Microbiology Monographs

Series Editor: Alexander Steinbüchel

Johannes H. P. Hackstein *Editor*

(Endo)symbiotic Methanogenic Archaea

Second Edition

Microbiology Monographs

Volume 19

Series Editor
Alexander Steinbüchel
Münster, Germany

More information about this series at http://www.springer.com/series/7171

Johannes H. P. Hackstein
Editor

(Endo)symbiotic Methanogenic Archaea

Second Edition

Springer

Editor
Johannes H. P. Hackstein
IWWR
Faculty of Science
Radboud University Nijmegen
Nijmegen, The Netherlands

ISSN 1862-5576 ISSN 1862-5584 (electronic)
Microbiology Monographs
ISBN 978-3-319-98835-1 ISBN 978-3-319-98836-8 (eBook)
https://doi.org/10.1007/978-3-319-98836-8

Library of Congress Control Number: 2018957565

© Springer Nature Switzerland AG 2010, 2018
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Methanogens are prokaryotic microorganisms that produce methane as end product of their metabolism. They are strictly anaerobic archaea belonging to the taxon Euryarchaeota. Methanogens occupy a wide variety of anaerobic environments, even extreme habitats characterized by high temperature, high salinity, and extreme pH (Liu and Whitman 2008). More temperate habitats include marine and freshwater sediments, flooded soils, landfills, anaerobic digesters, geothermal systems, and the heartwood of trees. Notably, methanogens also thrive in the cytoplasm of anaerobic unicellular eukaryotes and in the gastrointestinal tracts of animals and humans. Frequently, they attach to the internal surfaces of the gastrointestinal tracts and of the protists living in intestinal environments with the aid of special adhesion-like proteins (Liu and Whitman 2008). In insect guts, methanogens have to cope with a very special environment, since they are exposed to a continuous influx of oxygen through the gut wall that challenges the strictly anaerobic symbionts. Notwithstanding that methanogens are very diverge, they can use only a very restricted number of substrates. They are unable to use organic substances (with the exception of acetate and formate), and consequently, methanogens must rely on CO_2, methyl group containing compounds, and acetate, which are provided by the fermentations performed by complex anaerobic bacterial communities. The methanogenic substrates are predominantly metabolized with the aid of H_2 that is provided by syntrophic bacterial communities or, in the case of certain anaerobic protists, by the action of specialized mitochondrion-derived organelles, the hydrogenosomes. Since both hydrogenosomes and syntrophic bacterial communities rely on a low concentration of H_2, the interspecies hydrogen transfer that is mediated by methanogens is crucial for the proper functioning of hydrogenosomes and syntrophic anaerobic bacterial communities.

The synthesis of methane follows a complex biochemical pathway that is characterized by a number of unique coenzymes and membrane-bound enzyme complexes. It has been reviewed thoroughly by Hedderich and Whitman (2006). CO_2 is reduced to methane by H_2 in hydrogenotrophic methanogens, which represent the majority of the methanogens living in symbiosis with protists and multicellular

animals. The second type of substrate, methyl group-containing compounds including methanol, methylated amines, and methylated sulfides, is used by methylotrophic methanogens, which are predominantly found in the gastrointestinal tracts of mammals and insects. The third type of substrate is acetate, which is metabolized by acetoclastic methanogens. Although only two genera (Methanosarcina and Methanosaeta) use acetate as substrate, as much as two-thirds of the biologically generated methane is derived from acetate. Notably, acetoclastic methanogens are rare among the symbiotic methanogens. This is not surprising since symbiotic acetoclastic methanogens compete with their hosts for acetate. Notwithstanding, an acetoclastic methanogen has been identified as endosymbiont in the free-living protist *Metopus es* (Narayanan et al. 2009).

Methanogens are abundant in habitats where electron acceptors such as O_2, NO_3^-, Fe^{3+}, and SO_4^{2-} are limiting (Liu and Whitman 2008). In such methanogenic habitats, complex organic matter is degraded to methane by the syntrophic action of different groups of anaerobic bacteria. Organic polymers are degraded initially by specialized bacteria to sugars, lactate, short-chain fatty acids, and alcohols. These products are further fermented by other bacteria to acetate, formate, H_2, and CO_2, which are the substrates used by methanogens. These methanogens catalyze the terminal step in the anaerobic food chain by converting the various methanogenic substrates to methane, which is released into the atmosphere. More than 70% of the annual global methane emissions (ranging from 500 to 600 Tg CH_4/year) stem from biological methanogenesis and contribute significantly to global warming (Whitman et al. 2006). The symbiotic methanogens in the gastrointestinal tract of ruminants and other "methanogenic" mammals contribute significantly to the global methane budget. Especially the rumen hosts an impressive diversity of methanogens, which have been studied using culture-independent 16S rRNA methods (Janssen and Kirs 2008; Kittelmann and Janssen 2011; Kittelmann et al. 2013; St-Pierre and Wright 2013; Wright et al. 2004). Insects, in particular termites, also host very complex methanogenic communities, but they release much less methane due to the concomitant oxidation of methane in the soil and the termite mounds. Also, the contributions by the methanogenic endosymbionts of protists living in freshwater sediments can be neglected (van Hoek et al. 2006).

This monograph deals with methanogenic endosymbionts of free-living and symbiotic protists, episymbionts of rumen ciliates, methanogenic endosymbionts of ciliates and termite flagellates, which are sometimes accompanied by eubacterial endosymbionts, and methanogens in the gastrointestinal tract of vertebrates and arthropods. One review summarizes our knowledge about the genomic consequences of living together in symbiotic associations; another review discusses the role of methanogens in syntrophic degradation. Finally, the current state of information about hydrogenosomes has been reviewed.

We gratefully acknowledge the efforts of the authors who contributed excellent chapters to this volume of *Microbiology Monographs*. We thank Springer for publishing this monograph.

Nijmegen, The Netherlands — Johannes H. P. Hackstein
June 2018

References

Hedderich R, Whitman WB (2006) Physiology and biochemistry of methane-producing Archaea. In: Dworkin M et al (eds) The prokaryotes, vol 2, 3rd edn. Springer, New York, pp 1050–1079

Janssen PH, Kirs M (2008) Structure of the archaeal community of the rumen. Appl Environ Microbiol 74:3619–3625

Kittelmann S, Janssen PH (2011) Characterization of rumen ciliate community composition in domestic sheep, deer, and cattle feeding on varying diets, by means of PCR-DGGE and clone libraries. FEMS Microbiol Ecol 75:468–481

Kittelmann S, Seedorf H, Walters WA, Clemente JC, Knight R, Gordon JI, Janssen PH (2013) Simultaneous amplicon sequencing to explore co-occurence patterns of bacterial archaeal and eukaryotic microorganisms in rumen microbial communities. PLoS One 8(2):e47879

Liu Y, Whitman WB (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann NY Acad Sci 1125:171–189

Narayanan N, Krishnakumar B, Anupama VN, Manilal VB (2009) Methanosaeta sp., the major archaeal endosymbiont of Metopus es. Res Microbiol 160:600–607

St-Pierre B, Wright ADG (2013) Diversity of gut methanogens in herbivorous animals. Animal 7(s1):49–56

van Hoek AHAM, van Alen TA, Vogels GD, Hackstein JHP (2006) Contribution by the methanogenic endosymbionts of anaerobic ciliates to methane production in Dutch freshwater sediments. Acta Protozool 45:215–224

Whitman WB, Bowen T, Boone D (2006) The methanogenic bacteria. In: Dworkin M et al (eds) The prokaryotes, vol 3, 3rd edn. Springer, New York, pp 165–207

Wright ADG Williams AJ, Winder B, Christophersen CT, Rodgers SL, Smith KD (2004) Molecular diversity of rumen methanogens from sheep in Western Australia. Appl Environ Microbiol 70:1263–1270

Contents

Free-Living Protozoa with Endosymbiotic Methanogens 1
Tom Fenchel and Bland J. Finlay

Anaerobic Ciliates and Their Methanogenic Endosymbionts 13
Johannes H. P. Hackstein and Rob M. de Graaf

Symbiotic Methanogens and Rumen Ciliates 25
Kazunari Ushida

Methanogenic and Bacterial Endosymbionts of Free-Living Anaerobic Ciliates 37
Naoya Shinzato, Kazutaka Takeshita, and Yoichi Kamagata

Termite Gut Flagellates and Their Methanogenic and Eubacterial Symbionts 55
Yuichi Hongoh and Moriya Ohkuma

Methanogens in the Digestive Tract of Termites 81
Andreas Brune

Methanogenic Archaea in Humans and Other Vertebrates: An Update 103
Everly Conway de Macario and Alberto J. L. Macario

Methanogens in the Gastrointestinal Tract of Animals 121
Johannes H. P. Hackstein and Theo A. van Alen

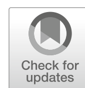
Syntrophy in Methanogenic Degradation 153
Nicolai Müller, Peer Timmers, Caroline M. Plugge, Alfons J.M. Stams, and Bernhard Schink

Hydrogenosomes 193
Johannes H. P. Hackstein and Aloysius G. M. Tielens

Evolution of Prokaryote-Animal Endosymbiosis from a Genomics Perspective 223
Rosario Gil, Amparo Latorre, and Andrés Moya

Index 257

Free-Living Protozoa with Endosymbiotic Methanogens



Tom Fenchel and Bland J. Finlay

Contents

1 Discovery ... 2
2 Distribution ... 3
3 Morphology and Life Cycles ... 5
4 Significance of the Association ... 5
5 Intracellular H_2-Tension and Methanogens ... 7
6 Symbiotic Consortia as Natural Chemostats ... 8
7 The Role of Symbiotic Methanogenesis in Natural Habitats ... 9
References ... 10

Abstract Methanogenic bacteria occur in many, but not all, free-living obligate anaerobic protozoa. This sort of symbiosis is especially common among anaerobic ciliates but is also found in a few species of amoebae and flagellates. Protozoa harbouring methanogens have a clostridium-type fermentative metabolism with H_2 as metabolite, the hydrogen generation taking place in special organelles, so-called hydrogenosomes. The relation between the host cells and their endosymbiotic methanogens is syntrophic hydrogen transfer. By removing the generated H_2, the methanogens stimulate host H_2 production, thus increasing the energetic yield of the energy metabolism. This sort of symbiosis has evolved independently in many cases and involves representatives of several major groups of methanogenic bacteria. Symbiotic methanogenesis of free-living anaerobic protozoa plays only a modest quantitative role in terms of CH_4 production in most habitats.

T. Fenchel (✉)
Marine Biological Laboratory, University of Copenhagen, Helsingør, Denmark
e-mail: tfenchel@bio.ku.dk

B. J. Finlay
River Laboratory, Queen Mary University of London, Dorset, UK
e-mail: b.j.finlay@qmul.ac.uk

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_1

1 Discovery

It was discovered early that sulphidic aquatic habitats rich in decaying organic matter—so-called sapropel—harbour special and characteristic protozoan biota (Lauterborn 1901), and throughout the twentieth century, a number of such sapropelic protozoa were described including flagellates, amoeboid organisms, and, not least, ciliates from such habitats including stratified water columns with an anaerobic hypolimnion, aquatic sediments beneath a certain depth, accumulations of sulphur bacteria, and sewage tanks. It later became clear that what characterises these habitats is primarily absence of oxygen. Later, it was demonstrated that many of these protozoa are true anaerobes in that they lack cytochrome oxidase (Fenchel et al. 1977), and in general they are sensitive to the presence of oxygen. Through motile chemosensory behaviour, they even avoid trace concentrations of O_2. The ciliates, in particular, are capable of O_2-uptake that is not coupled to energy conservation, but it allows the ciliates to maintain intracellular anaerobic conditions (Fenchel and Finlay 1990b). This is shown by the fact that low O_2-tension in the environment (up to 3–4% atmospheric saturation) does not entirely block methane production of the symbiotic methanogens (Fenchel and Finlay 1992). It was also found that anaerobic ciliates were characterised by the presence of ectosymbiotic or endosymbiotic bacteria and sometimes both (Fenchel et al. 1977). It was later demonstrated that the endosymbiotic bacteria are methanogens—which is evident by their blue fluorescence in violet light due to the presence of the coenzyme F_{420} (van Bruggen et al. 1983; Fig. 1a)—and later CH_4 production by these symbiotic consortia could be demonstrated directly (e.g. van Bruggen et al. 1986). Altogether some 40 species of anaerobic free-living ciliates are known to harbour methanogenic bacteria (Fenchel and Finlay 1991c).

The ectosymbiotic bacteria are never methanogens. They occur only in marine species, except for some anaerobic ciliates collected in a sulphate-rich solution lake (Fenchel and Finlay 1995). In two cases (the anaerobic ciliates *Metopus contortus* and *Caenomorpha levanderi*), it has been shown that the ectosymbionts are sulphate reducers, and this is also likely to be the case for other ectosymbionts of marine anaerobic ciliates (Fenchel and Ramsing 1992). They probably serve the same purpose for the host cells as do the methanogens, that is, to consume hydrogen which is produced as a metabolite of the host's fermentative metabolism.

An organelle originally found in the anaerobic parasitic flagellate *Trichomonas* (Müller 1980) was named the "hydrogenosome". The function of this organelle is in principle to ferment pyruvate into acetate and H_2, thus enhancing energy yield from fermentation. Hydrogenosomes have since been shown to be widely distributed, and they occur in most groups of anaerobic protozoa and also in some chytrids (Hackstein and Tielens 2018). With respect to the fermentative pathways, there is some variation among different groups. There is now evidence to show that hydrogenosomes derive from mitochondria although how certain enzymatic components such as hydrogenase were incorporated in them remains an open question (Akhmanova et al. 1998; Biagini et al. 1997; Embley and Martin 1998; Finlay and

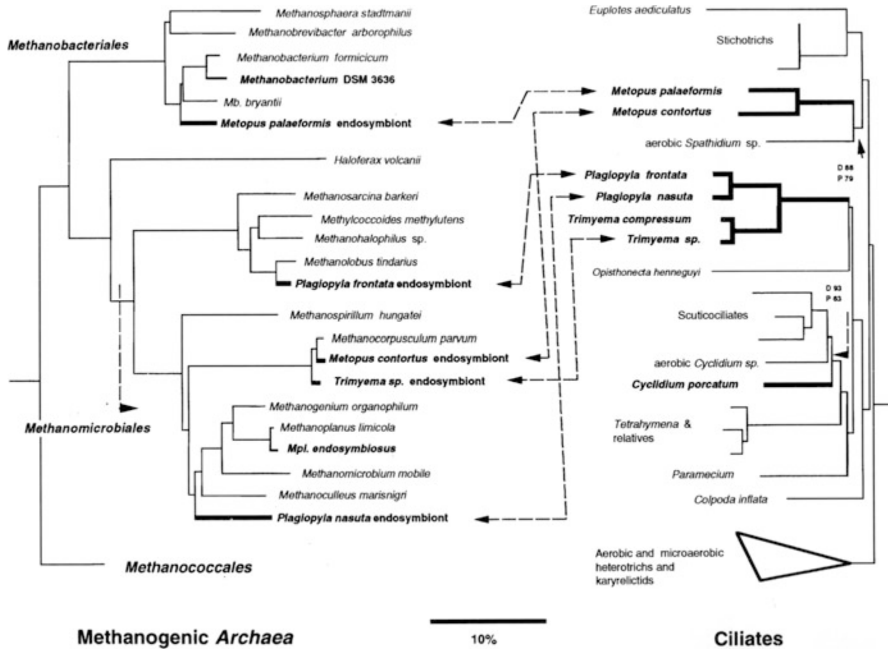


Fig. 1 Phylogenetic trees based on SSU rRNA for ciliates and methanogens. Anaerobic ciliates with hydrogenosomes and methanogens that occur as endosymbionts are printed with bold characters. From Embley and Finlay (1994)

Fenchel 1989; Hackstein et al. 1999). The fact that in many cases related sister groups of protozoa may include both aerobic forms with normal mitochondria and anaerobes with hydrogenosomes indicates that they have evolved independently within many groups of protozoa (e.g. Embley and Finlay 1994). The presence of hydrogenosomes and of methanogens has now also been established among different anaerobic protozoan symbionts in animals (see Hongoh and Ohkuma 2018; Ushida 2018).

Fermentation involving hydrogen production is thermodynamically feasible only if the external hydrogen tension does not exceed a certain level and if the presence of methanogens in anaerobic protozoa is understood as syntrophic hydrogen transfer (see Müller et al. 2018).

2 Distribution

Among protozoan groups that have anaerobic representatives, the ciliates have been studied in most detail. Following the most recent systematics of ciliates (Lynn 2008), free-living anaerobic representatives are found in at least eight orders, and among them six have representatives with methanogenic symbionts. Three of these orders

apparently include only anaerobes: Armophorida (e.g. *Metopus*, *Caenomorpha*), Plagiopylida (*Plagiopyla*, *Sonderia*, *Trimyema*), and Odonstomatida (e.g. *Myelostoma*, *Saprodinium*). Within five other orders, anaerobes are sister groups with aerobes, or in some cases (*Cyclidium* and other genera within Pleurostomatida and *Lacrymaria* within Haptorida), the genera include both aerobic forms and anaerobes with hydrogenosomes and symbiotic methanogens (Esteban and Finlay 1994; Esteban et al. 1993; Fenchel and Finlay 1995). This taxonomic diversity of ciliates with methanogenic symbionts is further increased if some symbiotic ciliates are included such as the intestinal commensal ciliate *Nyctotherus* and ciliates of the rumen (see Ushida 2018). Sequencing of rRNA genes provides an evolutionary tree that is rather consistent with ciliate taxonomy based on morphological criteria and also shows that adaptations to anaerobic life including hydrogenosomes have evolved independently within different groups (Embley and Finlay 1994; Hackstein and Tielens 2018; Van Hoek et al. 2000).

Several otherwise aerobic ciliates belonging to different taxonomic groups are capable of slow balanced growth under strict anaerobic conditions (Bernard and Fenchel 1996). It is therefore not particularly strange that strict anaerobes have evolved independently within different groups.

A phylogenetic tree for the symbiotic methanogens also indicates that the association between the methanogenic symbionts and their anaerobic hosts has evolved independently on several occasions (Embley and Finlay 1994; Lewis et al. 2018; Fig. 1; Hackstein and de Graaf 2018; Shinzato et al. 2018). The symbionts belong to different cardinal groups of methanogens, but they are not identical to any sequenced species of free-living methanogens. Even within ciliate genera of anaerobic ciliates (*Metopus*, *Trimyema*, and *Plagiopyla*), different species may harbour methanogens belonging to different major groups. The results of rRNA-gene sequencing of symbiotic methanogens of *Metopus contortus* and *Plagiopyla nasuta* are at variance with earlier claims that their methanogenic symbionts are "opportunistic symbionts", that is, otherwise free-living methanogens, and also that they have been isolated into pure cultures (van Bruggen et al. 1986; Goosen et al. 1988). Edgcomb et al. (2011) studied a karyorelictid ciliate, *Parduzia orbis*, recovered from the anoxic St. Barbara Basin off California. Through DNA extraction it was shown to harbour several prokaryote endosymbionts including methanogens and sulphate reducers. This is a rather intriguing finding that warrants further studies. Obligate anaerobes are otherwise not known among karyorelictid ciliates.

Among other groups of protozoa, the amoebae *Pelomyxa* and *Mastigella* harbour methanogenic symbionts (van Bruggen et al. 1985, 1988). This is strange in that these organisms do not have hydrogenosomes. *Pelomyxa* seems to have more types of endosymbiotic bacteria, of which one is not a methanogen. It has been speculated that this organism is responsible for producing the necessary H_2 on the basis of fermentative metabolites of the amoeba so that the protozoan-bacteria consortium should represent a three-step food chain, but this must be investigated closer.

While there are several free-living anaerobic flagellates, there is only one example, the genus *Psalteriomonas*, of a flagellate that has hydrogenosomes and harbours symbiotic methanogens. The genus includes two species and belongs to the family

Vahlkampfiidae; the species occur in eutrophic ponds (Broers et al. 1993; van Bruggen et al. 1988). The symbiotic flagellates in the termite gut also possess symbiotic methanogens.

3 Morphology and Life Cycles

There is variation with respect to morphology and behaviour among the symbiotic methanogens, but it is a common theme that they tend to remain in close contact or even being attached to hydrogenosomes (Fig. 2b-f) and that they are not enclosed in a membrane-covered vacuole. In the limnic *Metopus palaeformis*, the symbiotic methanogens, 300–400 per host cell, appear as long rods that are mainly found in the vicinity, but not attached to the hydrogenosomes (Finlay and Fenchel 1991). In the marine *Metopus contortus*, there are 6000–10,000 methanogens per host cell. They appear to undergo a polymorphic life cycle, and they seem to start off as ordinary short rods with a typical bacterial cell wall. When they make contact with a hydrogenosome, the cell wall is at least partially lost. The cells become larger and attain an irregular shape (Fig. 2b). Sequencing of rRNA genes shows that there is only a single species of methanogens related to the genus *Methanocorpusculum* (Finlay and Fenchel 1991; Embley et al. 1992). A related species occurs as the symbiont of a *Trimyema* sp. It has a similar life cycle, and the irregularly shaped bacteria eventually become embedded in aggregations of hydrogenosomes (Fig. 2.f; Finlay et al. 1993). A similar arrangement of hydrogenosomes and methanogens is found in the anaerobic ciliate *Cyclidium porcatum* (Fig. 2e; Esteban et al. 1993).

The most intimate relation between hydrogenosomes and methanogens is found in the marine ciliate *Plagiopyla frontata* (Fenchel and Finlay 1991b; Fig. 2c,d).

The hydrogenosomes and the methanogens are both disk-shaped, and they are arranged like a stack of coins with alternating methanogens and hydrogenosomes; the stacks are capped with hydrogenosomes at either end. There are altogether about 3500 methanogens and a similar number of hydrogenosomes per *Plagiopyla* cell during the entire growth phase of the ciliate (generation time 35–36 h). Prior to the division of the host cells, the hydrogenosomes divide, followed by a division of the methanogens so that the aggregates double, but they retain the characteristic arrangement of hydrogenosomes and symbionts.

In these and other examples, it is obvious that there is always a close physical contact between hydrogenosomes and the symbiont cells.

4 Significance of the Association

The compound 2-bromoethanesulfonic acid (BES) is a specific inhibitor of methanogenesis (Oremland and Capone 1988). When applied to ciliates with methanogens, methane evolution stops immediately. The fluorescence of the

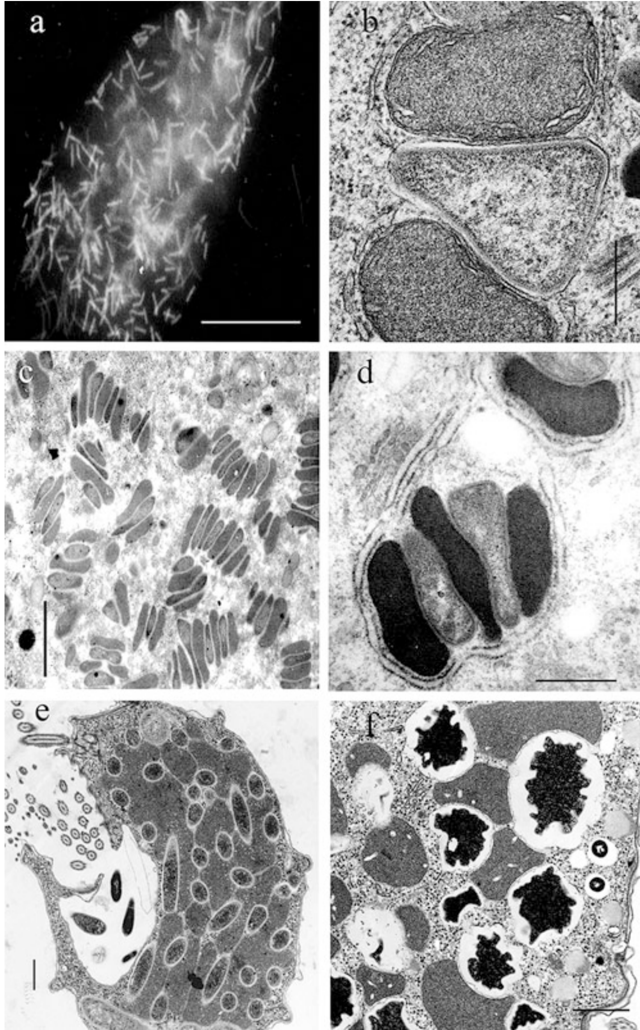


Fig. 2 (**a**): Fluorescence of methanogens in *Metopus palaeformis*; scale bar, 10 μm. (**b**) A methanogen sandwiched between two hydrogenosomes in *Metopus contortus*; scale bar, 0.5 μm. (**c**) and (**d**): Stacks of alternating hydrogenosomes (darker) and methanogens in *Plagiopyla frontata*; scale bars, 5 and 0.5 μm, respectively. (**e**): Complex of methanogens and hydrogenosomes in *Cyclidium porcatum*; scale bar, 0.5 μm. (**f**): Irregularly shaped methanogens in vacuoles surrounded by hydrogenosomes in *Trimyema* sp.; scale bar, 0.5 μm

methanogens is not affected, but the bacteria no longer divide, and so their number is halved for every subsequent division of the host cells. After eight cell divisions, the host cells are aposymbiotic, and the methanogens do not recover when the ciliates are transferred to a medium without BES. It has also proven impossible to reinfect the ciliates with water from the sampling locality or culture fluid from non-treated

cultures filtered through a 5 μm filter—nor from extract of homogenised ciliates with intact methanogens. The aposymbiotic cells survive and grow indefinitely, but they seem to have lost the capability to attain methanogenic symbionts again (Fenchel and Finlay 1991a). Taking into consideration (1) that methanogenic symbionts do not apparently occur as free-living, (2) that they have many special adaptations to life as endosymbionts, and (3) that aposymbiotic ciliates apparently cannot be reinfected with methanogens, it seems to indicate that the endosymbiotic methanogens have approached the status of organelles.

When BES is added to cultures of growing *Metopus contortus* or *Plagiopyla frontata*, the exponential growth rate constant immediately decreases to about 70% of the previous value. Aposymbiotic cells that have been kept without BES also grow with a growth rate constant that is 70% of that of cells with active methanogens, and the growth yield is also about 70% of that of normal cells. However, in similar experiments with *Metopus palaeformis*, BES did not seem to affect the growth rate constant significantly. In *Trimyema* Shinzato et al. (2018) also found that aposymbiotic cells showed a more limited negative effect on the growth rate relative to cells that harbours methanogens.

5 Intracellular H_2-Tension and Methanogens

The production of methane by the ciliates is closely coupled to their growth rate. In *Plagiopyla frontata* CH_4 production is about 4.5 pmol per cell h^{-1} during exponential growth. This figure decreases to about half that value during the last two cell divisions in batch cultures, and during the stationary phase, it drops to the detection limit after about 100 h (Fenchel and Finlay 1992).

The CH_4 production rate must be a measure of the H_2 production of the hydrogenosomes: it takes 4 H_2 to produce one CH_4. Measuring CH_4 and H_2 production of *Plagiopyla frontata* and *Metopus contortus* simultaneously showed that some hydrogen (about 5%) is not consumed by the methanogens, but diffuses out of the ciliates. Measuring H_2 production of aposymbiotic (previously BES-treated) cells could not, however, account for the CH_4 production of cells with active methanogens: in *Metopus* the measured H_2 production could account for about 70% of the CH_4 production of normal cells, and in *Plagiopyla* the corresponding figure was only about 45%. It is possible that some of the reduction equivalents produced by the hydrogenosomes is in the form of formate as has been shown for the anaerobic ciliate *Trimyema* (Goosen et al. 1990; Holler and Pfennig 1991). This was not tested in Fenchel and Finlay (1992), but it is likely that in the absence of methanogens, H_2-tension will build up in the ciliates and thus inhibit H_2 production in the hydrogenosomes which will instead excrete more reduced end products than acetate, such as lactate or propionate, and that the significance of the association between the host cells and their methanogenic symbionts is one of syntrophic H_2-transfer (Müller et al. 2018).

This is supported by simple calculations of the concentration of H_2 in a spherical cell in the absence of methanogens so that H_2 is lost only through diffusion. Using parameter values for a *Plagiopyla* cell, that is, its volume, its H_2 production rate under exponential growth, and the diffusion coefficient and solubility of H_2 in water, it could be shown that—in the absence of methanogens—the H_2-tension would increase to about 1.3 kPa, a value around a thousandfold higher than the ambient H_2-tension, and this is a H_2-tension that would be inhibitory to fermentative processes involving H_2-release (Fenchel and Finlay 1992, 1995).

6 Symbiotic Consortia as Natural Chemostats

It was noted that the volume fraction of methanogens in host cells is remarkably constant when comparing different species and in individual species under different growth conditions, that is, around 2%. This can be explained by describing the symbiotic consortium as a kind of chemostat (Finlay and Fenchel 1992; Fenchel and Finlay 1995).

It is assumed that the growth rate of the symbiont is dependent only on the H_2 production of the host and also that the cells are "diluted" due to the growth, that is, increase in cell volume of the host, which is also coupled to H_2 production. The system deviates from a real chemostat in that some H_2 is not diluted at the same rate as the bacteria, but is also to some small extent lost through diffusion across the cell surface of the host cells. Cell yield of methanogens (in terms of dry weight production per unit CH_4 produced) and maximum growth rate constants for methanogens were taken from the literature. Applying the model to data on *Plagiopyla frontata* and its methanogen symbionts predicted realistic values for the volume fraction of methanogens and also showed that over a rather wide range of host growth rates (up to 80% of the maximum growth rates for the methanogens), the volume fraction constituted by the symbionts is relatively stable (Fenchel and Finlay 1995).

An interesting aspect of the model is that it shows that an association between host cells and an intracellular bacterium that is solely dependent on some host metabolite can instantaneously become stable, and the generation time of the symbionts becomes identical to that of the host cell. As in a real chemostat, the bacteria will increase in number until competition for the substrate lowers their growth rate constant to become identical of that of the host. It is, therefore, not so difficult to imagine the origin of such associations. Once a bacterial cell has somehow evaded a food vacuole, it can grow and multiply in the cytoplasm of its future host on the basis of a host metabolite; then the association will be stable.

7 The Role of Symbiotic Methanogenesis in Natural Habitats

It can be asked what is the quantitative role of symbiotic methanogenesis in natural systems. Some theoretical consideration would suggest that in the case of anaerobic freshwater systems, this role is small. In such systems in the absence of sulphate, the terminal mineralization process is methanogenesis. Anaerobic protozoa are phagotrophs, and they have low growth efficiencies in comparison to aerobic phagotrophs—and consequently the biomass relative to the biomass of their food bacteria is low (Fenchel and Finlay 1990a). It was calculated that in such methanogenic systems, symbiotic methanogenesis could at most contribute about 3% of the produced methane (Fenchel 1993). This was demonstrated directly for lake sediments where it was found that the methanogenesis of anaerobic ciliates was negligible compared to methanogenesis caused by free-living bacteria (Van Hoek et al. 2006). Schwartz and Frenzel (2005) found that in rice paddies anaerobic ciliates with symbiotic methanogens contributed only a few percent of the total methane production.

The situation may be different in marine habitats. Seawater has a high content of sulphate, so the dominating terminal mineralization process under anaerobic conditions is sulphate reduction. In a sense, the host cells can be considered as a refuge for methanogens in anaerobic but sulphate-rich habitats. Otherwise, methanogenesis plays a significant role only when sulphate has been depleted, and this happens only when there is a very high input of degradable organic matter or at considerable depths in sediments. In seawater, symbiotic methanogenesis could therefore potentially play a larger relative role. Fenchel (1993) tested this by measuring total methanogenesis and that of methanogenic ciliates for different marine shallow water habitats and at different depths in sediments. In sandy sediments methanogenic ciliates contributed at the most 2–3% of the total CH_4 production. In masses of photosynthetic sulphur bacteria and especially in an accumulation of decaying sea grass leaves, higher values were found. In the latter case, where there were about 200 ciliates with methanogenic symbionts ml^{-1} down to about 20 cm depth, symbiotic methanogenesis contributed up to >80% of the total CH_4 production at one occasion, but in most cases it was around 20%. But this, first of all, reflects that the dominating terminal mineralization process was sulphate reduction at this site.

In general it can be concluded that symbiotic methanogenesis plays a modest role in a biogeochemical context—primarily because phagotrophy plays a modest quantitative role in anaerobic habitats.

References

Akhmanova A, Voncken F, van Alen T, van Hoek A, Boxma B, Vogels G, Veenhuis M, Hackstein JHP (1998) A hydrogenosome with a genome. Nature 396:527–528

Bernard C, Fenchel T (1996) Some microaerobic ciliates are facultative anaerobes. Eur J Protistol 32:293–297

Biagini GA, Finlay BJ, Lloyd D (1997) Evolution of the hydrogenosome. FEMS Microbiol Lett 155:133–140

Broers CAM, Meiers HM, Symens JC, Stumm CK, Vogels GD (1993) Symbiotic association of *Psalteriomonas vulgaris* n. spec. with *Methanobacterium formicicum*. Eur J Protostol 29:98–105

Edgcomb VP, Leadbetter ER, Bourland W, Beaudoin D, Bernhard JM (2011) Structured multiple endosymbiosis of bacteria and archaea in a ciliate from marine sulfidic sediments: a survival mechanisms in low oxygen, sulfidic sediments? Front Microbiol 2:55. https://doi.org/10.3389/fmicb2011.00055. eCollection 2011

Embley T, Finlay BJ (1994) The use of small subunit rRNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts. Microbiology 130:225–235

Embley TM, Martin W (1998) A hydrogen-producing mitochondrion. Nature 396:517–519

Embley TM, Finlay BJ, Brown S (1992) RNA sequence analysis shows that the symbionts in the ciliate *Metopus contortus* are polymorphs of a single methanogen species. FEMS Microbiol Lett 97:57–62

Esteban G, Finlay BJ (1994) A new genus of anaerobic scuticociliate with endosymbiotic methanogens and ectobiotic bacteria. Arch Protistol 144:350–356

Esteban G, Guhl BE, Clarke KJ, Embley TM, Finlay BJ (1993) *Cyclidium porcatum* n.sp.: a free-living anaerobic scuticociliate containing a stable complex of hydrogenosomes, eubacteria and archaebacteria. Eur J Protistol 29:262–270

Fenchel T (1993) Methanogenesis in marine shallow water sediments: the quantitative role of anaerobic protozoa with endosymbiotic methanogenic bacteria. Ophelia 37:67–82

Fenchel T, Finlay BJ (1990a) Anaerobic free-living protozoa: growth efficiencies and the structure of anaerobic communities. FEMS Microbiol Ecol 74:269–276

Fenchel T, Finlay BJ (1990b) Oxygen toxicity, respiration and behavioural responses to oxygen in free-living anaerobic ciliates. J Gen Microbiol 136:1953–1959

Fenchel T, Finlay BJ (1991a) Endosymbiotic methanogenic bacteria in anaerobic ciliates: significance for the growth efficiency of the host. J Protozool 38:18–22

Fenchel T, Finlay BJ (1991b) Synchronous division of an endosymbiotic methanogenic bacterium in the anaerobic ciliate *Plagiopyla frontata* Kahl. J Protozool 38:22–28

Fenchel T, Finlay BJ (1991c) The biology of free-living anaerobic ciliates. Eur J Protistol 26:201–215

Fenchel T, Finlay BJ (1992) Production of methane and hydrogen by anaerobic ciliates containing symbiotic methanogens. Arch Microbiol 157:475–480

Fenchel T, Finlay BJ (1995) Ecology and evolution in anoxic worlds. Oxford University Press, Oxford

Fenchel T, Ramsing NB (1992) Identification of sulphate-reducing ectosymbiotic bacteria from anaerobic ciliates using 16S rRNA binding oligonucleotide probes. Arch Microbiol 158:394–397

Fenchel T, Perry T, Thane A (1977) Anaerobiosis and symbiosis with bacteria in free-living ciliates. J Protozool 24:154–163

Finlay BJ, Fenchel T (1989) Hydrogenosomes in some anaerobic protozoa resemble mitochondria. FEMS Microbiol Lett 65:311–314

Finlay BJ, Fenchel T (1991) Polymorphic bacterial symbionts in the anaerobic ciliated protozoon *Metopus*. FEMS Microbiol Lett 79:187–190

Finlay JB, Fenchel T (1992) An anaerobic ciliate as a natural chemostat for the growth of endosymbiotic methanogens. Eur J Protistol 28:127–137

Finlay BJ, Embley TM, Fenchel T (1993) A new polymorphic methanogen, closely related to *Methanocorpusculum parvum*, living in stable symbiosis within the anaerobic ciliate *Trimyema* sp. J Gen Microbiol 139:371–378

Goosen NK, Horemans AMC, Hillebrand JW, Stumm CK, Vogels D (1988) Cultivation of the sapropelic ciliate *Plagiopyla nasuta* Stein and isolation of the endosymbiont *Methanobacterium formicicum*. Arch Microbiol 150:165–170

Goosen NK, van der Drift C, Stumm CK, Vogels GD (1990) End products of metabolism in the anaerobic ciliate *Trimyema compressum* in monoxenic cultures. FEMS Microbiol Lett 69:171–176

Hackstein JHP, de Graaf RM (2018) Anaerobic ciliates and their methanogenic endosymbionts. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Hackstein JHP, Tielens AGM (2018) Hydrogenosomes. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Hackstein JHP, Akhmanova A, Boxma B, Harhangi HR, Voncken FGJ (1999) Hydrogenosomes: eukaryotic adaptations to anaerobic environments. Trends Microbiol 7:441–447

Holler S, Pfennig N (1991) Fermentation products of the anaerobic ciliate *Trimyema compressum* in monoxenic cultures. Arch Microbiol 156:327–334

Hongoh Y, Ohkuma M (2018) Termite gut flagellates and their methanogenic and eubacterial symbionts. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Lauterborn R (1901) Die “sapropelische” Lebewelt. Zool Anz 24:50–55

Lewis WH et al (2018) Morphology and phylogeny of a new species of anaerobic ciliate Trimyema finlayi n.sp. with endosymbiotic methanogens. Front Microbiol 9:140

Lynn DH (2008) The ciliated protozoa. Springer, Berlin

Müller M (1980) The hydrogenosome. In: Gooday GW, Lloyd D, Trinci APJ (eds) The eukaryotic cell. Cambridge University Press, Cambridge

Müller N, Timmers, P Plugge CM, Stams AJM, Schink B (2018) Syntrophy in methanogenic degradation. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Oremland RS, Capone DG (1988) Use of “specific” inhibitors in biogeochemistry and microbial ecology. Adv Microb Ecol 10:285–383

Schwartz MVJ, Frenzel P (2005) Methanogenic symbionts of anaerobic ciliates and their contribution to methanogenesis in an anoxic rice field soil. FEMS Microbiol Ecol 52:93–99

Shinzato N, Takeshita K, Kamagata Y (2018) The methanogenic and eubacterial endosymbionts of *Trimyema*. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Ushida K (2018) Symbiotic methanogens and rumen ciliates. In: Hackstein JHP (ed) (Endo) symbiotic methanogenic archaea. Springer, Heidelberg

van Bruggen JJA, Stumm CK, Vogels GD (1983) Symbiosis of methanogenic bacteria and sapropelic protozoa. Arch Microbiol 136:89–95

van Bruggen JJA, Stumm CK, Zwart KB, Vogels GD (1985) Endosymbiotic methanogenic bacteria of the sapropelic amoeba *Mastigella*. FEMS Microbiol Ecol 31:187–192

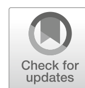
van Bruggen CAM, Zwart KB, Hermans JGF, van Hove EM, Stumm CK, Vogels GD (1986) Isolation and characterization of *Methanoplanus endosymbioticus* sp. nov, an endosymbiont of the marine sapropelic ciliate *Metopus contortus* Quennerstedt. Arch Microbiol 144:367–374

van Bruggen JJA, van Rens GLM, Geertman EJM, Zwart KB, Stumm CK, Vogels GD (1988) Isolation of a methanogenic endosymbiont of the sapropelic amoeba *Pelomyxa palustris* Greef. J Protozool 35:20–23

Van Hoek AHAM, van Alen TA, Sprakel VSI, Leunissen JAM, Brigge T, Vogels GD, Hackstein JHP (2000) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol Biol Evol 17:251–258

Van Hoek AHAM, van Alen TA, Vogels GD, Hackstein JHP (2006) Contribution by the methanogenic endosymbionts of anaerobic ciliates to methane production in Dutch freshwater sediments. Acta Protozool 45:215–224

Anaerobic Ciliates and Their Methanogenic Endosymbionts



Johannes H. P. Hackstein and Rob M. de Graaf

Contents

1 Introduction 14
2 Methanogenic Endosymbionts Are Transmitted Vertically 15
3 Studies of the SSU rRNA Genes of Host and Symbiont 15
4 Endosymbiont Replacements 19
References 21

Abstract Many anaerobic ciliates possess hydrogenosomes, and consequently, they have the potential to host endosymbiotic methanogens. The endosymbiotic methanogens are vertically transmitted, and even cyst stages carry methanogens. Accordingly, the analysis of the SSU rRNA genes of ciliates and their methanogenic endosymbionts revealed that the endosymbionts are specific for their hosts and not identical with free-living methanogens. Notably, the endosymbionts of a monophyletic group of ciliates that thrive in either freshwater environments or intestinal tracts are substantially different. Ciliates from freshwater sediments host methanogens belonging to the Methanomicrobiales, while ciliates thriving in the intestinal tracts of cockroaches, millipedes and frogs host methanogens that belong to the Methanobacteriales. Comparative analysis of free-living and gut-dwelling ciliates and their corresponding endosymbionts reveals only a limited co-evolution suggesting infrequent endosymbionts replacements. Such an endosymbiont replacement is clearly the reason for the very distant endosymbionts of free-living and gut-dwelling ciliates: the endosymbionts are related to the methanogens in the particular environments, in which the hosts live.

J. H. P. Hackstein (✉) · R. M. de Graaf
IWWR, Faculty of Science, Radboud University Nijmegen, Nijmegen, The Netherlands
e-mail: j.hackstein@science.ru.nl

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_2

1 Introduction

Anaerobic protists with hydrogenosomes have the potential to host endo- or episymbiotic methanogens (Hackstein et al. 2002; Hackstein and Tielens 2018; Fenchel and Finlay 2018; Ushida 2018). Anaerobic ciliates, in particular, are well known to have evolved hydrogenosomes repeatedly (7 out of 22 ciliate taxa, see Fenchel and Finlay 1995), and all of them seem to host endosymbiotic methanogens (Hackstein et al. 2002; Fig. 1). A few methanogenic endosymbionts have been isolated and cultured in vitro (van Bruggen et al. 1984, 1986; Goosen et al. 1988): these endosymbionts were found to be similar to free-living methanogens, for example, *Methanocorpusculum parvum* or *Methanobacterium formicicum.* However, the culturing techniques did not allow to decide whether the endosymbionts were specific for their hosts or identical with their free-living relatives. Analysis of the small subunit of the ribosomal genes (SSU rDNA) eventually revealed that the methanogenic endosymbionts were similar, but not identical to their free-living relatives (Embley and Finlay 1994; Embley et al. 1995; Fenchel and Finlay 1995, 2018). The endosymbionts belonged to different taxa of methanogens, and even the endosymbionts of closely related host species appeared to be very different. It was concluded that the observed symbioses were established several times independently, most likely along with the evolution of hydrogenosomes (Embley and Finlay 1994; Embley et al. 1995; Fenchel and Finlay 1995). The major conclusion was that

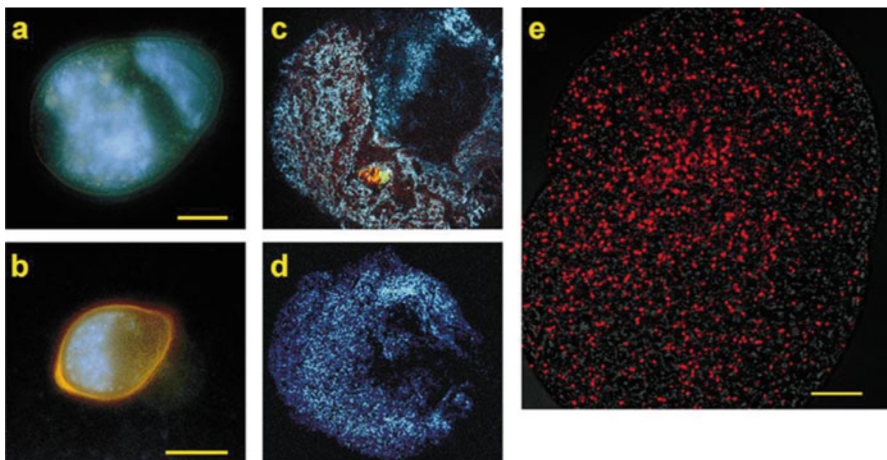


Fig. 1 Endosymbiotic methanogens of *Nyctotherus ovalis.* (**a**)–(**d**) F_{420} autofluorescence. (**e**) In situ hybridization. (**a**) *Nyctotherus ovalis* from *Blaberus* sp. var. Amsterdam. (**b**) Cyst of *N. ovalis* from the same isolate. (**c**) Squash preparation of *N. ovalis* from *Blaberus* sp. var. Amsterdam. (**d**) Squash preparation of *N. ovalis* from *Periplaneta americana* var. Amsterdam; note the rod shape of the methanogens. (**e**) Part of *N. ovalis* from *Blaberus* sp. var. Nijmegen; in situ hybridization with a probe specific for methanogenic archaea, labelled with Cy5. Confocal LSM. Bars indicate 25 μm in (**a**), (**c**) and (**d**), 20 μm in (**b**) and 10 μm in (**e**). Reproduced with permission by Oxford University Press from van Hoek et al. (2000)

the endosymbionts were specific for the particular host species and not representatives of opportunistic methanogens that could thrive in both aquatic and intracellular environments.

2 Methanogenic Endosymbionts Are Transmitted Vertically

This fits well with the observation that the methanogenic endosymbionts are "vertically" transmitted: at mitosis, the endosymbionts are distributed to the daughter cells, and even at encystation, the endosymbionts are retained (van Hoek et al. 2000; Fig. 1b). The behaviour of the endosymbionts at conjugation has not been studied to my knowledge, but it is likely that both exconjugants possess endosymbionts. If a species is known to host methanogenic endosymbionts, all members of an uncultivated population possess these endosymbionts. However, there are several reports that ciliates kept in culture tend to lose their endosymbionts (Shinzato et al. 2018). Certain strains of cultured ciliates lost their endosymbionts completely after some time, while other strains belonging to the same species retained the symbionts. Interestingly, Wagener et al. (1990) succeeded to reinfect such a symbiont-free strain of *Trimyema compressum* with *Methanobacterium formicicum.* This experiment revealed that *M. formicicum* can be regarded as an opportunistic methanogen that can be taken up by a symbiont-free ciliate. This consortium was functional, albeit with a lower efficiency than the original methanogenic endosymbionts (Wagener et al. 1990; Shinzato et al. 2018). The analysis of the SSU rRNA genes has shown that the uptake of methanogens from the environment is not a general phenomenon, but the experiments of Wagener et al. (1990) have shown that it is possible.

3 Studies of the SSU rRNA Genes of Host and Symbiont

In order to analyse the "vertical" inheritance of the methanogenic endosymbionts in more detail, van Hoek et al. (2000) studied the methanogenic endosymbionts of closely related anaerobic ciliates that thrive either in freshwater sediments or in the intestinal tracts of cockroaches, millipedes and frogs. van Hoek et al. (2000) amplified the SSU rRNA genes from both the endosymbionts and their hosts, using single-cell PCR. The phylogenetic analysis of the SSU rRNA genes of the hosts revealed the anticipated monophyly of the various cockroach-dwelling *Nyctotherus* species and strains that thrive in the guts of millipedes and frogs. Notably, the free-living species *Metopus* sp., *Brachonella* sp. and *Caenomorpha*-like belonged to the same monophyletic cluster. The *Caenomorpha* species formed a closely related but paraphyletic cluster (Fig. 2). The monophyly of the *Nyctotherus/Metopus/Brachonella* cluster was confirmed by the phylogenetic analysis of the 12S

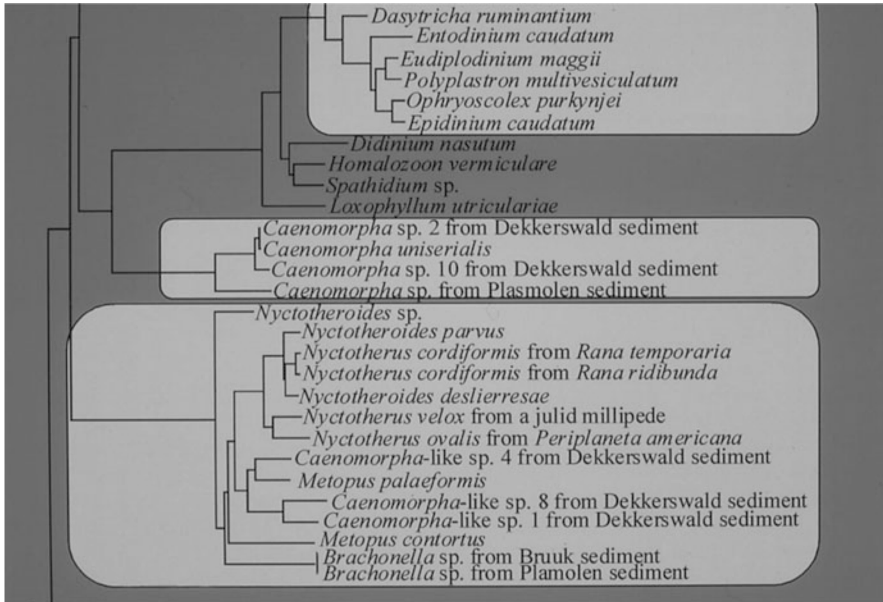


Fig. 2 Detail of a phylogenetic tree (Molphy Star Decomposition, Adachi and Hasegawa 1996) demonstrating the evolution of the relevant ciliate taxa on the basis of their 18S rDNA. Boxed species are anaerobes and possess hydrogenosomes. The unboxed species are aerobes with mitochondria. The *Nyctotherus/Nyctotheroides* and *Metopus/Brachonella/Caenomorpha-like* cluster is monophyletic (Armophoridae and Clevelandellids). The *Caenomorpha* species form a paraphyletic cluster

(SSU) rRNA genes located on the genomes of the hydrogenosomes (Boxma et al. 2005) and the corresponding hydrogenases (not shown). This means that the hydrogenosomes of these ciliates are monophyletic and, consequently, that these organelles had been acquired by the last common ancestor of this clade—before the various ciliate species adapted to their particular freshwater—or gut environments.

However, the analysis of the SSU rRNA genes of the methanogenic endosymbionts revealed an unexpected result (Fig. 3). The endosymbionts formed two clusters that belong to two different orders of methanogens (Methanobacteriales vs. Methanomicrobiales). One cluster contained the endosymbionts of the free-living ciliate species; the other contained the endosymbionts of the gut-dwelling ciliate species. Notably, the endosymbionts of the freshwater ciliates clustered among methanogens (Methanomicrobiales) living predominantly in freshwater sediments, whereas the endosymbionts of the intestinal ciliates clustered among predominantly intestinal or faecal methanogens (Methanobacteriales). Each group of endosymbionts was monophyletic, and each endosymbiotic methanogen was distinct from any known environmental methanogen. The endosymbionts were different from each other, given that they were from a different ciliate ribotype. Also, endosymbionts from different ciliate ribotypes living in the same pond were

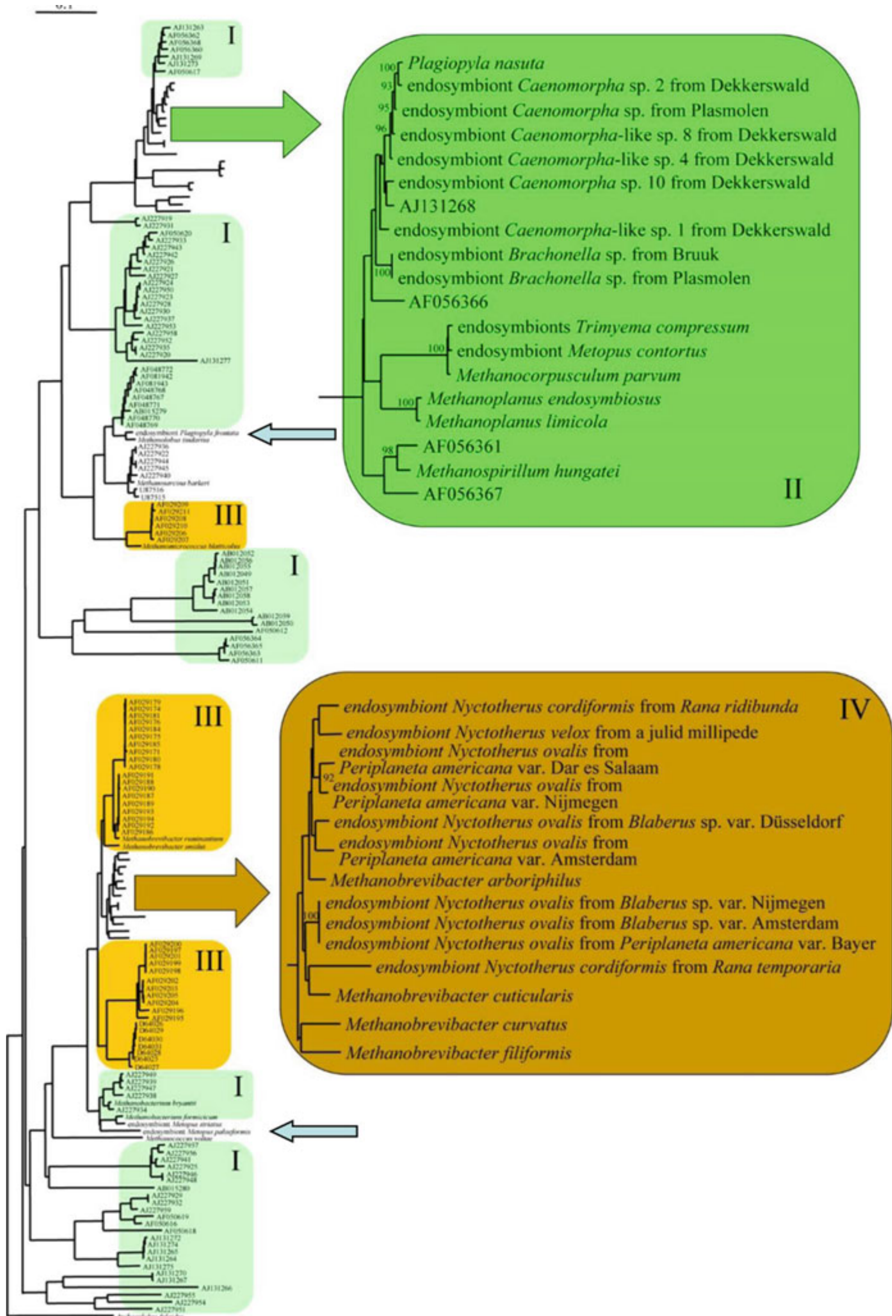


Fig. 3 Neighbour-joining tree (Saitou and Nei 1987) inferred from approximately 770 positions of the 16S rDNA of methanogenic archaea. The clades with the endosymbionts from freshwater (green, box II) and intestinal ciliates (yellow, box IV) are highlighted and enlarged. The light-green boxes

different, but endosymbionts from the same ciliate ribotype were identical—regardless of the sampling place. Methanogenic endosymbionts from earlier studies (all from free-living ciliates) clustered at different positions in the phylogenetic tree (Fig. 3) but always among methanogens from freshwater environments. There is one potential exception: the endosymbiont of *Trimyema compressum* strain S10 appeared to be similar to *Methanobrevibacter arboriphilus* that clusters among the endosymbionts of gut ciliates (Fig. 3; Shinzato et al. 2007, 2018), whereas endosymbionts from other *Trimyema* strains cluster among the freshwater methanogens (Fig. 3). However, the ciliate strain S10 had been isolated from a sewage installation, which is likely to harbour *M. arboriphilus*-like methanogens. Also, Narayanan et al. (2009) provided evidence for the presence of an acetoclastic *Methanosaeta* species as endosymbiont of *Metopus es*. This endosymbiont might be derived from an environmental free-living member of the Methanosaetaceae, which thrive in anaerobic digesters just as *Metopus es.* Recently, another symbiosis between a *Metopus* species from anaerobic granular sludge in a domestic wastewater treatment plant and *Methanoregula boonei* has been described. The methanogenic endosymbiont was accompanied by *Clostridium aminobutyricum* (Hirakata et al. 2015).

Metopus species most closely related to *Metopus palaeformis* predominated in the methane production in uranium of contaminated subsurface sediments. The activity of the endosymbionts was monitored by the analysis mcrA mRNA transcripts and revealed the importance of acetate for the metabolism in the sediments. Free methanogens were not identified (Holmes et al. 2014).

Thus, there is a clear correlation between the methanogenic endosymbionts and the free-living methanogens from the corresponding environments in which the ciliate host lives. This suggests that the endosymbionts stem from the environment, but the fact that the SSU rDNA sequences from the endosymbionts and the free-living methanogens are different argues against the existence of opportunistic symbionts. The substantial times of evolutionary divergence that result in a significant sequence divergence from environmental methanogens also argue for specific, long-lasting symbiotic associations. Other arguments against opportunistic symbionts are provided by the already mentioned vertical transmission of the symbionts and the failure to demonstrate an endosymbiont exchange in transfaunation experiments with *Nyctotherus* ciliates from different cockroach strains (van Hoek et al. 1999, 2000).

Fig. 3 (continued) (I) indicate predominantly free-living methanogens from environmental sources such as sediments and rice fields. The light-yellow boxes (III) mark predominantly uncultured intestinal methanogens. The small arrows indicate the endosymbionts of the free-living ciliates *Plagiopyla frontata* (upper) and of *Metopus striatus* and *Metopus palaeformis* (lower).The endosymbiont of *Trimyema compressum* strain S 10 is similar to *Methanobrevibacter arboriphilus* that is located in box IV. The distance data were bootstrap resampled 100 times (Felsenstein 1985). Only bootstrap values above 90% are displayed in the highlighted, enlarged boxes II and IV. Reproduced with permission by Oxford University Press from van Hoek et al. (2000)

4 Endosymbiont Replacements

To study this dilemma further, van Hoek et al. (2000) analysed the potential co-evolution between ciliates and their methanogenic endosymbionts at the level of their SSU rRNA genes. It had been shown earlier with the analysis of symbiotic associations between bacteria and insects that these symbioses exhibited a complete congruency between host and symbiont phylogenies (Baumann et al. 1995, 1997; Bandi et al. 1994, 1997). With respect to the ciliates, there was clearly no congruency between the phylogenies of the free-living and gut-dwelling ciliates and their endosymbionts. As already mentioned, the host environment determined the phylogenetic position of the endosymbiont (Fig. 3). To circumvent this problem, van Hoek et al. (2000) constructed separate phylogenetic trees for the free-living and intestinal ciliates and their endosymbionts (Fig. 4). Also these trees did not provide evidence for a strict congruency between host and symbiont trees. Only a few potential co-speciation events could be identified. The use of different tree-building algorithms and user-defined trees did not lead to a better match between host and symbiont phylogenies. Thus, the evolution of the anaerobic ciliates and their endosymbionts studied here cannot be completely vertical. Several times in evolution, a horizontal transfer of symbionts must have taken place, i.e. the evolution of the intestinal ciliates must have included a minimum of one endosymbiont replacement but potentially some more. As already mentioned, the last common ancestor of both the free-living and the intestinal ciliates hosted hydrogenosomes and, consequently, methanogenic endosymbionts. The nature of these ancestral endosymbionts is unknown, but one might assume that these endosymbionts were related to environmental methanogens. Adaptation of the ciliates to a different environment must have involved an endosymbiont replacement, since it has been shown that all ciliates studied so far possess endosymbionts that are related to free-living methanogens thriving in the corresponding environment (Fig. 5). Notably, the endosymbionts of ciliates living in the guts of frogs and their larvae are of the "intestinal" type, although the hosts of the ciliates, the frogs and their larvae, thrive in an environment that is crowded with free-living methanogens of the "freshwater sediment" type.

Ciliates radiating in the same ecological niche host methanogens that are distinct and different in DNA sequence from all known environmental methanogens. As already mentioned, the endosymbionts do not strictly co-speciate with their hosts, a trait that might be caused by accidental endosymbiont replacements within one and the same environment. However, the genetic distance to environmental methanogens suggest that such endosymbiont replacements are infrequent and followed by regular periods of strictly vertical transmission. A similar phenomenon has been observed in the symbiosis between proteobacteria and certain bivalves belonging to the genus *Solemya* (Krueger and Cavanaugh 1997; Distel 1998). Also here, endosymbiont replacements have been postulated. Since grazing ciliates regularly take up bacteria and free-living methanogens, it is reasonable to assume that one or the other methanogen will escape digestion and survive in the cytoplasm of the ciliate.

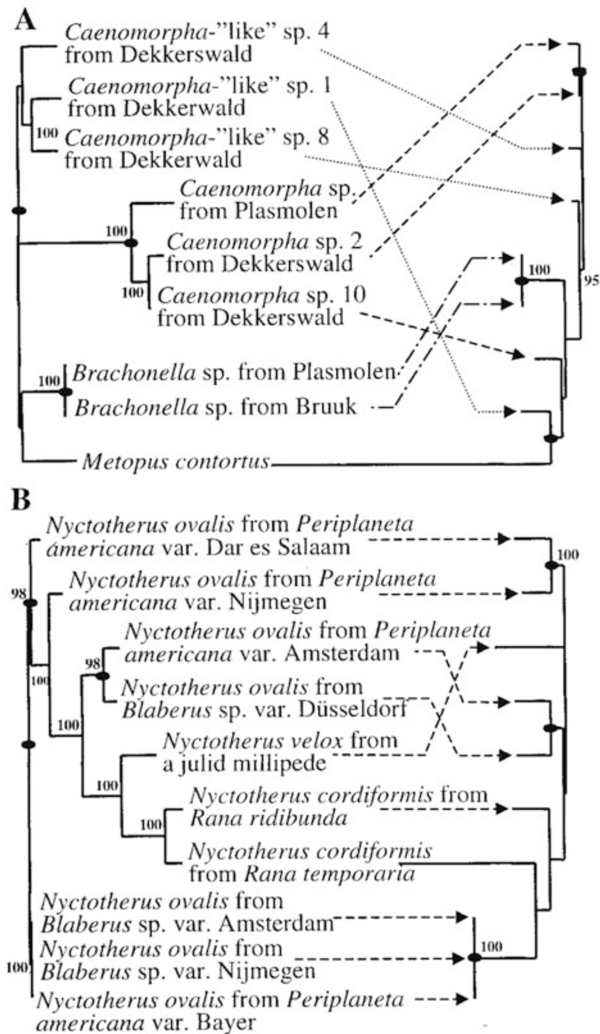


Fig. 4 TreeMap trees of hosts and symbionts (Page 1995) based on 460 positions of the 18S rDNA sequences of the ciliate hosts and 770 positions of the 16S rDNA sequences of the methanogenic endosymbionts. (**a**) Freshwater ciliates (left tree) and their methanogenic endosymbionts (right tree). (**b**) Intestinal ciliates (left tree) and their methanogenic endosymbionts (right tree). Corresponding pairs of ciliates and their endosymbionts are indicated by arrows. Only bootstrap values above 90% are displayed. Presumed co-speciation events are indicated by bullets. Reproduced with permission by Oxford University Press from van Hoek et al. (2000)

Eventually such a methanogen might replace an aged population of endosymbionts suffering from its genetic load due to the action of "Muller's ratchet" (c.f. Doolittle 1998). The successful introduction of *Methanobacterium formicicum* into symbiont-free cells of *Trimyema compressum* shows that such a mechanism must be possible (Wagener et al. 1990). Thus, this scenario can explain both the limited co-evolution between ciliates and their methanogenic endosymbionts and the obvious relationship between endosymbionts and environmental methanogens.

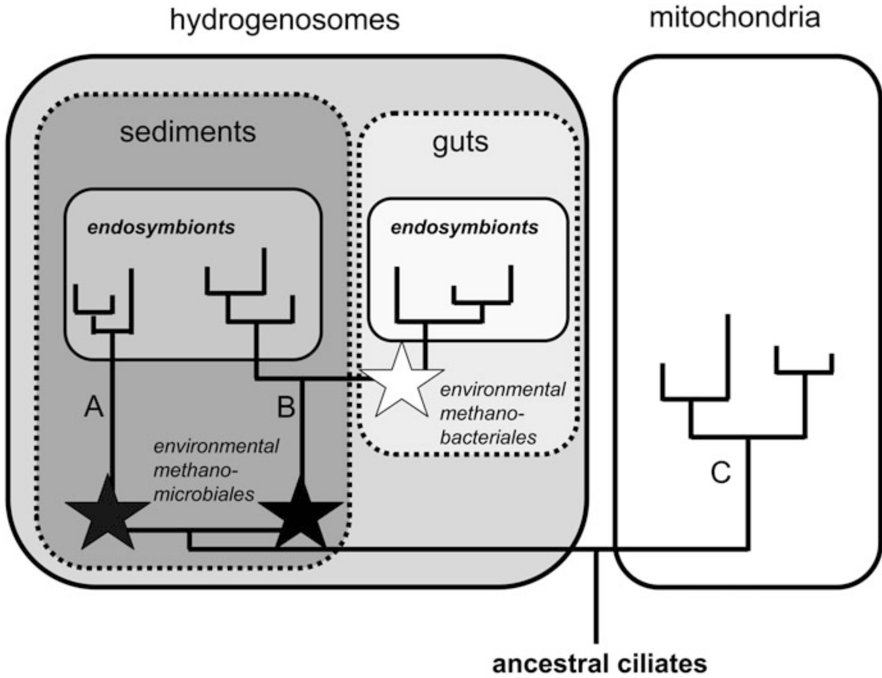


Fig. 5 Cartoon summarizing the evolution of anaerobic heterotrichous ciliates (**a**, Caenomorphidae; **b**, Armophoridae and Clevelandellids) and their endosymbiotic methanogens. Ancestral ciliates diverged into aerobic, mitochondria-bearing ciliates (most likely the Stichotrichs), (**c**) and anaerobic, hydrogenosome-bearing heterotrichs (**a**, **b**). The black asterisks identify the first acquisition of methanogenic endosymbionts that precedes the adaptation of the ciliates to the various ecological niches. Because it is not known whether the evolution of hydrogenosomes preceded the divergence of Caenomorphidae and Armophoridae and Clevelandellids, two different, independent acquisitions are possible (black asterisks). Subsequently, the ciliates diverge (black lines), and both Caenomorphids and part of the Armophoridae and Clevelandellids radiate in freshwater sediments. Their endosymbionts are closely related to environmental, free-living Methanomicrobiales. Those Armophoridae and Clevelandellids (**b**) that adapt to life in the gastro-intestinal tract acquire endosymbionts that are related to intestinal Methanobacteriales, thereby replacing the ancestral endosymbionts (white asterisk). Redrawn after Hackstein et al. (2002)

References

Adachi J, Hasegawa M (1996) MOLPHY Version 2.3. Programs for molecular phylogenetics based on maximum likelihood. Computer Science Monographs, no. 28. Institute of Statistical Mathematics, Tokyo, Japan

Bandi C, Damiani G, Magrassi L, Grigolo A, Fani R, Sacch L (1994) Flavobacteria as intracellular symbionts in cockroaches. Proc R Soc Lond B Biol Sci 257:42–48

Bandi C, Sironi M, Nalepa CA, Corona S, Sacchi L (1997) Phylogenetic distant intracellular symbionts in termites. Parasitologia 39:71–75

Baumann P, Lai C-Y, Baumann L, Rouhbakhsh D, Moran NA, Clark MA (1995) Mutualistic associations of aphids and prokaryotes: biology of the genus *Buchnera*. Appl Environ Microbiol 61:1–7

Baumann P, Moran NA, Baumann L (1997) The evolution and genetics of aphid endosymbionts. Bioscience 47:12–20

Boxma B, de Graaf RM, van der Staay GWM, van Alen TA, Ricard G, Gabaldon T, van Hoek AHAM, Moon-van der Staay SY, Koopman WJH, van Hellemond JJ, Tielens AGM, Friedrich T, Veenhuis M, Huynen MA, Hackstein JHP (2005) An anaerobic mitochondrion that produces hydrogen. Nature 434(7029):74–79

Distel DL (1998) Evolution of chemoautotrophic endosymbioses in bivalves. Bivalve-bacteria chemosymbioses are phylogenetically diverse but morphologically similar. BioScience 48:277–286

Doolittle WF (1998) You are what you eat: a gene transfer ratchet could account for bacterial genes in eukaryotic nuclear genomes. Trends Genet 14:307–311

Embley TM, Finlay BJ (1994) The use of small-subunit ribosomal-RNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts. Microbiology 140:225–235

Embley TM, Finlay BJ, Dyal PL, Hirt RP, Wilkinson M, Williams AG (1995) Multiple origins of anaerobic ciliates with hydrogenosomes within the radiation of aerobic ciliates. Proc R Soc Lond Ser B-Biol Sci 262(1363):87–93

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fenchel T, Finlay BJ (1995) Ecology and evolution in anoxic worlds. Oxford University Press, New York

Fenchel T, Finlay BJ (2018) Free-living protozoa with endosymbiotic methanogens. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Goosen NK, Horemans AMC, Hillebrand SJW, Stumm CK, Vogels GD (1988) Cultivation of the sapropelic ciliate *Plagiopyla nasuta* stein and isolation of the endosymbiont *Methanobacterium formicicum*. Arch Microbiol 150(2):165–170

Hackstein JHP, Tielens AGM (2018) Hydrogenosomes. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Hackstein JHP, van Hoek AHAM, Leunissen JAM, Huynen M (2002) Anaerobic ciliates and their methanogenic endosymbionts. In: Seckbach J (ed) Symbiosis: mechanisms and model systems. Kluwer Academic Publishers, Dordrecht, pp 451–464. ISBN 1-4020-0189-4

Hirakata Y, Oshiki M, Kuroda K, Hatamoto M, Kubota K, Yamaguchi T, Harada H, Araki N (2015) Identification and detection of prokaryotic symbionts in the ciliate *Metopus* from anaerobic granular sludge. Microb Environ 30:335–338

Holmes DE, Giloteaux L, Orellana R, Williams KH, Robbins MJ, Lovley DR (2014) Methane production from protozoan endosymbionts following stimulation of microbial metabolism within subsurface sediments. Front Microbiol. https://doi.org/10.3389/fmicb.2014.00366

Krueger DM, Cavanaugh CM (1997) Phylogenetic diversity of bacterial symbionts of *Solemya* hosts based on comparative sequence analysis of 16S rRNA genes. Appl Environ Microbiol 63:91–98

Narayanan N, Krishnakumar B, Anupama VN, Manilal VB (2009) *Methanosaeta* sp., the major archaeal endosymbiont of *Metopus es*. Res Microbiol 160:600–607

Page RDM (1995) Paralell phylogenies: reconstructing the history of host-parasite assemblage. Cladistics 10:155–173

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Shinzato N, Watanabe I, Meng XY, Sekiguchi Y, Tamaki H, Matsui T, Kamagata Y (2007) Phylogenetic analysis and fluorescence in situ hybridization detection of archaeal and bacterial endosymbionts in the anaerobic ciliate *Trimyema compressum*. Microb Ecol 54:627–636

Shinzato N, Takeshita K, Kamagata Y (2018) The methanogenic and eubacterial endosymbionts of *Trimyema*. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Ushida K (2018) Symbiotic methanogens and rumen ciliates. In: Hackstein JHP (ed) (Endo) symbiotic methanogenic archaea. Springer, Heidelberg

van Bruggen JJA, Zwart KB, van Assema RM, Stumm CK, Vogels GD (1984) *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus* McMurrich. Arch Microbiol 139(1):1–7

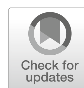
van Bruggen JJA, Zwart KB, Hermans JGF, van Hove EM, Stumm CK, Vogels GD (1986) Isolation and characterization of *Methanoplanus endosymbiosus* sp.nov, an endosymbiont of the marine sapropelic ciliate *Metopus contortus* Quennerstedt. Arch Microbiol 144(4):367–374

van Hoek AHAM, Sprakel VSI, Van Alen TA, Theuvenet APR, Vogels GD, Hackstein JHP (1999) Voltage-dependent reversal of anodic galvanotaxis in *Nyctotherus ovalis*. J Eukaryot Microbiol 46(4):427–433

van Hoek AHAM, van Alen TA, Sprakel VSI, Leunissen JAM, Brigge T, Vogels GD, Hackstein JHP (2000) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol Biol Evol 17(2):251–258

Wagener S, Bardele CF, Pfennig N (1990) Functional integration of *Methanobacterium formicicum* into the anaerobic ciliate *Trimyema compressum*. Arch Microbiol 153:496–501

Symbiotic Methanogens and Rumen Ciliates



Kazunari Ushida

Contents

1 The Rumen and Ciliated Protozoa 26
2 Methanogens Associated with Rumen Ciliates 27
3 Detection of Methanogens Associated with Ciliates 27
4 The Effect of Ciliated Protozoa on the Composition of Methanogenic Archaea in the Rumen 33
References 33

Abstract Ciliated protozoa are the principal component of the rumen microbiota. They contribute significantly the digestion of ruminants. As anaerobic fermentative microorganisms, rumen ciliated protozoa produce a significant amount of hydrogen and formate. Methanogenic archaea therefore associate closely with rumen ciliated protozoa. The presence of episymbiotic methanogens in rumen ciliated protozoa has been demonstrated as early as 1980s by microscopy. The number of ciliate-associated methanogens increases from the 10^0 level to 10^4/cell of ciliates after feeding. Enhancement of hydrogen and/or formate production from the ciliates by feeding attracts free-living methanogens. There are a couple of studies about the phylogeny of the ciliate-associated methanogens based on a molecular ecological approach. A range of methanogenic archaeal 16S rDNA, representing Methanobacteriales, Methanomicrobiales and Methanosarcinales, have been detected as ciliate-associated methanogens. However, it is still difficult to draw a conclusion about a potentially specific interaction between a particular ciliate species and a species of methanogenic archaea from these limited studies.

K. Ushida (✉)
Academy of Emerging Sciences, Chubu University, Kasugai, Aichi, Japan
e-mail: k_ushida@isc.chubu.ac.jp

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_3

1 The Rumen and Ciliated Protozoa

The rumen has a great capacity to digest plant polymers with the aid of anaerobic microbiota (Hungate 1966). This microbial ecosystem allowed ruminant animals to evolve into the predominant mammals in particular environments such as the semi-arid savannas (Hoffman 1973).

It is well established that the rumen ciliate protozoa have a significant impact on feed digestion in the ruminant animals, although the elimination of the ciliated protozoa does not impair the survival of the ruminant (Ushida et al. 1991).

As an anaerobic environment, the rumen microbial ecosystem requires an electron sink other than oxygen (Wolin 1975). Methane is the prevalent electron sink in this particular ecosystem (Hungate 1966). Fermentative microorganisms, therefore, create a specific relationship with hydrogenotrophic organisms to perform the different fermentation steps.

In the rumen, ciliated protozoa are known as potent hydrogen and formate producers. One cell of an axenic culture of rumen protozoa can produce 5 nmol of hydrogen per day (Ushida and Jouany 1996; Tokura et al. 1997). Potentially, this corresponds to a daily hydrogen production of approximately 50 L in the rumen of a sheep (Ushida et al. 1996). Besides hydrogen, one axenic ciliate cell produces 100 nmol of formate, which corresponds to a daily production of about 50 mol of formate in the rumen of a sheep. Such a concentration of hydrogen and formate attracts methanogens and makes the ciliate/methanogen consortium a predominant contributor for the ruminal methanogenesis. In fact, methanogenic bacteria associated with rumen ciliates were apparently responsible for 9–25% of methanogenesis in rumen fluid (Newbold et al. 1995). Consequently, elimination of the ciliated protozoa from the rumen, the defaunation, is associated with a 30–45% reduction of ruminal methanogenesis (Ushida et al. 1996).

In the case of rumen ciliates, the elimination of methanogens causes a decrease in the degradative capacities to some extent (Table 1) (Ushida and Jouany 1996). In particular, the elimination of methanogenesis increases the hydrogen and formate production from the ciliates at least by a factor of two but sometimes to a level several times higher. This slows the fermentation process down (Wolin 1975).

Table 1 Elimination of methanogenesis from ciliated protozoa affects the apparent dry matter degradation (%) in vitro (Ushida and Jouany 1996)

Fauna type	+M	−M
Mixed type A[a]	67.7	61.1
Epidinium spp.	50.4	42.4
Polyplastron multivesiculatum	49.2	47.0
Isotricha prostoma	27.8	22.7

[a]Mixed type A was defined by Eadie (1967) in which the rumen harbours *Polyplastron multivesiculatum* as a particular organism with common Entodinia and Isotrichids

2 Methanogens Associated with Rumen Ciliates

Methanogenic archaea associate closely with the rumen ciliates to facilitate the interspecies hydrogen transfer in the form of an episymbiosis or an endosymbiosis.

No free-living methanogens were detected in the protozoal fraction prepared by sedimentation (Sharp et al. 1998). Therefore, it was believed that all methanogens that are metabolically associated with the ciliates are present inside the cell or intimately attached to the cell surface of the ciliates.

Episymbiotic methanogens of rumen ciliates were microscopically observed as early as 1980 by their characteristic F_{420} autofluorescence (Vogels et al. 1980). Endosymbiotic methanogens were observed by an archaea-specific oligonucleotide probe approach (Finlay et al. 1994). It has been shown that these endosymbiotic methanogens are localized in the cytoplasm, not in digestive vacuoles, and adjacent to the hydrogenosomes. Interestingly, the number of the endosymbiotic methanogens exceeds the number of those attached on the cell surfaces of ciliates.

The number of ciliate-associated methanogens increased from the level of 10^0 to 10^4 most probable number (MPN)/cell of ciliate after feeding (Tokura et al. 1997). When the ciliated protozoa engulfed and fermented feed particles, the number of ciliate-associated methanogens increased. Since the maximal level was recorded shortly (1–2 h) after feeding, it is unlikely that endosymbionts grow to this level in this short period of time. Accordingly, such a rapid increase in the numbers of ciliate-associated methanogens may reflect the active attachment or vigorous engulfment of free-living methanogens. Indeed, the hydrogen supply from the ciliates strongly attracted free-living methanogens (Stumm et al. 1982).

Ciliated protozoa predate and digest engulfed bacteria as a major prey. If engulfed methanogens would be the source of the endosymbiotic methanogens, these methanogens need to be resistant against protozoal lytic activity, or they may escape from the digestion within food vacuoles. This point may be supported by the fact that anaerobic ciliates, *Metopus* spp. and *Nyctotherus* spp., which harbour methanogenic symbionts closely related to the free-living organisms (Embley and Finley 1993; van Hoek et al. 2000). One study evaluated the resistance of methanogenic archaea against the lytic activity of rumen protozoa. It was found that some of the methanogens are relatively resistant against the protozoal lytic activities; i.e. *Methanosarcina barkeri* DSM 800 was more resistant than *Methanobrevibacter* sp. MF1 (Newbold et al. 1996). DSM 800 could establish the interspecies hydrogen transfer with *Polyplastron multivesiculatum* (Ushida et al. 1997).

3 Detection of Methanogens Associated with Ciliates

There are a couple of studies about the phylogeny of the ciliate-associated methanogens based on molecular phylogenetic approaches (Sharp et al. 1998; Tokura et al. 1999b; Chagan et al. 1999; Ohene-Adjei et al. 2007; Regensbogenova et al. 2004; Irbis and Ushida 2004; Tymensen et al. 2012; Belanche et al. 2014).

However, little information is available about the methanogens isolated from ciliates. An isolation of methanogens from washed ciliated protozoa was tried, and the strain *Methanobrevibacter* sp. MB9 was isolated. This isolate was phylogenetically close to *Methanobrevibacter ruminantium*, on the basis of morphology and 16S rRNA phylogeny (Tokura et al. 1999a). Other attempts for the isolation of symbiotic methanogens have not been found in the literature probably due to the difficulty and tediousness of the isolation procedure. Even for the free-living methanogens, relatively few had been isolated from the rumen (Janssen and Kirs 2008). The isolate *Methanobrevibacter* sp. MB9 uses hydrogen, formate and small amounts of 2-propanol. This substrate use, 2-propanol, is not common for ruminal *Methanobrevibacter* species.

Partial sequences of 16S rDNA of ciliate-associated methanogens are available from studies in Japan and the UK (Chagan et al. 1999; Tokura et al. 1999b; Irbis and Ushida 2004; Regensbogenova et al. 2004). DNA was extracted from washed cells of ciliate protozoa. In some studies, DNA was extracted from a single cell of the ciliated protozoa. Table 2 shows the distribution of archaeal 16S sequences retrieved from the cells of ciliated rumen protozoa. The phylogenetic analyses are also shown in Fig. 1a, b. In this table, there are some unidentified strains of which strain 1Y is phylogenetically close to *Methanobrevibacter gottschalkii*, strain SM9 is close to *Methanobrevibacter millerae*, strain OCP is close to *Methanobrevibacter olleyae* and strain Z8 is close to *Methanobrevibacter ruminantium* (Rea et al. 2007; Evans et al. 2009). More recent studies (Tymensen et al. 2012) demonstrated that greater abundance of OTUs corresponding to the genus *Methanobrevibacter* in rumen protozoa associated methanogens (PAM) than free-living methanogens. In the latter, OTUs corresponding to the genus *Methanomicrobium* are present in abundance.

Methanobrevibacter-like sequences and those similar to *Methanomicrobium* were the predominant sequences detected in different studies. *Methanobrevibacter* sp. 1Y-like sequences were found in a range of protozoal species. Lastly, *Methanomicrobium mobile* (AY196679)-like sequences were detected in a range of protozoa both in Japanese and British studies. Methanogens belonging to the Methanobacteriales were detected predominantly in Japanese studies (Accession numbers start with AB; see Tokura et al. 1997, 1999a, b; Chagan et al. 1999; Irbis and Ushida 2004), while those belong to Methanomicrobiales were predominantly detected in a British study (Accession numbers start with AJ; see Regensbogenova et al. 2004). Interestingly enough, *Ophryoscolex caudatum* was studied in both the Japanese and British studies. This particular rumen protozoon harboured a variety of methanogens such as Methanobacteriales, Methanomicrobiales and Methanosarcinales. In addition to these methanogens, *Ophryoscolex caudatum* harboured also Thermoplasmatales. Other Entodiniomorphs like *Polyplastron multivesiculatum*, *Eudiplodinium maggii*, *Diplodinium dentatum*, *Metadinium medium* and *Entodinium furca* harboured relatively limited numbers of species of methanogens. In the case of holotrichs, *Isotricha intestinalis* harboured a phylogenetically broad range of methanogens similar to that shown in *Ophryoscolex caudatum*. An aquatic ciliate, *Metopus contortus*, can host a broad range of methanogens. Accordingly, this aquatic ciliate is defined as the generalist host for

Table 2 Registered archaeon 16S rDNA retrieved from rumen ciliate protozoa

Suggested nearest known isolate	Registered archaeon 16S rRNA sequence retrieved from rumen ciliate protozoa								
	P. multivesiculatum	*Eu. magii*	*O. caudatum*	*D. dentatum*	*E. furca*	*M. medium*	*I. prostoma*	*I. intestinalis*	Mixed population
Methanobacteriales									
Methanobrevibacter ruminantium MB9 (isolate)									AB017514
Methanobrevibacter smithii ALI	AB189858								
Methanobrevibacter millerae ZA-10							AB026169		AB022182, AB022185, AB022181
Methanobrevibacter wolinii SH	AB189856		AB189866					AB189861	
Methanobrevibacter sp. 1Y	AB026173-74	AB026171	AB189865						AB022183-84
Methanobrevibacter sp. SM9	AB189857		AB189864					AB189859, AB189861	
Methanobrevibacter sp. OCP							AB026168		
Methanobrevibacter sp. Z8		AB026170							
Methanobacterium sp. GH	AB026175								
Methanosphaera stadtmanae DSM 3091		AB026172							AB022186

(continued)

Table 2 (continued)

Suggested nearest known isolate	Registered archaeon 16S rRNA sequence retrieved from rumen ciliate protozoa								
	P. multivesiculatum	*Eu. magii*	*O. caudatum*	*D. dentatum*	*E. furca*	*M. medium*	*I. prostoma*	*I. intestinalis*	Mixed population
Methanomicrobiales									
Methanomicrobium mobile DSM 1539			AJ606400, AJ606411			AJ606405-10			
Methanomicrobium mobile (AY196679)			AJ606402, AB189867	AJ606418	AJ606412-17	AJ606401		AB189862	
Methanosarcinales									
Methanimicrococcus blatticola PAT			AJ606403						
Methanimicrococcus blatticola DSM 13328			AJ606404						
Methanosarcina sp. 2214B				AJ606419					
Thermoplasmatales									
Thermoplasma sp. XT107			AB189868					AB189863	

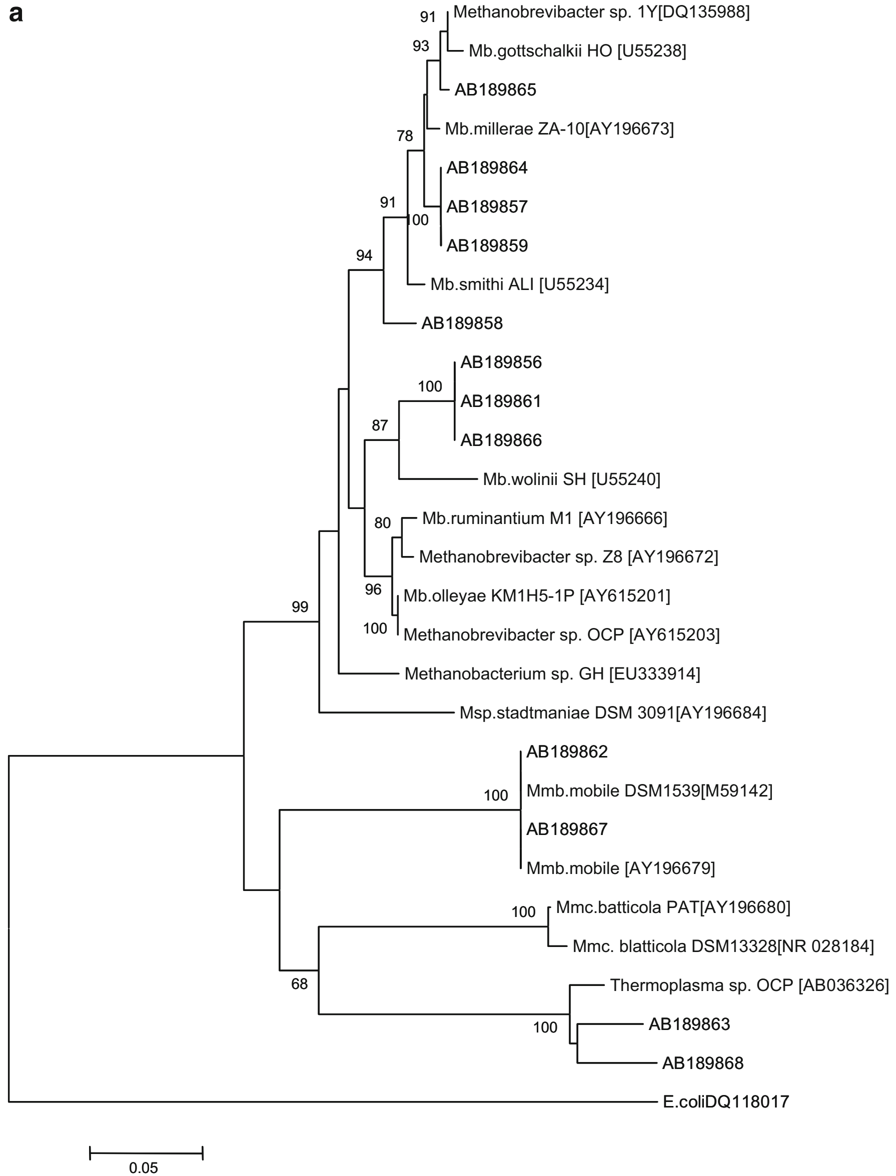


Fig. 1 Neighbour-joining tree computed from partial 16S DNA of methanogens associated with rumen ciliated protozoa by MEGA 4.0 (Tamura et al. 2007) with 500 replicates for bootstrap. (**a**) Partial sequences (*E. coli* [DQ118017]16S rDNA position 781–1233) are used to analyse ciliate-associated archaea (AB189856-AB189868); (**b**) (*E. coli* [DQ118017] position 218–798) are used to analyse ciliate-associated archaea (AB022181-AB022186, AB026168-026175, AJ606400-AJ606419)

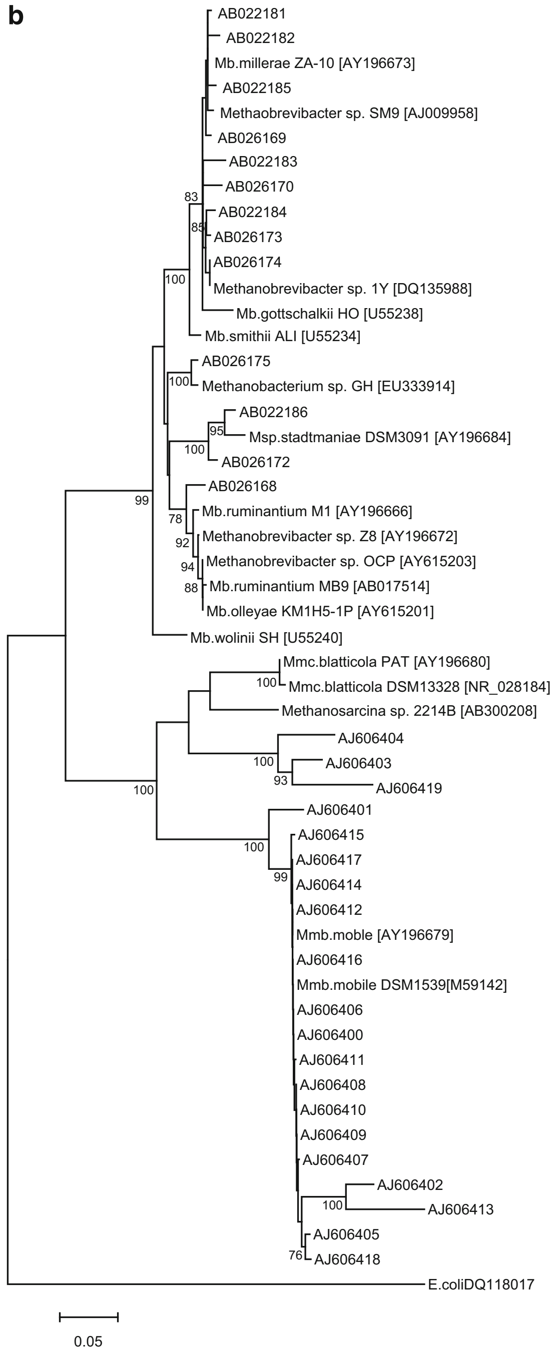


Fig. 1 (continued)

methanogens (Embley and Finlay 1993). Rumen ciliated protozoa like *Isotricha intestinalis* and *Ophryoscolex caudatum* can also be a generalist host for methanogens. However, it is still difficult to draw a conclusion about a potentially specific interaction between a particular ciliate species and a species of methanogenic archaea from these limited studies.

