chemiosmosis for growth (net chemical synthesis), taken together with the circumstance that the molecular machinery of harness-

ing ion gradients for energy conservation (ATPases) is far more universal and conserved among modern cells than the myriad of proteins involved in generating ion gradients, suggests that the ability to harness a preexisting ion gradient is older than the ability to generate an ion gradient with a chemistry that is specified by genes. If so, that would mean that early biochemical systems "learned" to harness naturally preexisting ion gradients before they "learned" to generate such gradients themselves. That is hardy an unreasonable thought because the chemically simpler process (harnessing) precedes the chemically more complex process (redox-coupled pumping). From that it would follow that the (biogeo)chemistry that gave rise to modern microbes most likely arose in an environment that was naturally chemiosmotic (more alkaline on the inside than on the outside) from the outset. That is what Mike Russell (see chapter by Russell in this volume) has been saying for about 20 years, and a closer look at bioenergetics in the context of early evolution, which is currently not a mainstream undertaking, indicates it to be a robust concept.

ATPases are proteins, and because there is no evident trace of an RNA world in ATPases, it would seem likely that they arose after the origin of translation and genes (which constitutes a big jump from the considerations in this paper so far), but that would not be an insurmountable problem if we assume that acetyl phosphate, supplied by an ancestral version of the acetyl-CoA pathway, was readily available to the chemical system assumed here. If this were like that, then prebiotic chemistry (and the RNA world) might have been raised on acetyl phosphate all the way up to the origin of genes and proteins, weaned of its dependence upon CO or methyl sulfide by the invention of genetically encoded proteinaceous ATPases, and might have learned to walk upright (made the transition to the free-living state) on H_2 and CO_2 with the invention of mechanisms to generate ion gradients with chemical reactions that exploit that redox potential and that are specifiable by genes.

The latter point brings us to the circumstance that the mechanisms and chemistry of ion gradient generation differs in the comparison of acetogens with methanogens, suggesting that the mechanisms and the proteins underlying those crucial inventions arose rather late in the overall scheme of biochemical evolution prior to the origin of free-living cells, and that the biochemical lineages that gave rise to eubacteria and archaebacteria made those discoveries independently. I find it far more reasonable to assume that the first archaebacteria and the first eubacteria emerged as free-living cells independently from a physically confined hydrothermal mound type of environment (Martin and Russell 2003) than to assume that archeabacteria arose from any group of eubacteria or *vice versa*, for the reason that those two main groups of prokaryotes share so few genes in common (Koonin and Martin 2005, Dagan and Martin 2007).

Hydrothermal mounds would have been the most focused interface of H_2 and CO_2 on the early Earth, providing good conditions for them to react. And what was the source of energy that provided the "charge separation" or redox potential behind the H_2/CO_2 couple so that acetate or methane might have been formed in an exergonic reaction at a hydrothermal vent? The answer would be geochemical (Martin and Russell 2007): oxidation of carbon to CO_2 at high temperature in volcanos, providing marine CO_2, and serpentinization in hydrothermal systems (Bach et al. 2006), providing H_2 from the reduction of water via oxidation of Fe(II) in olivine within the Earth's crust.

One objective of inferences about early evolution is to narrow the gap between the elements on the early Earth and real life in order to make that transition more tangible. In that sense, the redox potential of the H_2/CO_2 couple at a hydrothermal vent and the physiology

of organisms that use that couple as their main reaction today would seem to converge at the acetyl-CoA pathway. It would support these arguments and speculations if abiotically formed compounds such as acetate, methane, methyl sulfide, formate, and/or CO would be reported in the effluent of modern alkaline hydrothermal systems.

Addendum

Since this chapter was delivered in May 2007, a couple of developments have arisen that would seem worth appending to the text above. First, the statement on page 55 that there are only four known pathways of CO_2 fixation is no longer correct. With the recent discovery of the 3-hydroxypropionate/4-hydroxybutyrate pathway (Berg et al. 2007) there are now five (Thauer 2007). The status of the acetyl-CoA pathway as the most primitive of the five would appear, however, to be unaffected by that important finding. Second, the closing passage points out that reports of abiogenic formation of methane in hydrothermal vents would offer some support in evidence for the considerations put forth here. Such evidence has now been reported by Proskurowski et al. (2008), and serpentinization appears to be involved in the mechanism of methane formation.

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Hydrogenosomes, Mitochondria, and Mitosomes – Or the Final Proof of the Endosymbiont Theory

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With 3 Figures

Abstract

The analysis of mitochondrial genomes and proteomes, organellar chaperons, and ADP/ATP transporters has shown that mitochondria, hydrogenosomes, and mitosomes evolved from a common alpha-proteobacterial ancestor by reductive evolution through evolutionary "tinkering". The discovery of a missing link between mitochondria and hydrogenosomes removed the last doubts about the validity of the endosymbiont theory for the evolution of the eukaryotic cell.

Zusammenfassung

Die Analyse von mitochondriellen Genomen und Proteomen, aber auch von organellären Chaperonen und ADP/ATP-Transportern zeigte, dass Mitochondrien, Hydrogenosomen und Mitosomen von einem gemeinsamen alpha-proteobakteriellen Vorfahren abstammen. Die verschiedenen Organellen entstanden durch reduktive Evolution auf dem Wege evolutionärer „Basteleien". Die Entdeckung eines Bindeglieds (*missing link*) zwischen Mitochondrien und Hydrogenosomen konnte die letzten Zweifel an der Endosymbionten-Hypothese ausräumen.

One hundred years ago, C. Mereschkowsky (1905) published a notoriously ignored landmark paper: "Über Natur und Ursprung der Chromatophoren im Pflanzenreiche" ("On the nature and origin of the chromatophores in the plant kingdom"). Twenty years later, I. E. Wallin (1925) postulated a "bacterial nature of mitochondria". The reasons for Wallin's postulate were much less obvious, and his claim of having cultivated mitochondria *in vitro* turned out (of course) to be wrong. Eventually, the genomics era provided the tools to prove or disprove the endosymbiont hypothesis for the origin of the eukaryotic cell. The phylogenetic analysis of alpha-proteobacterial, cyanobacterial, mitochondrial, and plastidic genomes revealed that mitochondria and plastids are monophyletic, i.e., all present-day plastids seemed to be derived from one and the same cyanobacterial ancestor, whereas all present-day mitochondria appeared to stem from one and the same alpha-proteobacterial ancestor (Gray et al. 1999, Rodriguez-Ezpeleta et al. 2005). These observations confirmed that the evolution of the eukaryotic cell involved one (in the case of animals) or two (in the case of plants) singular, historical events that took place at least 1.5 billion and po-

tentially more than 2 billion years ago (DE DUVE 2005). Thus, after one century of biological, biochemical, classical genetic, and molecular studies, the serial endosymbiont hypothesis became a historical fact.

However, the mitochondria of the various eukaryotes are very different. There are anaerobic variants that do not use oxygen as terminal electron acceptor. Others lack components of the electron transfer chain, and some are functionally and genetically constrained. The same is true for plastids. Colorless, reduced plastids (apicoplasts) have been identified in parasitic organisms such as *Plasmodium,* the parasite causing malaria. The mitochondrion of *Plasmodium* has retained just 5 genes; in contrast, the mitochondrion of the flagellate *Reclinomonas americana* with its 97 genes has the largest coding capacity of all mitochondrial genomes studied to date. However, also this genome does not encode more than 67 different proteins, whereas the mitochondrial proteome consists of 500–1000 different proteins. Notably, the analysis of the mitochondrial proteomes of man and yeast has revealed that their mitochondria differ in about 50 % of their proteins. The vast majority of the mitochondrial proteins are encoded by the genes located in the nucleus, and less than 20 % are derived from the ancestral alpha-proteobacterium. The evolution from an alpha-

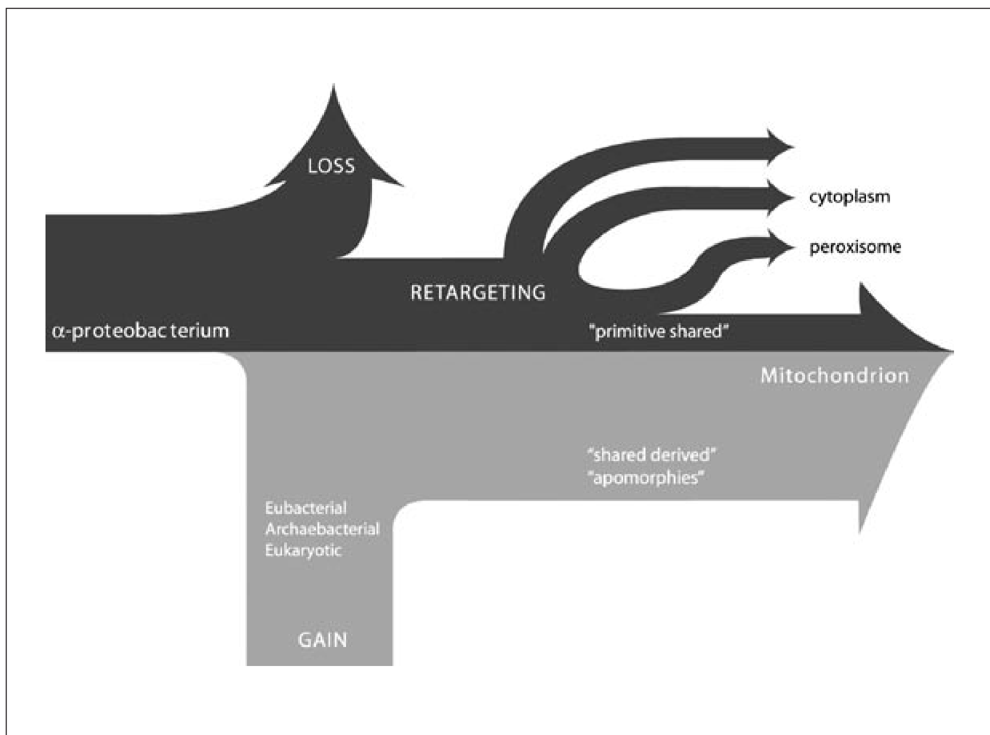

Fig. 1 Loss, gain and retargeting. During the evolution of mitochondria, the substantial loss of genes of the original endosymbiont was not only accompanied by a transfer of genes to the nucleus, but also by a remarkable retargeting of alpha-proteobacterial proteins to other cellular compartments. Consequently, mitochondria, and peroxisomes possess a similar fraction of proteins of alpha-proteobacterial origin (GALBADON et al. 2006), whereas up to 80 % of the mitochondrial proteome can be made up from proteins that have various non-alpha-proteobacterial origins. Modified from GABALDON and HUYNEN et al. 2004.

proteobacterium, with minimally 800 and potentially more than 8000 genes, into a mitochondrion involved not only a tremendous loss of genes and the transfer of many others to the nucleus, but also a substantial retargeting of alpha-proteobacterial proteins to other subcellular compartments, such as the peroxisomes, which are believed not to be of endosymbiotic origin at all. Peroxisomes were identified as consisting of as many alpha-proteobacterial proteins as "textbook" mitochondria (Fig. 1; GABALDON et al. 2006).

Moreover, in addition to mitochondria, there are a number of unusual organelles, i. e., hydrogenosomes, mitosomes (= cryptons), mitochondrial remnants, and modified mitochondria, which do not fit into the scheme of a textbook mitochondrion. These organelles are found predominantly in anaerobic eukaryotic microbes. For example, hydrogenosomes were defined as subcellular particles that make H_2 and ATP (MÜLLER 1993). Mitosomes are tiny double-walled organelles that make neither H_2 nor ATP. Mitochondrial remnants, which also make neither H_2 nor ATP, were thought to be degenerate mitochondria, just like the modified mitochondria, which have been identified as inconspicuous double-walled compartments in a few parasites. Also these organelles are likely to produce neither H_2 nor ATP. Remarkably, none of these organelles possesses an organelle genome. Given the above-mentioned promiscuity of organellar origins, the lack of a genome (which could provide straightforward evidence for their descent) makes it difficult to trace their potentially mitochondrial (alpha-proteobacterial) origins (Fig. 2).

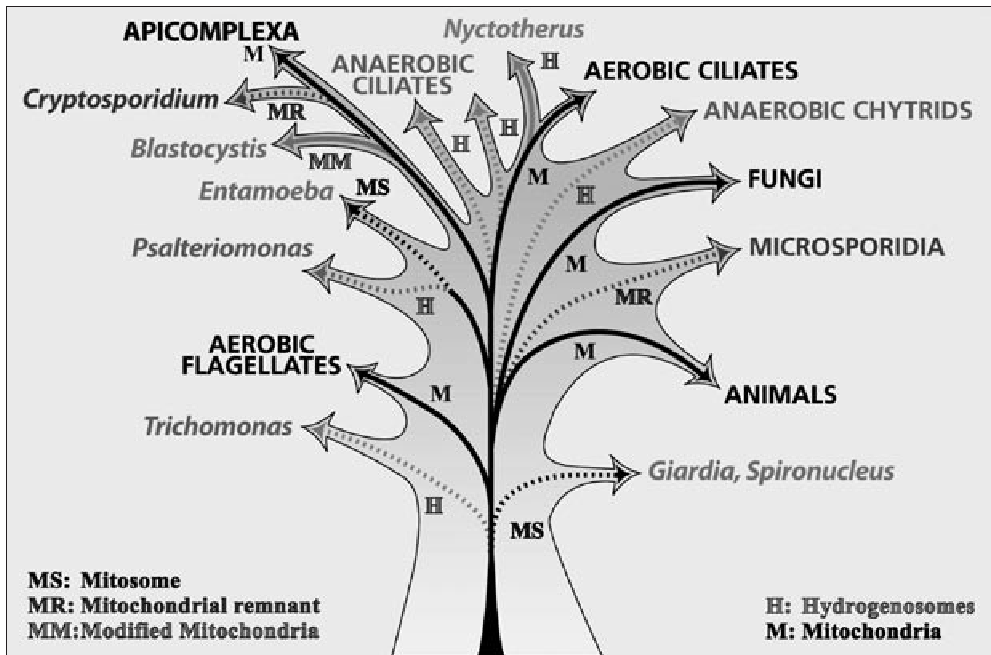

Fig. 2 Phylogenetic relationships between aerobic and anaerobic protists with a tentative evolutionary tree of mitochondria, modified mitochondria, mitochondrial remnants, and hydrogenosomes. The relationships are based on a variety of molecular data. The solid arrows indicate phylogenetic relationships based on the analysis of mitochondrial genomes. Dashed arrows indicate the loss of organellar genomes. The tentative phylogenetic relationships between the various types of organelles belonging to the mitochondrial family are therefore based on the analysis of nuclear genes encoding organellar proteins.