4 The Effect of Ciliated Protozoa on the Composition of Methanogenic Archaea in the Rumen

As indicated above, a cell of ciliated protozoa can harbour up to 10^4 methanogens.

This number may differ according to protozoal species, since protozoal size determined the number of PAM as big protozoa had 1.7–3.3 times more methanogen DNA than smaller protozoa (Belanche et al. 2014). Since the number of ciliates ranges from 10^5 to 10^6 cells/mL rumen fluid (Williams and Coleman 1991), they may encompass a methanogenic population as large as 10^{10} methanogens/mL. If there is a specific relationship between the ciliate species and their methanogenic symbionts, an increase in the number of ciliated protozoa should affect the methanogenic archaeal population as a selective pressure upon the methanogenic population. In an in vivo study, Ohene-Adjei et al. (2007) indicated that an inoculation of *Polyplastron multivesiculatum* into the rumen predominantly associated with the detection of methanogens closely related to *Methanobrevibacter bryantii*, *Methanobrevibacter ruminantium* and *Methanosphaera stadtmanae*. These authors also showed that inoculation of holotrich protozoa (Isotrichidae) into the rumen was primarily associated with the detection of methanogens closely related to *Methanobrevibacter smithii*. Although this Canadian in vivo study appears not to agree with the results shown in Table 2, it is likely that the presence of particular ciliate protozoa may promote the predominance of particular species of methanogens.

Again, the specificity of the host-methanogenic symbiont relationship is still difficult to be proven, because a long-term pure culture system for rumen ciliates has not been established so far. Without a pure culture of rumen ciliated protozoa, consisting of aposymbiotic ciliates, an inoculation study as reported for *Trimyema compressum* cannot be realized (Wagener et al. 1990; Holler and Pfennig 1991).

References

Belanche A, De la Fuente G, Newbold CJ (2014) Study of methanogen communities associated with different rumen protozoal populations. FEMS Microbiol Ecol 90:663–677

Chagan I, Tokura M, Jouany JP, Ushida K (1999) Detection of methanogenic archaea associated with rumen ciliate protozoa. J Gen Appl Microbiol 45:305–308

Eadie JM (1967) Studies on the ecology of certain rumen ciliate protozoa. J Gen Microbiol 49:173–194
Embley TM, Finlay BJ (1993) Systematic and morphological diversity of endosymbiotic methanogens in anaerobic ciliates. Antonie van Leeuwenhoek 64:261–271
Evans PN, Hinds LA, Sly LI, McSweeney CS, Morrison M, Wright ADG (2009) Community composition and density of methanogens in the foregut of the Tammar Wallaby (*Macropus eugenii*). Appl Environ Microbiol 75:2598–2602
Finlay BJ, Esteban G, Clarke KJ, Williams AG, Embley TM, Hirt RP (1994) Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol Lett 117:157–161
Hoffman RR (1973) Ruminant stomach. East African Literature Bureau, Nairobi, Kenya
Holler S, Pfennig N (1991) Fermentation products of the anaerobic ciliate *Trimyema compressum* in monoxenic cultures. Arch Microbiol 156:327–334
Hungate RE (1966) The rumen and its microbes. Academic Press, New York
Irbis C, Ushida K (2004) Detection of methanogens and proteobacteria from a single cell of rumen ciliate protozoa. J Gen Appl Microbiol 50:203–212
Janssen PH, Kirs M (2008) Structure of the archaeal community of the rumen. Appl Environ Microbiol 74:3619–3625
Newbold CJ, Lassalas B, Jouany JP (1995) The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. Lett Appl Microbiol 21:230–234
Newbold CJ, Ushida K, Morvan B, Fonty G, Jouany JP (1996) The role of ciliate protozoa in the lysis of methanogenic bacteria in rumen fluid. Lett Appl Microbiol 23:421–425
Ohene-Adjei S, Teather RM, Ivanj M, Forster RJ (2007) Postinoculation protozoan establishment and association patterns of Methanogenic archaea in the ovine rumen. Appl Environ Microbiol 73:4609–4618
Rea S, Bowman JP, Popovski S, Pimm C, Wright ADG (2007) *Methanobrevibacter millerae* sp. nov. and *Methanobrevibacter olleyae* sp. nov., methanogens from the ovine and bovine rumen that can utilize formate for growth. Int J Syst Evol Microbiol 57:450–456
Regensbogenova M, McEwan NR, Javorsky P, Kisidayova S, Michalowski T, Newbold CJ, Hackstein JHP, Pristas P (2004) A re-appraisal of the diversity of the methanogens associated with the rumen ciliates. FEMS Microbiol Lett 238:307–313
Sharp R, Ziemer CJ, Stern MD, Stahl DA (1998) Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiol Ecol 36:71–78
Stumm CK, Gijzen HJ, Vogels GD (1982) Association of methanogenic bacteria with ovine rumen ciliates. Br J Nutr 47:95–99
Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
Tokura M, Ushida K, Miyazaki K, Kojima Y (1997) Methanogens associated with rumen ciliates. FEMS Microbiol Ecol 22:137–143
Tokura M, Tajima K, Ushida K (1999a) Isolation of *Methanobrevibacter* sp. as a ciliate-associated ruminal methanogen. J Gen Appl Microbiol 45:43–47
Tokura M, Chagan I, Ushida K, Kojima Y (1999b) Phylogenetic study of methanogens associated with rumen ciliates. Curr Microbiol 39:123–128
Tymensen LD, Beauchemin KA, McAllister TA (2012) Structures of free-living and protozoa-associated methanogen communities in the bovine rumen differ according to comparative analysis of 16S rRNA and mcrA genes. Microbiology 158:1808–1817
Ushida K, Jouany JP (1996) Methane production from ciliated rumen protozoa and its effect on protozoal activity. Lett Appl Microbiol 23:129–132
Ushida K, Jouany JP, Demeyer DI (1991) Effects of presence or absence of rumen protozoa on the efficiency of utilization of concentrate and fibrous feeds. In: Tsuda T, Sasaki Y, Kawashima R (eds) Physiological aspects of digestion and metabolism in ruminants. Academic Press, San Diego, pp 626–655

Ushida K, Tokura M, Itabashi H, Takenaka A (1996) Ciliate protozoa and ruminal methanogenesis. In: Onodera R, Itabashi H, Ushida K, Yano H, Sasaki Y (eds) Rumen microbes and digestive physiology in ruminants. Japan Scientific Society Press/Karger, Tokyo, pp 209–220

Ushida K, Newbold CJ, Jouany JP (1997) Interspecies hydrogen transfer between the rumen ciliate *Polyplastron multivesiculatum* and *Methanosarcina barkeri*. J Gen Appl Microbiol 43:129–131

van Hoek AHAM, van Alen TA, Sprakel VSI, Leunissen JAM, Brigge T, Vogels GD, Hackstein JHP (2000) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol Biol Evol 17:251–258

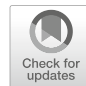
Vogels GD, Hoppe WF, Stumm CK (1980) Association of methanogenic bacteria with rumen ciliates. Appl Environ Microbiol 40:608–612

Wagener S, Bardele CF, Pfennig N (1990) Functional integration of *Methanobacterium formicicum* into the anaerobic ciliate *Trimyema compressum*. Arch Microbiol 153:496–501

Williams AG, Coleman GS (1991) The rumen protozoa. Springer, London

Wolin MJ (1975) Interactions between the bacterial species of the rumen. In: McDonald IW, Warner ACI (eds) Digestion and metabolism in the ruminant. University of New England Publ. Unit, Armidale, Australia

Methanogenic and Bacterial Endosymbionts of Free-Living Anaerobic Ciliates



Naoya Shinzato, Kazutaka Takeshita, and Yoichi Kamagata

Contents

1 Anaerobic Ciliate *Trimyema compressum* 38
2 Methanogenic Symbiont 40
3 Bacterial Symbiont 45
4 Genome Sequence of Bacterial Symbiont 47
5 Bacterial Symbiont in Other Anaerobic Ciliates 48
6 Perspectives 51
References 51

Abstract *Trimyema compressum* thrives in anoxic freshwater environments in which it preys on bacteria and grows with fermentative metabolisms. Like many anaerobic protozoa, instead of mitochondria, *T. compressum* possess hydrogenosomes, which are hydrogen-producing, energy-generating organelles characteristic of anaerobic protozoa and fungi. The cytoplasm of *T. compressum* harbours hydrogenotrophic methanogens that consume the hydrogen produced by hydrogenosome, which confers an energetic advantage to the host ciliate. Symbiotic associations between methanogenic archaea and *Trimyema* ciliates are thought to be established independently and/or repeatedly in their evolutional history. In addition to methanogenic symbionts, *T. compressum* houses bacterial symbiont TC1 whose function is unknown in its cytoplasm. Recently, we analysed whole-genome sequence of TC1 symbiont to investigate its physiological function in the tripartite

N. Shinzato
Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa, Japan

Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan

K. Takeshita
Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa, Japan

Y. Kamagata (✉)
Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan
e-mail: y.kamagata@aist.go.jp

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_4

symbiosis and found that fatty acid synthesis *fab* operon of TC1 symbiont lacked typical transcriptional repressor, which is normally coded on the upstream of the *fab* operon. The sequence data suggested that TC1 symbiont contributes to host *Trimyema* by the synthesis of fatty acid or its derivative. In this review, we summarize the early works and recent progress of the studies on *Trimyema* ciliates, including a stably cultivable model protozoa *T. compressum*, and discuss about symbiotic associations in oxygen-scarce environments.

1 Anaerobic Ciliate *Trimyema compressum*

Trimyema is an anaerobic ciliate that thrives in various anoxic aquatic environments, including marine, saltern, and hydrothermal vent (Lackey 1925; Augustin et al. 1987; Nerad et al. 1995; Baumgartner et al. 2002; Cho et al. 2008; Park and Simpson 2015). *Trimyema* ciliates prey on bacteria and grow with fermentative metabolisms. Morphological features characteristic of *Trimyema* are as follows: (1) the presence of a prominent caudal cilium, (2) a cytosome near the apical end of the cell, (3) somatic kineties in longitudinal rows forming several oblique ciliary girdles, and (4) a semicircular structuring of the oral ciliature (Augustin et al. 1987; Nerad et al. 1995; Baumgartner et al. 2002). On the basis of morphological features, *T. compressum*, *Trimyema koreanum*, and *Trimyema minutum* have been described so far, and recently *Trimyema finlayi* was newly reported (Lewis et al. 2018).

T. compressum is frequently found in anoxic freshwater sediments and is the best-studied species in *Trimyema* (Fig. 1). *T. compressum* has been cultured monoxenically or axenically from a polluted ditch and some wastewater treatment reactors using synthetic media supplemented with living or dead bacteria as food (Wagener and Pfennig 1987; Goosen et al. 1990a; Yamada et al. 1994; Shinzato

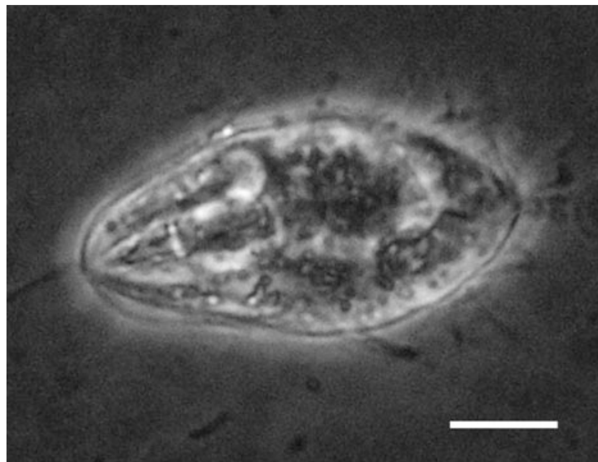


Fig. 1 Phase contrast image of living *T. compressum*. *T. compressum* swims actively in the medium and preys on food bacteria. Bar represents 10 μm

et al. 2007). Based on the literatures, the ciliate could grow in the temperature range of 10–35 °C, in which the optimum was ranging from 25 to 30 °C (Wagener and Pfennig 1987; Goosen et al. 1990a). The maximum cell density under optimum growth conditions reached approximately 2–3 × 10^3 cells ml^{-1} (Goosen et al. 1990a).

T. compressum was found to have some food selectivity as reported in many other protozoa. Schulz et al. (1990) tested the preferential use of various chemolithotrophic and phototrophic bacteria and concluded that only gram-negative bacteria supported the ciliate's growth (Schulz et al. 1990). However, Yamada et al. (1994) reported that *T. compressum* could prey on several types of bacteria and archaea belonging to the genera *Lactobacillus*, *Clostridium*, *Desulfovibrio*, *Enterobacter*, *Escherichia*, *Pelobacter*, and *Methanoculleus*. The maximum number of *T. compressum* cells varied depending on the species of food bacteria supplemented. The highest number of ciliates reached 9300 cells ml^{-1} after feeding on the cells of *Desulfovibrio vulgaris* (Yamada et al. 1994).

The observed food selectivity of *T. compressum* might be related to the nutritional effect of food bacteria on the ciliate. For example, some sterols such as stigmasterol, stigmastanol, or ergosterol have been reported to enhance the growth of *T. compressum* and to be essential for reproducible growth of symbiont-free strains of the ciliate (Wagener and Pfennig 1987). In addition to sterol requirement, *T. compressum* also needs unidentified growth factors in food bacteria. Broers et al. (1991) reported that *Bacteroides* and *Klebsiella* cells inactivated by γ-irradiation could support the ciliate growth; however, they could not be replaced with autoclaved bacterial cells, suggesting the involvement of unidentified heat-labile growth factors in these bacteria (Broers et al. 1991).

It is known that all of *Trimyema* species live in anoxic habitats and their energy metabolisms are highly adapted to oxygen-free environments. *T. compressum* possesses hydrogenosome, an anaerobic energy-producing organelle found in some lineages of anaerobic protozoa and fungi (Hackstein et al. 2008a, b). Hydrogenosome oxidizes pyruvate with substrate-level phosphorylation and disposes of the excess reducing equivalent derived from fermentative metabolisms by hydrogenase-mediated proton reduction (Müller 1993; Boxma et al. 2005). The hydrogen production and methylviologen-reducing activity are hallmark features of hydrogenosome (Zwart et al. 1988; Goosen et al. 1990a; Broers et al. 1991).

On the other hand, metabolite profiles of *T. compressum* indicated that they gain energy by digestion of food bacteria via fermentative metabolisms. Goosen et al. (1990b) examined fermentation products of *T. compressum* grown with *Bacteroides* sp. as food and detected ethanol, acetate, lactate, formate, CO_2, and hydrogen under anaerobic conditions in which ethanol was formed in substantial amounts representing 44% of the total carbon excreted (Goosen et al. 1990b). However, metabolite profiles fluctuated depending on the growth conditions such as anaerobicity, the species or amount of food bacteria (Goosen et al. 1990b; Holler and Pfennig 1991). For instance, under microaerobic conditions, *T. compressum* was shown to produce formate and CO_2 as major end products accompanying oxygen consumption, while no hydrogen, ethanol, or succinate was formed (Goosen et al.

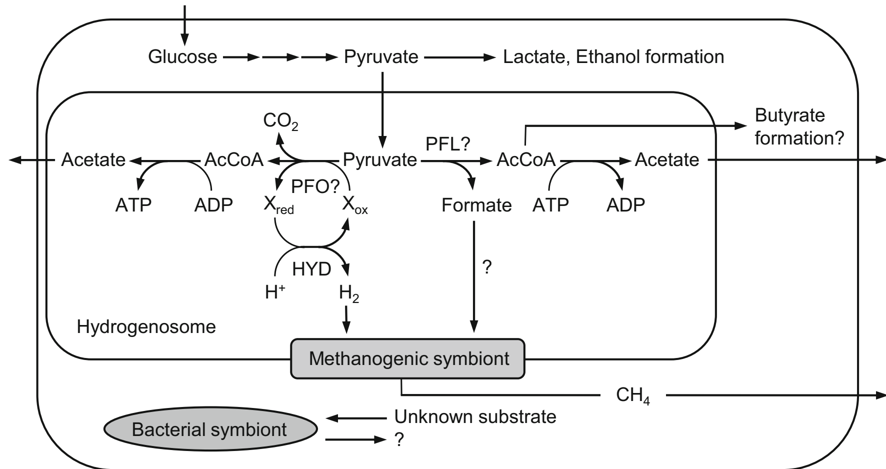


Fig. 2 Speculative metabolic schemes of carbohydrate metabolism in the symbiotic consortium of *T. compressum*. Abbriviations: AcCoA, acetyl-CoA; HYD, hydrogenase; PFL, pyruvate formate lyase; PFO, pyruvate ferredoxin oxidoreductase. Xox, red, unknown electron carrier. Methanogenic symbionts are capable to use both hydrogen and formate as the substrate for methanogenesis. Substrate and contribution of bacterial symbionts are unknown (modified from Fig. 5 of Hackstein et al. 2008a)

1990b). These observations suggested that *T. compressum* could use oxygen as a terminal electron acceptor depending on the situation.

The details of carbohydrate metabolism in *Trimyema*, however, remain to be elucidated, since no biochemical or molecular studies have been performed. Therefore, metabolite profiles are the only available information to allow the speculation of metabolic features of this ciliate. As mentioned previously, ethanol, lactate, acetate, formate, CO_2, and hydrogen have been reported as major fermentative products of *T. compressum*. Based on the metabolic profiles, Hackstein et al. (2008a) presented a speculative metabolic scheme of carbohydrate degradation pathway in *Trimyema* (Fig. 2). In this scheme, pyruvate formate lyase (PFL) is hypothesized to be involved in pyruvate oxidation, since apparent formate production has been found in *T. compressum* cultures. This type of carbohydrate metabolism resembles those of some anaerobic *Chytridiomycota* fungi (Boxma et al. 2004; Hackstein et al. 2008a).

2 Methanogenic Symbiont

Symbiotic associations between protozoa and methanogenic archaea are found in various anoxic environments (Hackstein and Vogels 1997). Methanogenic symbionts in protozoa can be easily detected by bluish-green fluorescence of coenzyme F_{420}, which is characteristic of methanogens (Doddema and Vogels 1978). The association of methanogenic symbionts is normally found in hydrogenosome-bearing protozoa,

and the hydrogen that evolved from the organelles is believed to be consumed by the endosymbiotic methanogen. As the oxidation of NADH and $FADH_2$ coupled to proton or bicarbonate reduction is thermodynamically feasible only at low hydrogen concentrations, methanogenic symbionts could facilitate anaerobic metabolism of the host protozoa by scavenging hydrogen (Stams 1994). Many free-living and intestinal species of anaerobic protists have been reported to harbour methanogenic symbionts (Embley and Finlay 1993, 1994; Van Hoek et al. 2000). Thus far, *Methanobacterium formicicum* and *Methanoplanus endosymbiosus* have been isolated from anaerobic ciliates and amoeba (Van Bruggen et al. 1984, 1986, 1988; Goosen et al. 1988).

In *Trimyema* species, *T. compressum* and *Trimyema* sp. were found to possess methanogenic symbionts in their cytoplasm (Wagener and Pfennig 1987; Finlay et al. 1993). *T. compressum* appeared to harbour them by nature, because the ciliates freshly cultured from environmental samples were always accompanied by methanogens (Wagener and Pfennig 1987; Goosen et al. 1990a; Yamada et al. 1994; Shinzato et al. 2007). The cell size of methanogenic symbionts in *T. compressum* was 0.65 μm wide and 1.6–3.3 μm long (Wagener and Pfennig 1987), and another study reported that they were 0.3–0.4 μm wide and 1.3–2.0 μm long (Shinzato et al. 2007). The number of methanogenic symbionts varies between zero and several hundreds in host cells (Wagener and Pfennig 1987). In the case of *T. compressum* strain S10 that we have long studied, single ciliate cell contained an average of 436 cells of methanogenic symbionts ($N = 20$), ranging from 272 to 769 (Shinzato et al. 2007).

Transmission electron microscopic observations of freshly cultured strains of *T. compressum* demonstrated that the methanogenic symbionts are located nearby or embedded in hydrogenosomes (Figs. 3 and 4). Such characteristic proximity between methanogen and hydrogenosome has been found in various anaerobic protozoa harbouring methanogenic symbionts (Embley and Finlay 1994). Because

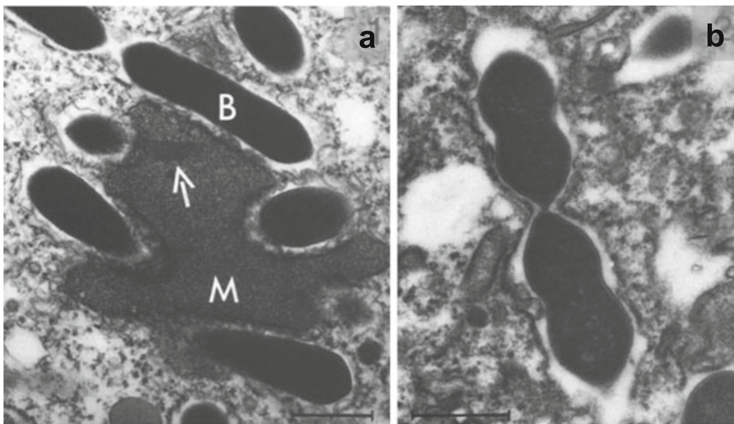


Fig. 3 Transmission electron micrograph of ultra-thin section of *T. compressum* strain N. (**a**) Methanogenic symbionts (B) associate with microbodies (M: hydrogenosomes). (**b**) Dividing cell of non-methanogenic (bacterial) symbiont, not associated with microbodies. Bars represent 0.5 μm. (reprinted from Fig. 1 and 2 of Goosen et al. (1990a, b) with permission of the publisher)

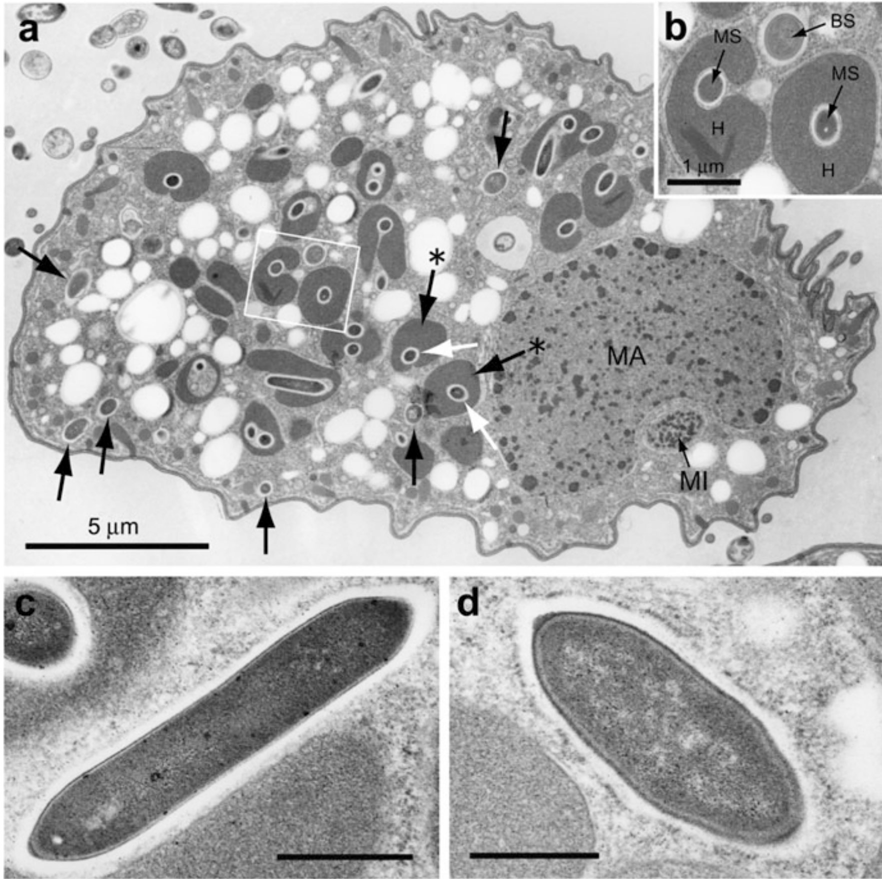


Fig. 4 Transmission electron micrographs of ultra-thin sections of *T. compressum* strain S10. (**a**) Whole view of *T. compressum* cell. The methanogenic symbionts (white arrows) were closely associated with hydrogenosomes (black arrows with asterisks), while the bacterial symbionts (black arrows) were distributed over the cytoplasm independent of hydrogenosomes. The macronucleus and micronucleus are shown with MA and MI, respectively. (**b**) Enlarged view of the boxed region in (a). Methanogenic symbionts (MS) were surrounded by hydrogenosomes (H). Bacterial symbionts are also shown (BS). (**c**) Longitudinal section of the methanogenic symbiont. (**d**) Longitudinal section of the bacterial symbiont. Bars represent 0.5 µm unless stated otherwise (reprinted from Fig. 1 of Shinzato et al. 2007 with permission of the publisher)

hydrogen has a large diffusion flux, the observed close contact between hydrogenosome and methanogenic symbiont is important to maximize the efficiency of interspecies hydrogen transfer. The molecular phylogenetic identification based on 16S rRNA gene of the symbionts in *T. compressum* strain S10 showed that they were closely related to *Methanobrevibacter arboriphilus* with 97.2% sequence similarity (in ca. 1300 bp) (Shinzato et al. 2007).

On the other hand, another species of *Trimyema* has been reported to harbour a distinct type of methanogenic symbionts. Finlay et al. (1993) examined the methanogenic symbionts in *Trimyema* sp., which was cultured from sediments of

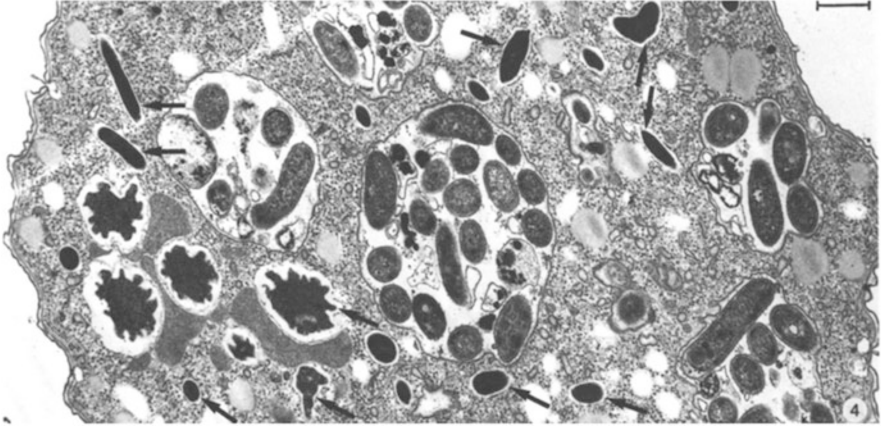


Fig. 5 Transmission electron micrographs of ultra-thin sections of *Trimyema* sp. Electron-dense methanogens with various shapes are enclosed individually in vacuoles within the ciliate cytoplasm (arrows). Polymorphic transformation of methanogens is observed from disc-shaped to stellate form, at which the methangens completely surrounded by hydrogenosomes. Several large vacuoles, each containing many (non-methanogen) food bacteria, are also shown. Bar represents 1 μm. (reprinted from Fig. 4 of Finlay et al. 1993 with permission of the publisher)

a productive pond in England, and showed that its methanogenic symbionts were relatively small and irregularly disc-shaped and distributed over the cytoplasm (Fig. 5; Finlay et al. 1993). However, they appeared to show polymorphic traits according to the degree of association with hydrogenosome, and those attached to hydrogenosome were significantly larger and profusely dentate. This morphological change is thought to facilitate efficient capture of hydrogen that evolved from the hydrogenosome. Molecular phylogenetic inspections of the methanogenic symbionts in *Trimyema* sp. based on 16S rRNA gene sequences revealed that they were closely related to the free-living methanogen *Methanocorpusculum parvum* with a 10-base difference in 840 compared bases. A relative of *M. parvum*, which was isolated and described as *M. endosymbiosus* (Van Bruggen et al. 1986), has also been reported from marine ciliate, *Metopus contortus* (Embley et al. 1992).

The association between *T. compressum* and methanogenic symbionts seems to be somewhat unstable and capricious, even though *T. compressum* cells freshly isolated from the environment always harbour methanogenic symbionts. Indeed, most monoxenic and axenic cultures of *T. compressum* lost their symbionts during continued cultivation as mentioned earlier (Wagener and Pfennig 1987; Goosen et al. 1990a; Yamada et al. 1997). *T. compressum* strain K isolated in Germany lost their symbionts during continued cultivation, especially under conditions with an abundant supply of food bacteria. In contrast, methanogen-bearing rate of the ciliate could be enhanced under food-limited conditions (Wagener and Pfennig 1987). The loss of symbionts may be due to the outgrowth of host cells, which disturbs the synchronization of growth of host and symbiont. The loss of symbionts has been also reported in other anaerobic ciliates. Finlay et al. (1993) mentioned the experience that some species of anaerobic ciliates lost methanogenic symbionts when the cultures were held at an elevated

temperature (27 °C), whereas methanogenic symbionts tended to be maintained in the ciliate when the cultures were kept at a low temperature (10 °C). This observation indicates that vigorous growth of the ciliates at elevated temperature might surpass the growth of methanogenic symbionts.

Methanogenic symbionts are generally believed to provide metabolic advantages to the host ciliate (Holler and Pfennig 1991). Yamada et al. (1997) examined the effect of methanogenic symbionts on the host and demonstrated that in the absence of symbionts, the major products shifted from acetate to butyrate and the maximum cell yield decreased from 3300 to 2700 cells ml^{-1} (Yamada et al. 1997). In anaerobic metabolism such as fermentation of carbohydrate, formation of more oxidized product yields more energy. Methanogenic symbionts therefore appeared to contribute to further substrate oxidation in *T. compressum* by scavenging hydrogen thus maintaining the hydrogen concentration at very low level. The contribution of the methanogenic symbionts for the host growth has also been examined in two hydrogenosomal ciliates, *Plagiopyla frontata* and *M. contortus*, by methanogen-curing experiments using bromoethane sulfonic acid (BES), a specific inhibitor for methanogenesis. The results showed that the absence of methanogenic symbionts reduced the growth yield by approximately 30% in both ciliates (Fenchel and Finlay 1991).

As described earlier, it appears that two types of methanogens have established symbiotic association with *Trimyema* species. The first one is a rod-shaped methanogen found in *T. compressum*, which was the relative of *Methanobacterium* or *Methanobrevibacter* in the order *Methanobacteriales* (Wagener and Pfennig 1987; Shinzato et al. 2007). The other one is not rod-shaped, polymorphic methanogen, which was found in *Trimyema* sp. It has been identified as the close relative of *Methanocorpusculum parvum* in the order *Methanomicrobiales* (Finlay et al. 1993). This indicates that *Trimyema* ciliates have established symbiotic associations with different species of methanogens independently in their particular niches as hypothesized in other anaerobic ciliates (Embley and Finlay 1993).

Trimyema is not the only ciliate that harbours phylogenetically distantly related methanogens as endosymbionts. A freshwater ciliate, *M. contortus*, harboured polymorphic methanogens closely related to the symbiotic methanogen found in *Trimyema* sp., while *Metopus palaeformis*, another ciliate species isolated from a municipal landfill, was found to be associated with rod-shaped, non-transforming *Methanobacterium* species (Embley et al. 1992). Likewise, relatives of the genera *Methanolobus* and *Methanoculleus* have been reported as endosymbionts in *P. frontata* and *Plagiopyla nasuta*, respectively (Embley and Finlay 1994). These disorderly combinations of methanogenic symbionts and host ciliates suggest that these symbioses may have been established independently in their particular niches after diversification of each ciliate species (Embley and Finlay 1994; Van Hoek et al. 2000). In addition, these events may have been accompanied by the replacement of methanogenic symbionts.

The possibility of symbiont replacement has been examined using an aposymbiotic strain of *T. compressum*. Wagener et al. (1990) attempted to reinfect aposymbiotic ciliates with two strains of *M. formicicum* strains DSM3636 and 3637, which had been originally isolated from *Metopus striatus* and *Pelomyxa palustris*, respectively, and successfully established new symbiotic associations using

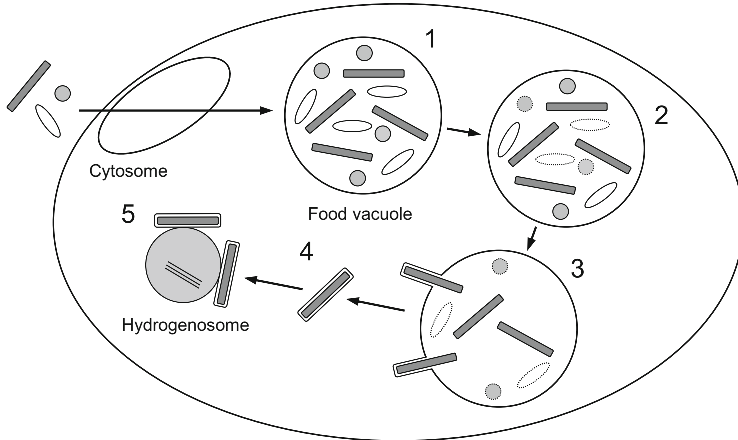


Fig. 6 Proposed process of establishment of a symbiotic association between *T. compressum* and methanogens. (1) uptake of bacteria into food vacuoles; (2) digestion of food bacteria; (3) separation of methanogens into the cytoplasm; (4) transport of the methanogens surrounded by a membrane; (5) localization of methanogens nearby hydrogenosome. The last step was not demonstrated in the experiments (redrawn from Fig. 5 of Wagener et al. 1990)

exogenous methanogens. In the course of symbiosis formation, methanogens ingested by food vacuoles were surrounded by a cytoplasmic membrane and eventually separated from the vacuoles (Fig. 6). The newly established consortium produced methane, and the growth of the host ciliate was significantly stimulated under food-limited conditions (Wagener et al. 1990). However, this consortium was readily dissolved by abundant food supply, which indicates its relatively flexible symbiotic association between methanogens and host ciliates. The success of symbiosis reconstruction suggests that methanogenic symbiont and host ciliate might recognize each other by not highly specific ways, which could allow frequent symbiont replacement of anaerobic ciliates. However, it is still unclear what factors are involved in the establishment of symbiotic associations between methanogens and anaerobic protozoa. Furthermore, methanogens engulfed in food vacuoles must be taken out and transported close to hydrogenosome, for which a specialized recognition and transport mechanism would be needed.

3 Bacterial Symbiont

In addition to methanogenic symbionts, *T. compressum* has been reported to harbour bacterial symbionts in its cytoplasm. The bacterial symbionts were reported first from two strains of *T. compressum*, which were isolated from different habitats in Europe (Goosen et al. 1990a). The bacterial symbionts reported were rod-shaped bacteria with 0.3–0.4 μm wide and 0.5–0.7 μm long (Fig. 3). Single ciliate cells possessed 20–100 bacterial symbionts in the

cytoplasm. In contrast to methanogenic symbionts, bacterial symbionts are located in the cytoplasm independently of hydrogenosomes. Bacterial symbionts having similar morphologies were also found in a Japanese strain, *T. compressum* strain S10, which was isolated from a sewage treatment reactor (Shinzato et al. 2007); therefore, these bacterial symbionts seem to be distributed widely in *T. compressum* populations. However, no description of bacterial symbionts was found in the report for a monoxenic culture of *T. compressum* established by Wagener and Pfennig (Wagener and Pfennig 1987). One might speculate that they have been eliminated from the ciliate cell during the purification process with antibiotic treatment (penicillin and streptomycin).

The bacterial symbiont found in *T. compressum* strain S10 was designated TC1 and morphologically and phylogenetically examined (Shinzato et al. 2007). TC1 were spherical rods with 0.3–0.6 μm wide and 0.8–2.0 μm long and persisted stably in the ciliate for 20 years of cultivation. Transmission electron microscopic observation showed that they were distributed throughout the cytoplasm in contrast to methanogenic symbionts, which associated consistently with hydrogenosomes (Fig. 4). Molecular phylogenetic identification based on 16S rRNA gene sequence and fluorescence in situ hybridization (FISH) revealed that they are a member of the order *Clostridiales* and affiliated with the lineage of *Syntrophomonadaceae*. The closest isolate was *Desulfitobacterium hafniense* with 85.7% of sequence identity indicating its uniqueness of the bacterial symbiont (unpublished result). The phylogenetic relationships among bacterial symbionts of *T. compressum* strains are unclear because phylogenetic information of the bacterial symbionts found in the two European strains is not available.

The physiological roles of these bacterial symbionts on ciliate survival are of great importance to understand the basis of this symbiosis. Goosen et al. (1990a) compared bacterial symbiont-bearing and symbiont-free strains in terms of the requirement of growth factors and the response to antibacterial drugs. The results showed that only bacterial symbiont-free strain required sterols for growth (stigmasterol, stigmastanol, or ergosterol) and that only bacterial symbiont-bearing strain was sensitive to chloramphenicol (100 μg ml^{-1}), in which the growth rate of bacterial symbiont-bearing strain was markedly decreased with a low maximum cell yield (40–50% of untreated control).

Likewise, antibiotic treatment (penicillin and streptomycin) for establishing the axenic culture of *T. compressum* caused a striking decrease in ciliate cell yield (50% of untreated ciliates) (Broers et al. 1991). Such growth suppression by antibiotic treatment was also reported in *T. compressum* strain S10. Although the bacterial symbiont-free strain of *T. compressum* generated from antibiotic treatment of its original strain could grow without any growth factors, their maximum cell yield decreased to 30% of that of original strain (Shinzato et al. 2007). Since antibiotic treatment is expected to eliminate the bacterial symbionts from the ciliates, these observations strongly suggest that the bacterial symbionts support the vigorous growth of *T. compressum*.

The axenic culture of *T. compressum* has been reported to require C24-alkylated sterols such as stigmasterol, stigmastanol, and ergosterol as growth stimulating

factors. Strictly anaerobic protozoa could not synthesize C24-alkylated sterols, since this reaction requires the participation of molecular oxygen (Nes and McKean 1977). On the other hand, a variety of bacteria contain hopanoids (pentacyclic triterpenes), which are structurally similar to sterols, the biosynthesis of which does not require molecular oxygen (Rohmer et al. 1979). Therefore, although the possible contribution of bacterial symbionts as providers of sterol precursors was not considered in previous reports, it must be advantageous for *T. compressum*, especially when they grow in the environments scarce in sterols or their precursors. However, it has not been tested whether hopanoids can support the growth of ciliate. On the other hand, *T. compressum* has been demonstrated to be able to produce a distinct sterol precursor, tetrahymanol, which can be synthesized without molecular oxygen, by itself (Holler et al. 1993).

Another possible contribution of the bacterial symbionts to the host ciliate is the role of the hydrogen scavenger, which could work as a backup to the methanogenic symbionts. Goosen et al. (1990a) examined hydrogenase activity in both bacterial symbiont-bearing and symbiont-free strains of *T. compressum* by cytochemical staining and showed that hydrogenase activity was detected only in bacterial symbiont-bearing strain even though both strains possessed methanogenic symbionts (Goosen et al. 1990a). Other physiological roles of bacterial symbiont in *T. compressum* remain to be elucidated.

4 Genome Sequence of Bacterial Symbiont

Whole-genome sequence data would be a great help for understanding the fundamental metabolism and physiological role of intracellular symbiont. In addition, comparative genome analysis between the symbiont and its free-living relatives could highlight the key metabolism of the symbiont, since the genes that are not essential for symbiotic associations would be promptly eliminated from the symbiont genome (Moran et al. 2009). To elucidate the physiological role of the bacterial symbiont TC1 in *T. compressum*, Shinzato et al. (2016) determined a complete genome sequence of TC1 symbiont using a single molecule sequencer (PacBio RSII, Pacific Biosciences, CA). A 20 Kb-fragment library prepared from two μg of the symbiont genome allowed to reconstruct a 1.59 Mb of circular chromosome and a 35.8 Kb of plasmid sequences (Shinzato et al. 2016).

The genome sequence of TC1 symbiont obviously represented erosive and deteriorated features, which are widely seen in the genome of intracellular symbionts (Table 1). For instance, the G+C content of the TC1 genome was 32.8%, which is fairly low compared with those of many free-living bacteria. It seems to be the result of an AT mutational bias generally observed in intracellular symbionts (McCutcheon and Moran 2012). Moreover, although the number of protein-coding genes in the chromosome reached 1694, about one-thirds of the genes (606) were expected to be pseudo-genes accounting for 55% of coding density, which is significantly lower than those of free-living bacteria with a typical range of 85–90% (McCutcheon and

Table 1 Genome feature of TC1 symbiont living in *T. compressum* strain S10

Size (bp)	GC (%)	Cording density (%)	CDSs	CDSs			CDS size (bp)	IS[a]	tRNA	rRNA operon
				Assigned	Hypothetical	Pseudogene				
1,586,453	32.8	55.0	1694	874	214	606	918	183	36	2

[a]IS: Number of insertion sequences detected by IS finder (Siguier et al. 2006)

Moran 2012). The genome of intracellular symbionts during genome reduction process tends to contain numerous insertion sequences (ISs), because ISs are expected to accelerate gene loss and genome rearrangement (Siguier et al. 2014). An in silico survey revealed that many IS-related ORFs (35 of complete and 147 of partial ISs) are distributed in the chromosome. These genome features clearly indicate that TC1 symbiont is in the course of extreme genome reduction process under intracellular habitat.

On the other hand, whole-genome sequence could provide insightful information to elucidate fundamental metabolisms and physiological role of the bacterial symbionts. Considering the gene repertoire of the genome, TC1 seems to utilize peptides and/or amino acids as carbon and energy sources, which are probably obtained from the host metabolites. The hypothesized sterol precursor synthesis and hydrogen-scavenging activity of TC1 symbiont are not supported by metabolic pathways deduced from its genome sequence. However, fatty acid or lipoate synthesis may be a key metabolism for elucidating the roles of bacterial symbiont in *T. compressum*. TC1 symbiont retains an entire gene set for fatty acid synthesis (*fab* operon) even in its deteriorated genome. Interestingly, this *fab* operon lacks a typical transcriptional repressor, which is highly conserved in the domain Bacteria (unpublished data). Although genome structure of the symbiont needs to be examined carefully, these observations suggest that TC1 symbiont may contribute to fatty acid metabolism of the host ciliate.

5 Bacterial Symbiont in Other Anaerobic Ciliates

Except for *T. compressum*, tripartite symbiotic association composed of anaerobic protozoa, methanogenic archaea, and bacteria has also been reported in some anaerobic ciliates. *Cyclidium porcatum*, an anaerobic scuticociliate, has been reported to contain both methanogenic and bacterial symbionts in the anterior part of the cell. They were associated with the hydrogenosome and formed a tightly organized complex (ca. 8 μm) (Esteban et al. 1993). The bacterial symbionts were relatively large and thick rods, which are distinguishable from the small methanogenic symbionts. Both prokaryotic symbionts were visualized by simultaneous FISH-staining using archaea- and bacteria-specific probes (Esteban et al. 1993). However, the molecular phylogeny of these symbionts has not been elucidated. Although physiological significance of the bacterial symbionts is still unclear, their close association to hydrogenosomes implies that molecular hydrogen (or uncertain substrate) is supplied by hydrogenosomes, which is different from the case of bacterial symbionts

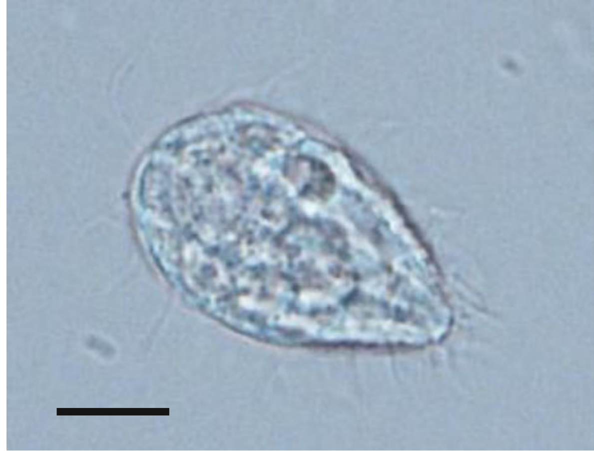


Fig. 7 Phase contrast image of unclassified anaerobic ciliate strain GW7. The ciliate was cultured from sewage treatment reactor with *Lactococcus* cells as food. Bar represents 10 μm

in *T. compressum*. As all constituents (hydrogenosome, methanogen, and bacterial symbionts) persisted in mostly the same ratios, these three components are thought to proliferate at the same rate, probably by synchronizing with the division rate of the ciliate (Esteban et al. 1993). Such complex symbiosis has also been reported in the giant amoeba *P. palustris*, in which both methanogenic and bacterial symbionts were held even in its cysts (Van Bruggen et al. 1983).

Recently, we attempted to cultivate *Cyclidium* ciliate to investigate its symbiotic associations between prokaryotic cells as reported previously and successfully obtained monoxenic culture of small ciliate named strain GW7 from sewage treatment reactor (Okinawa, Japan) using *Lactococcus* cells as food (Fig. 7). This ciliate is relatively smaller than *T. compressum* cells and morphologically resembles *Cyclidium* ciliates (approx. 17 μm wide and 29 μm long). We examined the 18S rRNA gene sequence of this ciliate and identified *C. porcatum* as the closest relative. However, phylogenetic analysis demonstrated that *C. porcatum* is located apart from the lineage of genus *Cyclidium*, which suggest that *C. porcatum* needs to be reclassified (data not shown). Requirement of taxonomic revision of this genus has also been posed by taxonomists (Gao et al. 2017).

Bacteria- or archaea-specific fluorescence in situ hybridization using each domain-targeting oligonucleotide probes and transmission electron microscopy of ultra-thin section of *C. porcatum* strain GW7 showed that both methanogenic archaea and bacterial symbionts lived in a single ciliate cell (Figs. 8 and 9). Both methanogenic and bacterial symbionts are closely associated with hydrogenosomes, which formed tripartite complex as reported in another strain of *C. porcatum* (Esteban et al. 1993). Although molecular identification of each intracellular symbiont remains to be elucidated, further investigation about this symbiotic association may reveal unknown microbe-microbe interactions in anoxic environments as such close association between bacterial symbiont and hydrogenosome has been scarcely observed.

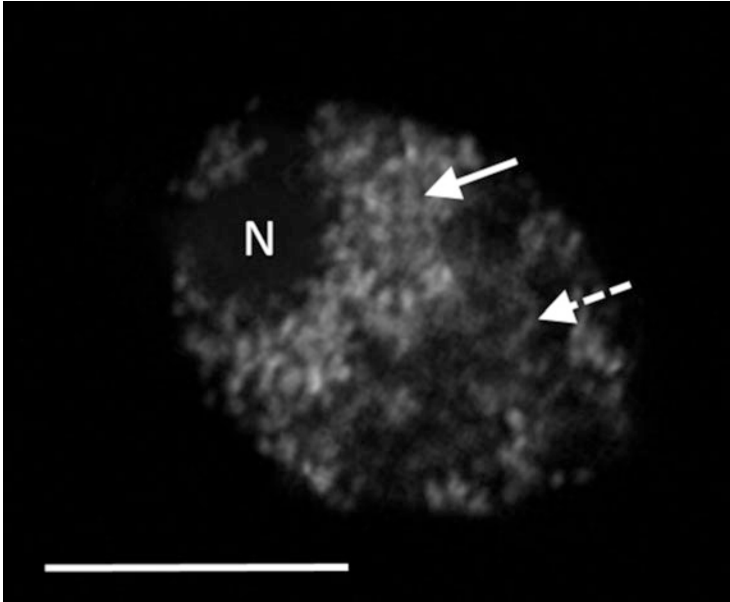


Fig. 8 Fluorescence in situ hybridization detection of bacterial and archaeal endosymbionts in anaerobic ciliate GW7. Bacterial (white arrow) and archaeal (broken arrow) endosymbionts were simultaneously visualized with each domain specific fluorescent-labeled oligonucleotide probe, N indicates ciliate nucleus. Bar represents 10 μm

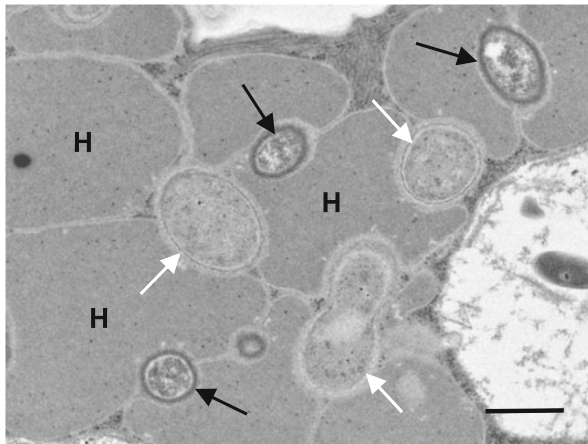


Fig. 9 Transmission electron micrographs of ultra-thin sections of anaerobic ciliate strain GW7. Methanogenic symbionts having thick cell wall (black arrows) and unclassified bacterial symbionts (white arrows) live in a single ciliate cell and embedded in the cluster of hydrogenosome (H). Bar represents 1 μm

6 Perspectives

Symbiotic associations between methanogenic archaea and anaerobic protozoa are widely distributed around anoxic environments on the earth. In the course of methanogen-bearing protozoa (ciliate) evolution, it is obvious that symbiont replacement has occurred on several protozoan lineages, which implies its relatively flexible and plastic host-symbiont partnership. Strictly obligate symbiotic associations between anaerobic protozoa and their methanogenic archaea have never been evidenced to date, which is not a typical co-evolutional process of intracellular symbiosis. On the other hand, for example, selection of partner methanogen, process of symbiosis establishment, and lost/reacquisition of methanogen by protozoa still remain to be investigated.

The tripartite symbiotic association found in *T. compressum* described in this chapter is one of the valuable research models for studying symbiotic interactions among bacteria, archaea, and protozoa. Monoxenic culture of *T. compressum* established in our laboratory has been maintained over 20 years without significant changes indicating that it is a stable model for studying symbiotic associations. On the other hand, whole-genome sequencing of bacterial symbiont revealed that its physiological basis and reductive genome feature characteristic of intracellular symbionts. In the future studies, overall metabolic interactions among symbionts and host protozoa and evolutional history of the symbiosis would be the next research subjects.

A diverse array of protozoa dwell in anaerobic environments in nature. In those environments, numbers of limitations in yielding energy or certain nutrients are likely to promote the formation of various types of symbiosis to survive in such environments. In this context, further investigation focusing on the oxygen-scarce environments will extend our knowledge about microbe-microbe interactions on the earth.

References

Augustin H, Foissner W, Adam H (1987) Revision of the genera *Acineria*, *Trimyema* and *Trochiliopsis* (Protozoa, Ciliophora). Bull Br Mus Nat Hist (Zool) 52:197–224

Baumgartner M, Stetter KO, Foissner W (2002) Morphological, small subunit rRNA, and physiological characterization of *Trimyema minutum* (Kahl, 1931), an anaerobic ciliate from submarine hydrothermal vents growing from 28°C to 52°C. J Eukaryot Microbiol 49:227–238

Boxma B, Voncken F, Jannink S, van Alen T, Akhmanova A, van Weelden SW, van Hellemond JJ, Ricard G, Huynen M, Tielens AG, Hackstein JHP (2004) The anaerobic chytridiomycete fungus *Piromyces* sp. E2 produces ethanol via pyruvate: formate lyase and an alcohol dehydrogenase E. Mol Microbiol 51:1389–1399

Boxma B, de Graaf RM, van der Staay GW, van Alen TA, Ricard G, Gabaldon T, van Hoek AH, Moon-van der Staay SY, Koopman WJ, van Hellemond JJ, Tielens AG, Friedrich T, Veenhuis M, Huynen MA, Hackstein JHP (2005) An anaerobic mitochondrion that produces hydrogen. Nature 434:74–79

Broers CA, Stumm CK, Vogels GD (1991) Axenic cultivation of the anaerobic free-living ciliate *Trimyema compressum*. J Protozool 38:507–511

Cho BC, Park JS, Xu K, Choi JK (2008) Morphology and molecular phylogeny of *Trimyema koreanum* n. sp., a ciliate from the hypersaline water of a solar saltern. J Eukaryot Microbiol 55:417–426

Doddema HJ, Vogels GD (1978) Improved identification of methanogenic bacteria by fluorescence microscopy. Appl Environ Microbiol 36:752–754

Embley TM, Finlay BJ (1993) Systematic and morphological diversity of endosymbiotic methanogens in anaerobic ciliates. Antonie Van Leeuwenhoek 64:261–271

Embley TM, Finlay BJ (1994) The use of small subunit rRNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts. Microbiology 140:225–235

Embley TM, Finlay BJ, Thomas RH, Dyal PL (1992) The use of rRNA sequences and fluorescent probes to investigate the phylogenetic positions of the anaerobic ciliate *Metopus palaeformis* and its archaeobacterial endosymbiont. J Gen Microbiol 138:1479–1487

Esteban G, Guhl BE, Clarke KJ, Embley TM, Finlay BJ (1993) *Cyclidium porcatum* n. sp.: a free-living anaerobic scuticociliate containing a stable complex of hydrogenosomes, eubacteria and archaeobacteria. Eur J Protistol 29:262–270

Fenchel T, Finlay BJ (1991) Endosymbiotic methanogenic bacteria in anaerobic ciliates: significance for the growth efficiency of the host. J Protozool 38:18–22

Finlay BJ, Embley TM, Fenchel T (1993) A new polymorphic methanogen, closely related to *Methanocorpusculum parvum*, living in stable symbiosis within the anaerobic ciliate *Trimyema* sp. J Gen Microbiol 139:371–378

Gao F, Huang J, Zhao Y, Li L, Liu W, Miao M, Zhang Q, Li J, Yi Z, El-Serehy HA, Warren A, Song W (2017) Systematic studies on ciliates (Alveolata, Ciliophora) in China: progress and achievements based on molecular information. Eur J Protistol 61:409–423

Goosen NK, Horemans AMC, Hillebrand SJW, Stumm CK, Vogels GD (1988) Cultivation of the sapropelic ciliate *Plagiopyla nasuta* Stein and isolation of the endosymbiont *Methanobacterium formicicum*. Arch Microbiol 150:165–170

Goosen N, Wagener S, Stumm CK (1990a) A comparison of two strains of the anaerobic ciliate *Trimyema compressum*. Arch Microbiol 153:187–192

Goosen NK, Van der Drift C, Stumm CK, Vogels CD (1990b) End products of metabolism in the anaerobic ciliate *Trimyema compressum*. FEMS Microbiol Lett 69:171–176

Hackstein JHP, Vogels GD (1997) Endosymbiotic interactions in anaerobic protozoa. Antonie Van Leeuwenhoek 71:151–158

Hackstein JHP, de Graaf RM, van Hellemond JJ, Tielens AGM (2008a) Hydrogenosomes of anaerobic ciliates. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes. Springer, Berlin, pp 97–112

Hackstein JHP, Baker SE, van Hellemond JJ, Tielens AGM (2008b) Hydrogenosomes of anaerobic chytrids: an alternative way to adapt to anaerobic environments. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes. Springer, Berlin, pp 148–162

Holler S, Pfennig N (1991) Fermentation products of the anaerobic ciliate *Trimyema compressum* in monoxenic cultures. Arch Microbiol 156:327–334

Holler S, Pfennig N, Neunlist S, Rohmer M (1993) Effect of a non-methanogenic symbiont and exogenous stigmasterol on the viability and tetrahymanol content of the anaerobic ciliate *Trimyema compressum*. Eur J Protistol 29:42–48

Lackey JB (1925) Studies on the biology of sewage disposal. N J Agr Exp Sta Bull 417:1–39

Lewis WH, Sendra KM, Embley TM, Esteban GF (2018) Morphology and phylogeny of a new species of anaerobic ciliate, *Trimyema finlayi* n. sp. with endosymbiotic methanogens. Front Microbiol 9:140

McCutcheon JP, Moran NA (2012) Extreme genome reduction in symbiotic bacteria. Nat Rev Microbiol 10:13–26

Moran NA, McLaughlin HJ, Sorek R (2009) The dynamics and time scale of ongoing genomic erosion in symbiotic bacteria. Science 323:379–382

Müller M (1993) The hydrogenosome. J Gen Microbiol 139:2879–2889

Nerad TA, Schaffer SA, Small EB, Mangold LA (1995) *Trimyema shoalsia* sp. n., an anaerobic, microaerotolerant marine ciliate from Appledore Island, Gulf of Maine, USA. Acta Protozool 34:289–298

Nes WR, McKean ML (1977) Biochemistry of steroids and other isopentenoids. University Park Press, Baltimore

Park JS, Simpson AG (2015) Diversity of heterotrophic protists from extremely hypersaline habitats. Protist 166:422–437

Rohmer M, Bouvier P, Ourisson G (1979) Molecular evolution of biomembranes: structural equivalents and phylogenetic precursors of sterols. Proc Natl Acad Sci U S A 76:847–851

Schulz S, Wagener S, Pfennig N (1990) Utilization of various chemotrophic and phototrophic bacteria as food by the anaerobic ciliate Trimyema compressum. Eur J Protistol 26:122–131

Shinzato N, Watanabe I, Meng XY, Sekiguchi Y, Tamaki H, Matsui T, Kamagata Y (2007) Phylogenetic analysis and fluorescence in situ hybridization detection of archaeal and bacterial endosymbionts in the anaerobic ciliate *Trimyema compressum*. Microb Ecol 54:627–636

Shinzato N, Aoyama H, Saitoh S, Nikoh N, Nakano K, Shimoji M, Shinzato M, Satou K, Teruya K, Hirano T, Yamada T, Nobu MK, Tamaki H, Shirai Y, Park S, Narihiro T, Liu WT, Kamagata Y (2016) Complete genome sequence of the intracellular bacterial symbiont TC1 in the anaerobic ciliate *Trimyema compressum*. Genome Announc 4:e01032–e01016

Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M (2006) ISfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res 34:D32–D36

Siguier P, Gourbeyre E, Chandler M (2014) Bacterial insertion sequences: their genomic impact and diversity. FEMS Microbiol Rev 38:865–891

Stams AJ (1994) Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie Van Leeuwenhoek 66:271–294

Van Bruggen JJA, Stumm CK, Vogels GD (1983) Symbiosis of methanogenic bacteria and sapropelic protozoa. Arch Microbiol 136:89–95

Van Bruggen JJA, Zwart KB, Van Assema RM, Stumm CK, Vogels GD (1984) *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus* McMurrich. Arch Microbiol 139:1–7

Van Bruggen JJA, Zwart KB, Hermans JGE, Van Hove EM, Stumm CK, Vogels GD (1986) Isolation and characterisation of *Methanoplanus endosymbiosus* sp. nov. an endosymbiont of the marine sapropelic ciliate *Metopus contortus* Quennerstedt. Arch Microbiol 144:367–374

Van Bruggen JJA, Van Rens GLM, Geertman EJM, Van Hove EM, Stumm CK (1988) Isolation of a methanogenic endosymbiont of the sapropelic ameba *Pelomyxa palustris* Greeff. J Protozool 35:20–23

Van Hoek AH, Van Alen TA, Sprakel VS, Leunissen JA, Brigge T, Vogels GD, Hackstein JH (2000) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol Biol Evol 17:251–258

Wagener S, Pfennig N (1987) Monoxenic culture of the anaerobic ciliate *Trimyema compressum* lackey. Arch Microbiol 149:4–11

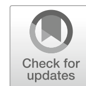
Wagener S, Bardele CF, Pfennig N (1990) Functional integration of *Methanobacterium formicicum* into the anaerobic ciliate *Trimyema compressum*. Arch Microbiol 153:496–501

Yamada K, Kamagata Y, Nakamura K, Inamori Y, Nakamura I (1994) Selectivity of food bacteria for the growth of anaerobic ciliate *Trimyema compressum*. Arch Microbiol 161:229–233

Yamada K, Kamagata Y, Nakamura K (1997) The effect of endosymbiotic methanogens on the growth and metabolic profile of the anaerobic free-living ciliate *Trimyema compressum*. FEMS Microbiol Lett 149:129–132

Zwart KB, Goosen NK, Van Schijndel MW, Broers CAM, Stumm CK, Vogels GD (1988) Cytochemical localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis*, *Plagiopyla nasuta*, and *Trimyema compressum*. J Gen Microbiol 134:2165–2170

Termite Gut Flagellates and Their Methanogenic and Eubacterial Symbionts



Yuichi Hongoh and Moriya Ohkuma

Contents

1 Introduction 56
2 Methanogenic Endosymbionts of Termite Gut Flagellates 59
  2.1 Phylogeny of Endosymbiotic Methanogens 59
  2.2 Predicted Functions of Endosymbiotic Methanogens 61
3 Eubacterial Symbionts of Termite Gut Flagellates 65
  3.1 Phylogeny of Ectosymbiotic Eubacteria 65
  3.2 Phylogeny of Endosymbiotic Eubacteria 67
  3.3 Predicted Functions of Eubacterial Ecto- and Endosymbionts 70
4 Concluding Remarks and Future Perspectives 73
References 75

Abstract Termites harbor an abundance and diversity of symbiotic microbes in their gut, which comprise all the three domains of life, Eucarya, Bacteria, and Archaea. One of the most prominent features of this microbiota is the cellular association of the gut flagellates with eubacteria and/or methanogenic archaea. The eubacterial and methanogenic symbionts are observed both inside and on the surface of the host flagellate cells. Recent technological advances in genomics have enabled researchers to predict the functions of these as-yet-uncultivable prokaryotic symbionts, in addition to their phylogenetic positions and specific localizations based on 16S rRNA analyses. Several complete and draft genome sequences of endo- and ectosymbionts of gut flagellates have shown consistency of their functions in spite of their taxonomic diversity.

Y. Hongoh (✉)
Department of Life Science and Technology, School and Graduate School of Life Science and Technology, Tokyo Institute of Technology, Tokyo, Japan

Japan Collection of Microorganisms (JCM), RIKEN BioResource Research Center, Tsukuba, Ibaraki, Japan
e-mail: yhongo@bio.titech.ac.jp

M. Ohkuma
Japan Collection of Microorganisms (JCM), RIKEN BioResource Research Center, Tsukuba, Ibaraki, Japan

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_5

1 Introduction

Termites are one of the most important decomposers in temperate to tropical regions (Sugimoto et al. 2000). Their ability to thrive on recalcitrant, nitrogen-poor lignocellulose is mostly attributable to the activity of the microbial community in the gut (Fig. 1) (Brune and Ohkuma 2011; Hongoh 2011; Brune 2014). In phylogenetically "lower" termites, the gut microbiota comprises both eukaryotes and prokaryotes, while "higher" termites (family Termitidae) generally harbor only prokaryotic gut microbes. The majority of these gut microbes are as-yet uncultivable; their phylogenetic and spatial distributions have been studied mainly by small subunit rRNA-based molecular analyses (Hongoh 2010; Brune 2014; Mikaelyan et al. 2015). However, the detailed symbiotic mechanism remains yet unclear due to their unculturability.

The eukaryotic gut symbionts comprise two distinct lineages of flagellated protists, belonging to either the phylum Parabasalia or the order Oxymonadida in the phylum Preaxostyla. They are unique to termites and the wood-feeding cockroach genus *Cryptocercus*, and each termite species possesses a specific set of flagellate species (Yamin 1979; Kitade 2004). Although the cultivation of these flagellates is very difficult, several studies on axenic or mixed cultures demonstrated that the flagellates are strictly anaerobic and ferment cellulose: $n(C_6H_{12}O_6) + n(2H_2O) \rightarrow n(2CH_3COOH + 2CO_2 + 4H_2)$ (Yamin 1980, 1981; Odelson and Breznak 1985a).

In addition to the cellulolytic flagellates, lower termites harbor diverse eubacteria in their gut. Several hundred or more species of eubacteria inhabit the gut of a single lower termite species, and the community structure is basically consistent within a host species (Hongoh et al. 2003, 2005; Boucias et al. 2013; Abdul Rahman et al. 2015; Tai et al. 2015). The eubacterial gut symbionts, distributed among more than 25 phyla, constitute one or more monophyletic clusters in each phylum, suggesting that they are not allochthonous, but autochthonous symbionts inherited from parents

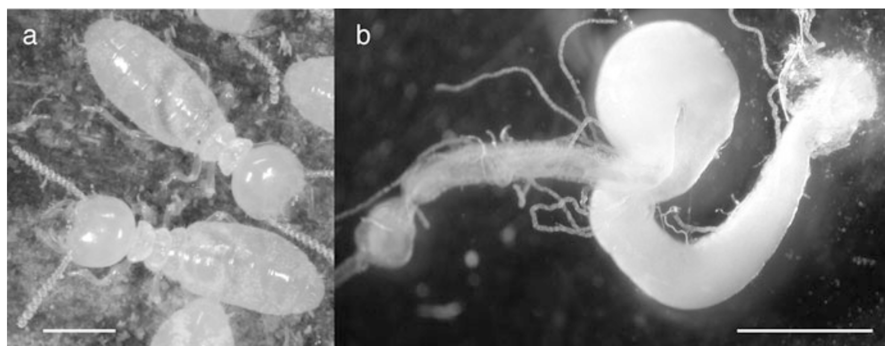


Fig. 1 Termites and a removed gut. (**a**) The lower termite *Reticulitermes speratus*. (**b**) A removed gut from *R. speratus*. Bars = 1 mm. Panel **b** was originally published in Hongoh et al. (2008a) as supporting information

to offspring through the proctodeal trophallaxis (i.e., transmission of gut contents from the anus of a donor to the mouth of a recipient) (Andrew 1930; Kitade et al. 1997; Hongoh et al. 2005).

Another domain of life, Archaea, is also found in termite guts. The majority are methanogens, which are less abundant and less diverse compared to eubacterial gut symbionts (Ohkuma et al. 1999; Shinzato et al. 1999; Brauman et al. 2001; Friedrich et al. 2001; Donovan et al. 2004; Brune 2018, this volume). To date, three methanogenic strains have been isolated from a lower termite, *Reticulitermes flavipes*, and described as *Methanobrevibacter cuticularis*, *Methanobrevibacter curvatus* (Leadbetter and Breznak 1996), and *Methanobrevibacter filiformis* (Leadbetter et al. 1998). All of these methanobrevibacters attach to the gut epithelium. The occurrence of methanobrevibacters and their attachment to the gut wall are observed in both lower and higher termites (Fig. 2a, b) (Tokura et al. 2000; Pester and Brune 2007). From a wood-feeding higher termite, a methanobrevibacter strain closely related to *Methanobrevibacter arboriphilus* has been isolated, and from higher termites of various feeding habits, three strains of the genus *Methanobacterium*, closely related to *Methanobacterium bryantii*, have also been isolated (Deevong et al. 2004).

In general, soil- and litter-feeding higher termites emit much more methane than wood-feeding higher and lower termites (Brauman et al. 1992; Sugimoto et al. 1998). In the gut of wood-feeding termites, unlike many other anoxic environments, H_2-dependent acetogenesis outcompetes methanogenesis as "H_2-sink" (Odelson and Breznak 1983; Breznak and Switzer 1986; Pester and Brune 2007). Methanogens account for 0–10% of the gut prokaryotic population (Leadbetter and Breznak 1996; Brauman et al. 2001) and the rate of CH_4 emission is only 10% of that of CO_2-reductive acetogenesis in the gut of lower termites (Pester and Brune 2007). Although acetogenesis from H_2 and CO_2 appears nutritionally more beneficial to termites than methanogenesis because acetate is their chief energy and carbon source, the physiological and physicochemical basis for the outcompetition of methanogenesis by acetogenesis is unclear. The localization of methanogens in the gut might account in part for this outcompetition (Breznak 2000; Tholen and Brune 2000), but the question why the localization of methanogens is restricted to the gut wall and the cells of the relatively few species of flagellates in the gut of lower termites remains unanswered. Methane oxidation has never been observed in termite guts (Pester et al. 2007).

Whereas numerous eubacteria and methanogens reside as free or wall-attached forms in the termite gut, it is known that the majority of the prokaryotic members in the gut of lower termites exist as endo- or ectosymbionts of the gut flagellates (Berchtold et al. 1999; Tai et al. 2015). Indeed, the physical association of cells between the flagellates and prokaryotes is one of the most prominent features of the termite gut microbiota (Brune and Stingl 2006; Ohkuma 2008). In this chapter, the studies on the cellular association between the flagellates and methanogenic archaea, as well as between the flagellates and eubacteria, in the termite gut are reviewed.

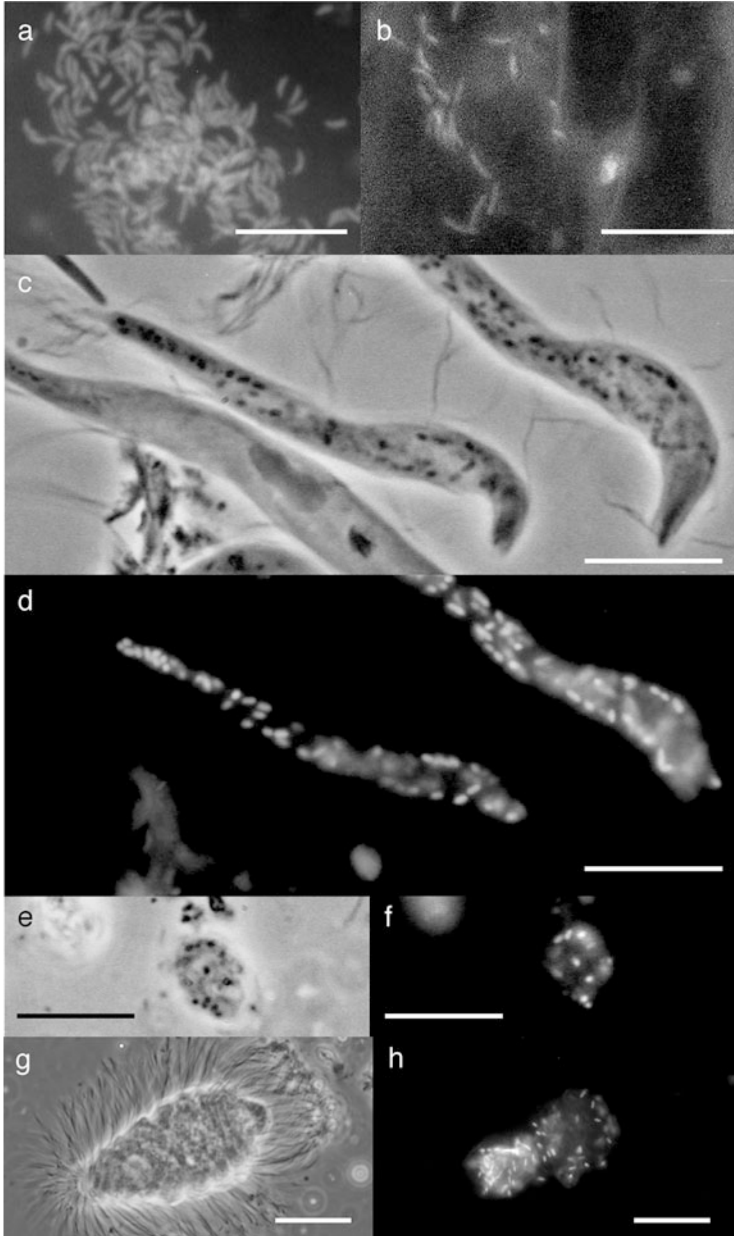


Fig. 2 Localization and morphology of methanogens detected by epifluorescence microscopy. (**a**) Epifluorescence image of methanogens on the gut epithelium of the lower termite *Reticulitermes speratus*. (**b**) Epifluorescence image of methanogens on the gut epithelium of the higher termite *Microcerotermes* sp. (**c**) Phase contrast image of the oxymonad protists *Dinenympha parva* from the gut of *R. speratus*. (**d**) Epifluorescence image of endosymbiotic methanogens in the *D. parva* cells. (**e**) Phase contrast image of the parabasalid flagellate *Microjoenia* sp. from the gut of

2 Methanogenic Endosymbionts of Termite Gut Flagellates

This section describes the phylogeny and predicted roles of the methanogenic endosymbionts of flagellates in the gut of lower termites. Although there have been fewer reports on the endosymbiotic methanogens of termite gut flagellates compared to those on the eubacterial endosymbionts, the reports contain valuable data, which help us to capture a general tendency of methanogenic endosymbionts on their phylogeny and host specificity and to elucidate their functional roles in the symbiosis with the flagellate host.

2.1 *Phylogeny of Endosymbiotic Methanogens*

The presence of methanogens inside the cells of certain flagellate species in the termite gut was evidenced for the first time by Lee et al. (1987). *Methanobrevibacter*-like rod-shaped cells were detected, on the basis of the autofluorescence from the cofactors F_{420} and F_{350}, inside the cells of the parabasalid flagellates *Trichomitopsis termopsidis*, *Tricercomitus termopsidis*, and *Hexamastix termopsidis* from the gut of the termite *Zootermopsis angusticollis*. The corresponding rod-shaped cells associated with these small parabasalids had been described by Kirby (1930). Endosymbiotic methanogens in these three flagellate species have also been reported in the congeneric termite *Zootermopsis nevadensis* (Pester and Brune 2007). No molecular data exist for these methanogens to date.

Tokura et al. (2000) discovered endosymbiotic methanogens inside the cells of the parabasalid flagellate *Microjoenia* sp. and the oxymonad flagellate *Dinenympha parva* in the gut of the termite *Reticulitermes speratus*, on the basis of the F_{420} and F_{350} autofluorescence (Fig. 2c–f). About 10–50 cells of methanogens were found constantly inside the cells of *Microjoenia* sp. and *D. parva*, respectively (Hara et al. 2004). The total numbers of methanogens associated with *Microjoenia* sp. and *D. parva* were 7.9×10^3 and 1.3×10^5 per gut, respectively. Methanogens were also observed in other species of *Dinenympha* and the oxymonad *Pyrsonympha* sp., but the association was occasional. In total, approximately 4% of the flagellate cells in *R. speratus* guts were found to be associated with methanogens (Tokura et al. 2000).

Fig. 2 (continued) R. speratus. (**f**) Epifluorescence image of endosymbiotic methanogens in the *Microjoenia* cell. (**g**) Phase contrast image of the parabasalid flagellate *Spirotrichonympha leidyi* from the gut of the lower termite *Coptotermes formosanus*. (**h**) Epifluorescence image of endosymbiotic methanogens in the *S. leidyi* cell. Bars = 10 µm. Panels a and b–f were originally published in Tokura et al. (2000) and slightly modified. Panels g and h were kindly provided by Jun-Ichi Inoue and a related study was published in Inoue et al. (2008)

In the termite *Hodotermopsis sjoestedti*, all the cells of *Dinenympha* and *Microjoenia* were found to be associated with methanogens. The population of the methanogen-associated flagellates was much larger than in *R. speratus*; they accounted for 42% of the total flagellate cells. In both termite species, *R. speratus* and *H. sjoestedti*, methanogens free in the gut luminal fluid were rarely found (Tokura et al. 2000), while many were observed on the gut epithelium as seen in *Reticulitermes flavipes*. *R. flavipes* and *Reticulitermes santonensis* [synonym of *R. flavipes*, found in European countries (Jenkins et al. 2001)] harbor no gut flagellates which are associated with methanogens (Leadbetter and Breznak 1996; Pester and Brune 2007).

To identify the phylogenetic positions of these endosymbiotic methanogens in *R. speratus* and *H. sjoestedti*, clone sequence analyses of archaeal 16S rRNA genes were performed. About 50 cells of *Dinenympha* and *Microjoenia*, respectively, were physically isolated by micromanipulation and directly used for PCR amplification. All of the sequenced clones were affiliated with the genus *Methanobrevibacter* (Fig. 3). In *R. speratus*, two phylotypes were obtained from *D. parva*, while a single phylotype was found from *Microjoenia* sp. Similarly, two phylotypes were from *Dinenympha* spp. and a single phylotype from *Microjoenia* sp. in *H. sjoestedti*. These phylotypes showed only 94.6–97.3% sequence identity to closest cultured species, suggesting that they are novel *Methanobrevibacter* species (Tokura et al. 2000). It is unclear whether the two methanogen phylotypes from *D. parva* inhabit the cells of an identical host strain or the cells of distinct strains of *D. parva*.

Interestingly, the methanogen phylotypes LRsD3 from *D. parva*, LRsM1 from *Microjoenia* sp., and RsW10 from the gut wall fraction of *R. speratus* showed >99.7% sequence similarity to one another (cluster A in Fig. 3) (Tokura et al. 2000). The localizations of the cluster A methanogens inside the cells of *D. parva* and *Microjoenia* sp. were confirmed by fluorescence in situ hybridization (FISH) analysis (Hara et al. 2004). It remains unknown whether these phylotypes represent an identical species which can change its habitat or represent similar but distinct lineages that have adapted specifically to the respective habitats. No evidence of cospeciation between the endosymbiotic methanogens and the flagellate hosts has been found.

Another endosymbiotic methanogen is observed inside the cells of the parabasalid flagellate *Spirotrichonympha leidyi* in the gut of the termite *Coptotermes formosanus* (Fig. 2g, h) (Tsunoda et al. 1993; Inoue et al. 2008). A single *S. leidyi* cell harbors about 80 cells of methanogens, accounting, in total, for 0.3% of the prokaryotic community in *C. formosanus* guts. The archaeal 16S rRNA sequences obtained from *S. leidyi* cells consisted of a single phylotype, SlMeN10, sharing >99% similarity with one another and 98% similarity with those of cluster A in Fig. 3 (Inoue et al. 2008).

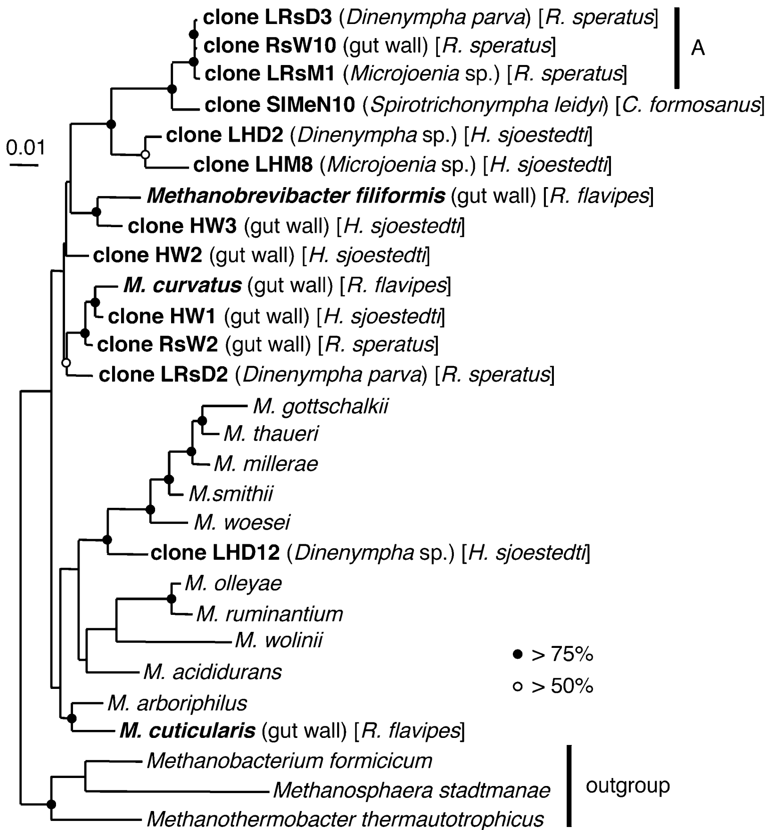


Fig. 3 Phylogenetic position of methanogens found from the gut of lower termites. Uncultured clones obtained by PCR from the gut of lower termites and type strains of described species belonging to the genus *Methanobrevibacter* were used to construct a phylogenetic tree. The localization or host protist species are shown with the host termite species in parentheses. Clones and isolates deriving from termite guts are shown in bold. The tree was constructed using PhyML v2.4.4 with the HKY nucleotide substitution model. Bootstrap confidence values were calculated by 100 resamplings

2.2 Predicted Functions of Endosymbiotic Methanogens

Endosymbiotic methanogens inside flagellate cells in the termite gut have never been cultured. In addition, no functional gene has been obtained from them. Therefore, there is no direct evidence for their functions. However, it is still possible to predict some of their functions from their taxonomic positions, localizations, and the data reported by Odelson and Breznak (1985b), who investigated the physiology of a cultured flagellate, *Trichomitopsis termopsidis*, with and without "endogenous" methanogens.

The cultured strains closest to the endosymbiotic methanogens are *Methanobrevibacter curvatus* and *Methanobrevibacter filiformis*, which have been isolated from the gut of *R. flavipes*. The energy source of these isolates is restricted to H_2 and CO_2, yielding CH_4 as the sole product: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$. They require one or more complex nutrients such as yeast extract and rumen fluid (Leadbetter and Breznak 1996; Leadbetter et al. 1998). Assuming that the endosymbiotic methanogens share basic physiologies with these isolates, a simple interpretation of the endosymbiosis is mutualism: the flagellate host provides H_2, CO_2, and other nutrients to the endosymbiotic methanogens, and in turn, the methanogens enhance the growth of the host flagellate by promoting the lignocellulose fermentation through the elimination of excess H_2.

The symbiosis mediated by interspecies transfer of H_2 between H_2-evolving fermentative anaerobes and H_2-consuming methanogens is not rare. In general, a high concentration of H_2 suppresses fermentation; the concentration of H_2 must be kept low (Müller et al. 2018, this volume). Actually, the growth rate of *Trichomitopsis termopsidis* 6057C, the sole axenic flagellate culture from the termite gut to date, was much enhanced when co-cultured with an H_2-consuming methanogen, *Methanospirillum hungatii* (Odelson and Breznak 1985b). In the co-cultivation, the produced gas was shifted from H_2 to CH_4, clearly suggesting the interspecies transfer of H_2. Heat-killed *M. hungatii* could not enhance the growth rate. Thus, it seems likely that the endosymbiotic methanogens benefit the flagellate host by lowering the H_2 concentration together with other H_2-consuming prokaryotes in the gut.

H_2 partial pressures, measured using agarose-embedded guts with microelectrodes, were very high in the dilated portion (paunch) of termite hindguts. In the paunch region, the values were 15–30 kPa in *R. santonensis* and 30–72 kPa in *Z. nevadensis* (Pester and Brune 2007), and 2 to 5 kPa in *R. flavipes* (Ebert and Brune 1997). However, since the hydrogen emission of the embedded guts was 30- to 50-fold higher than in living termites (Pester and Brune 2007), the actual values in vivo might be much lower. This discrepancy was possibly caused by the damages of the H_2-consuming bacteria and a limited concentration of O_2 utilized to oxidize H_2 compared to living termites (Pester and Brune 2007). It has been demonstrated that the Fe-hydrogenases of a termite gut flagellate retain more than a half of their H_2-evolving potential under 0 kPa if the H_2 partial pressure can be kept lower than 20 kPa (Inoue et al. 2007).

Considering that gut flagellates are the major source of H_2 and CO_2, produced during lignocellulose fermentation, it is reasonable that the methanogens have exploited the cytoplasm of the flagellates as their habitat. While the H_2 partial pressure is very high in the central region of a termite hindgut, it decreases toward the gut peripheral regions (Ebert and Brune 1997; Pester and Brune 2007). Messer and Lee (1989) demonstrated that exogenously supplied H_2 greatly enhanced the methanogenic activity of the termite *Z. angusticollis*, which harbors methanogen-associated flagellates as the main sites for CH_4 emission in the gut. In *Z. nevadensis*, a significant increase of CH_4 emission was also observed, even though the extent was smaller than in *Z. angusticollis* (Pester and Brune 2007). These findings suggest

that the H_2 concentration is a limiting factor for methanogenesis by the endosymbionts. In addition, the gut peripheral regions contain oxygen that suppresses methanogenesis, while the central region is almost completely anoxic (Ebert and Brune 1997). Therefore, the cytoplasm of the H_2-evolving flagellates, a habitat in close proximity to the H_2 source and probably protected from penetrating oxygen, seems an ideal habitat for stable, highly active methanogenesis. Indeed, anaerobic flagellates in various environments occasionally harbor endosymbiotic methanogens as described elsewhere in this book (Fenchel and Finlay 2018, this volume). The endosymbiosis seems also beneficial to the methanogens in the termite gut for avoiding washout because methanobrevibacters are generally nonmotile.

Although these factors reasonably explain the merit of the endosymbiosis to the methanogens, the interspecies H_2 transfer cannot fully explain the benefit to the flagellate hosts. Since H_2 diffuses rapidly through the hindgut of termites, it is questionable that the endosymbiotic methanogens can create a boundary layer with a significantly lower H_2 partial pressure around the host flagellate cells within such a high concentration of H_2 (Breznak 2000). While the elimination of H_2 by H_2-consuming prokaryotes including methanogens seems crucially important in the gut symbiotic system, the flagellates do not need to harbor them as intracellular symbionts which occupy large spaces in the cytoplasm. Actually, in *R. flavipes* and *R. santonensis*, the majority of H_2-consuming methanogens were found on the gut epithelium and not associated with the flagellate cells (Leadbetter and Breznak 1996; Pester and Brune 2007; Brune 2018, this volume).

A clue to a factor that may benefit the flagellate hosts by the endosymbiosis with methanogens has been implied in the study of the cultured flagellate *Trichomitopsis termopsidis* derived from the gut of *Z. angusticollis*. The cultivation of *T. termopsidis* had been achieved by Yamin (1978). He treated a mixed culture comprising *T. termopsidis* and diverse gut bacteria with penicillin and streptomycin, to acquire an axenic culture of *T. termopsidis*. Odelson and Breznak (1985b) used this *T. termopsidis* 6057 culture provided by Yamin, and they unexpectedly found that the "axenic" culture produced both CH_4 and H_2. This clearly indicated that the *T. termopsidis* 6057 culture contained methanogenic archaea, which are insensitive to penicillin and streptomycin.

In their study, the coexistence of the methanogens persisted for a long time, and the emission of CH_4 resumed even after 1-year suppression of methanogenesis by changing nutrients in the culture medium. Their attempts to isolate methanogens from the *T. termopsidis* 6057 culture failed, although methanogens were readily isolated from another "axenic" culture of a parabasalid flagellate, *Trichonympha sphaerica*, which had also been established from a *Z. angusticollis* gut by Yamin (1981) using the same culture condition and medium. Epifluorescence microscopy of the *T. termopsidis* culture failed to detect F_{420}-fluorescent cells. From these results, Odelson and Breznak (1985b) speculated that the *T. termopsidis* culture 6057 contained a small amount of "endogenous" methanogens inhabiting the cytoplasm of *T. termopsidis* as "energy parasites similar to chlamydiae."

To eliminate the "endogenous" methanogens, Odelson and Breznak (1985b) treated *T. termopsidis* 6057 with bromoethanesulfonate (BES), an analog of a

cofactor 2-mercaptoethanesulfonate (CoM), which inhibits methanogenesis, and they succeeded in establishing the putatively axenic culture *T. termopsidis* 6057C exhibiting no methanogenic activity. As described above, Lee et al. (1987) discovered that *T. termopsidis* in *Z. angusticollis* guts permanently harbors endosymbiotic methanogens. Hence, assuming that the "endogenous" methanogens were the intracellular symbionts that Lee and coworkers found later, the comparative experiments between *T. termopsidis* 6057 and 6057C conducted by Odelson and Breznak (1985b) should provide crucial information on the roles of the intracellular methanogens in the symbiosis with the flagellate host.

The elimination of methanogens with the BES treatment exhibited a drastic change in the growth of *T. termopsidis*. The growth rate of *T. termopsidis* 6057C decreased to 1/8 of that of *T. termopsidis* 6057. Interestingly, replacement of heat-killed rumen bacteria as a food source in the culture medium by a heat-killed eubacterial strain, *Bacteroides* sp. JW20, recovered the growth rate of *T. termopsidis* 6057C to a level comparable to that of *T. termopsidis* 6057. Various other strains of prokaryotes, including *Methanospirillum hungatii*, could not replace *Bacteroides* sp. JW20. This implies that the endosymbiotic methanogens provide certain essential growth factors to the flagellate host, which could be replaced to some extent by a specific strain of heat-killed bacteria as a nutritional source. Thus, it is likely that endosymbiotic methanogens are nutritionally essential to the efficient growth of the flagellate host. A hypothesized symbiotic system between intracellular methanogens and their flagellate host is outlined in Fig. 4.

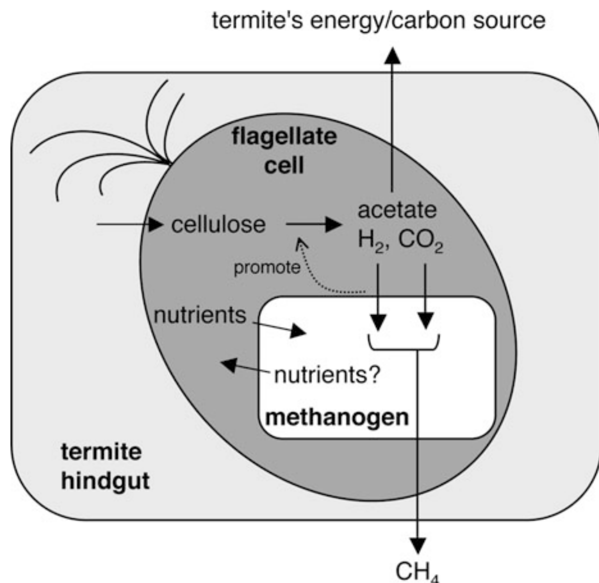


Fig. 4 Hypothesized roles of endosymbiotic methanogens. Endosymbiotic methanogens consume H_2 and CO_2, which are abundantly produced during the lignocellulose fermentation by the flagellate host, and they emit CH_4. The elimination of H_2 stimulates the fermentation process of the flagellate host. The host supplies certain nutrients to the methanogens, and, in turn, the latter supply essential growth factors to the former

3 Eubacterial Symbionts of Termite Gut Flagellates

Numerous reports have been published on the eubacterial symbionts that are physically associated with the cells of the flagellates in the gut of lower termites. This section reviews the recent achievements in the study of those ecto- and endosymbionts of the flagellates on their phylogeny, localization, coevolutionary history, and functions predicted mostly on the basis of their genome sequences.

3.1 Phylogeny of Ectosymbiotic Eubacteria

Most of the flagellates in the termite gut harbor ectosymbiotic eubacteria, which attach to the surface of the host cells laterally or with a tip. Numerous morphological studies have described the presence of ectosymbionts on the gut flagellates (Radek 1999), and a specific apparatus to hold the ectosymbionts has occasionally been found (Tamm 1980; Radek et al. 1996; Radek and Tischendorf 1999; Yuki et al. 2015). The application of culture-independent molecular techniques has enabled researchers to identify these ectosymbionts phylogenetically, which is otherwise almost impossible due to the unculturability.

Among the ectosymbionts, the most frequently found groups are the genus *Treponema* in the phylum *Spirochaetes* and the order *Bacteroidales* in the phylum *Bacteroidetes*. In various flagellate species in the termite gut, both groups of ectosymbionts are observed on a single host cell simultaneously (Fig. 5a, b) (Hongoh et al. 2007b; Izawa et al. 2017). Multiple phylotypes of treponemes have occasionally been detected on a single host flagellate cell (Noda et al. 2003), while only a single phylotype of *Bacteroidales* ectosymbionts is found on a single host cell in most cases (Stingl et al. 2004; Noda et al. 2006a, 2009; Desai et al. 2010). In the parabasalid flagellate *Caduceia versatilis* from the termite *Cryptotermes cavifrons*, a eubacterial phylotype belonging to the phylum *Synergistetes* has been discovered as a motility ectosymbiont (Hongoh et al. 2007a). All the cells of the host *C. versatilis* are covered with this ectosymbiont, named "*Candidatus* Tammella caduceiae," and also simultaneously covered with a phylotype belonging to *Bacteroidales* (Fig. 5c–f). Ectosymbionts belonging to the genus *Desulfovibrio* in the class *Deltaproteobacteria* (Sato et al. 2009; Strassert et al. 2012; Kuwahara et al. 2017) and the genus *Endomicrobium* in the phylum *Elusimicrobia* (Izawa et al. 2017) have also been reported from specific groups of gut flagellates.

Two studies attempted to elucidate the evolutionary history between the *Bacteroidales* ectosymbionts and their flagellate hosts. Noda et al. (2009) surveyed the gut flagellate community in several termite species for the presence of *Bacteroidales* ectosymbionts by FISH analysis using a *Bacteroidales*-specific probe. The *Bacteroidales*-associated flagellate species were collected using a micromanipulator and subjected to 16S rRNA gene clone analysis. In total, combined with previously published data, 31 taxa of *Bacteroidales* ectosymbionts from 17 flagellate

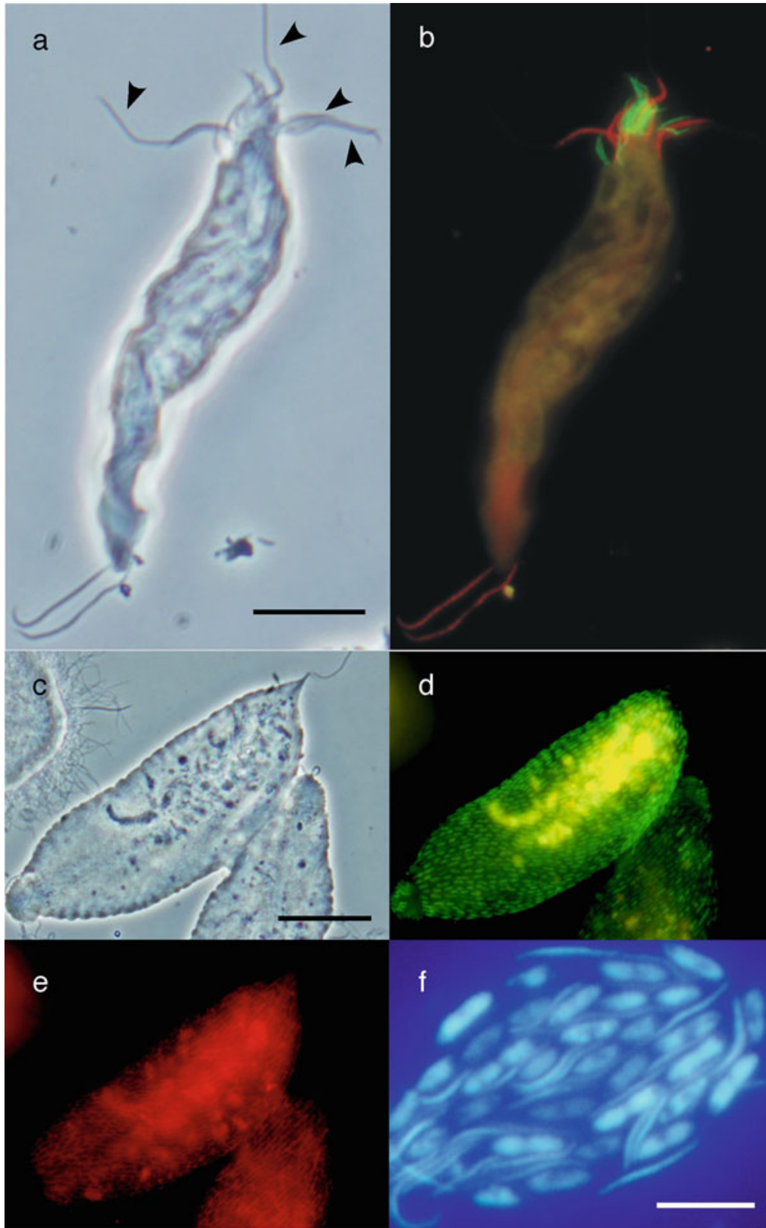


Fig. 5 Ectosymbiotic eubacteria discovered on the surface of flagellate cells from the gut of lower termites. (**a**) Phase contrast image of the oxymonad flagellate *Dinenympha porteri* from the gut of the termite *Reticulitermes speratus*. (**b**) FISH analysis of the ectosymbiotic spirochetes (red) and "*Ca*. Symbiothrix dinenymphae" (*Bacteroidales*) (green) using taxon-specific probes. Arrowheads in panel **a** indicate the true flagella of the flagellate host. Bar = 10 μm in panel **a**. (**c**) Phase contrast image of the parabasalid flagellate *Caduceia versatilis* from the gut of the termite *Cryptotermes*

genera in 10 families were used for phylogenetic analysis. The results clearly indicated multiple, independent acquisitions of the *Bacteroidales* ectosymbionts by different flagellate genera. Within a host genus, however, the ectosymbionts appear to have cospeciated with their host flagellates.

The cospeciation within a host genus was examined in detail by Desai et al. (2010). They focused on the relationship between the parabasalid flagellates belonging to the genus *Devescovina* and their *Bacteroidales* ectosymbionts and demonstrated that the devescovinids and the ectosymbionts have strictly cospeciated. On the other hand, they also found that the *Bacteroidales* ectosymbionts of the oxymonad flagellates *Oxymonas* spp. do not constitute a monophyletic cluster but derived from distantly related lineages. The latter result was consistent with a previous report that *Oxymonas* sp. from the termite *Neotermes koshunensis* harbors two distinct lineages of *Bacteroidales* ectosymbionts (Noda et al. 2006b). Hongoh et al. (2007a, b) found that a single phylotype sharing 97% sequence identity, designated as "*Candidatus* Symbiothrix dinenymphae," resides on the cells of several distinct flagellate species belonging to the genus *Dinenympha*. These data indicate a complex evolutionary history of the symbiosis between the gut flagellates and their ectosymbiotic eubacteria.

3.2 Phylogeny of Endosymbiotic Eubacteria

In addition to the ectosymbionts, the majority of the termite gut flagellates harbor endosymbiotic eubacteria. One of the most prevailing endosymbionts is the genus *Endomicrobium*, which was formerly named Termite Group I (TG1). This eubacterial group was reported for the first time in 1996 by Ohkuma and Kudo, based on 16S rRNA sequences which were obtained by PCR amplification from the gut homogenate of *R. speratus*. The specific localization of this group, i.e., inside the cells of various gut flagellate species, was later unveiled (Stingl et al. 2005; Ikeda-Ohtsubo et al. 2007; Ohkuma et al. 2007). In general, a single host flagellate cell contains tens to thousands of the cells of a single *Endomicrobium* phylotype (Stingl et al. 2005; Ohkuma et al. 2007; Zheng et al. 2015). As an example, "*Candidatus* Endomicrobium trichonymphae," an obligate intracellular symbiont of the flagellate *Trichonympha agilis* (Stingl et al. 2005; Hongoh et al. 2008a), is shown in Fig. 6a–c.

Similar to the case of the ectosymbionts belonging to the *Bacteroidales*, the evolutionary history of the symbiosis between *Endomicrobia* and their flagellate hosts appears to be complicated. While it has been demonstrated that *Endomicrobia*

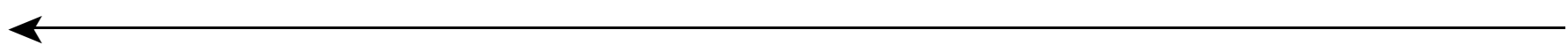


Fig. 5 (continued) cavifrons. (**d**) FISH analysis of ectosymbiotic "*Ca*. Tammella caduceiae" (*Synergistetes*) (green). (**e**) FISH analysis of an ectosymbiotic *Bacteroidales* phylotype. Bar = 50 μm in panel **c**. (**f**) DAPI-stained ectosymbiotic bacteria on the surface of a host flagellate cell. Bar = 5 μm in panel **f**. Panels **a** and **b** were originally published in Hongoh et al. (2007b). Panels **c**–**f** were originally published in Hongoh et al. (2007a)

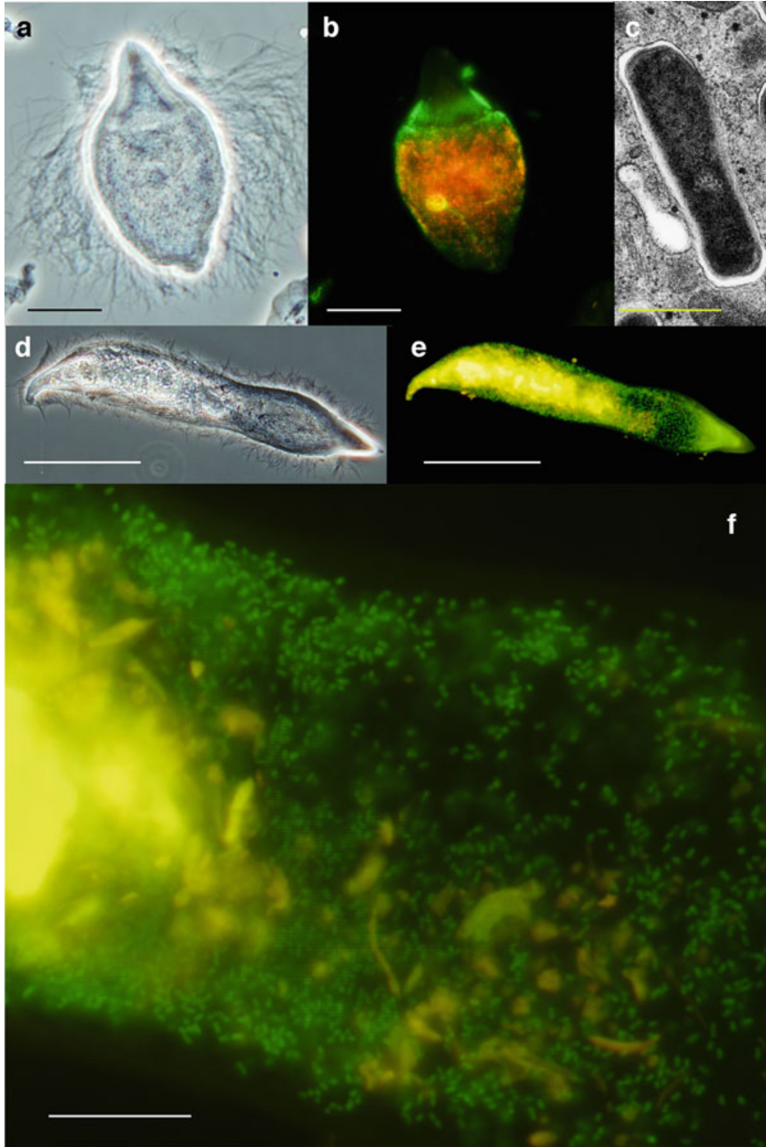


Fig. 6 Endosymbiotic eubacteria discovered inside the cells of flagellate in the gut of lower termites. (**a**) Phase contrast image of the parabasalid flagellate *Trichonympha agilis* from the gut of the termite *Reticulitermes speratus*. (**b**) FISH analysis of "*Ca*. Endomicrobium trichonymphae" (orange) and other eubacteria (green) using taxon-specific probes. The majority of the eubacteria detected with the green signals include ectosymbiotic "*Ca*. Desulfovibrio trichonymphae." Bars = 20 μm. (**c**) Transmission electron microscopy of "*Ca*. Endomicrobium trichonymphae". Bar = 0.5 μm. (**d**) Phase contrast image of the parabasalid flagellate *Pseudotrichonympha grassii* from the gut of the termite *Coptotermes formosanus*. (**e**) Fish analysis of the endosymbiotic *Bacteroidales* bacteria (green). The yellow color indicates the autofluorescence from phagocytosed

have strictly cospeciated with their host *Trichonympha* flagellates (Ikeda-Ohtsubo and Brune 2009), multiple acquisitions or horizontal transfers of *Endomicrobia* are suggested in other host lineages and at the level of host families (Ikeda-Ohtsubo et al. 2007; Ohkuma et al. 2007; Desai et al. 2010). Discoveries of multiple free-living and ectosymbiotic *Endomicrobium* lineages from termite guts distributing among endosymbiotic clades (Ikeda-Ohtsubo et al. 2010; Zheng et al. 2016; Izawa et al. 2017) support the view that multiple endosymbiotic events by free-living or ectosymbiotic species have occurred between this genus and various flagellate species (Brune 2017; Mikaelyan et al. 2017).

In contrast to *Endomicrobia*, which are endosymbionts of various species of gut flagellates, the endosymbionts belonging to the *Bacteroidales* inhabit only the cells of the parabasalid flagellates in the genus *Pseudotrichonympha* (Fig. 6d–f) (Noda et al. 2007). Further, *Pseudotrichonympha* flagellates are unique to termites in the family Rhinotermitidae (Yamin 1979; Kitade and Matsumoto 1998). Hence, the distribution of the endosymbiotic *Bacteroidales* bacteria is restricted. Their population, however, is huge, and they are predominant in the prokaryotic gut microbiota of rhinotermitid termites. In the termite *Coptotermes formosanus*, a single cell of *Pseudotrichonympha grassii* contains up to 10^5 cells of the *Bacteroidales* endosymbiont, designated as "*Candidatus* Azobacteroides pseudotrichonymphae," and in total, it accounts for two-thirds of the prokaryotic cells in *C. formosanus* guts (Noda et al. 2005; Hongoh et al. 2008b).

Noda et al. (2007) examined the evolutionary history of this triplex symbiosis among rhinotermitid termites, *Pseudotrichonympha* flagellates, and "*Ca.* Azobacteroides pseudotrichonymphae." Phylogenetic analyses showed that these three have almost completely cospeciated, implying the importance of "*Ca.* Azobacteroides pseudotrichonymphae" to the host *Pseudotrichonympha*, as well as the importance of the *Pseudotrichonympha* to the host termites. Indeed, it has been demonstrated by selective elimination of *P. grassii* from the gut microbiota that *P. grassii* is essential for the host *C. formosanus* to feed on wood materials (Yoshimura 1995).

The flagellate genera *Eucomonympha* and *Teranympha* which are relatives of *Pseudotrichonympha* harbor endosymbionts not belonging to the *Bacteroidales* but to the genus *Treponema* (Ohkuma et al. 2015). The spirochetal endosymbiont associated with the *Eucomonympha*, designated as "*Candidatus Treponema intracellularis*," is rod shaped, unlike a typical, spiral morphology. This endosymbiont phylogenetically clusters with ectosymbiotic phylotypes as in the case of "*Ca.* Azobacteroides pseudotrichonymphae" and its ectosymbiotic *Bacteroidales* relatives; these endosymbionts may have evolved from ectosymbionts.

Fig. 6 (continued) wood particles. Bars = 50 μm. (**f**) Magnified image of **e**. Bar = 10 μm. Panels **a**–**c** were originally published in Hongoh et al. (2008a), and panels **d**–**f** were originally published in Hongoh et al. (2008b) as supporting online materials

As seen in the ectosymbiosis, multiple species of endosymbionts occasionally coinhabit a single flagellate cell. For example, *Trichonympha collaris* in the gut of *Z. nevadensis* intracellularly harbors both "*Ca*. Endomicrobium trichonymphae" and "*Candidatus* Adiutrix intracellularis," which belong to a deep-branching clade of the *Deltaproteobacteria* (Ikeda-Ohtsubo et al. 2016). *T. collaris* also harbors a phylotype of the ectosymbiont "*Ca*. Desulfovibrio trichonymphae," proximal to the location of "*Ca*. Adiutrix intracellularis." Another ectosymbiotic phylotype of "*Ca.* Desulfovibrio trichonymphae," Rs-N31, coexists with "*Ca*. Endomicrobium trichonymphae" phylotype Rs-D17 in *T. agilis* in *R. speratus* guts (Fig. 6b) (Sato et al. 2009; Kuwahara et al. 2017). In case of *Trichonympha* species in the guts of the genus *Incisitermes*, an actinobacterial endosymbiont, "*Candidatus* Ancillula trichonymphae," in the order *Bifidobacteriales* inhabits the host cytoplasm instead of "*Ca*. Endomicrobium trichonymphae" (Strassert et al. 2012, 2016).

Eubacterial endosymbionts are found even in the nucleoplasm of various gut flagellate species. For example, *T. agilis* in *R. speratus* guts occasionally harbors two intranuclear bacteria, "*Candidatus* Nucleococcus trichonymphae" and "*Candidatus* Nucleococcus kirbyi" belonging to the order *Puniceicoccales* in the phylum *Verrucomicrobia* (Kirby 1944; Sato et al. 2014).

3.3 Predicted Functions of Eubacterial Ecto- and Endosymbionts

Because the eubacterial symbionts associated with termite gut flagellates have never been cultured thus far, their functions and symbiotic roles have been predicted on the basis of their genome sequences, in addition to their taxonomic positions, localizations, functional gene marker analyses, and fragmental physiological information.

The most intriguing and clearly demonstrated function of ectosymbionts, even without genomic data, is to provide the host flagellates with the motility. The parabasalid flagellate *Mixotricha paradoxa* in the gut of the termite *Mastotermes darwiniensis* harbors three species of treponemes on its cell surface (Wenzel et al. 2003). Surprisingly, *M. paradoxa* cannot swim by its own flagella, but is propelled solely by a synchronized movement of the ectosymbiotic treponemes (Cleveland and Grimstone 1964). Similarly, the flagellate *Caduceia versatilis* in the gut of *Cryptotermes cavifrons* swims only by movement of the bundled flagella of the ectosymbionts "*Ca.* Tammella caduceiae" (Fig. 5c–f) (Tamm 1982; Hongoh et al. 2007a). The detailed mechanism of these peculiar ectosymbioses, however, remains unknown.

The motility symbiosis has never been observed in other treponemal ectosymbionts, and instead, other functions have been hypothesized on the basis of the physiological data obtained from cultured strains of the genus *Treponema* isolated from termite guts. Leadbetter et al. (1999) have succeeded in axenically culturing *Treponema* strains from termite guts. The strains, ZAS-1 and ZAS-2 isolated from the gut of *Z. angusticollis*, later described as *Treponema primitia* (Graber and Breznak 2004; Graber et al. 2004), are homoacetogens which grow by mixotrophy using

sugars, or H_2 plus CO_2, and exhibit a low level of nitrogen-fixing activity. Another strain, ZAS-9 isolated also from a *Z. angusticollis* gut, later described as *Treponema azotonutricium* (Graber et al. 2004), grows by heterotrophy fermenting sugars to acetate, ethanol, CO_2, and H_2, and it exhibits a strong activity of nitrogen fixation (Lilburn et al. 2001). From these data, one can speculate that the ectosymbiotic treponemes might be involved in mutualism mediated by interspecies H_2 transfer as suggested in the endosymbiosis with methanogens, and/or they might contribute to the nitrogen metabolism of the host flagellates and termites by nitrogen fixation.

Although there have been no genome sequence or physiological data reported from ectosymbiotic treponemes thus far, in the endosymbiotic "*Ca*. Treponema intracellularis," these functions, i.e., reductive acetogenesis from H_2 and CO_2 and nitrogen fixation, were experimentally demonstrated and indicated also by single-cell genomics of the endosymbionts (Ohkuma et al. 2015). In *Hodotermopsis sjoestedti*, "*Ca*. Treponema intracellularis" residing within *Eucomonympha* cells accounts for most of the nitrogen-fixing activity of the termite host (Ohkuma et al. 2015).

Nitrogen fixation is conducted also by ectosymbiotic and endosymbiotic bacteria belonging to the *Bacteroidales*. The complete genome sequence of the endosymbiont "*Ca*. Azobacteroides pseudotrichonymphae" was obtained and analyzed, using whole genome amplification (WGA) from the bacterial population within a single *Pseudotrichonympha* cell in a *C. formosanus* gut (Hongoh et al. 2008b). The genome is small (1.1 Mb) but retained biosynthetic pathways for most proteinaceous amino acids and various cofactors and ability to fix dinitrogen and to recycle nitrogen wastes (urea and ammonia). The main carbon and energy sources are glucose, xylose, and uronates, which should be abundant in the host cytoplasm as a result of lignocellulose hydrolysis by the flagellate host (Hongoh et al. 2008b). In addition, Inoue et al. (2007) experimentally demonstrated that the fraction of "*Ca*. Azobacteroides pseudotrichonymphae" cells exhibited a strong uptake-type hydrogenase activity. Thus, the endosymbiont may also contribute to the elimination of H_2, although it does not possess a typical hydrogenase; a complex comprising an electron-bifurcating heterodisulfide reductase (HdrABC) and a flavin oxidoreductase (FlxABCD) might be responsible for the activity (Hongoh et al. 2008b; Ramos et al. 2015). A schematic metabolic pathway of this endosymbiont is shown in Fig. 7.

"*Candidatus* Armantifilum devescovinae," the *Bacteroidales* ectosymbiont of the flagellate genus *Devescovina*, also possesses genes responsible for nitrogen fixation (Noda et al. 2006a; Desai and Brune 2012). Since phagocytosis of *Bacteroidales* ectosymbionts by the host devescovinids can be observed (Noda et al. 2006b), it is conceivable that the ectosymbionts serve as a nutrient source for the flagellate hosts. Another *Bacteroidales* ectosymbiont, "*Candidatus* Vestibaculum sp.," associated with the flagellate genus *Barbulanympha* in the gut of the wood-feeding cockroach *Cryptocercus punctulatus*, also possesses genes for nitrogen fixation, and its activity was experimentally demonstrated (Tai et al. 2016). In contrast to these two ectosymbionts, no genes responsible for nitrogen fixation were found in the single-cell genomes of the *Bacteroidales* ectosymbiont "*Ca.* Symbiothrix dinenymphae" (Fig. 5b). This ectosymbiont instead equips an array of glycoside hydrolases, which should be involved in lignocellulose digestion (Yuki et al. 2015).

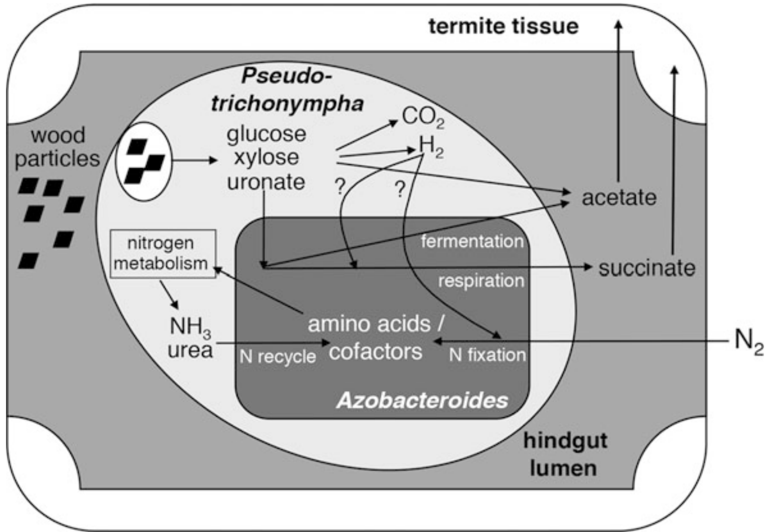


Fig. 7 Predicted functions of endosymbiotic. "*Ca*. Azobacteroides pseudotrichonymphae" phylotype CfPt1-2 from the gut of *Coptotermes formosanus*. The genome sequence data was analyzed and published in Hongoh et al. (2008b)

Trichonympha agilis in the gut of *R. speratus* simultaneously harbors the endosymbiont "*Ca*. Endomicrobium trichonymphae" phylotype Rs-D17 and the ectosymbiont "*Ca*. Desulfovibrio trichonymphae" phylotype Rs-N31, as described above (Fig. 6a–c). The Rs-N31 bacterium is buried in the surface layer of the host *T. agilis* and connected to the outside only through a ca. 40 nm pore or groove (Kuwahara et al. 2017). The complete genome sequences of both symbionts were obtained and analyzed (Hongoh et al. 2008a; Izawa et al. 2016; Kuwahara et al. 2017), and the predicted symbiotic system within a host *T. agilis* cell is outlined in Fig. 8. Both genomes are small, 1.1 Mb and 1.4 Mb in "*Ca.* E. trichonymphae" and "*Ca.* D. trichonymphae," respectively, and ca. 15% of the predicted protein-coding sequences are pseudogenized in both genomes. While "*Ca.* E. trichonymphae" ferments glucose 6-phosphate and uronates to ethanol, acetate, H_2, and CO_2, the latter three end products were used by "*Ca.* D. trichonymphae" as the main energy and carbon sources. Both bacteria can synthesize various amino acids and cofactors, although they do not possess genes for nitrogen fixation. The small pore or narrow groove that connects "*Ca.* D. trichonymphae" to the gut lumen may be necessary for uptake of sulfate to oxidize hydrogen, and in case that sulfate supply is not enough, the bacterium can conduct fumarate respiration by taking up malate from the host cell and excrete succinate.

The predicted functions of ecto- and endosymbionts, including "*Ca*. Adiutrix trichonymphae" (Ikeda-Ohtsubo et al. 2016) and "*Ca*. Ancillula trichonymphae" (Strassert et al. 2016), are summarized in Table 1.

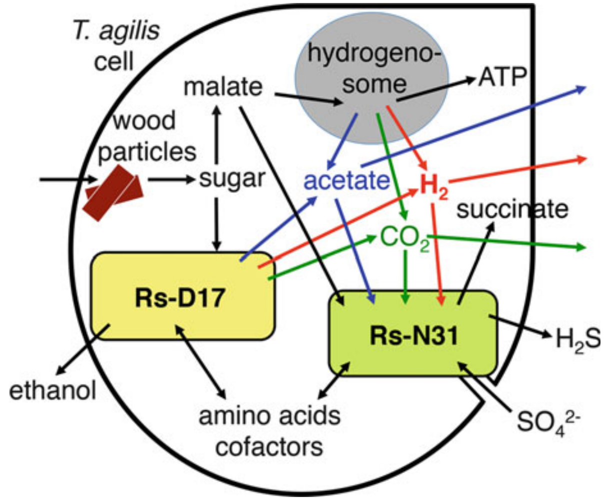


Fig. 8 Predicted symbiotic system within a *Trichonympha agilis* cell from the gut of *Reticulitermes speratus*. The data was based on the genome sequences of "*Ca*. Endomicrobium trichonymphae" phylotype Rs-D17 and "*Ca*. Desulfovibrio trichonymphae" phylotype Rs-N31, originally published in Hongoh et al. (2008a) and Kuwahara et al. (2017)

4 Concluding Remarks and Future Perspectives

Most of the flagellates in termite guts harbor ecto- and endosymbiotic eubacteria and/or methanogenic archaea. During these two decades, their phylogenetic and spatial structures have been revealed to some extent by using molecular analyses based on 16S rRNA sequences. In addition, genomic approaches using whole genome amplification, including single cell genomics, have been established; the functions of these ecto- and endosymbiotic bacteria have been gradually revealed, although genome analysis of endosymbiotic methanogens remains unachieved.

The ecto- and endosymbionts of gut flagellates comprise diverse taxa, including the phyla *Spirochaetes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Synergistetes*. Their symbiotic roles, however, show consistency (Table 1). The most common function is biosynthesis of various amino acids and cofactors, and several ecto- and endosymbionts additionally possess genes for nitrogen fixation and recycling. Because most lower termites feed exclusively on nitrogen-poor dead wood, these functions appear to be critical for the nutrition of the host termites and possibly the host flagellates although detailed physiology of the flagellates is almost unknown. Removal of hydrogen is also common; this activity will promote the lignocellulose fermentation by the flagellate hosts and gut bacteria. Thus, these ecto- and endosymbioses composed of diverse prokaryotic taxa appear to be the results of evolutionary convergence (Ohkuma et al. 2015; Strassert et al. 2016). The next step to decipher this complex symbiotic system may be functional analyses of the uncultured flagellate hosts and more detailed analyses of the ecto- and endosymbionts, using multi-omics technologies.

Table 1 Predicted symbiotic roles of ecto- and endosymbiotic eubacteria of termite gut flagellates

Bacterial symbiont	Localization	Host flagellate	Host termite	Predicted functions	References
Ca. Endomicrobium trichonymphae	Intracellular	*Trichonympha agilis*	*Reticulitermes speratus*	Synthesis of amino acids/cofactors	Hongoh et al. (2008a)
Ca. Desulfovibrio trichonymphae	Surface-embedded	*Trichonympha agilis*	*Reticulitermes speratus*	H_2-removal, synthesis of amino acids/cofactors	Kuwahara et al. (2017)
Ca. Adiutrix trichonymphae	Intracellular	*Trichonympha collaris*	*Zootermopsis nevadensis*	N-fixation, H_2-removal, synthesis of amino acids/cofactors	Ikeda-Ohtsubo et al. (2016)
Ca. Ancillula trichonymphae	Intracellular	*Trichonympha paraspiralis*	*Incisitermes marginipennis*	Synthesis of amino acids/cofactors	Strassert et al. (2016)
Ca. Azobacteroides pseudotrichonymphae	Intracellular	*Pseudotrichonympha grassii*	*Coptotermes formosanus*	N-fixation, N-recycle, H_2-removal ?, synthesis of amino acids/cofactors	Hongoh et al. (2008b)
Ca. Treponema intracellularis	Intracellular	*Eucomonympha* spp.	*Hodotermopsis sjoestedti*	N-fixation, N-recycle, H_2-removal, synthesis of amino acids/cofactors	Ohkuma et al. (2015)
Ca. Symbiothrix dinenymphae	Surface	*Dinenympha* spp.	*Reticulitermes speratus*	lignocellulose digestion, synthesis of amino acids/cofactors	Yuki et al. (2015)
Ca. Vestibaculum sp.	Surface	*Barbulanympha* sp.	*Cryptocercus punctulatus*	N-fixation, N-recycle, synthesis of amino acids/cofactors	Tai et al. (2016)
Ca. Armantifilum devescovinae	Surface	*Devescovina* spp.	Kalotermitids	N_2-fixation	Desai and Brune (2012)
Treponema spp.	Surface	*Mixotricha paradoxa*	*Mastotermes darwiniensis*	Motility	Cleveland and Grimstone (1964)
Ca. Tammella caduceiae	Surface	*Caduceia versatilis*	*Cryptotermes cavifrons*	Motility	Tamm (1982), Hongoh et al. (2007b)

References

Abdul Rahman N, Parks DH, Willner DL, Engelberktson AL, Goffredi SK, Warnecke F, Scheffrahn RH, Hugenholtz P (2015) A molecular survey of Australian and North American termite genera indicates that vertical inheritance is the primary force shaping termite gut microbiomes. Microbiome 3:e5

Andrew BJ (1930) Method and rate of protozoan refaunation in the termite *Termopsis angusticollis*. Hagen Univ Calif (Berkeley) Publ Zool 33:449–470

Berchtold M, Chatzinotas A, Schönhuber W, Brune A, Amann R, Hahn D, König H (1999) Differential enumeration and in situ localization of microorganisms in the hindgut of the lower termite *Mastotermes darwiniensis* by hybridization with rRNA-targeted probes. Arch Microbiol 172:407–416

Boucias DG, Cai Y, Sun Y, Lietze VU, Sen R, Raychoudhury R, Scharf ME (2013) The hindgut lumen prokaryotic microbiota of the termite *Reticulitermes flavipes* and its responses to dietary lignocellulose composition. Mol Ecol 22:1836–1853

Brauman A, Kane MD, Labat M, Breznak JA (1992) Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science 257:1384–1387

Brauman A, Dore J, Eggleton P, Bignell DE, Breznak JA, Kane MD (2001) Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. FEMS Microbiol Ecol 35:27–36

Breznak JA (2000) Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbioses, ecology. Kluwer Academic Publishers, Dordrecht, pp 209–232

Breznak JA, Switzer JM (1986) Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl Environ Microbiol 52:623–630

Brune A (2014) Symbiotic digestion of lignocellulose in termite guts. Nat Rev Microbiol 12: 168–180

Brune A (2017) Ectosymbiotic *Endomicrobia* – a transition stage towards intracellular symbionts? Environ Microbiol Rep 9:474–476

Brune A (2018) Methanogens in the digestive tract of termites. In: Hackstein JHP (ed) Microbiology monographs: (Endo)symbiotic methanogenic archaea. Springer-Nature, Heidelberg

Brune A, Ohkuma M (2011) Role of the termite gut microbiota in symbiotic digestion. In: Bignell DE, Roisin Y, Lo N (eds) Biology of termites: a modern synthesis. Springer, New York, pp 413–438

Brune A, Stingl U (2006) Prokaryotic symbionts of termite gut flagellates: phylogenetic and metabolic implications of a tripartite symbiosis. Prog Mol Subcell Biol 41:39–60

Cleveland LR, Grimstone AV (1964) The fine structure of the flagellate *Mixotricha paradoxa* and its associated micro-organisms. Proc R Soc Lond B 159:668–686

Deevong P, Hattori M, Yamada A, Trakulnaleamsai S, Ohkuma M, Noparatnaraporn N, Kudo T (2004) Isolation and detection of methanogens from the gut of higher termites. Microbes Environ 19:221–226

Desai MS, Brune A (2012) *Bacteroidales* ectosymbionts of gut flagellates shape the nitrogen-fixing community in dry-wood termites. ISME J 6:1302–1313

Desai MS, Strassert JFH, Meuser K, Hertel H, Ikeda-Ohtsubo W, Radek R, Brune A (2010) Strict cospeciation of devescovinid flagellates and *Bacteroidales* ectosymbionts in the gut of dry-wood termites (Kalotermitidae). Environ Microbiol 12:2120–2132

Donovan SE, Purdy KJ, Kane MD, Eggleton P (2004) Comparison of *Euryarchaea* strains in the guts and food-soil of the soil-feeding termite *Cubitermes fungifaber* across different soil types. Appl Environ Microbiol 70:3884–3892

Ebert A, Brune A (1997) Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower termite *Reticulitermes flavipes* (Kollar). Appl Environ Microbiol 63:4039–4046

Fenchel T, Finlay BJ (2018) Free-living protozoa with endosymbiotic methanogens. In: Hackstein JHP (ed) Microbiology monographs: (Endo)symbiotic methanogenic archaea. Springer-Nature, Heidelberg

Friedrich MW, Schmitt-Wagner D, Lueders T, Brune A (2001) Axial differences in community structure of *Crenarchaeota* and *Euryarchaeota* in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. Appl Environ Microbiol 67:4880–4890

Graber JR, Breznak JA (2004) Physiology and nutrition of *Treponema primitia*, an H_2/CO_2-acetogenic spirochete from termite hindguts. Appl Environ Microbiol 70:1307–1314

Graber JR, Leadbetter JR, Breznak JA (2004) Description of *Treponema azotonutricium* sp. nov. and *Treponema primitia* sp. nov., the first spirochetes isolated from termite guts. Appl Environ Microbiol 70:1315–1320

Hara K, Shinzato N, Oshima T, Yamagishi A (2004) Endosymbiotic *Methanobrevibacter* species living in symbiotic protists of the termite *Reticulitermes speratus* detected by fluorescent *in situ* hybridization. Microbes Environ 19:120–127

Hongoh Y (2010) Diversity and genomes of uncultured microbial symbionts in the termite gut. Biosci Biotechnol Biochem 74:1145–1151

Hongoh Y (2011) Toward the functional analysis of uncultivable, symbiotic microorganisms in the termite gut. Cell Mol Life Sci 68:1311–1325

Hongoh Y, Ohkuma M, Kudo T (2003) Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol Ecol 44:231–242

Hongoh Y, Deevong P, Inoue T, Moriya S, Trakulnaleamsai S, Ohkuma M, Vongkaluang C, Noparatnaraporn N, Kudo T (2005) Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl Environ Microbiol 71:6590–6599

Hongoh Y, Sato T, Dolan MF, Noda S, Ui S, Kudo T, Ohkuma M (2007a) The motility symbiont of the termite gut flagellate *Caduceia versatilis* is a member of the "*Synergistes*" group. Appl Environ Microbiol 73:6270–6276

Hongoh Y, Sato T, Noda S, Ui S, Kudo T, Ohkuma M (2007b) *Candidatus* Symbiothrix dinenymphae: bristle-like *Bacteroidales* ectosymbionts of termite gut protists. Environ Microbiol 9:2631–2635

Hongoh Y, Sharma VK, Prakash T, Noda S, Taylor TD, Kudo T, Sakaki Y, Toyoda A, Hattori M, Ohkuma M (2008a) Complete genome of the uncultured Termite Group 1 bacteria in a single host protist cell. Proc Natl Acad Sci USA 105:5555–5560

Hongoh Y, Sharma VK, Prakash T, Noda S, Toh H, Taylor TD, Kudo T, Sakaki Y, Toyoda A, Hattori M, Ohkuma M (2008b) Genome of an endosymbiont coupling N_2 fixation to cellulolysis within protist cells in termite gut. Science 322:1108–1109

Ikeda-Ohtsubo W, Brune A (2009) Cospeciation of termite gut flagellates and their bacterial endosymbionts: *Trichonympha* species and '*Candidatus* Endomicrobium trichonymphae'. Mol Ecol 18:332–342

Ikeda-Ohtsubo W, Desai M, Stingl U, Brune A (2007) Phylogenetic diversity of '*Endomicrobia*' and their specific affiliation with termite gut flagellates. Microbiology 153:3458–3465

Ikeda-Ohtsubo W, Faivre N, Brune A (2010) Putatively free-living '*Endomicrobia*' – ancestors of the intracellular symbionts of termite gut flagellates? Environ Microbiol Rep 2:554–559

Ikeda-Ohtsubo W, Strassert JF, Kohler T, Mikaelyan A, Gregor I, McHardy AC, Tringe SG, Hugenholtz P, Radek R, Brune A (2016) '*Candidatus* Adiutrix intracellularis', an endosymbiont of termite gut flagellates, is the first representative of a deep-branching clade of *Deltaproteobacter*ia and a putative homoacetogen. Environ Microbiol 18:2548–2564

Inoue J, Saita K, Kudo T, Ui S, Ohkuma M (2007) Hydrogen production by termite gut protists: characterization of iron hydrogenases of parabasalian symbionts of the termite *Coptotermes formosanus*. Eukaryot Cell 6:1925–1932

Inoue J, Noda S, Hongoh Y, Ui S, Ohkuma M (2008) Identification of endosymbiotic methanogen and ectosymbiotic spirochetes of gut protists of the termite *Coptotermes formosanus*. Microbes Environ 23:94–97

Izawa K, Kuwahara H, Kihara K, Yuki M, Lo N, Itoh T, Ohkuma M, Hongoh Y (2016) Comparison of intracellular "*Ca*. Endomicrobium trichonymphae" genomovars illuminates the requirement and decay of defense systems against foreign DNA. Genome Biol Evol 8:3099–3107

Izawa K, Kuwahara H, Sugaya K, Lo N, Ohkuma M, Hongoh Y (2017) Discovery of ectosymbiotic *Endomicrobium* lineages associated with protists in the gut of stolotermitid termites. Environ Microbiol Rep 9:411–418

Jenkins TM, Dean RE, Verkerk R, Forschler BT (2001) Phylogenetic analyses of two mitochondrial genes and one nuclear intron region illuminate European subterranean termite (Isoptera: Rhinotermitidae) gene flow, taxonomy, and introduction dynamics. Mol Phylogenet Evol 20:286–293

Kirby H (1930) Trichomonad flagellates from termites. Univ Calif (Berkeley) Publ Zool 33: 393–444

Kirby H (1944) The structural characteristics and nuclear parasites of some species of *Trichonympha* in termites. Univ Calif (Berkeley) Publ Zool 49:185–282

Kitade O (2004) Comparison of symbiotic flagellate faunae between termites and a wood-feeding cockroach of the genus *Cryptocercus*. Microbes Environ 19:215–220

Kitade O, Matsumoto T (1998) Characteristics of the symbiotic flagellate composition within the termite family Rhinotermitidae (Isoptera). Symbiosis 25:271–278

Kitade O, Maeyama T, Matsumoto T (1997) Establishment of symbiotic flagellate fauna of *Hodotermopsis japonica* (Isoptera: Termopsidae). Sociobiology 30:161–167

Kuwahara H, Yuki M, Izawa K, Ohkuma M, Hongoh Y (2017) Genome of '*Ca*. Desulfovibrio trichonymphae', an H_2-oxidizing bacterium in a tripartite symbiotic system within a protist cell in the termite gut. ISME J 11:766–776

Leadbetter JR, Breznak JA (1996) Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite *Reticulitermes flavipes*. Appl Environ Microbiol 62:3620–3631

Leadbetter JR, Crosby LD, Breznak JA (1998) *Methanobrevibacter filiformis* sp. nov., a filamentous methanogen from termite hindguts. Arch Microbiol 169:287–292

Leadbetter JR, Schmidt TM, Graber JR, Breznak JA (1999) Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283:686–689

Lee MJ, Schreurs PJ, Messer AC, Zinder SH (1987) Association of methanogenic bacteria with flagellated protozoa from a termite gut. Curr Microbiol 15:337–341

Lilburn TG, Kim KS, Ostrom NE, Byzek KR, Leadbetter JR, Breznak JA (2001) Nitrogen fixation by symbiotic and free-living spirochetes. Science 292:2495–2498

Messer AC, Lee MJ (1989) Effect of chemical treatments on methane emission by the hindgut microbiota in the termite *Zootermopsis angusticollis*. Microb Ecol 18:275–284

Mikaelyan A, Köhler T, Lampert N, Rohland J, Boga H, Meuser K, Brune A (2015) Classifying the bacterial gut microbiota of termites and cockroaches: a curated phylogenetic reference database (DictDb). Syst Appl Microbiol 38:472–482

Mikaelyan A, Thompson CL, Meuser K, Zheng H, Rani P, Plarre R, Brune A (2017) High-resolution phylogenetic analysis of *Endomicrobia* reveals multiple acquisitions of endosymbiotic lineages by termite gut flagellates. Environ Microbiol Rep 9:477–483

Müller N, Timmers P, Plugge CM, Stams AJM, Schink B (2018) Syntrophy in methanogenic degradation. In: Hackstein JHP (ed) Microbiology monographs: (Endo)symbiotic methanogenic archaea. Springer-Nature, Heidelberg

Noda S, Ohkuma M, Yamada A, Hongoh Y, Kudo T (2003) Phylogenetic position and in situ identification of ectosymbiotic spirochetes on protists in the termite gut. Appl Environ Microbiol 69:625–633

Noda S, Iida T, Kitade O, Nakajima H, Kudo T, Ohkuma M (2005) Endosymbiotic *Bacteroidales* bacteria of the flagellated protist *Pseudotrichonympha grassii* in the gut of the termite *Coptotermes formosanus*. Appl Environ Microbiol 71:8811–8817

Noda S, Inoue T, Hongoh Y, Kawai M, Nalepa CA, Vongkaluang C, Kudo T, Ohkuma M (2006a) Identification and characterization of ectosymbionts of distinct lineages in *Bacteroidales*

attached to flagellated protists in the gut of termites and a wood-feeding cockroach. Environ Microbiol 8:11–20

Noda S, Kawai M, Nakajima H, Kudo T, Ohkuma M (2006b) Identification and *in situ* detection of two lineages of *Bacteroidales* ectosymbionts associated with a termite gut protist, *Oxymonas* sp. Microbes Environ 21:16–22

Noda S, Kitade O, Inoue T, Kawai M, Kanuka M, Hiroshima K, Hongoh Y, Constantino R, Uys V, Zhong J-H, Kudo T, Ohkuma M (2007) Cospeciation in the triplex symbiosis of termite gut protists (*Pseudotrichonympha* spp.), their hosts, and their bacterial endosymbionts. Mol Ecol 16:1257–1266

Noda S, Hongoh Y, Sato T, Ohkuma M (2009) Complex coevolutionary history of symbiotic *Bacteroidales* bacteria of various protists in the gut of termites. BMC Evol Biol 9:e158

Odelson DA, Breznak JA (1983) Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl Environ Microbiol 45:1602–1613

Odelson DA, Breznak JA (1985a) Cellulase and other polymer-hydrolyzing activities of *Trichomitopsis termopsidis,* a symbiotic protozoan from termites. Appl Environ Microbiol 49: 622–626

Odelson DA, Breznak JA (1985b) Nutrition and growth characteristics of *Trichomitopsis termopsidis*, a cellulolytic protozoan from termites. Appl Environ Microbiol 49:614–621

Ohkuma M (2008) Symbioses of flagellates and prokaryotes in the gut of lower termites. Trends Microbiol 16:345–352

Ohkuma M, Kudo T (1996) Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. Appl Environ Microbiol 62:461–468

Ohkuma M, Noda S, Kudo T (1999) Phylogenetic relationships of symbiotic methanogens in diverse termites. FEMS Microbiol Lett 171:147–153

Ohkuma M, Sato T, Noda S, Ui S, Kudo T, Hongoh Y (2007) The candidate phylum 'Termite Group 1' of bacteria: phylogenetic diversity, distribution, and endosymbiont members of various gut flagellated protists. FEMS Microbiol Ecol 60:467–476

Ohkuma M, Noda S, Hattori S, Iida T, Yuki M, Starns D, Inoue J, Darby AC, Hongoh Y (2015) Acetogenesis from H_2 plus CO_2 and nitrogen fixation by an endosymbiotic spirochete of a termite-gut cellulolytic protist. Proc Natl Acad Sci USA 112:10224–10230

Pester M, Brune A (2007) Hydrogen is the central free intermediate during lignocellulose degradation by termite gut symbionts. ISME J 1:551–565

Pester M, Tholen A, Friedrich MW, Brune A (2007) Methane oxidation in termite hindguts: absence of evidence and evidence of absence. Appl Environ Microbiol 73:2024–2028

Radek R (1999) Flagellates, bacteria, and fungi associated with termites: diversity and function in nutrition – a review. Ecotropica 5:183–196

Radek R, Tischendorf G (1999) Bacterial adhesion to different termite flagellates: ultrastructural and functional evidence for distinct molecular attachment modes. Protoplasma 207:43–53

Radek R, Roesel J, Hausmann K (1996) Light and electron microscopic study of the bacterial adhesion to termite flagellates applying lectin cytochemistry. Protoplasma 193: 105–122

Ramos AR, Grein F, Oliveira GP, Venceslau SS, Keller KL, Wall JD, Pereira IA (2015) The FlxABCD-HdrABC proteins correspond to a novel NADH dehydrogenase/heterodisulfide reductase widespread in anaerobic bacteria and involved in ethanol metabolism in *Desulfovibrio vulgaris* Hildenborough. Environ Microbiol 17:2288–2305

Sato T, Hongoh Y, Noda S, Hattori S, Ui S, Ohkuma M (2009) *Candidatus* Desulfovibrio trichonymphae, a novel intracellular symbiont of the flagellate *Trichonympha agilis* in termite gut. Environ Microbiol 11:1007–1015

Sato T, Kuwahara H, Fujita K, Noda S, Kihara K, Yamada A, Ohkuma M, Hongoh Y (2014) Intranuclear verrucomicrobial symbionts and evidence of lateral gene transfer to the host protist in the termite gut. ISME J 8:1008–1019

Shinzato N, Matsumoto T, Yamaoka I, Oshima T, Yamagishi A (1999) Phylogenetic diversity of symbiotic methanogens living in the hindgut of the lower termite *Reticulitermes speratus* analyzed by PCR and in situ hybridization. Appl Environ Microbiol 65:837–840

Stingl U, Maass A, Radek R, Brune A (2004) Symbionts of the gut flagellate *Staurojoenina* sp. from *Neotermes cubanus* represent a novel, termite-associated lineage of Bacteroidales: description of '*Candidatus* Vestibaculum illigatum'. Microbiology 150:2229–2235

Stingl U, Radek R, Yang H, Brune A (2005) "*Endomicrobia*": cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. Appl Environ Microbiol 71:1473–1479

Strassert JFH, Köhler T, Wienemann THG, Ikeda-Ohtsubo W, Faivre N, Franckenberg S, Plarre R, Radek R, Brune A (2012) '*Candidatus* Ancillula trichonymphae', a novel lineage of endosymbiotic *Actinobacteria* in termite gut flagellates of the genus *Trichonympha*. Environ Microbiol 14:3259–3270

Strassert JF, Mikaelyan A, Woyke T, Brune A (2016) Genome analysis of '*Candidatus* Ancillula trichonymphae', first representative of a deep-branching clade of *Bifidobacteriales*, strengthens evidence for convergent evolution in flagellate endosymbionts. Environ Microbiol Rep. doi: https://doi.org/10.1111/1758-2229.12451

Sugimoto A, Inoue T, Tayasu I, Miller LR, Takeichi S, Abe T (1998) Methane and hydrogen production in a termite-symbiont system. Ecol Res 13:241–257

Sugimoto A, Bignell DE, Macdonald J (2000) Global impact of termites on the carbon cycle. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbioses, ecology. Kluwer Academic, Dordrecht, pp 409–436

Tai V, James ER, Nalepa CA, Scheffrahn RH, Perlman SJ, Keeling PJ (2015) The role of host phylogeny varies in shaping microbial diversity in the hindguts of lower termites. Appl Environ Microbiol 81:1059–1070

Tai V, Carpenter KJ, Weber PK, Nalepa CA, Perlman SJ, Keeling PJ (2016) Genome evolution and nitrogen fixation in bacterial ectosymbionts of a protist inhabiting wood-feeding cockroaches. Appl Environ Microbiol 82:4682–4695

Tamm SL (1980) The ultrastructure of prokaryotic-eukaryotic cell junctions. J Cell Sci 44:335–352

Tamm SL (1982) Flagellated ectosymbiotic bacteria propel a eukaryotic cell. J Cell Biol 94: 697–709

Tholen A, Brune A (2000) Impact of oxygen on metabolic fluxes and in situ rates of reductive acetogenesis in the hindgut of the wood-feeding termite *Reticulitermes flavipes*. Environ Microbiol 2:436–449

Tokura M, Ohkuma M, Kudo T (2000) Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. FEMS Microbiol Ecol 33: 233–240

Tsunoda K, Ohmura W, Yoshimura T, Tokoro M (1993) Methane emission by the termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) I. Effects of termite caste, population size and volume of test containers. Wood Res 79:34–40

Wenzel M, Radek R, Brugerolle G, König H (2003) Identification of the ectosymbiotic bacteria of *Mixotricha paradoxa* involved in movement symbiosis. Eur J Protistol 39:11–23

Yamin MA (1978) Axenic cultivation of the cellulolytic flagellate *Trichomitopsis termopsidis* (Cleveland) from the termite *Zootermopsis*. J Protozool 25:535–538

Yamin MA (1979) Flagellates of the orders Trichomonadida Kirby, Oxymonadida Grassé, and Hypermastigida Grassi and Foà reported from lower termites (Isoptera families Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae, and Serritermitidae) and from the wood-feeding roach *Cryptocercus* (Dictyoptera: Cryptocercidae). Sociobiology 4:1–120

Yamin MA (1980) Cellulose metabolism by the termite flagellate *Trichomitopsis termopsidis*. Appl Environ Microbiol 39:859–863

Yamin MA (1981) Cellulose metabolism by the flagellate *Trichonympha* from a termite is independent of endosymbiotic bacteria. Science 211:58–59

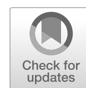
Yoshimura T (1995) Contribution of the protozoan fauna to nutritional physiology of the lower termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). Wood Res 82: 68–129

Yuki M, Kuwahara H, Shintani M, Izawa K, Sato T, Starns D, Hongoh Y, Ohkuma M (2015) Dominant ectosymbiotic bacteria of cellulolytic protists in the termite gut also have the potential to digest lignocellulose. Environ Microbiol 17:4942–4953

Zheng H, Dietrich C, Thompson CL, Meuser K, Brune A (2015) Population structure of Endomicrobia in single host cells of termite gut flagellates (*Trichonympha* spp.). Microbes Environ 30:92–98

Zheng H, Dietrich C, Radek R, Brune A (2016) *Endomicrobium proavitum*, the first isolate of *Endomicrobi*a class. nov. (phylum *Elusimicrobia*) – an ultramicrobacterium with an unusual cell cycle that fixes nitrogen with a Group IV nitrogenase. Environ Microbiol 18:191–204

Methanogens in the Digestive Tract of Termites



Andreas Brune

Contents

1 Introduction 82
2 Methane as a Product of Symbiotic Digestion 83
3 Diversity of Methanogens in Termite Guts 85
4 Structure of the Methanogenic Communities 87
5 Differences in Methanogenic Activities and Populations 88
6 Coexistence with Homoacetogens 89
7 Association with Gut Flagellates 91
8 Intercompartmental Transfer of Hydrogen 92
9 Relationship to Oxygen 94
10 Termites as a Source of Atmospheric Methane 95
11 Conclusions 96
References 96

Abstract Methanogenesis in termite guts is a product of symbiotic digestion, fueled by hydrogen and reduced one-carbon compounds that are formed during the fermentative breakdown of plant fiber and humus. Methanogens are restricted to the hindgut region and can be found in several distinct microhabitats. In lower termites, the methanogens belong almost exclusively to the genus *Methanobrevibacter*. They are either endosymbionts of flagellate protists or colonize the periphery of the hindgut, a habitat that is not fully anoxic. The oxygen-reducing capacities of the few isolates available so far indicate that they are well adapted to the continuous influx of oxygen across the gut wall. In higher termites, which lack gut flagellates, the hindgut is highly compartmented and characterized by strong differences in pH, redox potential, and other microenvironmental conditions. Here, the archaeal communities differ strongly between compartments and comprise not only *Methanobacteriales*, but also *Methanosarcinales*, *Methanomicrobiales*, and the recently discovered *Methanomassiliicoccales*. All methanogens in termite guts

A. Brune (✉)
Research Group Insect Gut Microbiology and Symbiosis, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany
e-mail: brune@mpi-marburg.mpg.de

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_6

belong to distinct phylogenetic clusters that are restricted to the intestinal tracts of insects and millipedes. Only few representatives have been isolated in pure culture. The high methane emissions of termites, together with their enormous biomass in the tropics, make them a significant natural source of this important greenhouse gas.

1 Introduction

Most insects that thrive on a fiber-rich diet harbor microbial symbionts that participate in digestion, but only termites, cockroaches, and the larvae of scarab beetles have been found to emit methane (Hackstein and van Alen 2018). This methane is produced by methanogenic archaea, which represent the last link in an anaerobic feeding chain of microorganisms located in the enlarged hindgut of these insects—microbial bioreactors that transform lignocellulosic matter to short-chain fatty acids, the major energy source for the host (Fig. 1). A detailed account of symbiotic digestion in termites can be found in other reviews of this topic (Brune and Ohkuma 2011; Brune 2014).

This chapter will provide an overview of the diverse aspects of methanogenesis in termites, including the role of methanogens in symbiotic digestion, the diversity and structure of the methanogenic community in different termite lineages, and advances

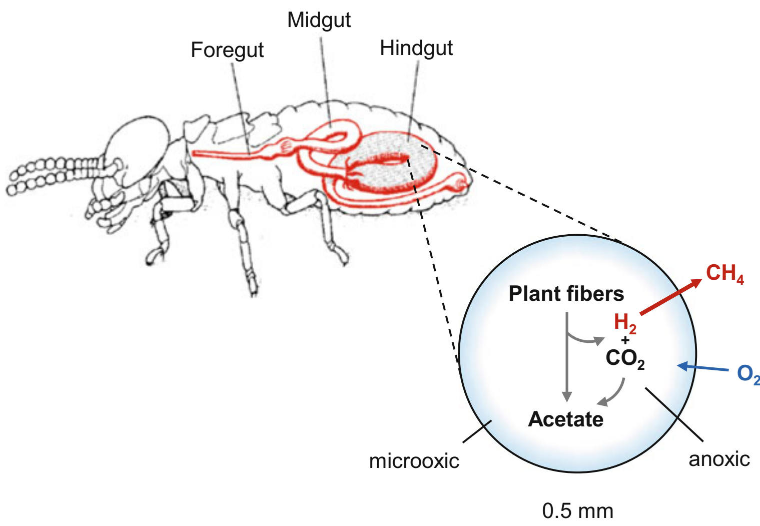


Fig. 1 The hindgut of termites is a microbial bioreactor that transforms lignocellulose to acetate and other short-chain fatty acids. Hydrogen formed during fermentation of plant fibers is the major substrate of both methanogenesis and reductive acetogenesis. The anoxic status of the hindgut lumen is maintained by the microorganisms colonizing the microoxic hindgut periphery, which consume the oxygen diffusing across the gut wall. Originally published in Brune (2010), reprinted with permission of ©Springer Nature

in understanding the interactions of methanogens with other gut microbiota and their physicochemical microenvironment. For detailed coverage of the older literature and broader surveys of methanogenesis in insects and millipedes and the associations between methanogens and termite gut flagellates, the reader is referred to other review articles (Breznak 2000; Ohkuma and Brune 2011; Brune 2018; Hongoh and Ohkuma 2018).

2 Methane as a Product of Symbiotic Digestion

Methane formation in the guts of termites had been suspected already more than 80 years ago. When Cook (1932) studied the respiratory gas exchange of *Zootermopsis nevadensis*, he found that the termite continued to form substantial amounts of an unidentified gas when the oxygen in the vessel was depleted. He was not able to analyze the gas, but—inspired by the situation in ruminants—proposed that the gas was most likely hydrogen or methane or a mixture of both. However, it took more than 40 years after Cook's initial observation until methane production in termite guts was finally recognized by Breznak and coworkers. While demonstrating nitrogenase activity in living termites and wood-feeding cockroaches with the acetylene reduction assay, the authors identified methane as an additional peak present in the gas chromatograms (Breznak et al. 1973, 1974)—a classic case of serendipity in science. Historical details have been reviewed elsewhere (Breznak 2000; Brune 2018).

Breznak (1975) had pointed out that the amount of methane produced by termites, if based on body weight, is in the same order of magnitude as that of ruminants. This observation immediately aroused the interest of atmospheric chemists studying the role of methane in radiative forcing of the atmosphere, who identified termites as a potential source of considerable strength of this greenhouse gas (see below). In the following years, methane production was documented for almost all termite species investigated (e.g., Brauman et al. 1992; Shinzato et al. 1992; Wheeler et al. 1996; Bignell et al. 1997; Sugimoto et al. 1998b), with marked differences in the methane emission rates between wood- and humus-feeding taxa (wood vs. humus; Fig. 2).

Methanogenic archaea form methane in two fundamentally different processes: (1) the reduction of CO_2 or methyl groups to CH_4 via the C_1 pathway (hydrogenotrophic methanogenesis); and (2) the cleavage of acetate to CH_4 and CO_2 via the acetyl-CoA pathway (aceticlastic methanogenesis) (Liu and Whitman 2008; Thauer et al. 2008). Interestingly, there is no evidence for aceticlastic methanogenesis in termite guts. As in the human gut and in the rumen, it is assumed that the relatively slow-growing aceticlastic species cannot cope with the short retention times of intestinal habitats (Liu and Whitman 2008). However, there is no explanation why they do not avoid washout by attaching to intestinal surfaces (see below).

The electron donors of hydrogenotrophic methanogenesis are hydrogen and reduced C_1 compounds, such as methanol and formate, which are formed during the fermentative breakdown of organic matter. In the hindguts of lower termites,

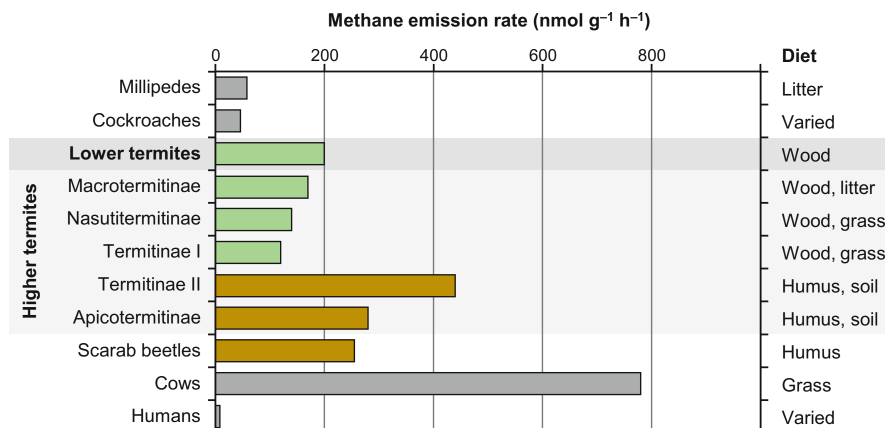


Fig. 2 Methane emission rates of lower and higher termites compared to those of other invertebrates, cows, and humans. The typical diet of the respective taxon is indicated; members of Termitinae were grouped into wood-feeding (green) and humus-feeding (brown) species. Values are averages, based on fresh weight, and were compiled from various sources (for details, see Brune 2018). Originally published in Brune (2010), adapted with permission of ©Springer Nature

hydrogen is the central free intermediate of lignocellulose degradation. It is a major fermentation product of cellulolytic flagellates and can accumulate to substantial concentrations (Ebert and Brune 1997; Pester and Brune 2007). Turnover rates are in the range of 7–16 μmol H_2 h^{-1} (g fresh weight)$^{-1}$ (Pester and Brune 2007)—on a volume basis, hydrogen fluxes in termite guts are in the same range as in the bovine rumen.

Methane production by lower termites strictly depends on the presence of (hydrogen-producing) gut flagellates (Odelson and Breznak 1983; Rasmussen and Khalil 1983; Messer and Lee 1989). However, the rates of methanogenesis are much lower than one would expect based on the large amount of hydrogen presumably produced by the microbial fermentations. If termites are fed with antibacterial drugs, their hydrogen and methane emission rates increase strongly, which indicates that methanogenic archaea compete with bacteria for hydrogen formed by the flagellates (Odelson and Breznak 1983).

In the phylogenetically higher termites (family Termitidae), which lack gut flagellates, the methanogenic substrates are most likely formed by fermenting bacteria. Here, methanogenesis in intact guts and gut homogenates is strongly stimulated by the supply of external hydrogen but also by formate (Brauman et al. 1992; Schmitt-Wagner and Brune 1999).

The most prominent process responsible for bacterial hydrogen oxidation in termite guts is the reduction of CO_2 to acetate (Breznak and Switzer 1986). It is a unique feature of termite guts that the bacteria responsible for reductive acetogenesis—at least in wood-feeding termite species—are members of the phylum *Spirochaetes* (Leadbetter et al. 1999; Ottesen and Leadbetter 2011). Although hydrogenotrophic methanogenesis occurs in most wood-feeding termites, it becomes

more important than reductive acetogenesis in the fungus-cultivating and humivorous taxa—both in gut homogenates and in situ (Breznak and Switzer 1986; Brauman et al. 1992; Tholen and Brune 1999, 2000; Pester and Brune 2007).

Despite the apparent substrate limitation of methanogenesis in termite guts, termites Hydrogen emission in considerable amounts (Zimmerman et al. 1982; Odelson and Breznak 1983; Ebert and Brune 1997; Sugimoto et al. 1998b; Schmitt-Wagner and Brune 1999; Pester and Brune 2007; Cao et al. 2010; Yanase et al. 2013), which indicates that production and consumption of hydrogen in the hindgut are not tightly coupled (see below).

3 Diversity of Methanogens in Termite Guts

The methanogens in termite guts belong to several genus-level lineages of the orders *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, and *Methanomassiliicoccales* (Fig. 3). However, not all lineages are represented by pure cultures. There are only three described species, all from the genus *Methanobrevibacter* (*Methanobacteriales*) and all from the same host species, namely, the lower termite *Reticulitermes flavipes* (Leadbetter and Breznak 1996; Leadbetter et al. 1998). Like other members of this genus found in the human gut or the rumen, they grow exclusively on H_2 + CO_2 (*Methanobrevibacter cuticularis* also grows, albeit poorly, on formate). Their genomes have been sequenced (Poehlein and Seedorf 2016).

Most of the *Methanobacteriales* clones from higher termites fall into the radiation of the genus *Methanobrevibacter*, but they are phylogenetically distinct from their relatives in lower termites and other insects. Several strains of *Methanobacteriales* in higher termites have been isolated in pure culture and characterized in some detail (Deevong et al. 2004). They comprise a *Methanobrevibacter* strain that is closely related to *Methanobrevibacter arboriphilus* and grows also on formate and several *Methanobacterium* strains that are close relatives of *Methanobacterium bryantii* and utilize also secondary alcohols. However, none of these strains were deposited in a culture collection.

The only other methanogen isolated from insect guts is *Methanomicrococcus blatticola* from the cockroach *Periplaneta americana*. It is the first cultivated representative of a lineage of *Methanosarcinales* and differs from the *Methanobrevibacter* species in its inability to grow on H_2 + CO_2. Instead, it is specialized on the hydrogen-dependent reduction of methanol or methylamines to methane (Sprenger et al. 2000). Its obligate requirement for hydrogen is explained by the inability to oxidize methyl groups to carbon dioxide (Sprenger et al. 2005). At low hydrogen concentrations, the use of methanol as the terminal electron acceptor in methanogenesis is thermodynamically more favorable than the use of carbon dioxide. As a consequence, the substrate affinity of *M. blatticola* for hydrogen is higher than those reported for hydrogenotrophic methanogens and that for methanol

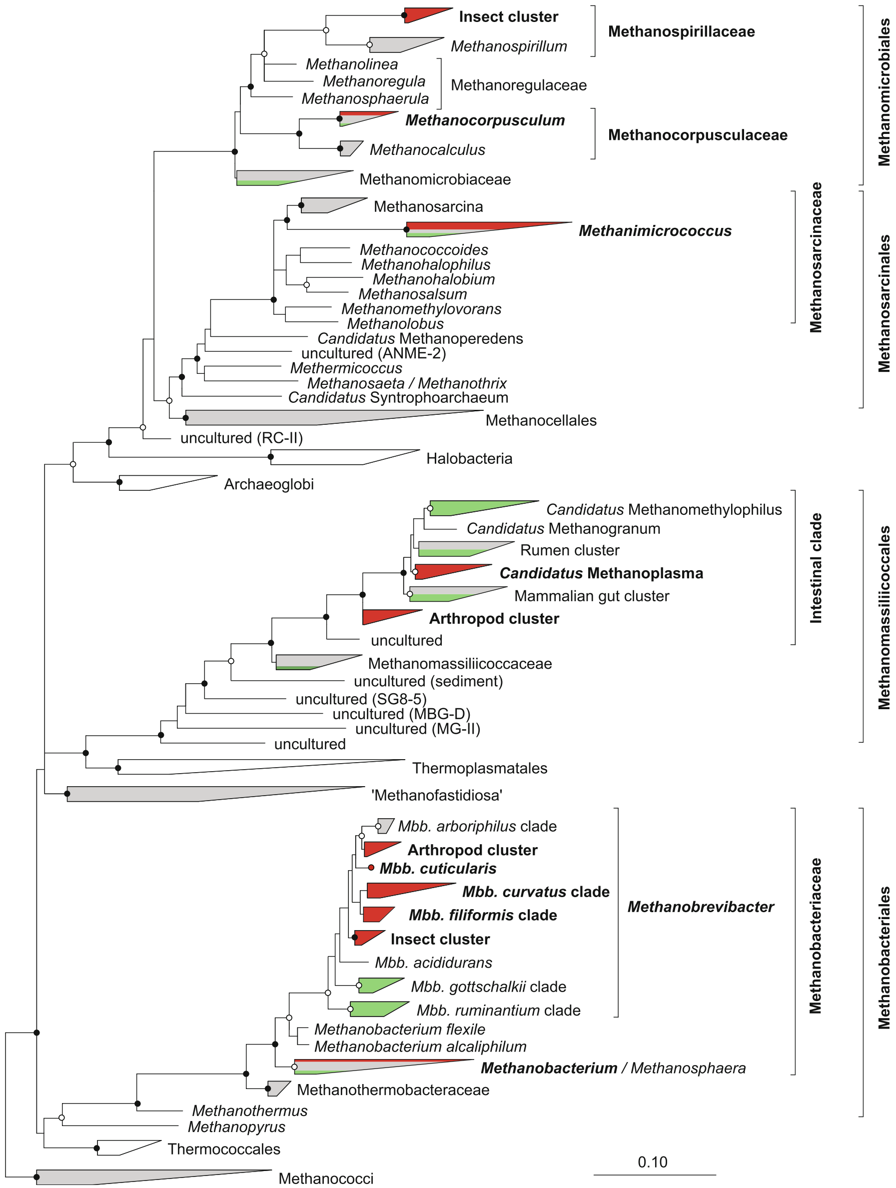


Fig. 3 Phylogenetic position of methanogens that occur in the guts of termites and other insects (red) or mammals (green) among other methanogenic (gray) and non-methanogenic (white) clades of *Euryarchaeota*. Taxa with representatives from insect guts are in boldface. Simplified version of a larger maximum-likelihood tree, based on a manually curated alignment of near full-length 16S rRNA gene sequences; symbols indicate node support (closed circle, >90%; open circle, >70%)

even surpasses those of other methylotrophic taxa (*Methanosphaera stadtmanae*, *Methanosarcina barkeri*) (Sprenger et al. 2007).

The uncultured members of *Methanosarcinales* encountered in higher termites (see below) fall into the radiation of the genus *Methanimicrococcus*, which comprises also clones recovered from other cockroaches and scarab beetle larvae (e.g., Hara et al. 2002; Egert et al. 2003). The same is true for the *Methanomicrobiales* clones obtained from higher termites, which form a sister group of the genus *Methanospirillum*; no representatives of this "insect cluster" have been brought into culture (Fig. 3).

Members of *Methanomassiliicocales* fall into the so-called intestinal clade and form several clusters that consist exclusively of clones from arthropod guts. A highly enriched culture has been obtained from the soil-feeding termite *Cubitermes ugandensis* (Paul et al. 2012). Physiological and ultrastructural characterization, combined with a comparative analysis of its genome, identified "*Candidatus* Methanoplasma termitum" as an obligately methyl-reducing hydrogenotroph without a cell wall (Lang et al. 2015). It shares a new mode of energy metabolism with the distantly related *Methanomassiliicoccales luminyensis* and other uncultured representatives of this order (Borrel et al. 2014; Lang et al. 2015). Since all *Methanomassiliicoccales* seem to lack coenzyme F_{420} (Lang et al. 2015), their cells cannot be visualized by epifluorescence microscopy.

4 Structure of the Methanogenic Communities

The methanogens colonizing the hindgut of lower termites belong almost exclusively to the genus *Methanobrevibacter* (*Methanobacteriales*). Cultivation-independent, 16S-rRNA-based surveys documented the presence of unique *Methanobrevibacter*-related phylotypes in each lower termite investigated (Ohkuma et al. 1995, 1999; Ohkuma and Kudo 1998; Shinzato et al. 1999, 2001). *Reticulitermes flavipes* harbors at least three species with distinct morphotypes, which colonize the hindgut cuticle and have been isolated in pure culture (Fig. 4). Also other lower termites harbor more than one lineage of *Methanobrevibacter*, and the phylotypes attached to the hindgut cuticle or to filamentous bacteria at the gut wall are phylogenetically distinct from those associated with the gut flagellates (Tokura et al. 2000; Hara et al. 2004; Inoue et al. 2008), which suggests an adaptation to the respective microhabitats (see below).

The methanogenic communities in the hindgut of higher termites are much more diverse and comprise members of *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, and *Methanomassiliicoccales*. Clone libraries of archaeal 16S rRNA genes are available for members of the genera *Alyscotermes* (Apicotermitinae), *Cubitermes* and *Ophiotermes* (Cubitermitinae), *Macrotermes* and *Odontotermes* (Macrotermitinae), *Nasutitermes* and *Trinervitermes* (Nasutitermitinae), *Pericapritermes*, and *Microcerotermes* (Termitinae) (Ohkuma et al. 1999; Friedrich et al. 2001; Donovan et al. 2004; Miyata et al. 2007; Paul et al. 2012;

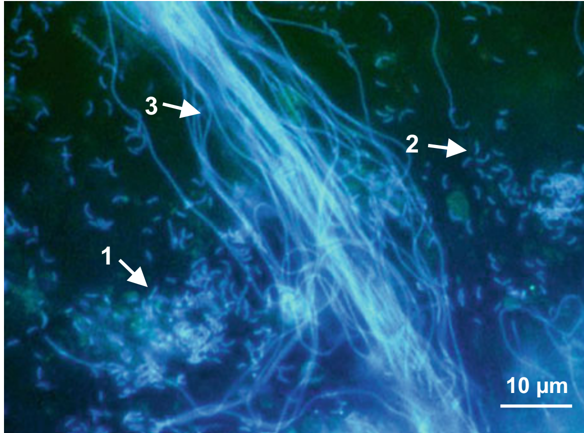


Fig. 4 Methanogens associated with the hindgut wall of *Reticulitermes flavipes*, visualized by the autofluorescence of their cofactor F_{420}. The arrows point to the characteristic morphotypes of *Methanobrevibacter cuticularis* (1), *Methanobrevibacter curvatus* (2), and *Methanobrevibacter filiformis* (3). Microphotograph courtesy of J. R. Leadbetter and J. A. Breznak. Originally published in Brune (2010), reprinted with permission of ©Springer Nature

Shi et al. 2015). Short-read amplicon libraries have been obtained for members of the genera *Nasutitermes* and *Tenuirostritermes* (Nasutitermitinae); *Drepanotermes*, *Gnathamitermes*, and *Macrognathotermes* (Termitinae); and *Syntermes* (Syntermitinae) (Rahman et al. 2015; Santana et al. 2015).

While representatives of all four orders have been recovered from Cubitermitinae, Syntermitinae, and Termitinae, clone libraries of Macrotermitinae yielded no *Methanomicrobiales*, and those of Apicotermitinae and Nasutitermitinae yielded no *Methanosarcinales*. The results obtained with short-read amplicon libraries differed between colonies of the same species and were not always consistent with those previously obtained for other members of the same subfamily, which indicates differences in community structure even between closely related taxa (Rahman et al. 2015). Coevolution between termites and methanogens is only diffuse and might be disturbed by rampant host switching, as observed for members of their bacterial microbiota (Bourguignon et al. 2018). However, the drivers of methanogenic community structure in termite guts remain unclear.

5 Differences in Methanogenic Activities and Populations

Information on the population sizes of methanogens in insect guts is scarce. Cultivation-based studies indicate that *Reticulitermes flavipes* harbors about 10^6 methanogens per gut, which is about 5% of the total cell count of prokaryotes (Leadbetter and Breznak 1996; Tholen et al. 1997). Such numbers are inherently inaccurate because of the uncertainties created by cultivation bias, the absence of

cofactor F_{420} from *Methanomassiliicoccales*, and the difficulties in enumerating prokaryotic cells attached to intestinal surfaces or intimately associated with the flagellate cells that occupy the bulk of the hindgut volume.

Hybridization of RNA extracted from the guts of a wide range of termite species with domain-specific oligonucleotide probes indicated that the average proportion of archaeal rRNA was only 1.5% of all prokaryotic rRNA (Brauman et al. 2001). The higher fraction of archaeal rRNA in soil-feeding species ($2.3 \pm 0.5\%$) than in wood-feeding and fungus-cultivating species ($0.9 \pm 0.5\%$) is in agreement with a general trend toward higher methane emission rates among termites with a humivorous lifestyle (Fig. 2), but it should be kept in mind that not all the archaea in termite guts are necessarily methanogenic (Friedrich et al. 2001).

Amplicon sequencing studies employing both universal and prokaryote primers indicated that archaeal reads obtained for *Reticulitermes* species range between 0.1 and 0.2% of the reads classified as prokaryotes (Boucias et al. 2013; Rahman et al. 2015). However, unrealistically high proportions of archaea obtained for termites from other genera (e.g., above 50% in *Porotermes*; Rahman et al. 2015) put into question the reliability of this approach.

Since soil-feeding termites—in contrast to their wood- and grass-feeding relatives—digest peptide-rich soil organic matter (Ji and Brune 2006; Brune and Ohkuma 2011), it is tempting to suggest that differences in methanogenic activity are diet related. However, information on the fermentative processes in the hindguts of humivorous insects is sparse, and also the substrate spectra of the mostly uncultivated methanogens in higher termites has to be better understood before a reasonable hypothesis can be proposed.

Such knowledge may also help to clarify whether the presence of methanogens provides benefits their respective hosts. So far, such evidence is lacking. In lower termites, methane emission rates can differ strongly among members of the same genus, and sometimes members of the same species are not consistently colonized by methanogens (e.g., Shinzato et al. 1992; Wheeler et al. 1996), indicating that the presence of methanogens provides no advantage. In *Zootermopsis angusticollis*, elimination of methanogens by feeding with bromoethanesulfonic acid (BES) does not affect the survival of the termites (Messer and Lee 1989).

6 Coexistence with Homoacetogens

The predominance of reductive acetogenesis over methanogenesis in most wood-feeding termites has puzzled microbiologists for the longest time. For thermodynamic reasons, methanogens should always outcompete homoacetogens for hydrogen, their common substrate—at least in a well-mixed system. However, the introduction of microsensor techniques into termite gut research led to the recognition that termite guts are spatially structured microenvironments characterized by steep diffusion gradients of metabolites (see Brune 1998; Brune and Friedrich 2000).

This conceptual advance allowed an explanation for the coexistence of methanogens and homoacetogens in this habitat.

Firstly, it turned out that hydrogen concentrations in termite guts are much higher than originally considered—far above the threshold concentrations at which methanogens can outcompete homoacetogens for hydrogen. At the hydrogen partial pressures observed in the hindgut proper of several lower termites (1–100 kPa; Ebert and Brune 1997; Pester and Brune 2007), both processes would operate at substrate saturation, and a direct competition for hydrogen cannot occur. Therefore, the previous hypothesis explaining the predominance of reductive acetogenesis in termite guts with an increased competitiveness of homoacetogens based on their ability to grow mixotrophically on H_2 and other substrates (Breznak 1994) was no longer tenable.

Secondly, high-resolution profiles of hydrogen concentration in the intestinal tracts of lower and higher termites (Ebert and Brune 1997; Schmitt-Wagner and Brune 1999; Pester and Brune 2007; Köhler et al. 2012) and rate measurements of reductive acetogenesis by microinjection of radiotracers (Tholen and Brune 1999, 2000; Pester and Brune 2007) documented that sources and sinks of hydrogen are not evenly distributed within the hindgut. The high hydrogen concentrations at the gut center of *Reticulitermes* spp., the steep hydrogen gradients toward the gut periphery, and the absence of any stimulatory effect of externally supplied hydrogen on the in situ rates of reductive acetogenesis indicate that the hydrogen-consuming, homoacetogenic spirochetes co-locate with the hydrogen-producing flagellates in the hindgut lumen. By contrast, the strong hydrogen sink at the hindgut wall, which is clearly caused by an anaerobic process (Ebert and Brune 1997), and the dense colonization of the cuticle with *Methanobrevibacter* species (Leadbetter and Breznak 1996; Leadbetter et al. 1998) explain the strong stimulation of methanogenesis by externally supplied hydrogen.

The spatial separation of the hydrogenotrophic processes—reductive acetogenesis in the gut lumen and methanogenesis in the periphery—avoids direct competition between homoacetogens and methanogens for their common substrate (Fig. 5). Nevertheless, it remains to be explained why the homoacetogens are able to colonize the hydrogen-rich gut lumen, whereas the methanogens (unless associated with gut flagellates) are not. In this context, it is important to recall that the termite gut is unusual not only with respect to the predominance of reductive acetogenesis over methanogenesis but also in the abundance of spirochetal life forms in wood-feeding termites (Lilburn et al. 1999; Breznak 2000). So far, the termite gut is the only habitat that harbors spirochetes capable of reductive acetogenesis (Leadbetter et al. 1999; Breznak and Leadbetter 2006).

Diversity studies and expression analysis of FTHFS genes, the functional markers of reductive acetogenesis, have revealed that termite gut treponemes predominate over homoacetogenic firmicutes in all wood-feeding termites studied to date (Salmassi and Leadbetter 2003; Ottesen et al. 2006; Pester and Brune 2006; Warnecke et al. 2007). Apparently, these highly motile spirochetes are well adapted to actively maintain their position in the hindgut lumen, whereas methanogens must attach to surfaces to prevent washout—they can colonize the gut lumen only by

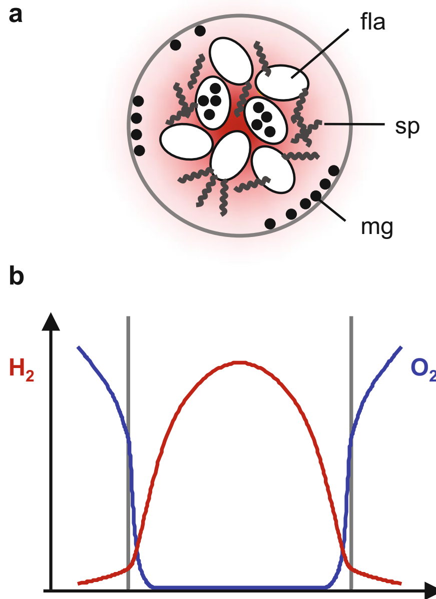


Fig. 5 Schematic cross section (**a**) of the hindgut of a wood-feeding lower termite, illustrating the location of methanogens (mg) at the hindgut wall and homoacetogenic spirochetes (sp) within the lumen of the paunch. In some termite species, methanogens are also associated with the gut flagellates (fla). Radial profiles (**b**) of oxygen and hydrogen partial pressure reveal that the respiratory activity of the gut microbiota maintains steep oxygen gradients within the gut periphery, rendering the center anoxic. Hydrogen formed by the flagellates accumulates at the gut center but is consumed toward the periphery. The strong hydrogen sink below the gut wall is probably caused by methanogens, which prevent larger amounts of H_2 from escaping into the atmosphere. Originally published in Brune (2010), reprinted with permission of ©Springer Nature

associating with the gut flagellates or (in some higher termites) by attaching to cuticular spines that protrude from the gut wall into the lumen (Bignell et al. 1980).

7 Association with Gut Flagellates

Methanogens colonizing intestinal environments are commonly associated with anaerobic protists (see Hackstein and van Alen 2018). The typical habitats of methanogens in termite guts are the hindgut cuticle and the surface of filamentous bacteria colonizing the hindgut wall (Hackstein and Stumm 1994; Leadbetter and Breznak 1996; Leadbetter et al. 1998), but also the gut flagellates of lower termites are frequently colonized by methanogenic symbionts (Odelson and Breznak 1985; Messer and Lee 1989; Shinzato et al. 1992; Hackstein and Stumm 1994; Radek 1994, 1997; Tokura et al. 2000; Hara et al. 2004).

Generally, only smaller species of termite gut flagellates are associated with methanogens. Lee et al. (1987) investigated the colonization of gut flagellates by methanogens in the hindgut of *Zootermopsis angusticollis* by epifluorescence microscopy and reported that only the small trichomonadid flagellates *Trichomitopsis termopsidis*, *Tricercomitus termopsidis*, and *Hexamastix termopsidis* were associated with cells showing the characteristic F_{420} autofluorescence of methanogens. The larger hypermastigotes, which appeared to be the major hydrogen source (Messer and Lee 1989), usually lacked methanogenic symbionts. Similar observations were made by Tokura et al. (2000) with *Reticulitermes speratus*, where the methanogens were regularly associated with the oxymonadid *Dinenympha parva* and a small hypermastigote *Microjoenia* sp., and with *Hodotermopsis sjoestedti*, where the methanogens were associated with *Dinenympha* and *Microjoenia* spp. in large abundance. In all cases, the methanogens seemed to be located within the host cells, which is in agreement also with ultrastructural data reported by Lee et al. (1987).