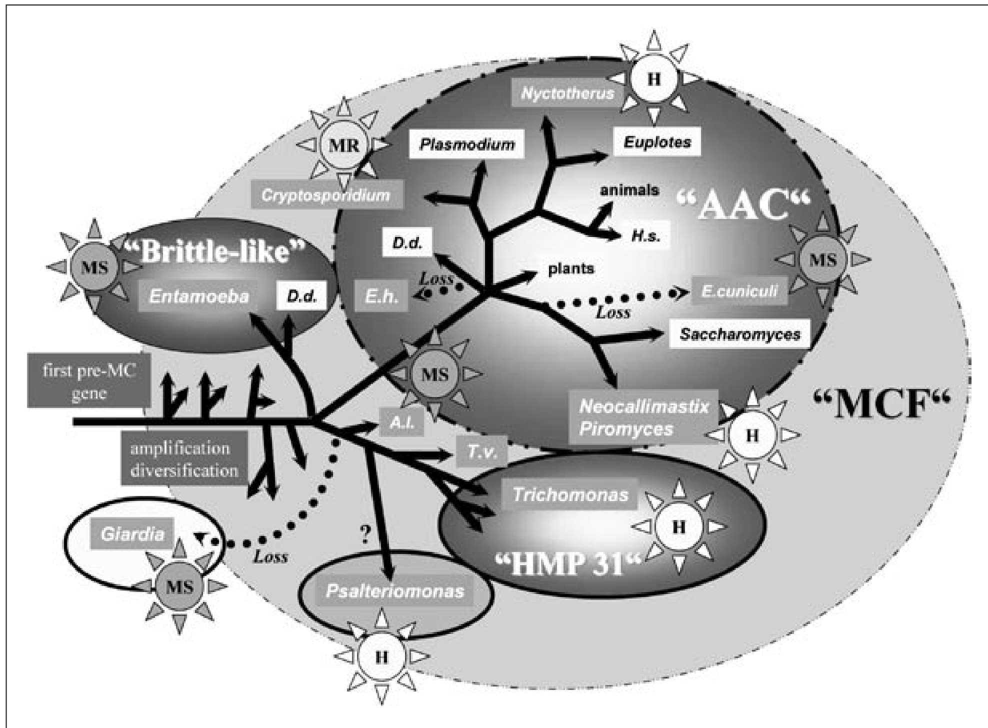

Fig. 3 Evolution of mitochondria, hydrogenosomes, mitosomes, modified mitochondria, and mitochondrial remnants. Phylogenetic analysis of the ADP/ATP transporters of the various organelles revealed that they evolved repeatedly and independently. Losses of true mitochondrial AACs were compensated by the evolution of alternative carriers from members of the mitochondrial carrier family, which is a eukaryotic invention. The position of *Psalteriomonas* is purely speculative, as is the evolution and function of the various carriers of *Trichomonas* (except for HMP 31, see Tjaden et al. 2004) and *Antonospora locustae* (Microsporidia). Tinkering and reductive evolution shaped the evolution of the ADP/ATP carriers of the various organelles. AAC, ATP/ADP carrier; *A. l.*, *Antonospora locustae*; *D. d.*, *Dictyostelium discoideum*; *E. h.*, *Entamoeba histolytica*; H, hydrogenosome; HMP 31, hydrogenosomal carrier protein 31 (of *T. vaginalis*); *H. s.*, *Homo sapiens*; MC, mitochondrial carrier; MCF, mitochondrial carrier family; MR, mitochondrial remnant; MS, mitosome; *T. v.*, *Trichomonas vaginalis*.

Phylogenetic analysis of nuclear-encoded, but organellar HSP 60 (cpn 60) chaperons and ADP/ATP carriers supports mitochondrial origins for the hydrogenosomes of the anaerobic chytrids *Piromyces* and *Neocallimastix*, and the anaerobic ciliate *Nyctotherus.* Interestingly, the HSP 60 proteins of *Trichomonas*, *Giardia*, and *Entamoeba* form a moderately supported cluster distinct from mitochondriate organisms (see Voncken et al. 2002, van der Giezen et al. 2003). Notably, the hydrogenosomes of *Trichomonas* and the mitosomes of *Entamoeba* use different, alternative members of the mitochondrial carrier family for the transport of ATP across their hydrogenosomal/mitosomal membranes (Fig. 3; Tjaden et al. 2004, Chan et al. 2005), whereas the diplomonad *Giardia* and the microsporidium *Encephalitozoon* have lost all mitochondrial nucleotide carriers. Consistently and in almost all cases, the ADP/ATP carriers of the genome-less organelles – regardless of whether they produce ATP or not – are related to a member of the mitochondrial carrier family from a closely related species that possesses a bona-fide mitochondrion (Fig. 3). Notably, this

does not apply to the hydrogenosomes of *Trichomonas* and the mitosomes of *Entamoeba*. The functional and phylogenetic analysis of the ADP/ATP transporters of the various mitochondria, hydrogenosomes, and mitosomes therewith strongly supports the hypothesis that all these organelles belong to the mitochondrial family and that they evolved repeatedly by evolutionary tinkering (bricolage) in the various evolutionary lines as adaptations to the specific requirements of the host organisms (Fig. 3).

The hydrogenosomes of the trichomonads were initially considered both morphologically and biochemically to be distinct from mitochondria (reviewed in Yarlett and Hackstein 2005, Hackstein et al. 2006). Subsequent biochemical and molecular studies have changed this view, in particular the discovery of proteins with homology to the 24 and 51 kDa subunits of a mitochondrial-type complex I (Hrdy et al. 2004; see, however, Dyall et al. 2004 for an alternative view). However, these proteins are not likely to function in a rudimentary mitochondrial complex I. Rather, the 24-kDa (NuoE) and 51-kDa (NuoF) subunits serve as a diaphorase or, alternatively, as an NADH dehydrogenase, which allows the reoxidation of NADH and FMN. In contrast to mitochondria described in textbooks, the trichomonad hydrogenosomes metabolize pyruvate through pyruvate : ferredoxin oxidoreductase and hydrogenase to acetate, carbon dioxide, and hydrogen (Müller 1993). Neither enzyme is of alpha-proteobacterial origin, and notably, anaerobic chytrids use pyruvate : formate lyase instead of a pyruvate : ferredoxin oxidoreductase or a pyruvate dehydrogenase to metabolize pyruvate (Boxma et al. 2004). On the other hand, the hydrogenosomes of the ciliate *Nyctotherus ovalis* use pyruvate dehydrogenase, like true mitochondria. For several reasons, these hydrogenosomes represent a missing link between ciliate mitochondria and hydrogenosomes. As the only hydrogenosome known to date, it possesses a mitochondrial genome with at least 5 genes encoding components of a functional mitochondrial complex I, several ribosomal proteins, and a tRNA (Boxma et al. 2005). The nuclear genome of *Nyctotherus* is likely to encode more than 200 mitochondrial proteins, not only additional components of mitochondrial complex I and II, but also a pyruvate dehydrogenase, several representatives of the mitochondrial carrier family, components of a mitochondrial import and protein synthesis machinery, TCA cycle enzymes, and enzymes involved in fatty acid and amino acid metabolism (Boxma et al. 2005, Hackstein et al. 2006).

In conclusion, mitochondria, hydrogenosomes, and mitosomes evolved from a common ancestor by evolutionary tinkering. Starting with a mitochondrial-type organelle, which, however, was clearly different from the mitochondria presented in textbooks (cf. Gabaldon and Huynen 2003), all the various types of mitochondria, hydrogenosomes, and mitosomes evolved to allow the adaptation of their hosts to their peculiar environments. All these organelles belong to a continuum that covers the whole range from mitochondria presented in textbooks to mitosomes that did not retain a respiratory chain, but still transport ATP into the large mitosomes of *Entamoeba* or into the inconspicuous mitosomes of *E. cuniculi* and *Giardia* that did not retain any gene encoding a mitochondrial carrier protein (Hackstein et al. 2006). Hydrogen might have played a role in the ancestral metabolism of the host, but it is unlikely that hydrogenase activity was one of the properties of the protomitochondrion, which contributed, however, much more than just parts of a TCA cycle and an electron transport chain (Gabaldon and Huynen 2003). The only protomitochondrial activity that seems to be shared by all organelles of the mitochondrial family is the synthesis of FeS clusters (Lill and Mühlenhoff 2005). Consequently, all available in-

formation supports the interpretation of mitochondria, hydrogenosomes, and mitosomes as members of one and the same (mitochondrial) family of organelles exemplifying reductive evolution through evolutionary tinkering.

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Evolution und Menschwerdung

Vorträge anlässlich der Jahresversammlung vom 7. bis 9. Oktober 2005
zu Halle (Saale)

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Evolution und Menschwerdung gehören noch immer zu den interessantesten Themen, mit denen sich die Naturwissenschaft auseinandersetzt und die die Öffentlichkeit faszinieren. Die Thematik verlangt eine interdisziplinäre Auseinandersetzung, für die eine Akademie wie die Leopoldina prädestiniert ist. Daher griff die Jahresversammlung 2005 verschiedene Aspekte hierzu auf.

Die Schwerpunkte der Tagung spiegeln den enormen Fortschritt der Erkenntnisse über das Evolutionsgeschehen und den veränderten Blickwinkel wider, der sich aufgrund des außerordentlich großen Wissenszuwachses und veränderter Diskussionsebenen in der Forschung, aber auch zwischen Wissenschaft und Gesellschaft ergeben. Die Evolution des Menschen und dessen physische, geistige und kulturelle Entwicklungstendenzen stehen dabei im Zentrum.

Der Band spannt den Bogen vom Urknall und der Bildung der Planetensysteme über die Entstehung des Lebens, die Entwicklung von Prokaryoten und Eukaryoten, die Evolution und das Sterben der Saurier, die Analyse von Insektenstaaten bis hin zu Fragen der Menschwerdung und Formen der menschlichen Kultur. Hier werden unter anderem „Das Sprachmosaik und seine Evolution”, die „Evolution durch Schrift”, Rituale, Religionen, Gemeinschaftsbildung und sozialer Wandel unter evolutionären Aspekten untersucht, aber auch „Bilder in Evolution und Evolutionstheorie” sowie die „Griechischen Anfänge der Wissenschaft” betrachtet.

Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Microorganisms in Symbioses and Pathogenesis

Nova Acta Leopoldina NF *96*, Nr. 356, 71–78 (2008)

Unravelling Microbial Diversity and Metabolism in Marine Sponges

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With 2 Figures

Abstract

Many marine sponges are associated with enormous numbers of microorganisms that reside internally, yet extracellularly within the sponge extracellular matrix. The microbial community is phylogenetically complex but highly sponge-specific and is vertically transmitted via the reproductive stages to the next sponge generation. Representatives of this elusive community have not been cultivated, and their metabolism is just now beginning to be understood in molecular studies. We present recent findings on the diversity and metabolic capacities of sponge-associated microbial consortia.

Zusammenfassung

Viele marine Schwämme sind mit einer großen Anzahl an Mikroorganismen assoziiert, die intern, jedoch außerhalb der Zellen in der extrazellulären Matrix des Schwammes vorliegen. Die mikrobielle Gemeinschaft ist phylogenetisch komplex, hochgradig Schwamm-spezifisch und wird vertikal über die reproduktiven Stadien (Embryos, Larven) an die nächste Schwammgeneration weitergegeben. Weil Vertreter dieser mikrobiellen Gemeinschaft bisher noch nicht kultiviert werden konnten, ist erst wenig über deren Stoffwechseleigenschaften bekannt. Dieser Übersichtsartikel stellt neue Erkenntnisse zur Diversität und zum Stoffwechsel der Schwamm-assoziierten mikrobiellen Konsortien vor.

1. Introduction

Sponges form one of the deepest radiations of the Metazoa, with a fossil record dating back 580 million years (Li et al. 1998). Owing to their unique body design – the lack of true tissues and organs – sponges were recognized as true animals only in 1766 (Pallas 1776). In spite of their rather simple morphological organization, their genomic repertoire is strikingly complex and resembles that of higher metazoans in many respects (Müller et al. 2001).

In the 1960s and 1970s, it was recognized that many species of Demospongiae, the largest class of sponges, are associated with enormous numbers of microorganisms (e.g., Vacelet et al. 1975). For examples, all species of the Demospongiae orders Verongida, Agelasida, Chondrosida, and Lithistida contain high numbers of microorganisms. The microbial communities of the high-microbial-abundance Demospon-

giae have been analyzed during the past two decades using molecular techniques centering around the 16S rRNA gene. The recent surge of interest in sponge microbiology has led to systematic investigations of marine sponges from tropical and subtropical regions, temperate waters such as the North Sea and the Antarctic, the deep sea, and even from a freshwater lake (for a review, see HENTSCHEL et al. 2006, TAYLOR et al. 2007).