Odelson and Breznak (1985) were the first to note that a putatively axenic culture of *Trichomitopsis termopsidis*, a gut flagellate isolated from a *Zootermopsis* species, contained a methanogenic symbiont. The symbiosis was not obligate because cultures continued to grow after they were cured of the methanogenic symbiont. Nevertheless, growth yields of *T. termopsidis* increased when the flagellate was cultivated in the presence of the methanogen *Methanospirillum hungatei*, which suggested that the flagellates may benefit in a similar manner from their methanogenic symbiont. There are reports that indicate that methanogens associated with eukaryotic partner organisms in other environments might benefit from interspecies hydrogen transfer, and the stimulation of fermentative processes by end product removal (hydrogen, formate) might even result in a mutual advantage (Schink 1997). However, considering the high hydrogen concentrations throughout the gut lumen of lower termites, it is not clear whether termite gut flagellates indeed benefit from the hydrogen-consuming activity of their methanogenic symbionts under in situ conditions. At the same time, this would mean that the methanogens associated with gut flagellates are never hydrogen limited as long as they can maintain their position in the hydrogen-rich gut lumen—no matter whether their particular host is producing hydrogen or not. From that perspective, the association of *Methanobrevibacter* species with gut flagellates might simply serve to maintain a stable position in the anoxic and hydrogen-rich hindgut lumen, an argument that may apply also to the hydrogenotrophic bacteria associated with such protists (see Hongoh and Ohkuma 2018).

8 Intercompartmental Transfer of Hydrogen

The guts of higher termites are characterized by the absence of cellulolytic flagellates and show (with the exception of the fungus-cultivating species) also a pronounced compartmentation, which goes hand in hand with remarkable dynamics of intestinal pH and redox potential (Brune and Kühl 1996; Kappler and Brune 2002; Köhler et al. 2012; Fig. 6). Since methanogenesis in termite guts is typically hydrogen

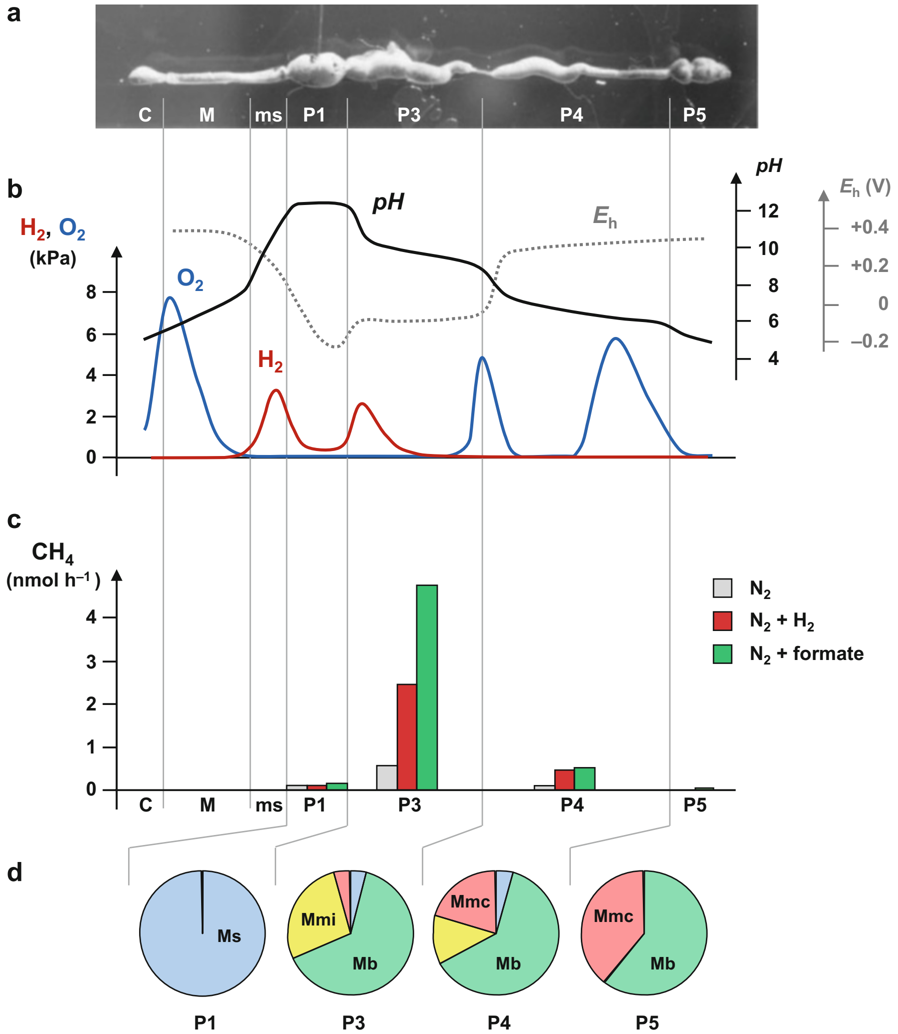


Fig. 6 Gut morphology (**a**) and microsensor profiles (**b**) of oxygen, hydrogen, pH, and redox potential (E_h) along the gut axis of a soil-feeding termite (*Cubitermes* spp.). Methanogenic capacities of individual compartments (**c**) were determined with isolated gut sections incubated under a N_2 headspace with or without addition of H_2 or formate. Relative abundance of methanogens (**d**) in 16S-rRNA-based clone libraries of the respective gut sections (Ms, *Methanosarcinales*; Mb, *Methanobacteriales*; Mmi, *Methanomicrobiales*; Mmc, *Methanomassiliicoccales*). Vertical lines indicate the borders between the different gut regions. Scheme based on various studies (Brune and Kühl 1996; Schmitt-Wagner and Brune 1999; Friedrich et al. 2001; Kappler and Brune 2002). Originally published in Brune (2010), reprinted with permission of ©Springer Nature

limited, Sugimoto et al. (1998b) suggested that differences in the rates of hydrogen and methane emission between termite species might reflect the particular location of the methanogens relative to the hydrogen source. In soil-feeding *Cubitermes* species, hydrogen production and consumption are spatially separated in different gut compartments (Schmitt-Wagner and Brune 1999; Tholen and Brune 1999). The strong stimulation of both methanogenesis and reductive acetogenesis in intact gut compartments by external hydrogen led to the hypothesis that hydrogen diffuses across the gut epithelia between hydrogen-producing and hydrogen-consuming gut regions, which are in close contact in situ. A cross-epithelial transfer of reducing equivalents has been experimentally documented in cockroaches and scarab beetle larvae (Lemke et al. 2001, 2003) and would explain the low hydrogen and high methane emissions of such soil-feeding termites. Since methanogenesis in the posterior hindgut is not only stimulated by hydrogen but also by formate, which accumulates to considerable concentrations in other gut compartments, there might also be intercompartmental transfer of reducing equivalents via the hemolymph (Schmitt-Wagner and Brune 1999).

A detailed analysis of the archaeal community structure in the gut compartmentation of *Cubitermes orthognathus* showed that the different phylogenetic groups are not evenly distributed among the different hindgut compartments (Friedrich et al. 2001). Each of the individual gut compartments harbors a distinct assemblage of Euryarchaeota (Fig. 6d). *Methanosarcinales* colonize the anterior, extremely alkaline compartment, whereas *Methanobacteriales*, *Methanomicrobiales*, and *Methanomassiliicoccales* are found in the posterior, less alkaline to neutral compartments. These gut regions harbor also the highest methanogenic capacities, and many of the microbial cells attached to the gut wall or to cuticular spines projecting from the hindgut wall into the lumen show the characteristic autofluorescence of methanogens (Schmitt-Wagner and Brune 1999).

9 Relationship to Oxygen

As obligate anaerobes, the methanogens in termites are restricted to the hindgut, the only gut region characterized by a negative redox potential (Ebert and Brune 1997; Kappler and Brune 2002; Köhler et al. 2012). It is not clear why they are regularly (in some cases exclusively) located at the hindgut wall, a microhabitat that experiences a constant influx of oxygen across the epithelium (Brune 1998; Brune and Friedrich 2000). Like all other methanogens, the three *Methanobrevibacter* species colonizing the gut epithelium of *Reticulitermes flavipes* (Leadbetter and Breznak 1996; Leadbetter et al. 1998) (and also *Methanomicrococcus blatticola* colonizing the hindgut epithelium of cockroaches; Sprenger et al. 2000) do not grow in media containing even traces of oxygen and are much more sensitive to oxygen accumulation than the homoacetogenic *Sporomusa* species isolated from termite guts (Boga and Brune 2003). However, *Methanobrevibacter* species remain metabolically active in dense cell suspensions that are exposed to controlled oxygen fluxes as

long as the influx of oxygen does not exceed their capacity for oxygen removal (Tholen et al. 2007), whereas reductive acetogenesis of *Sporomusa* species is inhibited even at the lowest oxygen fluxes (Boga and Brune 2003). It has been proposed that the redirection of electron flow from methanogenesis toward oxygen reduction enables *Methanobrevibacter* species to colonize the hindgut periphery of termites. The mechanisms of tolerance to reactive oxygen species and the biochemistry of oxygen reduction in *Methanobrevibacter* species have been discussed elsewhere (Brune 2018).

Nevertheless, the location of methanogens at the gut wall of lower termites, at the unfavorable end of the outwardly directed hydrogen gradient, remains enigmatic. It has been suggested that an attachment to the hindgut cuticle might protect against predation or prevent washout from the gut, which could compensate methanogens for the negative effects of hydrogen limitation and exposure to inflowing oxygen (Breznak 2000). In higher termites, the explanation for the colonization of the hindgut cuticle might lie also in the putative transfer of hydrogen between different compartments. The microorganisms located at the gut wall might be at the bottom end of the radial hydrogen flux from the gut proper but might benefit from external hydrogen entering the hindgut by cross-epithelial transfer from other compartments (see above).

10 Termites as a Source of Atmospheric Methane

Although the counter-gradients of methane and oxygen in the hindgut periphery provide seemingly ideal conditions for aerobic methane oxidation (Brune et al. 2000), there is no evidence for the presence of methanotrophic bacteria or their activities in termite guts (Pester et al. 2007). This means that the different methane emission rates of termites from different feeding guilds directly reflect differences in methane production within their intestinal tract. In the past, many attempts were made to extrapolate from the results of laboratory measurements of methane emissions to the contribution of termites to the global methane budget, but even the most recent estimates are still far from accurate and suffer from numerous biases (see Sanderson 1996; Bignell et al. 1997). Sugimoto and colleagues demonstrated that it is very important to consider methane oxidation in the mound material and the surrounding soil as an important factor mitigating methane production by termites at the environmental level (Sugimoto et al. 1998a, 2000). As a consequence, the net emissions of methane from intact colonies of soil-feeding termites are much lower than those of wood-feeding termites, even though the opposite would be predicted from the gross methane emission rates determined with individual termites in the laboratory.

In view of the grossly overestimated contribution of termites to global methane emissions into the atmosphere propagated in the older literature (reviewed by Collins and Wood 1984), it is important to note that the most recent estimates place these rates at probably less than 10 Tg/year (1.5–7.4 Tg; Sugimoto et al. 1998b) and

almost certainly below 20 Tg/year (a number that is still used in the last global budget published by the IPCC; Denman et al. 2007). Although termites remain a significant natural source of methane on the planet, their contribution to the total source strength (ca. 600 Tg/year) is certainly dwarfed by the sources under anthropogenic influence (such as the ruminants) (Kirschke et al. 2013). More detailed reviews of this subject can be found elsewhere (Bignell 2010; Brune 2018).

11 Conclusions

Termites are a significant source of methane in tropical ecosystems. Methane and short-chain fatty acids are formed from lignocellulosic matter by an anaerobic feeding chain of microorganisms located in the highly enlarged hindguts. However, termite hindguts are not purely anoxic fermentors. The gut habitat is characterized by the continuous influx of O_2 across the gut wall and steep hydrogen gradients between gut lumen and periphery. Despite the high hydrogen concentrations in the gut lumen, methanogens are not the predominant hydrogenotrophic microorganisms in lower termites. The ability to attach to biotic or abiotic surfaces or to colonize the cytoplasm of flagellate protists might be an important factor in the successful colonization of the intestinal tract. In higher termites, which lack gut flagellates, the increased methane production is correlated with a dietary shift from wood to humus. The assemblage of methanogenic archaea in higher termites is more diverse and changes between the major hindgut compartments, each of which differs with respect to the prevailing physicochemical conditions. The drivers determining archaeal community structure in the different microhabitats are not clear, but might involve the availability of and competition for methanogenic substrates and differences in adaptation to pH, oxygen, and other stresses imposed by the respective microenvironments. Since most of the methanogens in termite guts belong to lineages without any cultured representatives, more isolates are sorely needed to address these questions.

References

Bignell DE (2010) Termites. In: Reay D, Smith P, van Amstel A (eds) Methane and climate change. Earthscan, London, pp 62–73

Bignell DE, Oskarsson H, Anderson JM (1980) Specialization of the hindgut wall for the attachment of symbiotic microorganisms in a termite *Procubitermes aburiensis*. Zoomorphology 96: 103–112

Bignell DE, Eggleton P, Nunes L, Thomas KL (1997) Termites as mediators of carbon fluxes in tropical forests: budgets for carbon dioxide and methane emissions. In: Watt AB, Stork NE, Hunter MD (eds) Forests and insects. Chapman and Hall, London, pp 109–134

Boga HI, Brune A (2003) Hydrogen-dependent oxygen reduction by homoacetogenic bacteria isolated from termite guts. Appl Environ Microbiol 69:779–786

Borrel G, Parisot N, Harris HM, Peyretaillade E, Gaci N, Tottey W, Bardot O, Raymann K, Gribaldo S, Peyret P, O'Toole PW, Brugère JF (2014) Comparative genomics highlights the unique biology of *Methanomassiliicoccales*, a *Thermoplasmatales*-related seventh order of methanogenic archaea that encodes pyrrolysine. BMC Genomics 15:679

Boucias DG, Cai Y, Sun Y, Lietze V-U, Sen R, Raychoudhury R, Scharf ME (2013) The hindgut lumen prokaryotic microbiota of the termite *Reticulitermes flavipes* and its responses to dietary lignocellulose composition. Mol Ecol 22:1836–1853

Bourguignon T, Lo N, Dietrich C, Šobotník J, Sidek S, Roisin Y, Brune A, Evans TA (2018) Rampant host-switching shaped the termite gut microbiome. Curr Biol 28:649–654.e2

Brauman A, Kane M, Breznak JA (1992) Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science 257:1384–1387

Brauman A, Dore J, Eggleton P, Bignell D, Breznak JA, Kane MD (2001) Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. FEMS Microbiol Ecol 35:27–36

Breznak JA (1975) Symbiotic relationships between termites and their intestinal microbiota. Symp Soc Exp Biol 29:559–580

Breznak JA (1994) Acetogenesis from carbon dioxide in termite guts. In: Drake HL (ed) Acetogenesis. Chapman and Hall, New York, pp 303–330

Breznak JA (2000) Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer Academic, Dordrecht, pp 209–231

Breznak JA, Leadbetter JR (2006) Termite gut spirochetes. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The prokaryotes, Proteobacteria: delta and epsilon subclasses. Deeply rooting bacteria, vol 7, 3rd edn. Springer, New York, pp 318–329

Breznak JA, Switzer JM (1986) Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl Environ Microbiol 52:623–630

Breznak JA, Brill WJ, Mertins JW, Coppel HC (1973) Nitrogen fixation in termites. Nature 244: 577–580

Breznak JA, Mertins JW, Coppel HC (1974) Nitrogen fixation and methane production in a wood-eating cockroach, *Cryptocercus punctulatus* Scudder (Orthoptera: Blattidae). Univ Wisc Forest Res Notes 184:1–2

Brune A (1998) Termite guts: the world's smallest bioreactors. Trends Biotechnol 16:16–21

Brune A (2010) Methanogens in the digestive tract of termites. In: Hackstein JHP (ed) (Endo) symbiotic methanogenic archaea, 1st edn. Springer, Heidelberg, pp 81–100

Brune A (2014) Symbiotic digestion of lignocellulose in termite guts. Nat Rev Microbiol 12: 168–180

Brune A (2018) Methanogenesis in the digestive tracts of insects. In: Stams AJM, Sousa D (eds) Biogenesis of hydrocarbons. Handbook of hydrocarbon and lipid microbiology, 2nd edn. Springer, Cham

Brune A, Friedrich M (2000) Microecology of the termite gut: structure and function on a microscale. Curr Opin Microbiol 3:263–269

Brune A, Kühl M (1996) pH profiles of the extremely alkaline hindguts of soil-feeding termites (Isoptera: Termitidae) determined with microelectrodes. J Insect Physiol 42:1121–1127

Brune A, Ohkuma M (2011) Role of the termite gut microbiota in symbiotic digestion. In: Bignell DE, Roisin Y, Lo N (eds) Biology of termites: a modern synthesis. Springer, Dordrecht, pp 439–475

Brune A, Frenzel P, Cypionka H (2000) Life at the oxic−anoxic interface: microbial activities and adaptations. FEMS Microbiol Rev 24:691–710

Cao Y, Sun J-Z, Rodriguez JM, Lee KC (2010) Hydrogen emission by three wood-feeding subterranean termite species (Isoptera: Rhinotermitidae): production and characteristics. Insect Sci 17:237–244

Collins NM, Wood TG (1984) Termites and atmospheric gas production. Science 224:84–86

Cook SF (1932) The respiratory gas exchange in *Termopsis nevadensis*. Biol Bull 63:246–257

Deevong P, Hattori S, Yamada A, Trakulnaleamsai S, Ohkuma M, Noparatnaraporn N, Kudo T (2004) Isolation and detection of methanogens from the gut of higher termites. Microb Environ 19:221–226

Denman KL, Brasseur G, Chidthaisong A, Ciais P, Cox PM et al (2007) Couplings between changes in the climate system and biogeochemistry. In: Solomon S et al (eds) Climate change 2007: the physical science basis. Fourth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC). Cambridge University Press, Cambridge, pp 499–587

Donovan SE, Purdy KJ, Kane MD, Eggleton P (2004) Comparison of *Euryarchaea* strains in the guts and food-soil of the soil-feeding termite *Cubitermes fungifaber* across different soil types. Appl Environ Microbiol 70:3884–3892

Ebert A, Brune A (1997) Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower termite *Reticulitermes flavipes* (Kollar). Appl Environ Microbiol 63:4039–4046

Egert M, Wagner B, Lemke T, Brune A, Friedrich MW (2003) Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl Environ Microbiol 69:6659–6668

Friedrich MW, Schmitt-Wagner D, Lueders T, Brune A (2001) Axial differences in community structure of *Crenarchaeota* and *Euryarchaeota* in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. Appl Environ Microbiol 67:4880–4890

Hackstein JHP, Stumm CK (1994) Methane production in terrestrial arthropods. Proc Natl Acad Sci USA 91:5441–5445

Hackstein JHP, van Alen TA (2018) Methanogens in the gastro-intestinal tract of animals. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Hara K, Shinzato N, Seo M, Oshima T, Yamagishi A (2002) Phylogenetic analysis of symbiotic archaea living in the gut of xylophagous cockroaches. Microb Environ 17:185–190

Hara K, Shinzato N, Oshima T, Yamagishi A (2004) Endosymbiotic *Methanobrevibacter* species living in symbiotic protists of the termite *Reticulitermes speratus* detected by fluorescent in situ hybridization. Microb Environ 19:120–127

Hongoh Y, Ohkuma M (2018) Termite gut flagellates and their methanogenic and eubacterial symbionts. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Inoue J, Noda S, Hongoh Y, Ui S, Ohkuma M (2008) Identification of endosymbiotic methanogen and ectosymbiotic spirochetes of gut protists of the termite *Coptotermes formosanus*. Microb Environ 23:94–97

Ji R, Brune A (2006) Nitrogen mineralization, ammonia accumulation, and emission of gaseous NH_3 by soil-feeding termites. Biogeochemistry 78:267–283

Kappler A, Brune A (2002) Dynamics of redox potential and changes in redox state of iron and humic acids during gut passage in soil-feeding termites (*Cubitermes* spp.). Soil Biol Biochem 34:221–227

Kirschke S, Bousquet P, Ciais P, Saunois M, Canadell JG et al (2013) Three decades of global methane sources and sinks. Nat Geosci 6:813–823

Köhler T, Dietrich C, Scheffrahn RH, Brune A (2012) High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites (*Nasutitermes* spp.). Appl Environ Microbiol 78:4691–4701

Lang K, Schuldes J, Klingl A, Poehlein A, Daniel R, Brune A (2015) New mode of energy metabolism in the seventh order of methanogens as indicated by comparative genome analysis of “*Candidatus* Methanoplasma termitum”. Appl Environ Microbiol 81:1338–1352

Leadbetter JR, Breznak JA (1996) Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite *Reticulitermes flavipes*. Appl Environ Microbiol 62:3620–3631

Leadbetter JR, Crosby LD, Breznak JA (1998) *Methanobrevibacter filiformis* sp. nov., a filamentous methanogen from termite hindguts. Arch Microbiol 169:287–292

Leadbetter JR, Schmidt TM, Graber JR, Breznak JA (1999) Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283:686–689

Lee MJ, Schreurs PJ, Messer AC, Zinder SH (1987) Association of methanogenic bacteria with flagellated protozoa from a termite hindgut. Curr Microbiol 15:337–341

Lemke T, van Alen T, Hackstein JHP, Brune A (2001) Cross-epithelial hydrogen transfer from the midgut compartment drives methanogenesis in the hindgut of cockroaches. Appl Environ Microbiol 67:4657–4661

Lemke T, Stingl U, Egert M, Friedrich MW, Brune A (2003) Physicochemical conditions and microbial activities in the highly alkaline gut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl Environ Microbiol 69:6650–6658

Lilburn TG, Schmidt TM, Breznak JA (1999) Phylogenetic diversity of termite gut spirochaetes. Environ Microbiol 1:331–345

Liu Y, Whitman WB (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann N Y Acad Sci 1125:171–189

Messer AC, Lee MJ (1989) Effect of chemical treatments on methane emission by the hindgut microbiota in the termite *Zootermopsis angusticollis*. Microb Ecol 18:275–284

Miyata R, Noda N, Tamaki H, Kinjyo K, Aoyagi H, Uchiyama H, Tanaka H (2007) Phylogenetic relationship of symbiotic archaea in the gut of the higher termite *Nasutitermes takasagoensis* fed with various carbon sources. Microb Environ 22:157–164

Odelson DA, Breznak JA (1983) Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl Environ Microbiol 45:1602–1613

Odelson DA, Breznak JA (1985) Nutrition and growth characteristics of *Trichomitopsis termopsidis*, a cellulolytic protozoan from termites. Appl Environ Microbiol 49:614–621

Ohkuma M, Brune A (2011) Diversity, structure, and evolution of the termite gut microbial community. In: Bignell DE, Roisin Y, Lo N (eds) Biology of termites: a modern synthesis. Springer, Dordrecht, pp 413–438

Ohkuma M, Kudo T (1998) Phylogenetic analysis of the symbiotic intestinal microflora of the termite *Cryptotermes domesticus*. FEMS Microbiol Lett 164:389–395

Ohkuma M, Noda S, Horikoshi K, Kudo T (1995) Phylogeny of symbiotic methanogens in the gut of the termite *Reticulitermes speratus*. FEMS Microbiol Lett 134:45–50

Ohkuma M, Noda S, Kudo T (1999) Phylogenetic relationships of symbiotic methanogens in diverse termites. FEMS Microbiol Lett 171:147–153

Ottesen EA, Leadbetter JR (2011) Formyltetrahydrofolate synthetase gene diversity in the guts of higher termites with different diets and lifestyles. Appl Environ Microbiol 77:3461–3467

Ottesen EA, Hong JW, Quake SR, Leadbetter JR (2006) Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. Science 314:1464–1467

Paul K, Nonoh JO, Mikulski L, Brune A (2012) "*Methanoplasmatales*," *Thermoplasmatales*-related archaea in termite guts and other environments, are the seventh order of methanogens. Appl Environ Microbiol 78:8245–8253

Pester M, Brune A (2006) Expression profiles of *fhs* (FTHFS) genes support the hypothesis that spirochaetes dominate reductive acetogenesis in the hindgut of lower termites. Environ Microbiol 8:1261–1270

Pester M, Brune A (2007) Hydrogen is the central free intermediate during lignocellulose degradation by termite gut symbionts. ISME J 1:551–565

Pester M, Tholen A, Friedrich MW, Brune A (2007) Methane oxidation in termite hindguts: absence of evidence and evidence of absence. Appl Environ Microbiol 73:2024–2028

Poehlein A, Seedorf H (2016) Draft genome sequences of *Methanobrevibacter curvatus* DSM11111, *Methanobrevibacter cuticularis* DSM11139, *Methanobrevibacter filiformis* DSM11501, and *Methanobrevibacter oralis* DSM7256. Genome Announc 4:e00617–e00616

Radek R (1994) *Monocercomonoides termitis* n. sp., an oxymonad from the lower termite *Kalotermes sinaicus*. Arch Protistenkunde 144:373–382

Radek R (1997) *Spirotrichonympha minor* n. sp., a new hypermastigote termite flagellate. Eur J Protistol 33:361–374

Rahman NA, Parks DH, Willner DL, Engelbrektson AL, Goffredi SK, Warnecke F, Scheffrahn RH, Hugenholtz P (2015) A molecular survey of Australian and North American termite genera indicates that vertical inheritance is the primary force shaping termite gut microbiomes. Microbiome 3:5

Rasmussen RA, Khalil MAK (1983) Global production of methane by termites. Nature 301: 700–702

Salmassi TM, Leadbetter JR (2003) Molecular aspects of CO_2-reductive acetogenesis in cultivated spirochetes and the gut community of the termite *Zootermopsis angusticollis*. Microbiology 149:2529–2537

Sanderson MG (1996) Biomass of termites and their emissions of methane and carbon dioxide: a global database. Global Biogeochem Cycles 10:543–557

Santana RH, Catão ECP, Lopes FAC, Constantino R, Barreto CC, Krüger RH (2015) The gut microbiota of workers of the litter-feeding termite *Syntermes wheeleri* (Termitidae: Syntermitinae): archaeal, bacterial, and fungal communities. Microb Ecol 70:545–556

Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61:262–280

Schmitt-Wagner D, Brune A (1999) Hydrogen profiles and localization of methanogenic activities in the highly compartmentalized hindgut of soil-feeding higher termites (*Cubitermes* spp.). Appl Environ Microbiol 65:4490–4496

Shi Y, Huang Z, Han S, Fan S, Yang H (2015) Phylogenetic diversity of Archaea in the intestinal tract of termites from different lineages. J Basic Microbiol 54:1–8

Shinzato N, Yoshino H, Yara K (1992) Methane production by microbial symbionts in the lower and higher termites of the Ryukyu Archipelago. In: Sato S, Ishida M, Ishikawa H (eds) Endocytobiology V. Tübingen University Press, Tübingen, pp 161–166

Shinzato N, Matsumoto T, Yamaoka I, Oshima T, Yamagishi A (1999) Phylogenetic diversity of symbiotic methanogens living in the hindgut of the lower termite *Reticulitermes speratus* analyzed by PCR and in situ hybridization. Appl Environ Microbiol 65:837–840

Shinzato N, Matsumoto T, Yamaoka I, Oshima T, Yamagishi A (2001) Methanogenic symbionts and the locality of their host lower termites. Microb Environ 16:43–47

Sprenger WW, van Belzen MC, Rosenberg J, Hackstein JHP, Keltjens JT (2000) *Methanomicrococcus blatticola* gen. nov., sp., nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. Int J Syst Evol Microbiol 50:1989–1999

Sprenger WW, Hackstein JHP, Keltjens JT (2005) The energy metabolism of *Methanomicrococcus blatticola*: physiological and biochemical aspects. Antonie van Leeuwenhoek 87:289–299

Sprenger WW, Hackstein JH, Keltjens JT (2007) The competitive success of *Methanomicrococcus blatticola*, a dominant methylotrophic methanogen in the cockroach hindgut, is supported by high substrate affinities and favorable thermodynamics. FEMS Microbiol Ecol 60:266–275

Sugimoto A, Inoue T, Kirtibutr N, Abe T (1998a) Methane oxidation by termite mounds estimated by the carbon isotopic composition of methane. Global Biogeochem Cycles 12:595–605

Sugimoto A, Inoue T, Tayasu I, Miller L, Takeichi S, Abe T (1998b) Methane and hydrogen production in a termite-symbiont system. Ecol Res 13:241–257

Sugimoto A, Bignell DE, MacDonald JA (2000) Global impact of termites on the carbon cycle and atmospheric trace gases. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbioses, ecology. Kluwer Academic, Dordrecht, pp 409–435

Thauer RK, Kaster A-K, Seedorf H, Buckel W, Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. Nat Rev Microbiol 6:579–591

Tholen A, Brune A (1999) Localization and in situ activities of homoacetogenic bacteria in the highly compartmentalized hindgut of soil-feeding higher termites (*Cubitermes* spp.). Appl Environ Microbiol 65:4497–4505

Tholen A, Brune A (2000) Impact of oxygen on metabolic fluxes and *in situ* rates of reductive acetogenesis in the hindgut of the wood-feeding termite *Reticulitermes flavipes*. Environ Microbiol 2:436–449

Tholen A, Schink B, Brune A (1997) The gut microflora of *Reticulitermes flavipes*, its relation to oxygen, and evidence for oxygen-dependent acetogenesis by the most abundant *Enterococcus* sp. FEMS Microbiol Ecol 24:137–149

Tholen A, Pester M, Brune A (2007) Simultaneous methanogenesis and oxygen reduction by *Methanobrevibacter cuticularis* at low oxygen fluxes. FEMS Microbiol Ecol 62:303–312

Tokura M, Ohkuma M, Kudo T (2000) Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. FEMS Microbiol Ecol 33:233–240

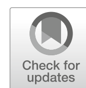
Warnecke F, Luginbühl P, Ivanova N, Ghassemian M, Richardson TH et al (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450: 560–565

Wheeler GS, Tokoro M, Scheffrahn RH, Su N-Y (1996) Comparative respiration and methane production rates in nearctic termites. J Insect Physiol 42:799–806

Yanase Y, Miura M, Fujii Y, Okumura S, Yoshimura T (2013) Evaluation of the concentrations of hydrogen and methane emitted by termite using a semiconductor gas sensor. J Wood Sci 59: 243–248

Zimmerman PR, Greenberg JP, Wandiga SO, Crutzen PJ (1982) Termites: a potentially large source of atmospheric methane, carbon dioxide, and molecular hydrogen. Science 218:563–565

Methanogenic Archaea in Humans and Other Vertebrates: An Update



Everly Conway de Macario and Alberto J. L. Macario

Contents

1 Scope and Objective 104
2 Introduction 104
3 Dynamics of Methanogens in Humans 107
4 Newly Described Methanogens in Humans 108
5 Methanogens in Human Disease 109
6 Trimethylaminuria 111
7 Methanogens in Animals 111
8 Methods 113
9 Basic Concepts and Facts 113
10 Conclusions and Perspectives 114
References 115

Abstract This article updates a previous one with the same title from 2010. It is not a comprehensive review but a summary of various reports published from 2011 onwards, illustrating advances in the field of methanogens in the microbiota of animals and humans. New data have provided a solid basis to the belief that the gut microbiota of animals and humans plays a key role in health and in various diseases. Efforts were made to elucidate the composition of the microbiota and its changes with age, diet, health status, disease stage, and other host and environmental factors. Also, experiments were conducted to dissect the components of the microbiota and to understand their interactions among themselves and with the immune and chaperoning systems of the host. Methanogenic archaea have been confirmed as an ever present component of the microbiota in animals and humans. In the latter and some animal species, *Methanobrevibacter smithii* is prevalent, but

E. Conway de Macario (✉) · A. J. L. Macario
Department of Microbiology and Immunology, School of Medicine, University of Maryland at Baltimore, Baltimore, MD, USA

Institute of Marine and Environmental Technology (IMET), Columbus Center, Baltimore, MD, USA

Euro-Mediterranean Institute of Science and Technology (IEMEST), Palermo, Italy
e-mail: econwaydemacario@som.umaryland.edu; ajlmacario@som.umaryland.edu

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_7

several other species have been added to the list of archaea inhabiting the various ecosystems in the body of animals and humans. Furthermore, while many studies focused on the microbiota of the gastrointestinal tract, others were directed to the identification of methanogenic archaea in other mucosal ecosystems such as the nose and lungs and in the skin. Thus the repertoire of ecosystems in the body that harbor archaea was extended and so was the range of archaeal species that inhabit those ecosystems. The currently known diversity of ecosystems and sub-ecosystems within each of the main ones, e.g., gastrointestinal tract, and of archaeal species that inhabit them seems to represent a small part of the whole, the tip of the iceberg so to speak. It may, therefore, be inferred that the study of archaea pertaining to the physiology and pathology of humans and animals is one that promises a wealth of key results and important contributions to medicine and environmental health.

1 Scope and Objective

Since the publication of Methanogenic Archaea in Humans and Other Vertebrates in 2010 (Conway de Macario and Macario 2010), many pertinent reports have appeared dealing with the topic of archaea in animals and humans and on the potential pathogenicity of these microbes (see, for instance, Gaci et al. 2014; Bang and Schmitz 2015; Hugon et al. 2016; Barko et al. 2017; Moissl-Eichinger et al. 2017; Seo et al. 2017; Chaudhary et al. 2018; Ghavani et al. 2018). Here, we present an update of the reference list of our previous article. This is not a comprehensive critical review but a selection of articles we thought illustrate new developments. We have searched the literature and databases and found reports focusing on a variety of issues, which we have grouped as follows: **dynamics of methanogens in humans, newly described methanogens in humans, methanogens in human disease, trimethylaminuria, methanogens in animals, methods, and basic concepts and facts.** Our purpose is to provide references, preferentially the latest publications, with a very brief explanation of each to easy the search for new knowledge.

2 Introduction

There is increasing interest in the microbiota of animals and humans because of its implications in health and disease (Conway de Macario and Macario 2009; Bellavia et al. 2013; Bang et al. 2014a; Brugère et al. 2014; Gaci et al. 2014; Huynh et al. 2015; Forbes et al. 2016; Barko et al. 2017). Methanogens are one of the components of the human microbiota, and, therefore, interest in these microbes is also steadily growing, as demonstrated by the rising number of pertinent scientific reports that can be found in a variety of journals. Furthermore, it is becoming increasingly clear that

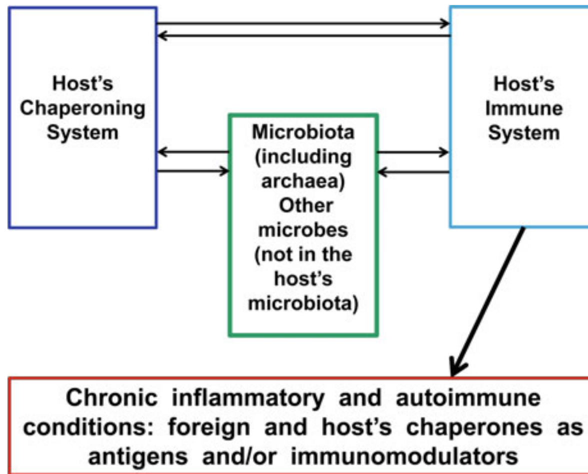


Fig. 1 The microbiota (including archaeal species) and other microbes that come in contact with humans and animals can interact with the host's chaperoning and immune systems. This interaction occurs normally, physiologically, but it can be altered and become pathogenic. For example, normal molecular chaperones, e.g., Hsp60, participate in many interactions between the two systems to maintain normal cell physiology, but, if they are perturbed, chaperones become mediators of pathologic events, namely, they become etiopathogenic factors. This switch to pathology may be initiated by genetic or acquired factors, which cause qualitative and/or quantitative modifications of the molecular chaperone, or immune cross-reactivity between the human and microbial chaperone orthologs, or a break in the balance between the pro- and anti-inflammatory actions of the chaperone. Thus, autoimmune and chronic inflammatory pathologies may occur, for example, when a foreign chaperone molecule, or products derived from it, originating in bacteria or archaea of the microbiota or sporadic infections, invades the body and enters the general circulation. See Cappello et al. (2009, 2010), Macario and Conway de Macario (2016), Marino Gammazza et al. (2014, 2017)

the microbiota and the immune and chaperoning systems are closely interrelated in physiology and in disease, Fig. 1 (Bellavia et al. 2013; Macario and Conway de Macario 2016; Forbes et al. 2016; Marino Gammazza et al. 2017). It follows that it is likely that methanogens, or products derived from them, also participate in the interaction between the microbiota and the immune and chaperoning systems. For example, it is known that some bacterial molecular chaperones invade the circulation of the human host and, thereby, elicit an immune response against the bacterial molecules. Since these bacterial chaperones are similar, some strongly, in sequence and structure to their human orthologs, the immune system reaction spills over so to speak on to the human molecules, generating autoimmune phenomena, Fig. 1 (Cappello et al. 2009, 2010; Marino Gammazza et al. 2014, 2017). The methanogenic archaea also have molecular chaperones that are similar to their bacterial and human counterparts (Macario et al. 2004; Large and Lund 2009; Large et al. 2009). Consequently, we can expect that archaeal chaperone molecules (or products thereof) like those from bacteria penetrate into the body (e.g., through the intestinal wall) and circulate which will elicit an immune response against them

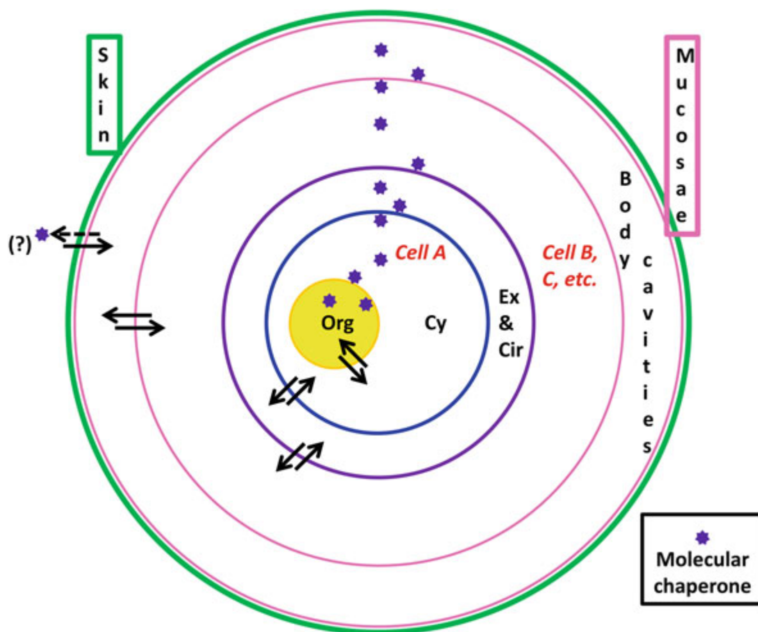


Fig. 2 The chaperoning system is comprised of the whole set of molecular chaperones, co-chaperones, chaperone cofactors, chaperone receptors, and other closely interacting molecules. A chaperone (indicated with a seven-pointed star icon) can reside in, and migrate to and from (as indicated by the pairs of arrows), an organelle (Org; e.g., nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, and microvesicles), the cytosol (Cy), extracellular space (Ex; e.g., the intercellular space in tissues or spaces such as the synovial and pleural spaces), body fluids and circulation (Cir; e.g., lymph, blood, and cerebrospinal fluid), cells (*B*, *C*, etc.) other than that (*Cell A*) in which it originated, and body cavities (e.g., the gastrointestinal genitourinary tracts and the airways). In these locations, chaperones may be free in solution or attached to particles of various types (e.g., ribosome, platelet, red and white cells, and exosome) or to membranes (e.g., cell wall on the inside or on the outside) or other surfaces (e.g., endothelium and various epithelia). Chaperones are also found in secretions, for instance, urine, sweat, and saliva. Therefore, chaperones whether endogenous (i.e., from the host) or foreign (i.e., from bacteria or archaea from the host's microbiota or sporadic infections) have many opportunities for interacting with molecules, membranes, and cells of various kinds, particularly those of the immune system. It is not yet established with certainty if chaperones can traverse the skin in any direction, as indicated by a question mark on the top left of the picture. See Macario and Conway de Macario (2009), Macario et al. (2010)

but cross-reactive with the human equivalents. This possibility opens a wide field of research in human microbiology and pathology, particularly with regard to human acquired chaperonopathies. These are diseases in which one (or more) component of the chaperoning system, Fig. 2, e.g., a chaperone, is abnormal and plays an etiopathogenic role: it participates in the mechanism generating disease (Macario and Conway de Macario 2005, 2016). In other words, chaperones can be a cause of disease, either primary or auxiliary. Chaperonopathies can be classified into genetic and acquired. The former are caused by a mutation in a chaperone gene-protein, while the acquired chaperonopathies are due to nongenetic alterations of the

chaperone molecule. These alterations can be quantitative (increase or decrease concentration; misplacement) or qualitative (decrease or lack of function, malfunction, gain of function). For instance, an acquired chaperonopathy may be caused by a microbial (e.g., a methanogen in the human gut) chaperone that cross-reacts with the human counterpart and, because of that, initiates an autoimmune condition involving the host's chaperone, which thus becomes pathogenic, Fig. 1. This situation ought to be investigated in what pertains to methanogens, considering the high frequency of these archaea in the human microbiota and their variations during development and aging, and in disease in relation to disease status.

3 Dynamics of Methanogens in Humans

The colonization of microbes in the human gastrointestinal tract was studied examining the succession of bacteria, archaea, and micro-eukaryotes during the first year of life (at days 1, 3, 5, 28, 150, and 365 postpartum), using quantitative real-time PCR and 16S and 18S rRNA gene amplicon sequencing (Wampach et al. 2017). *Methanosphaera* and *Methanobrevibacter* were found along with bacteria and micro-eukaryotes.

The factors that determine the acquisition of methanogenic archaea in humans are poorly defined. A study was carried out to identify the factors that determine the colonization in children (6–10 years of age) of the two main gastrointestinal archaeal species, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* (van de Pol et al. 2017). Environmental factors such as diet, lifestyle, hygiene, child rearing, and medication were recorded by repeated questionnaires. The relationship between these determinants and the presence and abundance of archaea was analyzed by logistic and linear regression, respectively. The consumption of organic yogurt and organic milk were positively associated with the presence of *M. smithii*. Milk products were identified as possible source for *M. smithii* but not for *M. stadtmanae*.

To elucidate when in life the most frequent human methanogen, *Methanobrevibacter smithii*, establishes itself in the human gastrointestinal tract, a multipronged strategy was developed, including microscopic observation by fluorescence in situ hybridization, polymerase-chain-reaction (PCR) sequencing detection, and microbial identification and culture, using gastric juice from 1-day-old newborns (Grine et al. 2017). The methanogen was found in all samples, and it was suggested that its source was in the mother's gut microbiota.

It is generally believed that about 33% of humans produce methane, which is measured in exhaled air. It is also thought that this exhaled methane is produced by methanogenic archaea in the human gut. A recent study tends to challenge these somewhat classical notions because it showed that all human examined (over 100 of various ages) did produce methane (Keppler et al. 2016). These results were obtained using laser absorption spectroscopy to measure with precision concentrations and stable carbon signatures of exhaled methane. It is suggested that there might be sources of methane other than gut methanogens in humans, for instance,

human cells, and that highly sensitive and accurate spectroscopy techniques ought to be utilized for measuring methane in human specimens.

The human gastrointestinal tract is a composite ecosystem inhabited by a complex microbiota, which includes archaea. The prevalence of the most commonly found archaeal species in the human gut, namely, *Methanobrevibacter smithii* of the Methanobacteriales and the relatively recently described species of the Methanomassiliicoccales, were assessed in healthy and obese children and in adults (Vanderhaeghen et al. 2015). Methanobacteriales were found in 89% and 65% of adults and children, respectively, while Methanomassiliicoccales were present in 50% of the adults and one child. Methanobacteriales coincided with the presence of bacteria involved in the trophic chain from carbohydrate degradation to hydrogen and formate formation.

Gut microbiota composition varies according to various factors among which diet seems to be one of the most important, and it is usually related to the geographical area in which the population under study resides. A study on this issue was carried out in Thailand on children from two parts of the country with distinctive diets each (La-Ongkham et al. 2015). The results showed differences in the bacterial components of the microbiota between the two groups, but no differences were found pertaining to the archaeal components.

Among the components of the human gut microbiota, the H_2-utilizing microbes comprise the methanogens (*Methanobrevibacter smithii* being the most prevalent), acetogens, and sulfate-reducing bacteria. In a study aimed at defining the metabolic roles, co-occurrence, and possible interactions of these 3 groups of microbes, fecal samples from 40 healthy adult female monozygotic (MZ) and 28 dizygotic (DZ) twin pairs were examined, using various methods (Hansen et al. 2011). Twenty-two bacterial species, of which 20 were Clostridiales, positively correlated with methanogens. The patterns of co-occurrence of species with methanogens were more similar among the MZ than in the DZ twin pairs. The *M. smithii* pangenome was found to contain 987 genes conserved in all strains and 1860 genes that varied among strains. Strains from MZ and DZ twin pairs had a similar degree of shared genes and SNPs and were significantly more similar than strains isolated from mothers or members of other families. The 101 adhesin-like proteins (ALPs) in the *M. smithii* pangenome showed strain-specific differences in expression and responsiveness to formate. It was hypothesized that *M. smithii* uses a variety of ALPs repertoires to generate diversity and, thereby, establish specific syntrophic associations with bacteria, typical of different metabolic niches in the gut.

4 Newly Described Methanogens in Humans

A methanogen previously undescribed was identified in human feces, and, based on genetic and phenotypic data, it was proposed to be a novel species of a new genus and named *Methanomassiliicoccus luminyensis* gen. nov., sp. nov. (Dridi et al.

2012a). The growth characteristics and methyltransferase genes were later described (Kröninger et al. 2017).

The genome of a methanogen present in the human gut was sequenced, and it was proposed to be a previously undescribed methanogenic archaeon belonging to a seventh order of methanogens and was named *Candidatus Methanomethylophilus alvus* Mx1201 (Borrel et al. 2012). Likewise, a methanogen, *Candidatus Methanomassiliicoccus intestinalis* Issoire-Mx1, found in the human gut, was determined by genome sequencing to be a Thermoplasmatales-related archaeon (Borrel et al. 2013a).

While the generally reported prevalence of *Methanobrevibacter smithii* in human stools reaches almost 100% and that of *Methanosphaera stadtmanae* is about 30%, the prevalence of *Methanomassiliicoccus luminyensis* is only 4% (Dridi et al. 2012b).

Analysis of sequence data pertaining to organisms from various ecosystems, including the human gastrointestinal tract, supports the existence of a seventh order of methanogens (Borrel et al. 2013b). Genomic data from members of this lineage, *Methanomassiliicoccus luminyensis* and *Candidatus Methanomethylophilus alvus*, reveal key features of the evolution and metabolic characteristics of these microbes. The data suggest a loss of genes involved in the first six steps of methanogenesis from H_2/CO_2 and the oxidative part of methylotrophic methanogenesis. Also, the data indicate that the archaea belonging to this lineage are able to use a wide range of methylated compounds.

Other distinctive features of the organisms belonging to the seventh order of methanogens confirmed that Methanomassiliicoccales are phylogenetically distant from all other orders of methanogens and belong to a large evolutionary branch including also lineages of non-methanogenic archaea (Borrel et al. 2014).

Recently, a halophilic archaeon was isolated from human feces (Khelaifia et al. 2018), but its role in this niche has yet to be elucidated, and efforts in this direction may prove rewarding in that unexpected roles for halophilic archaea in human health and disease may be uncovered.

5 Methanogens in Human Disease

As mentioned on the Sect. 2 (Fig. 1), the human gut microbiota and the immune system interact, and the consequences of this interaction can be beneficial or induce pathological processes. In recent work it was found that *Methanosphaera stadtmanae* and RNA derived from it are strong stimulant of innate immunity (Vierbuchen et al. 2017).

It was also reported that another methanogen present in the human gut, *Methanomassiliicoccus luminyensis* strain B10T, interacts with the immune system and induces release of pro-inflammatory cytokines (Bang et al. 2017).

It has been proposed that presence of certain methanogens in the intestine can contribute to constipation, and the effect of probiotics on this condition was tested

(Ojetti et al. 2017). The administration of *Lactobacillus reuteri* for 4 weeks was accompanied by a significant decrease of CH_4 production and by improvement of bowel movement.

Intestinal inflammation and changes in gut microbiota are believed to be associated with cystic fibrosis, and, recently, it was established that patients with the disease show a significant decrease of H_2-consuming microorganisms, which suggests that methanogens may be involved (Miragoli et al. 2017).

Since methanogens in the human gut produce methane gas and have an impact on the metabolism of the carrier, a study was carried out to examine metabolic parameters in methane-producing subjects before and after antibiotic treatment (Mathur et al. 2016). It was observed that diminution or eradication of breath methane and *M. smithii* reduction were associated with significant improvements in total cholesterol, LDL, and insulin levels and with lower glucose levels in prediabetic subjects with obesity.

The most prevalent methanogen in the human gut is *M. smithii*, which can produce flatulence and promote constipation, seems to be implicated also in the irritable bowel syndrome. In patients with this condition and constipation, *M. smithii* was increased by comparison with patients with the syndrome and diarrhea and with healthy controls (Ghoshal et al. 2016). It may be proposed that by reducing the load of the archaeon in the gut, one can ameliorate the symptoms in those patients with irritable bowel syndrome and constipation.

In 1988, methanogenic bacteria were found for the first time in human dental plaque (Belay et al. 1988). At the time, the methanogens identified were *M. smithii* and *Methanosphaera stadtmanae*. More recent studies, using different methods, demonstrated the presence also of *Methanobrevibacter oralis* (Huynh et al. 2015), thus extending the diversity of archaea that inhabit ecosystems of the human body.

The potential for interacting with the immune system of the two most common human gut-microbiota archaea, *M. smithii* and *Methanosphaera stadtmanae*, was evaluated (Blais Lecours et al. 2014). Mononuclear cells stimulated with either one or the other of these two archaea produced inflammatory factor TNF, but *M. stadtmanae* was a stronger stimulator than *M. smithii* and was present in higher concentrations than *M. smithii* in patients with inflammatory bowel disease (IBD). Along the same line, the immunomodulatory effect of *M. stadtmanae* and *M. smithii* was tested (Bang et al. 2014b). *M. stadtmanae* was a strong inducer of pro-inflammatory cytokines in monocyte-derived dendritic cells (moDCs), considerably more potent than *M. smithii*. The two archaea upregulated the cell-surface receptors CD86 and CD197 in moDCs and had an impact on the expression of antimicrobial peptide genes.

Biofilm formation is common among bacteria and represents a mechanism for pathogenicity in those microbes that can cause disease. The capacity for biofilm formation of three archaeal species, two present in mucosa ecosystems in humans and one pertinent to bioreactor technology, namely, *M. smithii* and *Methanosphaera stadtmanae* and *Methanosarcina mazei*, respectively, was investigated (Bang et al. 2014a). The three species did form mono- and multilayer biofilms on various types of surfaces, suggesting that this might be a mechanism that these archaea use to

adhere to natural substrates like the intestinal mucosa or the particulate support in bioreactors.

It is known that some antimicrobial peptides (AMP) can kill or inactivate bacteria. Whether AMPs are also effective against archaea was investigated, using as targets two archaea known to be part of the human microbiota, *M. smithii* and *M. stadtmanae*, and *Methanosarcina mazei* (Bang et al. 2012). All these three methanogens were highly sensitive to the action of derivatives of human cathelicidin, of porcine lysin, and of a synthetic antilipopolysaccharide peptide (Lpep), with some differences between the three species.

More recently, a similar investigation was carried out but directed to another methanogen also found in the human gut, *Methanomassiliicoccus luminyensis* (Bang et al. 2017). This methanogen was highly sensitive to LL32 (a derivative of human cathelicidin) but was resistant to the action of porcine lysin NK-2 and the synthetic antilipopolysaccharide peptide (Lpep). The results revealed that *M. luminyensis* is quite different from *M. smithii* and *M. stadtmanae* regarding resistance to the action of the compounds tested. Furthermore, *M. luminyensis* had a considerable weaker potency to stimulate production of pro-inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) and monocyte-derived dendritic cells (moDCs) as compared with *M. smithii* and *M. stadtmanae*.

6 Trimethylaminuria

A methanogen, *Methanomassiliicoccus luminyensis* B10, found in the human gastrointestinal tract seems to be restricted to utilizing only methyl compounds, including trimethylamine (TMA) (Dridi et al. 2012a; Brugère et al. 2014). This methanogen in culture was able to deplete TMA by reducing it with H_2 for methanogenesis. It was, therefore, suggested that this methanogen could be used to treat some conditions characterized by excess TMA and its accumulation, which is the basis for of trimethylaminuria (TMAU; fish-odor syndrome) (Brugère et al. 2014). TMA formed by the intestinal microbiota may also be implicated in atherosclerosis risk by affecting trimethylamine oxide (TMAO) production levels. Therefore, reducing the levels of TMA using the archaeon *M. luminyensis*, and others with the same metabolic characteristics that may be discovered (Borrel et al. 2017), seems a promising approach to treat patients with excess TMA (Brugère et al. 2014).

7 Methanogens in Animals

The fecal microbiota was studied in healthy Sprague Dawley rats at various ages, from 3 weeks to 2 years, under controlled environmental and dietary conditions (Flemer et al. 2017). Time-related variations were noted, including methanogenic archaea. It was also established that the microbiota of rats and mice were most

similar to each other but that of rats was the most similar to the human microbiota, which favors rats as the animals of choice to do experiments aiming to clarify issues pertaining to the human microbiota.

The fecal microbiota was examined in four kinds of primates and five kinds of carnivora (including herbivorous, omnivorous, and carnivorous) (Chen et al. 2017). The results showed that the phylogeny of the host had a greater influence on the microbiota than the host's diet. Forty-four bacterial phyla and two archaeal phyla were identified.

The rumen microbiota of domestic ruminants has been extensively examined, but very few studies on the rumen microbiota of wild ruminants have been published. An example of the later study pertains to the wild ruminant impala (*Aepyceros melampus melampus*) (Cersosimo et al. 2015). The methanogens found belonged to the genera *Methanobrevibacter* and *Methanosphaera* (94.3% and 4.0% of the classified sequences, respectively).

Demonstration of colonization of the intestinal ecosystem of various animal species by archaeal organisms has been done in many laboratories; some reports also deal with the changes over time of the archaeal microbiota profile, in relation to age, diet, and other parameters (Saengkerdsub and Ricke 2014). This type of research aiming at clarifying the dynamics of the archaeal microbiota in animals and human continues to develop. For example, in pigs it was demonstrated that *Methanobrevibacter smithii* was abundant at 28 days of age, dramatically reduced at 42 days, and undetectable at 63 days (Federici et al. 2015). In contrast, *Methanobrevibacter boviskoreani* showed a mirror curve of abundance: as *M. smithii* decreased with age, *M. boviskoreani* increased so that at 63 days of age was the most prevalent archaeal species in the piglets intestine.

The presence of methanogenic archaea in animal ecosystems, particularly the gastrointestinal tract, has been studied for decades. Results from 16 rRNA sequence comparison and analysis have provided information on methanogens occurring in a wide range of animals, including wild and domestic ruminants, camelids, marsupials, primates, birds, and reptiles (St-Pierre and Wright 2013). Interestingly, although methanogens are very diverse phylogenetically and in other aspects, only a few varieties have been found in the gut of animals, mostly pertaining to the genus *Methanobrevibacter*. Much less prevalent are species of the genus *Methanosphaera*, the order Methanomicrobiales, or the Thermoplasmatales. It has also been perceived that the host species, diet, and age are determinant factors for the type and abundance of archaea occurring in the gut of any given organism. Overall, it has been observed that the archaeal microbiota may differ strongly between breeds and be remarkably similar between unrelated species. In conclusion, the information thus far available indicates that archaea inhabiting the gastrointestinal tract of animals vary considerably but belong only to a limited number of genera.

The hindgut microbiota of white rhinoceros was surveyed in search of archaeal representatives by examining 153 archaeal 16S rRNA sequences obtained from PCR products from feces of seven adult animals (Luo et al. 2013). Sixty percent of the sequences were closely related to *Methanocorpusculum labreanum*, 27% of the sequences were closely related to *Methanobrevibacter smithii*, 4% of the sequences

could be assigned to *Methanosphaera stadtmanae*, while the rest of the sequences were distantly related to *Methanomassiliicoccus luminyensis* and were considered to be novel species or strains that have yet-to-be cultivated and characterized.

8 Methods

It is current knowledge that culturing methanogens is not easy and that the development of techniques for their identification and quantification in environmental, animal, and human samples is a necessity (Chaudhary et al. 2015). For example, in vitro methods have been standardized for reciprocal conversion of methanogenic and non-methanogenic microbiota from human fecal samples (Tottey et al. 2015). Efforts have been made to critically discuss the various methods available to detect, identify, and quantify archaea in samples from humans, for example, feces, subgingival plaque, and vaginal mucosa (Dridi 2012). Although it is well established that *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* are the most prevalent archaeal representatives in the human microbiota, others have also been found such as *Methanobrevibacter oralis* and *Methanomassiliicoccus luminyensis*. Furthermore, DNA sequences have been detected, using molecular probes, PCR, and metagenomics analyses, indicating that the diversity of archaea in the human microbiota may be wider than that it is currently known. More studies are necessary to elucidate the whole range of archaeal species present in the human microbiota and establish their respective roles in health and disease.

9 Basic Concepts and Facts

Recent advances in elucidating archaea diversity in the human microbiota included the application of protocols directed specifically to archaeal targets, using PCR-based methods to reveal archaea in the human gastrointestinal tract, lung, nose, and skin (Koskinen et al. 2017). It was discovered that *Methanobacterium* and *Woesearchaeota* (DPANN superphylum) are present in the human gastrointestinal tract and the human lung, respectively. Furthermore, it was found that the components and diversity of the archaea microbiota were distinctive of location, intestine, lung, nose, or skin.

Likewise, other investigations are showing an increase in the main branches of the archaeal phylogenetic tree, including novel phyla, classes, and orders (Adam et al. 2017). The data from various laboratories indicate that archaea are considerably more diverse than previously anticipated and occupy a very wide range of ecosystems, including various in the human body.

Very interesting developments have occurred in the area of direct interspecies electron transfer (DIET) (Lovley 2017). Electrons may be transported long distances (even measured in cm), and thus electron exchange between microbes can occur

even if their bodies are far apart on condition that some conductor joins them. Electric connections seem to occur also between gut microbes, including methanogens, which has revealed potential key roles for pili and c-type cytochromes in natural electrical grids. However, key data are still missing to elucidate the archaeal electrical connection that would facilitate DIET-based methane production and consumption.

Difficulties in sorting out archaea in animal and human microbiota are emerging since prokaryotes may not be amenable to classification by comparing a single gene or even groups of gene families because of the sequence exchanges and genome remodeling that occurred during evolution (Levasseur et al. 2017). The study of the archaic microbe Lokiarchaeum sp., considered to be an archaeal host close to the emergence of eukaryotes, revealed a mosaic constituted of genes from archaeal (∼36%), bacterial (∼28%), and eukaryotic (∼2%) origin. This mosaicism was also observed in another archaeon from the human gut, *Methanomassiliicoccus luminyensis*, whose genome was found to be composed of genes of archaeal (67%) and bacterial (22%) origin. These findings illustrate the problems in classifying prokaryotes, which seem to be mosaics, chimeric beings, composed of different life domains, and also shed doubts on the reality of the tree of life composed of well-defined phylogenetic branches, clearly separate from one another.

The origin of eukaryotes and the complexity of the eukaryotic cell with its various compartments and organelles have been investigated for some time, but convincing explanations are still rare. A recent study revealed that Asgard archaea contain a repertoire of eukaryotic genes, thus suggesting that the primitive host cell, possibly an archaeon, already had eukaryotic-like components when it received sequences from other sources (Zaremba-Niedzwiedzka et al. 2017).

10 Conclusions and Perspectives

Considerable progress took place over the last decade in the understanding of the human and animal microbiotas; their composition, variations in relation to diet, age, administration of antibiotics, and other factors; and roles in health and disease. These studies revealed that methanogenic archaea are characteristic components of human and animal microbiotas with one species being present in practically 100% of cases. Also, it became apparent that methanogens are not only present in the human gastrointestinal tract, including oral mucosa and intestine, and in the vagina, as it had been established many years ago, but also in the nose, lung, and skin. *Methanosarcina smithii* and *Methanosphaera stadtmaniae* were confirmed as the prevalent methanogens in humans, particularly the former as reported in the past, but other species-strains were added to the list of human methanogens.

The newly reported findings confirm the notion that study of the archaea inhabiting humans and animals is highly desirable for various reasons; for instance, it promises to reveal key steps of the mechanisms involved in the interactions between the microbiota and the immune and chaperoning systems. These

interactions are very important in the maintenance of health and, when altered, can cause disease. By elucidating the basic mechanisms of interaction between methanogens and bacterial partners in the human microbiota, and of the interaction of the latter with the immune and chaperoning systems, roads will be opened to developing treatment strategies directed to, or using, components of the microbiota and its products. No doubt methanogenic archaea will be at the center of these medical applications, as well as in the development of means for diagnosis and assessing prognosis and response to treatment in diseases involving the microbiota.

Acknowledgments E.C. de M. and A.J.L.M. were partially supported by IMET. This is IMET contribution number IMET 18-010).

References

Adam PS, Borrel G, Brochier-Armanet C, Gribaldo S (2017) The growing tree of Archaea: new perspectives on their diversity, evolution and ecology. ISME J 11(11):2407–2425. https://doi.org/10.1038/ismej.2017.122. Epub 2017 Aug 4. PMID:28777382

Bang C, Schmitz RA (2015) Archaea associated with human surfaces: not to be underestimated. FEMS Microbiol Rev 39(5):631–648. https://doi.org/10.1093/femsre/fuv010. Epub 2015 Apr 22. Review. PMID:25907112

Bang C, Schilhabel A, Weidenbach K, Kopp A, Goldmann T, Gutsmann T, Schmitz RA (2012) Effects of antimicrobial peptides on methanogenic archaea. Antimicrob Agents Chemother 56 (8):4123–4130. https://doi.org/10.1128/AAC.00661-12. Epub 2012 May 14. PMID:22585226

Bang C, Ehlers C, Orell A, Prasse D, Spinner M, Gorb SN, Albers SV, Schmitz RA (2014a) Biofilm formation of mucosa-associated methanoarchaeal strains. Front Microbiol 5:353. https://doi.org/10.3389/fmicb.2014.00353. eCollection 2014. PMID:25071757

Bang C, Weidenbach K, Gutsmann T, Heine H, Schmitz RA (2014b) The intestinal archaea *Methanosphaera stadtmanae* and *Methanobrevibacter smithii* activate human dendritic cells. PLoS One 9(6):e99411. https://doi.org/10.1371/journal.pone.0099411. eCollection 2014. PMID:24915454

Bang C, Vierbuchen T, Gutsmann T, Heine H, Schmitz RA (2017) Immunogenic properties of the human gut-associated archaeon *Methanomassiliicoccus luminyensis* and its susceptibility to antimicrobial peptides. PLoS One 12(10):e0185919. https://doi.org/10.1371/journal.pone.0185919. eCollection 2017. PMID:28982164

Barko PC, McMichael MA, Swanson KS, Williams DA (2017) The gastrointestinal microbiome: a review. J Vet Intern Med. https://doi.org/10.1111/jvim.14875. [Epub ahead of print]. Review. PMID:29171095

Belay N, Johnson R, Rajagopal BS, Conway de Macario E, Daniels L (1988) Methanogenic bacteria from human dental plaque. Appl Environ Microbiol 54(2):600–603. PMID:3355146

Bellavia M, Tomasello G, Romeo M, Damiani P, Lo Monte AI, Lozio L, Campanella C, Marino Gammazza A, Rappa F, Zummo G, Cocchi M, Conway de Macario E, Macario AJL, Cappello F (2013) Gut microbiota imbalance and chaperoning system malfunction are central to ulcerative colitis pathogenesis and can be counteracted with specifically designed probiotics. Med Microbiol Immunol 202(6):393–406. https://doi.org/10.1007/s00430-013-0305-2. Epub 2013 Jul 18. PMID:23864544

Blais Lecours P, Marsolais D, Cormier Y, Berberi M, Haché C, Bourdages R, Duchaine C (2014) Increased prevalence of *Methanosphaera stadtmanae* in inflammatory bowel diseases. PLoS One 9(2):e87734. https://doi.org/10.1371/journal.pone.0087734. eCollection 2014. PMID:24498365

Borrel G, Harris HM, Tottey W, Mihajlovski A, Parisot N, Peyretaillade E, Peyret P, Gribaldo S, O'Toole PW, Brugère JF (2012) Genome sequence of "*Candidatus Methanomethylophilus alvus*" Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens. J Bacteriol 194(24):6944–6945. https://doi.org/10.1128/JB.01867-12. PMID: 23209209

Borrel G, Harris HM, Parisot N, Gaci N, Tottey W, Mihajlovski A, Deane J, Gribaldo S, Bardot O, Peyretaillade E, Peyret P, O'Toole PW, Brugère JF (2013a) Genome sequence of "*Candidatus Methanomassiliicoccus intestinalis*" Issoire-Mx1, a third Thermoplasmatales-related methanogenic archaeon from human feces. Genome Announc 1(4). https://doi.org/10.1128/genomeA.00453-13. pii: e00453-13. PMID:23846268

Borrel G, O'Toole PW, Harris HM, Peyret P, Brugère JF, Gribaldo S (2013b) Phylogenomic data support a seventh order of Methylotrophic methanogens and provide insights into the evolution of methanogenesis. Genome Biol Evol 5(10):1769–1780. https://doi.org/10.1093/gbe/evt128. PMID:23985970

Borrel G, Parisot N, Harris HM, Peyretaillade E, Gaci N, Tottey W, Bardot O, Raymann K, Gribaldo S, Peyret P, O'Toole PW, Brugère JF (2014) Comparative genomics highlights the unique biology of Methanomassiliicoccales, a Thermoplasmatales-related seventh order of methanogenic archaea that encodes pyrrolysine. BMC Genomics 15:679. https://doi.org/10.1186/1471-2164-15-679. PMID:25124552

Borrel G, McCann A, Deane J, Neto MC, Lynch DB, Brugère JF, O'Toole PW (2017) Genomics and metagenomics of trimethylamine-utilizing Archaea in the human gut microbiome. ISME J 11(9):2059–2074. https://doi.org/10.1038/ismej.2017.72. Epub 2017 Jun 6. PMID:28585938

Brugère JF, Borrel G, Gaci N, Tottey W, O'Toole PW, Malpuech-Brugère C (2014) Archaebiotics: proposed therapeutic use of archaea to prevent trimethylaminuria and cardiovascular disease. Gut Microbes 5(1):5–10. https://doi.org/10.4161/gmic.26749. Epub 2013 Oct 31. PMID:24247281

Cappello F, Conway de Macario E, Di Felice V, Zummo G, Macario AJL (2009) *Chlamydia trachomatis* infection and anti-Hsp60 immunity: the two sides of the coin. PLoS Pathog 5(8): e1000552. https://doi.org/10.1371/journal.ppat.1000552. Epub 2009 Aug 28. PMID:19714222

Cappello F, Marino Gammazza A, Zummo L, Conway de Macario E, Macario AJL (2010) Hsp60 and AChR cross-reactivity in myasthenia gravis: an update. J Neurol Sci 292(1–2):117–118. https://doi.org/10.1016/j.jns.2010.02.021. Epub 2010 Mar 16. No abstract available. PMID:20236663

Cersosimo LM, Lachance H, St-Pierre B, van Hoven W, Wright AD (2015) Examination of the rumen bacteria and methanogenic archaea of wild impalas (*Aepyceros melampus melampus*) from Pongola, South Africa. Microb Ecol 69(3):577–585. https://doi.org/10.1007/s00248-014-0521-3. Epub 2014 Oct 29. PMID:25351144

Chaudhary PP, Gaci N, Borrel G, O'Toole PW, Brugère JF (2015) Molecular methods for studying methanogens of the human gastrointestinal tract: current status and future directions. Appl Microbiol Biotechnol 99(14):5801–5815. https://doi.org/10.1007/s00253-015-6739-2. Epub 2015 Jun 19. Review. PMID:26088176

Chaudhary PP, Conway PL, Schlundt J (2018) Methanogens in humans: potentially beneficial or harmful for health. Appl Microbiol Biotechnol 102(7):3095–3104. https://doi.org/10.1007/s00253-018-8871-2. Epub 2018 Mar 1. Review. PMID:29497795

Chen X, Li QY, Li GD, Xu FJ, Han L, Jiang Y, Huang XS, Jiang CL (2017) The distal gut bacterial community of some primates and carnivora. Curr Microbiol. https://doi.org/10.1007/s00284-017-1368-x. [Epub ahead of print]. PMID:29127454

Conway de Macario E, Macario AJL (2009) Methanogenic archaea in health and disease: a novel paradigm of microbial pathogenesis. Int J Med Microbiol 299(2):99–108. https://doi.org/10.1016/j.ijmm.2008.06.011. Epub 2008 Aug 30. PMID:18757236

Conway de Macario E, Macario AJL (2010) Methanogenic archaea in humans and other vertebrates. Chapter 7. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea, Microbiology monographs, vol 19. Springer, Berlin, pp 101–113. https://doi.org/10.1007/978-3-642-13615-3_7

Dridi B (2012) Laboratory tools for detection of archaea in humans. Clin Microbiol Infect 18 (9):825–833. https://doi.org/10.1111/j.1469-0691.2012.03952.x. Review. PMID:22897827

Dridi B, Fardeau ML, Ollivier B, Raoult D, Drancourt M (2012a) *Methanomassiliicoccus luminyensis* gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. Int J Syst Evol Microbiol 62(Pt 8):1902–1907. https://doi.org/10.1099/ijs.0.033712-0. PMID:22859731

Dridi B, Henry M, Richet H, Raoult D, Drancourt M (2012b) Age-related prevalence of *Methanomassiliicoccus luminyensis* in the human gut microbiome. APMIS 120(10):773–777. https://doi.org/10.1111/j.1600-0463.2012.02899.x. Epub 2012 Mar 23. PMID:22958284

Federici S, Miragoli F, Pisacane V, Rebecchi A, Morelli L, Callegari ML (2015) Archaeal microbiota population in piglet feces shifts in response to weaning: *Methanobrevibacter smithii* is replaced with *Methanobrevibacter boviskoreani*. FEMS Microbiol Lett 362(10). https://doi.org/10.1093/femsle/fnv064. pii: fnv064. Epub 2015 Apr 22. PMID:25903267

Flemer B, Gaci N, Borrel G, Sanderson IR, Chaudhary PP, Tottey W, O'Toole PW, Brugère JF (2017) Fecal microbiota variation across the lifespan of the healthy laboratory rat. Gut Microbes 8(5):428–439. https://doi.org/10.1080/19490976.2017.1334033. Epub 2017 Jun 6. PMID:28586297

Forbes JD, Van Domselaar G, Bernstein CN (2016) The gut microbiota in immune-mediated inflammatory diseases. Front Microbiol 7:1081. https://doi.org/10.3389/fmicb.2016.01081. eCollection 2016. PMID:27462309

Gaci N, Borrel G, Tottey W, O'Toole PW, Brugère JF (2014) Archaea and the human gut: new beginning of an old story. World J Gastroenterol 20(43):16062–16078. https://doi.org/10.3748/wjg.v20.i43.16062. Review. PMID:25473158

Ghavami SB, Rostami E, Sephay AA, Shahrokh S, Balaii H, Aghdaei HA, Zali MR (2018) Alterations of the human gut *Methanobrevibacter smithii* as a biomarker for inflammatory bowel diseases. Microb Pathog 117:285–289. https://doi.org/10.1016/j.micpath.2018.01.029. Epub 2018 Feb 22. PMID:29477743

Ghoshal U, Shukla R, Srivastava D, Ghoshal UC (2016) Irritable bowel syndrome, particularly the constipation-predominant form, involves an increase in *Methanobrevibacter smithii*, which is associated with higher methane production. Gut Liver 10(6):932–938. https://doi.org/10.5009/gnl15588. PMID:27458176

Grine G, Boualam MA, Drancourt M (2017) *Methanobrevibacter smithii*, a methanogen consistently colonising the newborn stomach. Eur J Clin Microbiol Infect Dis. https://doi.org/10.1007/s10096-017-3084-7. [Epub ahead of print]. PMID:28823095

Hansen EE, Lozupone CA, Rey FE, Wu M, Guruge JL, Narra A, Goodfellow J, Zaneveld JR, McDonald DT, Goodrich JA, Heath AC, Knight R, Gordon JI (2011) Pan-genome of the dominant human gut-associated archaeon, *Methanobrevibacter smithii*, studied in twins. Proc Natl Acad Sci U S A 108(Suppl 1):4599–4606. Epub 2011 Feb 11. PMID:21317366

Hugon P, Lagier JC, Colson P, Bittar F, Raoult D (2016) Repertoire of human gut microbes. Microb Pathog. https://doi.org/10.1016/j.micpath.2016.06.020. pii: S0882-4010(15)30231-X. [Epub ahead of print]. Review. PMID:27317857

Huynh HT, Pignoly M, Nkamga VD, Drancourt M, Aboudharam G (2015) The repertoire of archaea cultivated from severe periodontitis. PLoS One 10(4):e0121565. https://doi.org/10.1371/journal.pone.0121565. eCollection 2015. PMID:25830311

Keppler F, Schiller A, Ehehalt R, Greule M, Hartmann J, Polag D (2016) Stable isotope and high precision concentration measurements confirm that all humans produce and exhale methane. J Breath Res 10(1):016003. https://doi.org/10.1088/1752-7155/10/1/016003. PMID:26824393

Khelaifia S, Caputo A, Andrieu C, Cadoret F, Armstrong N, Michelle C, Lagier JC, Djossou F, Fournier PE, Raoult D (2018) Genome sequence and description of Haloferax massiliense sp. nov., a new halophilic archaeon isolated from the human gut. Extremophiles 22 (3):485–498. https://doi.org/10.1007/s00792-018-1011-1. Epub 2018 Feb 12. PMID:29435649

Koskinen K, Pausan MR, Perras AK, Beck M, Bang C, Mora M, Schilhabel A, Schmitz R, Moissl-Eichinger C (2017) First insights into the diverse human archaeome: specific detection of Archaea in the gastrointestinal tract, lung, and nose and on skin. MBio 8(6). https://doi.org/10.1128/mBio.00824-17. pii: e00824-17. PMID:29138298

Kröninger L, Gottschling J, Deppenmeier U (2017) Growth characteristics of *Methanomassiliicoccus luminyensis* and expression of methyltransferase encoding genes. Archaea 2017:2756573. https://doi.org/10.1155/2017/2756573. eCollection 2017. PMID:29230105

La-Ongkham O, Nakphaichit M, Leelavatcharamas V, Keawsompong S, Nitisinprasert S (2015) Distinct gut microbiota of healthy children from two different geographic regions of Thailand. Arch Microbiol 197(4):561–573. https://doi.org/10.1007/s00203-015-1089-0. Epub 2015 Feb 3. PMID:25644242

Large AT, Lund PA (2009) Archaeal chaperonins. Front Biosci 14:1304–1324. PMID:19273132

Large AT, Goldberg MD, Lund PA (2009) Chaperones and protein folding in the archaea. Biochem Soc Trans 37(Pt 1):46–51. https://doi.org/10.1042/BST0370046. PMID:19143600

Levasseur A, Merhej V, Baptiste E, Sharma V, Pontarotti P, Raoult D (2017) The rhizome of Lokiarchaeota illustrates the mosaicity of archaeal genomes. Genome Biol Evol 9 (10):2635–2639. https://doi.org/10.1093/gbe/evx208. PMID:29048529

Lovley DR (2017) Happy together: microbial communities that hook up to swap electrons. ISME J 11(2):327–336. https://doi.org/10.1038/ismej.2016.136. Epub 2016 Nov 1. PMID:27801905

Luo YH, Wright AD, Li YL, Li H, Yang QH, Luo LJ, Yang MX (2013) Diversity of methanogens in the hindgut of captive white rhinoceroses, *Ceratotherium simum*. BMC Microbiol 13:207. https://doi.org/10.1186/1471-2180-13-207. PMID:24228793

Macario AJL, Conway de Macario E (2005) Sick chaperones, cellular stress, and disease. N Engl J Med 353(14):1489–1501. PMID:16207851

Macario AJL, Conway de Macario E (2009) The chaperoning system: physiology and pathology. Exp Med Rev 2008/2009 2–3:9–21

Macario AJL, Conway de Macario E (2016) The chaperoning and the immune systems with the microbiome integrate a matrix that supports health: when one of them is disturbed the others suffer and disease ensues. Life Saf Secur 4(6):101–123. https://doi.org/10.12882/2283-7604.2016.4.6 http://www.iemest.eu/life-safety-and-security/images/Doc/ARTICOLI/2016/macario_24/Macario04.pdf

Macario AJL, Malz M, Conway de Macario E (2004) Evolution of assisted protein folding: the distribution of the main chaperoning systems within the phylogenetic domain archaea. Front Biosci 9:1318–1332. PMID:14977547

Macario AJL, Cappello F, Zummo G, Conway de Macario E (2010) Chaperonopathies of senescence and the scrambling of interactions between the chaperoning and the immune systems. Ann N Y Acad Sci 1197:85–93. https://doi.org/10.1111/j.1749-6632.2010.05187.x. PMID:20536837

Marino Gammazza A, Rizzo M, Citarrella R, Rappa F, Campanella C, Bucchieri F, Patti A, Nikolic D, Cabibi D, Amico G, Conaldi PG, San Biagio PL, Montalto G, Farina F, Zummo G, Conway de Macario E, Macario AJL, Cappello F (2014) Elevated blood Hsp60, its structural similarities and cross-reactivity with thyroid molecules, and its presence on the plasma membrane of oncocytes point to the chaperonin as an immunopathogenic factor in Hashimoto's thyroiditis. Cell Stress Chaperones 19(3):343–353. https://doi.org/10.1007/s12192-013-0460-9. Epub 2013 Sep 22. PMID:24057177

Marino Gammazza A, Caruso Bavisotto C, David S, Barone R, Rappa F, Campanella C, Conway de Macario E, Cappello F, Macario AJL (2017) HSP60 is a ubiquitous player in the physiological and pathogenic interactions between the chaperoning and the immune systems. Curr Immunol Rev 13(1):44–55. https://doi.org/10.2174/1573395513666170412170540 http://www.eurekaselect.com/151566

Mathur R, Chua KS, Mamelak M, Morales W, Barlow GM, Thomas R, Stefanovski D, Weitsman S, Marsh Z, Bergman RN, Pimentel M (2016) Metabolic effects of eradicating breath methane using antibiotics in prediabetic subjects with obesity. Obesity (Silver Spring) 24(3):576–582. https://doi.org/10.1002/oby.21385. Epub 2016 Feb 1. PMID:26833719

Miragoli F, Federici S, Ferrari S, Minuti A, Rebecchi A, Bruzzese E, Buccigrossi V, Guarino A, Callegari ML (2017) Impact of cystic fibrosis disease on archaea and bacteria composition of gut microbiota. FEMS Microbiol Ecol 93(2). pii: fiw230. Epub 2016 Nov 2. PMID:27810876

Moissl-Eichinger C, Pausan M, Taffner J, Berg G, Bang C, Schmitz RA (2017) Archaea are interactive components of complex microbiomes. Trends Microbiol. https://doi.org/10.1016/j.tim.2017.07.004. pii: S0966-842X(17)30174-9. [Epub ahead of print]. Review. PMID:28826642

Ojetti V, Petruzziello C, Migneco A, Gnarra M, Gasbarrini A, Franceschi F (2017) Effect of Lactobacillus reuteri (DSM 17938) on methane production in patients affected by functional constipation: a retrospective study. Eur Rev Med Pharmacol Sci 21(7):1702–1708. PMID:28429333

Saengkerdsub S, Ricke SC (2014) Ecology and characteristics of methanogenic archaea in animals and humans. Crit Rev Microbiol 40(2):97–116. https://doi.org/10.3109/1040841X.2013.763220. Epub 2013 Feb 21. Review. PMID:23425063

Seo M, Heo J, Yoon J, Kim SY, Kang YM, Yu J, Cho S, Kim H (2017) Methanobrevibacter attenuation via probiotic intervention reduces flatulence in adult human: a non-randomised paired-design clinical trial of efficacy. PLoS One 12(9):e0184547. https://doi.org/10.1371/journal.pone.0184547. Collection 2017. PMID:28937980

St-Pierre B, Wright AD (2013) Diversity of gut methanogens in herbivorous animals. Animal 7 (Suppl 1):49–56. https://doi.org/10.1017/S1751731112000912. Epub 2012 Apr 27. PMID:22717175

Tottey W, Gaci N, Borrel G, Alric M, O'Toole PW, Brugère JF (2015) In-vitro model for studying methanogens in human gut microbiota. Anaerobe 34:50–52. https://doi.org/10.1016/j.anaerobe.2015.04.009. Epub 2015 Apr 14. PMID:25887578

van de Pol JA, van Best N, Mbakwa CA, Thijs C, Savelkoul PH, Arts IC, Hornef MW, Mommers M, Penders J (2017) Gut colonization by methanogenic archaea is associated with organic dairy consumption in children. Front Microbiol 8:355. https://doi.org/10.3389/fmicb.2017.00355. eCollection 2017. PMID:28344572

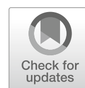
Vanderhaeghen S, Lacroix C, Schwab C (2015) Methanogen communities in stools of humans of different age and health status and co-occurrence with bacteria. FEMS Microbiol Lett 362(13): fnv092. https://doi.org/10.1093/femsle/fnv092. Epub 2015 May 29. PMID:26025070

Vierbuchen T, Bang C, Rosigkeit H, Schmitz RA, Heine H (2017) The human-associated archaeon *Methanosphaera stadtmanae* is recognized through its RNA and induces TLR8-dependent NLRP3 inflammasome activation. Front Immunol 8:1535. https://doi.org/10.3389/fimmu.2017.01535. eCollection 2017. PMID:29181003

Wampach L, Heintz-Buschart A, Hogan A, Muller EEL, Narayanasamy S, Laczny CC, Hugerth LW, Bindl L, Bottu J, Andersson AF, de Beaufort C, Wilmes P (2017) Colonization and succession within the human gut microbiome by archaea, bacteria, and microeukaryotes during the first year of life. Front Microbiol 8:738. https://doi.org/10.3389/fmicb.2017.00738. eCollection 2017. PMID:28512451

Zaremba-Niedzwiedzka K, Caceres EF, Saw JH, Bäckström D, Juzokaite L, Vancaester E, Seitz KW, Anantharaman K, Starnawski P, Kjeldsen KU, Stott MB, Nunoura T, Banfield JF, Schramm A, Baker BJ, Spang A, Ettema TJ (2017) Asgard archaea illuminate the origin of eukaryotic cellular complexity. Nature 541(7637):353–358. https://doi.org/10.1038/nature21031. Epub 2017 Jan 11. PMID:28077874

Methanogens in the Gastrointestinal Tract of Animals



Johannes H. P. Hackstein and Theo A. van Alen

Contents

1 Introduction 122
2 Vertebrates 122
3 Conclusions: Vertebrates 131
4 Arthropods 132
5 Conclusions: Arthropods 146
References 148

Abstract Nearly all vertebrates host methanogens in their gastrointestinal tracts. However, a great fraction of vertebrates emits only traces of methane from their faeces (approx. 1 nmol/g faeces/h) and has no significant amounts of methane in their breath. In contrast, many animals host some 100 times more methanogens in their gastrointestinal tract and emit methane with their breath. These substantial differences are not caused by different feeding habits; rather a genetic factor controls the presence of large amounts of methanogens. The attribute "methane production" is evolutionary stable, and the loss of this character obeys Dollo's law: once lost in the course of evolution, this character cannot be acquired another time.

Also invertebrates can host methanogens in their gastrointestinal tract. In contrast to the vertebrates, only a few taxa of arthropods emit methane: millipedes, termites, cockroaches, and scarab beetles. All other arthropods in our study did not emit methane and did not host even traces of methanogens. As in vertebrates, the diet of the animals is not crucial for the presence of methanogens. Again, a genetic factor seems to control the presence or absence of methanogens. Methanogenesis is also a prerequisite for the presence of intestinal anaerobic protozoa with endosymbiotic methanogens, but not for the presence of impressive structural differentiations of the hindgut epithelium, which—in methanogenic taxa—host enormous amounts of methanogens.

J. H. P. Hackstein (✉) · T. A. van Alen
IWWR, Faculty of Science, Radboud University Nijmegen, Nijmegen, The Netherlands
e-mail: j.hackstein@science.ru.nl

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_8

1 Introduction

Methanogens are the dominating archaeal organisms and they possess a great phylogenetic and ecological diversity (Woese et al. 1990; Liu and Whitman 2008). They occupy a broad spectrum of ecological niches, including the cytoplasm of unicellular anaerobic eukaryotes and the gastrointestinal tract of various animals (Lange et al. 2005). Most methanogens have not been cultured yet, and their diversity can only be deduced from the analysis of their 16S rRNA genes. Methanogens in complex anaerobic environments are frequently involved in interspecies hydrogen transfer thereby improving fermentations and electron transfer in syntrophic communities of bacteria and archaea (Schink 1997; Stams and Plugge 2009; Müller et al. 2018). Earlier studies have suggested that the endosymbiotic methanogens of protozoa occur only in protists with hydrogenosomes, i.e. hydrogen-producing organelles of mitochondrial descent (Fenchel and Finlay 1995; Hackstein et al. 2006a; Hackstein and Tielens 2018). On the other hand, hydrogenosomes are not the same, and methanogens are not always present in protists with hydrogenosomes; sometimes, endosymbiotic methanogens are present in protists without hydrogenosomes (Fenchel and Finlay 2018; Hackstein and Tielens 2018). It has been shown that methanogens in protists exhibit a certain host specificity (Fenchel and Finlay 1995), but on the other hand, endosymbiont replacements seem to be possible, especially in evolutionary timescales (van Hoek et al. 2000). Notably, methanogens in the gastrointestinal tracts of animals are not found everywhere; in some animals methanogens are abundant; in others they are only of very low abundance or even completely absent. In this review, we will discuss the elusive distribution of methanogens in the gastrointestinal tract of vertebrates and arthropods.

2 Vertebrates

Vertebrates are born (or hatch from the egg) with a sterile gastrointestinal (GI) tract. Soon after birth or hatching, the GI tract becomes colonized by bacteria and archaea. Eventually, after reaching adultness, vertebrates, and especially mammals, host a very complex and numerous microbiota in their guts (Zoetendal et al. 2006; Liu and Whitman 2008). These microbiota are host-specific and clearly different from free-living bacterial communities (Ley et al. 2008a, b). Virtually all these microbiota include methanogens (Miller and Wolin 1986; Hackstein and van Alen 1996; Hackstein et al. 1996). However, the number of methanogens varies at least by two orders of magnitude between species, with the consequence that the faeces of certain species emit less than 1 nmol/g/h of methane while the faeces of other species produce much more than 100 nmol/g/h. A systematic analysis of more than 250 species of vertebrates reveals a bimodal distribution of the methane emissions from faeces with about 85 species producing less than 5 nmol/g/h and about 123 producing

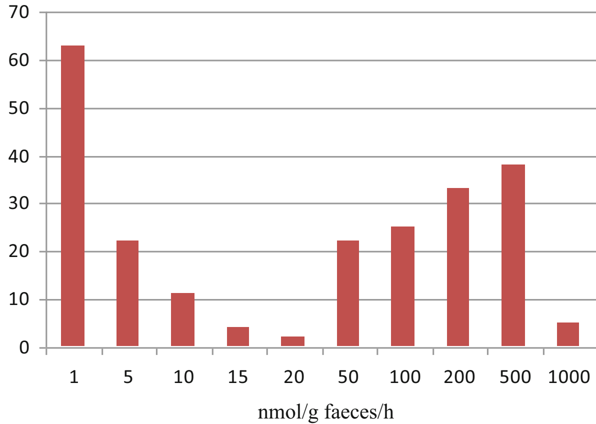


Fig. 1 Histogram of the mean methane emission rates (nmol/g faeces/h, abscissa) by the faeces of 225 amniotes. Ordinate: number of species

more than 50 nmol/g/h (Fig. 1; Hackstein and van Alen 1996). Only a few species produce intermediate amounts of methane. If one assumes that one methanogenic archaeon produces approximately 1 fmol methane per hour (10^{-15} mol/h), then 1 g of faeces of the low producers could host not more than 10^6 methanogens. Accordingly, high methane producers could host more than 10^8 methanogens in 1 g of faeces. This range has been confirmed by enumeration (Miller and Wolin 1982; Doré et al. 1995; El Oufir et al. 1996; Liu and Whitman 2008). These differences have physiological consequences: while the high methane producers emit significant amounts of methane with their breath, the concentrations of methane in the breath of low producers do not exceed the atmospheric background concentration (Hackstein and van Alen 1996). Therefore, we will name the species with faecal emissions of less than 3 nmol/g/h "non-producers" and those with emissions above 20 nmol/g/h "producers".

Using advanced DNA methods, it is possible to analyse the composition of the microbiome which is characteristic for a particular host (Zoetendal et al. 2004; Regensbogenova et al. 2004a; Ley et al. 2008a, b; Kittelmann and Janssen 2011; Kittelmann et al. 2013; St-Pierre and Wright 2013). In this study we will neither analyse nor discuss the various microbiomes; we will also not discriminate between the general composition of plain methanogens from the GI tract and the particular contribution of rumina or hindgut differentiations. We will also not concentrate on the contributions by protists in the GI tract to the methane production (Regensbogenova et al. 2004b; Ushida 2018).

Methane producers and non-producers are not randomly distributed (Table 1), and it is not the case that *all* animals with a plant-based diet produce methane and that animals with a protein-rich diet do not produce methane. Remarkably, crocodiles, boas and ant-eaters emit methane, while chiropters, the great panda and the red panda do not make methane (Hackstein and van Alen 1996).

With a few exceptions, individuals of the same species share their methane status, as well as representatives of closely related species. This raises the question as to whether it is possible to identify the reasons for the presence or absence of high

Table 1 Methane production in vertebrates

	# of species	Methane producer	Non-producer	Mean methane production Producer/non-producer[a]
Xenopus laevis	1	1	–	Methane in breath
Emydidae	1	1		Methane in breath
Testudines	4	4	–	30–109/–
Caiman crocodilus	1	1	–	181/–
Iguanidae	3	2	1	275–322/5
Pythonidae	3	3	–	59–/–
Ratites	8	4	4	93–414/0.2–0.7
Anser anser	1	1	–	79/–
Anas platyrhynchos	1	1	–	70/–
Galli	2	2	–	102–142/–
Columba livia	1	–	1	–/0.3
Passer domesticus	1	–	1	–/0.7
Strigiformes	3	–	3	–/0.2–0.5
Tachyglossus	1	1	–	44/–
Marsupialia	9	8	1	6–499/0.03
Choleopinae	2	2	–	7–290/–
Myrmecophagidae	2	2	–	134–208
Tenrecidae	2	–	2	–/0.1–0.4
Erinaceinae	2	–	2	–/0.2–0.8
Sorex sp.	1	–	1	No methane in breath
Talpa europaea	1	–	1	No methane in breath
Tupaia bergeri	1	–	1	–/1
Chiroptera	4	–	4	–/1–2
Macroscelides	1	–	1	–/0.2
Cheirogaleus medius	1	1	–	5[b]/–
Lemuridae	10	10	–	7–505/–
Loridae	3	3	–	11–79/–
Galagonidae	2	1	1	4/3
Alouatta caraya	1	1	–	73/–
Aotinae	3	3	–	113–373/–
Atelinae	3	3	–	155–347/–
Cebinae	2	–	2	–/0.2–0.4
Callimico goeldii	1	–	1	–/1
Pitheciinae	2	2	–	129–433/–
Callitrichidae	10	4	6	7–77/0.5–3
Cercopithecinae	11	11	–	31–530/–
Colobinae	6	6	–	219–459/–
Hylobatidae	4	4	–	234–433/–
Hominidae	4	4	–	135–417/–
Canidae	4	–	4	–/0.1–4
Felis silvestris	1	–	1	–/0.1

(continued)

Table 1 (continued)

	# of species	Methane producer	Non-producer	Mean methane production Producer/non-producer[a]
Procyon lotor	1	–	1	–/0.3
Ailuridae	4	–	4	–/0.2–2
Ursinae	6	–	6	–/0.3–3
Viverridae	4	–	4	–/0.1–1
Delphinapterus leucas	1	–	1	No methane in breath
Tursiops truncatus	1	–	1	No methane in breath
Trichechus manatus	1	1	–	51/–
Proboscidea	2	2	–	9–41/–
Equidae	2	2	–	30–118/–
Tapiridae	2	2	–	66–311/–
Rhinoceros unicornis	1	1	–	8/–
Procavia capensis	1	1	–	257/–
Orycteropus afer	1	1	–	15/–
Suidae	3	3	–	30–68/–
Tayassu tajacu	1	1	–	329/–
Choeropsis liberiensis	1	1	–	76/–
Camelidae	3	3	–	73–121/–
Giraffidae	2	2	–	21–41
Ovibos moschatus	1	1	–	72/–
Cervinae	5	5	–	69–423/–
Odocoileinae	3	3	–	53–138
Alces alces	1	1	–	110/–
Cephalophus monticola	1	1	–	29/–
Tragelaphinae	3	3	–	66–435/–
Bovinae	2	2	–	116–226/–
Caprinae	6	6	–	21–4230/–
Reduncinae	2	2	–	14–59/–
Manis tricuspis	1	–	1	–/0.2
Sciuridae	17	7	10	8–142/0.01–4
Castor fiber	1	–	1	–/1
Jaculus jaculus	1	–	1	–/0.3
Cricetinae	8	2	6	9/0.3–2
Cricetomys gambianus	1	1	–	100/–
Gerbillinae	2	–	2	–/0.1–0.4
Murinae	7	1	6	26/0.1–2
Myoxidae	2	–	2	–/0.1–2
Graphiurus murinus	1	–	1	–/0.6
Hystricidae	3	3	–	29–108/–
Thryonomys swinderianus	1	1	–	3[b]/–
Erethizontidae	2	2	–	90–583/–

(continued)

Table 1 (continued)

	# of species	Methane producer	Non-producer	Mean methane production Producer/non-producer[a]
Chinchilla lanigera	1	1	–	128/–
Caviinae	4	4	–	7–237/–
Dolichotis patagonum	1	1	–	25/–
Hydrochoerus hydrochaeris	1	1	–	311/–
Dasyproctidae	3	3	–	87–176/–
Octodontidae	2	2	–	2[b]–8/–
Capromys pilorides	1	1	–	28/–
Myocastor coypus	1	1	–	440/–
Lagomorpha	3	3	–	4[b]–42/–

For a more extended version of this table, see Hackstein and van Alen (1996)
[a]Range of the mean emissions of producers/mean emissions of non-producers as nmol methane/g faeces/h
[b]Classified as methane producer on the basis of their maximal emissions

amounts of methanogens in the GI tract. As already mentioned, it had been assumed that a plant-based diet rich in fibres provides the basis for the presence of high numbers of methanogens (Miller and Wolin 1986). This is clearly the case in ruminants such as cattle, sheep and goats. These animals, but also hindgut fermenters such as horse and elephant, produce high amounts of methane (Table 1). On the other hand, bamboo-eating pandas do emit only traces of methane, and also vegetarian chiropters do not produce methane. In contrast, carnivorous crocodiles, giant snakes and ant-eating species such as the great ant-eater, the tamandua, and the aardvark release large amounts of methane from their faeces; armadillos emit low, but still significant, concentrations of methane. Only one species of ant-eating animal, the pangolin, is a non-producer (Table 1). Therefore, a plant-based, fibre-rich diet cannot be the primary reason for the presence of large numbers of methanogens. Also the presence of a highly differentiated GI tract does not necessarily predispose for methane production: while African and South American ostriches do produce methane, their Australian/New Zealander relatives emu and cassowary, which possess a GI tract of similar complexity and which use a comparable diet, are non-producers (Table 1; Fig. 2). Also dolphins and whales, which possess complex foregut differentiations, do not produce methane (Table 1). Remarkably, Baleen whales, which prey on animals (fish and crustaceans), host a unique gut microbiome with similarities to both carnivores and herbivores (Sanders et al. 2015). Thus, neither the presence of a highly differentiated GI tract nor a fibre-rich diet predisposes automatically for the presence of high numbers of methanogens.

The just-mentioned example of the methane-producing and non-producing ostriches provides evidence for the intrinsic reasons for the presence/absence of

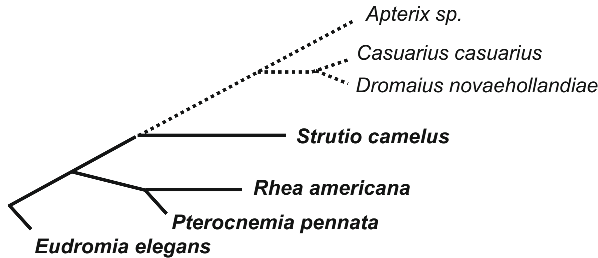


Fig. 2 Evolution of ratites: faeces of emu (*Dromaius novaehollandiae*), cassowary (*Casuarius casuarius*) and kiwi (*Apteryx* sp.) do not emit significant amounts of methane (max. 2 nmol/g/h). Faeces of ostrich (*Struthio camelus*), nandu (*Rhea americana*), Darwin nandu (*Pterocnemia pennata*) and tinamou (*Eudromia elegans*) produce between 137 and 414 nmol/g/h methane. The tree is based on mitochondrial 12S rDNA sequence data of Cooper et al. (1992). Redrawn after Hackstein and van Alen (1996)

methanogens. A phylogenetic tree of the ostriches based on the mitochondrial 12S rRNA genes (Fig. 2) reveals that these birds are monophyletic. The methane-emitting African/South American ostriches occupy a basal position in the phylogenetic tree, while the non-producing Australian/New Zealander ostriches and ratites are found in the top of the tree. The latter ostriches and ratites share a recent common ancestor, and this ancestor obviously lost the property to host methanogens, a property that is shared by all its descendants. This argues that a heritable, genetic property provides the basis for the presence of large numbers of methanogens in the GI tract. Also the study of South American apes supports this interpretation. While all old-world apes and monkeys are methane producers, the methane status of the New World apes can differ by species, even by subspecies. For example, the marmoset *Leontopithecus rosalia rosalia* produces only 1 nmol/g/h methane, whereas the closely related *Leontopithecus rosalia chrysomelas* is a producer of some 70–500 nmol/g/h faecal methane. Among the Callitrichidae (marmosets and tamarins), which include the two *Leontopithecus* species, four species are producers and six species are non-producers in our screen (Table 1; Hackstein and van Alen 1996). Moreover, seven species of monkeys belonging to the Cebidae produce large amounts of methane by their faeces, while two *Cebus* species emit only traces. This means that the property "methane production" can be lost at the species level in the absence of any significant differences in diet or other physiological parameters.

Also, among the Rodentia the character "methane production" can be lost at the species, subspecies or even at the population level. For example, 7 of the 17 species of sciurids studied produced methane, while 10 did not (Table 1). The species *Sciurus vulgaris* was identified as methane producer, but a highly inbred population of ten individuals did not produce methane. Since differences in the diet can be excluded, a dietary basis for the character "methane production" can be excluded.

This holds also true for the Muridae, where non-producers predominate. From the nine species of Cricetidae and Cricetomyinae studied, only three species (golden hamster, muskrat and giant pouched rat) emitted methane. From the seven species

belonging to the Murinae, only one, *Leopoldamys sabanus*, produced methane (Table 1). It has also been shown that certain strains of laboratory rat are methanogenic while other strains are non-methanogenic (Florin et al. 2000). In clear contrast, all species of carnivores, chiropters and insectivores did not produce methane irrespective of their diet, whereas all artiodactyls and perissodactyls studied produced methane. Also all caviomorphs, hystricomorphs (e.g. capybara) and lagomorphs tested produced methane (Table 1). In contrast, the beaver (also a rodent) produces only traces of methane. Illumina sequencing revealed that the archaeal community is very limited, with more than 99% of the archaeal sequences corresponding to a single species, *Methanosphaera stadtmanae* (Gruninger et al. 2016).

Thus, a direct correlation between diet and methane production can be excluded, and an alternative explanation for the significant differences found among the species examined seems difficult. Notably, a lack of infection by methanogens in "non-producers" can also be excluded since virtually all species tested produce at least traces of methane indicating the presence of methanogens (Table 1). However, if we incorporate the property "methane producer"/"non-producer" in a phylogenetic tree that is based on the analysis of mammalian protein sequences (Miyamoto and Goodman 1986), a rationale for the phenomenon "methane production" becomes evident (Fig. 3). Non-producers cluster as whole branches, or they are found in terminal positions of the tree. With other words, methane production is a primitive-shared (plesiomorphic) character, while the loss of methane production is a shared-derived (synapomorphic) character. The loss of methane production clearly obeys Dollo's rule: once lost in the course of evolution, the competence for methanogenesis cannot be restored. This holds also true for those losses at lower taxonomic levels (e.g. species level) that could not be included into the phylogenetic tree. The integration of the methane data into other phylogenetic trees, for example, the "classical" tree of Novacek (1992), or the molecular trees of Li et al. (1990) or of Janke et al. (1994) does not lead to a different interpretation. Consequently, the loss of the competence to host large numbers of methanogens is an evolutionary stable character that must have a heritable, genetic basis. This can also explain the loss of methanogenesis in populations where the Hardy-Weinberg equation describes the distribution of genetic characters. Evidence for the presence of producers and non-producers in a species has been described here for the species *Sciurus vulgaris* (Table 1) but are also well known for strains of laboratory rats and, notably, human populations (Miller and Wolin 1982; Segal et al. 1988; Brusa et al. 1993; Hudson et al. 1993; Doré et al. 1995; Florin et al. 2000; Levitt et al. 2006).

At the moment, one can only speculate about the physiological or biochemical basis for the presence of large amounts of methanogens in the guts and faeces of many animals and their significantly lower number in certain other species. Different levels of bile acids have been assumed as modulators of the methane production (Florin and Jabbar 1994), but a general function of bile acids as physiological control of methanogenesis seems unlikely. The presence of a receptor for methanogenic archaea or some other adherence mechanism could potentially explain the prolonged persistence of high numbers of methanogens in the gut. In the absence of the

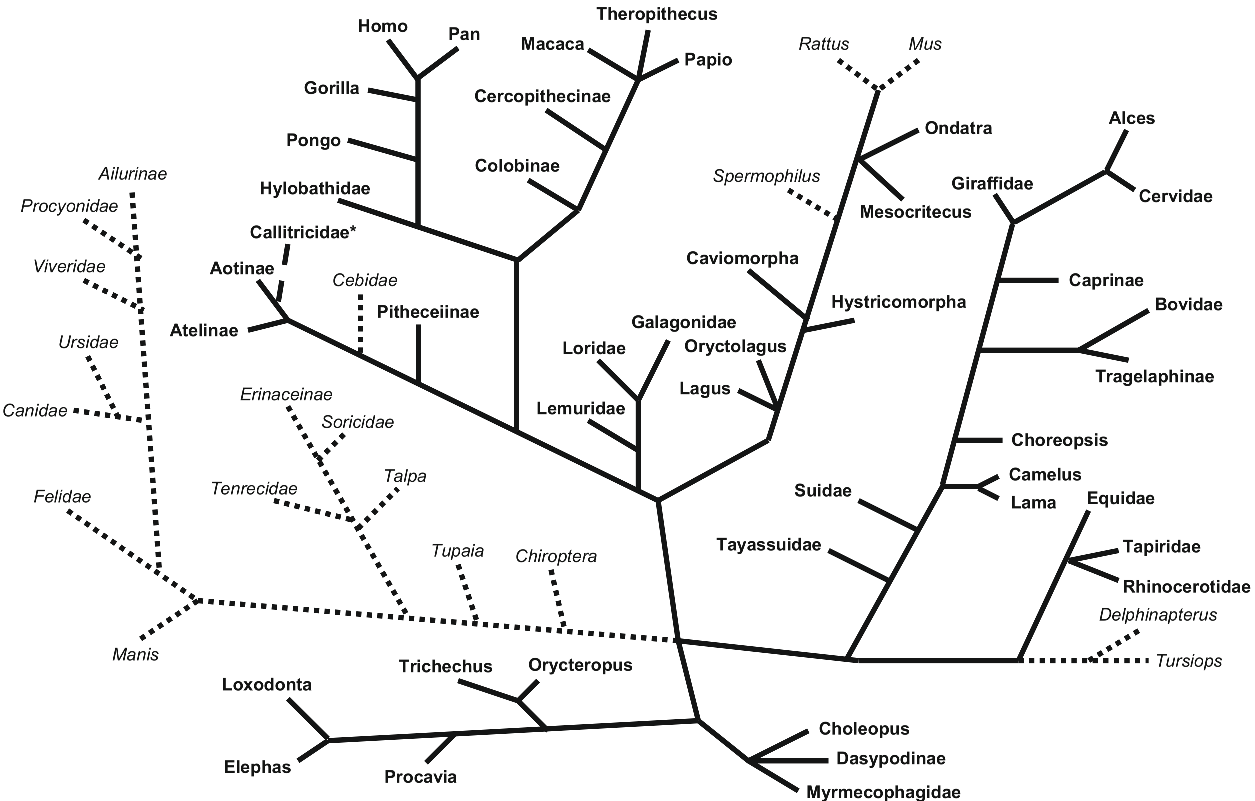


Fig. 3 Integration of information about methane production into the protein sequence-based phylogenetic tree of Miyamoto and Goodman (1986). Producers, roman; non-producers, italics; asterisk only four out of the ten species are methane producers. Redrawn after Hackstein and van Alen (1996)

receptor-mediated adhesion to the gut wall, methanogens are easily removed from the GI tract in the course of digestion, compensated only by high division rates of the methanogens. This might allow to maintain only titres of methanogens that are 30–100 times lower than in species with an adhesion mechanism for methanogens.

It is surprising that so many animals host high numbers of methanogens that cause a significant emission of methane. Most of these methanogenic animals possess "alloenzymatic" intestines that depend in their function on the presence of complex symbiotic microbial associations (Langer 1988, 1991, 1994; Langer and Snipes 1991). Alloenzymatic intestines are characterized by well-developed hindguts and caeca, but also by the evolution of rumina and other forestomach fermenting organs without rumination. Recently, the analysis of the microbiomes of the various animals has provided evidence for the existence of characteristic "foregut" and "hindgut" microbiota (Ley et al. 2008b) supporting the anatomical studies. Notably, there is evidence that the postnatal development of these differentiations depends on the presence of effective microbial fermentations, in particular the presence of certain fermentation products such as propionate and butyrate (Jesse et al. 1994). In contrast, "autoenzymatic" intestines do not have the need for microbial symbionts for their digestions, and consequently, they do not possess the differentiations that are characteristic for the alloenzymatic guts. However, they host-specific microbial communities that are characteristic for "simple" GI tracts (Ley et al. 2008b). Most of these intestines are found among the animals that belong to the category of "non-producers" of methane. This does not mean that monogastric, autoenzymatic intestines are devoid of any significant microbiota. Our measurements have shown that virtually all of the non-producers of methane emit significant amounts of hydrogen (Hackstein and van Alen 1996), which is indicative of intensive microbial fermentations. But as mentioned above, these fermentations do not contribute to the digestion of resistant biopolymers, and they do not induce any of the intestinal differentiations that are found in the methanogenic animals with alloenzymatic digestion. Even a fibre-rich diet of a non-methanogenic animal does not correlate with any alloenzymatic differentiation of the GI tract. The best example is the giant panda, which relies completely on a bamboo diet, but does not possess any fermentative intestinal differentiation. His gut microbiome classifies his GI tract as belonging to the "simple" type (Ley et al. 2008b). Notably, evolution allowed the development of panda's famous additional thumb, but not of a well-developed caecum or colon. On the other hand, the leaf-eating, methanogenic colobid monkeys evolved a foregut fermenting structure similar to the rumen of the ruminants with a microbiome that resembles that of ruminants (Ley et al. 2008a, b).

A secondary loss of methanogenesis is possible in alloenzymatic animals with foregut differentiations, e.g. dolphins and whales (Fig. 3), but also in animals with hindgut differentiations, e.g. many murids and certain New World monkeys and apes. Monogastric, autoenzymatic animals are primarily non-methanogenic (Fig. 3). The correlation between the presence of high numbers of methanogens and the

presence of intestinal differentiations is striking and for sure not accidental. Obviously, methanogens fulfil a crucial role in intestinal fermentations that allow the digestion of plant polymers (Schink 1997; Stams and Plugge 2009). It is likely that this role cannot be taken over by other hydrogen-consuming bacteria such as sulphate-reducing bacteria (Hansen et al. 2011). Notably, the presence of high numbers of methanogens must be controlled by one or several genetic factors, since the evolutionary loss of the capacity to host high numbers of methanogens cannot be restored. Also the population-specific distributions of methane producers and non-producers in human populations are indicative of a genetic basis (Segal et al. 1988; Hudson et al. 1993; Brusa et al. 1993; Levett et al. 2006). Furthermore, the analysis of the trait "methane production" in pedigrees reveals inheritance patterns that are compatible with the interpretation of an autosomal dominant inheritance (Hackstein et al. 1995). Twin studies that rejected a genetic influence on the methane status of humans might be erroneous due to a statistical analysis that seems not suitable for the detection of different classes of methane producers/non-producers (Florin et al. 2000). Moreover, the study was predominantly performed with adolescent twins who are known to be problematic. A recent study with adult monozygotic vs. dizygotic twins revealed a twin pair concordance of 74% for carriage of methanogens (Hansen et al. 2011). It has also been discussed that a lack of proper infection with methanogens can be excluded for the explanation of the bimodal distribution of producers and non-producers, since also non-producers exhibit a low level of methanogenesis in their faeces. Thus, the presence of high numbers of methanogens in the GI tract of vertebrates is still elusive but obviously under the control of one or several genetic factors. It is for sure not the consequence of particular dietary habits or the presence of intestinal differentiations.

3 Conclusions: Vertebrates

Most, but not all, methane producers have a plant-based diet. In contrast, there are animals with a high protein diet that emit methane. Also, certain non-producer species (and their relatives) have a plant-based diet, and most animals with a high protein diet do not emit methane. Remarkably, there are individuals of otherwise methanogenic animal species that have lost their capacity of methane production. Certain individuals can be non-methanogenic, while other individuals of the same species produce methane. Problems with methanogen infections are unlikely because virtually all (non-methanogenic) animals studied here host at least minor amounts of methanogens. Therefore, the distribution of methane producers/non-producers is likely to rely on the genetic background—and not on diet, behaviour and infection problems.

4 Arthropods

Arthropods represent by far the largest global biodiversity of all multicellular animals. Despite their small size and the tiny volumes of their intestinal tracts, many arthropods host a complex microbiota in their guts (Bayon 1980; Hackstein and Stumm 1994; Cazemier et al. 1997; Hackstein 1997; Egert et al. 2003; Brune 2006; Hackstein et al. 2006b; Warnecke et al. 2007). Already in 1953, Paul Buchner in his seminal monograph (Buchner 1953) described the fascinating world of symbiotic associations between arthropods and bacteria. He emphasized not only the enormous diversity of differentiations of the intestinal tract, but also the more direct associations between bacteria and their hosts involving specialized tissues and organs (e.g. "bacteriomes"). There were a lot of speculations about the contribution of the symbionts to the host's nutrition, but only recently the progress in molecular biological techniques and bioinformatics allowed unravelling of the molecular basis of some of these symbiotic associations (Moran 2003, 2007; Moran and Baumann 2000; Hoffmeister and Martin 2003; Canback et al. 2004; Dillon and Dillon 2004; Dale and Moran 2006; Moya et al. 2008; Ruby 2008; Gil et al. 2018). In remarkable contrast to the situation in vertebrates, the role of methanogenic archaea is very limited in the arthropod world. While nearly all vertebrates host at least traces of methanogens, the vast majority of the arthropod taxa is completely devoid of methanogens. In principle, the detection of methanogens in the GI tract of arthropods is easy, since arthropods exhale intestine-born methane with their breath (Bijnen et al. 1996). Due to their small size, the methane production of arthropods can be measured non-invasively by incubating the intact specimen in stoppered glass vials. With a standard gas chromatograph, it is possible to detect sub-nanomolar concentrations of methane after the prolonged incubation of individual or several specimen. That way, the presence of less than 10^6 methanogens in the GI tract of a single arthropod can be detected. Moreover, epifluorescence microscopy allows the unequivocal identification of single methanogens.

In a first experiment, we screened more than 110 representatives of 35 higher taxa of terrestrial arthropods for methane and hydrogen emissions (Hackstein and Stumm 1994; Table 2). In a second experiment, we analysed some 70 strains of cockroaches representing 44 different species (Hackstein 1997; Hackstein et al. 2006b; Table 3). To confirm the presence or absence of methanogens in the GI tracts, individual arthropods were dissected and subjected to an analysis with the aid of epifluorescence microscopy (Hackstein and Stumm 1994). Epifluorescence microscopy allows the detection of individual methanogenic archaea due to their blue F_{420} autofluorescence that is characteristic for methanogens (Doddema and Vogels 1978; Figs. 4 and 5).

Our analysis revealed that only representatives of 4 out of the 35 taxa studied emitted methane: millipedes, cockroaches, termites and scarab beetles. All other species did not produce methane, but sometimes hydrogen instead (Table 2). Also the microscopical inspection did not provide evidence for the presence of any methanogen in the arthropods belonging to a non-methanogenic taxon (Hackstein

Table 2 Methane and hydrogen production in invertebrates

	Common name	Methane	Hydrogen	Protists
Araneae	Spiders			
Araneus diadematus[a] (A)		–	+	–
Acari	Mites and ticks			
Boophilus microplus		–	–	–
Isopoda	Sow bugs			
Oniscus asellus[a] (A)		–	–	–
Porcellio scaber (A)		–	–	–
Chilopoda	Centipedes			
Lithobius forficatus (A)		–	–	–
Diplopoda	Millipedes			
Chicobolus sp. (J)		+	+	–
Mestosoma hylaeicum (A)		–	–	–
Orthoporus sp. (J)		+	+	C
Pycnotropis acuticollis (A)		+	+	nd
Rhapidostreptus virgator (A)		+	+	C
Unidentified A (J)		+	+	–
Unidentified B (J)		+	+	–
Unidentified D (J)		+	+	C
Unidentified K (J)		+	+	C
Glomeris sp.[a] (A)		–	+	–
Julus sp.[a] (A)		–	–	–
Polydesmus sp.[a] (A)		–	–	–
Tachypodoiulus niger[a] (A)		–	–	–
Thysanura	Bristletails			
Lepisma saccharina (A)		–	–	–
Collembola	Springtails			
Folsomia candida (J, A)		–	–	–
Acrididae	Short-horned grasshopper			
Locusta migratoria (A)		–	–	–
Schistocerca gregaria (A)		–	–	–
Unidentified[a] (A)		–	–	–
Gryllidae	Crickets			
Acheta domesticus (A)		–	–	–
Decticus sp.[a] (A)		–	–	–
Gryllus bimaculatus (A)		–	–	–
Vetralla quadrata (A)		–	–	–
Phasmidae	Stick and leaf insects			
Eurycantha calcarata (A)		–	–	–
Pharnacia acanthopus (A)		–	–	–
Sipyloidea sipylus (A)		–	–	–
Mantidae	Mantids			
Hierodula membranacea (A)		–	–	–

(continued)

Table 2 (continued)

	Common name	Methane	Hydrogen	Protists
Blattidae	Cockroaches			
Blaberus craniifer (A)		+	+	C
Blaberus fuscus (L, A)		+	–	C
Blaberus giganteus (L)		+	+	C
Blatta orientalis (A)		+	+	–
Blattella germanica (A)		+	+	–
Ectobius sp.[a] (A)		–	–	–
Gromphadorhina portentosa (L, A)		+	+	C
Leucophaea sp. (A)		+	+	–
Panchlora nivea (A)		–	–	–
Periplaneta americana (L, A)		+	–	C
Periplaneta australasia (L, A)		+	+	C
Pycnoscelus surinamensis (L, A)		+	+	C
Supella supellectilium (L, A)		+	–	F
Isoptera	Termites			
Cryptotermes brevis (A)		+	–	nd
Heterotermes indicola (A)		+	–	F
Mastotermes darwiniensis (A)		+	–	F
Reticulitermes santonensis (A)		+	–	F
Dermaptera	Earwigs			
Forficula auricularia[a] (A)		–	–	–
Heteroptera	Bugs			
Dysdercus intermedius (L, A)		–	+	–
Oncopeltus fasciatus (L, A)		–	–	–
Platymeris biguttatus (A)		–	–	–
Pyrrhocoris apterus (L, A)		–	–	–
Cicadoidea	Cicadas			
Nephotettix cincticeps (A)		–	–	–
Aphididae				
Aphis fabae (L, A)		–	–	–
Apidae				
Apis mellifera (A)		–	–	–
Carabidae	Ground beetles			
Carabus sp.[a] (A)		–	–	nd
Pterostichus niger[a] (A)		–	–	nd
Silphidae	Carrion beetles			
Nicrophorus vespillo[a] (A)		–	–	nd
Dermestidae	Dermestid beetles			
Dermestes frischi (A)		–	–	nd
Tenebrionidae	Darkling beetles			
Oryzaephilus sp. (L, A)		–	–	nd
Scarus tristis (L)		–	–	nd

(continued)

Table 2 (continued)

	Common name	Methane	Hydrogen	Protists
Tenebrio sp. (L)		–	–	nd
Tribolium confusum (L, A)		–	–	nd
Zophobas morio (L, A)		–	–	nd
Cryptophagidae	Silken fungus beetles			
Alphitobius diapecurius (L, A)		–	–	nd
Bostrichidae	Branch and twig borers			
Acanthoscelides panaceae (L, A)		–	–	nd
Rhyzopertha dominica (L, A)		–	–	nd
Sitophilus granarius (L, A)		–	–	nd
Anobiidae	Death-watch beetles			
Anobium punctatum (L)		–	–	nd
Oligomerus ptilinoides (L)		–	–	nd
Ptilinus pectinicornis (L)		–	–	nd
Stegobium paniceum (A)		–	–	nd
Xestobium rufovillosum (L)		–	–	nd
Lyctinae	Powder-post beetles			
Lyctus africanus (L)		–	–	nd
Lyctus brunneus (L)		–	–	nd
Minthea rugicollis (L)		–	–	nd
Dynastinae	Rhinoceros beetles			
Dynastes hercules (L)		+	+	F
Cetoniinae	Rose chafers			
Cetonia aurata (L)		+	+	F
Dicronorhina micans (L)		+	+	F
Eudicella gralli (L, A)		+	+	F
Eudicella smithii (L)		+	+	F
Pachnoda bhutana (L, A)		+	+	F
Pachnoda ephippiata (L)		+	+	F
Pachnoda marginata (L)		+	–	F
Pachnoda nachtigali (L, A)		+	–	F
Pachnoda savignyi (L, A)		+	–	F
Potosia cuprea (L, A)		+	+	F
Phyllopertha horticola[a] (A)		–	–	–
Geotrupinae	Dung beetles			
Geotrupes sp.[a] (A)		–	+	nd
Geotrupes sp.[a] (A)		–	–	nd
Cerambycidae	Longhorn beetles			
Hylotrupes bajulus (L)		–	–	–
Chrysomelidae	Leaf beetles			
Crioceris asparagi[a] (A)		–	–	nd
Diabrotica balteata (A)		–	–	nd
Leptinotarsa decemlineata (A)		–	+	nd

(continued)

Table 2 (continued)

	Common name	Methane	Hydrogen	Protists
Phaedon cochleariae (L, A)		–	+	nd
Curculionidae	Weevils			
Otiorhynchus sulcatus (A)		–	+	nd
Lepidoptera	Butterflies and moths			
Aphomia sociella[a] (L)		–	+	–
Bombyx mori (L)		–	–	nd
Caligo memnon (L)		–	–	nd
Danaus plexippus (L)		–	+	nd
Ephestia kühniella (L)		–	–	nd
Galleria mellonella[a] (L, A)		–	–	–
Heliothis virescens		–	–	nd
Pieris brassicae[a] (L)		–	–	nd
Plutella xylostella (L)		–	–	nd
Spodoptera frugiperda (L)		–	–	nd
Trabala vishnou (L)		–	–	nd
Diptera	Flies			
Hylemya antiqua (L)		–	–	–
Musca domestica (P, A)		–	–	–
Tipula sp.[a] (L)		–	+	–
Siphonaptera	Fleas			
Ctenocephalides felis (L)		–	–	nd

nd not determined, *C* ciliates, *F* flagellates, *L* larva, *A* adult
[a]Endemic European species from the field

and Stumm 1994). A correlation with the diets of various arthropods was not evident since even species with a strong plant- and fibre-rich diet were completely non-methanogenic (e.g. crickets, locusts, stick insects). A size factor could also be excluded since even tiny larvae of methanogenic species with a gut volume of about 1 μl emitted methane. Thus, as in vertebrates, methane production is characteristic for certain taxa and, therefore, controlled by an intrinsic, hereditary, genetic property of the host. This assumption is supported by the observation that certain species of cockroaches (which belong to a methanogenic taxon) have lost the capacity to host methanogens. These species belong predominantly to the Blattellinae and Plectopterinae (Table 3). Even non-methanogenic strains of *Blattella germanica* and *Periplaneta americana* were found. These non-methanogenic strains could be transiently infected with methanogens by co-culture with methanogenic species. However, soon after the removal of the donor insects, the methanogens in the recipients were lost. This means that these non-methanogenic strains had definitively lost the capacity to maintain permanently methanogens in their GI tract. Trials to infect non-methanogenic species that belong to a non-methanogenic taxon were unsuccessful: sow bugs and crickets could not be infected with methanogens—not even transiently (Hackstein and Stumm 1994; Hackstein et al. 1996).

Table 3 Methane emission in cockroaches

Species	Methane emissions	Hindgut differentiation	Protists in hindgut
Blattoidea			
Blattidae			
Blatta orientalis	+	+	C
Deropeltis sp.	+	+	C
Periplaneta americana	+	+	C
Periplaneta australasiae	+	+	C
Periplaneta brunnea	+	+	C
Periplaneta fulginosa	+	+	C
Polyzosteriinae			
Eurycotis floridana	+	+	C
Blaberoidea			
Polyphagidae			
Polyphaginae			
Polyphaga aegyptiaca	+	+	C
Blattellidae			
Plectopterinae			
Eudromiella sp. (Costa Rica)	–	–	–
Lupparia sp. (Luzon, Philippines)	–	–	–
Supella longipalpa	–	–	–
Supella supellectilium	+	+	F
Blattellinae			
Blattella germanica	+ and –	+/–	–
Ischnoptera sp.	nd	–	–
Loboptera decipiens	nd	–	–
Parcoblatta lata	+	nd	nd
Shawella couloniana	–	–	–
Symploce pallens	–	nd	nd
Ectobiinae			
Ectobius sylvestris	–	–	–
Ectobius sp.	–	–	–
Nyctiborinae			
Nyctibora sp. (Costa Rica)	+	+	–
Blaberidae			
Blaberoid complex			
Zetoborinae			
Schultesia lampyridiformis	+	+	–
Blaberinae			
Archimandrita sp.	+	+	–
Blaberus craniifer	+	nd	nd
Blaberus fuscus	+	+	C
Blaberus discoidalis	+	+	C

(continued)

Table 3 (continued)

Species	Methane emissions	Hindgut differentiation	Protists in hindgut
Blaberus giganteus	+	+	C
Blaberus sp. *CR*	+	nd	nd
Byrsotria fumigata	+	+	C
Eublaberus distanti	+	+	–
Eublaberus posticus	+	+	–
Blaptica sp.	+	+	C+F
Panchloroid complex			
Pycnoscelinae			
Pycnoscelus surinamensis	+	+	C+F
Diplopterinae			
Diploptera punctata	+	+	C
Panchlorinae			
Panchlora nivea	–	–	–
Oxyhaloinae			
Gromphadorhina chopardi	+	+	C
Gromphadorhina portentosa	+	+	C
Leucophaea maderae	+	+	–
Nauphoeta cinerea	+	+	F
Gen. near Griffiniella	+	+	C
Epilamproid complex			
Epilamprinae			
Rhabdoblatta sp.	+	+	–

Hindgut differentiations—presence of an enlarged, well-differentiated hindgut
nd not determined, *C* ciliates, *F* flagellates

Therefore, we analysed the GI tract and the intestinal surfaces in more detail. The complete GI tracts of the arthropods were dissected and studied by epifluorescence, phase contrast and differential interference contrast (DIC) microscopies. Arthropod guts are clearly compartmentalized in the anterior-posterior direction. In general, it is possible to identify an oesophagus, crop, midgut and hindgut (Dettner and Peters 2003). The intestinal tract of millipedes and cockroaches is structured relatively simple (Fig. 6a), while the GI tract of cetonid and scarabaeid larvae is dominated by a voluminous midgut and hindgut (Fig. 6c). The GI tract of termites, especially of humivorous species, is highly structured and consists of compartments with a very variable pH value (Fig. 6b). Notably, in all methanogenic arthropods studied so far, the methanogens are restricted to the hindgut (Hackstein and Stumm 1994), also the methanogens, which are associated with anaerobic, gut-dwelling protozoa. Only parasites, such as gregarines (lacking methanogenic endosymbionts), can be found in the midgut, which, however, in the methanogenic arthropods hosts a complex and numerous microbiota of (facultatively) anaerobic bacteria. The strongly alkaline pH in the midgut of humivorous insects could explain the absence of methanogens and symbiotic protozoa, which depend on habitats with a moderate, near neutral pH as

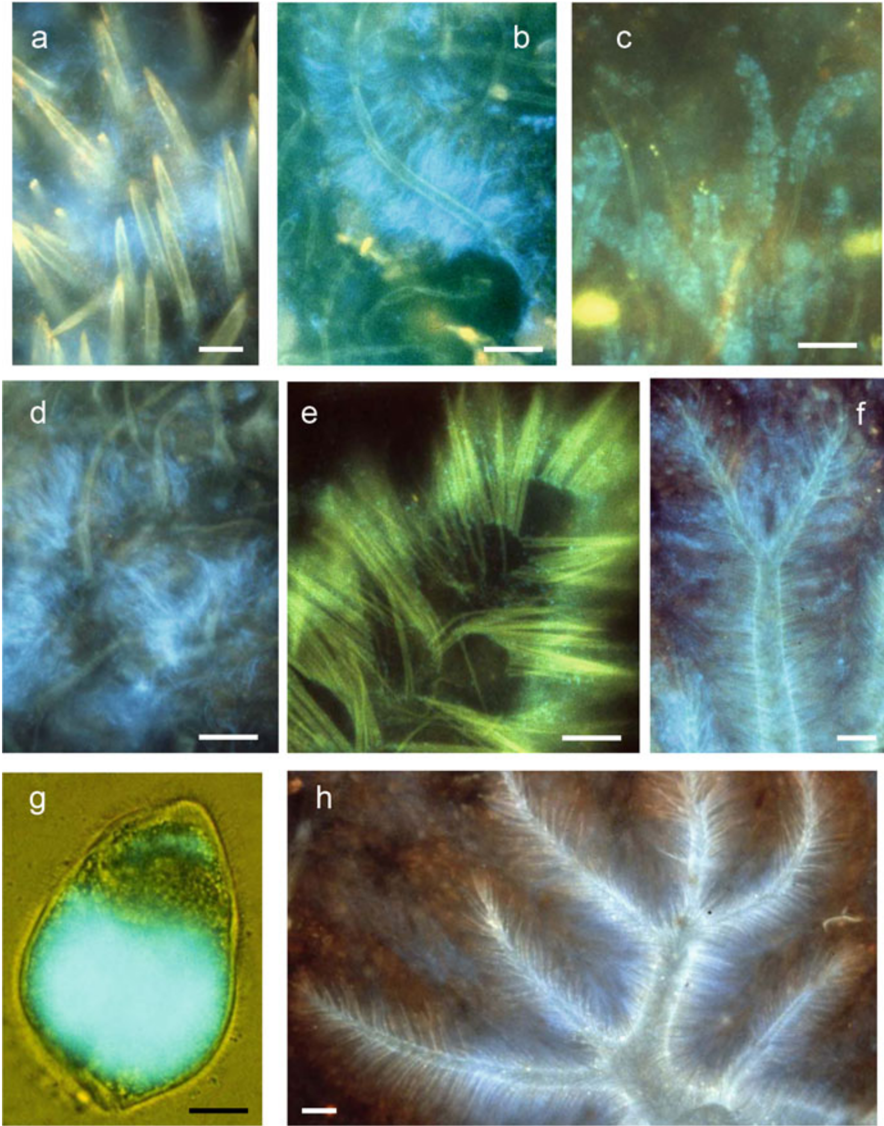


Fig. 4 Methanogenic archaea in the hindguts of the various arthropods, detected by fluorescence microscopy. The blue autofluorescence caused by the cofactor F_{420} indicates the presence of methanogens. The yellowish/greenish fluorescence originates from the chitinous cuticular structures of the arthropod hosts. (**a**) Filamentous methanogens loosely associated with cuticular hairs in the anterior part of the hindgut of the cockroach *Diploptera punctata.* Bar: 10 µm. (**b**) Filamentous methanogens adhering with their tips to cuticular hairs of the hindgut of the cockroach *Nauphoeta cinerea.* Bar: 10 µm. (**c**) Coccoid methanogens closely associated with cuticular hairs of the hindgut of the cockroach *Leucophaea maderae.* Bar: 10 µm. (**d**) Filamentous methanogens in the posterior hindgut of *Diploptera punctata.* There is no evidence for a close association with cuticular hindgut structures. Bar: 10 µm. (**e**) Small, coccoid methanogens between cuticular hairs (yellowish

found in the hindguts. However, the midguts of cockroaches possess a more or less neutral pH. Nevertheless, methanogens are completely absent from this compartment. Whether the peritrophic membrane (Dettner and Peters 2003), which wraps the gut contents during their passage through the anterior parts of the GI tract, prohibits the colonization by methanogens remains unclear. However, it is noteworthy to mention that the peritrophic membrane becomes disintegrated in hindgut.

The methanogens occur free floating in the hindgut lumen, attached to food particles, adhering to the gut wall, or as endosymbionts of protists. In certain insects, cuticular differentiations such as trichomes or complex epithelial differentiation of the gut wall (e.g. "pseudosetae", Figs. 4f, h, 7, and 8) enlarge the inner surface of the hindgut by several orders of magnitude. These structures provide attachment sites for a complex microbiota, which includes methanogens as a dominant component (Figs. 4f, h and 7). However, the presence of such elaborated differentiations of the gut wall does not per se enable the colonization by methanogens. Also non-methanogenic insects possess such structures—without any trace of a methanogenic archaeon (Fig. 5a, b). Notably anaerobic protozoa with endosymbiotic methanogens were also found exclusively in the hindgut of many (but not all) methanogenic arthropods (Figs. 4g and 5d–h). Such protozoa were never found in the GI tract of non-methanogenic animals.

The morphology of both the intestinal and the endosymbiotic methanogens is rather variable, suggesting the presence of various species of methanogens. Only three species from termite guts and one from a cockroach gut have been isolated and cultured in vitro (Leadbetter and Breznak 1996; Leadbetter et al. 1998; Sprenger et al. 2000). All four species of methanogens adhere to the internal surface of the hindgut. PCR- and T-RFLP-guided profiling studies in termites and cetonids confirmed the anticipated diversity of intestinal archaea, which are clearly different from non-gut communities (Ohkuma et al. 1995, 1999; Shinzato et al. 1999, Tokura et al. 2000; Brauman et al. 2001; Friedrich et al. 2001; Egert et al. 2003; Donovan et al. 2004; Miyata et al. 2007). Also the *Nyctotherus*-like ciliates from the hindgut of methanogenic cockroaches and millipedes and their methanogenic endosymbionts are different at the 16S rDNA level from each other and from free-living gut methanogens (van Hoek et al. 1998, 2000). The endosymbiotic methanogens are similar to but distinct from gut-dwelling *Methanobrevibacter* species. The free-living relatives of *Nyctotherus* host different methanogens belonging to the

Fig. 4 (continued) autofluorescence) covering the hindgut of *Nyctibora* sp. Note that many methanogens are found at the basis of the hairs, adhering to the cuticle of the hindgut, at a distance of only a few micrometres to the tracheoles, which support aerobic mitochondrial metabolism in the hindgut epithelium. Bar: 10 µm. (**f**) Coccoid methanogens closely associated with a "pseudoseta" from the hindgut of a larva of the scarab beetle *Pachnoda marginata*. Bar: 10 µm. (**g**) An anaerobic nyctotheroid ciliate from the hindgut of the cockroach *Byrsotria fumigata*. Note the intensive autofluorescence of F_{420} originating from endosymbiotic methanogens. Bar: 10 µm. (**h**) Filamentous methanogens closely associated with a pseudoseta from the hindgut of a larva from the scarab beetle *Pachnoda bhutana*. Bar: 10 µm. Reproduced with permission from Hackstein et al. (2006b)

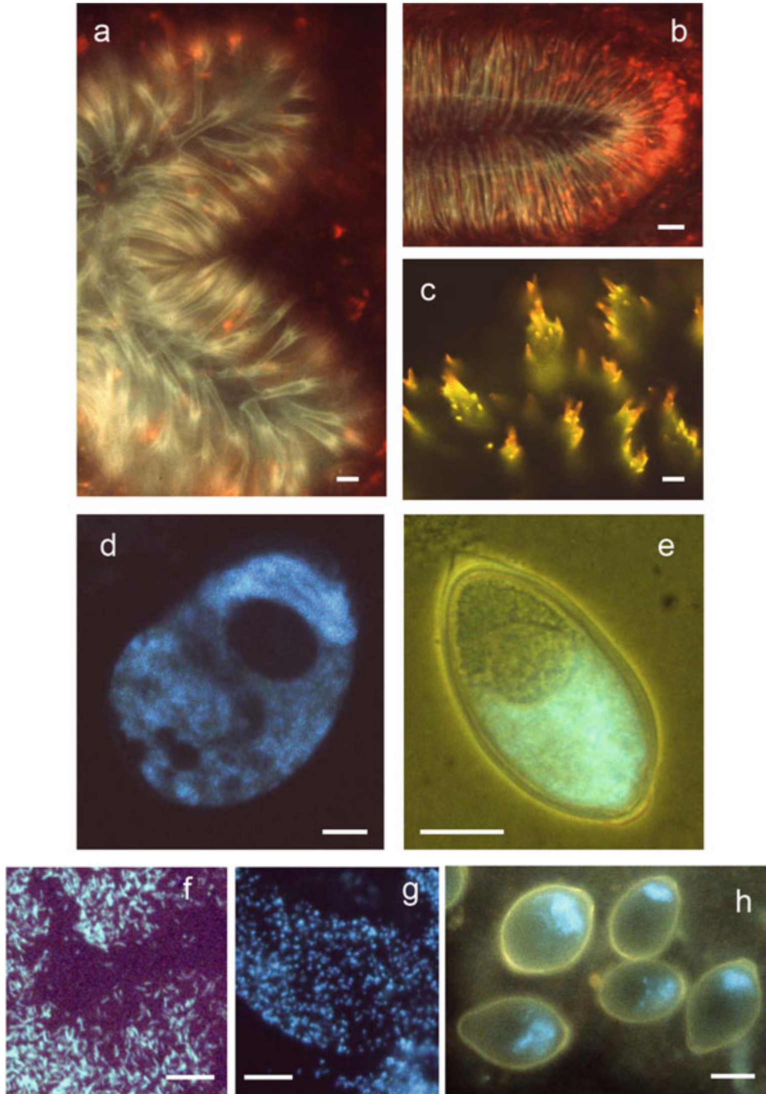


Fig. 5 (**a**) and (**b**) Differentiations of the hindgut epithelium of short-horned grasshoppers and crickets. Note the complete absence of blue-fluorescing methanogens. Both taxa do not produce methane. (**a**) *Phaeophylacris bedoides,* a cave-dwelling cricket (bar 10 μm). (**b**) Unidentified, European short-horned grasshopper (bar 10 μm). (**c**) Cuticular structures at the junction between midgut and hindgut of the cockroach *Rhabdoblatta* sp. These chitinous bracts are likely to have a function in disrupting the peritrophic membrane before the gut contents enter the hindgut (bar 10 μm). (**d**) An anaerobic nyctotheroid ciliate from the hindgut of the cockroach *Deropeltis* sp. The blue autofluorescence stems from numerous endosymbiotic methanogens. The dark spot identifies the location of the macronucleus, which does not contain methanogens (bar 10 μm). (**e**) Cyst (resting stage) of the ciliate shown in Fig. 4g (i.e. from the hindgut of the cockroach *Byrsotria fumigata*). The blue autofluorescence discloses the presence of methanogens also in cysts (bar 10 μm). (**f**) and (**g**) Endosymbiotic methanogens from ciliates thriving in the hindgut of the cockroach *Periplaneta americana* (strain Amsterdam) (**f**) and the cockroach *Blaberus* sp. (strain

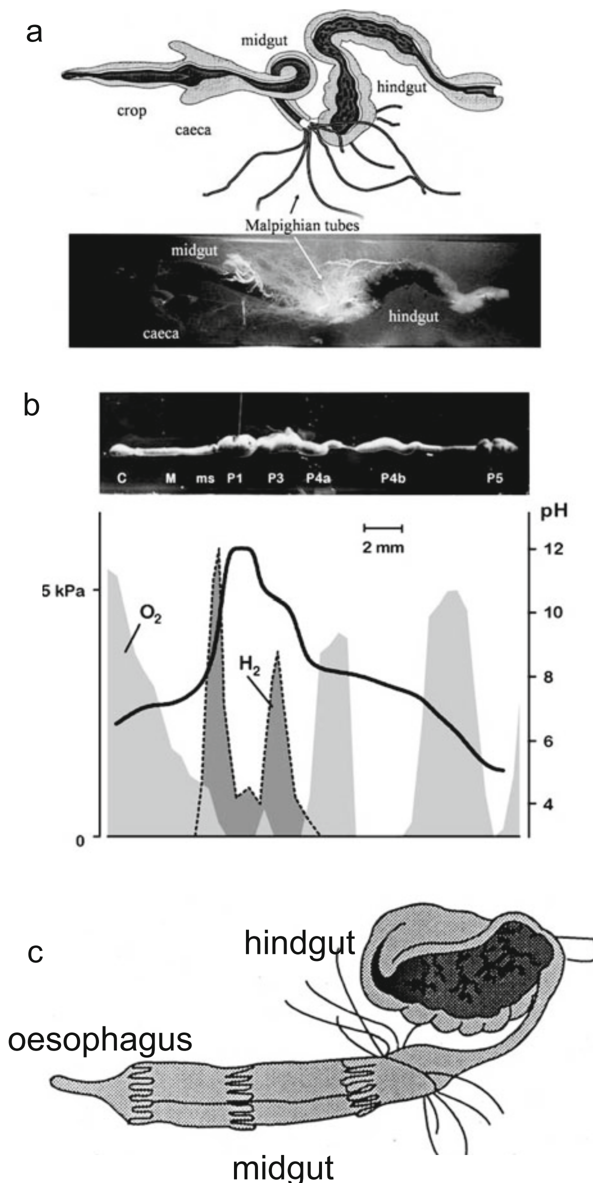


Fig. 6 Macroscopical views of the intestinal tract of cockroaches (**a**), termites (**b**) and larvae of scarab beetles (**c**). (**a**) Above: cartoon of the intestinal tract of a cockroach (*Periplaneta americana*). Below: a picture of a gut of *Periplaneta americana,* which has been embedded into agarose for microsensor measurements (after removal of the crop). (**b**) The unravelled intestinal tract of a termite (*Cubitermes* sp.) to demonstrate the complex longitudinal compartmentalisation of the termite gut. A microsensor is inserted into compartment P1. The plot below displays the longitudinal variations in pH (solid line) and the partial pressures of O_2 and H_2. C, crop; M, midgut; ms, mixed segment; P1–P5, proctodeal regions. Reproduced with permission from Brune and Friedrich (2000). (**c**) A cartoon demonstrating the gross organization of the intestinal tract of the larva of the scarab beetle *Pachnoda* sp. The midgut is highly alkaline. The interior of the hindgut is shown to indicate the location of the pseudosetae (black structures; c.f. Figs. 4f, h and 7). Reproduced with permission from Hackstein et al. (2006b)

Fig. 5 (continued) Amsterdam) (**g**). The methanogens were released from the ciliates by gentle squashing. Note the different shapes of the methanogens (bar 5 μm). (**h**) Cysts of ciliates from the hindgut of a cockroach belonging to the Oxyhaloinae (genus near *Griffiniella*) containing endosymbiotic methanogens (blue autofluorescence) (bar 10 μm). Reproduced with permission from Hackstein et al. (2006b)

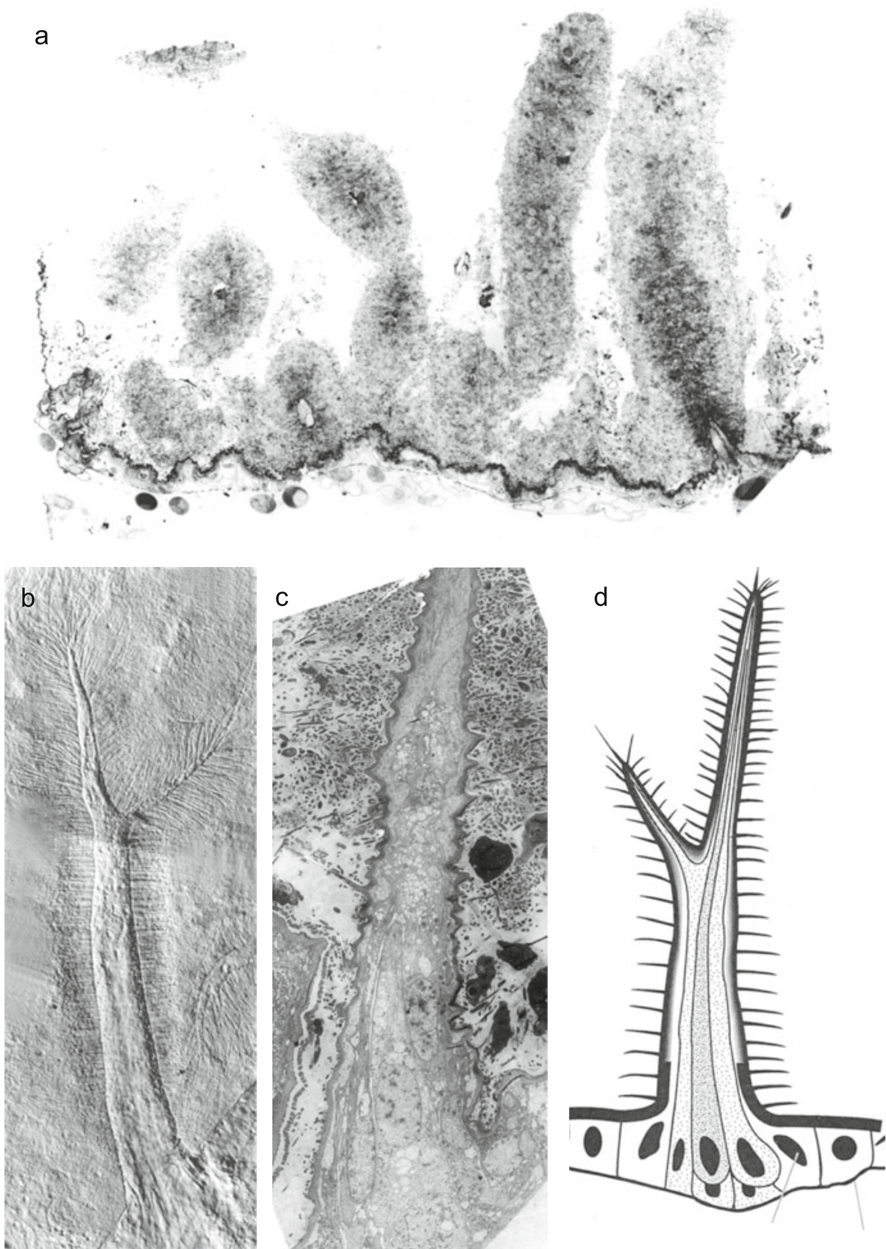


Fig. 7 (**a**) Light micrograph (semi-thin section) of the hindgut epithelium of a larva of *Dynastes hercules* (Scarabaeidae). Villus-like structures, measuring between 200 and 500 μm, protrude into the lumen of the hindgut. These structures, which we have named "pseudosetae", are composed of several elongated cells of the hindgut epithelium and covered by a complex prokaryotic microbiota,

Methanomicrobiales (van Hoek et al. 2000). The ciliates and their endosymbionts predominantly co-speciate suggesting a vertical inheritance of the endosymbionts. The exceptions from the co-speciation argue for infrequent endosymbiont replacements (van Hoek et al. 2000; Hackstein et al. 2002).

The adherence of the methanogens to the internal surfaces and the supporting structures of the hindgut might explain the persistence of methanogens in the arthropod guts. It is conceivable that their adherence is under genetic control explaining the occurrence of methanogens in certain taxa, their absence in other taxa and the non-methanogenic strains of otherwise methanogenic arthropods.

The adherence of the methanogens to the gut wall and the small size of the guts create a problem for the survival of the methanogens. Methanogens are strictly anaerobic (Liu and Whitman 2008), but at the gut wall, they experience a continuous influx of oxygen. Due to their small size, arthropod guts possess a large surface to volume ratio (Brune and Friedrich 2000; Hackstein et al. 2006b) that makes it difficult to maintain anaerobic conditions inside the gut. However, aerobic and facultatively anaerobic bacteria located close to the gut wall sequester the oxygen and generate a steep oxygen gradient across the gut wall with the consequence that the lumen, but not the wall of the gut, becomes completely anaerobic. In lower termites, the centre of the gut is populated by flagellates that generate hydrogen, which accumulates in the centre of the gut and diffuses outwards through the gut wall, where methanogens and other bacteria create a hydrogen sink (Fig. 9a). Thus, in lower termites the methanogens at the gut wall occupy a position between an inside-directed oxygen gradient and an outside-directed hydrogen gradient. In cockroaches, there is no outside-directed hydrogen gradient, since the gut ciliates possess endosymbiotic methanogens and since the free-living methanogens are more evenly distributed throughout the hindgut (Fig. 9b). Nevertheless, also in cockroaches, the hindgut microbiota generate a steep oxygen gradient and produce hydrogen in the lumen of the gut that is consumed in situ by interspecies hydrogen transfer. The lack of accumulation of hydrogen even after incubation of hindguts in a hydrogen atmosphere indicates that hydrogen is limiting methanogenesis in the hindgut (Fig. 9b).

Interestingly, we detected hydrogen emissions in many of the methanogenic species in our screen (Table 2; Hackstein and Stumm 1994). The explanation for

Fig. 7 (continued) including methanogens (c.f. Fig. 4f, h). (**b**) Light micrograph (differential interference contrast) of a single pseudoseta from the hindgut of a *Pachnoda marginata* (Scarabaeidae) larva after the removal of the bacteria adhering to this structure. The surface of the pseudoseta is covered with a cuticle, which carries numerous hairs (trichomes) enhancing the surface by about two orders of magnitude. Sizes 100–300 μm. (**c**) and (**d**) Electron micrograph (**c**) and cartoon (**d**) of a single pseudoseta. Note that tracheae and tracheoles as well as mitochondria are lacking in the distal parts of the pseudosetae. The vacuoles are most likely involved in the transport of fermentation products (mainly short chain fatty acids) generated in the lumen of the hindgut to the hindgut epithelium and eventually to the haemolymph. Black ovals in D indicate the nuclei of the hindgut epithelium and the pseudoseta. Reproduced with permission from Hackstein et al. (2006b)

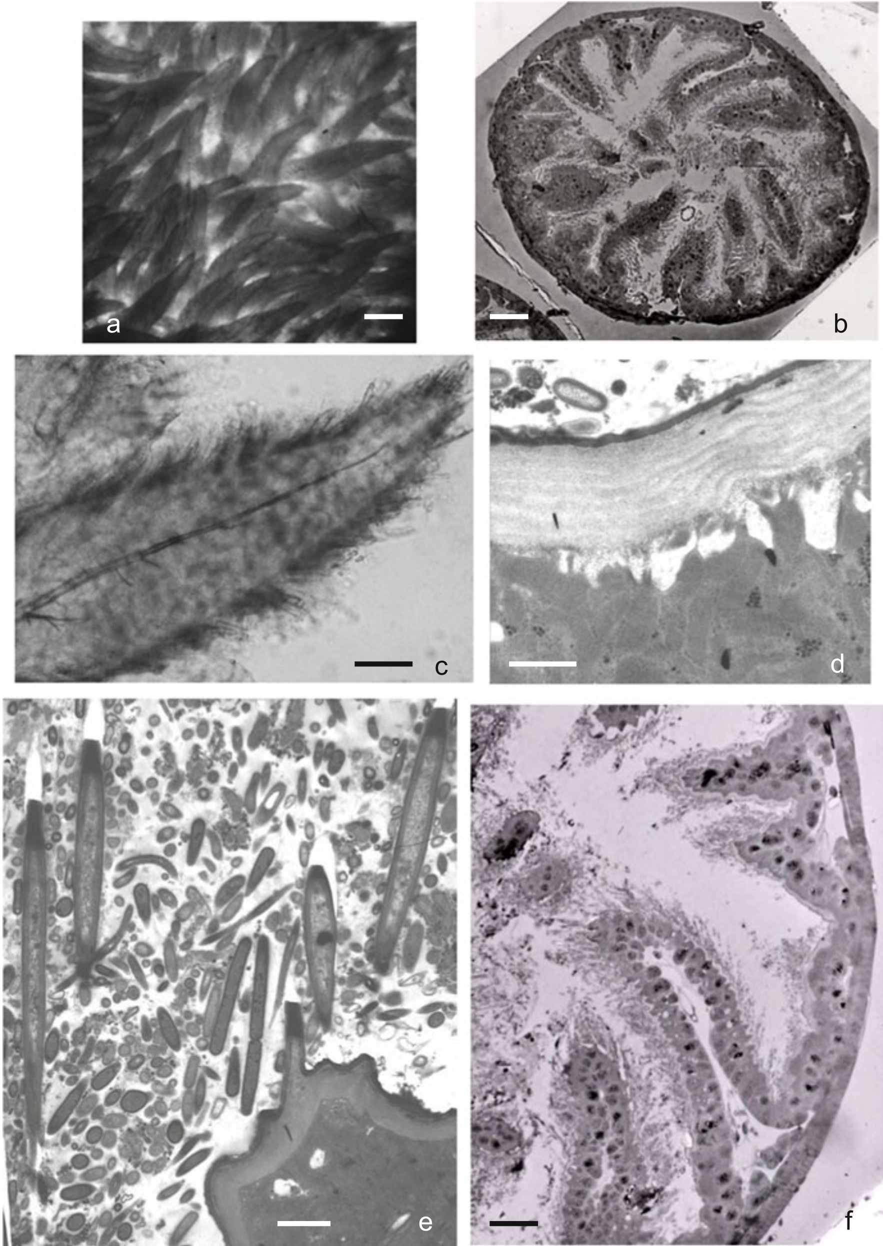


Fig. 8 (**a**)–(**f**) Various aspects of the hindgut epithelium of the cockroach *Nyctibora* sp. (**a**) Low magnification light microscopy reveals that the inner surface of the hindgut is covered by villus-like protrusions of the hindgut epithelium (bar 200 μm). (**b**) A cross-section of the hindgut shows that these villi fill nearly the whole volume of the gut (bar 200 μm). (**f**) The same aspect at higher magnification

this paradox lies in the fact that dense populations of (facultatively) anaerobic bacteria in the midgut generate substantial amounts of hydrogen that—in the absence of methanogens—diffuses out of the midgut. Part of this hydrogen is exhaled with the breath, while another, substantial part is transferred to the methanogenic hindgut by intercompartment hydrogen transfer (Lemke et al. 2001). The anatomy of the intestinal tract of termites, cockroaches and scarab beetle larvae favours such an intercompartment hydrogen transfer (Lemke et al. 2001). We estimated that the hydrogen, which is transferred to the hindgut, contributes to some 25–30% of the methane production in the hindgut (Lemke et al. 2001; Hackstein et al. 2006b).

5 Conclusions: Arthropods

Methanogenic archaea in the intestinal tract of arthropods are found exclusively in four taxa: millipedes, cockroaches, termites and the larvae of scarab beetles. Methanogens are found only in the hindgut: free floating or attached to food particles, attached to the hindgut wall or to specific differentiations of the hindgut wall such as setae (bristles) and pseudosetae, which are composed out of bundles of setae. Species, populations and genera of a methane-forming taxon can lose the ability to host methanogens. This has been shown for cockroaches and—more recently—also for millipedes (Šustr et al. 2014). For cockroaches it could be shown that cocultivation of methane-free animals with methanogenic donors can lead to a transient methane formation by the non-methanogenic host. There was no permanent transformation possible, indicating that non-methane producers had definitively lost the ability to harbour methanogens. Thus, as in the case of methanogenic vertebrates, there is strong evidence that also the presence of methanogens in the GI tract of arthropods is genetically controlled.

Fig. 8 (continued) (bar 100 μm) reveals the presence of tracheae inside of these villi. (**c**) A light micrograph at higher magnification (bar 100 μm), which shows that tracheae and tracheoles are present in each of the villi. (**d**) Mitochondria with many cristae are found just below the cuticle, which covers the epithelial cells at the luminal side (bar 1 μm). (**e**) Electron micrograph of a villus, which is associated with numerous bacteria forming a complex microbiota strongly adhering to the villus with its trichomes (several of which are cut). Note the trachea inside the epithelial cell (close to letter “e”) (bar 2 μm). Reproduced with permission from Hackstein et al. (2006b)

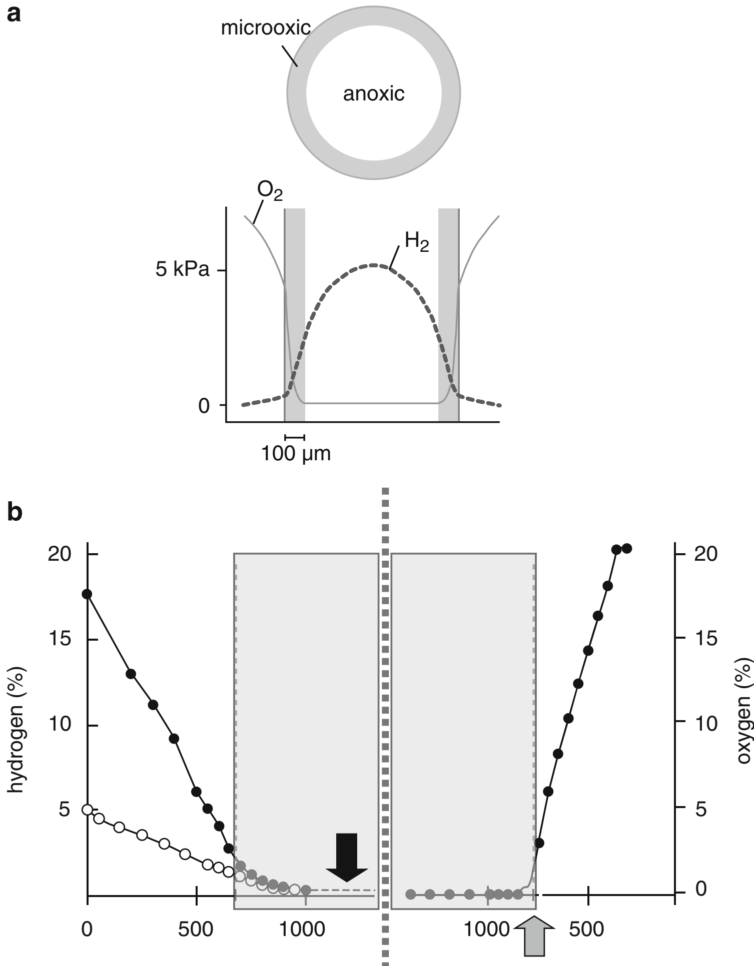


Fig. 9 Cartoons illustrating the radial profiles of H_2 and O_2 partial pressures in termite (**a**) and cockroach guts (**b**), respectively, as measured with the aid of microsensors at explanted guts, which had been embedded into agarose (Brune et al. 1995; for such a set-up, see Fig. 6a, b). Very steep O_2 gradients (**a**, and right part of **b**) are caused by the respiration of facultatively aerobic microbiota with the consequence of small microoxic zones at the periphery of the hindgut lumen (cross-section in (**a**), and grey arrow in (**b**), right panel). The H_2 peak in the termite hindgut is caused by hydrogen-producing protozoa. The hydrogen diffuses out of the gut, being partially consumed by methanogens colonizing the hindgut wall. In (**b**), hydrogen is generated throughout the hindgut lumen, but the presence of methanogens throughout the lumen keeps the partial pressure of hydrogen low (black arrow at the left panel). The hydrogen-consuming communities are not saturated, since even the application of external hydrogen at a partial pressure of 18% does not cause higher than background levels of hydrogen in the gut lumen [left panel, open circles (5% H_2) and black circles (18% H_2)]. The shaded areas indicate the location of the left and right halves of the hindgut, respectively. Abscissa: distance to the surface of the agarose in micrometres. Reproduced with permission from Brune and Friedrich (2000)—(**a**). Reproduced with permission from Hackstein et al. (2006b)—(**b**)

References

Bayon C (1980) Volatile fatty-acids and methane production in relation to anaerobic carbohydrate fermentation in *Oryctes nasicornis* larvae (Coleoptera, Scarabaeidae). J Insect Physiol 26:819–828

Bijnen FGC, Harren FJM, Hackstein JHP, Reuss J (1996) Intracavity CO laser photoacoustic trace gas detection: cyclic CH4, H2O and CO2 emission by cockroaches and scarab beetles. Appl Optics 35:5357–5368

Brauman A, Dore J, Eggleton P, Bignell D, Breznak JA, Kane MD (2001) Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. FEMS Microbiol Ecol 35:27–36

Brune A (2006) Symbiotic associations between termites and prokaryotes. In: Dworkin M, Falkow S, Rosenberg E, Schliefer K-H, Stackebrandt E (eds) The prokaryotes, Symbiotic associations, biotechnology, applied microbiology, vol 1, 3rd edn. Springer, New York, pp 439–474

Brune A, Friedrich M (2000) Micrcoecology of the termite gut: structure and function on a microscale. Curr Opin Microbiol 3:263–269

Brune A, Emerson D, Breznak J (1995) The termite gut microflora as an oxygen sink – microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl Environ Microbiol 61:2681–2687

Brusa T, Canzi E, Allievi L, Delpuppo E, Ferrari A (1993) Methanogens in the human intestinal-tract and oral cavity. Curr Microbiol 27:261–265

Buchner P (1953) Endosymbiose der Tiere mit pflanzlichen Mikroorganismen. Verlag Birkhäuser, Basel (English translation: Endosymbiosis of animals with plant microorganisms. Interscience, New York, 1965)

Canback B, Tamas I, Andersson SGE (2004) A phylogenomic study of endosymbiotic bacteria. Mol Biol Evol 21:1110–1122

Cazemier AE, Hackstein JHP, op den HJM C, Rosenberg J, van der Drift C (1997) Bacteria in the intestinal tract of different species of arthropods. Micro Ecol 33:189–197

Cooper A, Mourer-Chauvire C, Chambers GK, von Haeseler A, Wilson A, Pääbo S (1992) Independent origins of New Zealand Moas and Kiwis. Proc Natl Acad Sci USA 89:8741–8744

Dale C, Moran NA (2006) Molecular interactions between bacterial symbionts and their hosts. Cell 126:453–465

Dettner K, Peters W (2003) Lehrbuch der Entomologie Gustav Fischer Stuttgart 2. Auflage

Dillon RJ, Dillon VM (2004) The gut bacteria of insects: nonpathogenic interactions. Ann Rev Entomol 49:71–92

Doddema HJ, Vogels GD (1978) Improved identification of methanogenic bacteria by fluorescence microscopy. Appl Environ Microbiol 36:752–754

Donovan SE, Purdy KJ, Kane MD, Eggleton P (2004) Comparison of Euryarchaea strains in the guts and food-soil of the soil-feeding termite *Cubitermes fungifaber* across different soil types. Appl Environ Microbiol 70:3884–3892

Doré J, Pochart P, Bernalier A, Goderel I, Morvan B, Rambaud JC (1995) Enumeration of H_2-utilizing methanogenic archaea, acetogenic and sulfate-reducing bacteria from human feces. FEMS Microbiol Ecol 17:279–284

Egert M, Wagner B, Lemke T, Brune A, Friedrich MW (2003) Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera : Scarabaeidae). Appl Environ Microbiol 69:6659–6668

El Oufir L, Flourie B, des Varannes SB, Barry JL, Cloarec D, Bornet F, Galmiche JP (1996) Relations between transit time, fermentation products, and hydrogen consuming flora in healthy humans. Gut 38:870–877

Fenchel T, Finlay BJ (1995) Ecology and evolution in anoxic worlds. Oxford University Press, Oxford

Fenchel T, Finlay BJ (2018) Free-living protozoa with endosymbiotic methanogens. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Florin THJ, Jabbar IA (1994) A possible role for bile-acid in the control of methanogenesis and the accumulation of hydrogen gas in the human colon. J Gastroent Hepatol 9:112–117

Florin THJ, Zhu G, Kirk KM, Martin NG (2000) Shared and unique environmental factors determine the ecology of methanogens in humans and rats. Am J Gastroenterol 95:2872–2879

Friedrich MW, Schmitt-Wagner D, Lueders T, Brune A (2001) Axial differences in community structure of Crenarchaeota and Euryarchaeota in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. Appl Environ Microbiol 67:4880–4890

Gil R, Latorre A, Moya A (2018) Evolution of prokaryote-animal endosymbiosis from a genomics perspective. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Gruninger RJ, McAllister TA, Forster RJ (2016) Bacterial and archaeal diversity in the gastrointestinal tract of the North American beaver (*Castor canadensis*). PLoS One. https://doi.org/10.1371/journal.pone.0156457

Hackstein JHP (1997) Eukaryotic molecular biodiversity: systematic approaches for the assessment of symbiotic associations. Antonie van Leeuwenhoek 72:63–76

Hackstein JHP, Stumm CK (1994) Methane production in terrestrial arthropods. Proc Natl Acad Sci USA 91:5441–5445

Hackstein JHP, Tielens AGM (2018) Hydrogenosomes. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Hackstein JHP, van Alen TA (1996) Fecal methanogens and vertebrate evolution. Evolution 50:559–572

Hackstein JHP, van Alen TA, op den HJM C, Smits A, Mariman E (1995) Intestinal methanogenesis in primates – a genetic and evolutionary approach. Dtsch Tierärztl Wschr 36:152–154

Hackstein JHP, Langer P, Rosenberg J (1996) Genetic and evolutionary constraints for the symbiosis between animals and methanogenic bacteria. Environ Monit Assess 42:39–56

Hackstein JHP, van Hoek AHAM, Leunissen JAM, Huynen M (2002) Anaerobic ciliates and their methanogenic endosymbionts. In: Seckbach J (ed) Symbiosis: mechanisms and model systems. Kluwer Academic Publishers, Dordrecht, pp 451–464. ISBN 1-4020-0189-4

Hackstein JHP, Tjaden J, Huynen M (2006a) Mitochondria, hydrogenosomes and mitosomes: products of evolutionary tinkering! Curr Genet 50:225–245

Hackstein JHP, van Alen TA, Rosenberg J (2006b) Methane production by terrestrial arthropods. In: König H, Varma A (eds) Intestinal microorganisms of termites and other invertebrates. Soil biology, Manual for soil analysis, vol 6. Springer, Heidelberg, pp 155–180

Hansen EE, Lozupone CA, Rey FE, Wu M, Guruge JL, Narra A, Goodfellow J, Zaneveld JR, McDonald DT, Goodrich JA, Heath AC, Knight R, Gordon JI (2011) Pan-genome of the dominant human gut-associated archaeon, *Methanobrevibacter smithii,* studied in twins. Proc Natl Acad Sci USA 108(Suppl 1):4599–4606

Hoffmeister M, Martin W (2003) Interspecific evolution: microbial symbiosis, endosymbiosis and gene transfer. Env Microbiol 5:641–649

Hudson MJ, Tomkins AM, Wiggins HS, Drasar BS (1993) Breath methane excretion and intestinal methanogenesis in children and adults in rural Nigeria. Scand J Gastroenterol 28:993–998

Janke A, Feldmaier-Fuchs G, Thomas WK, von Haeseler A, Pääbo S (1994) The marsupial mitochondrial genome and the evolution of placental mammals. Genetics 137:243–256

Jesse BW, Wang L-Q, Baldwin RI (1994) Genetic regulation of postnatal metabolic development. Proc Soc Nutr Physiol 3:287–288

Kittelmann S, Janssen PH (2011) Characterization of rumen ciliate community composition in domestic sheep, deer, and cattle feeding on varying diets, by means of PCR-DGGE and clone libraries. FEMS Microbiol Ecol 75:468–481

Kittelmann S, Seedorf H, Walters WA, Clemente JC, Knight R, Gordon JI, Janssen PH (2013) Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial archaeal and eukaryotic microorganisms in rumen microbial communities. PLoS One 8(2):e47879

Lange M, Westerman P, Ahring BK (2005) Archaea in protozoa and metazoa. Appl Microbiol Biotechnol 66:465–474

Langer P (1988) The mammalian herbivore stomach. Comparative anatomy, function, and evolution. Gustav Fischer Verlag, Stuttgart

Langer P (1991) Evolution of the digestive tract in mammals. Verh Dtsch Zool Ges 84:169–193

Langer P (1994) Food and digestion of cenozoic mammals in Europe. In: Chivers DJ, Langer P (eds) The digestive systems in mammals: food, form, and function. Cambridge University Press, Cambridge, pp 9–24

Langer P, Snipes RL (1991) Adaptations of gut structure to function in herbivores. In: Tsuda T, Sasaki Y, Kawashima R (eds) Physiological aspects of digestions and metabolism in ruminants. Academic Press, San Diego, pp 349–384

Leadbetter JR, Breznak JA (1996) Physiological ecology of *Methanobrevibacter cuticularis* sp nov and *Methanobrevibacter curvatus* sp nov, isolated from the hindgut of the termite *Reticulitermes flavipes*. Appl Environ Microbiol 62:3620–3631

Leadbetter JR, Crosby LD, Breznak JA (1998) *Methanobrevibacter filiformis* sp. nov., a filamentous methanogen from termite hindguts. Arch Microbiol 169:287–292

Lemke T, van Alen T, Hackstein JHP, Brune A (2001) Cross-epithelial hydrogen transfer from the midgut compartment drives methanogenesis in the hindgut of cockroaches. Appl Environ Microbiol 67:4657–4661

Levitt MD, Furne JK, Kuskowski M, Ruddy J (2006) Stability of human methanogenic flora over 35 years and a review of insights obtained from breath methane measurements. Clin Gastroenterol Hepatol 4:123–129

Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI (2008a) Evolution of mammals and their gut microbes. Science 320:1647–1651

Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI (2008b) Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol 6:776–788

Li WH, Gouy M, Sharp PM, Uigin CO, Yang YW (1990) Molecular phylogeny of rodentia, lagomorpha, primates, artiodactyla, and carnivora and molecular clocks. Proc Natl Acad Sci USA 87:6703–6707

Liu YC, Whitman WB (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. In: Wiegel J, Maier RJ, Adams MWW (eds) Incredible anaerobes: from physiology to genomics to fuels. Ann NY Acad Sci 1125:171–189

Miller TL, Wolin MJ (1982) Enumeration of *Methanobrevibacter smithii* in human feces. Arch Microbiol 131:14–18

Miller TL, Wolin MJ (1986) Methanogens in human and animal intestinal tracts. Sys Appl Microbiol 7:223–229

Miyamoto MM, Goodman M (1986) Biomolecular systematics of eutherian mammals – phylogenetic patterns and classification. Syst Zool 35:230–240

Miyata R, Noda N, Tamaki H, Kinjyo K, Aoyagi H, Uchiyamai H, Tanaka H (2007) Phylogenetic relationship of symbiotic archaea in the gut of the higher termite *Nasutitermes takasagoensis* fed with various carbon sources. Microb Environ 22:157–164

Moran NA (2003) Tracing the evolution of gene loss in obligate bacterial symbionts. Curr Opin Microbiol 6:512–518

Moran NA (2007) Symbiosis as an adative process and source of phenotypic complexity. Proc Natl Acad Sci USA 104(Suppl 1):8627–8633

Moran NA, Baumann P (2000) Bacterial endosymbionts in animals. Curr Opin Microbiol 3:270–275

Moya A, Pereto J, Gil R, Latorre A (2008) Learning how to live together: genomic insights into prokaryote-animal symbioses. Nat Rev Genet 9:218–229

Müller N, Timmers P, Plugge CM, Stams AJM, Schink B (2018) Syntrophy in methanogenic degradation. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea. Springer, Heidelberg

Novacek MJ (1992) Mammalian phylogeny – shaking the tree. Nature 356:121–125

Ohkuma M, Noda S, Horikoshi K, Kudo T (1995) Phylogeny of symbiotic methanogens in the gut of the termite *Reticulitermes speratus*. FEMS Microbiol Lett 134:45–50

Ohkuma M, Noda S, Kudo T (1999) Phylogenetic relationships of symbiotic methanogens in diverse termites. FEMS Microbiol Lett 171:147–153

Regensbogenova M, Pristas P, Javorsky P, Moon-van der Staay SY, van der Staay GWM, Hackstein JHP, Newbold CJ, McEwan NR (2004a) Assessment of ciliates in the sheep rumen by DGGE. Lett Appl Microbiol 39:144–147

Regensbogenova M, McEwan NR, Javorsky P, Kisidayova S, Michalowski T, Newbold CJ, Hackstein JHP, Pristas P (2004b) A re-appraisal of the diversity of the methanogens associated with the rumen ciliates. FEMS Microbiol Lett 238:307–313

Ruby EG (2008) Symbiotic conversations are revealed under genetic interrogation. Nat Rev Microbiol 6:752–762

Sanders JG, Beichman AC, Roman J, Scott JJ, Emerson D, McCarthy JJ, Girguis PR (2015) Baleen whales host a unique gut microbiome with similarities to both carnivores and herbivores. Nat Commun. https://doi.org/10.1038/ncomms9285

Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61:262–280

Segal I, Walker ARP, Lord S, Cummings JH (1988) Breath methane and large bowel-cancer risk in contrasting African populations. Gut 29:608–613

Shinzato N, Matsumoto T, Yamaoka I, Oshima T, Yamagishi A (1999) Phylogenetic diversity of symbiotic methanogens living in the hindgut of the lower termite *Reticulitermes speratus* analyzed by PCR and in situ hybridization. Appl Environ Microbiol 65:837–840

Sprenger WW, van Belzen MC, Rosenberg J, Hackstein JHP, Keltjens JT (2000) *Methanomicrococcus blatticola* gen. nov., sp nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana*. Int J Syst Evol Microbiol 50:1989–1999

Stams AJM, Plugge CM (2009) Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat Rev Microbiol 7:568–577

St-Pierre B, Wright ADG (2013) Diversity of gut methanogens in herbivorous animals. Animal 7 (s1):49–56

Šustr V, Chroňáková A, Semanová S, Tajovský K, Šimek M (2014) Methane production and methanogenic archaea in the digestive tracts of Millipedes (Diplopoda). PLoS One 9(7): e102659

Tokura M, Ohkuma M, Kudo T (2000) Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. FEMS Microbiol Ecol 33:233–240

Ushida K (2018) Symbiotic methanogens and rumen ciliates. In: Hackstein JHP (ed) (Endo) symbiotic methanogenic archaea. Springer, Heidelberg

van Hoek AHAM, van Alen TA, Sprakel VSI, Hackstein JHP, Vogels GD (1998) Evolution of anaerobic ciliates from the gastrointestinal tract: phylogenetic analysis of the ribosomal repeat from *Nyctotherus ovalis* and its relatives. Mol Biol Evol 15:1195–1206

van Hoek AHAM, van Alen TA, Sprakel VSI, Leunissen JAM, Brigge T, Vogels GD, Hackstein JHP (2000) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol Biol Evol 17:251–258

Warnecke F, Luginbühl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT, Cayouette M, McHardy AC, Djordjevic G, Aboushadi N, Sorek R, Tringe SG, Podar M, Martin HG, Kunin V, Dalevi D, Madejska J, Kirton E, Platt D, Szeto E, Salamov A, Barry K, Mikhailova N, Kyrpides NC, Matson EG, Ottesen EA, Zhang XN, Hernandez M, Murillo C, Acosta LG, Rigoutsos I, Tamayo G, Green BD, Chang C, Rubin EM, Mathur EJ, Robertson DE,

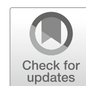
Hugenholtz P, Leadbetter JR (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450:560–565

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms – proposal for the domains archaea, bacteria, and eucarya. Proc Natl Acad Sci USA 87:4576–4579

Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR (2004) Molecular ecological analysis of the gastrointestinal microbiota: a review. J Nutr 134(2):465–472

Zoetendal EG, Vaughan EE, de Vos WM (2006) A microbial world within us. Mol Microbiol 59:1639–1650

Syntrophy in Methanogenic Degradation



Nicolai Müller, Peer Timmers, Caroline M. Plugge, Alfons J. M. Stams, and Bernhard Schink

Contents

1 Introduction 154
2 Syntrophic Culture Systems, Microbiology, Biochemistry, and Molecular Biology 159
2.1 Ethanol 159
2.2 Butyrate 161
2.3 Propionate 164
2.4 Acetate 167
2.5 Branched Fatty Acids 168
2.6 Benzoate 169
2.7 Sugars 170
2.8 Amino Acids 171
2.9 Carrier Systems 174
2.10 Alternative Substrates for Pure Cultures, Technical Systems to Replace Methanogens, and Other Culturing Approaches for Syntrophs 175
2.11 Anaerobic Methane Oxidation 177
3 Spatial Organization of Syntrophic Communities 180
4 Concluding Remarks 181
References 182

Abstract This chapter deals with microbial communities of bacteria and archaea which closely cooperate in methanogenic degradation and perform metabolic functions in this community that neither one of them could carry out alone. The

N. Müller · B. Schink (✉)
Microbial Ecology, Department of Biology, University of Konstanz, Konstanz, Germany
e-mail: Nicolai.Mueller@uni-konstanz.de; Bernhard.Schink@uni-konstanz.de

P. Timmers
Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands

Wetsus, European Centre of Excellence for Sustainable Water Technology, Leeuwarden
The Netherlands
e-mail: Peer.Timmers@wetsus.nl

C. M. Plugge · A. J. M. Stams
Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands
e-mail: Caroline.plugge@wur.nl; Fons.stams@wur.nl

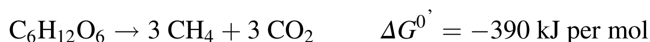
© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_9

methanogenic degradation of fatty acids, alcohols, most aromatic compounds, amino acids, and others is performed in partnership between fermenting bacteria and methanogenic Archaea. The energy available in these processes is very small, attributing only fractions of an ATP unit per reaction run to every partner. The biochemical strategies taken include in most cases reactions of substrate-level phosphorylation combined with various kinds of reversed electron transport systems in which part of the gained ATP is reinvested into thermodynamically unfavorable electron transport processes. Altogether, these systems represent fascinating examples of energy efficiency at the lowermost energy level that allows microbial life.

1 Introduction

In oxygen-limited environments such as lake sediments or the lower layers of eutrophic lakes in summertime, biomass oxidation has to be coupled to alternative electron acceptors such as nitrate, Mn(IV), Fe(III), sulfate, or CO_2 (which is reduced to methane) (Zehnder 1978; Schink 1989). The relative importance of these alternative electron acceptors depends on their availability in the respective habitat; most freshwater sediments are rich in iron oxides, and marine sediments are well supplied with sulfate due to the high sulfate content of seawater (28 mM). Only methanogenesis is independent of external electron acceptors because the methanogenic degradation of biomass is actually a dismutation of organic carbon:



$$C_6H_{12}O_6 \rightarrow 3\ CH_4 + 3\ CO_2 \qquad \Delta G^{0'} = -390\ \text{kJ per mol}$$

Whereas aerobic, nitrate-reducing, or manganese-reducing bacteria typically are able to degrade polymeric organic compounds via the respective monomers to CO_2 and other inorganic products in one single cell, the conversion of complex organic matter by iron reducers or sulfate reducers requires a cooperation with fermenting bacteria which feed the respective terminal oxidizers with classical fermentation products such as fatty acids, alcohols, and others. Methanogenic degradation of organic matter is even more complex and requires cooperation of three different metabolic groups (guilds) of microbes, including primary fermenters, secondary fermenters, and methanogens (Bryant 1979; Fig. 1a). Primary fermenting bacteria are known for long times and have been isolated by classical procedures with all kinds of polymeric or monomeric substrates. Also anaerobic protozoa including flagellates and ciliates can operate in this manner (see chapter by Fenchel and Finlay 2018, this volume). Different from iron reducers or sulfate reducers, methanogenic archaea use only very few substrates, including hydrogen, CO_2, other C_1 compounds, and acetate. In one exceptional case, a methanogen can also oxidize isopropanol and ethanol (Widdel et al. 1988). Thus, the majority of classical fermentation products, such as alcohols, fatty acids, branched-chain fatty acids, and aromatic fatty acid residues from partial degradation of amino acids,

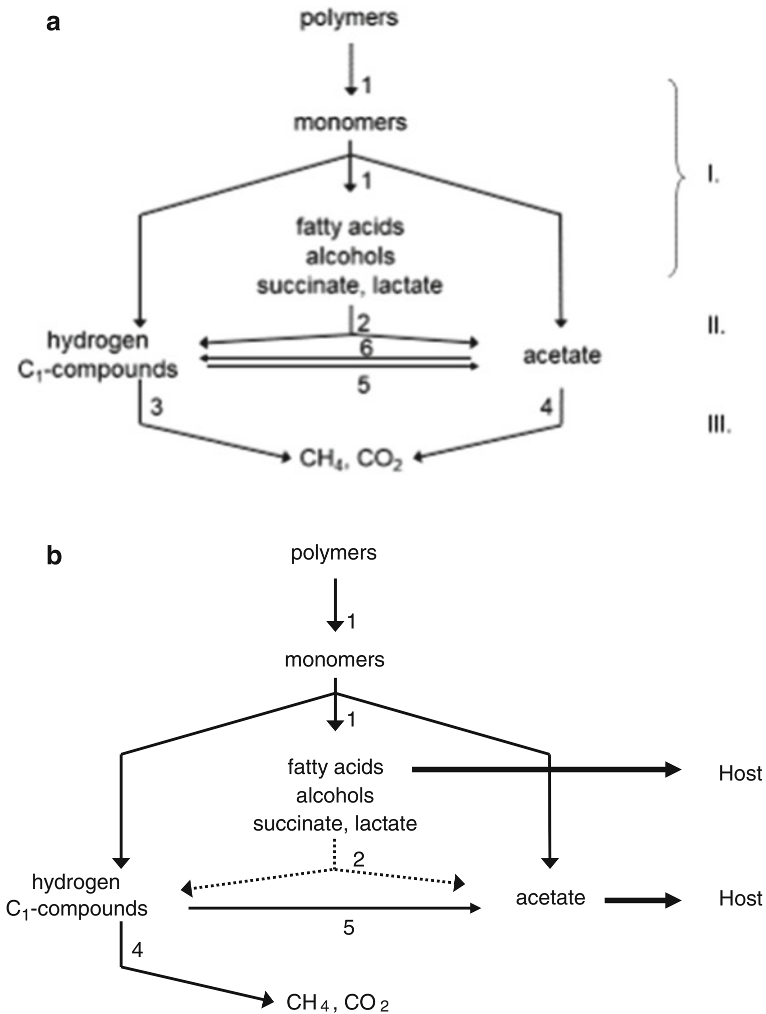


Fig. 1 Methanogenic degradation of complex organic matter by cooperation of different metabolic groups. (**a**) Electron flow in a freshwater sediment or biogas reactor. (**b**) Electron flow in plant digestion in the rumen. Metabolic groups of organisms involved: primary fermenters (1), secondary fermenters (2), hydrogen and C_1 compounds using methanogens (3), acetoclastic methanogens (4), homoacetogenic bacteria (5), and syntrophic acetate oxidizers (6) (modified after Schink 1997)

long-chain fatty acids from lipid hydrolysis, heterocyclic aromatic compounds deriving from nucleic acids, etc., all need to be fermented further to those substrates that methanogens can use (Bryant 1979; Schink 1997; Schink and Stams 2002; McInerney et al. 2008; Stams and Plugge 2009; Schink et al. 2017). This is the function of the secondary fermenting bacteria which depend on close cooperation with methanogenic partners and are subject of this chapter.