2. Microbial Diversity

Microorganisms can contribute up to 40–60% of the biomass of the high-microbial-abundance demosponges. These "bacteriosponges" literally harbor their own microcosm within their mesohyl matrix. The Mediterranean sponge *Aplysina aerophoba* (Verongida, Demospongiae) (Fig. 1) can be considered as a model bacteriosponge as its microbiology has been studied in detail for more than three decades. The bacteriosponge community consists of as many as eight different bacterial phyla, as shown by 16S rRNA gene analysis: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria, Proteobacteria* (*Alpha-*, *Gamma-*, *Delta-*), *Gemmatimonadetes*, *Chloroflexi*, and *Nitrospira* (HENTSCHEL et al. 2002). Interestingly, each bacterial phylum harbors one or more

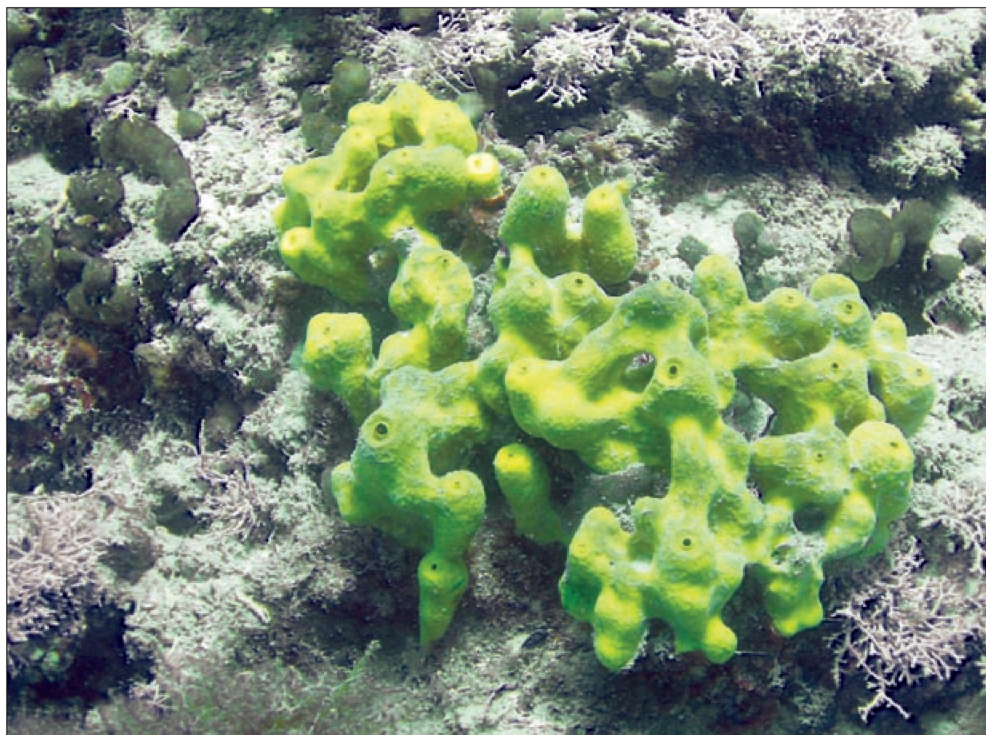

Fig. 1 The Mediterranean sponge *Aplysina aerophoba* (underwater photography by T. WINTER, Würzburg).

sponge specific clades that are exclusively derived from geographically and taxonomically disparate sponges and are missing in marine sediments, seawater, and other environments.

In addition to the eight known bacterial phyla, a new candidate phylum, "*Poribacteria*", was discovered (Fieseler et al. 2004) and is to date exclusively associated with marine sponges. Phylogenetically, the *Poribacteria* comprise a superphylum together with their closest relatives, the phyla *Planctomycetes*, *Verrucomicrobia,* and *Chlamydiae* (Wagner and Horn 2006). Transmission electron micrographs and fluorescence *in situ* hybridization (FISH) studies show a nucleoid-like structure within the poribacterial cell, a property that has been so far observed only in the *Planctomycetes* (Lindsay et al. 1997, Fieseler et al. 2004). Sponge-specific archaea, represented by the *Cenarchaeum symbiosum* clade, have also been discovered in sponges using cultivation-independent studies (e.g., Holmes and Blanch 2007, Pape et al. 2006). Members of the sponge-specific microbial community described above have not been cultivated to date.

The associations between sponges and complex microbial consortia have been shown to be remarkably stable over time and space, yet the mechanisms by which these associations are formed and maintained are still under debate (Hentschel et al. 2006). The first indications of vertical transmission of microbes via sponge reproductive stages came from electron microscopy studies in the 1960s and 1970s that documented the presence of microorganisms in oocytes, embryos, and larvae of different sponge species (e.g., Levi and Levi 1976). Subsequent studies revealed at least four different modes of microbial incorporation ranging from phagocytosis of microbes by oocytes directly from the mesohyl to the incorporation of microorganisms from follicle cells into embryos (Ereskovsky et al. 2005 and references cited therein). Moreover, these studies showed that oviparous and viviparous species likewise use vertical transmission to transfer microorganisms between generations.

Three recent studies applied molecular methods (16S rDNA library, DGGE, and FISH) and demonstrated the presence of the above-mentioned, sponge-specific lineages of up to six different phyla in the reproductive stages of each of the three sponges *Corticium* sp., *Ircinia felix*, and *Mycale laxissima* (Enticknap et al. 2006, Schmitt et al. 2007, Sharp et al. 2007). From these cumulative microscopy and molecular data, it can be concluded that members of the sponge-specific microbial communities are passed through the reproductive stages to the next sponge generation and that vertical transmission is an important mechanism for the maintenance of the sponge-microbe association.

3. From Diversity to Function: Microbial Metabolism

Because sponge-associated microorganisms have not been cultivated, their metabolic properties and physiological demands are still largely unknown. Provided that the mesohyl microbiota can avoid being digested, the interior of sponges should be a nutritionally rich and stable habitat compared to the oligotrophic waters of the tropical and subtropical oceans in which they live. Organic carbon is likely supplied to the microbial community as a by-product of the host's extensive phagocytosis of food bacteria filtered in from the seawater. Ammonium is probably made available as an end product of the host's metabolism.

Accordingly, heterotrophic microbial metabolism appears likely but has not been investigated. The sponge-associated microorganisms are clearly metabolically active, as inferred from the bright signal intensities obtained by FISH. In the following, the current knowledge on the physiology and the autotrophic metabolism of sponge-associated microorganisms is presented.

3.1 Photosynthesis

The presence of Cyanobacteria in sponges, represented by the widely abundant "*Synechococcus spongiarum*" clade, is well documented (Usher et al. 2004, Steindler et al. 2005). Because these bacteria are typically found in the outer, light-exposed surface, their role in the protection of sponges from high light intensities has been proposed (Wilkinson 1983). From a nutritional point of view, it appears that the photosynthetically active cyanobacterial symbionts could provide their sponge host with the metabolites glycogen or glycerol (Wilkinson 1983) (Fig. 2). Indeed, the bulk of the total energy requirements of the Great Barrier Reef sponge *Phyllospongia lamellosa* are provided by phototrophic symbionts (Cheshire et al. 1997). Furthermore, cyanobacterial uptake of inorganic nitrogen via nitrogen fixation and subsequent transfer of metabolites to the sponge host (Wilkinson and Fay 1979) might allow the animals to thrive in nutrient-poor environments, such as tropical reefs.

3.2 Nitrification

Since ammonium is available to the sponge microbiota as a metabolic waste product resulting from the host's metabolism, it has been postulated that sponges are suitable niches for nitrifying microorganisms. Nitrification is the conversion of ammonia to nitrite by ammonia-oxidizing bacteria (AOB) or archaea (AOA) and subsequently to nitrate by nitrite-oxidizing bacteria (NOB) for energy conservation. In fact, 16S rRNA genes of ammonia-oxidizing bacteria (*Nitrosospira*, *Nitrosococcus*) and archaea (*Cenarchaeum symbiosum*) as well as nitrite-oxidizing bacteria (*Nitrospira*, *Nitrospina*) have been detected in several Mediterranean and Caribbean demosponges (e. g. Hentschel et al. 2002, Bayer et al. 2007, Diaz and Ward 1997, Holmes and Blanch 2007; Fig. 2). In addition, the functional genes coding for bacterial and archaeal ammonia monooxygenase, the key enzyme of nitrification, have been cloned from *A. aerophoba* and other Mediterranean demosponges (Bayer et al. 2008). Moreover, genome sequence analysis of the *C. symbiosum* sponge symbiont revealed the genomic repertoire for nitrification (Hallam et al. 2006), and indirect support for this ability was provided by the cultivation of a closely related archaeon, *Nitrosopumilus maritimus*, and the confirmation of nitrification in this isolate (Könneke et al. 2005). The molecular data are corroborated by physiological studies that showed that live sponges excrete both ammonium and nitrate into the surrounding seawater (Corredor et al. 1988, Diaz and Ward 1997, Davy et al. 2002, Bayer et al. 2007, 2008).

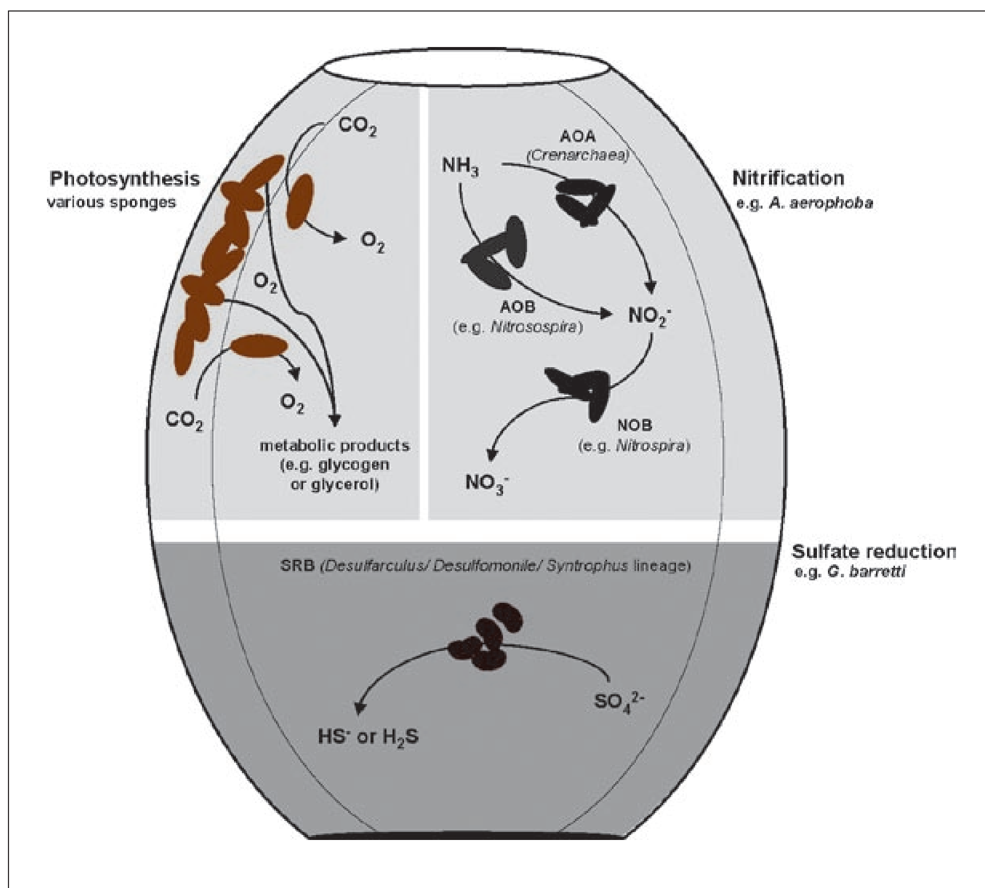

Fig. 2 The known metabolic pathways of sponge-associated microorganisms. The light-shaded background depicts aerobic pathways and the dark-shaded background depicts anaerobic pathways in the sponge mesohyl. A thin line separates the outer, light-exposed pinacoderm layer from the inner mesohyl core. AOB, ammonia-oxidizing bacteria; AOA, ammonia-oxidizing archaea; NOB, nitrite-oxidizing bacteria; SRB, sulfate-reducing bacteria.

3.3 Sulfate Reduction

As an anaerobic process, sulfate reduction occurs predominantly in marine sediments. Anoxic conditions are found in the mesohyl of some sponges, e.g., *Geodia barretti* and in well-aerated species such as *A. aerophoba* during periods of non-pumping (Hoffmann et al. 2005, Hoffmann et al. 2008). The isolation of facultatively anaerobic microorganisms from sponge tissues (e.g., Santavy et al. 1990), the visualization of anaerobic, sulfate-reducing bacteria in sponges by FISH (Schumann-Kindel et al. 1997, Hoffmann et al. 2005), and the discovery of anaerobic, methanogenic euryarchaeotes in sponges (Webster et al. 2001) support the observations of permanently or temporarily anoxic conditions in the sponge interior at some sponges. Hoffmann et al. (2005) were the first to document

bacterial sulfate-reduction processes in *G. barretti* (Fig. 2), and sulfate reduction rates of 1200 nmol cm^{-3} d^{-1} to occur in sponge tissue. *G. barretti* carries bacteria of a sulfate-reducing clade related to the *Desulfarculus*/*Desulfomonile*/*Syntrophus* lineage, as shown by FISH. Future studies will provide insights into the distribution of sulfate reducers in other sponge species, possibly identify taxonomically new sulfate-reducing species, and elucidate the general microbial sulfur metabolism in sponges.

4. Conclusions

Microbial diversity studies have provided a wealth of information regarding the complexity of sponge-associated microbiota, but the metabolic interactions between sponges and their associated microbes and between the different microorganisms of the community are just beginning to be understood. Certainly, the sponge anatomy (shape, size), the sponge behavior (pumping activity), and seasonal changes (temperature) will be important parameters that determine microbial metabolism within the mesohyl. Much remains to be discovered about the diversity, metabolic repertoire, and mechanisms of interactions between sponges and their associated microbial communities.

Acknowledgements

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Viruses – The Overlooked Players

Markus G. Weinbauer (Villefranche-sur-Mer)

With 2 Figures

Abstract

Viruses are typically ten times more abundant than prokaryotes in the water column of aquatic systems. Despite the finding that viruses cause prokaryotic mortality, which is on average as significant as grazing-mediated mortality, their impact has only been studied in a limited number of environments. Using an approach controlling the number of viruses in communities, we have shown that the presence of viruses often increases the number of prokaryotic phylotypes detected, whereas the effect on prokaryotic production is more variable. Thus, viruses seem to sustain host diversity in the environment and affect prokaryote-mediated ecosystem functions.

Zusammenfassung

Die Abundanz von Viren in der Wassersäule aquatischer Systeme ist normalerweise zehnfach höher als die der Prokaryoten. Obwohl bekannt ist, dass Viren Mortalität bei Prokaryoten verursachen, die im Durchschnitt etwa so bedeutend ist wie Mortalität durch Abweiden (*grazing*), so wurde der Einfluss ersterer nur in einer begrenzten Anzahl von Ökosystemen untersucht. Unter Verwendung eines Forschungsansatzes, der die Abundanz der Gesamtzahl der Viren in einer Gemeinschaft manipuliert, kann gezeigt werden, dass bei Anwesenheit von Viren die Anzahl der detektierbaren prokaryotischen Phylotypen sehr oft erhöht ist, während der Effekt für die prokaryotische Produktion variabel ist. Daraus kann geschlossen werden, dass Viren die Wirtsdiversität aufrecht erhalten und gleichzeitig die auf Prokaryoten zurückgehenden Ökosystemfunktionen beeinflussen.

Viruses were shown at the end of the 1970s to be much more abundant in aquatic systems than previously thought. These findings, however, were not recognized by virologists, who were concentrating on isolated phage–host systems, and by microbial ecologists, who were developing methods to quantify the number and activity of bacteria and establishing the principles of a microbial food web. Only a decade later, when microbial ecology was firmly established and the smallest fractions of the plankton was recognized, were viruses shown in a number of studies to be more abundant than prokaryotes (Bergh et al. 1989) and to cause significant mortality (Proctor and Fuhrman 1990, Suttle et al. 1990); hence, they were likely to impact biogeochemical cycles. This gave rise to the discipline of viral community ecology, which has resulted in one of the latest developments in studies of the metagenomics of viruses (Breitbart et al. 2002). A concept developed in which phages keep the winners of prokaryotic competitions for resources in check; this “killing

the winner" hypothesis predicts that viral lysis sustains prokaryotic diversity (Thingstad and Lignell 1997).

Here I will review the distribution of viral life strategies in aquatic environments, tackle the question of whether the abundance and diversity of phages in the environment is due to lytic or lysogenic infection, and present a synopsis of the effect of phages on the link between diversity and activity of prokaryotic communities. Unpublished data from my research group will also be presented.

Studies of phage–host systems predict that lysogeny should predominate when the host diversity is low or when the environment is too hostile for free phages to sustain lytic infection. In natural communities, the percentage of lysogens in the entire prokaryotic community is generally higher in the oligotrophic deep ocean than in the more eutrophic coastal systems or anoxic environments, where phages seem to be the major cause of mortality of bacterioplankton (Weinbauer et al. 2003) (Fig. 1). Phage production measurements and estimations of induced phage production based on known induction frequencies and temperate phage replication rates within the host indicate that lytic phages out-compete temperate phages in more-productive environments. These results also indicate that the effect of the lytic versus lysogenic lifestyle can be studied at the community level. This statement is not trivial since it suggests that the potentially vast variety of viral life strategies is not evenly distributed and that the environment is a force factor. From the few data published, it can be estimated that 5.2×10^{28} cells or 46 % of the planktonic prokaryotes in the world's oceans contain a functional genome and thus represent a huge potential for virus-mediated gene transfer.

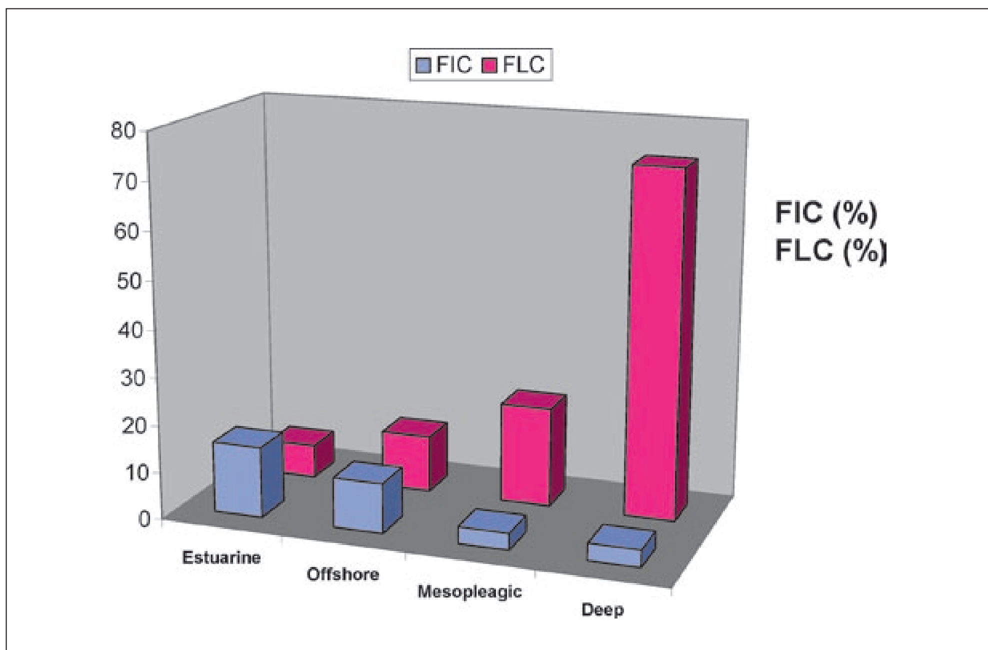

Fig. 1 Frequency of infected cells (FIC) and frequency of lysogenic cells (FLC) in major marine environments. Data from the Gulf of Mexico, the Mediterranean Sea, and the Gulf of Mexico are averaged. FIC was estimated using transmission electron microscopy, and FLC was estimated by using mitomycin C to induce phage formation.

Methods used to estimate phage production and lysogeny in bacterioplankton are not very precise, and this problem arises often when entire communities are studied. However, most studies – at least when calculations and predictions instead of evidence are used – suggest that induction is typically, albeit not always, much less significant than lytic phage production. It can thereby be hypothesized that the majority of phages in the environment are lytic.

Little is known about whether phage diversity *in situ* is due to lytic or temperate phages. *In situ* data on the co-variation of virus and host diversity are rare. During the phytoplankton bloom in 2004 in Villefranche Bay (northwestern Mediterranean Sea, France), we followed the changes in the diversity of cyanophages using primers targeting the DNA encoding capsid protein g20 and the diversity of cyanobacteria using primers targeting the DNA encoding RuBisCo. The number of bands after denaturing gradient gel electrophoresis (DGGE) ranged from 5 to 18 for cyanophages and 8 to18 for cyanobacteria. The positive correlation of the number of cyanophage bands and the number of cyanobacterial bands suggested a co-variation of phage and host diversity. This type of relationship is more suggestive of a lytic than a lysogenic lifestyle. When samples were treated with mitomycin C as an inducer of phage formation, we obtained a PCR product for cyanophages, which suggested that cyanobacteria in this environment also contain inducible prophages. A genetic fingerprint analysis is planned. Prophages in cyanobacteria have been detected using isolates, genomic data, and induction studies with natural communities (Ortmann et al. 2002).

In parallel, we carried out a study on the co-variation of viral genome size distribution using pulsed-field gel electrophoresis (PFGE), and 16S rRNA gene primers and DGGE. Viral genomes sizes typically occurred in three size ranges: 25–35, 50–70, and 180–250 kb. The number of bands ranged from 12 to 21 for viruses and from 36 to 47 for bacteria. The number of PFGE bands was well correlated to the number of DGGE bands before and after the phytoplankton bloom but not during the bloom. During the bloom, a maximum in bacterial richness was followed by a maximum in viral richness. Interestingly, lysogeny dropped from 23 to 5 % during the bloom. At the same time, a virus of ca. 39 kb, usually only detected when samples were treated with mitomycin C, was found *in situ* but was not found in mitomycin C-treated samples. Another phage of ca. 25 kb was detected *in situ* during the bloom for the first time and was also detected in the samples treated with mitomycin C. These results suggest mass lysis caused by an induction event, and this could have contributed to the decrease in bacterial richness and the increase in viral richness. Another phage with an unusually large genome size of ca. 195 kb was found in samples treated with mitomycin C but not *in situ*. This result indicates that viral diversity is typically due to lytic phages but that sometimes a mass induction event can contribute to viral diversity.

The "killing the winner" hypothesis is based on a lytic lifestyle of phages. The results obtained suggest that this is the most common lifestyle of phages in marine waters. Laboratory studies of samples with and without viruses were used to test this hypothesis for bacteria (Schwalbach et al. 2004) and archaea (Winter et al. 2004). The studies were typically performed in the dark after removal of grazers by filtration. Viruses were found to have an effect on prokaryotic diversity. A stimulation of richness was not detected, but rather a change in the phylotypes present or even a reduction in the number of phylotypes occurred. In other studies using this approach, the presence of viruses usually reduced bacterial pro-

duction, based on ^{3}H-leucine or ^{3}H-thymidine incorporation, which led to the "reduction of biomass accumulation" hypothesis; however, sometimes the bacterial production was stimulated. Such a stimulation has been predicted by models (Fuhrman 1999) in which lysis reduces the carbon flow to higher trophic levels via grazing and the lysis products stimulate growth of non-infected cells; however, this was assumed when the entire microbial community was present.

To investigate how phages influence the diversity and activity of bacterioplankton and the link between diversity and activity, we carried out experiments in the southwestern Mediterranean gyre (two experiments each in the mixed layer and in the deep chlorophyll maximum layer) and in three environments in the Chinese Sea around Hong Kong (coastal water of Clearwater Bay, polluted Victoria Harbor, and river-influenced Peng Chau; two experiments each). In the Mediterranean offshore environment, viruses stimulated bacterial richness by 21 % and bacterial production was reduced (average 54 %); the effect on archaea, which was only detected in the deep chlorophyll maximum layer, was inconclusive. In the environments near Hong Kong, prokaryotic richness was always higher in the presence of viruses (21 % average); production was repressed in four experiments (34 % average) and stimulated in two experiments (Fig. 2). These data support the "killing the winner" hypothesis and suggest that the "reduction of biomass accumulation" hypothesis is typically but not always true.

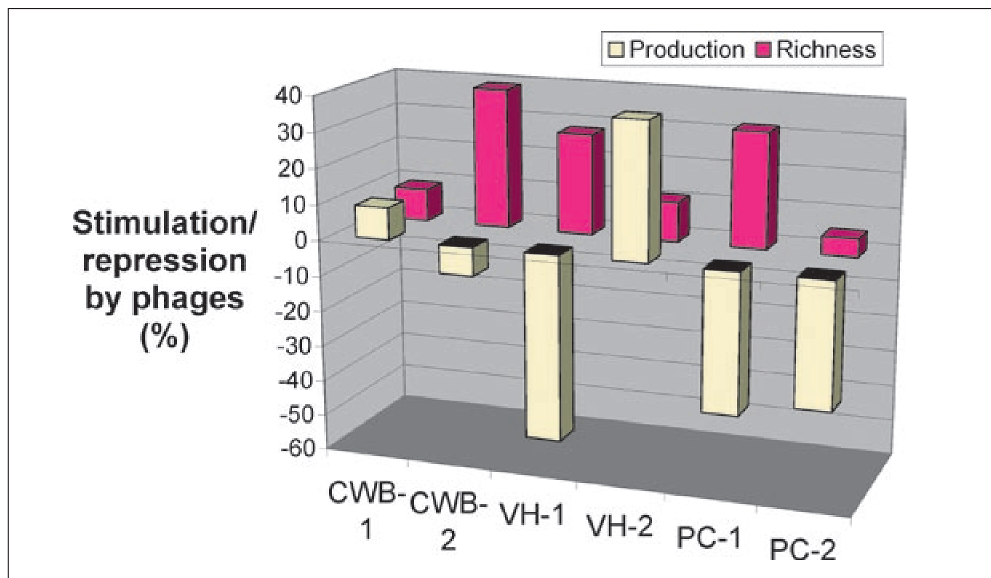

Fig. 2 Effect of viruses on bacterial diversity and production. Data are given as the percentage of stimulation or repression in the samples treated with viruses compared to that of the virus-free control. Data are from three environments in the Chinese Sea around Hong Kong representing polluted (Victoria Harbor, VH), river-influenced (PC), and "clean" (Clear Water Bay, CWB) coastal waters. Experiments were repeated within ca. 1 day (1 and 2). Diversity was judged as richness, i. e., the number of bands on a denaturing gradient gel after 16S rRNA gene PCR amplification, and bacterial production was measured using ^{3}H-leucine as precursor.

The difference in our results and those of other studies might be caused by the different experimental approaches used. In other studies, a concentrate of the natural virus community was added to bacteria in virus-free water. In our study, we inoculated the bacterial community at 10 % of the *in situ* abundance into virus-rich (0.2-µm-filtered water) and virus-free seawater fractions (filtered water, 100-kDa cutoff). This dilution stimulates bacterial growth, which might have resulted in a better detection of the effect of the presence and absence of viruses. Overall, our data indicate that viruses affect the link between the diversity and function of prokaryotes by stimulating diversity and reducing biomass accumulation.

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350 Jahre Leopoldina – Anspruch und Wirklichkeit

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Metabolic Interactions between the Carpenter Ant *Camponotus floridanus* and its Endosymbiont *Blochmannia*

Heike Feldhaar, Evelyn Zientz, and Roy Gross (Würzburg)

Abstract

Whole genome sequencing of bacteria allows a cultivation-independent bottom-up approach to an understanding of their metabolic capabilities – or in the case of endosymbiotic bacteria a prediction of their function in the host organism. The genome sequence in combination with gene expression studies of *Blochmannia floridanus*, the obligate intracellular endosymbiont of the carpenter ant *Camponotus floridanus*, enabled us to predict the nutritional function of the bacterium inside its host. Hypotheses based on the genome sequence were tested specifically with host ants *in vivo* using feeding experiments. Our results strongly suggest a role of the bacterium in the nutritional upgrading of the ant's diet by providing essential amino acids and a role in the recycling of nitrogen.

Zusammenfassung

Die Sequenzierung der Genome von Bakterien ermöglicht eine kultivierungsunabhängige Untersuchung ihrer metabolischen Fähigkeiten. Aus der Genomsequenz lässt sich vorhersagen, welche Funktionen z. B. endosymbiontische Bakterien für ihre Wirte haben können. Weiterhin ermöglicht ein solches Verfahren ein gezieltes Prüfen der Hypothesen zur Bedeutung einer solchen Symbiose. Die Auswertung der Genomsequenz sowie zusätzliche Genexpressionsstudien an *Blochmannia floridanus*, dem obligat intrazellulären Endosymbionten der Roßameise *Camponotus floridanus*, wiesen auf eine nahrungsaufwertende Funktion des Bakteriums hin. Fütterungsexperimente mit Ameisenkolonien unterstützen die Hypothese, dass *Blochmannia* seine Wirte tatsächlich mit essentiellen Aminosäuren versorgt und auch eine Rolle beim Stickstoffrecycling spielen kann.