Methanogenic environments are widely distributed in nature. Wetlands, freshwater sediments, swamps, and digestive tracts of ruminants and insects are environments that produce high amounts of methane. Man-made systems, such as rice paddies and anaerobic bioreactors and landfills, are other important sources of biogenic methane production.

Methanogenic archaea catalyze the final step in the overall anaerobic degradation of organic material to methane and CO_2. One metabolic group of methanogenic Archaea converts CO_2 plus hydrogen or formate to methane while others use acetate or methanol. Acetate, the most important intermediate in anaerobic digestion, accounts for approximately two-thirds of all methane produced, while the last third is produced from the reduction of CO_2 with electrons derived from the oxidation of hydrogen or formate (Ferry 1992; Liu and Whitman 2008). Currently, only two types of acetoclastic methanogens have been identified: *Methanosaeta* (*Methanothrix*) spp. and *Methanosarcina* spp. *Methanosarcina* sp. is a genus of versatile methanogens, including species capable of growing with different substrates including acetate, methanol, methylamines, and H_2/CO_2, whereas *Methanosaeta* sp. uses only acetate. *Methanosaeta* sp. is widely distributed in nature, and, because of its high affinity for acetate, it outcompetes *Methanosarcina* sp. in low-acetate environments (Conklin et al. 2006). In rumen and other animal gastrointestinal tracts, however, *Methanosarcina* sp. are typically present, due to the high acetate concentrations occurring in these environments (see chapter by Conway de Macario and Macario 2018, this volume). Both acetoclastic archaea grow very slowly, with doubling times of 1–12 (*Methanosaeta*) and 0.5–2 (*Methanosarcina*) days (Jetten et al. 1992). Despite their restricted substrate range (H_2/CO_2, formate, and methylated C_1 compounds), methanogenic archaea are phylogenetically very diverse. They are classified in seven orders (Borrel et al. 2012, 2013). Representatives of the orders Methanobacteriales, Methanomicrobiales, and Methanomassiliicoccales are commonly present in animal gastrointestinal tracts.

The importance of the secondary fermenting bacteria varies with the kind of substrate utilized and with the efficiency of the methanogenic partners at the end of the anaerobic feeding chain. If the methanogens maintain a low concentration of hydrogen and acetate, numerous classical primary fermentations are shifted to the formation of hydrogen, CO_2, and acetate and produce much less reduced side products such as fatty acids than they do in pure culture (Iannotti et al. 1973; Tewes and Thauer 1980; Schink and Zeikus 1982). Thus, the majority of electrons from substrate degradation will flow through the outer lines of the scheme depicted in Fig. 1a, and the electron flow through the central part may be only of minor importance to ensure complete degradation of biopolymers as this is typical for freshwater lake sediments, swamps, or sewage sludge digesters.

The situation is basically different in the fermentations proceeding inside the gastrointestinal tracts of animals There, the host organism is not favored by complete degradation of biopolymers inside the gut to methane and CO_2 but uses a substantial part of the overall electron input for its own support, e.g., in the form of fatty acids.

Since the retention time of the feed inside the guts is limited to a few hours or 2 days at maximum, neither the slow-growing acetate-utilizing methanogens nor the fatty acid-degrading syntrophic associations will establish. Therefore, these fatty acids accumulate in the gut and are taken up by the host. In ruminants, this acid transfer proceeds at concentrations of 60 mM acetate, 20 mM propionate, and 10 mM butyrate (Bryant 1977); in termite guts, only acetate is produced to major amounts (Breznak and Kane 1990; Brune 2007). Methanogenesis in these intestinal tracts is restricted to hydrogen utilization, therefore, in order to shift the overall electron flow mainly toward fatty acids production and to minimize unwanted side fermentations such as alcohol formation (Fig. 1b). Several studies have recognized the importance of adequate coefficients of production rate of these individual fatty acids to accurately predict CH_4 formation in the rumen (van Lingen et al. 2016). Under common rumen conditions, fatty acid dynamics rather than methanogenesis is controlled by the hydrogen pressure, and the thermodynamic control on the type of VFA formed is significant. In a modeling study, van Lingen et al. (2016) predicted the NAD^+ to NADH ratio as a key controller of the type of fatty acid produced and the associated amount of H_2 being formed that is consequently available for methanogenesis. Taking the NAD^+ to NADH ratio into account in dynamic rumen models may allow to improve the prediction of type of fatty acid formed as well and CH_4 formed. Moreover, a comprehensive study focused on the diurnal profiles of gaseous and dissolved metabolites (including lactate and ethanol) in the rumen, along with H_2 and CH_4 emission rates (van Lingen et al. 2017). The large variation observed in diurnal patterns of rumen metabolites, and the substantial increase of the hydrogen pressure rapidly after feeding followed by the occurrence of shifts in fermentation toward ethanol, lactate, and propionate at the expense of acetate, correlated with the redox state of NAD^+. This highlights the importance of including diurnal dynamics in rumen fermentation studies to further increase our understanding of fatty acid, hydrogen, and CH_4 production.

The ability to transfer electrons to a partner organism is an important metabolic feature associated with many physiologically diverse microorganisms. This trait is usually referred to as syntrophism. Syntrophism is a special type of symbiosis between (at least) two microorganisms which depend on each other via interspecies metabolite transfer for bioenergetic reasons. Especially among anaerobic microorganisms, cooperation of several metabolic types of bacteria in the feeding chain is a common feature. The mutual dependence can be explained calculating the changes in Gibbs' free energy ($\Delta G^{0'}$) for the oxidation of, e.g., ethanol to hydrogen, CO_2, and acetate (Bryant et al. 1967): under defined standard conditions with gases at 10^5 Pa pressure, 1 M concentration of products/substrates, at pH 7.0 and 298 K, the Gibbs' free energy value for ethanol oxidation is positive with +9.6 kJ/reaction (Table 1). This indicates that the reaction cannot take place, nor can any microbe gain energy from this oxidation. However, the Gibbs' free energy becomes negative when the hydrogen partial pressure (pH_2) decreases. This example of interspecies hydrogen transfer is characteristic for the way how organic matter is degraded in methanogenic habitats.

Table 1 Equations and standard free energy changes for relevant reactions described in the chapter (Gibbs free energy changes are taken from Thauer et al. 1977)

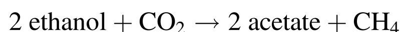
(a) Equations and free energy changes for secondary fermentation reactions	
Reaction	$\Delta G^{o\prime}$ [kJ/reaction]
Glucose → 2 acetate$^-$ + 2 H^+ + 2 CO_2 + 4 H_2	−216
Ethanol + H_2O → acetate$^-$ + H^+ + $2H_2$	+9.6
Propionate$^-$ + 2 H_2O → acetate$^-$ + CO_2 + 3 H_2	+76
Butyrate$^-$ + $2H_2O$ → 2 acetate$^-$ + H^+ + $2H_2$	+48
Crotonate$^-$ + $2H_2O$ → 2 acetate$^-$ + H^+ + H_2	−6.2
Acetate$^-$ + $2H_2O$ → 2 CO_2 + 4 H_2	+96
Benzoate$^-$ + $6H_2O$ → 3 acetate$^-$ + CO_2 + $2H^+$ + $3H_2$	+49.5
Phenol + $5H_2O$ → 3 acetate$^-$ + 3 H^+ + $2H_2$	+5.7
Acetone + CO_2 → 2 acetate$^-$ + $2H^+$	−34
Alanine + $3H_2O$ → acetate$^-$ + H^+ + HCO_3^- + NH_4^+ + $2H_2$	+7.5
Isoleucine + $3H_2O$ → 2-methylbutyrate$^-$ + H^+ + HCO_3^- + NH_4^+ + $2H_2$	+7.5
Valine + $3H_2O$ → isobutyrate$^-$ + H^+ + HCO_3^- + NH_4^+ + $2H_2$	+9.7
Leucine + $3H_2O$ → isovalerate$^-$ + H^+ + HCO_3^- + NH_4^+ + 2 H_2	+4.2
Leucine + $3H_2O$→ α-ketoisocaproate$^-$ + NH_4^+ + H_2	+51
α-Ketoisocaproate$^-$ → isovalerate$^-$ + H_2	−56
Glutamate$^-$ + $2H_2O$ → acetate$^-$ + $0.5H^+$ + HCO_3^- + NH_4^+ + 0.5 butyrate$^-$	−58
Glutamate$^-$ + $4H_2O$ → propionate$^-$ + $2HCO_3^-$ + H^+ + NH_4^+ + $2H_2$	−5.8
Glutamate$^-$ + $3H_2O$ → 2 acetate$^-$ + H^+ + HCO_3^- + NH_4^+ + H_2	−34
Aspartate$^-$ + 3 H_2O → acetate$^-$ + H^+ + 2 HCO_3^- + NH_4^+ + $2H_2$	−14
Serine + $2H_2O$ → acetate$^-$ + H^+ + HCO_3^- + NH_4^+ + H_2	−90
(b) Equations and free energy changes for reactions of methanogenic archaea	
Reaction	$\Delta G^{o\prime}$ [kJ/reaction]
$4H_2$ + CO_2 → CH_4 + $2H_2O$	−131
4 formate$^-$ + $4H^+$ → CH_4+ $3CO_2$ + $2H_2O$	−145
4CO + $2H_2O$ → CH_4 + $3CO_2$	−211
Acetate$^-$ + H^+ → CH_4 + CO_2	−35
4 methanol → $3CH_4$ + CO_2 + $2H_2O$	−106
H_2 + methanol → CH_4 + H_2O	−113
CO_2 + H_2O → H^+ + HCO_3^-	+4.8
CO_2 + H_2 → formate$^-$ + H^+	−4.5
(c) Equations and free energy changes for hydrogen-consuming reactions	
Reaction	$\Delta G^{o\prime}$ [kJ/reaction]
Crotonate$^-$ + H_2 → butyrate$^-$	−75
Pentenoate$^-$ + H_2 → valerate$^-$	−75
Glycine + H_2 → acetate$^-$ + NH_4^+	−78
CO_2 + H_2 → formate$^-$ + H^+	−4.5

2 Syntrophic Culture Systems, Microbiology, Biochemistry, and Molecular Biology

Life depends on the availability of energy, which is stored inside the cell in the form of ATP. Under physiological conditions, including heat losses, the synthesis of ATP requires 60–70 kJ per mol (Thauer et al. 1977). Membrane-bound ATPases couple the hydrolysis or synthesis of ATP to the transport of protons (in some cases also Na^+ ions) across the cytoplasmic membrane. Depending on the stoichiometry of the ATPase system in question, the ratio of ions translocated versus ATP synthesized or hydrolyzed may vary between 3 and 5; in most cases, a ratio of 3:4 appears to be justified (Engelbrecht and Junge 1997; Cherepanov et al. 1999). As a consequence, the smallest amount of energy that can still be converted to ATP—and with this into metabolic activity and growth—is equivalent to one third or one fourth of an ATP unit, i.e., in the range of −15 to −20 kJ per mol reaction (Schink 1997; Schink and Stams 2002). For syntrophic associations operating, e.g., in anoxic lake sediments at very little energy supply, one can assume that their energy charge is lower than that of a typical batch culture in the lab and that ATP can even be synthesized at energy spans as little as 10–12 kJ per mol reaction (Lever et al. 2015). It is this minimum increment of energy with which syntrophically fermenting methanogenic communities most often have to operate.

2.1 *Ethanol*

The biochemistry of syntrophic oxidation of ethanol, although the oldest syntrophic system known, has still not been elucidated in detail. Early work on the so-called S organism indicated that ethanol is oxidized via acetaldehyde to acetyl-CoA and further to acetate, including ATP synthesis by acetate kinase (Reddy et al. 1972a, b, c). This concept was confirmed by similar studies on the ethanol-oxidizing bacteria *Pelobacter acetylenicus* and *P. carbinolicus* (Schink 1985; Eichler and Schink 1986). Nonetheless, the energetics of this reaction chain are still unclear. The overall reaction



$$2 \text{ ethanol} + CO_2 \rightarrow 2 \text{ acetate} + CH_4$$

yields $\Delta G^{0\prime} = -112$ kJ per mol, which leaves a total of about −40 kJ per ethanol oxidation reaction for the syntrophic ethanol oxidizer, indicating that part of the ATP formed by substrate-level phosphorylation has to be invested into reversed electron transport. Oxidation of acetaldehyde in *Pelobacter* spp. can occur through two different enzyme systems (Schmidt et al. 2014). An acetylating acetaldehyde dehydrogenase converts acetaldehyde and coenzyme A to acetyl-CoA ($E^{0\prime} = -370$ mV) while reducing NAD^+. Acetyl-CoA is then phosphorylated to acetyl-phosphate, which serves as a substrate for acetate kinase allowing phosphorylation of ADP to

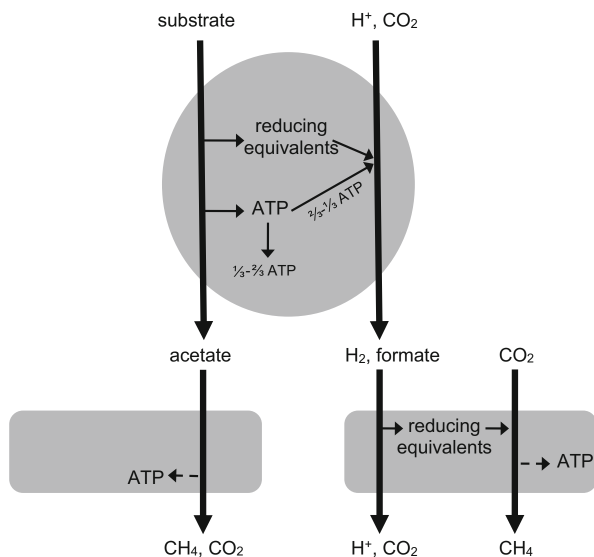


Fig. 2 Conversions performed by a secondary fermenting bacterium (top), a hydrogen- and formate-using methanogen (bottom right), and an acetoclastic methanogen (bottom left)

ATP. As such, the acetylating acetaldehyde dehydrogenase allows the synthesis of ATP by substrate-level phosphorylation but yields NADH which cannot be reoxidized with protons or protons and carbon dioxide easily and requires a to-date unidentified reversed electron transport system or simply occurs through NAD-dependent hydrogenases or formate dehydrogenases at sufficiently low hydrogen partial pressure or low formate concentration (Schmidt et al. 2014). The genome of *Pelobacter carbinolicus* harbors several potential candidate genes that could be responsible for the expression of reversed electron transport systems; however, none of them could yet be identified in biochemical or proteome studies (Aklujkar et al. 2012; Schmidt et al. 2014). Another enzyme system identified in *Pelobacter* spp., a tungsten-dependent non-acetylating acetaldehyde dehydrogenase, oxidizes acetaldehyde most likely with ferredoxin. A relevant function of the enzyme in the degradation of ethanol in *Pelobacter* spp. has not been identified yet, and it was speculated that this enzyme system probably serves as an "overpressure valve," i.e., preventing accumulation of acetaldehyde at the expense of ATP not generated through acetylating acetaldehyde dehydrogenase to enable endergonic oxidation of ethanol with NAD^+ (Schmidt et al. 2014). The energetically difficult reaction is the transfer of electrons from the acetaldehyde/ethanol couple (−196 mV) to hydrogen formation via NADH. Such a reaction requires energy input in the form of, e.g., a reversed electron transport, a feature that is common to all syntrophically fermenting bacteria studied so far (Fig. 2). Energy input to drive ethanol oxidation in *Pelobacter* spp. could therefore mean acetaldehyde removal without ATP formation and hence poses an indirect form of reversed electron transport. Since *P. acetylenicus* also contains a menaquinone-like electron carrier (Strohm and Schink, unpublished), a basically similar reversed electron transport system as suggested for *Syntrophomonas wolfei* (discussed in Sect. 2.2) can be anticipated, but experimental

evidence has not been provided yet, and recent evidence demonstrates that NAD^+ is the most important electron acceptor for the initial oxidation of ethanol to acetaldehyde (Schmidt et al. 2014). A comproportionating [FeFe]-hydrogenase as described for *Thermotoga maritima* (Schut and Adams 2009) could finally release the electrons from NADH and ferredoxin toward proton reduction. *T. maritima* ferments glucose to acetate, CO_2, and H_2 via the Embden-Meyerhof pathway, generating two NADH and four reduced ferredoxins per molecule of glucose. In order to reoxidize these carriers, a proposed bifurcating [FeFe]-hydrogenase uses electrons from NADH and reduced ferredoxin in a 1:1 ratio to produce H_2. Schut and Adams (2009) found genes with sequence similarity to this [FeFe]-hydrogenase in several other microorganisms, including the ethanol-degrading *P. carbinolicus*, the butyrate-degrading *S. wolfei* (discussed in Sect. 2.2), and the propionate-degrading *Syntrophobacter fumaroxidans* (discussed in Sect. 2.3). Even though [FeFe]-hydrogenases of high similarity to the one in *T. maritima* were identified both in the genome and proteome of *Pelobacter* spp., biochemical evidence of involvement of such a bifurcating hydrogenase is still missing (Aklujkar et al. 2012; Schmidt et al. 2014). Besides hydrogen, also formate was identified as a key metabolite in interspecies electron transfer between syntrophic partners in cocultures of *Pelobacter* spp. and *Methanospirillum hungatei*, and the importance of formate in syntrophic interactions is increasingly recognized (Schmidt et al. 2014; Schink et al. 2017).

Also sulfate reducers like *Desulfovibrio vulgaris* have been shown to couple ethanol oxidation to acetate with electron transfer to methanogenic partners (Bryant et al. 1977). However, this activity appears not to be coupled to ATP synthesis by the sulfate reducer because *D. vulgaris* does not grow in such cocultures (Kremer et al. 1988). The classical sulfate reducers oxidize ethanol via acetaldehyde directly to acetate without intermediate formation of an activated acetyl residue; thus, no ATP is formed by substrate-level phosphorylation and the cultures do not grow (Kremer et al. 1988). Nonetheless, syntrophic growth of an ethanol-degrading sulfate reducer in the absence of sulfate with a methanogen as electron scavenger has been documented (Walker et al. 2009). Ethanol-degrading sulfate reducers that grow in the absence of sulfate in coculture with methanogens either have to form ATP through substrate-level phosphorylation via acetyl-CoA or acetyl phosphate as intermediates, or the electron transfer from the intermediate carriers to hydrogen or formate as extracellular electron carriers has to be coupled to some kind of net ion translocation which is coupled to ATP synthesis.

2.2 Butyrate

Anaerobic butyrate degraders known to date belong to only two groups of bacteria, the genus *Syntrophomonas* within the phylum *Firmicutes* and the genus *Syntrophus* within the order *Syntrophobacterales* of the phylum *Proteobacteria* (Table 2). Fermentation of butyrate to acetate and hydrogen is endergonic (Table 1) and occurs only at very low hydrogen partial pressures, e.g., in the presence of methanogenic

Table 2 Propionate-degrading syntrophic bacteria (modified after McInerney et al. 2008)

Organism	Growth possible with				Phylogenetic position	Reference
	Propionate + sulfate	Propionate + fumarate	Propionate + Fumarate	Propionate + syntrophic partner		
Syntrophobacter fumaroxidans	+	+	+	+	*δ-Proteobacteria*	Harmsen et al. (1998)
Syntrophobacter pfennigii	+	–	–	+	*δ-Proteobacteria*	Wallrabenstein et al. (1995)
Syntrophobacter sulfatireducens	+	–	–	+	*δ-Proteobacteria*	Chen et al. (2005)
Syntrophobacter wolinii	+	ND	ND	+	*δ-Proteobacteria*	Boone and Bryant (1980)
Pelotomaculum schinkii	–	–	–	+	Low G+C Gram positives	De Bok et al. (2005)
Pelotomaculum thermopropionicum	–	+	+	+	Low G+C Gram positives	Imachi et al. (2002)
Pelotomaculum propionicicum	–	–	–	+	Low G+C Gram positives	Imachi et al. (2007)
Smithella propionica	ND	ND	ND	+		Liu et al. (1999)
Desulfotomaculum thermobenzoicum thermosyntrophicum	+	–	–	+	Low G+C Gram positives	Plugge et al. (2002b)

archaea (Schink 1997). Syntrophic butyrate oxidizers use only very few substrates. Beyond oxidation of saturated fatty acids in coculture with methanogens, axenic growth is possible only with unsaturated fatty acids such as crotonate (Schink 1997; McInerney et al. 2008). They cannot use external electron acceptors for growth, thus reflecting the high degree of specialization of these bacteria for syntrophic cooperation (Schink 1997).

Butyrate is oxidized via β-oxidation to acetate yielding one mole ATP per mole of butyrate. The reducing equivalents are transferred to flavoenzymes and NAD^+ (Wofford et al. 1986). Reoxidation of these electron carriers of a relatively positive redox potential with protons to form hydrogen is energetically difficult. Of course, a low hydrogen partial pressure helps to facilitate those reactions, but no known methanogen is able to maintain a hydrogen partial pressure low enough (10^{-10} atm) to allow direct proton reduction with these electrons (Thauer and Morris 1984; Schink 1997). Therefore, it was postulated that syntrophic butyrate degraders have to invest energy into a reversed electron transport, thus leaving only a fraction of an ATP for growth of the bacterium (Thauer and Morris 1984).

Müller et al. (2009) showed that an enzyme system similar to the comproportionating [FeFe]-hydrogenase of *Thermotoga maritima* is essential in butyrate oxidation by *Syntrophomonas wolfei*. The comproportionating [FeFe]-hydrogenase of *T. maritima* drives the endergonic reduction of protons to hydrogen with NADH by exergonic reduction of another couple of protons with reduced ferredoxin which is produced in pyruvate oxidation during growth on glucose (Schut and Adams 2009). In butyrate oxidation by *S. wolfei,* no such ferredoxin-reducing reaction is involved. Nonetheless, hydrogen formation from NADH is likely catalyzed by a [FeFe]-hydrogenase homologue in *S. wolfei.* This reaction is possible already at a hydrogen partial pressure of 10^{-3} atm (Schink 1997; Müller et al. 2009). Since the enzyme found in *S. wolfei* is associated with a formate dehydrogenase-like protein analogous to its homologue in *Eubacterium acidaminophilum,* interspecies electron transfer may occur via either hydrogen and/or formate, depending on the environmental conditions (Graentzdoerffer et al. 2003; Müller et al. 2009). High metabolic flexibility of *S. wolfei* is also indicated by the fact that different electron-releasing enzyme systems in syntrophically grown *S. wolfei* were identified by two different laboratories. This is likely caused by different cultivation conditions or the substrate preference of the syntrophic partner organism (Crable et al. 2016; Sieber et al. 2014, 2015; Schmidt et al. 2013). NADH-dependent hydrogenase and formate dehydrogenase were considered to be the enzymes responsible for NADH-reoxidation in cells grown in the presence of yeast extract and without cysteine in the cultivation media (Schmidt et al. 2013). Yet, NADH-dependent hydrogenase Hyd1ABC was identified as the most important electron-releasing enzyme system in *S. wolfei* grown without complex supplements and in the presence of both cysteine and sulfide as reducing agents by Sieber et al. (2014, 2015), and Crable et al. (2016), and formate dehydrogenases were found neither in the proteome nor as enzyme activities in cell lysates by the latter authors. Hyd1ABC is a multimeric [FeFe]-hydrogenase similar to the comproportionating hydrogenase in *T. maritima*; however it was demonstrated that the enzyme in

S. wolfei uses NADH as sole electron donor for hydrogen production and is independent of ferredoxin (Losey et al. 2017).

The thermodynamically most difficult step in butyrate oxidation is the transfer of electrons derived from butyryl-CoA oxidation to formate or hydrogen for which a redox potential difference of at least +200 mV has to be overcome (Schink 1997). It was hypothesized that electrons from butyrate oxidation are transferred to quinones in the membrane and that the reduced quinones are reoxidized with NAD^+ (Wallrabenstein and Schink 1994). Such a reaction would require energetization by, e.g., a proton gradient, which was found to be essential for hydrogen formation from butyrate by *S. wolfei* (Wallrabenstein and Schink 1994). Such an enzyme system was identified in the genome of *S. wolfei*, i.e., a membrane-spanning FeS-oxidoreductase, which accepts electrons from an electron-transferring flavoprotein reduced by butyryl-CoA dehydrogenase and transfers them to a menaquinone in the membrane (Sieber et al. 2010). This FeS-oxidoreductase is specifically expressed under syntrophic butyrate-oxidizing conditions (Schmidt et al. 2013; Sieber et al. 2015; Crable et al. 2016). A different hydrogenase, Hyd2ABC, which is located toward the outer space of *S. wolfei*, was identified as responsible enzyme for the reoxidation of reduced menaquinone (Crable et al. 2016). On the other hand, a periplasmic formate dehydrogenase serves as an alternative electron-release enzyme under varying cultivation conditions (Schmidt et al. 2013). It was also postulated that a proton gradient formed by ATPase drives the endergonic reduction of protons or protons and CO_2 with menaquinol; however, such a redox loop system has not been proven in vitro yet (Crable et al. 2016; Schmidt et al. 2013).

Another possible mechanism for reversed electron transport during butyrate oxidation was postulated for *Syntrophus aciditrophicus* based on genome data. Here, an Rnf complex could oxidize NADH and transfer electrons to ferredoxin, driven by influx of protons or sodium ions into the cell (McInerney et al. 2008). Electrons that arise during butyryl-CoA oxidation could be transferred to components of the membrane where NAD^+ is reduced in a similar manner as postulated for *S. wolfei* (McInerney et al. 2008). With the Rnf complex, *S. aciditrophicus* has the potential prerequisites for producing reduced ferredoxin during butyrate degradation, which may drive comproportionating reactions such as NADH oxidation by [FeFe]-hydrogenases or bifurcating reactions such as butyryl-CoA oxidation by the Bcd/EtfAB complex (Li et al. 2008; Herrmann et al. 2008). In contrast, genes that code for the Rnf complex are not present in the genome of *S. wolfei*, indicating that the pathway of butyrate degradation is different in both organisms and does not include reduced ferredoxin in *S. wolfei* (Müller et al. 2009).

2.3 Propionate

All currently identified syntrophic propionate-oxidizing bacteria are affiliated with the class of *Deltaproteobacteria* within the phylum of *Proteobacteria* (McInerney et al. 2005) or the low G+C Gram-positive bacteria in the class *Clostridia* within the

Table 3 Fatty acid-degrading syntrophic bacteria (modified after McInerney et al. 2008)

Organism	Substrate range	Phylogenetic position	Reference
Syntrophomonas wolfei subsp. *wolfei*	$C_{4:1}$–$C_{6:1}$, C_4–C_8	Low G+C Gram positives	McInerney et al. (1979, 1981)
Syntrophomonas sapovorans	$C_{16:1}$, $C_{18:1}$, $C_{18:2}$, C_4–C_{18}	Low G+C Gram positives	Roy et al. (1986)
Syntrophomonas bryantii	$C_{4:1}$, C_4–C_{11}, 2-methylvalerate	Low G+C Gram positives	Stieb and Schink (1985)
Syntrophomonas curvata	$C_{4:1}$, $C_{18:1}$, C_4–C_{18}	Low G+C Gram positives	Zhang et al. (2004)
Syntrophomonas erecta subsp. *erecta*	$C_{4:1}$, C_4 + $C_{5:1}$, C_4–C_8	Low G+C Gram positives	Zhang et al. (2005)
Syntrophomonas zehnderi	$C_{16:1}$, $C_{18:1}$, $C_{18:2}$, C_4–C_{18}	Low G+C Gram positives	Sousa et al. (2007)
Syntrophomonas cellicola	$C_{4:1}$, C_4–C_8, C_{10}	Low G+C Gram positives	Wu et al. (2006)
Thermosyntropha lipolytica	$C_{4:1}$, C_4–C_{18}, $C_{18:1}$, $C_{18:2}$, triglycerides, yeast extract, tryptone, casamino acids, betaine, pyruvate, ribose, xylose		Svetlitshnyi et al. (1996)
Syntrophothermus lipocalidus	$C_{4:1}$, C_4–C_{10}, isobutyrate		Sekiguchi et al. (2000)
Syntrophus aciditrophicus	$C_{4:1}$, fatty acids	*δ-Proteobacteria*	Jackson et al. (1999)
Syntrophus gentianae	$C_{4:1}$	*δ-Proteobacteria*	Szewzyk and Schink (1989)
Syntrophus buswellii	$C_{4:1}$	*δ-Proteobacteria*	Mountfort and Bryant (1982)

phylum *Firmicutes* (Imachi et al. 2002; Plugge et al. 2002b; de Bok et al. 2005) (Table 3). Some of the *Syntrophobacter* sp. are able to use sulfate as the electron acceptor for propionate oxidation (McInerney et al. 2005). In addition, they can grow by fermentation of pyruvate or fumarate. *Smithella propionica* is phylogenetically related to the genus *Syntrophus* (Liu et al. 1999) and lacks the ability to reduce sulfate. *S. propionica* does not oxidize propionate but ferments it to acetate plus butyrate and grows in pure culture on crotonate (de Bok et al. 2001; Liu et al. 1999). These substrates or substrate combinations have been used to obtain axenic cultures of the syntrophs since they bypass the energetically unfavorable steps in propionate oxidation. *Pelotomaculum schinkii*, however, could not be obtained in pure culture until today, nor could it grow on any other compound but propionate. Thus, this bacterium is a true obligately syntrophic bacterium (de Bok et al. 2005).

The question whether hydrogen or formate is transferred in syntrophic cocultures has been studied in propionate-degrading *Syntrophobacter fumaroxidans* cocultures (Fig. 3). Thermodynamic calculations, flux measurements in defined cocultures and

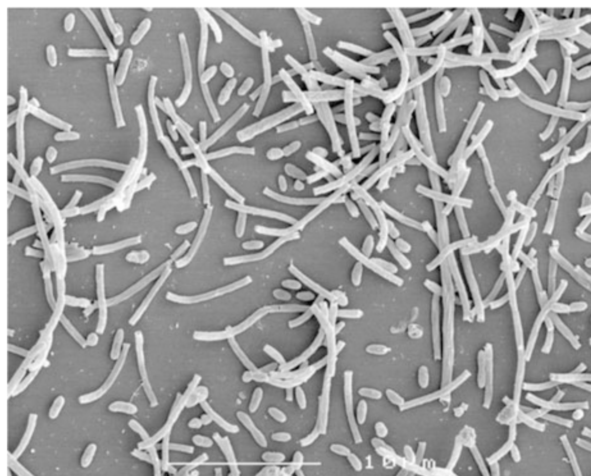


Fig. 3 Scanning electron micrograph of a syntrophic propionate-degrading coculture of *S. fumaroxidans* (lemon or oval shaped) and *M. hungatei* (rod-shaped)

enzyme measurements all confirmed that interspecies formate transfer is an essential mechanism in syntrophic propionate degradation in suspended cultures (Dong et al. 1994a; Dong and Stams 1995). The terminal reductases were studied in detail and biochemical evidence for formate transfer was found (De Bok et al. 2002). Two formate dehydrogenases were isolated and characterized. In contrast to most formate dehydrogenases which contain molybdenum, one formate dehydrogenase (CO_2-reductase) of *S. fumaroxidans* contains tungsten and has an unusually high specific activity both in the formate oxidation and the CO_2 reduction assay (Reda et al. 2008). Full genome sequences that became available allowed to study the metabolism of syntrophic propionate degraders in detail (Plugge et al. 2012; Müller et al. 2010; Kato et al. 2009; Kosaka et al. 2008). A comparative genome analysis of syntrophs and non-syntrophs pointed to an essential role of formate dehydrogenase in interspecies electron transfer (Worm et al. 2014). Transcriptome analysis with cocultures of *S. fumaroxidans* showed the presence of multiple hydrogenases, formate dehydrogenases, and hydrogen formate lyases in *S. fumaroxidans* (Worm et al. 2011). It was hypothesized that the large set of terminal reductases provides metabolic flexibility in these bacteria that are constrained by the thermodynamic limitations they have to cope with (Worm et al. 2011). Likely therefore, the relative role of formate and hydrogen is not easy to assess, especially because at in situ conditions the interconversion of hydrogen + carbon dioxide to formate is close to equilibrium (Dong and Stams 1995; Montag and Schink 2016; Felchner-Zwirello et al. 2013).

The microorganisms involved in propionate degradation are genuine specialists in obtaining metabolic energy for growth, since they have to grow under thermodynamically very unfavorable conditions (Leng et al. 2018). The standard Gibbs free energy change of the complete degradation of propionate to methane and CO_2 is about −60 kJ which is approximately equivalent to the amount of energy needed to produce one mol of ATP. A community of three microorganisms brings about this conversion: one bacterium that degrades propionate to acetate, CO_2, and hydrogen and two methanogenic Archaea, one that cleaves the acetate and another one that

uses hydrogen and/or formate. The actual energy that is available for each member of the community depends on the in situ concentrations of substrate, intermediates, and products and will vary during growth.

With the exception of *S. propionica*, all propionate degraders employ the methylmalonyl-CoA pathway to degrade propionate. In this route, one ATP is formed in the conversion of pyruvate to acetate via substrate-level phosphorylation. Reducing equivalents are released at three different redox levels: reduced ferredoxin is formed in the conversion of pyruvate to acetate, while NADH and enzyme-bound $FADH_2$ are formed in the oxidation of malate and succinate, respectively. These intracellular redox mediators need to be reoxidized by reduction of protons or CO_2. The oxidation of reduced ferredoxin ($E^{0'}$ Fd(ox)/Fd (red) = −398 mV) and NADH ($E^{0'}$ NAD^+/NADH = −320 mV) can be coupled to reduction of protons ($E^{0'}$ = −414 mV) or CO_2 (−432 mV) only if the hydrogen or formate concentration is kept low by methanogens.

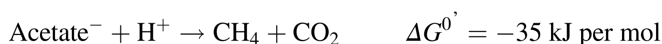
The oxidations of succinate and malate with protons are endergonic even at a hydrogen partial pressure as low as 1 Pa (the minimum level that can be achieved by methanogens). To drive these reactions, input of metabolic energy via reverse electron transport is required. The mechanism that drives succinate oxidation to fumarate ($E^{\circ\prime}$ = +33 mV) during syntrophic growth is similar to the mechanism of energy conservation in fumarate respiration by *Wolinella succinogenes* (Kröger et al. 2002), but operating in reverse (Worm et al. 2011). Experimental evidence was obtained that 2/3 ATP is needed to drive this conversion (van Kuijk et al. 1998). As such, the net ATP gain for the bacterium is 1/3 mol ATP per mol of propionate converted. However, this value may differ depending on the growth condition and the physiological state of the cells.

Oxidation of malate to oxaloacetate with NAD^+ is an endergonic reaction. Nonetheless, the purified malate dehydrogenase of *S. fumaroxidans* exhibits a very high K_m value toward oxaloacetate and NADH, and as such, the organism may be able to efficiently perform this conversion (van Kuijk and Stams 1996). *S. fumaroxidans* and *P. thermopropionicum* contain [FeFe]-hydrogenases that are homologues to the comproportionating [FeFe]-hydrogenase of *T. maritima*. This suggests that NADH and ferredoxin that are generated in the methylmalonyl-CoA pathway are simultaneously reoxidized with the reduction of protons. These novel bifurcating enzyme complexes may be essential in these syntrophic fermentations. Additionally, a membrane-bound Rnf complex in *S. fumaroxidans* is used to oxidize NADH coupled to reduced ferredoxin formation, a process that is driven by a proton gradient. Subsequently, ferredoxin-dependent hydrogenases form hydrogen (Worm et al. 2011).

2.4 Acetate

Although acetate can be used directly by certain methanogens such as *Methanosarcina* spp. and *Methanosaeta* spp. and is converted by these organisms to methane and CO_2, this situation is typical only for systems at moderate

temperature and low salt content. At enhanced temperature, acetate can be oxidized to $2CO_2$ + 4 pairs of reducing equivalents (H_2 or formate) in a reaction analogous to a reversal of homoacetate fermentation (see Table 1), and the electrons thus released are used by a methanogenic partner. This phenomenon has been observed first in a thermophilic reactor system (Zinder and Koch 1984) and later also at lower temperature in sludge of enhanced ammonia content (Schnürer et al. 1996). The overall reaction



$$\text{Acetate}^- + H^+ \rightarrow CH_4 + CO_2 \qquad \Delta G^{0'} = -35 \text{ kJ per mol}$$

can hardly feed two organisms. The energy yield increases with rising temperature (Schink 1997); at 60 °C, ΔG' is −42 kJ per mol, which is just sufficient to allow two organisms to grow with this process. At lower temperatures, the energy supply becomes a serious problem, and doubling times increase to the range of several weeks (Schnürer et al. 1996). Indications were reported that also at slightly acidic conditions, e.g., in bogs, acetate is degraded in a syntrophic cooperation (Metje and Frenzel 2007); at pH 5.0, the $\Delta G = -46$ kJ per mol.

The biochemistry of syntrophic acetate oxidation appears to be basically a reversal of the homoacetogenic acetate formation pathway (so-called Wood-Ljungdahl pathway or CO-dehydrogenase pathway). Acetate is activated to acetyl-CoA and cleaved by a CO-dehydrogenase/acetate synthase to a methyl and a carbonyl residue which are oxidized separately through well-described pathways (Schnürer et al. 1997; Hattori et al. 2000, 2005). The question remains how the bacterium couples this pathway to ATP synthesis. Since acetate activation through acetate kinase and phosphotransacetylase requires one ATP which is regained in the conversion of formyl tetrahydrofolate to formate, there is no net ATP formation by substrate-level phosphorylation. Moreover, the oxidation of methyl tetrahydrofolate to methylene tetrahydrofolate releases electrons at a rather positive redox potential (−200 mV) which would need an energy input for delivery as molecular hydrogen to a partner organism. Genome analyses of syntrophically acetate-oxidizing bacteria indicate the presence of numerous hydrogenases and formate dehydrogenases that might be involved in these syntrophic associations (Oehler et al. 2012; Manzoor et al. 2015, 2016) but the question of net ATP synthesis remains open so far.

2.5 *Branched Fatty Acids*

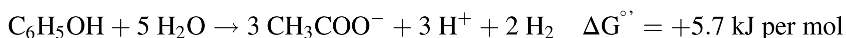
Branched-chain fatty acids are formed during degradation of amino acids. Oxidative decarboxylation of valine leads to 2-methylpropionate (isobutyrate), leucine forms 3-methylvalerate (isovalerate), and isoleucine forms 2-methylbutyrate (neovalerate). Whereas neovalerate can be degraded easily by beta-oxidation to an acetyl and a propionyl residue, the other two acids pose some mechanistic difficulties. While isobutyrate, after activation to isobutyryl-CoA, can be isomerized to butyryl-CoA in

a B_{12}-dependent reaction and subsequently cleaved to two acetyl residues (Stieb and Schink 1989), isovalerate degradation includes a carboxylation and subsequent formation of three acetyl residues (Stieb and Schink 1986). In all cases, the degradation of the branched carbon skeletons is slow, and these branched fatty acids, similar to the corresponding residues of aromatic amino acids, accumulate in anoxic environments to a certain extent and may be taken up again by other anaerobic bacteria for reductive amination to amino acids (Allison and Bryant 1963), thus saving a lot of biosynthetic effort into amino acid synthesis.

2.6 Benzoate

Aromatic compounds were considered for a long time not to be degradable in the absence of oxygen, and reliable reports on their degradation in methanogenic ecosystems date back only into the late 1970s (Healy and Young 1978). The best-studied system is the syntrophic oxidation of benzoate by species of the genus *Syntrophus*, i.e., *S. buswellii*, *S. aciditrophicus*, and *S. gentianae*. Benzoate degradation in these bacteria proceeds via an initial activation to benzoyl-CoA by a ligase reaction, partial reduction to a cyclohexadiene derivative, addition of water, and subsequent beta-oxidative ring cleavage and degradation to three acetyl moieties plus CO_2 (Schöcke and Schink 1997). Whereas nitrate-reducing bacteria invest two ATP equivalents into the partial reduction of benzoyl-CoA, strict anaerobes such as the iron-reducing bacterium *Geobacter metallireducens* use a less energy-expensive way (Fuchs et al. 2011; Philipp and Schink 2012), and a similar path is used by the syntrophically fermenting bacterium *Syntrophus aciditrophicus* (Fuchs 2008; Boll et al. 2016). The overall ATP yield has been calculated on the basis of growth yields with *S. gentianae* to be ⅓–⅔ ATP equivalents (Schöcke and Schink 1999).

Phenol is another important aromatic compound which is degraded anaerobically through carboxylation to a 4-hydroxybenzoyl derivative and subsequent dehydroxylation to benzoyl-CoA. In nitrate-reducing bacteria, the initial carboxylation consumes the equivalent of two ATP units. The energetic situation of fermentative phenol degradation is very tight:



$$C_6H_5OH + 5\ H_2O \rightarrow 3\ CH_3COO^- + 3\ H^+ + 2\ H_2 \quad \Delta G^{\circ\prime} = +5.7\ \text{kJ per mol}$$

Even in syntrophic cooperation with a hydrogen-oxidizing partner, the phenol degrader obtains only little energy (~−40 kJ per mol phenol; Schink 1997), thus keeping the overall energy budget small and hardly allowing to spend two ATP into the initial carboxylation reaction. So far, the details of the biochemistry of syntrophic phenol degradation have not been studied in detail yet; a defined coculture of a syntrophically phenol-degrading bacterium has been isolated (Qiu et al. 2008).

2.7 Sugars

Sugars can be fermented by numerous groups of bacteria and archaea. The biochemical pathways of sugar oxidation are diverse but in most cases end up with pyruvate as a key metabolite. Most bacteria degrade sugars by converting mono- or disaccharides from polysaccharide cleavage into fructose or glucose which are oxidized to pyruvate through the Embden-Meyerhof-Parnas pathway. Pyruvate can be further oxidized to acetate, or be oxidized to CO_2 by anaerobic respiration, or be used as internal electron acceptor for fermentative production of a variety of acids or solvents.

Fermentation of sugars via the Embden-Meyerhof-Parnas pathway with subsequent acetyl-CoA phosphorylation usually yields acetate, CO_2, and hydrogen. Formation of only acetate, CO_2, and hydrogen would require formation of four moles of ATP per mole of glucose, two in glycolysis, and two in the acetate kinase reaction. However, the reaction provides a negative free reaction enthalpy of only −216 kJ per mole (Table 1) which is not sufficient for formation of 4 ATP. This fermentation would need to reoxidize the glycolysis-derived NADH with protons which is endergonic under standard conditions. Most mesophilic sugar fermenters cope with this problem by releasing various reduced side products such as organic acids or alcohols. Thus, NADH can be reoxidized without hydrogen formation, but on the other hand, only 2–3 moles of ATP per mole of glucose can be gained. In the presence of hydrogen-scavenging methanogenic partners, the formation of only acetate, CO_2, and hydrogen is favored (Schink 1997). For example, the glucose-fermenting *Ruminococcus albus* shifts its fermentation pattern from acetate plus ethanol under axenic growth conditions to acetate, CO_2, and hydrogen in syntrophic coculture (Iannotti et al. 1973). Obviously, the bacterium optimizes its ATP gain which is maximal if the hydrogen partial pressure is low enough to shift the thermodynamic equilibrium of glucose oxidation toward a more negative free reaction enthalpy, thus allowing the formation of 4 ATP.

The facultatively anaerobic *Bacillus stamsii* (*Bacillus sp*. BoGlc83) grows anaerobically with glucose as substrate in the presence of a methanogenic partner (Müller et al. 2008). At first, this organism did not release reduced side products and appeared to be forced to cooperate with methanogens, therefore. Later it was shown that in cocultures with *Methanospirillum hungatei*, traces of lactate and succinate are formed besides acetate and methane at temperatures higher than 20 °C and at glucose concentrations higher than 2 mM (Müller et al. 2008). Regarding that the natural habitats of *B. stamsii* are cold profundal sediments, e.g., of Lake Constance, it seems likely that production of lactate and succinate is a stress response to unusual heat and high substrate concentrations much different from the cold and nutrient-poor natural environment of this organism. Besides its ability to degrade glucose syntrophically, *B. stamsii* is also able to ferment glucose anaerobically in pure culture, but only in the presence of 10 mM pyruvate (Müller et al. 2015). Under these conditions, lactate is produced as primary fermentation product from glucose and only small amounts of pyruvate are degraded (Müller et al. 2015).

Similar observations were made before with *Bacillus subtilis* where mixed acid fermentation from glucose was stimulated by pyruvate (Nakano et al. 1997). However, *B. subtilis* as well as *Bacillus jeotgali* and the scarab beetle larvae isolate *Bacillus* PeC11 grow anaerobically with glucose as sole energy source to a certain extent, while *B. stamsii* obligately depends on either a methanogenic partner or addition of pyruvate to anaerobically degrade glucose (Nakano et al. 1997; Müller et al. 2015). It was hypothesized that this inability could be explained by the lack of biosynthetic pathways in *B. stamsii*, as well as the absence of an ethanol-producing pathway to release electrons derived from NADH, as ethanol was always produced as a side product of glucose fermentation in *B. subtilis*, *B. jeotgali*, and *Bacillus* sp. PeC11 (Nakano et al. 1997; Müller et al. 2015). This strict dependency of *B. stamsii* on pyruvate and also on complex supplements such as yeast extract in pure culture indicate that under syntrophic conditions and in the absence of pyruvate and complex supplements, the methanogenic partner not only keeps hydrogen and formate levels low but could also provide essential growth factors and as such influences both dissimilation and assimilation in *B. stamsii* (Müller et al. 2015).

2.8 Amino Acids

Much of our knowledge on anaerobic protein and amino acid degradation has been obtained through studies on ruminants, since protein is an important dietary product for ruminants (Allison 1970; Bryant 1977; Hobson and Wallace 1982). Proteins in the rumen are hydrolyzed by extracellular proteases and intracellular peptidases (Hazlewood and Nugent 1978) to single amino acids, peptides, and ammonia. Also in anaerobic digesters, the input of proteins coming from different sources of waste (e.g., slaughterhouses, beer breweries, dairy industries) can be large. Proteins are composed of about 20 structurally different amino acids which require distinct biochemical pathways for degradation. As such, anaerobic degradation of amino acids by mixed methanogenic consortia is very complex and is performed by many fermentative microorganisms. Degradation involves oxidation and reduction reactions of one or more amino acids. Some amino acids are degraded preferentially via oxidation; others can also serve as electron acceptors. The combined oxidation and reduction of pairs of amino acids (Stickland reaction) is a well-known mechanism by which proteolytic clostridia degrade amino acids (Stickland 1934; Barker 1981; Stams 1994). In the Stickland reaction, the oxidation of one amino acid is coupled to the reduction of another one. In the oxidative branch, alanine and many other amino acids can be partly degraded; glycine is a classical electron acceptor in the reductive branch via a selenium-dependent glycine reductase (Andreesen 1994, 2004) (Table 1). Other couples have been described in the past (Barker 1981). Amino acid degradation is significantly affected by the presence of methanogens. Methanogens can act as scavengers of reducing equivalents in the oxidation of amino acids, taking over the role of the reductive branch of the Stickland reaction. Nagase and Matsuo (1982) observed that in mixed methanogenic communities, the

degradation of alanine, valine, and leucine was inhibited by inhibition of methanogens, and Nanninga and Gottschal (1985) could stimulate the degradation of these amino acids by the addition of hydrogen-utilizing anaerobes. Several anaerobic bacteria have been described that grow syntrophically on amino acids in coculture with methanogens (McInerney 1988; Stams 1994; Plugge and Stams 2005).

Usually the first step in the degradation of amino acids is a deamination (Barker 1981; McInerney 1988; Andreesen et al. 1989). Deamination can be performed by anaerobic bacteria in three ways. Oxidative NAD(P)-dependent deamination of alanine, valine, leucine, or isoleucine leads to the corresponding keto acid. The $\Delta G^{0\prime}$ of the deamination of alanine, valine, leucine, and isoleucine to the corresponding keto acids when coupled to hydrogen formation is around +60 kJ/mol (Table 1). As a consequence, methanogens are needed to pull the reaction in a similar fashion as described for other syntrophic oxidations above. The keto acid is then converted via oxidative decarboxylation to a fatty acid releasing electrons at $E^{\circ\prime} = -470$ mV, which can easily be transferred via ferredoxin to protons. Overall, the oxidative deamination of the four mentioned amino acids to fatty acids, ammonia, and hydrogen is slightly endergonic. The second mechanism is a reductive deamination and is found only in anaerobes (McInerney 1988; Andreesen et al. 1989; Andreesen 1994). Reducing equivalents are used to convert the amino acid to its corresponding fatty acid, with concomitant production of ammonia. An example is the reduction of glycine to acetate via the selenium-dependent glycine reductase (Stickland 1934; Andreesen 2004). The third mechanism, a redox-neutral reaction, results in the production of the corresponding keto acid. An example is the conversion of serine to pyruvate plus ammonia by the action of serine ammonia lyase or the C-C rearrangement of glutamate to 3-methylaspartate (Buckel and Barker 1974).

Glutamate is an abundant amino acid in proteins (McInerney 1988). In methanogenic habitats, glutamate can be metabolized in several different ways, leading to different growth yields. The effect of hydrogen removal by methanogenic partners on the metabolism of amino acid-fermenting anaerobes has been studied best with glutamate. Glutamate fermentation is carried out by a variety of anaerobes, including a number of *Clostridium* species, *Peptostreptococcus asaccharolyticus,* and *Acidaminococcus fermentans* (Gottschalk 1986; Boiangiu et al. 2005). These microorganisms ferment glutamate to acetate and butyrate by either the β-methylaspartate or the hydroxyglutarate pathway (Buckel and Barker 1974). In this fermentation, reducing equivalents formed in the oxidation of glutamate to acetate are disposed of, either partly or completely, by reductive formation of butyrate from acetyl residues. *Anaeromusa acidaminophila* ferments glutamate to acetate plus propionate (Nanninga et al. 1987). In this bacterium, reducing equivalents are disposed of by reduction of pyruvate to propionate. Besides acetate, butyrate, and propionate, also traces of hydrogen (up to 20 kPa) are formed during glutamate fermentation via the β-methylaspartate and the hydroxyglutarate pathway. Work in the lab of W. Buckel has unraveled the mechanisms underlying this hydrogen production (Buckel 2001a, b; Boiangiu et al. 2005). Pyruvate is oxidatively decarboxylated to acetyl-CoA by pyruvate-ferredoxin oxidoreductase. Reoxidation of reduced ferredoxin proceeds in two ways: the majority (up to 80%)

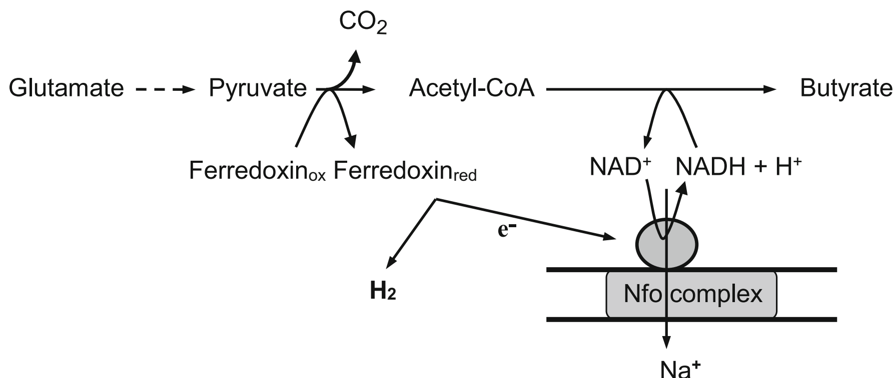


Fig. 4 Model of NADH-ferredoxin oxidoreductase (after Boiangiu et al. 2005)

is reoxidized during the synthesis of butyrate from two acetyl-CoA, and the remaining 20% is used to reduce protons to hydrogen. This reaction is catalyzed by an iron-only hydrogenase. For butyrate synthesis from two acetyl-CoA, however, reduced NADH is necessary. For *Clostridium tetanomorphum*, it was postulated that NAD^+ is reduced by a membrane-bound NADH-ferredoxin oxidoreductase (Fig. 4) (Buckel 2001b; Boiangiu et al. 2005).

Several *Bacteria* have been isolated which during growth on glutamate release reducing equivalents exclusively as hydrogen, in the form of acetate, the form of propionate, or both. Microorganisms that ferment glutamate to acetate only include *C. coolhaasii* (Plugge et al. 2000) and *C. proteoclasticus* (Tarlera et al. 1997). Propionate as the only product is formed from glutamate by *Aminobacterium colombiense* (Baena et al. 1998) and *Gelria glutamica* (Plugge et al. 2002a). *Acidaminobacter hydrogenoformans* (Stams and Hansen 1984; Meijer et al. 1999), *Thermanaerovibrio acidaminovorans* (Cheng et al. 1992; Baena et al. 1999a), and *Aminomonas paucivorans* (Baena et al. 1999b) form both acetate and propionate from glutamate. In syntrophy with methanogens, the hydrogen pressure can be lowered to 1 Pa, and glutamate degradation to CO_2, acetate or propionate, and hydrogen becomes feasible (Plugge et al. 2002a). Hence, both under these conditions in the acetate and in the propionate-forming pathway, energy conservation to the extent of 1 ATP per mole glutamate is thermodynamically possible.

According to ^{13}C-labeling studies with 1-^{13}C- and 3-^{13}C-glutamate, the pathway of glutamate fermentation to acetate in *Caloramator coolhaasii* proceeds via 3-methylaspartate and pyruvate. *T. acidaminovorans* forms propionate by oxidation of glutamate followed by decarboxylation of succinyl-CoA via methylmalonyl-CoA to propionyl-CoA (Plugge et al. 2001). Operation of the citric acid cycle can be excluded since no 2,3-double labeled propionate could be detected; obviously, neither fumarate nor free succinate was formed as intermediates. The formation of [2,3-^{13}C] succinate indicated that glutamate is directly oxidized to succinyl-CoA, in which part of the succinyl-CoA is converted to succinate and excreted, and the majority is further converted to propionate.

Members of the *Cloacimonetes* were the third dominant group of bacteria detected in a methanogenic UASB bioreactor. The *Cloacimonetes*, including candidate division WWE1 (Waste Water of Evry 1), are a subdominant group of bacteria found in multiple environments, including anaerobic digesters and the intestinal tracts of animals (Rinke et al. 2013; Limam et al. 2014). So far, all attempts to cultivate representatives of the *Cloacimonetes* were unsuccessful beyond enrichments, probably due to their need for obligatory symbiotic or syntrophic relationships with other microorganisms. However, using metagenomic sequence data and genomic assembly procedures, the genome of a representative bacterium *Candidatus Cloacimonas acidaminovorans* has been constructed (Pelletier et al. 2008). Based on sequence analyses, the candidate division WWE1 bacteria are regarded as syntrophs that are capable of amino acid fermentation, propionate and butyrate oxidation, as well as cellulose degradation (Pelletier et al. 2008; Rinke et al. 2013; Limam et al. 2014), but experimental proof of this claim is missing.

2.9 Carrier Systems

Depending on the type of syntrophic conversion, the carrier system that transfers electrons from the producer to the consumer may vary. The best-studied and best-accepted electron carrier is hydrogen. However, formate is considered to be an important agent in interspecies electron transfer during propionate conversion as already discussed in Sect. 2.3. Formate can also act as electron carrier in syntrophic butyrate conversion by *S. wolfei* since this bacterium contains a formate dehydrogenase with high homology to a formate dehydrogenase of *Eubacterium acidaminophilum* (FdhA-II) that was suggested to play a role also in interspecies formate transfer (Müller et al. 2009; Schmidt et al. 2013). Analysis of hydrogen and formate pools in various methanogenic environments indicates that both carriers are energetically equivalent and are most often used simultaneously (Schink et al. 2017).

In syntrophic acetone-degrading methanogenic cultures, acetate was identified as the only interspecies carrier compound (Platen and Schink 1987; Platen et al. 1994). In this syntrophic culture, growth and conversion of acetone to acetate proceeded until acetate had accumulated to ~10 mM. Addition of an active acetoclastic methanogen (*Methanosaeta* sp.) greatly enhanced the acetone degradation rate (Platen and Schink 1987).

Interspecies electron cycling through sulfur and sulfide has been described for *Desulfuromonas acetoxidans* in syntrophic cultures with *Chlorobium limicola*, a phototrophic green sulfur bacterium (Pfennig and Biebl 1976; Biebl and Pfennig 1978). A similar sulfur-cycle-mediated electron transfer was described in an artificial coculture which syntrophically oxidized acetate to CO_2 with concomitant reduction of nitrate (Kaden et al. 2002).

The discovery of bacterial nanowires and identification of presumed electron transfer components required for electrical conductivity in these pili-like structures provided a novel view at mechanisms involved in interspecies electron transfer

(Gorby et al. 2006; Reguera et al. 2005). Pili-like structures have been identified in a number of pure and mixed cultures, and also syntrophic cocultures of propionate-oxidizing *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus* produced these pili-like structures. Analysis of the conductive properties of pili indicated that they could transfer electrons between cells of *Geobacter sulfurreducens* and the surface of Fe(III) oxides (Reguera et al. 2005). These pili-like structures were not required for attachment to the insoluble electron acceptor; rather, they are interpreted to function as channels for electron transfer to the Fe(III) oxides, extending the electron transfer capabilities of the cells well beyond their outer surface (Reguera et al. 2005). Pili "nanowires" also were reported to serve as electric conduits to mediate long-range electron transfer across biofilms formed on anode electrodes in microbial fuel cells which could maximize current production per unit of anode surface area (Reguera et al. 2005).

Also other carriers have been discussed through the recent years as possible electron transfer systems between syntrophic partners. These include iron oxides, humic acid analogues such as anthraquinone disulfonate (Lovley et al. 1998), activated carbon and biochar (Liu et al. 2012; Chen et al. 2014), and various iron oxides (Kato et al. 2012; Viggi et al. 2014; Zhou et al. 2014). There is even evidence of direct electron transfer between syntrophic partners, either via direct interspecies electron transfer (Shrestha et al. 2013; Rotaru et al. 2014a,b; Li et al. 2015) or via nanowires (Shresta and Rotaru 2014). In all these cases, addition of the respective carrier system caused a substantial increase of interspecies electron transfer rates. Nonetheless, one has to keep in mind that some of these electron carriers have standard redox potentials of −200 mV and higher. Coupling interspecies electron transfer to methanogenesis requires a sufficiently low redox potential to allow a minimum amount of energy to be conserved on the side of the methanogens; thus, electrons have to be delivered by the partner at an average E' of at max. −270 mV. Electron transfer through carriers of substantially higher redox potential (e.g., anthraquinone disulfonate with $E_0' = -184$ mV) may work in short-time experiments but cannot secure a sufficient energy supply for growth and population maintenance of the methanogenic partners.

2.10 Alternative Substrates for Pure Cultures, Technical Systems to Replace Methanogens, and Other Culturing Approaches for Syntrophs

Outside the laboratory, bacterial communities are nearly always communities composed of a wide variety of species. It is appropriate to consider the relevance of these interspecies interactions to the outcome of activity assays and the cultivability in the laboratory. Defined cultures of syntrophically fermenting bacteria are required for detailed physiological and molecular studies and to understand their significant role in nature. To obtain such cultures, technical systems can be used to replace the

methanogenic partner, or alternative substrates can be supplied to bypass the energetically unfavorable steps occurring in syntrophic conversions.

The first axenic culture of an obligatory syntrophic bacterium was *S. wolfei* (Beaty et al. 1987). Studies on the butyrate metabolism of syntrophic cocultures of *S. wolfei* and *M. hungatei* revealed a high activity of β-oxidation enzymes (Wofford et al. 1986). With this knowledge, Beaty and coworkers grew *S. wolfei* on agar plates containing crotonate as the sole source of carbon and energy. The pure culture obtained dismutated crotonate to butyrate and acetate but exhibited butyrate oxidation only after reassociation with a syntrophic partner. Later it was shown that *S. wolfei* and *Syntrophospora bryantii* could grow in pure culture on butyrate plus 3-pentenoate (Amos and McInerney 1990; Dong et al. 1994b). Butyrate plus 3-pentenoate were converted to valerate, acetate, and propionate.

The first successful axenic culture of a syntrophic propionate-degrading bacterium was obtained from an enrichment culture by inhibiting the methanogens with bromoethanesulfonic acid (an analogue of Coenzyme M) and subsequently adding fumarate as external electron acceptor. This allowed to isolate *S. fumaroxidans* (Stams et al. 1993; Harmsen et al. 1998) and to study the pathway of propionate oxidation (Plugge et al. 1993). Phylogenetically, *S. fumaroxidans* is very closely related to sulfate-reducing bacteria.

Some sulfate-reducing bacteria can alter their metabolism and act as syntrophically fermenting partners if sulfate becomes depleted (see above; Bryant et al. 1977; Scholten et al. 2007; Walker et al. 2009). Although this metabolic flexibility may be helpful for the enrichment and isolation of syntrophic bacteria, it can be applied only to already highly enriched syntrophic cultures.

A strategy for isolation of syntrophs could be stepwise: from enrichment culture via molecular characterization ("omics"-based) to a strategic choice of substrate, electron acceptor, or unsaturated compound for the isolation of the microorganism. Examples of such unsaturated compounds used are fumarate, crotonate, pentenoate, and benzoate.

Moreover, metagenome data can be used to resolve interspecies relationships. Using metagenomic sequence data and a specific genome assembly procedure, the genome of a representative bacterium of the candidate phylum *Cloacimonetes*, *Candidatus* "*Cloacimonas acidaminovorans*" was reconstructed, and its metabolic properties indicated that it can grow as a syntroph (Pelletier et al. 2008).

A cultivation apparatus capable of maintaining very low H_2 (<0.01 Pa) pressures by mechanical means was developed by Valentine et al. (2000). This apparatus provided a method to study interspecies hydrogen transfer by externally providing the thermodynamic requirement for very low hydrogen concentrations, thus preventing the need for use of cocultures to study the metabolic pathways. The culture vessel is constructed of glass and operates by sparging a liquid culture with purified gases, which remove hydrogen directly as it is produced. The culture device was constructed to decouple the syntrophic relationship in an ethanol-oxidizing methanogenic enrichment culture, allowing ethanol oxidation to dominate the methane production. Moreover, the culture apparatus was successfully used to grow pure cultures of the ethanol-oxidizing, proton-reducing *P. acetylenicus* (Valentine et al. 2000). This culture

apparatus may have a potential to study also other forms of syntrophic metabolism; however, we have to realize that fatty acid oxidation requires hydrogen pressures substantially lower than needed for ethanol oxidation.

Assessing phenotypes and enrichment of hard-to-culture microorganisms is characterized by an increased miniaturization and throughput. "Omics" strategies allow gaining insight into population phenotypes at the milliliter and microliter scale, using lab-on-chip technologies that allow ultrahigh-throughput experiments at the single cell level, as well as cultivation starting from single-sorted cells (Vervoort et al. 2017). These lab-on-chip applications require optimization as many of them are not yet adapted to work with strict anaerobes or syntrophs.

A miniaturized, disposable microbial culture chip has been fabricated by microengineering a highly porous ceramic sheet with up to one million growth compartments (Ingham et al. 2007). A range of disposable, surface-culture, microbial growth chips, or "micro-Petri dishes," were created by using a microengineered mechanical system approach to engineer growth compartments on top of porous aluminum oxide, which acts as the surface on which an exceptional number of microbial samples can be grown, assayed, and recovered. High-throughput screening of >200,000 isolates from Rhine water based on metabolism of a fluorogenic organophosphate compound resulted in the recovery of 22 microcolonies with the desired phenotype. These isolates were predicted, on the basis of rRNA sequence, to include six new species. Through control of individual microwells, the micro-Petri dish can also be integrated in a more complex lab-on-a-chip system, with opportunities for a variety of applications, including anaerobic incubation. With this micro-Petri dish, direct integration of culture-based assays and enrichment with molecular detection and other technologies can be established.

2.11 Anaerobic Methane Oxidation

In most syntrophic processes described so far, a methanogenic partner receives electrons from a bacterium and produces methane to make the overall process energetically favorable. During anaerobic oxidation of methane (AOM) coupled to sulfate reduction, ANaerobic MEthanotrophic (ANME) archaea oxidize methane via reversal of the methanogenic pathway and donate electrons to sulfate-reducing bacteria (SRB) (Hinrichs et al. 1999; Boetius et al. 2000; Orphan et al. 2001). Sulfate-dependent AOM was first discovered in marine sediments and is thought to be a major contributor to the global carbon and sulfur cycle (reviewed in: Knittel and Boetius 2009). The overall Gibbs free energy yield of the reaction (reaction 1) is at the limit of what is energetically possible for sustaining life, with estimates between -18 and -35 kJ mol^{-1} (reviewed in Thauer 2011). Doubling times of the involved microorganisms were between 1.1 and 7.5 months (reviewed in: Timmers et al. 2017). Three clades of ANME have been described so far: ANME-1, ANME-2, and ANME-3 (Knittel and Boetius 2009). Some of these clades were

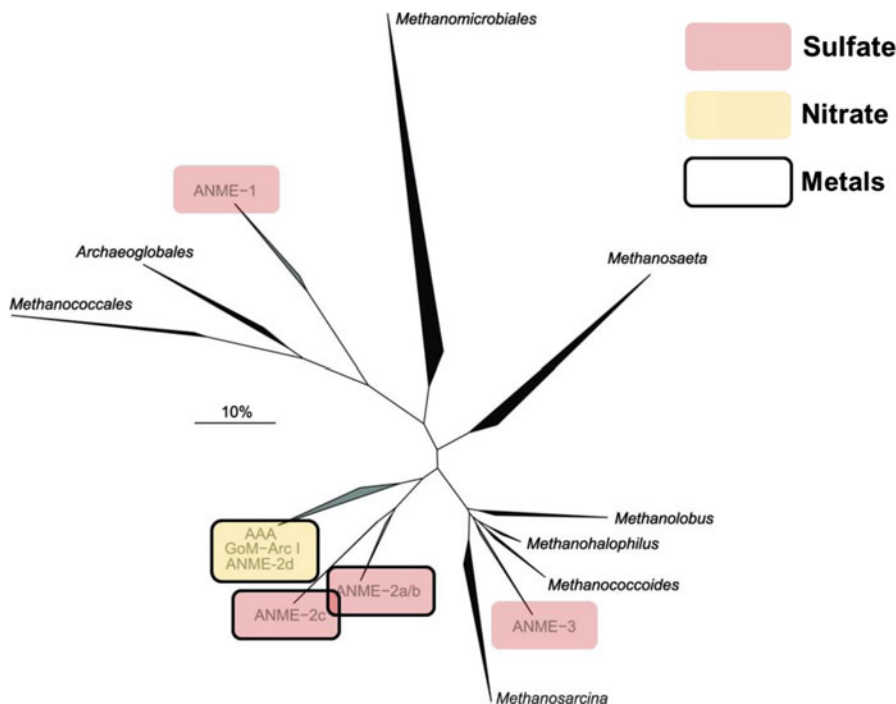


Fig. 5 Phylogenetic tree of full-length 16S rRNA sequences of all methanotrophic anaerobic clades so far described and their relation with other archaeal clades. The legend indicates the electron acceptors that ANME can use for AOM. The tree was reproduced and adapted from Timmers et al. (2017)

subdivided into phylogenetic subclusters (a, b, and c), using retrieved environmental 16S rRNA sequences (Fig. 5).

$$\text{Reaction 1}: CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad \Delta G^0 = -16.3\,\text{kJ}\,\text{mol}^{-1}$$

For long, it was a mystery which interspecies electron carrier (IEC) was used between ANME and the partner SRB. Experimental and modeling studies excluded methanogenic substrates such as hydrogen, formate, acetate, and methanol as IEC (Nauhaus et al. 2002; Treude et al. 2007; Orcutt and Meile 2008; Alperin and Hoehler 2009; Meulepas et al. 2010; McGlynn et al. 2015). Recent research indicated that both ANME-1 and ANME-2 transfer electrons directly to SRB via multiheme cytochromes (MHCs) (McGlynn et al. 2015; Wegener et al. 2015), and the SRB partner of ANME-1 produced pili-derived nanowires additionally (Wegener et al. 2015).

Members of another clade, ANME-2d (previously named GoM-Arc I and the Anaerobic methane oxidation Associated Archaea, AAA), have recently been shown

to oxidize methane coupled to nitrate reduction (reaction 2) (Haroon et al. 2013). Nitrate-dependent AOM was first also thought to be syntrophic (Raghoebarsing et al. 2006), but it was revealed that the ANME consume methane and nitrate alone (Haroon et al. 2013) and the bacterial partner consumed the toxic nitrite that is produced by the ANME. This explained the enrichment of interdependent consortia of ANME-2d with anammox bacteria (Haroon et al. 2013) or with the nitrite-dependent methane oxidizing bacterium "*Ca. Methylomirabilis oxyfera*" of the NC10 phylum (Raghoebarsing et al. 2006; Ettwig et al. 2008). The natural occurrence and activity of ANME-2d and the contribution to the carbon and nitrogen cycle is not known, but ANME-2d members have been found in a variety of environments (reviewed in: Welte et al. 2016).

$$\text{Reaction 2}: CH_4 + 4NO_3^- \rightarrow HCO_3^- + 4NO_2^- + H_2O + H^+ \quad \Delta G^0 = -517.2 \text{kJmol}^{-1}$$

AOM coupled to metal(oxide) reduction is energetically feasible as well and environmental, and enrichment studies gave indications that it indeed occurs (reviewed in: He et al. 2018). Today, there is evidence that enrichments of ANME-2a- and ANME-2c coupled AOM to reduction of AQDS, humic acids, and soluble iron (Scheller et al. 2016). ANME-2d enrichment cultures coupled AOM to reduction of dissolved iron and iron or manganese oxide minerals (Ettwig et al. 2016). Metal-dependent AOM also appears to be non-syntrophic where the ANME deposit electrons directly on the metal (oxides). This mechanism is not understood, but ANME-2 genomes encode for very large MHCs (i.e., many heme-binding sites per cytochrome) (McGlynn et al. 2015) and in the highest quantity so far reported for prokaryotes (i.e., highest amount of cytochromes in the genome) (Kletzin et al. 2015). At least some of these MHCs are probably responsible for metal-dependent AOM, since these are known to be required for extracellular electron transfer (Lovley 2017).

Metagenome, metatranscriptome, and metaproteome analysis of ANME enrichment cultures showed that all genes necessary for the reversal of the methanogenic pathway were present and expressed in both ANME-2a and ANME-2d. ANME-1 lacks the gene that encodes for N^5,N^{10}-methylene-tetrahydromethanopterin (H_4MPT) reductase (Mer), and some bypass mechanisms have been proposed (reviewed in: Timmers et al. 2017). Interestingly, another form of syntrophy has been discovered recently where the archaeon is the electron donating partner. Here, the archaeon oxidizes butane and transfers electrons to an SRB (Laso-Perez et al. 2016). Even more intriguing, these archaea also use (part of) the methanogenic pathway in reverse and use methyl coenzyme M reductase to activate butane, an enzyme formerly presumed to be unique to methanogenic and methanotrophic archaea. These findings indicate that syntrophic processes with archaea as the electron donating partner exist in a variety of forms of which many probably still await discovery.

3 Spatial Organization of Syntrophic Communities

The close cooperation of two metabolically different organisms during syntrophic degradation requires short transport paths between the partners to optimize metabolite transfer, especially at low overall energy yields. The metabolite flux from one organism to the other one is an inverse linear function of the diffusion distance (Schink and Thauer 1988). One should assume, therefore, that optimal transfer is ensured in mixed communities in which the partners are homogeneously mixed. Syntrophic cocultures show a defined tendency to form mixed aggregates also in defined laboratory cultures (Fig. 6). However, since the respective partners are different organisms, they multiply separately and will form sooner or later nests of genetically identical organisms which compete with each other within the nests and

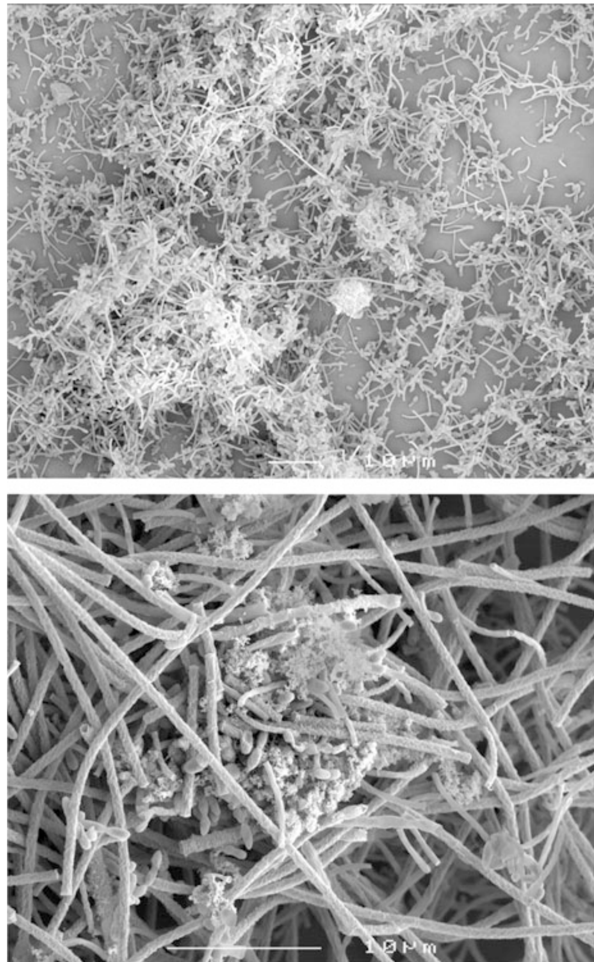


Fig. 6 Scanning electron micrographs of a propionate-converting (**a**) coculture of *S. fumaroxidans* (oval-shaped) and *M. hungatei* (rod-shaped) and (**b**) triculture of *S. fumaroxidans* (oval-shaped), *M. hungatei* (rod-shaped), and *M. concilii* (long filaments) showing the close proximity of the syntrophic partners

have only limited exchange to the partner nests outside. One has to assume that such communities should mix through each other to maintain optimal metabolite transfer at short distances. Microscopic pictures of methanogenic communities in biogas reactors have shown that nests, as described, really do exist within such structures but that in other areas, the partners appear to be fairly well mixed (Grotenhuis et al. 1991; Fang et al. 1995; Harmsen et al. 1996). It is still an open question how such mixing can be accomplished by organisms that appear to be basically immotile and do not show any means of gliding motility.

4 Concluding Remarks

The interrelationship of different trophic groups (guilds) within methanogenic microbial communities is a fascinating object to study, with perspectives to ecology, physiology, biochemistry, and energetics. These organisms cooperate in a very complex process, and they do so with minimum increments of energy for sustainment of life. These energy increments are at the lowermost range of energy that can be converted into ATP at all, and with this, these organisms are interesting model subjects to study energy starvation on a broader basis.

The question arises why nature designed methanogenic degradation in such a modular structure instead of having few types of organisms, which could convert polymeric substrates all the way down to methane plus CO_2. Theoretical considerations suggested that metabolic pathways can be efficient only up to a limited length of reaction chains (Costa et al. 2006), and this may apply as well to, e.g., cellulose degradation to methane. One can argue as well that the strategy taken in these anaerobic communities is simply to establish a complex network of functions by independent modular units. This makes regulation easy for every single unit which acts only in a single function rather than combining many different metabolic tasks in one.

Methanogenesis in bioreactors is a sustainable technology to produce biogas from organic waste. More than 80 % of the chemical energy in organic waste components is conserved as methane, which in aerobic conversion would have been lost. Presently, much research is done to replace fossil fuels to alternative sustainable (CO_2-neutral) energy sources. Microbial methane formation from waste and wastewater will contribute to this development. From the technological viewpoint, it will be important to produce methane at a high rate and to convert all organic compounds to biomass. The proper functioning and structuring of syntrophic communities of anaerobic bacteria and archaea will be important in this respect. Further research is needed to get insight into the factors that regulate methane formation by syntrophic communities.