An intracellular life style of bacteria within a eukaryotic host cell has developed many times during evolution (Buchner 1953). The best investigated cases are those of pathogenic bacteria which invade the eukaryotic host and exhibit parasitic phenotypes; these include important human pathogens such as the facultative intracellular members of the genera *Salmonella*, *Shigella* and *Listeria*, or obligately intracellular bacteria of the genus *Chlamydia*. On the other hand, intracellular interactions can be also beneficial for both partners, the bacterium and its host (Goebel and Gross 2001). Well known examples are interactions of intracellular bacteria with insects thriving on a diet scarce of important nutrients such as essential amino acids or vitamins (Douglas 1998). Such symbiotic interactions of bacteria with arthropods are very widespread, and it was estimated that up to 20 %

of insects carry such companions probably enriching their diet with essential nutrients (Buchner 1953). A common feature of these endosymbiotic bacteria of insects is their restricted occurrence within the host, i.e. only in specialized host cells, the so-called bacteriocytes, in addition to the reproductive tissue (Baumann et al. 1995, Buchner 1953). Among these interactions, those of *Buchnera aphidicola* with plant sap-sucking aphids and of *Wigglesworthia glossinidiae* with the blood-sucking tsetse flies have gained much attention (Baumann et al. 1995, Douglas 1998, Aksoy 1995). However, the first endosymbiotic interaction of bacteria with an animal host ever described was that of an intracellular bacterium with ants of the genus *Camponotus* (carpenter ants) which was reported in the 80s of the 19th century by Friedrich Blochmann. He observed these bacteria in the midgut tissue and in the reproductive organs of the infected animals (Blochmann 1882, Sauer et al. 2002). For many decades, the biological function of these bacteria remained a mystery because the carpenter ants were considered omnivorous, and a nutritional role of these bacteria for the host as mentioned above was not immediately obvious. The endosymbiotic bacteria of carpenter ants were recently classified in the novel genus *Blochmannia* in honour of their discoverer, Friedrich Blochmann (Sauer et al. 2000, Schröder et al. 1996). In addition to *Camponotus*, the bacteria were recently also identified in the related genera *Polyrhachis* and *Colobopsis*, all belonging to the subfamily *Formicinae* (Sameshima et al. 1999).

The genus *Camponotus* is among the most species-rich genera of ants and extremely successful; it virtually exists in most terrestrial habitats and is among the dominant ants in the canopies of tropical rain forests. The genus comprises more than 1,000 species, and all species investigated so far (>30) harbor *Blochmannia*, suggesting an important function of these bacteria in their host (Bolton 1996, Sameshima et al. 1999). The appearance of *Blochmannia* in closely related genera within the ant subfamily *Formicinae* suggests an age of the endosymbiosis of around 50 Million years. Phylogenetic analysis of the bacteria based on 16S rDNA sequences revealed a close relationship with other insect endosymbionts such as *Buchnera* and *Wigglesworthia* (Sauer et al. 2002, Sameshima et al. 1999). Like the latter two, these bacteria belong to the family *Enterobacteriaceae* within the Gammaproteobacteriae to which many environmental and pathogenic bacteria such as *E. coli*, *Salmonella* and *Yersinia* belong. The capacity to invade and survive in eukaryotic host cells is frequently observed with members of this family including the human pathogens mentioned above (Goebel and Gross 2001). In contrast to these mostly free-living bacteria, the endosymbiotic bacteria of insects could not be cultivated so far outside of their hosts. Therefore their endosymbiosis is considered to be obligate. Like other insect endosymbionts, *Blochmannia* is transmitted vertically between host generations via the germ line, leading to congruent phylogenetic trees of the bacteria and their host animals and indicating a strict co-evolution (Aksoy 1995, Baumann et al. 1995, Sauer et al. 2000, Wernegreen 2002).

Modern genome technology opens new approaches to investigate the biology of uncultured microorganisms such as the bacteriocyte endosymbionts. To date, the genomes of eight endosymbiotic bacteria residing in insects have been published: the genomes of three *Buchnera* species, *Wigglesworthia glossinidiae*, two *Blochmannia* species, *B. floridanus* and *B. pennsylvanicus* (Akman et al. 2002, Degnan et al. 2005, Gil et al. 2003, Shigenobu et al. 2000, Tamas et al. 2002, van Ham et al. 2003). These genomes revealed several common features:

(*i*) an extremely reduced genome size of between 600 and 800 kbp;
(*ii*) an extraordinary base composition with an extreme AT-bias (between 70 and 80 %);
(*iii*) the virtual absence of DNA-repair and recombination functions;
(*iv*) an extremely reduced repertoire of signal transduction and regulatory functions;
(*v*) a highly conserved genome structure and gene order within a genus; and
(*vi*) a high percentage of open reading frames having their closest orthologs in other members of the *Enterobacteriaceae*, indicating the exclusion of these bacteria from horizontal gene transfer events for millions of years (Wernegreen 2002).

This extreme reduction of genomic competence probably reflects the high degree of adaptation to a single ecological niche, namely to their insect host cell. On the other hand, the extreme genome reduction facilitates predictions concerning the biological role of these bacteria, because the functions retained in these "streamlined" genomes are expected to be essential for the endosymbiotic interaction with the host (Zientz et al. 2004).

A striking finding of these analyses is the fact that the metabolic capacity of these bacteria is very different from but in accordance with their respective host's nutritional requirements. For example, all *Buchnera* genomes encode genes required for the biosynthesis of amino acids essential to their aphid hosts, while those for non-essential amino acids were largely lost (Shigenobu et al. 2000, Tamas et al. 2002, van Ham et al. 2003). This is in line with the fact that the phloem sap, the sole diet of the host, is very poor in nitrogen compounds, in particular in essential amino acids. It is therefore likely that the bacteria enrich the poor diet of the host with essential nutrients; this has been suggested previously on the basis of feeding experiments with aposymbiotic animals which lost their bacterial companions, e. g. due to antibiotic treatment. In contrast, *Wigglesworthia*, the endosymbiont of tsetse flies, lost most genes encoding for amino acid biosynthesis while it retained many functions involved in the biosynthesis of cofactors and vitamins, functions which conversely have been lost in *Buchnera* (Akman et al. 2002, Nogge 1978). This is again in accordance with previous reports which suggested the supplementation of the vitamin-poor blood diet of the tsetse fly with these essential co-factors.

A nutritional basis of the ant – *Blochmannia* symbiosis is not obvious at first glance as the ants are considered to be omnivorous. However, the metabolic capacities of the two *Blochmannia* species sequenced so far show a remarkable similarity to those of *Buchnera*; biosynthetic pathways for essential amino acids are present while those of several non-essential amino acids have been lost (Gil et al. 2003, Zientz et al. 2004). Feeding experiments using a holidic artificial diet that allows specific exclusion of nutrients are in support of the hypothesis that *Blochmannia* – like *Buchnera* – provides its ant-host with essential amino acids (Straka 2006, Feldhaar unpublished). Ants that were fed with a diet lacking essential amino acids over a period of nine weeks were able to produce the same amount of brood as ants that were fed with a complete diet. When, in addition, ants were treated with antibiotics in order to reduce the number of endosymbiotic bacteria, the ants receiving a complete diet still raised a considerable amount of brood, whereas the ants fed with the diet lacking the essential amino acids raised significantly less brood. Thus supplementation of the diet with essential amino acids partially rescued the ants' ability to raise brood in aposymbiotic hosts.

A feature that is unique to *Blochmannia* in comparison to other endosymbiotic bacteria is the presence of a gene cluster encoding structural subunits and accessory factors of ure-

ase (Degnan et al. 2005, Gil et al. 2003). By means of quantitative RT-PCR we recently demonstrated that the expression of the *ureF* gene encoding an accessory factor required for maturation of a functional urease by nickel incorporation is differentially expressed during the development of the host animal, while the *ureA* gene encoding a structural urease subunit is constitutively expressed. *ureF* expression is lowest in the egg but strongly induced during the pupal stage of the animal. An expression pattern similar to *ureF* is followed by the *glnA* gene encoding glutamine synthetase which catalyzes the entrance of ammonium, a product of urease activity, into the cellular nitrogen pool (Zientz and Gross, unpublished). These observations indicate that a functional urease is present mainly during metamorphosis and that urease and glutamine synthetase act synergistically and play an important role in the development of the host by detoxification of toxic nitrogen compounds and nitrogen recycling during phases of high metabolic activity but shortage of food. In line with this presumptive high metabolic activity of the bacteria during metamorphosis, a strong replication of the bacteria was observed in particular during this phase (Wolschin et al. 2004). Moreover, these data indicate that despite of the very limited number of regulatory proteins encoded on the genome of *Blochmannia* at least some genes such as *ureF* are regulated on the transcriptional level. These data indicate that by their contribution to the nitrogen metabolism the bacteria may play an important role during host development. As ants are social insects with a complex colony structure, the endosymbionts may contribute to host fitness not only on the individual level but also on the colony level. Additionally, the importance of nutritional upgrading may vary during the host's ontogeny as food requirements change with life stage. In general, adult ants require a carbohydrate-rich diet to maintain their activity levels, whereas the brood as well as the queen require a protein-rich diet for growth or egg production, respectively (Wheeler 1994).

In accordance with their nutritional requirements, the bacteria appear to be less important for adult animals, since elimination of the bacteria from the animals by antibiotics did not impair the adult animals and their longevity (Sauer et al. 2002). In fact, histological investigation of the midgut tissue of old individuals indicates a degeneration of the symbiosis with increasing age of the animals. However, experiments with workers treated with antibiotics revealed that the success of brood-raising by the resulting aposymbiotic workers is significantly reduced, which may be due either to the reduction of the endosymbiotic population in the brood itself or in the workers caring for the brood. When treatment with antibiotics was stopped and workers were given untreated (not treated with antibiotics) brood to care for, they still had a lower capacity to raise brood in comparison to control colonies; this indicated that the presence of *Blochmannia* is not only important within the larvae, but may still be important in the adult workers caring for the brood (Feldhaar and Gross, unpublished). Under natural conditions only younger workers that still house *Blochmannia* at high level care for the brood; older workers containing fewer endosymbionts forage for food outside of the nest and no longer take care of the brood. Apparently, the symbiosis degrades in older workers as soon as it is no longer advantageous for themselves and the colony.

A role of *Blochmannia* in nutritional upgrading is supported by recent studies on the trophic level of arboreal ants. These studies revealed that *Camponotus* as well as *Polyrhachis* species – at least in tropical areas where these two genera are among the most species-rich and abundant ones – show stable isotope signatures similar to insects with a strictly herbivorous diet, as for example aphids (Blüthgen et al. 2003, Davidson et al. 2003). Ants

of the genera *Camponotus* and *Polyrhachis* should therefore largely rely on plant-derived resources like floral or extrafloral nectar or honeydew produced by aphids and other plant sap-sucking insects. Such exudates are very rich in carbohydrates but very poor in nitrogen compounds. Thus, due to the symbiosis with *Blochmannia*, these ants may have been less dependent on a steady influx of prey for colony development and were then able to successfully occupy nitrogen-limited niches like the canopy in tropical rain forests. The endosymbiosis may thus have contributed significantly to the success and radiation of the members of the genera *Camponotus* and *Polyrhachis* that together comprise approximately 15 % of all hitherto described ant species.

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Photorhabdus Endosymbiosis and Pathogenesis

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With 2 Figures

Abstract

Photorhabdus luminescens is a Gram-negative enterobacterium with a life cycle involving both symbiotic and pathogenic interactions. Insect pathogenicity by *Photorhabdus* is mediated by a variety of toxins and exoenzymes whose expression and mechanisms of action are still under characterization.

Zusammenfassung

Photorhabdus luminescens ist ein Gram-negatives Enterobakterium mit einem Lebenszyklus, der sowohl symbiotische als auch pathogene Wechselbeziehungen beinhaltet. Die Pathogenität gegenüber Insekten wird durch verschiedene Toxine und Exoenzyme vermittelt, deren Expression und Wirkungsmechanismen gegenwärtig charakterisiert werden.

Photorhabdus luminescens can establish symbiosis with entomopathogenic nematodes belonging to the Heterorhabditidae family. Bacteria are found colonizing the gut of the free-living nematode which is in the infective juvenile stage (IJ). At this stage the nematode can persist in the soil or invade the insect host. The *Heterorhabditis* infective juvenile is able to enter insects via natural openings such as the spiracles, mouth or anus and migrate to the hemocoel (open circulatory system) where they regurgitate the bacteria. A single IJ is enough to establish a successful infection in the insect (FORST and CLARKE 2001). Once in the hemocoel, *Photorhabdus* replicates (DABORN et al. 2001) and delivers toxins that rapidly kill the insect. *P. luminescens* is known to colonize the *Manduca* hemocoel (open circulatory system) and gut prior to insect death. The bacteria spread within the insect body colonizing the fat body, gut and other tissues (30 to 42 hours post infection; SILVA et al. 2002). During these stages, the bacteria release several toxins and exoenzymes that play a role in insect death and bioconversion of the insect body. Bioconversion of the cadaver is essential for bacteria replication and, in turn, for successful reproduction of the nematodes. Thus, *Heterorhabditis* can only grow in the insect when *Photorhabdus* is present (GERRITSEN and SMITS 1997, GERRITSEN et al. 1998, HAN and EHLERS 2000). After several cycles of growth and reproduction, the insect cadaver is exhausted and the infective juveniles reacquire the bacteria and leave the host to colonize new insects.

Several strains of *Photorhabdus luminescens* (TT01, W14) and *Photorhabdus temperata* (K122) have been found to carry out the above described life cycle, but a third species has been isolated from human wounds: *Photorhabdus asymbiotica*. Several clinical isolates have been reported mainly from Australia and the United States (Gerrard et al. 2004). *P. asymbiotica* is also pathogenic to insects, and a nematode symbiont has been recently described (Gerrard et al. 2006). Sequencing of *P. asymbiotica* strain ATCC43949 genome is nearly completed at the Sanger Centre (Cambridge, UK), and the genome sequence of *P. luminescens* strain TT01 is also available (http://genolist.pasteur.fr/PhotoList/). These genomic tools will allow comparisons within different species of *Photorhabdus* and with other related bacteria (such as *Yersinia*) and will help us to understand the evolutionary steps for diversification, and the requirements to become pathogens to different hosts.

An interesting feature in *Photorhabdus* genome is the presence of pathogenicity islands (PAIs). These regions in the genome contain genes encoding toxins or pathogenicity factors. Four pathogenicity islands (PAIs I – IV) are found in *P. luminescens* containing the following genes: *tc* (toxin complex), *mcf* (makes caterpillar floppy), *pnf* (putative necrotizing factor) and TTSS (type three secretion system) (Waterfield et al. 2004).

Pathogenicity island I contains the *toxin complex* (*tc*) genes, which are found in variable copy number in all *Photorhabdus* strains. Homologues of the *tc* genes are present in other bacteria, some of which interact with insects (*Serratia entomophila* and *Yersinia pestis*) and others with not known insect association (*Pseudomonas syringae* and *Fibrobacter succinogenes*) (Waterfield et al. 2001, ffrench-Constant et al. 2003).