References

Aklujkar M, Haveman SA, DiDonato R, Chertkov O, Han CS, Land ML, Brown P, Lovley DR (2012) The genome of *Pelobacter carbinolicus* reveals surprising metabolic capabilities and physiological features. BMC Genomics 13:690

Allison MJ (1970) Nitrogen metabolism in rumen microorganisms. In: Phillipson AT (ed) Physiology and digestion and metabolism in the ruminant. Oriel Press, Stockfield, pp 456–473

Allison MJ, Bryant MP (1963) Biosynthesis of branched-chain amino acids from branched-chain fatty acids by rumen bacteria. Arch Biochem Biophys 101:269–277

Alperin MJ, Hoehler TM (2009) Anaerobic methane oxidation by archaea/sulfate-reducing bacteria aggregates: 1. thermodynamic and physical constraints. Am J Sci 309:869–957

Amos DA, McInerney MJ (1990) Growth of *Syntrophomonas wolfei* on unsaturated short chain fatty acids. Arch Microbiol 154:31–36

Andreesen JR (1994) Glycine metabolism in anaerobes. Antonie Van Leeuwenhoek 66:223–237

Andreesen JR (2004) Glycine reductase mechanism. Curr Opin Chem Biol 8:454–461

Andreesen JR, Bahl H, Gottschalk G (1989) Introduction to the physiology and biochemistry of the genus *Clostridium*. In: Minton NP, Clarke DC (eds) *Clostridia*. Plenum Press, New York, pp 27–62

Baena S, Fardeau M-L, Labat M, Ollivier B, Garcia J-L, Patel BKC (1998) *Aminobacterium colombiense*, gen. nov., sp. nov., an amino-acid degrading anaerobe isolated from anaerobic sludge. Anaerobe 4:241–250

Baena S, Fardeau M-L, Ollivier B, Labat M, Thomas P, Garcia J-L, Patel BKC (1999a) *Aminomonas paucivorans* gen. nov., sp. nov., a mesophilic, anaerobic, amino-acid-utilizing bacterium. Int J Syst Bacteriol 49:975–982

Baena S, Fardeau M-L, Woo THS, Ollivier B, Labat M, Patel BKC (1999b) Phylogenetic relationships of three amino-acid-utilizing anaerobes, *Selenomonas acidaminovorans*, "*Selenomonas acidaminophila*" and *Eubacterium acidaminophilum*, as inferred from partial 16S rDNA nucleotide sequences and proposal of *Thermanaerovibrio acidaminovorans* gen. nov., comb. nov. and *Anaeromusa acidaminophila*, gen. nov., sp. nov., comb. nov. Int J Syst Bacteriol 49:969–974

Barker HA (1981) Amino acid degradation by anaerobic bacteria. Annu Rev Biochem 50:23–40

Beaty PS, Wofford NQ, McInerney MJ (1987) Separation of *Syntrophomonas wolfei* from *Methanospirillum hungatei* in syntrophic cocultures by using percoll gradients. Appl Environ Microbiol 53:1183–1185

Biebl H, Pfennig N (1978) Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. Arch Microbiol 117:9–16

Boetius A, Ravenschlag K, Schubert CJ, Rickert D, Widdel F, Giesecke A, Amann R, Jorgensen BB, Witte U, Pfannkuche O (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature 407:623–626

Boiangiu CD, Jayamani E, Brügel D, Herrmann G, Kim J, Forzi L, Hedderich R, Vgenopolou I, Pierik AJ, Steuber J, Buckel W (2005) Sodium ion pumps and hydrogen production in glutamate fermenting anaerobic bacteria. J Mol Microbiol Biotechnol 10:105–119

Boll M, Kung JW, Ermler U, Martins BM, Buckel W (2016) Fermentative cyclohexane carboxylate formation in *Syntrophus aciditrophicus*. J Mol Microbiol Biotechnol 26:165–179

Boone DR, Bryant MP (1980) Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. Appl Environ Microbiol 40:626–632

Borrel G, Harris HMB, Tottey W, Mihajlovski A, Parisot N, Peyretaillade E, Peyret P, Gribaldo S, O'Toole PW, Brugère JF (2012) Genome sequence of "*Candidatus* Methanomethylophilus alvus" Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens. J Bacteriol 194:6944–6945. https://doi.org/10.1128/JB.01867-12

Borrel G, O'Toole PW, Harris HMB, Peyret P, Brugère JF, Gribaldo S (2013) Phylogenomic data support a seventh order of methylotrophic methanogens and provide insights into the evolution of methanogenesis. Genome Biol Evol 5(10):1769–1780. https://doi.org/10.1093/gbe/evt128

Breznak JA, Kane MD (1990) Microbial H_2/CO_2 acetogenesis in animal guts: nature and nutritional significance. FEMS Microbiol Rev 7(3–4):309–313

Brune A (2007) Microbiology: woodworker's digest. Nature 450:487–488

Bryant MP (1977) Microbiology of the rumen. In: Stevenson MJ (ed) Duke's physiology of domestic animals, 9th edn. Cornell University Press, Itaca, pp 287–304

Bryant MP (1979) Microbial methane production – theoretical aspects. J Anim Sci 48:193–201

Bryant MP, Wolin EA, Wolin MJ, Wolfe RS (1967) *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch Mikrobiol 59:20–31

Bryant MP, Campbell LL, Reddy CA, Crabill MR (1977) Growth of *Desulfovibrio* in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl Environ Microbiol 33:1162–1169

Buckel W (2001a) Unusual enzymes involved in five ways of glutamate fermentation. Appl Microbiol Biotechnol 57:263–273

Buckel W (2001b) Sodium ion-translocating decarboxylases. Biochem Biophys Acta 1505:15–27

Buckel W, Barker HA (1974) Two pathways of glutamate fermentation by anaerobic bacteria. J Bacteriol 117:1248–1260

Chen S, Liu X, Dong X (2005) *Syntrophobacter sulfatireducens* sp. nov., a novel syntrophic, propionate-oxidizing bacterium isolated from UASB reactors. Int J Syst Evol Microbiol 55:1319–1324

Chen S, Rotaru AE, Shrestha PM (2014) Promoting Interspecies Electron Transfer with Biochar. Sci Rep 4:5019

Cheng G, Plugge CM, Roelofsen W, Houwen FP, Stams AJM (1992) *Selenomonas acidaminovorans* sp.nov., a versatile thermophilic proton-reducing anaerobe able to grow by the decarboxylation of succinate to propionate. Arch Microbiol 157:169–175

Cherepanov DA, Mulkidjanian AY, Junge W (1999) Transient accumulation of elastic energy in proton translocating ATP synthase. FEBS Lett 449:1–6

Conklin A, Stensel HD, Ferguson J (2006) Growth kinetics and competition between *Methanosarcina* and *Methanosaeta* in mesophilic anaerobic digestion. Water Environ Res 78:486–496

Conway de Macario E, Macario AJL (2018) Methanogenic archaea in humans and other vertebrates. In: Hackstein JHP (ed) (Endo)symbiotic methanogens. Springer, Berlin, pp 103–119

Costa E, Perez J, Kreft JU (2006) Why is metabolic labour divided in nitrification? Trends Microbiol 14:213–219

Crable BR, Sieber JR, Mao X, Alvarez-Cohen L, Gunsalus RP, Ogorzalek Loo RR, Nguyen H, McInerney MJ (2016) Membrane complexes of *Syntrophomonas wolfei* involved in syntrophic butyrate degradation and hydrogen formation. Front Microbiol 7:1795. https://doi.org/10.3389/fmicb.2016.01795

De Bok FAM, Stams AJM, Dijkema C, Boone DR (2001) Pathway of propionate oxidation by a syntrophic culture of *Smithella propionica* and *Methanospirillum hungatei*. Appl Environ Microbiol 67:1800–1804

De Bok FAM, Luijten MLGC, Stams AJM (2002) Biochemical evidence for formate transfer in syntrophic propionate oxidizing cocultures of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*. Appl Environ Microbiol 68:4247–4252

De Bok FAM, Harmsen JM, Plugge CM, de Vries MC, Akkermans ADL, de Vos WM, Stams AJM (2005) The first true obligately syntrophic propionate-oxidizing bacterium, *Pelotomaculum schinkii* sp. nov., cocultured with *Methanospirillum hungatei,* and emended description of the genus *Pelotomaculum*. Int J Syst Evol Microbiol 55:1697–1703

Dong X, Stams AJM (1995) Evidence for H_2 and formate formation during syntrophic butyrate and propionate degradation. Anaerobe 1:35–39

Dong X, Plugge CM, Stams AJM (1994a) Anaerobic degradation of propionate by a mesophilic acetogenic bacterium in co- and triculture with different methanogens. Appl Environ Microbiol 60:2834–2838

Dong X, Cheng G, Stams AJM (1994b) Butyrate oxidation by *Syntrophospora bryantii* in coculture with different methanogens and in pure culture with pentenoate as electron acceptor. Appl Microbiol Biotechnol 42:647–652

Eichler B, Schink B (1986) Fermentation of primary alcohols and diols, and pure culture of syntrophically alcohol-oxidizing anaerobes. Arch Microbiol 143:60–66

Engelbrecht S, Junge W (1997) ATP synthase: a tentative structural model. FEBS Lett 414:485–491

Ettwig KF, Shima S, van de Pas-Schoonen KT, Kahnt J, Medema MH, op den Camp HJM, MSM J, Strous M (2008) Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. Environ Microbiol 10:3164–3173

Ettwig KF, Zhu B, Speth D, Keltjens JT, Jetten MS, Kartal B (2016) Archaea catalyze iron-dependent anaerobic oxidation of methane. Proc Natl Acad Sci U S A 113:12792–12796

Fang HHP, Chui HK, Li YY (1995) Microstructural analysis of UASB granules treating brewery wastewater. Water Sci Technol 31:129–135

Felchner-Zwirello M, Winter J, Gallert C (2013) Interspecies distances between propionic acid degraders and methanogens in syntrophic consortia for optimal hydrogen transfer. Appl Microbiol Biotechnol 97:9193–9205

Fenchel T, Finlay BJ (2018) Free-living protozoa with endosymbiotic methanogens. In: Hackstein JHP (ed) (Endo)symbiotic methanogens. Springer, Berlin, pp 1–11

Ferry JG (1992) Methanogenesis: ecology, physiology, biochemistry & genetics. Chapman & Hall, New York

Fuchs G (2008) Anaerobic metabolism of aromatic compounds. Ann N Y Acad Sci 1125:82–99

Fuchs G, Boll M, Heider J (2011) Microbial degradation of aromatic compounds – from one strategy to four. Nat Rev Microbiol 9:803–816

Gorby YA, Yanina S, McLean JS, Rosso KM, Moyles D, Dohnalkova A, Beveridge TJ, Chang IS, Kim BH, Kim KS, Culley DE, Reed SB, Romine MF, Saffarini DA, Hill EA, Shi L, Elias DA, Kennedy DW, Pinchuk G, Watanabe K, Ishii S, Logan B, Nealson KH, Fredrickson JK (2006) Electrically conductive nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. Proc Natl Acad Sci 103:11358–11363

Gottschalk G (1986) Bacterial metabolism, 2nd edn. Springer, New York

Graentzdoerffer A, Rauh D, Pich A, Andreesen JR (2003) Molecular and biochemical characterization of two tungsten- and selenium-containing formate dehydrogenases from *Eubacterium acidaminophilum* that are associated with components of an iron-only hydrogenase. Arch Microbiol 179:116–130

Grotenhuis JTC, Smit M, Plugge CM, Xu YS, Van Lammeren AAM, Stams AJM, Zehnder AJB (1991) Bacteriological composition and structure of granular sludge adapted to different substrates. Appl Environ Microbiol 57:1942–1949

Harmsen HJM, Akkermans ADL, Stams AJM, de Vos WM (1996) Population dynamics of propionate-oxidizing bacteria under methanogenic and sulfidogenic conditions in anaerobic granular sludge. Appl Environ Microbiol 62:2163–2168

Harmsen HJM, Van Kuijk BLM, Plugge CM, Akkermans ADL, de Vos WM, Stams AJM (1998) Description of *Syntrophobacter fumaroxidans* sp.nov., a syntrophic propionate-degrading sulfate-reducing bacterium. Int J Syst Bacteriol 48:1383–1387

Haroon MF, Hu SH, Shi Y, Imelfort M, Keller J, Hugenholtz P, Yuan ZG, Tyson GW (2013) Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature 500:567–570

Hattori S, Kamagata Y, Hanada S, Shoun H (2000) *Thermacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. Int J Syst Evol Microbiol 50:1601–1609

Hattori S, Galushko AS, Kamagata Y, Schink B (2005) Operation of the CO dehydrogenase/acetyl-CoA pathway in both acetate oxidation and acetate formation by the syntrophically acetate-oxidizing bacterium *Thermacetogenium phaeum*. J Bacteriol 187:3471–3476

Hazlewood GP, Nugent JHA (1978) Leaf fraction 1 protein as a nitrogen source for the growth of a proteolytic rumen bacterium. J Gen Microbiol 106:369–371

He Z, Zhang Q, Feng Y, Luo H, Pan X, Gadd GM (2018) Microbiological and environmental significance of metal-dependent anaerobic oxidation of methane. Sci Total Environ 610–611:759–768

Healy JB, Young LY (1978) Catechol and phenol degradation by a methanogenic population of bacteria. Appl Environ Microbiol 35(1):216–218

Herrmann G, Jayamani E, Mai G, Buckel W (2008) Energy conservation via electron-transferring flavoprotein in anaerobic bacteria. J Bacteriol 190:784–791

Hinrichs KU, Hayes JM, Sylva SP, Brewer PG, DeLong EF (1999) Methane-consuming archaebacteria in marine sediments. Nature 398:802–805

Hobson PN, Wallace RJ (1982) Microbial ecology and activities in the rumen. Crit Rev Microbiol 9:253–320

Iannotti EL, Kafkewitz D, Wolin MJ, Bryant MP (1973) Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: changes caused by interspecies transfer of H_2. J Bacteriol 114:1231–1240

Imachi H, Sekiguchi Y, Kamagata Y, Hanada S, Ohashi A, Harada H (2002) *Pelotomaculum thermopropionicum* gen. nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. Int J Syst Evol Microbiol 52:1729–1735

Imachi H, Sakai S, Ohashi A, Harada H, Hanada S, Kamagata Y, Sekiguchi Y (2007) Pelotomaculum propionicicum sp nov., an anaerobic, mesophilic, obligately syntrophic propionate-oxidizing bacterium. Int J Syst Evol Microbiol 57:1487–1492

Ingham CJ, Sprenkels A, Bomer JG, Molenaar D, van den Berg A, van Hycklama Vlieg JET, de Vos WM (2007) A highly subdivided microbial growth chip constructed on a porous ceramic support. Proc Natl Acad Sci U S A 46:18217–18222

Jackson BE, Bhupathiraju VK, Tanner RS, Woese CR, McInerney MJ (1999) *Syntrophus aciditrophicus* sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. Arch Microbiol 171:107–114

Jetten MSM, Stams AJM, Zehnder AJB (1992) Methanogenesis from acetate: a comparison of the acetate methabolism of *Methanothrix soehngenii* and *Methanosarcina* spp. FEMS Microbiol Rev 88:181–198

Kaden J, Galushko AS, Schink B (2002) Cysteine-mediated electron transfer in syntrophic acetate oxidation by cocultures of *Geobacter sulfurreducens* and *Wolinella succinogenes*. Arch Microbiol 178:53–58

Kato S, Hashimoto K, Watanabe K (2012) Methanogenesis facilitated by electric syntrophy via (semi)conductive iron-oxide minerals. Environ Microbiol 14:1646–1654

Kletzin A, Heimerl T, Flechsler J, van Niftrik L, Rachel R, Klingl A (2015) Cytochromes c in archaea: distribution, maturation, cell architecture, and the special case of *Ignicoccus hospitalis*. Front Microbiol 6:439

Knittel K, Boetius A (2009) Anaerobic oxidation of methane: progress with an unknown process. Annu Rev Microbiol 63:311–334

Kosaka T, Kato S, Shimoyama T, Ishii S, Abe T, Watanabe K (2008) The genome of *Pelotomaculum thermopropionicum* reveals niche-associated evolution in anaerobic microbiota. Genome Res 18:442–448

Kremer DR, Nienhuis-Kuiper HE, Hansen TA (1988) Ethanol dissimilation in *Desulfovibrio*. Arch Microbiol 150:552–557

Kröger A, Biel S, Simon J, Gross R, Unden G, Lancaster CRD (2002) Fumarate respiration of *Wolinella succinogenes*: enzymology, energetics and coupling mechanism. Biochim Biophys Acta 1553:23–38

Laso-Perez R, Wegener G, Knittel K, Widdel F, Harding KJ, Krukenberg V, Meier DV, Richter M, Tegetmeyer HE, Riedel D, Richnow HH, Adrian L, Reemtsma T, Lechtenfeld OJ, Musat F (2016) Thermophilic archaea activate butane via alkyl-coenzyme M formation. Nature 539:396–401

Leng L, Yang P, Singh S, Zhuang H, Xu L, Chen WH, Dolfing J, Li D, Zhang Y, Zeng H, Chu W, Lee PH (2018) A review on the bioenergetics of anaerobic microbial metabolism close to the thermodynamic limits and its implications for digestion applications. Bioresour Technol 247:1095–1106

Lever MA, Rogers K, Karyn L, Lloyd KG, Overmann J, Schink B, Thauer RK, Hoehler TM, Jørgensen BB (2015) Life under extreme energy limitation: a synthesis of laboratory- and field-based investigations. FEMS Microbiol Ecol 39:688–728

Li F, Hinderberger J, Seedorf H, Zhang J, Buckel W, Thauer RK (2008) Coupled ferredoxin and crotonyl coenzyme a (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. J Bacteriol 190:843–850

Li H, Chang J, Liu P, Fu L, Ding D, Lu Y (2015) Direct interspecies electron transfer accelerates syntrophic oxidation of butyrate in paddy soil enrichments. Environ Microbiol 17:1533–1547

Limam RD, Chouari R, Mazéas L, Wu TD, Li T, Grossin-Debattista J, Guerquin-Kern J-L, Saidi M, Landoulsi A, Sghir A, Bouchez T (2014) Members of the uncultured bacterial candidate division WWE1 are implicated in anaerobic digestion of cellulose. Microbiol Open 3:157–167. https://doi.org/10.1002/mbo3.144

Liu Y, Whitman WB (2008) Metabolic, phylogenetic and ecological diversity of methanogenic archaea. In: Wiegel J, Maier MJ, Adams MW (eds) Incredible anaerobes: from physiology to genomics to fuels, vol 1125. Annals of the New York Academy of Sciences, New York, pp 171–189

Liu Y, Balkwill DL, Aldrich HC, Drake GR, Boone DR (1999) Characterisation of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov, sp. nov. and *Syntrophobacter wolinii*. Int J Syst Bacteriol 49:545–556

Liu FH, Rotaru AE, Shrestha PM, Malvankar NS, Nevin KP, Lovley DR (2012) Promoting direct interspecies electron transfer with activated carbon. Energy Environ Sci 5:8982–8989

Losey NA, Mus F, Peters JW, Le HM, McInerney MJ (2017) Syntrophomonas wolfei uses an NADH-dependent, ferredoxin-independent [FeFe]-hydrogenase to reoxidize NADH. Appl Environ Microbiol 83:e01335-17

Lovley DR (2017) Syntrophy goes electric: direct interspecies electron transfer. Annu Rev Microbiol 71:643–664

Lovley DR, Fraga JL, Blunt-Harris EL, Hayes LA, Phillips EJP, Coates JD (1998) Humic substances as a mediator for microbially catalyzed metal reduction. Acta Hydrochim Hydrobiol 26 (3):152–157

Manzoor S, Muller B, Niazi A, Schnurer A, Bongcam-Rudloff E (2015) Working draft genome sequence of the mesophilic acetate oxidizing bacterium *Syntrophaceticus schinkii* strain Sp3. Stand Genomic Sci 10:99. https://doi.org/10.1186/s40793-015-0092-z

Manzoor S, Bongcam-Rudloff E, Schnurer A, Muller B (2016) Genome-guided analysis and whole transcriptome profiling of the mesophilic syntrophic acetate oxidising bacterium *Syntrophaceticus schinkii*. PLoS One 11:e0166520. https://doi.org/10.1371/journal.pone.0166520

McGlynn SE, Chadwick GL, Kempes CP, Orphan VJ (2015) Single cell activity reveals direct electron transfer in methanotrophic consortia. Nature 526:531–U146

McInerney MJ (1988) Anaerobic hydrolysis and fermentation of fats and proteins. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. Wiley, New York, pp 373–415

McInerney MJ, Bryant MP, Pfennig N (1979) Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch Microbiol 122:129–135

McInerney MJ, Bryant MP, Pfennig N (1981) *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Appl Environ Microbiol 41:1029–1039

McInerney MJ, Stams AJM, Boone DR (2005) Genus *Syntrophobacter*. In: Staley JT, Boone DR, Brenner DJ, de Vos P, Garrity GM, Goodfellow M, Krieg NR, Rainey FA, Schleifer KH (eds) Bergey's manual of systematic bacteriology, vol 2, 2nd edn. Springer, New York, pp 1021–1027

McInerney MJ, Struchtemeyer CG, Sieber J, Mouttaki H, Stams AJM, Schink B, Rohlin L, Gunsalus RP (2008) Physiology, ecology, phylogeny and genomics of microorganisms capable of syntrophic metabolism. In: Wiegel J, Maier MJ, Adams MW (eds) Incredible anaerobes: from physiology to genomics to fuels, vol 1125. Annals of the New York Academy of Sciences, New York, pp 58–72

Meijer WG, Nienhuis-Kuiper ME, Hansen TA (1999) Fermentative bacteria from estuarine mud: phylogenetic position of *Acidaminobacter hydrogenoformans* and description of a new type of Gram-negative, propionigenic bacterium as *Propionibacter pelophilus* gen. nov., sp. nov. Int J Syst Bacteriol 49:1039–1044

Metje M, Frenzel P (2007) Methanogenesis and methanogenic pathways in a peat from subarctic permafrost. Environ Microbiol 9:954–964

Meulepas RJ, Jagersma CG, Khadem AF, Stams AJ, Lens PN (2010) Effect of methanogenic substrates on anaerobic oxidation of methane and sulfate reduction by an anaerobic methanotrophic enrichment. Appl Microbiol Biotechnol 87:1499–1506

Montag D, Schink B (2016) Biogas process parameters--energetics and kinetics of secondary fermentations in methanogenic biomass degradation. Appl Microbiol Biotechnol 100:1019–1026

Mountfort DO, Bryant MP (1982) Isolation and characterization of an anaerobic syntrophic benzoate-degrading bacterium from sewage sludge. Arch Microbiol 133:249–256

Müller N, Stingl U, Griffin BM, Schink B (2008) Dominant sugar utilizers in sediment of Lake Constance depend on syntrophic cooperation with methanogenic partner organisms. Environ Microbiol 10:1501–1511

Müller N, Schleheck D, Schink B (2009) Involvement of NADH:acceptor oxidoreducase and butyryl-CoA dehydrogenase in reversed electron transport during syntrophic butyrate oxidation by *Syntrophomonas wolfei*. J Bacteriol 191:6167–6177

Müller N, Worm P, Schink B, Stams AJM, Plugge CM (2010) Syntrophic butyrate and propionate oxidation processes: from genomes to reaction mechanisms. Environ Microbiol Rep 2:489–499

Müller N, Scherag FD, Pester M, Schink B (2015) *Bacillus stamsii* sp. nov., a facultatively anaerobic sugar degrader that is numerically dominant in freshwater lake sediment. Syst Appl Microbiol 38:379–389

Nagase M, Matsuo T (1982) Interaction between amino-acid degrading bacteria and methanogenic bacteria in anaerobic digestion. Biotechnol Bioeng 24:2227–2239

Nakano MM, Dailly YP, Zuber P, Clark DP (1997) Characterization of anaerobic fermentative growth of Bacillus subtilis: identification of fermentation end products and genes required for growth. J Bacteriol 179:6749–6755

Nanninga HJ, Gottschal JC (1985) Amino acid fermentation and hydrogen transfer in mixed cultures. FEMS Microbiol Ecol 31:261–269

Nanninga HJ, Drenth WJ, Gottschal JC (1987) Fermentation of glutamate by *Selenomonas acidaminophila*, sp. nov. Arch Microbiol 147:152–157

Nauhaus K, Boetius A, Krüger M, Widdel F (2002) In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area. Environ Microbiol 4:296–305

Oehler D, Poehlein A, Leimbach A, Müller N, Daniel R, Gottschalk G, Schink B (2012) Genome-guided analysis of physiological and morphological traits of the fermentative acetate oxidizer *Thermacetogenium phaeum*. BMC Genomics 13:723. https://doi.org/10.1186/1471-2164-13-723 URL http://www.biomedcentral.com/1471-2164/13/723

Orcutt B, Meile C (2008) Constraints on mechanisms and rates of anaerobic oxidation of methane by microbial consortia: process-based modeling of ANME-2 archaea and sulfate reducing bacteria interactions. Biogeosciences 5:1587–1599

Orphan VJ, House CH, Hinrichs KU, McKeegan KD, DeLong EF (2001) Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. Science 293:484–487

Pelletier E, Kreymeyer A, Bocs S, Rouy Z, Gyapay G, Chouari R, Riviere D, Ganesan A, Daegelen P, Sghir A, Cohen GN, Medigue C, Weissenbach J, Paslier DL (2008) "Candidatus Cloacamonas acidaminovorans": genome sequence reconstruction provides a first glimpse of a new bacterial division. J Bacteriol 190:2572–2579. https://doi.org/10.1128/JB.01248-07

Pfennig N, Biebl H (1976) *Desulfuromonas acetoxidans* gen. nov. sp. nov., a new anaerobic, sulfur-reducing acetate-oxidizing bacterium. Arch Microbiol 110:3–12

Philipp B, Schink B (2012) Different strategies in anaerobic biodegradation of aromatic compounds: nitrate reducers versus strict anaerobes. Environ Microbiol Rep 4:469–478. https://doi.org/10.1111/j.1758-2229.2011.00304

Platen H, Schink B (1987) Methanogenic degradation of acetone by an enrichment culture. Arch Microbiol 149:136–141

Platen H, Janssen PH, Schink B (1994) Fermentative degradation of acetone by an enrichment culture in membrane-separated culture devices and in cell suspensions. FEMS Microbiol Lett 122:27–32

Plugge CM, Stams AJM (2005) Syntrophism among Clostridiales. In: Duerre P (ed) Handbook on Clostridia. CRC Press, Boca Raton, pp 769–784

Plugge CM, Dijkema C, Stams AJM (1993) Acetyl-CoA cleavage pathway in a syntrophic propionate-oxidizing bacterium growing on fumarate in the absence of methanogens. FEMS Microbiol Lett 110:71–76

Plugge CM, Zoetendal EG, Stams AJM (2000) *Caloramator coolhaasii*, sp. nov. a glutamate-degrading, moderately thermophilic anaerobe. Int J Syst Bacteriol 50:1155–1161

Plugge CM, van Leeuwen JM, Hummelen T, Balk M, Stams AJM (2001) Elucidation of the pathways of catabolic glutamate conversion in three thermophilic anaerobic bacteria. Arch Microbiol 176:29–36

Plugge CM, Balk M, Zoetendal EG, Stams AJM (2002a) *Gelria glutamica*, gen. nov., sp. nov., a thermophilic obligate syntrophic glutamate-degrading anaerobe. Int J Syst Evol Microbiol 52:401–406

Plugge CM, Balk M, Stams AJM (2002b) *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* subsp. nov., a thermophilic syntrophic propionate-oxidizing spore-forming bacterium. Int J Syst Evol Microbiol 52:391–399

Plugge CM, Henstra AM, Worm P, Swarts DC, Paulitsch-Fuchs AH, Scholten JCM, Lykidis A, Lapidus AL, Goltsman E, Kim E, McDonald E, Rohlin L, Crable BR, Gunsalus RP, Stams AJM, McInerney MJ (2012) Complete genome sequence of *Syntrophobacter fumaroxidans* strain MPOB^T. Stand Genomic Sci 10:91–106

Qiu YL, Hanada S, Ohashi A, Harada H, Kamagata Y, Sekiguchi Y (2008) *Syntrophorhabdus aromaticivorans* gen. nov., sp. nov., the first cultured anaerobe capable of degrading phenol to acetate in obligate syntrophic associations with a hydrogenotrophic methanogen. Appl Environ Microbiol 74:2051–2058

Raghoebarsing AA, Pol A, van de Pas-Schoonen KT, Smolders AJP, Ettwig KF, Rijpstra WIC, Schouten S, Damste JSS, Op den Camp HJM, Jetten MSM, Strous M (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. Nature 440:918–921

Reda T, Plugge CM, Abram NJ, Hirst J (2008) Reversible interconversion of carbon dioxide and formate by an electroactive enzyme. PNAS 105:10654–10658

Reddy CA, Bryant MP, Wolin MJ (1972a) Characteristics of S organism isolated from *Methanobacillus omelianskii*. J Bacteriol 109:539–545

Reddy CA, Bryant M, Wolin MJ (1972b) Ferredoxin- and nicotinamide adenine dinucleotide-dependent H_2 production from ethanol and formate in extracts of S organism isolated from *Methanobacillus omelianskii*. J Bacteriol 110:126–132

Reddy CA, Bryant MP, Wolin MJ (1972c) Ferredoxin-dependent conversion of acetaldehyde to acetate and H_2 in extracts of S organism. J Bacteriol 110:133–138

Reguera G, McCarthy KD, Mehta T, Nicoll JS, Tuominen MT, Lovley DR (2005) Extracellular electron transfer via microbial nanowires. Nature 435:1098–1101

Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng J-F, Darling A, Malfatti S, Swan BK, Gies EA, Dodsworth JA, Hedlund BP, Tsiamis G, Sievert SM, Liu W-T, Eisen JA, Hallam SJ, Kyrpides NC, Stepanauskas R, Rubin EM, Hugenholtz P, Woyke T (2013) Insights into the phylogeny and coding potential of microbial dark matter. Nature 499:431–437. https://doi.org/10.1038/nature12352

Rotaru AE, Shrestha PM, Liu FH, Shrestha M, Shrestha D, Embree M, Zengler K, Wardman C, Nevin KP, Lovley DR (2014a) A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon dioxide to methane. Energy Environ Sci 7(1):408–415

Rotaru AE, Shrestha PM, Liu F, Markovaite B, Chen S, Nevin KP, Lovley DR (2014b) Direct interspecies electron transfer between *Geobacter metallireducens* and *Methanosarcina barkeri*. Appl Environ Microbiol 80(15):4599–4605

Roy F, Samain E, Dubourgier HC, Albagnac G (1986) *Syntrophomonas sapovorans* sp. nov., a new obligately proton reducing anaerobe oxidizing saturated and unsaturated long chain fatty acids. Arch Microbiol 145:142–147

Scheller S, Yu H, Chadwick GL, McGlynn SE, Orphan VJ (2016) Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction. Science 351:703–707

Schink B (1985) Fermentation of acetylene by an obligate anaerobe, Pelobacter acetylenicus sp. nov. Arch Microbiol 142:295–301

Schink B (1989) Mikrobielle Lebensgemeinschaften in Gewässersedimenten. Naturwissenschaften 76:364–372

Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61:262–280

Schink B, Stams AJM (2002) Syntrophism among prokaryotes. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The prokaryotes: an evolving electronic resource for the microbiological community, 3rd edn. Springer, New York, p 25

Schink B, Thauer RK (1988) Energetics of syntrophic methane formation and the influence of aggregation. In: Lettinga G et al (eds) Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, pp 5–17

Schink B, Zeikus JG (1982) Microbial ecology of pectin decomposition in anoxic lake sediments. J Gen Microbiol 128:393–404

Schink B, Montag D, Keller A, Müller N (2017) Hydrogen or formate – alternative key players in methanogenic degradation. Environ Microbiol Rep 9:189–202

Schmidt A, Müller N, Schink B, Schleheck D (2013) A proteomic view at the biochemistry of syntrophic butyrate oxidation in *Syntrophomonas wolfei*. Plos One. http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0056905

Schmidt A, Frensch M, Schleheck D, Schink B, Müller N (2014) Degradation of acetaldehyde and its precursors by *Pelobacter carbinolicus* and *P. acetylenicus*. PLoS One 9(12):e115902. https://doi.org/10.1371/journal.pone.0115902

Schnürer A, Schink B, Svensson BH (1996) *Clostridium ultunense* sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. Int J Syst Bacteriol 46:1145–1152

Schnürer A, Svensson BH, Schink B (1997) Enzyme activities in and energetics of acetate metabolism by the mesophilic syntrophically acetate-oxidizing anaerobe *Clostridium ultunense*. FEMS Microbiol Lett 154:331–336

Schöcke L, Schink B (1997) Energetics of methanogenic benzoate degradation by *Syntrophus gentianae* in syntrophic coculture. Microbiology 143:2345–2351

Schöcke L, Schink B (1999) Energetics and biochemistry of fermentative benzoate degradation by *Syntrophus gentianae*. Arch Microbiol 171:331–337

Scholten JCM, Culley DE, Brockman FJ, Wu G, Zhang W (2007) Evolution of the syntrophic interaction between *Desulfovibrio vulgaris* and *Methanosarcina barkeri*: involvement of an ancient horizontal gene transfer. Biochem Biophys Res Commun 5:48–54

Schut GJ, Adams MW (2009) The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. J Bacteriol 191:4451–4457

Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A, Harada H (2000) *Syntrophothermus lipocalidus* gen. nov., sp. nov., a novel thermophilic, syntrophic, fatty-acid-oxidizing anaerobe which utilizes isobutyrate. Int J Syst Evol Microbiol 50:771–779

Shresta PM, Rotaru AE (2014) Plugging in or going wireless: strategies for interspecies electron transfer. Front Microbiol. https://doi.org/103389/fmicb.2014.00237

Shrestha PM, Rotaru AE, Aklujkar M, Liu F, Shrestha M, Summers ZM, Malvankar N, Flores DC, Lovley DR (2013) Syntrophic growth with direct interspecies electron transfer as the primary mechanism for energy exchange. Environ Microbiol Rep 5(6):904–910

Sieber JR, Sims DR, Han C, Kim E, Lykidis A, Lapidus AL, McDonnald E, Rohlin L, Culley DE, Gunsalus RP, McInerney MJ (2010) The genome of *Syntrophomonas wolfei*: new insights into syntrophic metabolism and biohydrogen production. Environ Microbiol 12:2289–2301

Sieber JR, Le H, McInerney MJ (2014) The importance of hydrogen and formate transfer for syntrophic fatty, aromatic and alicyclic metabolism. Environ Microbiol 16:177–188

Sieber JR, Crable BR, Sheik CS, Hurst GB, Rohlin L, Gunsalus RP, McInerney MJ (2015) Proteomic analysis reveals metabolic and regulatory systems involved the syntrophic and axenic lifestyle of *Syntrophomonas wolfei*. Front Microbiol 6:115. https://doi.org/10.3389/fmicb.2015.00115

Sousa DZ, Smidt H, Alves MM, Stams AJM (2007) *Syntrophomonas zehnderi* sp. nov., an anaerobe that degrades long-chain fatty acids in co-culture with *Methanobacterium formicicum*. Int J Syst Evol Microbiol 57:609–615

Stams AJM (1994) Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie Van Leeuwenhoek 66:271–294

Stams AJM, Hansen TA (1984) Fermentation of glutamate and other compounds by *Acidaminobacter hydrogenoformans* gen. nov., sp. nov., an obligate anaerobe isolated from black mud. Studies with pure cultures and mixed cultures with sulfate reducing and methanogenic bacteria. Arch Microbiol 137:329–337

Stams AJM, Plugge CM (2009) Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat Rev Microbiol 7:568–577

Stams AJM, van Dijk J, Dijkema C, Plugge CM (1993) Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl Environ Microbiol 59:1114–1119

Stickland LH (1934) The chemical reaction by which *Cl. sporogenes* obtains its energy. Biochem J 28:1746–1759

Stieb M, Schink B (1985) Anaerobic oxidation of fatty acids by *Clostridium bryantii* sp. nov., a sporeforming, obligately syntrophic bacterium. Arch Microbiol 140:387–390

Stieb M, Schink B (1986) Anaerobic degradation of isovalerate by a defined methanogenic coculture. Arch Microbiol 144:291–295

Stieb M, Schink B (1989) Anaerobic degradation of isobutyrate by methanogenic enrichment cultures and by a *Desulfococcus multivorans* strain. Arch Microbiol 151:126–132

Svetlitshnyi V, Rainey F, Wiegel J (1996) *Thermosyntropha lipolytica* gen. nov., sp. nov., a lipolytic, anaerobic, alkalitolerant, thermophilic bacterium utilizing short- and long-chain fatty acids in syntrophic coculture with a methanogenic archaeum. Int J Syst Bacteriol 46:1131–1137

Szewzyk U, Schink B (1989) Degradation of hydroquinone, gentisate, and benzoate by a fermenting bacterium in pure or defined mixed culture. Arch Microbiol 151:541–545

Tarlera S, Muxi L, Soubes M, Stams AJM (1997) *Caloramator proteoclasticus* sp. nov., a moderately thermophilic anaerobic proteolytic bacterium. Int J Syst Bacteriol 47:651–656

Tewes FJ, Thauer RK (1980) Regulation of ATP synthesis in glucose fermenting bacteria involved in interspecies hydrogen transfer. In: Gottschalk G, Pfennig N, Werner H (eds) Anaerobes and anaerobic infections. G Fischer, Stuttgart, pp 97–104

Thauer RK (2011) Anaerobic oxidation of methane with sulfate: on the reversibility of the reactions that are catalyzed by enzymes also involved in methanogenesis from CO_2. Curr Opin Microbiol 14:292–299

Thauer RK, Morris JG (1984) Metabolism of chemotrophic anaerobes: old views and new aspects. In: Kelly DP, Carr NG (eds) The microbe 1984: prokaryotes and eukaryotes part II. Cambridge University Press, Cambridge, pp 123–168

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180

Timmers PHA, Welte CU, Koehorst JJ, Plugge CM, Jetten MS, Stams AJ (2017) Reverse methanogenesis and respiration in methanotrophic archaea. Archaea 2017:1654237. https://doi.org/10.1155/2017/1654237

Treude T, Orphan V, Knittel K, Gieseke A, House CH, Boetius A (2007) Consumption of methane and CO_2 by methanotrophic microbial mats from gas seeps of the anoxic Black Sea. Appl Environ Microbiol 73:2271–2283

Valentine DL, Reeburgh VS, Blanton DC (2000) A culture apparatus for maintaining H_2 at sub-nanomolar concentrations. J Microbiol Methods 39:243–251

Van Kuijk BLM, Stams AJM (1996) Purification and characterization of malate dehydrogenase from the syntrophic propionate-oxidizing bacterium strain MPOB. FEMS Microbiol Lett 144:141–144

Van Kuijk BLM, Schlösser E, Stams AJM (1998) Investigation of the fumarate metabolism of the syntrophic propionate-oxidizing bacterium strain MPOB. Arch Microbiol 169:346–352

van Lingen HJ, Plugge CM, Fadel JG, Kebreab E, Bannink A, Dijkstra J (2016) Thermodynamic driving force of hydrogen on rumen microbial metabolism: a theoretical investigation. PLoS One 11:10. https://doi.org/10.1371/journal.pone.0161362

Van Lingen HJ, Edwards JE, Vaidya JD, van Gastelen S, van den Bogert B, Saccenti E, Bannink A, Smidt H, Plugge CM, Dijkstra J (2017) Diurnal dynamics of gaseous and dissolved metabolites and microbiota composition in the bovine rumen. Front Microbiol 8:425

Vervoort Y, Gutiérrez Linares A, Roncoroni M, Liu C, Steensels J, Verstrepen KJ (2017) High-throughput system-wide engineering and screening for microbial biotechnology. Curr Opin Biotechnol 46:120–125

Viggi CC, Rosetti S, Fazi S, Paiano P, Majone M, Aulenta F (2014) Magnetite particles triggering a faster and more robust syntrophic pathway of methanogenic propionate degradation. Environ Sci Technol 48(13):7536–7543

Walker CB, He Z, Yang ZK, Ringbauer JA Jr, Zhou J, Voordouw G, Wall JD, Hazen TC, Arkin AP, Stahl DA (2009) The electron transfer system of syntrophically grown *Desulfovibrio vulgaris*. J Bacteriol 191:5793–5801

Wallrabenstein C, Schink B (1994) Evidence of reversed electron transport involved in syntrophic butyrate and benzoate oxidation by *Syntrophomonas wolfei* and *Syntrophus buswellii*. Arch Microbiol 162:136–142

Wallrabenstein C, Hauschild E, Schink B (1995) *Syntrophobacter pfennigii* sp. nov., new syntrophically propionate-oxidizing anaerobe growing in pure culture with propionate and sulfate. Arch Microbiol 164:346–352

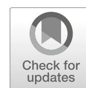
Wegener G, Krukenberg V, Riedel D, Tegetmeyer HE, Boetius A (2015) Intercellular wiring enables electron transfer between methanotrophic archaea and bacteria. Nature 526:587–U315

Welte CU, Rasigraf O, Vaksmaa A, Versantvoort W, Arshad A, Op den Camp HJ, Jetten MS, Luke C, Reimann J (2016) Nitrate- and nitrite-dependent anaerobic oxidation of methane. Environ Microbiol Rep 8:941–955

Widdel F, Rouviere PE, Wolfe RS (1988) Classification of secondary alcohol-utilizing methanogens including a new thermophilic isolate. Arch Microbiol 150:477–481

Wofford NQ, Beaty PS, McInerney MJ (1986) Preparation of cell-free extracts and the enzymes involved in fatty acid metabolism in *Syntrophomonas wolfei*. J Bacteriol 167:179–185
Worm P, Stams AJM, Cheng X, Plugge CM (2011) Growth- and substrate-dependent transcription of formate dehydrogenase and hydrogenase coding genes in *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*. Microbiology 157:280–289
Worm P, Koehorst JJ, Visser M, Sedano-Núñez VT, Schaap PJ, Plugge CM, Sousa DZ, Stams AJM (2014) A genomic view on syntrophic versus non-syntrophic lifestyle in anaerobic fatty acid degrading communities. Biochim Biophys Acta 1837:2004–2016
Wu C, Liu X, Dong X (2006) *Syntrophomonas cellicola* sp. nov., a spore-forming syntrophic bacterium isolated from a distilled-spirit-fermenting cellar, and assignment of *Syntrophospora bryantii* to *Syntrophomonas bryantii* comb. nov. Int J Syst Evol Microbiol 56:2331–2335
Zehnder AJB (1978) Ecology of methane formation. In: Mitchell R (ed) Water pollution microbiology, vol 2. Wiley, London, pp 349–376
Zhang C, Liu X, Dong X (2004) *Syntrophomonas curvata* sp. nov., an anaerobe that degrades fatty acids in co-culture with methanogens. Int J Syst Evol Microbiol 54:969–973
Zhang C, Liu X, Dong X (2005) *Syntrophomonas erecta* sp. nov., a novel anaerobe that syntrophically degrades short-chain fatty acids. Int J Syst Evol Microbiol 55:799–803
Zhou S, Xu J, Yang G, Zhuang L (2014) Methanogenesis affected by the co-occurrence of iron(III) oxides and humic substances. FEMS Microbial Ecol 88:107–120
Zinder SH, Koch M (1984) Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch Microbiol 138:263–272

Hydrogenosomes



Johannes H. P. Hackstein and Aloysius G. M. Tielens

Contents

1 Introduction 194
2 The Hydrogenosomes of the Trichomonadina 198
3 The Hydrogenosomes of Chytridiomycete Fungi 200
3.1 Mitochondria Versus Hydrogenosomes 201
3.2 Chytrids Perform a “Mixed Acid Fermentation” 202
3.3 The Hydrogenosomal Metabolism 202
3.4 The Role of the Hydrogenosomes in the Energy Metabolism of *Piromyces* sp. E2 205
3.5 The Evolution of Hydrogenosomes from Fungal Mitochondria 205
4 The Hydrogenosomes of Anaerobic Ciliates 207
4.1 *Nyctotherus ovalis* 207
4.2 In Silico Reconstruction of the Basal Hydrogenosomal Metabolism of *N. ovalis* 210
4.3 The Hydrogenosomes of Other Ciliates 211
4.4 Can the Methanogenic Symbionts Tell Us More About the Origin and Function of Ciliate Hydrogenosomes? 212
4.5 Evolutionary Aspects 213
5 Conclusions 215
References 215

Abstract “Hydrogenosomes” are mitochondrion-related, double membrane-bounded organelles that produce hydrogen and ATP. These properties discriminate them from the canonical aerobic functioning mitochondria known from standard textbooks and from the likewise mitochondrion-related “mitosomes” that produce neither hydrogen nor ATP. Hydrogenosomes and mitosomes are found in a broad

J. H. P. Hackstein (✉)
IWWR, Faculty of Science, Radboud University Nijmegen, Nijmegen, The Netherlands
e-mail: j.hackstein@science.ru.nl

A. G. M. Tielens
Department of Medical Microbiology and Infectious Diseases, Erasmus MC, University Medical Center, Rotterdam, The Netherlands

Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
e-mail: a.tielens@erasmusmc.nl

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*, Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_10

spectrum of rather unrelated species of unicellular, anaerobic eukaryotes, suggesting that hydrogenosomes and mitosomes evolved repeatedly and independently in the various taxonomic groups. Most of the known hydrogenosomes lack a genome and an electron-transport chain, which makes it sometimes difficult to trace their origins back to their mitochondrial origins. However, genomic evidence, EST studies and the analysis of the organellar metabolism clearly reveal both a mitochondrial descent and individual differences in the properties of the various organelles. In this chapter, we describe the diversity of hydrogenosomes. We also pay attention to the fact that certain hydrogenosomes are found in close association with endosymbiotic methanogens.

1 Introduction

It is now generally accepted that different types of mitochondrion-related organelles (MROs) evolved from an ancestral endosymbiont. Furthermore, all eukaryotes contain MROs, and all MROs are descendants of one ancestral endosymbiont (Embley and Martin 2006; Hackstein et al. 2006; Tielens and Van Hellemond 2007; Howe 2008; van der Giezen 2009; Müller et al. 2012). By differential loss and gain of metabolic functions, the organellar evolution resulted in a mosaic of mitochondrial functions in the various eukaryotic lineages (Hjort et al. 2010; Maguire and Richards 2014; Makiuchi and Nozaki 2014; Stairs et al. 2015).

A classification of these diverse organelles into five classes was proposed on the basis of their energy metabolism, as that was supposedly the original driving force for the endosymbiotic event (Müller et al. 2012). The various classes of MRO can be classified depending on whether or not they produce ATP, possess a proton-translocating electron-transport chain and use oxygen, hydrogen or another compound as final electron acceptor (Table 1). Although this classification based on energy metabolism is straightforward and possible, it should be realized that within each class, large variations in metabolic capacities exist. Furthermore, within one particular eukaryotic species, it can occur that the class of MRO depends on the stage in the life cycle or the environmental conditions.

Class 1 is formed by all canonical aerobic mitochondria that produce ATP using oxygen as final electron acceptor in their proton-translocating electron-transfer chain (Table 1). Mitochondria typically possess a genome that encodes components of the electron-transport chain. Usually mitochondria are depicted as organelles that use oxygen as terminal electron acceptor in their process of ATP production. However, many mitochondria exist that can produce ATP without using any oxygen. Mitochondria of anaerobic eukaryotes that produce ATP with the help of proton-pumping electron transport (i.e. oxidative phosphorylation), but use terminal electron

Table 1 Classification of the various types of MROs based on their energy metabolism

		ATP production	electron transport chain	final electron acceptor		
				O_2	H^+	'fumarate'
Class 1	aerobic mitochondrion	+	+	+	–	–
Class 2	anaerobic mitochondrion	+	+	–	–	+
Class 3	H_2-producing anaerobic mitochondrion	+	+	–	+	+
Class 4	hydrogenosome	+	–	–	+	–
Class 5	mitosome	–	–	–	–	–

Red colour indicates an aerobic energy metabolism, blue an anaerobic one, and yellow indicates that these MROs are not involved in ATP production

acceptors other than oxygen, such as fumarate, form class 2 (Tielens et al. 2002; Müller et al. 2012). Class 3 MROs is formed by the anaerobic functioning mitochondria that possess a proton-translocating electron-transport chain but can also donate electrons of substrate oxidation via a hydrogenase to protons, which makes them hydrogen-producing mitochondria. Class 4 is formed by another type of anaerobic ATP-producing organelle, the hydrogenosome. Hydrogenosomes are double membrane-bounded organelles of a size of 0.3–2 μm that are characterized by the production of hydrogen with the aid of a hydrogenase that donates electrons originating from the oxidation of substrates to protons (Müller 1993). Class 5 is formed by mitosomes which are, in contrast to mitochondria and hydrogenosomes, not involved

in the production of ATP. They are double membrane-bounded and are present only in anaerobic eukaryotic organisms that lack mitochondria and hydrogenosomes. Mitosomes do not produce hydrogen. Their function is elusive, and most of them share with mitochondria and hydrogenosomes only the presence of components of an iron-sulphur cluster synthesizing machinery (Tachezy and Dolezal 2007; Peña-Diaz and Lukeš 2018). Mitosomes and hydrogenosomes are found exclusively in anaerobic, unicellular organisms.

The discovery in the ciliate *Nyctotherus ovalis* of a hydrogen-producing MRO with a mitochondrial genome and parts of an electron-transport chain disclosed the existence of a link between hydrogenosomes and mitochondria (Boxma et al. 2005; Martin 2005; De Graaf et al. 2011). Notably, another example of such a link with a genome and an electron-transport chain has been discovered thereafter in the unrelated Stramenopile *Blastocystis* sp. (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008; Gentekaki et al. 2017).

Hydrogenosomes and mitosomes are found in a broad spectrum of species, suggesting that hydrogenosomes and mitosomes evolved repeatedly in rather unrelated taxonomic groups (Fig. 1). Even within the Excavata, at least three different rather unrelated species with hydrogenosomes were identified, i.e. the heterolobosean amoeboflagellate *Psalteriomonas lanterna*, the preaxostylid flagellate *Trimastix pyriformis* and the parabasalid flagellate *Trichomonas vaginalis* with its relatives *Tritrichomonas foetus*, *Monocercomonas* sp. and *Histomonas meleagridis.* In addition, *Giardia lamblia* (Excavata) is a species belonging to the diplomonads that hosts mitosomes. Among the Chromalveolata various anaerobic ciliate species with hydrogenosomes evolved repeatedly from different aerobic ciliate progenitors (see below). One of them, *Nyctotherus ovalis* possesses a hydrogenosome with a genome (Akhmanova et al. 1998a; Boxma et al. 2005). Also, the Stramenopile *Blastocystis* sp. possesses a hydrogenosome-like, genome-bearing organelle (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008; Gentekaki et al. 2017), and *Cryptosporidium* sp., which belongs to the Apicomplexa, hosts a mitochondrion-remnant (mitosome) that lacks a genome and a hydrogenase (Keithly 2008). Among the Unikonta, the anaerobic chytridiomycete fungi *Piromyces* sp. and *Neocallimastix* sp. possess hydrogenosomes (Müller 1993), while fungi-related Microsporidia such as *Encephalitozoon cuniculi* (Katinka et al. 2001), *Antonospora locustae* (Williams and Keeling 2005) and *Trachipleistophora hominis* (Williams et al. 2002) possess mitosomes. Mitosomes are also found in the rather unrelated species *Entamoeba histolytica* (Tovar et al. 1999) and *Mastigamoeba balamuthi* (Gill et al. 2007). These obviously diverse origins of the organelles (and hydrogenosomes in particular) strongly suggest that neither the various mitosomes nor the hydrogenosomes are the same. The various organelles, even if they belong to the same type, can be structurally and metabolically different. Lastly, on the basis of electron microscope studies, quite a number of potential hydrogenosome/mitosome-like organelles have been identified in anaerobes such as *Spironucleus elegans, Chilomastix cuspidata, Andalucia incarcerata, Lyromonas vulgaris, Monopylocystis visvesvarai, Sawyeria*

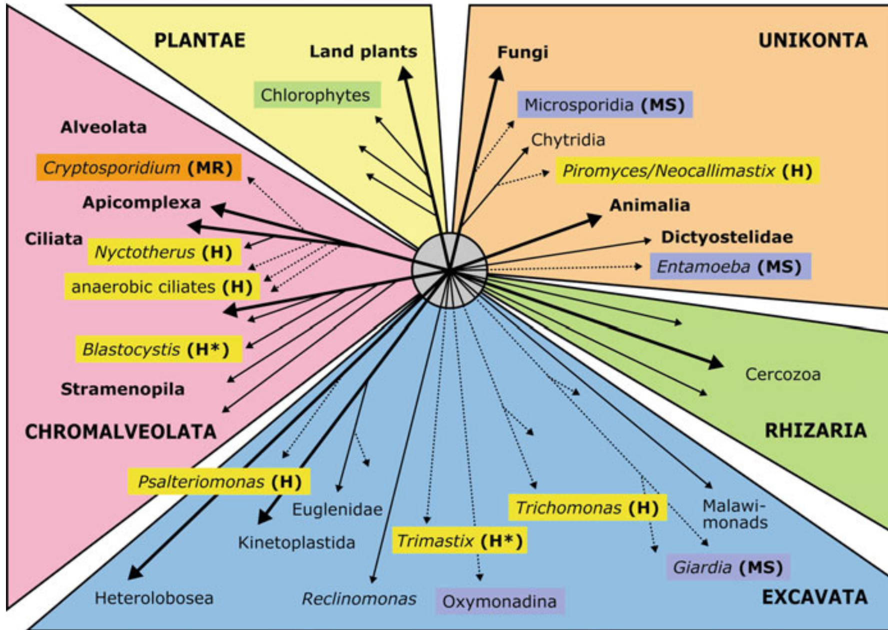


Fig. 1 Cartoon interpreting the evolution of mitochondria and related organelles (*H* hydrogenosomes, *MS* mitosomes, *MR* mitochondrial remnants). *H** indicates that hydrogenase activity has not yet been demonstrated in *Blastocystis*. The *solid lines* represent the evolutionary descent based on the presence of an organellar genome; the *broken lines* indicate the absence (loss) of a genome. The monophyly of the mitochondria has been derived from the phylogenetic analysis of more than 444 alpha-proteobacterial and 2061 mitochondrial genomes (February 2010). Since the branching order is not resolved so far, the cartoon indicates solely the common origin by the "origin" in the centre. A later loss of the organellar genome is only indicated if additional data argue for a loss of the organelle genome after the diversification of the hosts. The phylogenetic relationships between the hosts are still discussed controversially. Therefore, the smallest common denominator is used to display an unrooted "tree" of the most basic taxonomic arrangement agreed by most biologists: Plantae, Uniconta (animals and fungi), Rhizaria, Chromalveolata and Excavata. No attempts were made to root this tree nor to give any indication of a potential branching order. Certain green algae possess "normal" mitochondria but express a plastidic hydrogenase under anaerobic conditions ("chlorophytes"). Substantial differences in metabolism have been established for the hydrogenosomes of *Trichomonas*, *Piromyces/Neocallimastix* and the various ciliates. From Hackstein et al. (2006, modified)

marylandensis, Carpediemonas membranifera, Dysnectes brevis, Vahlkamfia anaerobica, Percolomona descissus, Postgaardi mariagerensis and *Breviata anathema* (Hampl and Simpson 2008).

As described above, in all five classes of MROs that can be discriminated based on their energy metabolism, numerous variations of the organelles exist within each class. In this review, we will focus on hydrogenosomes and hydrogen-producing mitochondria and describe a few different examples in the following in some detail.

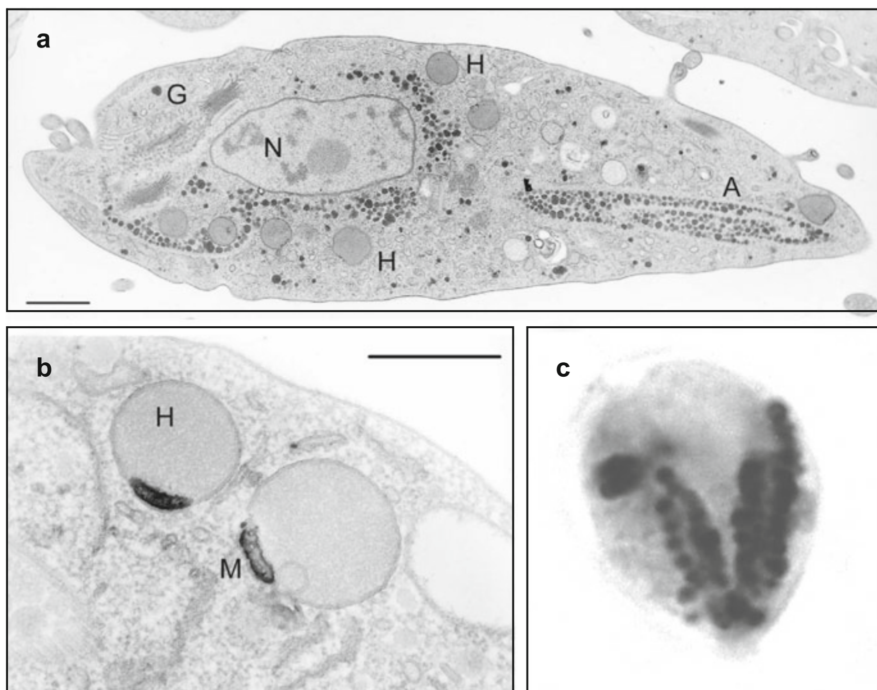


Fig. 2 (**a**) Electron micrograph of *Tritrichomonas foetus:* seven hydrogenosomes (*H*) can be identified in the cytoplasm (*N* nucleus; *G* Golgi apparatus; *A* axostyl). (**b**) A higher magnification reveals that a double membrane surrounds the hydrogenosomes (*M* marginal plate). A and B were kindly provided by M. Benchimol, Rio de Janeiro. *Bar*: in a and b 1 μm. (**c**) *Trichomonas vaginalis*, light microscopical picture of cell stained with BSTP following Zwart et al. (1988) to demonstrate hydrogenase activity; natural size approximately 10 × 45 μm. Courtesy C.K. Stumm, Nijmegen

2 The Hydrogenosomes of the Trichomonadina

Trichomonads are microaerophilic, parasitic flagellates that belong to the Parabasalia. The hydrogenosomes of Trichomonads are the best studied organelles of this kind. They were discovered in 1973 in *Tritrichomonas foetus,* 1974 in *Monocercomonas* sp., 1975 in *Trichomonas vaginalis* and 2008 in *Histomonas meleagridis* (Lindmark and Müller 1973, 1974; Lindmark et al. 1975; Mazet et al. 2008). These organelles are double membrane-bounded and about 0.3 μm in diameter in *T. foetus* and *T. vaginalis* (Fig. 2)*,* 0.3–0.6 μm in diameter in *H. meleagridis* (Mazet et al. 2008) and up to 2 μm in length in *Monocercomonas* (Benchimol 2008). They do not contain an organellar genome (Clemens and Johnson 2000). Most of the recent studies focussed on the human parasite *T. vaginalis.* In 2007 the complete (nuclear) genome has been published allowing a reconstruction of the hydrogenosomal metabolism that corroborates the earlier enzymatic studies (Carlton et al. 2007). In addition, the proteome of the hydrogenosomes has been analysed.

Together with the genome data, the analysis of the proteome suggests that the hydrogenosome of *T. vaginalis* consists of at least 200 different proteins (Henze 2008). This is considerably less than the 700–800 proteins predicted for yeast mitochondria (Sickmann et al. 2003) and suggests a significantly lower complexity of the hydrogenosomes in comparison to mitochondria.

The metabolism of the hydrogenosomes of *T. vaginalis* has been described in detail by Carlton et al. (2007), Hrdy et al. (2008), Huang et al. (2017) and Westrop et al. (2017); it will be summarized below. The trichomonad hydrogenosomes import pyruvate and malate (Hrdy et al. 2008). The latter is decarboxylated to pyruvate by a NAD-dependent malic enzyme inside the hydrogenosome. The initial step in the catabolism of pyruvate is the oxidative decarboxylation by the pyruvate: ferredoxin oxidoreductase (PFO) (Fig. 3) to acetyl-CoA and CO_2. The reduced ferredoxin is reoxidized by a [FeFe] hydrogenase; the genome of *T. vaginalis* encodes five hydrogenases with hydrogenosomal targeting signals. It is assumed that one or the other hydrogenase reacts directly with NADH, alternatively under the involvement of ferredoxin and the 24 kDa and 51 kDa proteins that are homologous to the corresponding subunits of a mitochondrial complex I (Hrdy et al. 2004).

The next step in the catabolism of pyruvate is the formation of acetate from acetyl-CoA with the simultaneous transfer of the CoA moiety to succinate (Fig. 3). This reaction is catalysed by the enzyme ASCT (acetate:succinate CoA-transferase). The corresponding gene was not identified in the draft genome version. However, thereafter, the gene and the enzyme have been identified and characterized in detail (van Grinsven et al. 2008).

The succinyl-CoA synthetase (SCS, also known as succinate thiokinase) uses the energy-rich CoA bond for the generation of ATP/GTP from ADP/GDP. It is the only enzyme of *Trichomonas* also known from the Krebs cycle of aerobic mitochondria. It regenerates succinate for the reaction with ASCT. It is unknown to date whether the acetate, which is formed by the action of ASCT, is excreted via diffusion or by a so far unidentified transporter.

ATP and ADP are exchanged by a member of the mitochondrial carrier family, HMP 31 (Tjaden et al. 2004). Besides this ATP-generating pathway, several other metabolic pathways have been identified. Most importantly, several components of the Fe-S cluster synthesizing machinery were identified—a function that is shared with mitochondria, the hydrogenosomes of *Blastocystis* and several mitosomes (Burri et al. 2006; Tachezy and Dolezal 2007). In addition, a glycine-decarboxylase complex (GDC, also known as glycine cleavage system, GCS) has been found. It allows the formation of THF-CH_2 (Methylene tetrahydrofolate), which is a key compound in the C_1 metabolism. Also this enzyme complex is found in mitochondria and the hydrogenosomes of *Nyctotherus* and *Blastocystis.* Furthermore, several enzymes with a function in the protection against reactive oxygen species (ROS) have been identified. Lastly, quite a number of proteins (Hmp35, HPP, Sam50, Pam18, Mdj1, Mge, Hsp10, Hsp60, Hsp70) involved in the import of proteins into the organelle were detected (Hrdy et al. 2008).

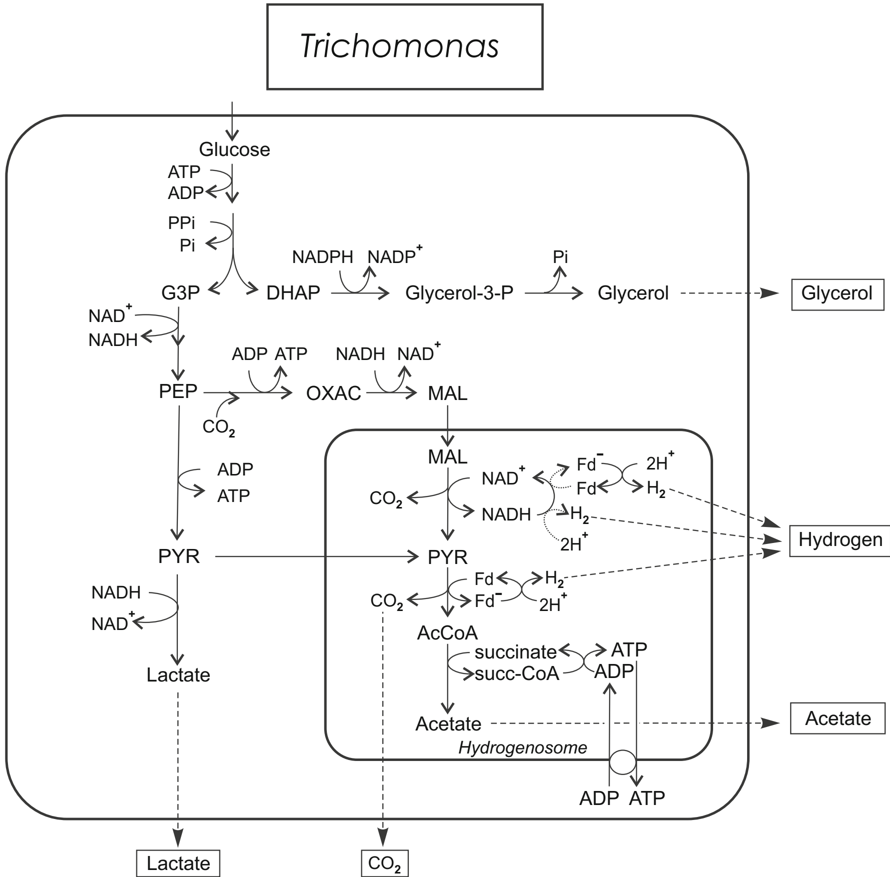


Fig. 3 Energy metabolism of *Trichomonas vaginalis* Shown is a scheme of the metabolic pathways involved in the production of the major end products. End products are in boxes. Abbreviations AcCoA, acetyl-CoA; DHAP, dihydroxyacetone phosphate; Fd, ferredoxin; G3P, glyceraldehyde-3-phosphate; MAL, malate; OXAC, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; Succ-CoA, succinyl-CoA (adapted from Carlton et al. 2007 and Hrdy et al. 2008)

3 The Hydrogenosomes of Chytridiomycete Fungi

Fungi form a very diverse group of eukaryotes belonging to the Unikonta. The majority of investigated fungi contain mitochondria and are capable of oxidative phosphorylation. On the other hand, there are anaerobically functioning chytridiomycete fungi that contain hydrogenosomes (Hackstein et al. 2008a).

Anaerobic chytridiomycete fungi are important symbionts in the gastrointestinal tract of herbivorous mammals. A flagellated rumen-dwelling organism, *Neocallimastix frontalis*, was described in 1975 by Colin Orpin (1975). Two years later,

Orpin published a report showing that *N. frontalis* and two other anaerobes had cell walls that contained chitin, indicating that these rumen-dwelling organisms are fungi (Orpin 1977).

The diversity of the anaerobic chytridiomycete fungi is large, and they are found in the gastrointestinal tract of nearly all large herbivores, ranging from ruminants such as cattle, sheep, goat, deer and antelopes to the foregut-fermenting marsupials and camelids on the one hand and hindgut-fermenting species such as horse, elephant, rhinoceros, mara (Patagonian hare) and capybara ("water pig", the world's largest rodent) on the other hand. Anaerobic chytridiomycetes can be isolated from rumen fluid or faeces, and they are maintained in anaerobic culture, most of them as pure axenic cultures. In the rumen of cattle or sheep, these anaerobic fungi can be as frequent as 7.6×10^8 thallus-forming units; in the faeces there are still 4.2×10^4 units per g dry weight (Trinci et al. 1994). Anaerobic chytrids are not truly host-specific since it is possible to transfaunate various host animals with isolates from different hosts. On the other hand, the various isolates are not the same, even if collected from the same host species and assigned to the same chytrid species. The patterns of utilization of substrates and the metabolic properties are different from isolate to isolate (Trinci et al. 1994).

3.1 Mitochondria Versus Hydrogenosomes

The majority of the cultured fungi belonging to the taxa Ascomycota, Basidiomycota and Zygomycota contain mitochondria. These mitochondria host a genome of varying size, which characteristically encodes only a handful of proteins (Bullerwell and Lang 2005). This implies that the vast majority of the 700–800 mitochondrial proteins (Sickmann et al. 2003) is nuclear encoded, synthesized in the cytoplasm and imported into the organelles. Interestingly, certain cultivars of mitochondriate species are able to maintain mitochondria in the absence of a mitochondrial genome. Such yeasts are known as "petites"; they are viable but respiration deficient and, consequently, incapable of growing on non-fermentable substrates (Contamine and Picard 2000). In this respect these mitochondria are similar to the genome-less chytrid hydrogenosomes.

On the other hand, two natural isolates of fission yeasts, *Schizosaccharomyces japonicus* var. *japonicus* and *S. japonicus* var. *versatilis*, lack detectable cytochromes and are respiration deficient but nevertheless retained fully functional mtDNA (Bullerwell and Lang 2005). These fission yeasts are considered to be an intermediate evolutionary stage in between respiratory-competent fungi and those that completely lack mitochondrial DNA. The mitochondria of these yeast species might be similar to the genome-containing hydrogenosomes of the anaerobic ciliate *Nyctotherus ovalis* that are described in detail below. Therefore, these respiration-deficient mitochondria represent an evolutionary intermediate between classical mitochondria and the hydrogenosomes of the chytridiomycete fungi.

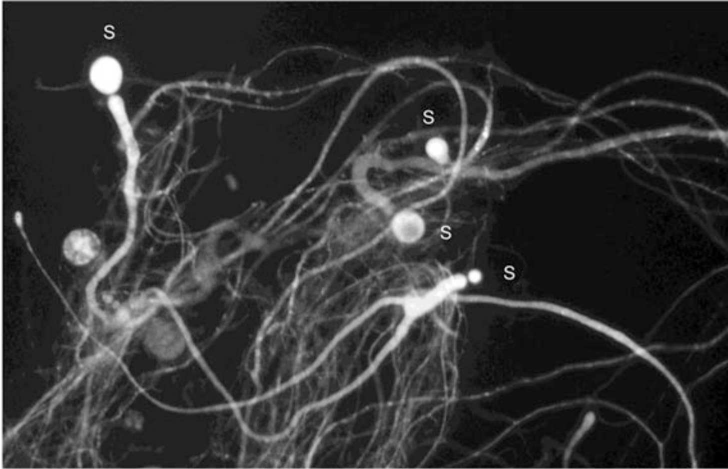


Fig. 4 Epifluorescence micrograph of *Piromyces* sp. E2 originally isolated from the faeces of an Indian elephant. Magnification about x400. The organism was vitally stained with Rhodamine 123. S: young sporangia. Reproduced with permission from Hackstein et al. (2008a)

3.2 Chytrids Perform a "Mixed Acid Fermentation"

Notably, the members of the phylum Chytridiomycota, for example, *Piromyces* (Fig. 4) and *Neocallimastix*, which possess hydrogenosomes, lack both mitochondria and an organellar genome (van der Giezen et al. 1997). These hydrogenosomes of chytrid fungi are double membrane-bounded compartments up to 1 μm in size (Fig. 5) that produce ATP by substrate-level phosphorylation together with hydrogen, CO_2, formate and acetate as end products of the organellar metabolism (Marvin-Sikkema et al. 1993, 1994; Akhmanova et al. 1999; Hackstein et al. 2001; Voncken 2001). The intact organism produces succinate, lactate and ethanol in addition when growing on cellulose, glucose or fructose as a carbon source (Julliand et al. 1998). Such a "mixed acid fermentation" is very similar to bacterial mixed acid fermentations that are, for example, well known for facultative anaerobic enteric bacteria, such as *Escherichia coli.*

3.3 The Hydrogenosomal Metabolism

The hydrogenosomal metabolism has been studied in more detail in the chytridiomycetes *Piromyces* and *Neocallimastix*. Notably, the hydrogenosomes of these organisms are clearly different from those known of Trichomonads and anaerobic ciliates, structurally (Fig. 5) and metabolically. Most importantly, the hydrogenosomes of *Neocallimastix* sp. L2 and *Piromyces* sp. E2 contain PFL as key enzyme (Akhmanova et al. 1999) and not PDH (as in *N. ovalis*) or PFO (as in *Trichomonas*

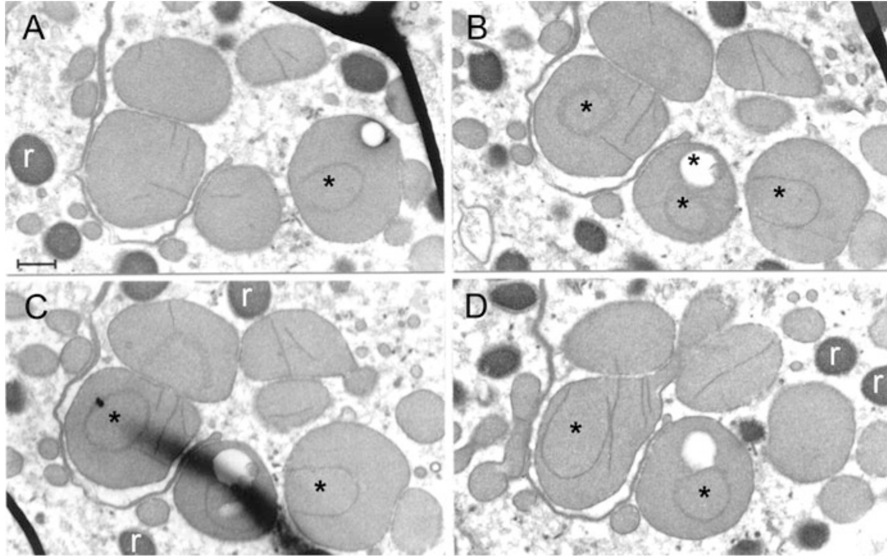


Fig. 5 Electron micrographs of the hydrogenosomes of *Neocallimastix* sp. L2. Serial sectioning, (**a**–**d**). Bar = 0.5 micrometre; r, ribosome globules (Munn et al. 1988). Asterisk, internal vesicular structures of the hydrogenosomes. Reproduced with permission from Hackstein et al. (2008a)

vaginalis and many other anaerobic organisms). Accordingly, PFO is lacking as the analysis of a large collection of ESTs reveals.

As discussed above, these chytridiomycetes produce formate, acetate, succinate, lactate, ethanol, hydrogen and carbon dioxide (Fig. 6). However, the ratio of these excreted end products is not constant, as it was shown that the growth of *Piromyces* sp. E2 in the presence of increasing concentrations of fructose is accompanied by changes in the fermentation pattern (Boxma et al. 2004). Increasing the fructose concentration from 0.1 to 0.5% resulted in a threefold increase in degradation of this substrate to end products. It is remarkable that the relative fluxes of fructose degradation through the various pathways were not constant during changing fructose concentrations. Although the absolute amounts of hydrogen formed in the incubations during growth at these increasing concentrations of fructose remained constant, the relative flux of malate into the hydrogenosomes and hence the relative flux to hydrogen decreased from 47 to 15% (Boxma et al. 2004). In contrast, the relative fluxes in the formation of the cytosolic end products lactate, ethanol and succinate increased severalfold. These observations show that increasing amounts of a fermentable carbon source result in an increased metabolism without an increased production of hydrogen. This implicates a relative shift from a hydrogenosomal carbon metabolism to a cytosolic one.

Metabolic experiments using labelled glucose indicated that an incomplete Krebs cycle operates in the reductive mode allowing the formation of succinate from oxaloacetate via a malate intermediate (Fig. 6). Since the formation of significant amounts of labelled CO_2 could be excluded while formate and acetate plus ethanol

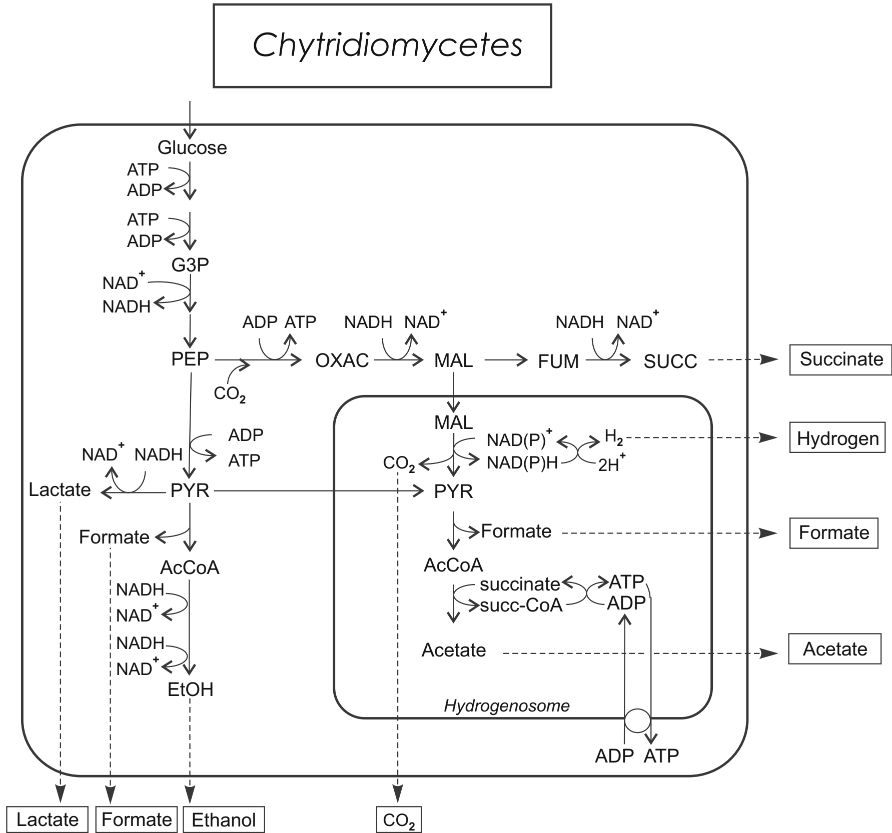


Fig. 6 Energy metabolism of *Chytridiomycetes* Shown is a scheme of the metabolic pathways involved in the production of the major end products. End products are in boxes. Abbreviations AcCoA, acetyl-CoA; EtOH, ethanol; FUM, fumarate; G3P, glyceraldehyde-3-phosphate; MAL, malate; OXAC, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; SUCC, succinate (adapted from Boxma et al. 2004)

were formed in a 1:1 ratio, it must be concluded that PFL and not pyruvate: ferredoxin oxidoreductase (PFO) or pyruvate dehydrogenase (PDH) play the central role in the hydrogenosomal metabolism (Boxma et al. 2004). Moreover, experiments with isolated hydrogenosomes of *Piromyces* have shown that acetate and formate are formed in equimolar amounts confirming the activity of PFL in the hydrogenosomes (Akhmanova et al. 1999).

Coculturing of *Piromyces* and the associated methanogen *Methanobrevibacter thaueri* resulted in a shift of the final metabolic end products to much less formate and hydrogen, which were consumed by the methanogens, while acetate was hardly consumed (Li et al. 2016).

3.4 The Role of the Hydrogenosomes in the Energy Metabolism of **Piromyces** *sp. E2*

The observation that the hydrogenosomal PFL and the cytoplasmic ADHE are the key enzymes in the degradation of carbohydrates by anaerobic chytrids reveals that the metabolism of these hydrogenosomes is fundamentally different from the hydrogenosomal metabolism in both trichomonads and *N. ovalis*-like ciliates. Obviously, anaerobic chytrids chose their own way to adapt to anaerobic environments by evolutionary tinkering. The metabolic scheme displayed in Fig. 6 shows a generalized metabolism involving substrate-level ATP formation with the aid of ASCT and SCS. A quantitative analysis revealed that (i) PFL must be present and (ii) that, under certain conditions, hydrogen formation can become marginal (Boxma et al. 2004). The evolutionary strategy of chytrids apparently tends to avoid the formation of reduction equivalents by using PFL instead of PFO or PDH (Akhmanova et al. 1999; Hackstein et al. 1999, 2006; Voncken 2001).

The major role of the chytrid hydrogenosomes seems to be the generation of ATP by substrate-level phosphorylation. The presence of PFL in the absence of hydrogenosomal ADHE most probably directs all organellar pyruvate into substrate-level ATP formation. A possible presence of ADHE inside the hydrogenosomes would compromise this function of the hydrogenosome as an energy-generating organelle. In the cytoplasm, however, ADHE might allow regulation of PFL activity, thus saving pyruvate (and its metabolites) for anabolic pathways. A partial Krebs cycle with links to anabolic pathways operates in the cytoplasm (Akhmanova et al. 1998b). This hypothesis is supported by the observation that several mitochondrial enzymes, which are involved in anabolic reactions, e.g. malate dehydrogenase, aconitase, isocitrate dehydrogenase and acetohydroxyacid reductoisomerase, have been retargeted to the cytoplasm in *Piromyces* sp. E2 (Akhmanova et al. 1998b; Hackstein et al. 1999). Consequently, compartmentalization of the energy metabolism seems to enhance the possibilities for regulation of the metabolic pathways of this organism.

3.5 The Evolution of Hydrogenosomes from Fungal Mitochondria

Using 18S rDNA phylogenies, or the phylogenies of mitochondrial genes from aerobic chytrids, a monophyletic origin of all chytrids becomes evident (Bullerwell and Lang 2005). There is no doubt about a fungal origin of the chytrids—regardless as to whether they are thriving in oxic or anoxic environments. The aerobic representatives possess mitochondria: phylogenetic analysis of their nuclear and

mitochondrial genomes reinforces their fungal origin (Bowman et al. 1992; Paquin et al. 1995; Paquin and Lang 1996). Also an analysis of biochemical and morphological traits consistently establishes a close relationship between chytrids and other fungi (Ragan and Chapman 1978). Akhmanova et al. (1998b) demonstrated that several enzymes of mitochondrial origin, which lack putative targeting signals, were retargeted to the cytoplasm (in active form) in the hydrogenosome-bearing chytrid *Piromyces*.

Chytrid hydrogenosomes look rather different from the pictures of mitochondria in textbooks (Fig. 5). However, electron microscopical analysis revealed a structure resembling the ultrastructure of mitochondria from particular diseased human patients (Frey and Mannella 2000; Hackstein et al. 2001; Voncken et al. 2002). Also, the relict mitochondrion (mitosome) of *Cryptosporidium parvum* looks very similar (Keithly et al. 2005). Apparently, in these cases, the inner membrane undergoes a derangement in the mechanism that normally stabilizes the crista junctions (Mannella 2006).

Because of their intrinsic function in the organelle, ADP/ATP carriers (AACs) and chaperonins are the best indicators for the phylogenetic analysis of an organelle of mitochondrial origin. Phylogenetic analysis of the AACs and chaperonins of anaerobic chytrids unequivocally revealed a fungal mitochondrial ancestry (Voncken 2001; Voncken et al. 2002; van der Giezen et al. 2002, 2003). Moreover, the spectrum of responses against the various inhibitors is quite specific and differentiates these AACs clearly from other adenine transporters—regardless as to whether these transporters are from mitochondrial or hydrogenosomal origin (Hackstein et al. 2006). While the AACs are eukaryotic “inventions” that allowed the exploitation of the ATP formed inside the organelle after the organelle formation, the chaperonins tend to trace the ancestry of the organelle back to the endosymbiont that gave rise to the mitochondrion. Also, the phylogenetic analysis of Hsp 60 and the Hsp 70 clearly reveals a clustering with their fungal mitochondrial relatives and not with the alpha-proteobacterial cluster (Hackstein et al. 1999; Voncken et al. 2002; van der Giezen et al. 2003).

Genomic analyses of the hydrogenosomal enzyme succinyl-CoA synthetase, SCS, (Dacks et al. 2006) and of two additional hydrogenosomal enzymes involved in arginine biosynthesis (Gelius-Dietrich et al. 2007) further confirm the fungal mitochondrial origin of the *Neocallimastix* hydrogenosome. Most of the other hydrogenosomal genes have not been identified so far.

We now know that the anaerobic chytrids comprise many species that are integral in the rumen ecosystem and crucial in the digestion of plant material to simple sugars. Moreover, they produce hydrogen needed for the growth of methanogenic bacteria (reviewed in Williams et al. 1994). However, there is no evidence for endo- or episymbiotic associations between anaerobic chytrids and methanogenic archaea. Notwithstanding, coculture of chytrids and methanogens has profound effects on the overall metabolism of the chytrids (Marvin-Sikkema et al. 1990).

4 The Hydrogenosomes of Anaerobic Ciliates

Ciliates represent an extremely species-rich, monophyletic group of highly complex unicellular eukaryotes. They are characterized by a nuclear dimorphism and rather complex patterns of morphologically distinct cortical cilia. Most of the ciliates thrive in aerobic environments and possess mitochondria, but anaerobic species evolved in at least 8 of the 22 orders of ciliates as classified by Corliss (Corliss 1979; Fenchel and Finlay 1995). Certain ciliates in seven of these eight orders possess "hydrogenosomes" (Hackstein et al. 2008b; Fig. 7). However, the identification of many of these hydrogenosomes was based solely on the presence of intracellular methanogenic archaea. Such a symbiotic association is indicative of an interspecies hydrogen transfer and could reveal the presence of intracellular hydrogen sources, i.e. hydrogenosomes (Hackstein et al. 2002).

The development of fluorescence microscopy, electron microscopy, cytobiochemistry and techniques for cellular fractionation allowed the discovery of hydrogenosomes in free-living anaerobic ciliates such as *Plagiopyla, Trimyema* and *Metopus* (van Bruggen et al. 1983, 1984, 1986; Goosen et al. 1988, 1990; Zwart et al. 1988; Finlay and Fenchel 1989; Fenchel and Finlay 1995; Biagini et al. 1997; Fenchel and Finlay 2018, this volume; Shinzato et al. 2018, this volume). Hydrogenosomes were also identified in ciliates thriving in the gastrointestinal tract of ruminants and marsupials (e.g. *Isotricha, Dasytricha, Epidinium, Eudiplodinium, Polyplastron, Amylovorax*) (Vogels et al. 1980; Snyers et al. 1982; Yarlett et al. 1981, 1982, 1983, 1984, 1985; Lloyd et al. 1989; Paul et al. 1990; Ellis et al. 1991a,b,c; Cameron and O'Donoghue 2002a). They were also found in *Nyctotherus ovalis* that lives in the hindgut of cockroaches (Gijzen et al. 1991; Akhmanova et al. 1998a; Boxma et al. 2005). Figure 7 shows the distribution of hydrogenosomes and mitochondria in the various orders of ciliates. All these hydrogenosomes are surrounded by a double membrane, and under optimal fixation conditions, in a number of ciliates species, cristae-like protrusions can be seen in these organelles, and in that way they clearly resemble mitochondria (Fig. 8).

4.1 Nyctotherus ovalis

Nyctotherus species (Armophorea) are anaerobic, heterotrichous ciliates with hydrogenosomes that thrive in the intestinal tract of cockroaches, millipedes, frogs and reptiles. *N. ovalis* from the hindgut of cockroaches is the only species that has been studied in more detail (van Hoek et al. 1998, 1999, 2000b). Notably, the presence of a mitochondrial genome has been demonstrated in the hydrogenosomes of *N. ovalis* (Akhmanova et al. 1998a; van Hoek et al. 2000a, Boxma et al. 2005; de Graaf et al. 2011). This genome was shown to be a typical mitochondrial genome of

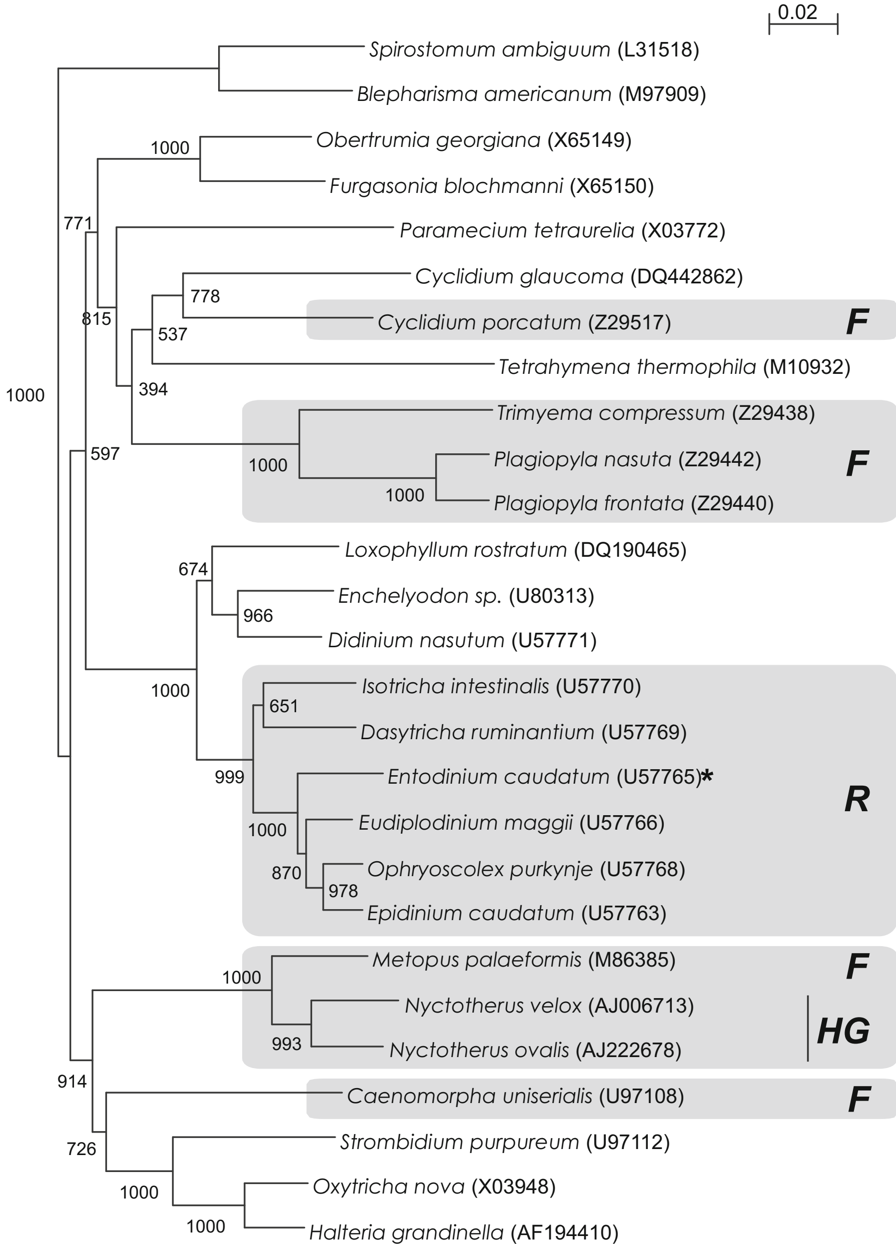


Fig. 7 Neighbour-joining phylogenetic tree of 18S ribosomal RNA of ciliates. Ribosomal RNA sequences were aligned using Clustal X (Jeanmougin et al. 1998) and phylogenetic trees were prepared by neighbour-joining (Saitou and Nei 1987). Shown are the accession numbers of used sequences and the bootstrap values for 1000 independent analyses. Shaded boxes indicate anaerobic ciliates with hydrogenosomes, whereas all other ciliates contain mitochondria and function aerobically. The natural habitat of the hydrogenosome-containing ciliates is indicated by the following abbreviations: F, free-living; HG, hindgut; R, rumen. Ciliate species that might possess mitosomes instead of hydrogenosomes are indicated by an asterisk. Reproduced with permission from Hackstein et al. (2008b)

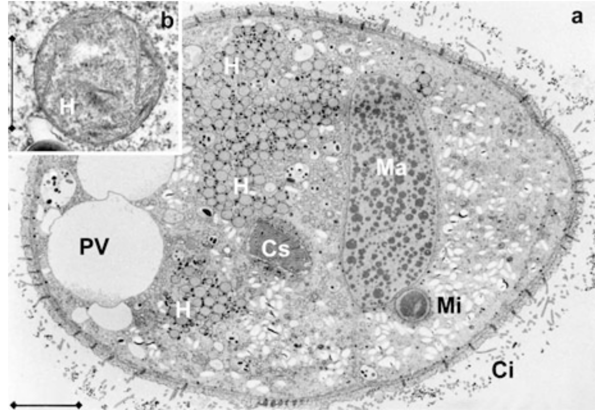


Fig. 8 Electron micrograph of *N. ovalis* (**a**) with a close-up view of a hydrogenosome (**b**) Bar in a, 10 micrometre; bar in b, 0,5 micrometre H, hydrogenosomes; Ma, macronucleus; Mi, micronucleus; Cs, cytostome; PV, pulsating vacuole. Reproduced with permission from Hackstein et al. (2008b)

ciliate origin (Boxma et al. 2005). This ciliate origin is reinforced by the analysis of some 90 genes that encode mitochondrial proteins (Boxma et al. 2005; Ricard 2008; de Graaf et al. 2011).

Metabolic studies revealed that a small part of the glucose was degraded to typical end products of a glycolytic fermentation: approximately 24% of the degraded glucose was excreted as lactate and 5% as ethanol (Boxma et al. 2005). The major part of the glucose was degraded via the hydrogenosomes to acetate and succinate. Those studies showed that *N. ovalis* does not use a complete Krebs cycle for the degradation of glucose and does not use pyruvate formate lyase (PFL) activity in its pyruvate metabolism, as is the case in hydrogenosomes of anaerobic chytrids. The product of glycolysis in the cytosol, pyruvate, is apparently either converted into lactate or ethanol or transported into the hydrogenosome to be converted into acetate or succinate. For the production of acetate, this pyruvate is decarboxylated by a pyruvate dehydrogenase complex (PDH) and not by a pyruvate:ferredoxin oxidoreductase (PFO) (Boxma et al. 2005). The excretion of significant amounts of succinate indicated that endogenously produced fumarate is used as a terminal electron acceptor. Protons act as another hydrogenosomal electron acceptor, which results in the formation of hydrogen. Fumarate reduction is most likely catalysed by a membrane-bound fumarate reductase (an anaerobically functioning variant of complex II), coupled to complex I of the electron-transport chain via quinones. Consistent with the biochemical/biophysical requirements (Tielens et al. 2002), small amounts of rhodoquinone 9 and menaquinone 8 were detected, whereas ubiquinone 7 and 8 (which are found in large amounts in the aerobic ciliates *Euplotes* and *Tetrahymena,* respectively) were not detected in *N. ovalis* (Boxma et al. 2005).

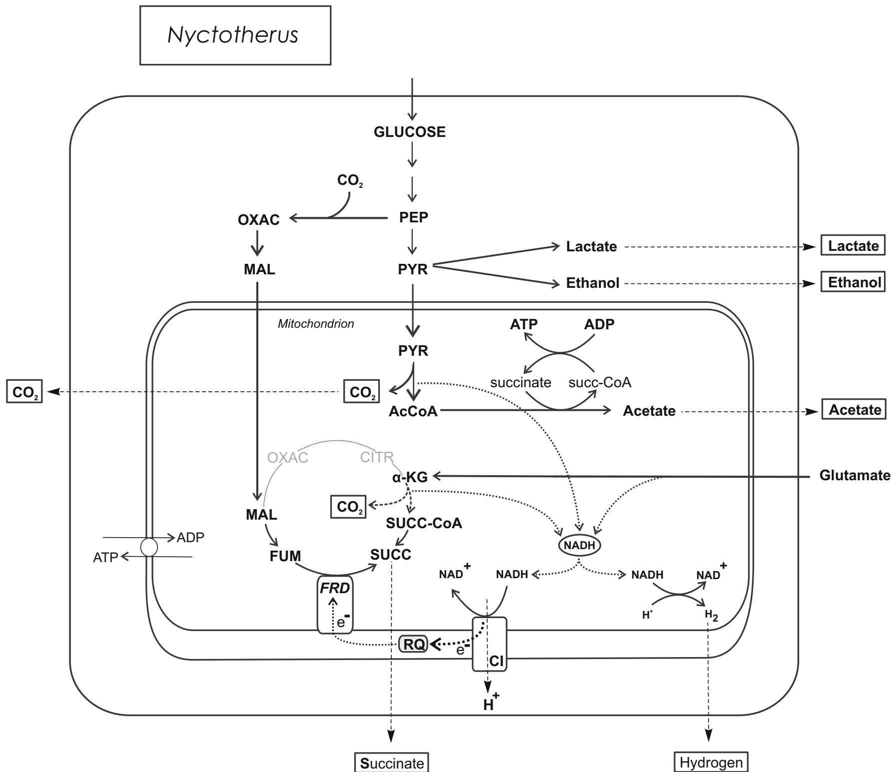


Fig. 9 Energy metabolism of *Nyctotherus ovalis* Shown is a scheme of the metabolic pathways involved in the production of the major end products. End products are in boxes. Abbreviations: AcCoA, acetyl-CoA; CI, complex I; FRD, fumarate reductase; FUM, fumarate; α-KG, α-ketoglutarate; MAL, malate; OXAC, oxaloacetate; PEP, phosphoenolpyruvate carboxykinase; PYR, pyruvate, RQ, rhodoquinone; SUCC, succinate; SUCC-CoA, succinyl-CoA (After de Graaf et al. 2011)

4.2 *In Silico Reconstruction of the Basal Hydrogenosomal Metabolism of* N. ovalis

The significance of these experimental data might be circumstantial without molecular support (Boxma et al. 2005). Genes for all four subunits of a PDH are present and are expressed. In addition, a gene was detected for acetyl-CoA synthase, an enzyme for the production of acetate from acetyl-CoA, and also several genes, which are predicted to encode enzymes of the Krebs cycle, i.e. malate dehydrogenase, succinate dehydrogenase (2 subunits), succinyl-CoA synthetase and alpha-ketoglutarate dehydrogenase (1 subunit). Thus, basically, the core energy metabolism of a typical ciliate mitochondrion was detected, albeit in an anaerobic version (Fig. 9). In fact, *N. ovalis* contains hydrogen-producing mitochondria (Boxma et al. 2005).

There is no evidence for genes encoding components of mitochondrial complexes III and IV. Notably, these complexes are also absent in the electron-transport chains of anaerobic mitochondria and the hydrogenosomes of *Blastocystis* (Tielens et al. 2002; Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008; Gentekaki et al. 2017). Therefore, it is likely that these hydrogenosomes gain their energy by the generation of a PMF through proton pumping by the mitochondrial complex I. Of the subunits of a mitochondrial complex I, 12 out of the 14 subunits that form the core of a bacterial complex I were cloned and sequenced until now (de Graaf et al. 2011). Accordingly, imaging studies using inhibitors and fluorescent dyes not only demonstrated the presence of a functional complex I in these hydrogenosomes but also indicated the absence of functional complexes III and IV and the absence of a plant-like alternative terminal oxidase (Boxma et al. 2005).

Also, no homologs of an F_oF_1-ATP synthase have been discovered so far, as in the organelles of *Blastocystis* and *Trichomonas* (Carlton et al. 2007; Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008).

In addition, components of a mitochondrial amino acid metabolism were identified, including a glycine cleavage system. Moreover, components of fatty acid metabolism, an AAC, several members of the mitochondrial solute carriers family, a malate-oxoglutarate translocator, components of a mitochondrial protein import and processing machinery, components of a protein synthesizing machinery and proteins belonging to a ROS defence systems were found. Several proteins originated from lateral gene transfer (Ricard 2008). Thus, the hydrogenosome of *N. ovalis* is not simply a rudimentary mitochondrion. It is a highly specialized organelle of considerable complexity.

4.3 The Hydrogenosomes of Other Ciliates

Metabolic studies have also been carried out on the hydrogenosomes of rumen ciliates such as *Dasytricha, Isotricha, Epidinium* and *Eudiplodinium*. All rumen ciliates form a monophyletic group (Fig. 7; Strüder-Kypke et al. 2006), but not all of them possess hydrogenosomes (Yarlett et al. 1984, 1985). Certain rumen ciliates utilize cellulose and starch—others predate on bacteria and smaller protozoa. Glucose is the major monosaccharide liberated by degradation of plant polymers and can be used by these rumen protozoa as fermentation substrate. The main end products of the metabolism of exogenously added glucose as well as of intracellular amylopectine of rumen ciliates with hydrogenosomes are hydrogen, acetate, lactate, butyrate and CO_2 (Yarlett et al. 1985; Ellis et al. 1991a, b, c). The ratio in which these end products are formed is influenced by O_2 and CO_2 concentrations similar to those present in the rumen. The investigated rumen ciliates are able to use oxygen as terminal electron acceptor. The nature of this terminal oxidase is still unknown, but cytochromes appear not to be involved. *Dasytricha ruminantium* is the best studied rumen ciliate, but even the knowledge of the metabolism of this rumen ciliate is still far from complete. The enzyme used for the degradation of pyruvate to acetyl-CoA

in this protozoon is suggested to be PFO, which has been identified tentatively in the hydrogenosomal fraction (Yarlett et al. 1981, 1982, 1985). This acetyl-CoA is the substrate for the hydrogenosomal formation of acetate but seems also to be exported from the hydrogenosomes for the formation of butyrate (Yarlett et al. 1985; Ellis et al. 1991b). These aspects make this hydrogenosome of rumen ciliates very different from that of *Nyctotherus* (Fig. 9) and also different from the hydrogenosomes of *Trichomonas* (Fig. 3) and the anaerobic chytrids (Fig. 6).