Four "*tc*" loci are found in *P. luminescens* (*tca, tcb, tcc* and *tcd*), each containing genes encoding for different modules: the "A" genes encode toxins and "BC" potentiators (i. e. *tcdA*, *tcdB*, and *tccC*). Recent experiments suggest that "B" genes also encode toxins and "C" a protein involved in processing/modification of the "B" toxin, effectively synergizing the "A" toxins. Furthermore, a BC pair can cross-synergize an A toxin encoded elsewhere in the genome (Waterfield et al. 2005).

The Tc toxins were originally identified as high molecular weight insecticidal complexes present in the supernatant of *P. luminescens* strain W14 (Bowen et al. 1998, Bowen and Ensign 1998). Tc toxins have oral and injectable activity against *Manduca sexta* (Waterfield et al. 2001a,b). The oral activity against caterpillars makes them valuable alternatives to the *Bacillus thuringiensis* toxin (Bt) for the development of transgenic crops (Waterfield et al. 2005).

Although the powerful effects of these toxins have been proved, we have limited knowledge about when and where the *tc* genes are expressed in bacterial cultures and, in particular, within the host during infection. The expression of *tcaB* (encoding an "A" toxin) and *tcdB* ("B" toxin) was analyzed *in vivo* and *in vitro* (Fig. 1) by RT-PCR. *tcaB* was expressed in late exponential/early stationary phase, correlating with the insect death, while *tcdB* is expressed in stationary phase corresponding to the time of bioconversion of the insect body (Daborn et al. 2001). The production of Tc proteins *in vivo* has been documented by immunolocalization experiments. During *Manduca* infection, the TcaC protein is found in the gut niche, in close proximity to *P. luminescens* cells, suggesting that it is expressed on the outer surface of bacteria (Fig. 2) (Silva et al. 2002). It is possible that Tc's have a role in adhesion to the insect gut, but the specific mode of action remains to be elucidated.

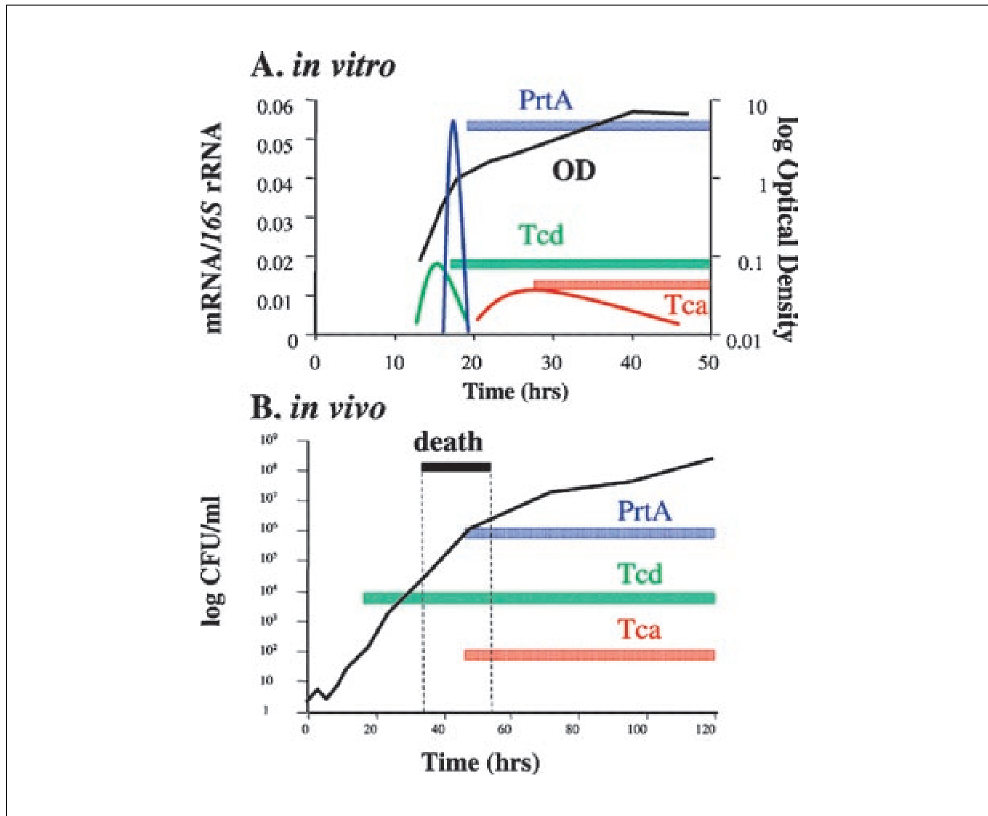

Fig. 1 Measuring the expression of candidate virulence factors *in vitro* (bacterial culture) and *in vivo* (insect infection). (*A*): *In vitro* mRNA production measured by quantitative RT-PCR is indicated by solid lines: blue for *prtA*, green for *tcd* and red for *tca*. The shaded boxes with the same colour codes correspond to measures of protein production. (*B*): *In vivo*, virulence factor proteins detected within infected *Manduca*. Tcd is produced before the insect dies, suggesting that it may play a role in insect death, while Tca is produced after insect death suggesting a role in bioconversion. PrtA protease activity is detected after insect death supporting its role in bioconversion (ffrench-Constant et al. 2003).

The *mcf1* (*makes caterpillars floppy*) gene was identified by screening a cosmid library, and it is located in Pathogenicity island II. Mcf1 toxin is very potent and causes loss of body turgor (floppy phenotype) in *Manduca* larvae within 12 hours and death around 24 hours post injection (Daborn et al. 2002). The predicted sequence of Mcf1 protein has a consensus BH3 domain-like motif. Proteins containing only this domain are pro-apoptotic. Mcf1 triggers apoptosis in both hemocytes and the midgut epithelium of *Manduca sexta*, and Mcf1 expression in *E. coli* is sufficient to allow persistence of bacteria in the insect and to cause host death (Daborn et al. 2002). Experiments using three different mammalian cell lines showed that *E. coli* expressing Mcf1 caused apoptosis, inducing nuclear fragmentation and activation of caspase-3 (an apoptosis marker) (Dowling et al. 2004). A second gene *mcf2* encoding a similar toxin was found elsewhere in the genome. The predicted Mcf2 protein sequence is similar to Mcf1 in the central and carboxy terminal domains. In

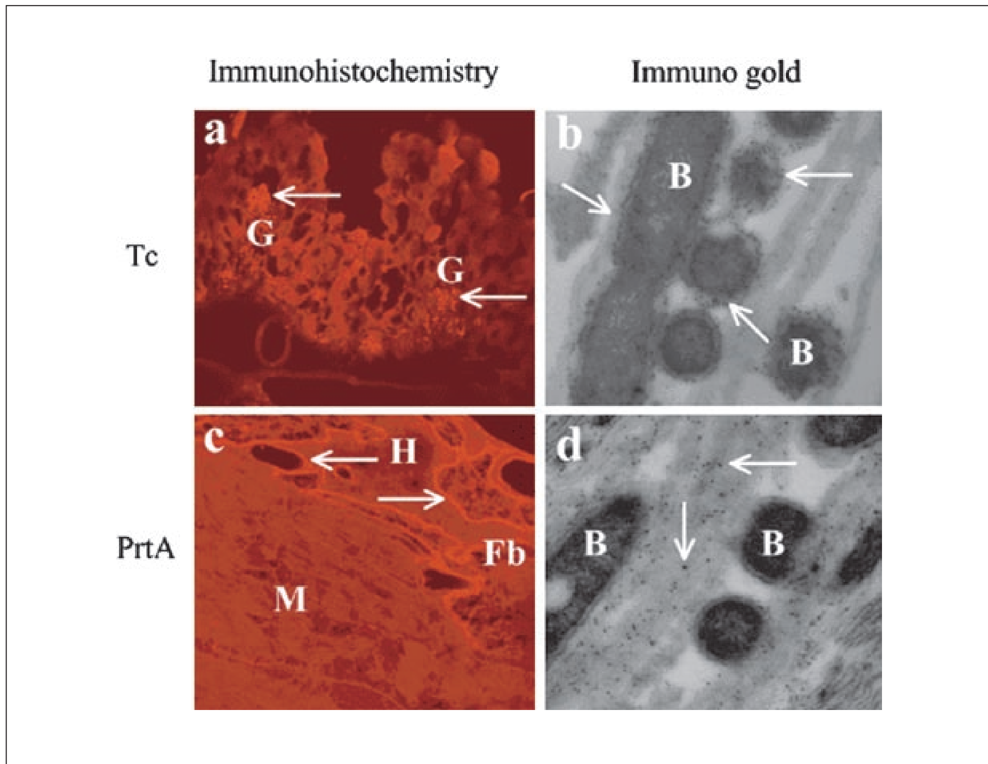

Fig. 2 Immunocytochemistry of the TcaC toxin component and the PrtA protease. Detection of anti-Tca and anti-PrtA immunoreactivity using either a fluorescent (*a*, *c*) or gold particle antibody conjugate (*b*, *d*). (*a*): Tca is detected in the folds or grooves (G) of the midgut epithelium where bacteria accumulate. (*b*): Examination of individual bacteria (B) shows that Tca is displayed on the bacterial surface. (*c*): PrtA is detected on the basal lamina of structures within the hemocoel (H), such as fat body (Fb) and muscle (M). (*d*): Detailed examination shows that PrtA is associated with sheets of lamina surrounding *Photorhabdus* cells (B) within their midgut niche (FFRENCH-CONSTANT et al. 2003).

the amino terminal domain, Mcf2 is similar to HrmA an avirulence protein from the plant pathogen *Pseudomonas syringae*, lacking the BH3 motif present in Mcf1. *E. coli* expressing Mcf2 can persist into and kill insects (WATERFIELD et al. 2003). The functional redundancy of Mcf1 and Mcf2 raises the question of whether these toxins are addressed to different insects or they might have different sites of action within the host.

A third PAI (PAI III) encodes a homologue of a cytotoxic necrotising factor (CNF)-like toxin gene named *pnf*. The CNF1 protein from urophatogenic *E. coli* causes cytoskeleton rearrangements in the host cells (FLATAU et al. 1997, SUGAI et al. 1999). The function of Pnf in *Photorhabdus* is unknown, but the carboxy-terminal region and the active site are conserved (BUETOW et al. 2001). Other genes in this PAI III are phage related and have similarity with genes from the cyanobacterium *Nostoc* sp. PCC7120 (WATERFIELD et al. 2004). This region seems to represent a novel group of mobile elements and have been named ***P**hotorhabdus* **V**irulence **C**asettes (PVC). *pnf* and other putative virulence factors are encoded in a carriage region adjacent to the conserved PVCs. Homologues of the PVC genes

are found in a large plasmid in *Serratia entomophila* and confer anti-feeding activity on beetle larvae. Injection of *Photorhabdus* PVCs destroys insect hemocytes, which undergo actin cytoskeleton condensation (Yang et al. 2006).

The fourth region (PAI IV) encodes a type III secretion system (TTSS). *Pseudomonas aeruginosa* and *Yersinia pestis* have similar TTSS islands. In *Y. pestis* the YopBD and YopR effectors are secreted and necessary for infection in the mouse model (Fields et al. 1999). The *lopT*, *lscH* and *lopBD* genes in *Photorhabdus* are homologues of the *Yersinia yopT*, *yopR* and *yopBD* genes, respectively. Heterologous expression of LopT in *Yersinia* has showed that this protein is translocated into mammalian cells in an active form. Furthermore, the *lopT* gene is only switched on at sites of cellular defense reaction during infection, such as insect nodules. A *Photorhabdus* mutant in TTSS did not induce the formation of nodules in insects and bacteria were phagocytosed by insect macrophages (Brugirard-Ricaud et al. 2005). These data show a direct implication of TTSS in *Photorhabdus* pathogenicity, indicating a role for LopT in avoiding phagocytosis during insect infection.

In addition to toxins, exoenzymes also play an important role in infection of the host. The number and different types of proteases can vary among strains. During the post-exponential growth phase, *Photorhabdus* produces a variety of exoenzymes the best studied of which is PrtA (Fig. 1) (Wang and Dowds 1993, Clarke and Dowds 1995, Daborn et al. 2001). The localization of PrtA in the insect gut during *Photorhabdus* infection is restricted to the basal laminae of the internal structures of the insect (Fig. 2). The basal laminae form a barrier between the fat body and the hemocoel. The PrtA protease could be involved in the destruction of this barrier allowing bacteria to access other tissues (ffrench-Constant et al. 2003).

Finally, a number of questions remain to be addressed in future experiments. Which is the mode of action of these toxins and proteases in the insect at cellular level? When and where toxin genes are expressed during the infection and bioconversion processes? And how the expression patterns relate to the insect phenotypes? Which are the regulators involved in the switching between symbiosis and pathogenesis? What is the role of these toxins in other organisms?

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BSE – Status quo und Quo vadis?

Gemeinsames Symposium der Deutschen Akademie der Naturforscher Leopoldina und der Österreichischen Akademie der Wissenschaften (ÖAW) vom 25. bis 26. Juli 2005 in Wien

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TSEs (Transmissible spongiforme Enzephalopathien) sind übertragbare Hirnerkrankungen mit schwammartigen degenerativen Veränderungen bei Mensch und Tier, die stets tödlich verlaufen. Besonders bekannt wurde die Bovine spongiforme Enzephalopathie (BSE). Noch immer gibt es weder eine verlässliche Prophylaxe noch Therapie. Seit 1996 hat die Deutsche Akademie der Naturforscher Leopoldina die Problematik wiederholt aufgegriffen und eine verbesserte und international koordinierte Tierseuchenbekämpfung, unabhängig von nationalstaatlichen Vorgaben, gefordert. Ziel der Veranstaltung 2005 war es, einen Überblick des gegenwärtig verfügbaren Wissens und des Stands der Forschung zu liefern. Der Band diskutiert die Auswirkungen der BSE-Problematik auf Landwirtschaft, Veterinärmedizin und Pharmaindustrie sowie die Wahrnehmung des „Rinderwahnsinns" in Medien und Öffentlichkeit. Behandelt werden außerdem die neuesten Forschungen zur Prion-Hypothese und zu Fragen von Anfälligkeit/Resistenz sowie zu genetischen Komponenten bei verschiedenen Tierarten, darüber hinaus werden Pathogenese-Modelle und medizinisch-therapeutische Ansätze sowie Maßnahmen zur Sicherheit in den Nahrungs- und Futtermittelketten erörtert. Die BSE-Thematik ist auch nach über zehn Jahren intensiver Forschung noch von hoher wissenschaftlicher, gesellschaftlicher und politischer Brisanz.

Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

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Evolution of Bacterial Virulence

Jörg Hacker ML, Ulrich Dobrindt, Tobias Ölschläger,
Hilde Merkert, and Gabriele Blum-Oehler (Berlin/Würzburg)

With 2 Figures

Abstract

A microbe–host interaction can be a symbiosis, commensalism, or pathogenic process. Pathogenic processes involve the presence and expression of virulence factors. Such factors have evolved through gene transfer, rearrangements, deletions, and point mutations. Bacteria–host interactions have been studied in much detail at the molecular level in *Escherichia coli*.

Zusammenfassung

Bei einer Mikroben-Wirts-Wechselwirkung kann es sich um eine Symbiose, einen Kommensalismus oder einen pathogenen Prozess handeln. Bei Infektionsprozessen spielen Pathogenitätsfaktoren eine große Rolle. Bei deren Evolution spielen Genübertragungen, Deletionen, DNA-Rearrangements sowie Punktmutationen eine zentrale Rolle. Mikroben-Wirts-Interaktionen wurden sehr detailliert auf molekularer Ebene in *Escherichia coli* untersucht.

1. Bacteria–Host Interactions

Bacteria–host interactions can lead to symbiosis, commensalism, and pathogenesis, and many examples are known. During symbiosis, e. g., of bacteria and sponges, at least one partner benefits from the other. During commensalism, the partners share physical space; there is, however, no direct evidence for benefit or detriment; the interaction between *Legionella* and amoebian cells is an example of commensalism. Pathogenesis, by definition, leads to the detriment of one partner following the action of the other (see Hacker and Carniel 2001). For example, various enterobacteria, such as *Shigella*, *Salmonella*, *Yersinia*, and *Escherichia coli* can infect various hosts.

2. *E. coli* as a Tool for Study of Pathogenesis

E. coli represents an excellent model system to study the molecular processes of commensalism as well as pathogenesis. As a commensal bacterium, it colonizes the intestine of hu-

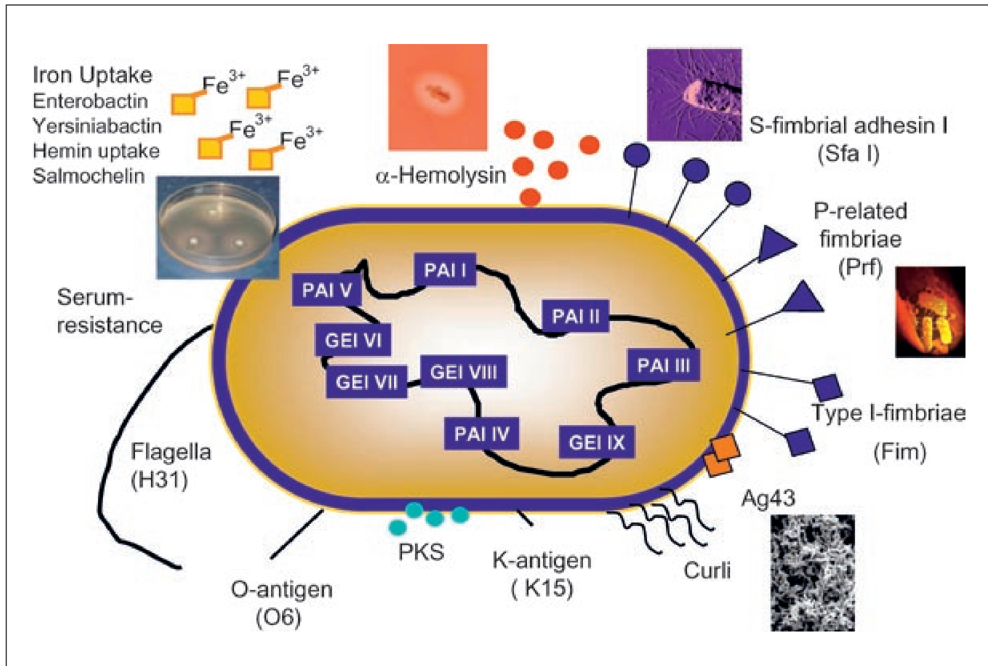

Fig. 1 Various pathogenicity factors of the pathogenic *E. coli* strain 536. The encoding genes are located on pathogenicity islands (PAI I–V) and genomic islands (GEI VI–IX).

mans and many animals and can even contribute to the host metabolism. As a pathogen, it can infect the gut and other organs and tissues, and lead to, for example, urinary tract infections, sepsis, and newborn meningitis.

Since the 1980s, it is known that pathogenic *E. coli* produce various virulence factors or pathogenicity factors that contribute to the infection process (Fig. 1). The pathogen produces specific adherence molecules that can lead to the colonization and thereby infection of the urinary tract. Capsules, O-antigens, outer membrane proteins, and toxins are involved in immune evasion and tissue destruction. Furthermore, modulins and invasins play a role in invasion, which may lead to bloodstream infections and infections of the brain. The specific interplay between the different virulence factors and host structures can result in different types of infection, sometimes developing from an acute to a chronic state.

3. Genome Analysis of Pathogenic and Commensal Isolates

To learn more about the pathogenic processes during urinary tract infections and the evolution of microbial pathogenesis, the complete genomes of different *E. coli* isolates have been sequenced over the past few years. Mobile elements comprise up to 30 % of the gene pool of both pathogens and commensals (Brzuszkiewicz et al. 2006). In pathogens, mobile genetic elements are often associated with genes necessary for the production of virulence factors. These genes are often found in pathogenicity islands – large DNA regions in the genomes of pathogenic *E. coli.* Pathogenicity islands were acquired by horizontal

gene transfer and evolved from former mobile DNA, such as bacteriophages and plasmids, which play a role as carriers of these virulence genes in intestinal *E. coli* (Schubert et al. 2004). Together with the Göttingen Genomics Laboratory, we sequenced the complete genome of the pathogenic *E. coli* strain 536. This strain carries five pathogenicity islands (Dobrindt et al. 2002, 2003, Brzuszkiewicz et al. 2006).

DNA regions similar to pathogenicity islands are also part of the genomes of commensal isolates (Grozdanov et al. 2004). However, in commensal bacteria, these genomic islands probably do not contribute to infections, but they might contribute to the fitness of the respective strains and to the adaptation of the microbes to particular environments. The commensal *E. coli* strain Nissle 1917, for instance, carries genomic islands encoding iron uptake systems and specific adherence molecules. In contrast to the classic pathogenicity islands of pathogenic strains, the genomic islands of commensal strains do not encode toxin determinants and gene clusters encoding adherence molecules (Grozdanov et al. 2002, Nougayrède et al. 2006). There appears to be a continual conversion of pathogenic to non-pathogenic strains, and *vice versa*.

4. Evolutionary Aspects

These differences in the genomes of pathogenic and non-pathogenic bacteria lead to the conclusion that genome variability is a key process in the evolution of pathogenic bacteria.

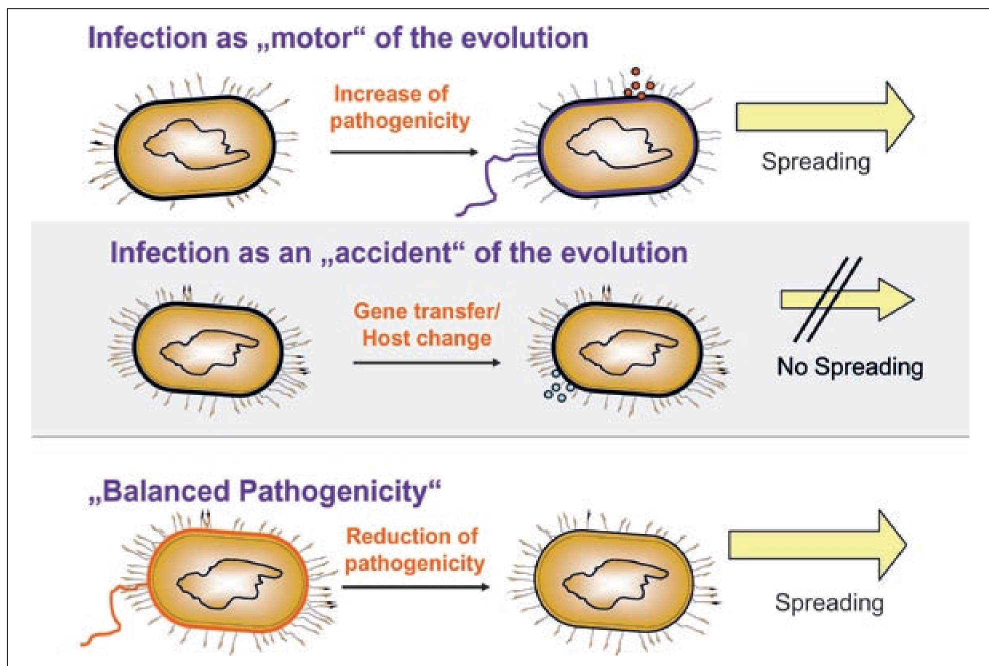

Fig. 2 Evolution of bacterial pathogens. The increase of pathogenicity can act as a “motor of evolution”. Zoonotic interactions very often represent an “accident” of evolution. In addition, various pathogens lose pathogenic potency during evolution.

Horizontal gene transfer via plasmids and bacteriophages, which results in the establishment of pathogenicity islands, represents a key event in the evolution of pathogenic bacteria (Hochhut et al. 2006). Furthermore, DNA rearrangements, deletions, and point mutations contribute to the evolution of pathogens. Some of the pathogenic bacteria increase their pathogenic potency as they evolve. Intestinal pathogens and respiratory pathogens might upgrade their fitness by increasing their pathogenic potency. In the majority of cases, however, there has been a reduction in pathogenicity during evolution (Middendorf et al. 2004). Thus, many pathogens, including urinary tract and skin pathogens, show a reduced pathogenicity in the long term, leading to the development of commensal organisms. This concept is termed "balanced pathogenicity" (Fig. 2).

Furthermore, serious pathogens are often transferred from animals to humans. Such zoonotic infections, e. g., salmonellosis (like the viral HIV infection and influenza), may be considered as "accidents of evolution" since they do not result from a co-evolution of pathogenic microbes and host organisms, but rather from a transfer from one host to the other. Many questions regarding the evolution of pathogens and commensals remain open. The powerful methods of DNA sequencing and functional genomics should result in new insights into the evolution of microbial pathogens.

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BVD – eine (un)heimliche Rinderseuche

Gemeinsames Symposium
der Deutschen Akademie der Naturforscher Leopoldina und
der Österreichischen Akademie der Wissenschaften (ÖAW)
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Die Rinderseuche BVD, die Bovine Virusdiarrhoe, mit ihrer letalen Form, der *Mucosal Disease*, ist, im Gegensatz zur BSE, der Bovinen spongiformen Enzephalopathie, keine Zoonose, sie ist für den Menschen an sich ungefährlich, aber dennoch für Rinderhalter von beträchtlicher wirtschaftlicher Bedeutung. Der BVD-Virus nutzt für seine Weiterverbreitung den noch vom Immunsystem ungeschützten fetalen Organismus als Eintrittspforte. Die Seuche lebt in den persistent infizierten Tieren weiter.
Der Band gibt einen Überblick des gegenwärtigen Forschungsstandes zu BVD. Er behandelt den BVD-Virus und die Infektion, die Erkrankung, Immunität und Immunisierung, die BVD-Erregerdiagnostik sowie die erforderlichen Maßnahmen der Tierseuchenbekämpfung.

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The Problem of Endemic Bacteria

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Abstract

Multilocus sequence typing (MLST) represents a method to describe the genetic diversity of organisms at the allelic level. Until now, more than 35 bacterial species have been analyzed by applying MLST schemes. The MLST technique is based on the sequence analysis of DNA sequences of internal fragments of bacterial housekeeping genes which mirrors the allelic diversity rather than the sequence diversity of particular species. Thus, different isolates can be grouped into different sequence types (ST) which form so-called ST complexes. One problem with this approach is that it works well if highly recombinogenic organisms are compared but in bacteria for which recombination is rare allele-based analysis is sometimes misleading. Nevertheless, distinct population structures can be described by MLST analysis. In principle, bacteria can be separated into species that use extensive homologous recombination (e. g. *Helicobacter pylori*) and groups of low and intermediate levels of recombination (e. g. *Escherichia coli*). A third population structure (e. g. *Yersinia pestis*) is specific for young groups of bacteria. In this article, the advantages and limitations of MLST will be discussed with a focus on endemic bacteria. The pathogenic bacteria *Yersinia pestis*, *Escherichia coli* and *Helicobacter pylori* are described as particular examples.

Zusammenfassung

Die Methode des „multilocus sequence typing“ (MLST) besitzt den Vorteil, dass die genetische Diversität von Organismen auf dem Allel-Level beschrieben werden kann. Momentan sind über 35 bakterielle Arten im Hinblick auf ihre MLST-Muster analysiert worden. Die MLST-Technik ist vor allem geeignet, um evolutionäre Entwicklungen von Mikroorganismen auf Grundlage der Allel-Diversität zu untersuchen. Meist werden die DNA-Sequenzen von sieben Haushaltsgenen bestimmt, und aus den unterschiedlichen allelen Varianten lassen sich dann spezifische Sequenztypen (STs) unterscheiden, die in sogenannte ST-Komplexe gruppiert werden. Ein Problem bei der Anwendung der MLST-Technik ergibt sich aus dem Einfluss der Häufigkeit von Rekombinationsereignissen auf die verwandschaftlichen Beziehungen. Mit Hilfe der STs ist es möglich, distinkte Populationsstrukturen aufzuspüren und Arten zu unterscheiden im Hinblick auf eine extensive homologe Rekombination (z. B. *Helicobacter pylori*) bzw. eine niedrige oder intermediäre Rekombinationsrate (z. B. *Escherichia coli*). Eine dritte Populationsstruktur bilden die sogenannten „jungen Bakterien-Arten“ (z. B. *Yersinia pestis*). In dem vorliegenden Artikel werden die Vorteile und Limitationen der MLST-Technik diskutiert und am Beispiel von endemisch vorkommenden Bakterien, insbesondere von *Yersinia pestis*, *Escherichia coli* und *Helicobacter pylori*, beschrieben.

Since its initial description in 1998 (MAIDEN et al. 1998), multilocus sequence typing (MLST) has been used to describe the genetic diversity at the allelic level of at least 35 bacterial species (http://pubmlst.org/databases.shtml). Characterization of individual isolates and long-term epidemiology are among the primary goals of many of these schemes but the results

also provide valuable sources of sequence data that can assist the elucidation of population structures and evolutionary mechanisms (Urwin and Maiden 2003, Feil 2004). The focus of MLST on allelic diversity rather than sequence diversity is partially because it is easy to implement and defend: each unique multilocus sequence type (ST) receives a unique designation and sets of related STs that differ sequentially by no more than a limited number of alleles (typically two of the seven examined) are grouped into so-called ST complexes. Sequence-based analyses present greater problems due to homologous recombination: the concurrent acquisition of multiple nucleotides can distort genetic relationships and obscure branch structure (Holmes et al. 1999, Schierup and Hein 2000). Indeed, due to extensive homologous recombination within multiple species, sequence-based trees often do not reliably recover the original phylogeny (Feil et al. 2001) and even allele-based approaches face problems due to merging of ST complexes as databases grow larger that require novel approaches.

Analyses of different species that have been examined by MLST indicate that there is no single "problem of endemic bacteria", but instead, as many distinct problems as there are distinct population structures. Furthermore, even single bacterial species may encompass multiple, distinct population structures. Yet, one might be tempted to separate bacteria in general into those with very extensive homologous recombination (e.g. *Helicobacter pylori*) (Suerbaum et al. 1998, Falush et al. 2001) *versus* those with low to intermediate levels of recombination (e.g. *Escherichia coli*) (Feil et al. 2001). Still other organisms (e.g. *Yersinia pestis*) represent a third population structure, that of young bacteria where the analysis of populations structures is difficult due to exceedingly low levels of sequence diversity (Sreevatsan et al. 1997, Achtman et al. 1999).

MLST provides no resolution whatsoever within *Y. pestis*, where six housekeeping gene fragments yielded no sequence diversity among 26 isolates from a global sample (Achtman et al. 1999). High resolution fingerprinting techniques have been used to subdivide this species; these include variable numbers of tandem repeats (VNTRs, MLVA) whose length varies among different isolates (Girard et al. 2004, Pourcel et al. 2004), genome-wide microarrays to detect genes and gene clusters that are variably present (Hinchliffe et al. 2003), and the presence or absence of insertion elements (IS elements) at multiple genomic locations (Motin et al. 2002). None of these techniques is ideal for resolving species-wide population structures. VNTRs and IS elements are plagued by high levels of homoplasies, which result in serious distortions of branch order (Achtman et al. 2004). Surprisingly, the resolution provided by microarrays is not particularly high, and microarray analyses face technical problems and occasional homoplasies as well. We have focused on single nucleotide polymorphisms (SNPs) that were revealed by genomic comparisons (Achtman et al. 2004). A comparison of three 5 MB genomes revealed a total of only 76 synonymous SNPs (sSNPs), that have accumulated over the past 20,000 years and whose distribution defined three branches and multiple populations within this species. In recent, still unpublished analyses we have screened 269 gene fragments among 200 isolates from China and global sources. These analyses have defined ≥9 distinct populations and additional subpopulations whose distribution shows phylogeographical structure that may reflect geographical differences among the host and vector species within which *Y. pestis* is endemic. Further progress will be facilitated by the multiple genome sequences that are currently in progress.

MLST is eminently suitable for analyzing the population structure of species such as *E. coli* with intermediate levels of genetic diversity and recombination. The population size of this species underwent a major contraction approximately 10–40 Myrs ago that elimi-

nated much of the preexisting ancestral diversity (Wirth et al. 2006). Most modern isolates reflect a subsequent expansion of four ancestral populations over the past 5 Myrs. Traces of the four ancestral populations are still evident in many modern isolates, leading to the assignments to groups called A, B1, B2 and D by a variety of methods, including MLST. But homologous recombination has been so frequent that one third of modern isolates possess considerable ancestry from two or more of these ancestral populations. Unlike *Y. pestis* or *H. pylori*, there is no discernable phylogeographic pattern of genetic diversity within *E. coli*. Instead, STs and ST Complexes correlate to a certain degree with virulence and pathogen type. One group of ST Complexes is specific for *Shigella*, and a second group is specific for EIEC (Enteroinvasive *E. coli*) strains, both of which cause dysentery. Bacteria expressing the K1 capsular polysaccharide which are isolated from newborn meningitis, urinary tract infections and invasive diseases of domesticated poultry belong to other ST Complexes while still other ST Complexes contain EPEC (Enteropathogenic *E. coli*) and EHEC (Enterohemorragic *E. coli*) bacteria that cause gastroenteritis and haemolytic uremic syndrome. Each of these pathogen types has evolved on multiple occasions.

Even basic genetic parameters such as mutation and recombination rate are heterogeneous within *E. coli* and seem to increase with the degree of virulence. We propose that increased virulence correlates with increased exposure to the host immune system, resulting in increased selection for the rate of evolution, possibly associated with higher frequencies of mutator strains.

Sequences of *H. pylori* have been subjected to so much homologous recombination that it is extremely rare to find identical. 500 bp sequences in isolates from unrelated individuals (Falush et al. 2003). In addition, transmission seems to be predominantly associated with close contact between their human hosts, resulting in extremely strong correlations between the genetic sequences of *H. pylori* and the geographic sources of the host. As a result, traces of human migrations within the last several thousand years are readily detectable among these bacteria (Falush et al. 2003) and their genetic diversity is in accord with human history in remote areas (Wirth et al. 2004, 2005) or aboriginal populations (Ghose et al. 2002, Yamaoka 2002). Thus, the study of population structure within this species becomes a study of the history of anatomically modern humans. Our recent results (Linz et al. 2007) suggest that *H. pylori* has accompanied human migrations over the last 100,000 years and contains signals of the "Out of Africa" human migrations followed by multiregional expansion in Africa and Asia.

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Legionella Type IV Effectors that Target Conserved Host Proteins

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Abstract

Legionella pneumophila is an intracellular pathogen that replicates in evolutionarily diverse hosts. The Dot/Icm transporter is a protein secretion apparatus that is essential for *L. pneumophila* pathogenesis. This transporter has the ability to inject bacterial proteins into eukaryotic host cells. These proteins are predicted to regulate the formation of a vacuole that supports intracellular growth of *L. pneumophila*. Here, we show that the Dot/Icm system injects proteins into host cells that modulate the function of evolutionarily conserved eukaryotic proteins involved in vesicle transport.

Zusammenfassung

Legionella pneumophila ist ein intrazelluläres pathogenes Bakterium, das sich in Wirten diverser evolutionärer Abstammung vermehrt. Bei dem Dot/Icm-Transporter handelt es sich um einen für den Pathogenitätsmechanismus von *L. pneumophila* essentiellen Protein-Sekretionsapparat. Dieser kann bakterielle Proteine in eukaryotische Wirtszellen injizieren. Vorhersagegemäß regulieren diese Proteine die Ausbildung einer Vakuole, welche das intrazelluläre Wachstum von *L. pneumophila* fördert. In der vorliegenden Studie zeigen wir, dass das Dot/Icm-System in die Wirtszelle Proteine injiziert, welche die Funktion evolutionär konservierter eukaryotischer Proteine modulieren; letztere sind am Vesikeltransport beteiligt.

Legionella pneumophila strains are Gram-negative bacteria that have the unique ability to replicate within evolutionarily diverse phagocytes. In nature, *L. pneumophila* is found ubiquitously in fresh water environments (Fields 1996). Free-living protozoa are the primary hosts for *L. pneumophila* in these aquatic reservoirs. Biofilms containing *L. pneumophila* can form in man-made water systems when antimicrobial treatment measures are not adequately maintained. When aerosols are generated from a contaminated source of water, *L. pneumophila* can gain access to the human lung. In this new environment these bacteria will grow within alveolar macrophages (Fraser et al. 1977, Horwitz and Silverstein 1980, McDade et al. 1977). If macrophages containing *L. pneumophila* are not cleared from the lung, a serious infection can occur that results in a potentially fatal pneumonia called Legionnaires' disease (Fraser et al. 1977). The elderly, heavy smokers, alcoholics, and individuals undergoing chemotherapy appear to be most susceptible to Legionnaires' disease. This is most likely due to a combination of immunosuppression and reduced mucocili-

ary action which prevent clearance of these bacteria from the lung. *L. pneumophila* does not appear to grow extracellularly in the host. This is supported by the observation that *L. pneumophila* mutants that are defective for intracellular growth in macrophages are also avirulent in animal models of disease (Horwitz 1987, Marra et al. 1992). Thus, *L. pneumophila* have an obligately intracellular lifestyle.

The life cycle of *L. pneumophila* was first defined in a series of morphological and cellular studies by Horwitz et al. (1980). The initial step in *L. pneumophila* pathogenesis occurs when the bacteria are ingested by macrophages. Uptake of the bacteria can be enhanced by opsonizing agents such as immunoglobulins or complement. Some strains of *L. pneumophila* are ingested by a coiling phagocytosis process (Horwitz 1984). This uptake phenomenon does not appear to dictate intracellular routing of bacteria because heat-killed and formalin-treated bacteria are still internalized by coiling phagocytosis, yet traffic to lysosomes (Horwitz 1984). Although uptake of *L. pneumophila* into non-phagocytic cells occurs, the efficiency of uptake is comparable to that of non-pathogenic lab strains of *E. coli*. Those *L. pneumophila* that are internalized by non-phagocytic host cells, however, establish an intracellular niche that supports replication.

Phagosomes containing *L. pneumophila* avoid processing by the endocytic machinery of the host cell. After uptake, the pH of the membrane-bound phagosome in which *L. pneumophila* reside remains neutral and this phagosome evades fusion with lysosomes (Horwitz 1983a, Horwitz and Maxfield 1984). In contrast, phagosomes containing an inert particle or an avirulent bacterium will be rapidly acidified and fuse with lysosomes. Over the next two hours, phagosomes containing *L. pneumophila* associate with smooth vesicles, mitochondria, and the endoplasmic reticulum in a sequential fashion (Swanson and Isberg 1995, Horwitz 1983b). The phagosome is eventually routed to a perinuclear position in the cell where it appears as a ribosome-studded vacuole on electron micrographs (Horwitz 1983b). Bacterial replication is initiated after formation of this ribosome-decorated compartment; therefore, this mature structure is referred to as the replicative phagosome. Intimate interactions with cellular organelles play an important role in converting the endocytic vacuole, in which *L. pneumophila* resides initially, into a replicative niche that is similar if not identical to the host rough ER (RER) (Tilney et al. 2001).

L. pneumophila cell division can be observed 4–6 hours after uptake and the doubling-time for these bacteria is approximately two hours inside macrophages (Marra and Shuman 1992). The bacteria will continue to grow inside the host cell for roughly 24 h at which time the replicative phagosome and then the macrophage cytoplasmic membrane will burst, releasing bacteria. It is not known whether lysis is caused by exceeding a physical restriction on the size of the replicative phagosome or by the specific activity of a bacterial gene product. There are mutants of *L. pneumophila* that reportedly have a defect in egress from host cells, however, identification of the proteins required for this process awaits further genetic characterization.

The first avirulent mutants of *L. pneumophila* were obtained by multiple passage of a wild type strain on synthetic medium containing sodium chloride (Horwitz 1987). For reasons that are not clear, wild type strains of *L. pneumophila* are sensitive to sodium ions. Most of these “salt resistant” mutants were unable to cause disease in an animal model of infection, could not replicate intracellularly; most interestingly, these mutants were found to traffic to lysosomes after macrophage uptake (Horwitz 1987). One of these mutants, called 25D, was further investigated. A plasmid was isolated from a wild type *L. pneumo-*

phila genomic library that could complement all of the virulence defects in 25D (MARRA et al. 1992). The cloned region was given the designation *icm*, short for **i**ntra**c**ellular **m**ultiplication. Several *L. pneumophila* intracellular growth mutants with phenotypes similar to 25D were isolated using a thymine-less death enrichment strategy (BERGER and ISBERG 1993). These were called *dot* mutants because of **d**efects in **o**rganelle **t**rafficking that were observed by electron microscopy (BERGER and ISBERG 1993, BERGER et al. 1994). Most *dot* mutants of *L. pneumophila* were found in lysosomes 8 h after uptake by macrophage-like U937 cells. From these data, it became apparent that evasion of phagosome lysosome fusion and intracellular growth were linked virulence traits. However, it was unclear whether these *dot/icm* mutants trafficked to lysosomes because they could not maintain a replicative organelle, or whether they could not grow because they were unable modulate vesicle transport to avoid fusion with lysosomes.

Extensive mutagenesis studies conducted by both the SHUMAN and ISBERG groups led to the identification of additional *icm* and *dot* genes respectively (SEGAL and SHUMAN 1997, VOGEL et al. 1996). Genetic analysis revealed that these two groups had overlapping sets of genes that have been identified independently. In total, 24 *dot/icm* genes have been identified. There are 17 *dot/icm* genes clustered in one region of the bacterial chromosome whereas 7 *dot/icm* genes are located in a second region. Most importantly, sequencing results indicate that 15 of the *dot/icm* products are similar to structural components of bacterial type IV secretion machines. These specialized protein secretion systems are found in a variety of Gram-negative bacteria (CHRISTIE 2001). One of the most interesting aspects of these type IV secretion systems is that most have the capacity to inject endogenously derived proteins into foreign cells. For instance, it has been demonstrated that a type IV transporter encoded by the *virB* genes of *Agrobacterium tumefaciens* can inject bacterial proteins into plant cells (CITOVSKY et al. 1992, VERGUNST et al. 2000). Type IV secretion systems have also been shown to direct the transfer of proteins and plasmid DNA between bacterial cells during conjugation (POHLMAN et al. 1994).

It is believed that the *L. pneumophila* Dot/Icm transporter operates similarly to other type IV systems. Accordingly, the primary function of this transporter would be to inject proteins into phagocytic host cells during bacterial infection that would alter phagosome trafficking by acting on host cellular factors that regulate biogenesis of membrane-bound organelles. Because *L. pneumophila* grow in evolutionarily diverse hosts, the prediction would be that the bacterial proteins injected would target host proteins that remain evolutionarily conserved.

In brief, data in support of this hypothesis have been presented at this symposium. Specifically, it has been shown that a *L. pneumophila* protein termed RalF functions as an exchange factor for the eukaryotic small GTP binding protein Arf. In addition, injection of RalF into host cells by the *L. pneumophila* Dot/Icm system has been demonstrated. Because Arf is highly conserved between hosts as diverse as yeast and humans, where it functions to regulate transport of membrane-bound vesicles, these data provide direct evidence that the Dot/Icm transporter is capable of injecting effector molecules into host cells that can affect proteins regulating vesicle transport.

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