The only other published metabolic studies on hydrogenosomes of ciliates deal with the free-living plagiopylid ciliate *Trimyema* (Goosen et al. 1988, 1990; Holler and Pfennig 1991; Shinzato et al. 2018, this volume). *Trimyema* consumes oxygen under micro-aerobic conditions and is reported to produce formate as the major end product with minor amounts of acetate and lactate (Goosen et al. 1990; Holler and Pfennig 1991). Under those micro-aerobic conditions, hydrogen and ethanol are not produced. Under strictly anaerobic conditions, however, ethanol is the main end product, while acetate, lactate, formate and hydrogen are then formed in minor amounts (Goosen et al. 1990; Holler and Pfennig 1991). This pattern of anaerobic fermentation products resembles the one found in anaerobic chytridiomycete fungi (Boxma et al. 2004; see above). These fungi perform a bacterial type mixed acids fermentation, using PFL for the degradation of pyruvate, instead of PDH or PFO, which is used by *N. ovalis* and *Trichomonads*, respectively. Albeit that no additional biochemical data are available and that no cell fractionation studies have been performed, it is likely that the plagiopylids evolved a type of hydrogenosome that is clearly different from those of *Nyctotherus* and *Dasytricha.*

4.4 Can the Methanogenic Symbionts Tell Us More About the Origin and Function of Ciliate Hydrogenosomes?

As mentioned before and described by Fenchel and Finlay (2018), this volume, and Ushida (2018), this volume, anaerobic ciliates are frequently associated with symbiotic methanogens. The nature of the methanogenic symbionts supports the conclusion that different ciliates host different types of hydrogenosomes. While *Nyctotherus* and *Metopus,* but also *Plagiopyla* and *Trimyema*, host different endosymbiotic methanogens (Fenchel and Finlay 1995; Fenchel and Finlay 2018, this volume; van Hoek et al. 2000b; Hackstein et al. 2002), certain rumen ciliates seem to host episymbiotic methanogens. Whether this episymbiotic association is specific and whether there is any rumen ciliate (except *Dasytricha* and *Isotricha*) with *endo*symbiotic methanogens is still a matter of debate (Fenchel and Finlay 1995; Tokura et al. 1999; Regensbogenova et al. 2004; Ushida 2018, this volume).

Because the methanogens (regardless of being endo- or episymbiotic) rely on substrates provided by the host, the properties of the endosymbiont might provide some information about the metabolic characteristics of the host. The group of Vogels and Stumm succeeded in cultivating a number of putative methanogenic

endosymbionts from the anaerobic ciliates *Metopus striatus, Metopus contortus* and *Plagiopyla nasuta*, from the amoeboflagellate *Psalteriomonas(Lyromonas) vulgaris* and the giant amoeba *Pelomyxa palustris* (van Bruggen et al. 1984, 1986, 1988; Goosen et al. 1988: see Fenchel and Finlay 1995 for more references and discussion). The conclusion from these studies was that certain endosymbionts were similar if not identical to well-known free-living methanogens, e.g. *Methanobacterium formicicum*. Only the putative endosymbiont from *Metopus contortus* seemed to represent a new type of methanogen, i.e. *Methanoplanus endosymbiosus*. The latter host, *Metopus*, belongs to the same taxon as *N. ovalis*, which makes it likely that this ciliate (*Metopus*) possesses a similar mode of pyruvate metabolism. The metabolic properties of the methanogenic endosymbiont *M. formicium*, however, suggested that this methanogen might be capable of using other substrates besides hydrogen and CO_2, e.g. formate (Dong et al. 1994). Notably, Narayanan et al. (2009) provided evidence for the presence of an acetoclastic *Methanosaeta* species as endosymbiont of *Metopus es* suggesting that methanogenic endosymbionts might be able to use acetate excreted by the hydrogenosomes. This argues again for the metabolic diversity among ciliate hydrogenosomes and their methanogenic endosymbionts. This metabolic diversity could provide additional arguments for multiple origins of the hydrogenosomes, but unfortunately, metabolic data of both, hosts and symbionts, are scarce.

4.5 Evolutionary Aspects

There exists a rather broad agreement that the anaerobic ciliates evolved secondarily from aerobic ancestors since several ciliate taxa comprise both aerobic and anaerobic species. Phylogenetic studies suggest that hydrogenosomes have arisen independently at least three to four times in ciliates (Fig. 7; Clarke et al. 1993; Embley and Finlay 1994; Embley et al. 1995, 2003; Fenchel and Finlay 1995; Hirt et al. 1998; Hackstein et al. 2001, 2002). The existence in *N. ovalis* and in *Blastocystis* of a "missing link", an organelle with characteristics of mitochondria as well as of hydrogenosomes, demonstrates that hydrogenosome-bearing ciliates can evolve from mitochondriate ciliates (Martin 2005; Ricard 2008; Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008). Albeit that the patchy distribution of hydrogenosomes alone is not sufficient to prove multiple, independent origins of ciliate hydrogenosomes, the existence of a missing link like the hydrogenosome of *N. ovalis* provides a clear scenario for the evolution of hydrogenosomes from mitochondria. Apparently, hydrogenosomes in ciliates can evolve "easily" by evolutionary tinkering from mitochondria in the course of the adaptation of their hosts to anaerobic/micro-aerobic environments. This happened several times independently in the evolution of ciliates—at least three independent origins are supported by the existence of three different types of hydrogenosomes in the few ciliates that have been studied so far.

It remained unclear until now whether or not all anaerobic ciliates possess hydrogenosomes, in particular those anaerobes that do not possess endosymbiotic methanogens. Theoretically, anaerobic ciliates might possess anaerobic mitochondria (Tielens et al. 2002) and hydrogenosomes or they could even have lost ATP-generating organelles completely. In this case, they most likely host mitochondrial remnants, mitosomes, just like *Giardia* and *Entamoeba* spp., which are completely dependent on cytosolic reactions for the production of ATP (see, e.g. Hackstein et al. 2006 and Hjort et al. 2010 for discussion). However, the presence of these elusive organelles has not been studied systematically and in more detail in ciliates so far—with a few remarkable exceptions to be discussed below.

It has already been addressed that at least among the rumen ciliates, species with mitosomes might exist, because there is evidence that certain rumen ciliates, for example, *Entodinium simplex*, *Entodinium caudatum*, *Diploplastron affine*, *Ophryoscolex caudatus*, *Eremoplastron bovis* and *Ostracodinium obtusum bilobum*, did not exhibit detectable hydrogenase activity in the particulate cell fraction (Yarlett et al. 1984). Also, electron microscopy did not reveal the presence of mitochondrial-shaped organelles or typical hydrogenosomes in certain species of rumen and marsupial gut ciliates; a systematic search for mitosomes, however, has not been performed (Williams and Coleman 1992; Cameron and O'Donoghue 2002b). The observation that also PFO and malate dehydrogenase (decarboxylating) activities (Yarlett et al. 1984) are not enhanced in the particulate cell fraction, together with a low cytoplasmic hydrogenase activity, might argue for the absence of hydrogenosomes and potentially for the presence of mitosomes. However, until now, there are no additional data that could support this speculation.

The adaptation to an anaerobic lifestyle with the aid of hydrogenosomes required the acquisition of an (oxygen-sensitive) hydrogenase. The evolution of fumarate respiration in *N. ovalis* shows that an adaptation to life in anaerobic environments can occur in steps—by evolutionary tinkering. Once anaerobiosis could be tolerated by the invention of fumarate respiration, it became possible to acquire a hydrogenase. The [FeFe] hydrogenase of *N. ovalis* most likely has been obtained by lateral gene transfer from anaerobic (sulphate-reducing) bacteria (Boxma et al. 2007; de Graaf et al. 2009). The peculiar 24 and 51 kDa subunits of this complex hydrogenase are paralogous to the corresponding proteins of the mitochondrial complex I (which is functional in *N. ovalis*) and have a different (most likely beta proteobacterial) origin. The acquisition of this hydrogenase obviously allows a fine tuning of the NADH pool, which is crucial for the maintenance of homeostasis under anaerobic conditions. Thus, *N. ovalis* not only turns out to be a missing link; it also demonstrates that the adaptation to anaerobic environments can involve several steps to allow the evolution of multiple levels for the control of homeostasis.

5 Conclusions

In the recent years, it has become clear that there are many mitochondria that do not function as described in most biochemical textbooks (Tielens et al. 2002). Moreover, there are mitochondrion-related organelles such as hydrogenosomes, mitosomes, mitochondrial remnants and mitochondrion-like organelles (Fig. 1; Hackstein et al. 2006). All these organelles exhibit a large diversity of metabolic properties (Tielens et al. 2002; Tielens and van Hellemond 2007; van der Giezen 2009; Hjort et al. 2010). In this review, the diversity of hydrogenosomes, bona fide hydrogenosomes and hydrogenosome/mitochondrion-like organelles has been described. It has been shown that the metabolism of all these organelles is rather different corroborating their independent evolution from mitochondria and mitochondrion-like organelles. Their diversity is the consequence of their independent evolution in different anaerobic niches from organelles that were already adapted to different aerobic environments. The diversity of the hydrogenosomes described here is remarkable. Since there are many such organelles awaiting a more detailed analysis, an even larger diversity of hydrogenosomes might be expected.

References

Akhmanova A, Voncken F, van Alen T, van Hoek A, Boxma B, Vogels G, Veenhuis M, Hackstein JHP (1998a) A hydrogenosome with a genome. Nature 396(6711):527–528

Akhmanova A, Voncken FGJ, Harhangi H, Hosea KM, Vogels GD, Hackstein JHP (1998b) Cytosolic enzymes with a mitochondrial ancestry from the anaerobic chytrid *Piromyces* sp. E2. Mol Microbiol 30(5):1017–1027

Akhmanova A, Voncken FGJ, Hosea KM, Harhangi H, Keltjens JT, den Camp HJMO, Vogels GD, Hackstein JHP (1999) A hydrogenosome with pyruvate formate-lyase: anaerobic chytrid fungi use an alternative route for pyruvate catabolism. Mol Microbiol 32(5):1103–1114

Benchimol M (2008) Struture of the hydrogenosome. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes, Microbiol monographs, vol 9. Springer, Berlin, pp 75–96

Biagini GA, Hayes AJ, Suller MTE, Winters C, Finlay BJ, Lloyd D (1997) Hydrogenosomes of *Metopus contortus* physiologically resemble mitochondria. Microbiology 143:1623–1629

Bowman BH, Taylor JW, Brownlee AG, Lee J, Lu SD, White TJ (1992) Molecular evolution of the fungi – relationship of the basidiomycetes, ascomycetes, and chytridiomycetes. Mol Biol Evol 9(2):285–296

Boxma B, Voncken F, Jannink S, van Alen T, Akhmanova A, van Weelden SWH, van Hellemond JJ, Ricard G, Huynen M, Tielens AGM, Hackstein JHP (2004) The anaerobic chytridiomycete fungus *Piromyces* sp E2 produces ethanol via pyruvate: formate lyase and an alcohol dehydrogenase E. Mol Microbiol 51(5):1389–1399

Boxma B, de Graaf RM, van der Staay GWM, van Alen TA, Ricard G, Gabaldon T, van Hoek AHAM, Moon-van der Staay SY, Koopman WJH, van Hellemond JJ, Tielens AGM, Friedrich T, Veenhuis M, Huynen MA, Hackstein JHP (2005) An anaerobic mitochondrion that produces hydrogen. Nature 434(7029):74–79

Boxma B, Ricard G, van Hoek AHAM, Severing E, Moon-van der Staay SY, van der Staay GW, van Alen TA, de Graaf RM, Cremers G, Kwantes M, McEwan NR, Newbold CJ, Jouany J-P, Michalowski T, Pristas P, Huynen MA, Hackstein JHP (2007) The [FeFe] hydrogenase of *Nyctotherus ovalis* has a chimeric origin. BMC Evol Biol 7:230

Bullerwell CE, Lang BF (2005) Fungal evolution: the case of the vanishing mitochondrion. Curr Opin Microbiol 8(4):362–369

Burri L, Williams BAP, Bursac D, Lithgow T, Keeling PJ (2006) Microsporidian mitosomes retain elements of the general mitochondrial targeting system. Proc Natl Acad Sci U S A 103: 15916–15920

Cameron SL, O'Donoghue PJ (2002a) The ultrastructure of *Amylovorax dehorityi* comb. Nov. and erection of the Amylovoracidae fam. Nov. (Ciliophora: Trichostomatia). Eur J Protistol 38: 29–44

Cameron SL, O'Donoghue PJ (2002b) The ultrastructure of *Macropodinium moiri* and revised diagnosis of the Macropodiniidae (Litostomatea: Trichostomatia). Eur J Protistol 38(2):179–194

Carlton JM, Hirt RP, Silva JC, Delcher AL, Schatz M, Zhao Q, Wortman JR, Bidwell SL, Alsmark UC, Besteiro S, Sicheritz-Ponten T, Noel CJ, Dacks JB, Foster PG, Simillion C, Van de Peer Y, Miranda-Saavedra D, Barton GJ, Westrop GD, Muller S, Dessi D, Fiori PL, Ren Q, Paulsen I, Zhang H, Bastida-Corcuera FD, Simoes-Barbosa A, Brown MT, Hayes RD, Mukherjee M, Okumura CY, Schneider R, Smith AJ, Vanacova S, Villalvazo M, Haas BJ, Pertea M, Feldblyum TV, Utterback TR, Shu CL, Osoegawa K, de Jong PJ, Hrdy I, Horvathova L, Zubacova Z, Dolezal P, Malik SB, Logsdon JM Jr, Henze K, Gupta A, Wang CC, Dunne RL, Upcroft JA, Upcroft P, White O, Salzberg SL, Tang P, Chiu CH, Lee YS, Embley TM, Coombs GH, Mottram JC, Tachezy J, Fraser-Liggett CM, Johnson PJ (2007) Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis*. Science 315:207–212

Clarke KJ, Finlay BJ, Esteban G, Guhl BE, Embley TM (1993) *Cyclidium porcatum* N.sp. – a free-living anaerobic scuticociliate containing a stable complex of hydrogenosomes, eubacteria and archaeobacteria. Eur J Protistol 29(2):262–270

Clemens DL, Johnson PJ (2000) Failure to detect DNA in hydrogenosomes of *Trichomonas vaginalis* by nick translation and immunomicroscopy. Mol Biochem Parasitol 106:307–313

Contamine V, Picard M (2000) Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. Microbiol Mol Biol Rev 64(2):281–315

Corliss JO (1979) The ciliated protozoa: characterization, classification, and guide to the literature. Pergamon Press, London

Dacks JB, Dyal PL, Embley TM, van der Giezen M (2006) Hydrogenosomal succinyl-CoA synthetase from the rumen-dwelling fungus *Neocallimastix patriciarum*; an energy-producing enzyme of mitochondrial origin. Gene 373:75–82

de Graaf RM, Duarte I, van Alen TA, Kuiper JWP, Schotanus K, Rosenberg J, Huynen MA, Hackstein JHP (2009) The hydrogenosomes of *Psalteriomonas lanterna*. BMC Evol Biol 9:287

de Graaf RM, Ricard G, van Alen TA, Duarte I, Dutilh BE, Burgtorf C, Kuiper JWP, van der Staay GWM, Tielens AGM, Huynen MA, Hackstein JHP (2011) The organellar genome and metabolic potential of the hydrogen- producing mitochondrion of *Nyctotherus ovalis*. Mol Biol Evol 28:2379–2391

Dong XZ, Plugge CM, Stams AJM (1994) Anaerobic degradation of propionate by a mesophilic acetogenic bacterium in coculture and triculture with different methanogens. Appl Environ Microbiol 60(8):2834–2838

Ellis JE, McIntyre P, Saleh M, Williams AG, Lloyd D (1991a) Influence of CO_2 and low concentrations of O_2 on fermentative metabolism of the ruminal ciliate *Polyplastron multivesiculatum*. Appl Environ Microbiol 57(5):1400–1407

Ellis JE, McIntyre P, Saleh M, Williams AG, Lloyd D (1991b) Influence of CO_2 and low concentrations of O_2 on fermentative metabolism of the rumen ciliate *Dasytricha ruminantium*. J Gen Microbiol 137:1409–1417
Ellis JE, McIntyre P, Saleh M, Williams AG, Lloyd D (1991c) The influence of ruminal concentrations of O_2 and CO_2 on fermentative metabolism of the rumen entodiniomorphid ciliate *Eudiplodinium maggii*. Curr Microbiol 23(5):245–251
Embley TM, Finlay BJ (1994) The use of small-subunit ribosomal-RNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts. Microbiology 140:225–235
Embley TM, Martin W (2006) Eukaryotic evolution, changes and challenges. Nature 440(7084): 623–630
Embley TM, Finlay BJ, Dyal PL, Hirt RP, Wilkinson M, Williams AG (1995) Multiple origins of anaerobic ciliates with hydrogenosomes within the radiation of aerobic ciliates. Proc R Soc Lond Ser B Biol Sci 262(1363):87–93
Embley TM, van der Giezen M, Horner DS, Dyal PL, Bell S, Foster PG (2003) Hydrogenosomes, mitochondria and early eukaryotic evolution. IUBMB Life 55(7):387–395
Fenchel T, Finlay BJ (1995) Ecology and evolution in anoxic worlds. Oxford University Press, New York
Fenchel T, Finlay BJ (2018) Free-living protozoa with endosymbiotic methanogens. In: Hackstein JHP (ed) (Endo)symbiotic methanogens, 2nd edn. Springer, Berlin
Finlay BJ, Fenchel T (1989) Hydrogenosomes in some anaerobic protozoa resemble mitochondria. FEMS Microbiol Lett 65(3):311–314
Frey TG, Mannella CA (2000) The internal structure of mitochondria. Trends Biochem Sci 25(7): 319–324
Gelius-Dietrich G, Ter Braak M, Henze K (2007) Mitochondrial steps of arginine biosynthesis are conserved in the hydrogenosomes of the chytridiomycete *Neocallimastix frontalis*. J Eukaryot Microbiol 54(1):42–44
Gentekaki E, Curtis BA, Stairs CW, Klimeš V, Eliáš M, Salas-Leiva DE, Herman EK, Eme L, Arias MC, Henrissat B, Hilliou F, Klute MJ, Suga H1, Malik SB, Pightling AW, Kolisko M, Rachubinski RA, Schlacht A, Soanes DM, Tsaousis AD, Archibald JM, Ball SG, Dacks JB, Clark CG, van der Giezen M, Roger AJ (2017) Extreme genome diversity in the hyper-prevalent parasitic eukaryote Blastocystis. PLoS Biol 15(9):e2003769. https://doi.org/10.1371/journal.pbio.2003769
Gijzen HJ, Broers CAM, Barughare M, Stumm CK (1991) Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl Environ Microbiol 57(6):1630–1634
Gill EE, Diaz-Trivino S, Barbera MJ, Silberman JD, Stechmann A, Gaston D, Tamas I, Roger AJ (2007) Novel mitochondrion-related organelles in the anaerobic amoeba *Mastigamoeba balamuthi*. Mol Microbiol 66(6):1306–1320
Goosen NK, Horemans AMC, Hillebrand SJW, Stumm CK, Vogels GD (1988) Cultivation of the sapropelic ciliate *Plagiopyla nasuta* Stein and isolation of the endosymbiont *Methanobacterium formicicum*. Arch Microbiol 150(2):165–170
Goosen NK, Van der Drift C, Stumm CK, Vogels GD (1990) End products of metabolism in the anaerobic ciliate *Trimyema compressum*. FEMS Microbiol Lett 69(1–2):171–175
Hackstein JHP, Akhmanova A, Boxma B, Harhangi HR, Voncken FGJ (1999) Hydrogenosomes: eukaryotic adaptations to anaerobic environments. Trends Microbiol 7(11):441–447
Hackstein JHP, Akhmanova A, Voncken F, van Hoek A, van Alen T, Boxma B, Moon-van der Staay SY, van der Staay G, Leunissen J, Huynen M, Rosenberg J, Veenhuis M (2001) Hydrogenosomes: convergent adaptations of mitochondria to anaerobic environments. Zool Anal Complex Sys 104(3–4):290–302
Hackstein JHP, van Hoek AHAM, Leunissen JAM, Huynen M (2002) Anaerobic ciliates and their methanogenic endosymbionts. In: Seckbach J (ed) Symbiosis: mechanisms and model systems. Kluwer Academic, Doordrecht, pp 451–464. ISBN 1-4020-0189-4

Hackstein JHP, Tjaden J, Huynen M (2006) Mitochondria, hydrogenosomes and mitosomes: products of evolutionary tinkering! Curr Genet 50(4):225–245

Hackstein JHP, Baker SE, van Hellemond JJ, Tielens AGM (2008a) Hydrogenosomes of anaerobic chytrids: an alternative way to adapt to anaerobic environments. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes, Microbiol monographs, vol 9. Springer, Berlin, pp 147–162

Hackstein JHP, de Graaf RM, van Hellemond JJ, Tielens AGM (2008b) Hydrogenosomes of anaerobic ciliates. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes, Microbiol monographs, vol 9. Springer, Berlin, pp 97–112

Hampl V, Simpson AGB (2008) Possible mitochondria-related organelles in poorly studied "amitochondriate" eukaryotes. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes, Microbiol monographs, vol 9. Springer, Berlin, pp 265–282

Henze K (2008) The proteome of *T. vaginalis* hydrogenosomes. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes, Microbiol monographs, vol 9. Springer, Berlin, pp 163–178

Hirt RP, Wilkinson AG, Embley TM (1998) Molecular and cellular evolution of ciliates: a phylogenetic perspective. In: Coombs GH, Vickerman K, Sleigh MA, Warren A (eds) Evolutionary relationships among protozoa. Chapman and Hall, London, pp 327–340

Hjort K, Goldberg AV, Tsaousis AD, Hirt RP, Embley TM (2010) Diversity and reductive evolution of mitochondria among microbial eukaryotes. Philos Trans Roy Soc B 365:713–727

Holler S, Pfennig N (1991) Fermentation products of the anaerobic ciliate *Trimyema compressum* in monoxenic cultures. Arch Microbiol 156:327–334

Howe CJ (2008) Cellular evolution: what's in a mitochondrion? Curr Biol 18:R429–R431

Hrdy I, Hirt RP, Dolezal P, Bardonová L, Foster PG, Tachezy J, Embley TM (2004) Trichomonas hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I. Nature 432:618–622

Hrdy I, Tachezy J, Müller M (2008) Metabolism of Trichomonad hydrogenosomes. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes, Microbiol monographs, vol 9. Springer, Berlin, pp 113–145

Huang KY, Ong SC, Wu CC, Hsu CW, Lin HC, Fang YK, Cheng WH, Huang PJ, Chiu CH, Tang P (2017) Metabolic reprogramming of hydrogenosomal amino acids in *Trichomonas vaginalis* under glucose restriction. J Microbiol Immunol Infect. S1684-1182(17)30241-4. https://doi.org/10.1016/j.jmii.2017.10.005

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trends Biochem Sci 23:403–405

Julliand V, Riondet C, de Vaux A, Alcaraz G, Fonty G (1998) Comparison of metabolic activities between *Piromyces citronii*, and equine fungal species, and *Piromyces communis*, a ruminal species. Anim Feed Sci Technol 70(1–2):161–168

Katinka MD, Duprat S, Cornillot E, Metenier G, Thomarat F, Prensier G, Barbe V, Peyretaillade E, Brottier P, Wincker P, Delbac F, El Alaoui H, Peyret P, Saurin W, Gouy M, Weissenbach J, Vivares CP (2001) Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. Nature 414:450–453

Keithly JS (2008) The mitochondrion-related organelle of *Cryptosporidium parvum*. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes, Microbiol monographs, vol 9. Springer, Berlin, pp 231–253

Keithly JS, Langreth SG, Buttle KF, Mannella CA (2005) Electron tomographic and ultrastructural analysis of the *Cryptosporidium parvum* relict mitochondrion, its associated membranes, and organelles. J Eukaryot Microbiol 52(2):132–140

Li Y, Jin W, Cheng Y, Zhu W (2016) Effect of the associated methanogen *Methanobrevibacter thaueri* on the dynamic profile of end and intermediate metabolites of anaerobic fungus *Piromyces* sp. F1. Curr Microbiol 73:434–441

Lindmark DG, Müller M (1973) Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus*, and its role in pyruvate metabolism. J Biol Chem 248:7724–7728

Lindmark DG, Müller M (1974) Superoxide-dismutase in anaerobic flagellates, *Tritrichomonas foetus* and *Monocercomonas* sp. J Biol Chem 249:4634–4637

Lindmark DG, Müller M, Shio H (1975) Hydrogenosomes in *Trichomonas vaginalis*. J Parasitol 61:552–554

Lloyd D, Hillman K, Yarlett N, Williams AG (1989) Hydrogen-production by rumen holotrich protozoa – effects of oxygen and implications for metabolic control by in situ conditions. J Protozool 36(2):205–213

Maguire F, Richards TA (2014) Organelle evolution: a mosaic of 'mitochondrial' functions. Curr Biol 24:R518–R520

Makiuchi T, Nozaki T (2014) Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa. Biochimie 100:3–17

Mannella CA (2006) The relevance of mitochondrial membrane topology to mitochondrial function. Biochim Biophys Acta 1762(2):140–147

Martin W (2005) The missing link between hydrogenosomes and mitochondria. Trends Microbiol 13(10):457–459

Marvin-Sikkema FD, Richardson AJ, Stewart CS, Gottschal JC, Prins RA (1990) Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. Appl Environ Microbiol 56:3793–3797

Marvin-Sikkema FD, Gomes TMP, Grivet JP, Gottschal JC, Prins RA (1993) Characterization of hydrogenosomes and their role in glucose-metabolism of *Neocallimastix* sp L2. Arch Microbiol 160(5):388–396

Marvin-Sikkema FD, Driessen AJM, Gottschal JC, Prins RA (1994) Metabolic energy generation in hydrogenosomes of the anaerobic fungus *Neocallimastix* – evidence for a functional-relationship with mitochondria. Mycol Res 98:205–212

Mazet M, Diogon M, Alderete JF, Vivares CP, Delbac F (2008) First molecular characterisation of hydrogenosomes in the protozoan parasite *Histomonas meleagridis*. Int J Parasitol 38:177–188

Müller M (1993) The hydrogenosome. J Gen Microbiol 139:2879–2889

Müller M, Mentel M, van Hellemond JJ, Henze K, Woehle C, Gould SB, Yu RY, van der Giezen M, Tielens AGM, Martin WF (2012) Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. Microbiol Mol Biol Rev 76:444–495

Munn EA, Orpin CG, Greenwood CA (1988) The ultrastructure and possible relationships of 4 obligate anaerobic chytridiomycete fungi from the rumen of sheep. Biosystems 22(1):67–81

Narayanan N, Krishnakumar B, Anupama VN, Manilal VB (2009) *Methanosaeta* sp., the major archaeal endosymbiont of *Metopus es*. Res Microbiol 160:600–607

Orpin CG (1975) Studies on rumen flagellate *Neocallimastix frontalis*. J Gen Microbiol 91:249–262

Orpin CG (1977) Occurrence of chitin in cell-walls of rumen organisms neocallimastix-frontalis, piromonas-communis and sphaeromonas-communis. J Gen Microbiol 99:215–218

Paquin B, Lang BF (1996) The mitochondrial DNA of *Allomyces macrogynus*: the complete genomic sequence from an ancestral fungus. J Mol Biol 255(5):688–701

Paquin B, Forget L, Roewer I, Lang BF (1995) Molecular phylogeny of *Allomyces macrogynus* – congruency between nuclear ribosomal RNA and mitochondrial protein-based trees. J Mol Evol 41(5):657–665

Paul RG, Williams AG, Butler RD (1990) Hydrogenosomes in the rumen entodiniomorphid ciliate *Polyplastron multivesiculatum*. J Gen Microbiol 136:1981–1989

Peña-Diaz P, Lukeš J (2018) Fe–S cluster assembly in the supergroup Excavata. J Biol Inorg Chem. https://doi.org/10.1007/s00775-018-1556-6

Perez-Brocal V, Clark CG (2008) Analysis of two genomes from the mitochondrion-like organelle of the intestinal parasite *Blastocystis*: complete sequences, gene content, and genome organization. Mol Biol Evol 25:2475–2482

Ragan MA, Chapman DJ (1978) A biochemial phylogeny of the protists. Academic Press, New York

Regensbogenova M, McEwan NR, Javorsky P, Kisidayova S, Michalowsky T, Newbold CJ, Hackstein JHP, Pristas P (2004) A re-appraisal of the diversity of the methanogens associated with the rumen ciliates. FEMS Microbiol Lett 238(2):307–313

Ricard G (2008) Evolution and genome structure of anaerobic ciliates. Thesis Radboud University, Nijmegen, The Netherlands

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Shinzato N, Takeshita K, Kamagata Y (2018) The methanogenic an eubacterial endosymbionts of *Trimyema*. In: Hackstein JHP (ed) (Endo)symbiotic methanogens, 2nd edn. Springer, Berlin

Sickmann A, Reinders J, Wagner Y, Joppich C, Zahedi R, Meyer HE, Schonfisch B, Perschil I, Chacinska A, Guiard B, Rehling P, Pfanner N, Meisinger C (2003) The proteome of *Saccharomyces cerevisiae* mitochondria. Proc Natl Acad Sci U S A 100(23):13207–13212

Snyers L, Hellings P, Bovy-Kesler C, Thines-Sempoux D (1982) Occurrence of hydrogenosomes in the rumen ciliates Ophryoscolecidae. FEBS Lett 137(1):35–39

Stairs CW, Leger MM, Roger AJ (2015) Diversity and origins of anaerobic metabolism in mitochondria and related organelles. Philos Trans R Soc Lond Ser B Biol Sci 370:20140326

Stechmann A, Hamblin K, Perez-Brocal V, Gaston D, Richmond GS, van der Giezen M, Clark CG, Roger AJ (2008) Organelles in Blastocystis that blur the distinction between mitochondria and hydrogenosomes. Curr Biol 18(8):580–585

Strüder-Kypke MC, Wright ADG, Foissner W, Chatzinotas A, Lynn DH (2006) Molecular phylogeny of litostome ciliates (Ciliophora, Litostomatea) with emphasis on free-living Haptorian genera. Protist 157(3):261–278

Tachezy J, Dolezal P (2007) Iron-sulfur proteins and iron-sulfur cluster assembly in organisms with hydrogenosomes and mitosomes. In: Martin WF, Müller M (eds) Origin of mitochondria and hydrogenosomes. Springer, Berlin, pp 105–133

Tielens AGM, van Hellemond JJ (2007) Anaerobic mitochondria:properties and origins. In: Martin WF, Müller M (eds) Origin of mitochondria and hydrogenosomes. Springer, Berlin, pp 85–103

Tielens AGM, Rotte C, van Hellemond JJ, Martin W (2002) Mitochondria as we don't know them. Trends Biochem Sci 27(11):564–572

Tjaden J, Haferkamp I, Boxma B, Tielens AGM, Huynen M, Hackstein JHP (2004) A divergent ADP/ATP carrier in the hydrogenosomes of *Trichomonas gallinae* argues for an independent origin of these organelles. Mol Microbiol 51:1439–1446

Tokura M, Chagan I, Ushida K, Kojima Y (1999) Phylogenetic study of methanogens associated with rumen ciliates. Curr Microbiol 39:123–128

Tovar J, Fischer A, Clark CG (1999) The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite Entamoeba histolytica. Mol Microbiol 32(5):1013–1021

Trinci APJ, Davies DR, Gull K, Lawrence MI, Nielsen BB, Rickers A, Theodorou MK (1994) Anaerobic fungi in herbivorous animals. Mycol Res 98:129–152, Part 2

Ushida K (2018) Symbiotic methanogens and rumen ciliates. In: Hackstein JHP (ed) (Endo)symbiotic methanogens, 2nd edn. Springer, Berlin

van Bruggen JJA, Stumm CK, Vogels GD (1983) Symbiosis of methanogenic bacteria and sapropelic protozoa. Arch Microbiol 136(2):89–95

van Bruggen JJA, Zwart KB, van Assema RM, Stumm CK, Vogels GD (1984) *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus* McMurrich. Arch Microbiol 139(1):1–7

van Bruggen JJA, Zwart KB, Hermans JGF, van Hove EM, Stumm CK, Vogels GD (1986) Isolation and characterization of *Methanoplanus endosymbiosus* sp.nov, an endosymbiont of the marine sapropelic ciliate *Metopus contortus* Quennerstedt. Arch Microbiol 144(4):367–374

van Bruggen JJA, van Rens GLM, Geertman EJM, Zwart KB, Stumm CK, Vogels GD (1988) Isolation of a methanogenic endosymbiont of the sapropelic ameba *Pelomyxa palustris* Greeff. J Protozool 35(1):20–23

van der Giezen M (2009) Hydrogenosomes and mitosomes: conservation and evolution of functions. J Eukaryot Microbiol 56:221–231

van der Giezen M, Sjollema KA, Artz RRE, Alkema W, Prins RA (1997) Hydrogenosomes in the anaerobic fungus *Neocallimastix frontalis* have a double membrane but lack an associated organelle genome. FEBS Lett 408(2):147–150

van der Giezen M, Slotboom DJ, Horner DS, Dyal PL, Harding M, Xue GP, Embley TM, Kunji ERS (2002) Conserved properties of hydrogenosomal and mitochondrial ADP/ATP carriers: a common origin for both organelles. EMBO J 21(4):572–579

van der Giezen M, Birdsey GM, Horner DS, Lucocq J, Dyal PL, Benchimol M, Danpure CJ, Embley TM (2003) Fungal hydrogenosomes contain mitochondrial heat-shock proteins. Mol Biol Evol 20(7):1051–1061

van Grinsven KW, Rosnowsky S, van Welden SWH, Pütz S, van der Giezen M, Martin W, van Hellemond JJ, Tielens AGM, Henze K (2008) Acetate:Succinate CoA-transferase in the hydrogenosomes of *Trichomonas vaginalis*. J Biol Chem 283:1411–1418

van Hoek AHAM, van Alen TA, Sprakel VSI, Hackstein JHP, Vogels GD (1998) Evolution of anaerobic ciliates from the gastrointestinal tract: phylogenetic analysis of the ribosomal repeat from *Nyctotherus ovalis* and its relatives. Mol Biol Evol 15(9):1195–1206

van Hoek AHAM, Sprakel VSI, Van Alen TA, Theuvenet APR, Vogels GD, Hackstein JHP (1999) Voltage-dependent reversal of anodic galvanotaxis in *Nyctotherus ovalis*. J Eukaryot Microbiol 46(4):427–433

van Hoek AHAM, Akhmanova AS, Huynen M, Hackstein JHP (2000a) A mitochondrial ancestry of the hydrogenosomes of *Nyctotherus ovalis*. Mol Biol Evol 17(1):202–206

van Hoek AHAM, van Alen TA, Sprakel VSI, Leunissen JAM, Brigge T, Vogels GD, Hackstein JHP (2000b) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol Biol Evol 17(2):251–258

Vogels GD, Hoppe WF, Stumm CK (1980) Association of methanogenic bacteria with rumen ciliates. Appl Environ Microbiol 40(3):608–612

Voncken F (2001) Hydrogenosomes: eukaryotic adaptations to anaerobic environments. PhD Thesis, University of Nijmegen (ISBN 90-9014868-x)

Voncken F, Boxma B, Tjaden J, Akhmanova A, Huynen M, Verbeek F, Tielens AGM, Haferkamp I, Neuhaus HE, Vogels G, Veenhuis M, Hackstein JHP (2002) Multiple origins of hydrogenosomes: functional and phylogenetic evidence from the ADP/ATP carrier of the anaerobic chytrid *Neocallimastix* sp. Mol Microbiol 44(6):1441–1454

Wawrzyniak I, Roussel M, Diogon M, Couloux A, Texier C, Tan KSW, Vivares CP, Delbac F, Wincker P, El Alaoui H (2008) Complete circular DNA in the mitochondria-like organelles of *Blastocystis hominis*. Int J Parasitol 38:1377–1382

Westrop GD, Wang L, Blackburn GJ, Zhang T, Zheng L, Watson DG, Coombs GH (2017) Metabolomic profiling and stable isotope labelling of *Trichomonas vaginalis* and *Tritrichomonas foetus* reveal major differences in amino acid metabolism including the production of 2-hydroxyisocaproic acid, cystathionine and S-methylcysteine. PLoS One. https://doi.org/10.1371/journal.pone.0189072.eCollection2017

Williams AG, Coleman GS (1992) The rumen protozoa. Springer, New York

Williams BAP, Keeling PJ (2005) Microsporidian mitochondrial proteins: expression in *Antonospora locustae* spores and identification of genes coding for two further proteins. J Eukaryot Microbiol 52:271–276

Williams AG, Withers SE, Naylor GE, Joblin KN (1994) Interactions between the rumen chytrid fungi and other microorganisms. In: Mountfort DO, Orpin CG (eds) Anaerobic fungi. Biology, ecology, and function. Marcel Dekker, New York, pp 191–227

Williams BAP, Hirt RP, Lucocq JM, Embley TM (2002) A mitochondrial remnant in the microsporidian *Trachipleistophora hominis*. Nature 418:865–869

Yarlett N, Hann AC, Lloyd D, Williams A (1981) Hydrogenosomes in the rumen protozoan *Dasytricha ruminantium* Schuberg. Biochem J 200(2):365–372

Yarlett N, Lloyd D, Williams AG (1982) Respiration of the rumen ciliate *Dasytricha ruminantium* Schuberg. Biochem J 206(2):259–266

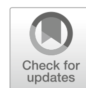
Yarlett N, Hann AC, Lloyd D, Williams AG (1983) Hydrogenosomes in a mixed isolate of *Isotricha prostoma* and *Isotricha intestinalis* from ovine rumen contents. Comp Biochem Physiol B 74(2):357–364

Yarlett N, Coleman GS, Williams AG, Lloyd D (1984) Hydrogenosomes in known species of rumen entodiniomorphid protozoa. FEMS Microbiol Lett 21(1):15–19

Yarlett N, Lloyd D, Williams AG (1985) Butyrate formation from glucose by the rumen protozoan *Dasytricha ruminantium*. Biochem J 228(1):187–192

Zwart KB, Goosen NK, van Schijndel MW, CAM B, Stumm CK, Vogels GD (1988) Cytochemical-localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis*, *Plagiopyla nasuta* and *Trimyema compressum*. J Gen Microbiol 134:2165–2170

Evolution of Prokaryote-Animal Endosymbiosis from a Genomics Perspective



Rosario Gil, Amparo Latorre, and Andrés Moya

Contents

1 Introduction 224
2 Survival, Replication, and Transmission: The Three Biological Processes Involved in the Establishment of a Permanent Symbiotic Association 232
3 Early Stages in the Symbiotic Relationship 233
 3.1 Facultative Symbionts 233
 3.2 Mobile Elements, Shaping the First Steps Toward an Obligate Endosymbiosis 236
4 Long-Established P-Endosymbioses 239
 4.1 Genomic Features 239
 4.2 Tiny Genomes 241
5 Complementation or Replacement 243
 5.1 Complementation and the Establishment of Microbial Consortia 243
 5.2 Replacement 245
6 Archaeal Ectosymbionts and Their Reductive Genome Evolution 246
7 Concluding Remarks 247
References 248

Abstract Symbioses involving prokaryotes living in close relationship with eukaryotic cells have been widely studied from a genomic perspective, especially in the case of insects. In the process toward host accommodation, symbionts undergo major genetic and phenotypic changes that can be detected in comparison with free-living relatives. But, as expected, several scenarios allowed the evolution of symbiotic associations, from the free-living stage, through secondary and facultative symbiosis, toward the final step of obligate primary endosymbiosis. Particular relevance has the association formed by the coexistence of several endosymbionts

R. Gil · A. Latorre · A. Moya (✉)
Institut de Biologia Integrativa de Sistemes (I2SysBio), Universitat de València, Paterna (València), Spain

Departament de Genètica, Universitat de València, Paterna (València), Spain

Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO), València, Spain
e-mail: rosario.gil@uv.es; amparo.latorre@uv.es; andres.moya@uv.es

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*, Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8_11

into a given host. A summary of findings in this field, as well as the evolutionary scenarios to explain these changes, is presented in this chapter.

1 Introduction

The term symbiosis refers to the close ecological relation between two (or more) species, able to report benefits to all (mutualism) or some of the implied organisms, with or without harm of one of the involved species (parasitism or commensalism, respectively). Symbiosis is an important source of evolutionary innovation, with examples in the entire biosphere, being even at the origin of the eukaryotic cell (Margulis 1993). Since then, stable symbioses have evolved independently many times in diverse groups of eukaryotes (Moya et al. 2008; Toft and Andersson 2010). Most symbioses have a demonstrated biochemical basis: in some cases, one of the partners benefits from organic compounds produced by the other; in others, its waste products (mainly nitrogen compounds) are recycled by the other. In mutualistic symbioses, matter and energy flow in both directions, so that both partners benefit from the association.

Numerous eukaryotic groups maintain a mutualistic relationship with prokaryotic cells, especially because many eukaryotic lineages present limited metabolic capabilities. Animal metabolism, in particular, is relatively narrow, and essential molecules (such as amino acids, vitamins, or fatty acids) must be retrieved from the environment for survival. Animals with specialized feeding behaviors tend to establish symbiotic associations with microorganisms, which provide the nutrients that are deficient in their diets. In fact, most intracellular mutualistic symbioses between bacteria and animals that have been analyzed at the genomic level (involving mainly insects, but also nematodes and deep-sea animals) are related to nutrient provision (Dubilier et al. 2008; Taylor et al. 2012; Sudakaran et al. 2017). Regarding insects, the most studied and diverse invertebrate group on Earth, the presence of such associations throughout most of their evolutionary history suggests that symbiosis has been a driving force in the diversification of the group.

A high proportion of mutualistic symbiotic relationships established by insects imply the participation of bacteria. The main features of the bacterial symbiont's genomes cited in this review are summarized in Table 1. Frequently, the association is so tight that it is called endosymbiosis, when the bacteria (endosymbiont) obligatorily live inside specialized eukaryotic cells (bacteriocytes), which can even form a specialized organ (the bacteriome), located inside the abdominal cavity of the insect. For this reason, obligate endosymbionts cannot be cultured outside their eukaryotic hosts. It has been estimated that up to 15% of all insect species carry bacterial endosymbionts (Douglas 1998; Baumann 2005), attributing to them the great adaptive success of the class Insecta, by making possible the colonization of new ecological niches and allowing them to feed on restricted diets (such as plant sap,

Table 1 Remarkable data of the relevant complete genomes of mutualistic insect endosymbionts mentioned in the text

Stage of symbiosis	Bacteria	Class	Insect host	Symbiotic function	Genome size (kb)	GC (%)	CDS	Accession number (s)
Facultative	*Sodalis glossinidius* morsitans	Gammaproteobacteria	*Glossina morsitans* (tsetse fly)	Unclear	4293	54.7	3259	AP008232-AP008235
	Serratia symbiotica Tucson[a]	Gammaproteobacteria	*Acyrthosiphon pisum* (aphid)	Heat tolerance	2789	51.7	1715	AENX01[d]
	Hamiltonella defensa 5AT[a]	Gammaproteobacteria	*Acyrthosiphon pisum* (aphid)	Resistance to parasitoid wasps	2169	40.0	1915	CP001277, CP001278
	Regiella insecticola LSRa[a]	Gammaproteobacteria	*Acyrthosiphon pisum* (aphid)	Resistance to parasitoid wasps and fungi	2067	42.2	1658	ACYF01[d], CM000957
	Wolbachia wCle	Alphaproteobacteria	*Cimex lectularius* (bedbug)	Nutrient provision	1482	34.2	981	AP013028
	Wolbachia pipientis wMel	Alphaproteobacteria	*Drosophila melanogaster* (fruit fly)	Reproductive parasite	1268	35.2	1103	AE017196
Single obligate	*Sodalis pierantonius* SOPE[a]	Gammaproteobacteria	*Sitophilus oryzae* (grain weevil)	Nutrient provision	4513	56.1	3153	CP006568
	Blochmannia floridanus[a]	Gammaproteobacteria	*Camponotus floridanus* (ant)	Nutrient provision and N storage	706	27.4	587	BX248583
	Wigglesworthia glossinidia	Gammaproteobacteria	*Glossina brevipalpis* (tsetse fly)	Nutrient provision	703	22.5	636	BA000021, AB063523

(continued)

Table 1 (continued)

Stage of symbiosis	Bacteria	Class	Insect host	Symbiotic function	Genome size (kb)	GC (%)	CDS	Accession number (s)
	Buchnera aphidicola APS	Gammaproteobacteria	*Acyrthosiphon pisum* (aphid)	Nutrient provision	656	26.4	574	BA000003, AP001070, AP001071
	Blattabacterium cuenoti Bge	Flavobacteria	*Blattella germanica* (cockroach)	Nutrient provision and N storage	641	27.1	577	CP001487, CP002849
	Tremblaya phenacola PAVE[a]	Betaproteobacteria	*Phenacoccus avenae* (mealybug)	Nutrient provision	172	42.1	175	CP003982, CP003983
	Tremblaya phenacola PPER[a]	Beta/ Gammaproteobacteria (chimera)	*Phenacoccus peruvianus* (mealybug)	Nutrient provision	198+7[b]	35.6	196	MKGN01[d]
	Carsonella ruddii PV [a]	Gammaproteobacteria	*Pachypsylla venusta* (psyllid)	Nutrient provision	160	16.6	182	AP009180
Obligate consortium	***Buchnera aphidicola* BCt**	**Gammaproteobacteria**	***Cinara tujafilina* (aphid)**	**Nutrient provision**	**453**[c]	**23.0**	**361**[c]	**CP001817, AY438024**
	***Serratia symbiotica* SCt**[a]	**Gammaproteobacteria**			**2500**	**52.1**	**1695**	**GCA_900002265**[e]
	Buchnera aphidicola BCc	Gammaproteobacteria	*Cinara cedri* (aphid)	Nutrient provision	425[c]	20.2	369[c]	CP000263, AY438025, EU660486
	[a]*Serratia symbiotica* SCc	Gammaproteobacteria			1763	29.2	672	CP002295
	***Carsonella ruddii* DC**[a]	**Gammaproteobacteria**		**Nutrient provision**	**174**	**17.6**	**207**	**CP003467**

	***Profftella armatura* DC**[a]	**Betaproteobacteria**	***Diaphorina citri* (psyllid)**	**Defensive symbiont**	**465**	**24.2**	**358**	**CP003468, CP003469**
	Carsonella ruddii CE [a]	Gammaproteobacteria	*Ctenarytaina eucalypti* (psyllid)	Nutrient provision	163	14.0	190	CP003541
	Sodalis-like endosymbiont	Gammaproteobacteria			1441	43.3	918	CP003546
	***Sulcia muelleri* CARI**[a]	**Flavobacteria**	***Clastoptera arizonana* (spittlebug)**	**Nutrient provision**	**277**	**21.1**	**246**	**CP002163**
	***Zinderia insecticola* CARI**[a]	**Betaproteobacteria**			**209**	**13.5**	**206**	**CP002161**
	Sulcia muelleri Sulcia-ALF[a]	Flavobacteria	*Macrosteles quadrilineatus* (leafhopper)	Nutrient provision	191	24.0	191	CP006060
	Nasuia deltocephalinicola NAS-ALF[a]	Betaproteobacteria			112	17.1	138	CP006059
	***Sulcia muelleri* GWSS**[a]	**Flavobacteria**	***Homalodisca vitripennis* (sharpshooter)**	**Nutrient provision**	**246**	**22.0**	**227**	**CP000770**
	***Baumannia cicadellinicola* Hc**[a]	**Gammaproteobacteria**			**686**	**33.2**	**595**	**CP000238**
	Sulcia muelleri SMDSEM[a]	Flavobacteria	*Diceroprocta semicincta* (cicada)	Nutrient provision	276	22.0	242	CP001605
	Hodgkinia cicadicola Dsem[a]	Alphaproteobacteria			144	58.4	169	CP001226
	***Sulcia muelleri* SMMAGTRE**[a]	**Flavobacteria**	***Magicicada tredecim* (cicada)**	**Nutrient provision**	**269**	**21.7**	**224**	**CP010828**
	***Hodgkinia cicadicola* MAGTRE**[a]	**Alphaproteobacteria**			**1571**	**29.1**	**252**	**NXGT01**[d]

(continued)

Table 1 (continued)

Stage of symbiosis	Bacteria	Class	Insect host	Symbiotic function	Genome size (kb)	GC (%)	CDS	Accession number (s)
	Tremblaya princeps PCVAL[a]	Betaproteobacteria	*Planococcus citri* (mealybug)	Nutrient provision	139	59.0	116	CP002918
	Moranella endobia PCVAL[a]	Gammaproteobacteria			538	43.5	414	CP003881

Genome information has been retrieved from GenBank
[a]These bacteria are named *Candidatus*
[b]The numbers indicate the size of the unique sequences plus the sum of all repeats once
[c]The number does not correspond to the one indicated in GenBank because one of the plasmids is not included in the "Genome assembly and annotation report" page of this genome
[d]The accession number corresponds to the whole-genome sequence (WGS) project
[e]The accession number corresponds to the genome assembly

cereals, or blood), poor in some essential nutrients, that are provided by the endosymbionts. There are cases, however, in which the insects are omnivorous and still harbor endosymbionts. Their genome sequences revealed that they are involved in nitrogen metabolism (Gil et al. 2003; López-Sánchez et al. 2009). The elimination of these bacteria, consequently, critically diminishes the biological fitness of the host, affecting to its growth, fertility, and/or longevity.

The first to notice the link between a restricted diet and the presence of endosymbiotic bacteria in insects was Paul Buchner (Buchner 1965), who coined the terms of primary (P-) endosymbiont and facultative or secondary (S-) symbiont, based on its morphologic characterization and its presence among the individuals of a certain taxonomic group. This classification has later been validated by means of molecular genetic techniques and the complete sequencing of genomes of an increasing number of endosymbiotic bacteria (reviewed in Lo et al. 2016; Latorre and Manzano-Marín 2017). Buchner classified as P-endosymbionts those bacteria of a unique morphological type that are present in all the insects of a defined taxonomic group, confined inside specialized insect cells located in the abdominal cavity. Such P-endosymbionts are essential for its host fitness and survival. On the other side, the S-symbionts were identified as morphologically diverse bacteria, without a defined spatial distribution in the host body and whose sporadic presence in some individuals of a defined taxon suggested that they were not essential for host survival. In fact, S-symbionts vary in number and distribution among species and among individuals of the same species and can live outside of the eukaryotic cells. The congruence between the phylogenetic trees based on host and their corresponding P-endosymbionts sequences indicates that each endosymbiont derives from a single infection of the ancestor of the host by the ancestors of its P-endosymbiont and follows a path of vertical evolution, promoted by their exclusively maternal transmission between insect generations (Munson et al. 1992). On the contrary, the topological incongruence between the phylogenetic trees based on sequences of the S-symbionts and their hosts and the polyphyletic character of such bacteria suggest the existence of multiple events of infection and/or the horizontal transfer of these bacteria among insects (Russell et al. 2003; Henry et al. 2013).

Buchner and the early researchers of prokaryote-eukaryote symbioses did not differentiate between the two prokaryotic domains, since the existence of archaea was not recognized until the 1990s of the past century (Woese et al. 1990). Therefore, certain early symbioses described as involving “bacteria” were, in fact, involving archaea (Hackstein et al. 2006). This was the case for many methanogenic symbionts hosted by protists that were described in termite and cockroach guts in the 1980s. These archaea have also been found in millipedes and scarabs and are always restricted to the hindgut, where they can appear free in the gut lumen, attached to digesta or to the hindgut wall, or as endosymbionts of anaerobic ciliated protozoa that occupy the same gut compartment (Wrede et al. 2012; Brune and Dietrich 2015). Little is known about the function of methanogenic archaea in the guts of arthropods, besides their role in lowering H_2 partial pressure by producing methane. Phylogenetic studies have been performed on anaerobic heterotrichous ciliates that keep an endosymbiotic association with methanogenic archaea (van Hoek et al. 2000). This is an

interesting study group because they live in the most divergent niches, such as marine and freshwater sediments and the intestinal tract of animals. The topology of the phylogenetic trees indicates that, although coevolution of host and endosymbiont is frequent, and probably hydrogenosome-bearing ciliates acquired methanogenic endosymbionts at the very beginning of their evolution toward anaerobiosis, prior to the anaerobic heterotrichous ciliate radiation, endosymbiont replacements must have accompanied the evolution of these protists (van Hoek et al. 2000; Hackstein et al. 2002). Whole-genome studies will help to identify other possible benefits of endosymbiotic methanogenic archaea to their hosts. However, even though a great effort has been made to expand the amount of archaea genomes available (Parks et al. 2017), a detailed study of the sequenced genomes is lacking in most cases, and, to our knowledge, no complete genome of a methanogenic archaea from a ciliate living in an insect gut has been published to date.

Even though new data are accumulating on prokaryotic symbionts of animals, most analyses concentrate on nutritional and physiological aspects (summarized in Latorre and Manzano-Marín 2017; Sudakaran et al. 2017). At the beginning of the genomic era, research on prokaryote endosymbionts of eukaryotic cells focused on a limited group of arthropods, which have been for a while the main models used to define the evolutionary and molecular aspects of prokaryote-animal symbioses. They are mostly sap-sucking insects (Hemiptera: Sternorrhyncha), such as aphids, white flies, psyllids, and mealybugs, but also the mammalian blood-feeding tsetse fly (Diptera: Glossinidae) and the omnivorous carpenter ants (Hymenoptera: Apocrita) and cockroaches (Blattodea). The research was further extended to other insect groups of order Hemiptera, including Auchenorrhyncha (cicadas, planthoppers, and spittlebugs), Coleorrhyncha (moss bugs), Heteroptera (stink bugs, kissing bugs), Coleoptera (weevils), and Phthiraptera (lice). Due to the amount of information accumulated, we will focus mostly on bacterial endosymbionts of insects to detangle the molecular aspects of these symbioses from a genomics perspective, paying special attention to the genomic changes experienced by the bacterium in their adaptation to an intracellular lifestyle.

The advent of high-throughput sequencing technologies allowed the complete sequencing of genomes and the development of metagenomic methods, making possible the study of environmental samples and non-cultivable microorganisms, thus offering new opportunities for symbiosis research. The availability of many genomes of bacterial endosymbionts opened the door to comparative analyses among them, unveiling common molecular aspects regarding the establishment and maintenance of symbiotic associations. In order to completely understand the different steps of genomic evolution of bacterial endosymbionts, it became necessary to analyze and compare genome sequences from several strains of the same species in different stages of their symbiotic integration, from a free-living bacterium to an obligate mutualistic endosymbiont (Moya et al. 2009; Toft and Andersson 2010; Latorre and Manzano-Marín 2017) (Fig. 1). The first step toward the establishment of an

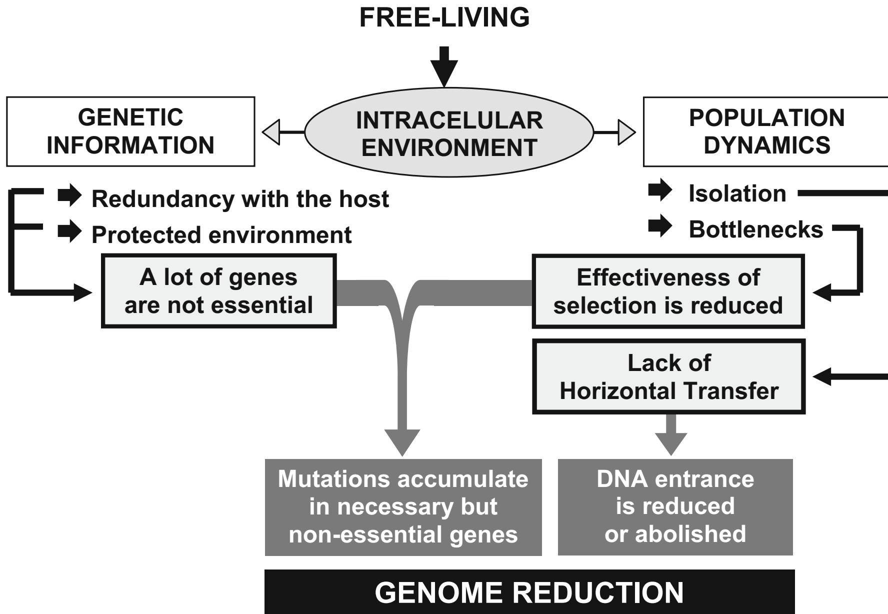


Fig. 1 Genetic and population factors involved in the genome reduction syndrome experienced by mutualistic endosymbionts. At the beginning of endosymbiosis, the new, rich, protected, and stable intracellular niche provided by the host makes superfluous some gene functions, which become redundant (since they can be contributed by the host) or unnecessary in a stable and protected environment but forces the preservation of genes required for the maintenance and viability of the partnership. The decreased efficiency of the purifying selection causes a fast accumulation of slightly deleterious mutations on nonessential genes, increasing the rates of genomic evolution. In addition, the drastic reduction of the bacterial effective population size between successive insect generations increases the relative influence of random genetic drift. Furthermore, the obligatory intracellular lifestyle prevents the entrance of genetic material by HGT, making the losses irreversible

obligate intracellular mutualistic symbiosis takes place when a free-living bacterium infects a eukaryotic host. From this point, both organisms will coevolve to adapt to the new situation. The host develops specialized cells to harbor the bacterium, which in turn provides benefits to the host that end up being essential. From an evolutionary point of view, this new stable situation triggers a cascade of changes that model the shape and content of the bacterial genome. In the course of this chapter, we will see how genomics and metagenomics helped researchers on the field to detangle the physiological and evolutionary changes undergone by bacteria in their way toward an obligatory mutualistic intracellular symbiosis with eukaryotic hosts.

2 Survival, Replication, and Transmission: The Three Biological Processes Involved in the Establishment of a Permanent Symbiotic Association

Mutualism and parasitism are two sides of the same coin. At the very beginning, it is not possible to determine if the relationship that would be established will be parasitic or mutualistic, since this distinction is based on the effect of the bacterium in the eukaryotic host, but, from the bacterial point of view, the biological processes needed to successfully infect hosts are largely the same for both types of microorganisms (Gil et al. 2004a). In both cases, it will be necessary to overcome the physical, cellular, and molecular barriers presented by the host, to achieve internalization, survival, and proficient replication of the prokaryote inside the eukaryotic host cell. No matter if the interaction is harmful, neutral, or beneficial to the host, natural selection will favor the bacteria that achieve this goal (Ochman and Moran 2001). Most evolutionary transitions leading toward symbiotic lifestyles involve gene loss and horizontal gene transfer (HGT) of virulence genes within bacterial lineages (Toft and Andersson 2010; Gil and Latorre 2012). Genomic analyses indicate that, in many cases, the same molecular factors are involved both in pathogenic and mutualistic relationships although, in the case of mutualism, traditionally considered parasitic traits, at some point, became beneficial for both partners. In facultative symbionts, toxins that are known or suspected to target eukaryotic cells are involved in protecting the host against natural enemies (Oliver et al. 2009, 2014). Most toxins are encoded by genes present in lysogenic bacteriophages, which also act as hot spots for nonhomologous recombination events that allow gene exchange of virulence cassettes among heritable symbionts (Degnan and Moran 2008). But even endosymbiotic bacteria with a long-time established relationship with their hosts, which have suffered a dramatic genome size reduction (as will be discussed below), maintain genes that encode essential endosymbiotic factors that are proposed to be virulence associated in bacterial pathogens, such as type III secretion systems and urease (Shigenobu et al. 2000; Goebel and Gross 2001; Gil et al. 2003; López-Sánchez et al. 2009). In many free-living bacteria, genes encoding the type III secretion system are located within pathogenicity islands that have been acquired by HGT. This system is present in many insect endosymbiotic bacteria where it has been proposed to be essential to invade the host cells, thus playing an essential role in the establishment of the symbiosis (Dale et al. 2002). On the other hand, the role of the urease is to metabolize urea to ammonium that can be recycled to amino acids. More recently, a defensive symbiont, producing a protective toxin against natural enemies was described: *Candidatus* Profftella armatura, endosymbiont of the Asian citrus psyllid *Diaphorina citri*, produces a polyketide toxin, a capability that apparently has acquired through HGT from a *Pseudomonas* (Nakabachi et al. 2013).

The establishment of a permanent intracellular association necessarily implies the development of efficient mechanisms for bacterial survival and replication inside the host cell. The bacteria must adapt their replication, so that their growth rates are

coordinated with the development of their hosts in a way that depends on their location inside the host cell. In *Buchnera aphidicola*, which lives confined in vacuole-like organelles inside the aphid bacteriocytes, there is a tight coupling of bacterial cell number and aphid growth, with the bacteria showing a doubling time of approximately 2 days, much longer than the maximum exhibited by many free-living bacteria (Baumann and Baumann 1994). Several endosymbiont genomes lack a canonical origin of replication *ori*C, which is consistent with the absence of *dna*A, the gene that encodes the essential DNA replication initiation protein in bacteria. This feature was first described for *Blochmannia floridanus* and *Wigglesworthia glossinidia* (Gil et al. 2003), but it is also common in very reduced genomes, such as those of *Carsonella ruddii*, *Hodgkinia cicadicola*, *Moranella endobia*, *Sulcia muelleri*, *Tremblaya princeps*, and *Zinderia insecticola* (summarized in López-Madrigal et al. 2013). It has been suggested that this could imply the existence of a more direct control of DNA replication of the symbionts by the host (Gil et al. 2003).

An efficient transmission of the bacteria to the offspring must also be guaranteed. The acquisition of mechanisms ensuring maternal transmission to the host progeny allows the association to be heritable, resulting in the emergence of a new composite organism host-endosymbiont. The fine-tuning of this process detected in long-established obligate mutualistic symbioses suggests a long history of selection favoring host adaptations that help to maintain the association (Moran and Telang 1998).

3 Early Stages in the Symbiotic Relationship

The whole-genome sequencing of many bacteria living in symbiosis with eukaryotic hosts has allowed the comparison among the evolutionary innovations carried out by these bacteria on their way from free-living to varied stages of integration with their respective hosts. To detangle the changes involved in each stage, over the next paragraphs, we will follow the path from facultative symbiosis to early obligate endosymbiosis, as it has been revealed by molecular studies and comparative genomics over the past years.

3.1 Facultative Symbionts

Many different types of facultative or S-symbionts have been described in arthropods, and have been extensively studied in several lineages of aphids, but also in psyllids, whiteflies, leafhoppers, tsetse flies, fruit flies, louse flies, coleopteran, hymenopteran, and mosquitoes (Table 1; Lo et al. 2016). They can be maternally transmitted between host generations, but, unlike P-endosymbionts, they can also be horizontally transferred among host individuals and species, and, therefore, they do

not share long evolutionary histories with their hosts. S-symbionts do not reside exclusively in specialized cells and organs and can also be found in gut tissues, glands, or body fluids, and when a P-endosymbiont is also present, they can occupy cells surrounding the P-bacteriocytes or even invade them. Phylogenetic studies indicate that facultative symbionts have established relatively recent associations with their hosts (Dale and Moran 2006; Manzano-Marín et al. 2017). Thus, their genomes may resemble those in the early stages of a transition from a free-living lifestyle to an obligate mutualism. Their uneven presence among species and individuals of the same species indicates that, contrary to P-endosymbionts, S-symbionts are dispensable and their influence on host fitness is variable. A range of effects, from negative to beneficial, have been described (Lo et al. 2016).

Some known S-symbionts have negative effects on growth and reproduction to the host or may establish neutral or parasitic associations. Heritable S-symbionts can spread among lineages by manipulating host reproduction to enhance matrilineal transmission through parthenogenesis, male killing, feminization of genetic males, or cytoplasmic incompatibility. This is the case of *Wolbachia* infecting arthropods, where it undergoes transfer among host lineages (McGraw and O'Neill 2004). Remarkably, *Wolbachia* appears as a typical P-endosymbiont in filarial nematodes, where it is required for normal development (Grote et al. 2017) and it has been suggested to be essential to complement the symbiotic functions of *B. aphidicola* in the banana black aphid *Pentalonia nigronervosa* (De Clerk et al. 2015) and to contribute to host fitness in the bedbug *Cimex lectularius* through the provisioning of B vitamins (Nikoh et al. 2014), just to mention some examples. A comprehensive review of known cases of facultative and obligate relationships between *Wolbachia* and arthropods can be found in Zug and Hammerstein (2015). The complete genomes of 9 different *Wolbachia* strains are already available, and 15 more have been published as draft genomes with different assembly levels, allowing unraveling the molecular basis of their interaction with their respective hosts by comparative genomics. Most of them correspond to reproductive parasites of arthropods, including fruit flies, mosquitoes, and wasps (Wu et al. 2004; Klasson et al. 2008, 2009; Ellegaard et al. 2013; Lindsey et al. 2016). Differences in genome size (from 0.96 to 1.54 Mb) are mostly due to the presence of repeated elements, especially to the amplification of the WO prophage and genes encoding ankyrin repeats. Furthermore, these repeated elements can undergo intragenic recombination (Bordenstein and Wernegreen 2004). There are evidences suggesting that several loci contained within the WO-B prophage correlate with the effects of the bacterial strain as reproductive parasite. The presence of such genes has been associated with cytoplasmic incompatibility (CI) and/or male killing, even in the absence of a complete WO prophage (Metcalf et al. 2014), while the presence of truncated ORFs in the WO-B elements can be on the bases of the loss of the CI-inducing phenotype (Sutton et al. 2014) and the conversion from CI to parthenogenesis-inducing lifestyle in some *Wolbachia* (Lindsey et al. 2016).

The first completely sequenced genome of an S-symbiont with no clear negative or positive effect corresponded to *Sodalis glossinidius* (Toh et al. 2006), the S-symbiont of the tsetse fly, and the first endosymbiont to be successfully cultured,

an indication that the association with its host is yet reversible (Dale and Maudlin 1999). Its genome size (4.17 Mb) is close to that of free-living bacteria, but its coding capacity is highly diminished by the presence of a big amount of pseudogenes (Belda et al. 2010). The genome also contains certain amounts of repetitive and mobile DNA, such as transposable elements and bacteriophages. Therefore, it appears that this bacterium is at the early stages in the reductive process affecting symbiont genomes. Even though the relationship between *S. glossinidius* and the tsetse fly is facultative, its elimination from females has been associated with a reduction in the longevity of their offspring (Dale and Welburn 2001). This positive effect can explain the spread of this symbiont in tsetse fly populations.

Many other S-symbionts described in insects confer beneficial effects on the survival and reproduction rates of their hosts. Their roles have been extensively studied in recent years both in silico (i.e., through the analysis of their genomes) and in vivo (based on correlation studies between the presence of a given symbiont and a particular trait, both in natural populations and in populations that have been experimentally cured of the bacteria; Feldhaar 2011; Su et al. 2013). Most of these studies have been performed on aphids, where three main S-symbionts have been described and analyzed at the genomic level. *Serratia symbiotica* and *Regiella insecticola* (Moran et al. 2005), which have only been found in aphids, and *Hamiltonella defensa*, also present in other sap-feeding insects such as psyllids and whiteflies (Clark et al. 1992; Moran et al. 2005; Rao et al. 2012). A variety of beneficial ecological traits have been associated with the presence of these S-symbionts, including resistance to natural enemies, enhanced heat tolerance, and insect-plant interactions (reviewed in Feldhaar 2011). In some cases, they can even compensate the loss of the essential endosymbiont (Koga et al. 2003).

The genome of *H. defensa* 5AT, S-symbiont of the pea aphid *Acyrthosiphon pisum* (Degnan et al. 2009), confirmed its beneficial role by protecting its host from the attack by parasitoid wasps, based on the identification of genes that encode for toxins, effector proteins, and two type III secretion systems. Its 2.17 Mb genome has undergone significant reduction in size relative to its closest free-living relatives, and important gene losses have been detected (it relies on the P-endosymbiont *B. aphidicola* for the synthesis of eight of the ten essential amino acids), which indicates that the reductive genome process is already advanced. Yet, it contains considerable amounts of genes involved in regulation of virulence factors and quorum sensing, which indicates that it still retains some ability to deal with changing environments and invasion of new host species. This genome also contains important amounts of repetitive DNA (21% of the genome), including insertion sequences (IS), group II introns, prophages, and plasmids. The degree of protection provided by *H. defensa* is highly variable among bacterial strains. Part of this variation must be due to the *H. defensa* chromosomal gene content (Martinez et al. 2014a, b), but it is also greatly influenced by the presence of a lysogenic lambda-like bacteriophage, designated "*A. pisum* secondary endosymbiont" phage (APSE; van der Wilk et al. 1999). The different variants of APSE identified harbor a specific virulence cassette region, encoding toxins that target eukaryotic tissues (Oliver et al. 2009). Therefore, the beneficial role of the phage toxins for the insect host fitness is

contributing to the spread and maintenance of *H. defensa* in host populations. Furthermore, the APSE lysis region is a hot spot for nonhomologous recombination of novel virulence cassettes, allowing gene exchange among S-symbionts by horizontal transmission (Degnan and Moran 2008).

Regiella insecticola is not only involved in resistance to parasitoid wasps, similarly to *H. defensa*, but also to fungal pathogens (Scarborough et al. 2005). Most of the genome of strain LSR1 (about 2.07 Mb) has been sequenced and compared with the close relative *H. defensa* 5AT (Degnan et al. 2010), showing that, even though they are sister species, their genomes are highly rearranged. The complete genome assembly was not possible due to the presence of high amounts of repetitive DNA, mostly IS elements. Its comparison with the genome of *R. insecticola* 5.15, which is unable of protecting its host against parasitoids, revealed that *R. insecticola* LSR1 possess five pathogenicity factors and translocation systems that are missing in the later, which might be involved in its protective function (Hansen et al. 2012).

Serratia symbiotica was first described in *A. pisum*, where it has been involved in protection against heat stress (Montllor et al. 2002; Russell and Moran 2006). It has been suggested that this phenotype is due to an enhanced preservation of bacteriocytes and, therefore, *B. aphidicola*'s survival at high temperatures (Burke et al. 2010). At present, the genomes of five strains from different aphids are available, although with different degrees of completeness. Some of these strains appear as typical facultative symbionts. This is the case of *S. symbiotica* CWBI-2.3^{T} from the aphid *Aphis fabae*, the second S-symbiont able to be grown in the laboratory (Foray et al. 2014), and *S. symbiotica* str. Tucson from *A. pisum* (Burke and Moran 2011). On the contrary, strains from aphids belonging to subfamily Lachninae have established a permanent and stable cooperative consortium with the host and the P-endosymbiont, *B. aphidicola* (Lamelas et al. 2011; Manzano-Marín and Latorre 2014; Manzano-Marín et al. 2017), thus becoming essential for the maintenance of the fitness of all three partners (see Sect. 5). Again, as seen in the previously mentioned facultative symbionts, the abundance of repeats (mainly IS) hampered full-genome assembly of the facultative *S. symbiotica* strains.

3.2 Mobile Elements, Shaping the First Steps Toward an Obligate Endosymbiosis

It has been postulated that soon after the establishment of obligate symbiosis, a massive gene loss must occur, probably by means of large deletion events that cause the elimination of series of contiguous genes (Moran and Mira 2001). Comparative genomics revealed that, later on, genome shrinkage proceeds through a process of gradual pseudogenization and gene loss scattered throughout the genome (Silva et al. 2001; Gómez-Valero et al. 2004). However, the mechanism involved in the large deletion events was unknown at that time. The identification of the genome changes

that occur in these initial stages of the adaptation toward endosymbiosis required the genomic study of clades of bacteria that have recently established such associations. In the last years, as seen in Sect. 3.1, genomes of different strains of the same species and in different degrees of adaptation to the intracellular live have become available, providing clues on the genome features driving genome reduction. By comparing the genomes of facultative S-symbiont strains, it was observed that they have very different genome architectures, suggesting a period of large-scale rearrangements, and all of them possess abundant copies of transposable elements (mainly IS) and prophages. Repeated elements provide numerous sites for homologous recombination between them, catalyzing large inversion and deletions (Aras et al. 2003).

Repetitive DNA is common in free-living bacteria, although in moderate amounts, and its presence increases in bacteria that have recently evolved as specialized pathogens (e.g., the enteric bacteria *Shigella* and *Salmonella enterica* Typhi; Jin et al. 2002; Wei et al. 2003) and intracellular parasites (e.g., *Wolbachia* strains parasitizing arthropods; Klasson et al. 2008, 2009; Wu et al. 2004). The increase in transposable elements, which could cause genome rearrangements, is also a common trait among bacteria that have recently established mutualistic relationships with their hosts (see examples in Sect. 3.1) and must have subsequent effects on the outcome of the symbiotic process (Bordenstein and Reznikoff 2005; Moran and Plague 2004). In fact, when the first three available *Wolbachia* genomes were compared, a high degree of rearrangements was observed, revealing the most highly recombining obligate intracellular bacterial community examined at that time (Klasson et al. 2009). Most recently, the genome of several clades from the genera *Sodalis* and *Serratia* has been compared, covering the spectrum from extracellular free-living to P-endosymbiont (*Sodalis* spp.) in the case of weevils and tsetse flies (Clayton et al. 2012; Oakeson et al. 2014) or a co-obligate in aphids (*S. symbiotica* strains SCt, SCc, and STs; Manzano-Marin and Latorre 2016). The size of the main chromosome of the facultative *S. glosinidius* (4.17 Mb) is close to that of its free-living relative *Sodalis praecaptivus* (4.71 Mb). Similarly, the genome sizes of the facultative *S. symbiotica* str. CWBI-2.3^T (3.58 Mb) and Tucson (2.79 Mb) are closer to the genome of free-living *Serratia*, such as *S. marcescens* Db11 (5.11 Mb) than to the reduced co-obligate *S. symbiotica* SCc (1.76 Mb) or STs (0.65 Mb). In comparison with their free-living relatives, the genomes of these facultative strains show a great enrichment in mobile elements and pseudogenes, together with a moderate number of rearrangements, consistent with the hypothesis that the increase in mobile elements can promote genomic shuffling. In addition, mobile elements can also promote gene inactivation (pseudogenization), further contributing to genome erosion, as it has been described in *S. glosinidius* (Belda et al. 2010) and *S. symbiotica* (Manzano-Marín and Latorre 2014).

IS are the most abundant and simplest transposable elements in nature (Touchon and Rocha 2007). They are able to move between replicons of a certain genome and can also be transferred between genomes of different organisms by HGT. Its persistence is usually explained by an intense ability for intergenomic mobilization and to its more or less efficient infecting capacity. The massive presence of IS must be related with some of the syndromes that appear at the beginning of the

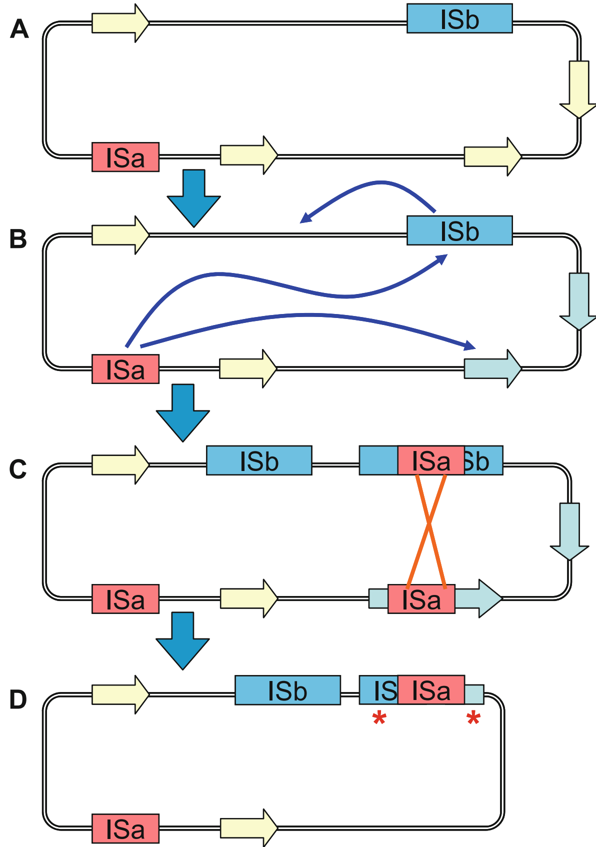


Fig. 2 An evolutionary scenario for the implication of IS in gene inactivation, genome reduction, and chromosomal rearrangements. (**a**) Free living cell. (**b**) Beginning endosymbiosis: many genes become superfluous or redundant. Massive transposition. (**c**) IS can be a source of genomic recombination. (**d**) Interrupted genes and IS degenerated by mutation

intracellular life (Fig. 2). IS elements are widespread in free-living bacteria, but their transposition is tightly controlled, so that only a few copies of a limited number of categories appear in each genome. The dramatic increase of these elements in intracellular bacteria must reflect an enhanced replicative transposition of elements that were already present at the onset of symbiosis and can then act as a source of gene inactivation and chromosomal rearrangements. After the establishment of the symbiosis, the decrease in the selective pressure caused by functional conditions and population dynamics in the new environment can favor the uncontrolled proliferation of such elements, which could be involved in the inactivation of nonessential genes. The high abundance of very similar (or even identical) repetitive elements in direct orientation can then serve as a substrate for unequal recombination, which would lead to a loss of the region between two elements, thus promoting genome size reduction in early stages. Additionally, the presence of these elements in opposite orientation will lead to genome rearrangements.

In this scenario, after the establishment of an obligate endosymbiont lifestyle, it was assumed that repetitive DNA tends to diminish until its total disappearance in long-term P-endosymbionts (Manzano-Marín and Latorre 2016). There are,

however, some striking genomes that do not follow the rule, as it is the case of two recent obligate endosymbionts that still retain features of their facultative stage, *Sodalis pierantonius* SOPE (Oakeson et al. 2014) and *S. symbiotica* SCt (Manzano-Marin and Latorre 2014). The obligate P-endosymbiont of the rice weevils, *S. pierantonius* SOPE, possesses a 4.51 Mb genome, similar to that of *S. glosinidius* or *S. praecaptivus.* The sequencing of the whole genome revealed that IS elements occupy about one third of its genome and a massive number of rearrangements were detected when comparing the three genomes (Oakeson et al. 2014). This impressive amount of repetitive DNA was not expected in an obligate mutualistic endosymbiont. The association of insects of the genus *Sitophilus* and their present endosymbionts is not antique. Some data indicate a recent endosymbiont replacement of an ancestral endosymbiont in the family Dryophtoridae to which the rice and maize weevils belong (Lefèvre et al. 2004). On the other hand, the comparison of the genome of *S. symbiotica* SCt, the co-obligate endosymbiont of the thuja tree aphid, together with *B. aphidicola* with the facultative *S. symbiotica* Tucson, revealed that their genomes are very similar in size (2.49 Mb vs. 2.57 Mb), as well as in gene content and functions (Manzano-Marín and Latorre 2014). Both strains hold a high number of IS elements, although belonging to different families, and a high number of rearrangements have been found between them.

4 Long-Established P-Endosymbioses

4.1 Genomic Features

In general, endosymbionts with a long-established relationship with their hosts have genomes 8–10 times smaller than those of their free-living relatives. In bacteria, whose genomes are highly compact, gene content correlates quite well with genome size (Casjens 1998). Therefore, the reduced size of endosymbiont genomes reflects the presence of a smaller number of genes than those of free-living bacteria. Several additional genome features have traditionally been associated with the degenerative syndrome affecting endosymbiotic bacteria. These include almost total absence of recombination, increased rate of nucleotide substitution, high A+T content (although as it will be discussed later, this no longer can be considered a general trait), accumulation of deleterious mutations by random genetic drift, loss of codon bias toward A or T, and accelerated sequence evolution (Andersson and Kurland 1998; Clark et al. 1999; Moya et al. 2002; Wernegreen 2015). Most of these characteristics are linked with the abovementioned informational and demographic factors affecting bacteria that live in close association with eukaryotic cells, although the accommodation to symbiotic life varies according to the age of the association, the host lifestyle, and the way of living within the host.

Comparative genomics analyses among several strains of *Buchnera*, *Blochmannia*, *Blattabacterium*, *Tremblaya*, and *Carsonella* revealed that all strains of each clade present a highly conserved genome architecture (Latorre and

Manzano-Marín 2017). However, when compared with the corresponding close free-living relatives and facultative symbionts, it was shown that the massive gene loss that took place in the process toward the last common symbiotic ancestor (LCSA) of each species was accompanied by many chromosomal rearrangements. The former presence of repetitive elements, already absent in most known endosymbiont genomes, might explain such genome reorganizations. The current lack of repetitive sequences, with a great potential as recombination sites, as well as the loss of *loci* needed for the catalysis of such recombination events in early stages of the symbiosis (except for the *Tremblaya* case that will be detailed later), appears to be in the origin of the high genomic architecture stability in old endosymbionts, quite unusual among the prokaryotes (Silva et al. 2003). In fact, genes needed for DNA repair and recombination are among the first losses detected in early symbiotic stages, thus contributing to genomic stasis in further steps in the endosymbiotic evolutionary path. The loss of the genes coding for the enzymes RecA and RecF in *S. pierantonius* and *S. glossinidius* (Dale et al. 2003; Oakeson et al. 2014) supports this idea.

The progressive loss of transposable elements might have been favored by the energetic benefit of decreasing transposase activity and avoiding the increase in genome size derived from the proliferation of these elements or by the need to control the mutagenizing effect of its mobilization. It is presumable that, at some point, IS elements expansion will be deleterious and these elements would be also affected by the process of genome degradation that these genomes suffer. The sexual isolation of P-endosymbionts and the loss of recombination genes must also have participated in the process, since HGT is the way of entrance of these elements in prokaryotic genomes (Touchon and Rocha 2007). Furthermore, due to the same two factors, the genome size cannot increase by acquisition of foreign DNA (Silva et al. 2003). Nevertheless, some recombination events can still take place in these reduced genomes, probably involving the RecBCD system, which in the absence of RecA might serve as a general exonuclease repair enzyme (Sabater-Muñoz et al. 2004), as revealed by the great plasticity of the plasmids involved in the biosynthesis of leucine in different lineages of *B. aphidicola*, showing that several events of insertion from a plasmid to the main chromosome have occurred since the divergence of these strains (Latorre et al. 2005).

In general, smaller genomes correlate with longer obligate associations. The differences in host lifestyle are also introducing changes in this degenerative process among strains of the same endosymbiont species. The small genomes of *B. aphidicola* are still suffering this reductive process, as evidenced by the fact that strains from several aphid subfamilies showed differences up to 200 kb (Gil et al. 2002; Manzano-Marín et al. 2017) and the presence of pseudogenes in the *B. aphidicola* genomes that have been sequenced and deposited in genome databases (24 complete genomes of 15 different aphid species). In addition, the degenerative process is randomly affecting different genes in each genome, conditioning the essentiality of the rest of the genes that are present in these reduced genomes.

Therefore, although we can hypothesize that the LCSA of *B. aphidicola* suffered a drastic genome reduction at the beginning of the symbiotic integration, since then, the different strains of the bacteria have undergone a reductive process in a way that correlates with their hosts.

In addition to changes in genome size, obligate and facultative endosymbionts of different insect hosts also differ in nucleotide composition. P-endosymbionts with an old association with their hosts have in general an A+T content higher than 70%, while P-endosymbionts with a younger association and S-symbionts have an A+T percentage intermediate with respect to older P-endosymbionts and free-living relatives (Moya et al. 2002; Wernegreen 2015). The loss of the bias in codon usage in these obligate intracellular bacteria, highly mitigated in P-endosymbionts with larger genomes and in S-symbionts and almost absent in *B. aphidicola*, is considered to be a consequence of this base composition bias (Moya et al. 2002; Rispe et al. 2004). This notable enrichment in A+T has been related to the loss of DNA repair enzymes, since the most common chemical changes in DNA (cytosine deamination and guanosine oxidation) led to changes in GC pairs leading to AT. However, several cases that do not follow this nucleotide composition rule have been described. The available genome sequences from eight strains of *Tremblaya princeps*, P-endosymbiont of pseudococcinae mealybugs, have a G+C content between 57.8% and 61.8%, much higher than expected for an endosymbiont (McCutcheon and von Dohlen 2011; López-Madrigal et al. 2011; Husnik and McCutcheon 2016). Another unusual case corresponds to the genomes of several *Hodgkinia cicadicola* strains (McCutcheon et al. 2009; van Leuven et al. 2014; Campbell et al. 2015, 2017), P-endosymbionts of cicadas, which have a wide range of G+C content, from 28.41% to 58.4%. Therefore, it has been proposed that, while gene loss associated with genome reduction is a critical step in endosymbiont genome evolution, mutational pressure favoring A+T is not. These two endosymbiotic species present several unusual features, including extremely reduced and inefficient genomes, and will be presented in more detail in the following sections.

4.2 Tiny Genomes

The final step of the genome minimization process might, in theory, lead to the loss of all genes except those that are essential for keeping the host-bacterial interaction reproducing. Therefore, it was expected that even the most reduced genome must retain those genes involved in the symbiotic relationship, as well as a reduced repertoire of genes necessary to maintain the three essential functions that define a living cell: maintenance, reproduction, and evolution (Luisi et al. 2002). Our group has performed some of the most comprehensive efforts to define the minimal core of essential genes (Gil et al. 2004b; Gil 2014; Gil and Peretó 2015). These studies allowed the identification of genes involved in informational processes that must be

present in any living cell, while the essential genes devoted to the symbiotic association can be deduced by the knowledge of the host's needs for survival and reproduction. However, most extremely reduced genomes (collectively known as tiny genomes; McCutcheon 2010; Moran and Bennett 2014) described have lost part of such essential functions. In most cases, as it will be presented in Sect. 5, genome degradation can proceed over the expected limit because of the implication of a second endosymbiont on the relationship. But there is an intriguing case: *C. ruddii* PV, the single P-endosymbiont of the psyllid *Pachypsylla venusta* (Nakabachi et al. 2006). Although a second bacterial symbiont has not been found in this psyllid, the 160 kb genome of *C. ruddii* PV does not seem to fulfill the conditions to be considered a mutualistic endosymbiont, not even a living organism (Tamames et al. 2007). Most genes for DNA replication, transcription, and translation are completely absent, and gene shortening causes, in some cases, the loss of essential domains and functional residues needed to fulfill these and other vital functions. In addition, it is unable to provide three essential amino acids (i.e., histidine, phenylalanine, and tryptophan) to its phloem-feeding host. Other *Carsonella* strains from different psyllids present also highly reduced genomes (from 158 to 174 kb), but a second P-endosymbiont is also found in these insects (Nakabachi et al. 2013; Sloan and Moran 2012). It has been hypothesized that the role of the missing genes could have been taken over by the co-optation/functional plasticity of remaining endosymbiont and host genes, some of which could have been acquired by the host through HGT from bacterial donors, as it has been suggested in other endosymbiotic systems (McCutcheon 2010; Price and Wilson 2014; Wilson and Duncan 2015). Sloan et al. (2014) identified the presence of ten bacterial genes in the nuclear genome of *P. venusta*, some of which appear to compensate for *C. ruddii* functional losses. Similarly to present organelles, this strain of *C. ruddii* is not able to sustain its own vital functions and seems to take advantage of bacterial genes that have been transferred to the host nuclear DNA. Therefore, it can be viewed as a further step toward the degeneration of the former P-endosymbiont and its transformation in a subcellular new entity between living cells and organelles, the symbionelle (Reyes-Prieto et al. 2014). In fact, now that there are paired insect-/host-endosymbiont genomes available, it has been seen that many host genomes carry bacterial genes acquired through HGT from their endosymbionts or, more frequently, from transient S-symbionts and/or former, already replaced, P-endosymbionts (López-Madrigal and Gil 2017). Many transferred bacterial genes are involved in metabolic collaboration between host and symbiont to synthesize essential molecules and might play a key role in the fine-tuning of mechanisms for the maintenance and regulation of insect-bacteria nutritional symbioses. Additionally, the eventual acquisition of these genes by the nuclear host genome might facilitate the extreme genome reduction found in several clades of long-term P-endosymbionts (Sloan et al. 2014; Luan et al. 2015 and references therein). Most of these genomes correspond to bacteria that have established a consortium with another endosymbiont inside the same host and will be described in the next section.

5 Complementation or Replacement

Eventually, after the establishment of a permanent symbiotic association between a bacterium and an animal host, a second bacterial species can join the association. Although initially this new association can be facultative (as seen in Sect. 3.1), if the second bacterium provides benefits to the organization, with time, it can become essential for host fitness. The involvement of two bacteria in the fitness of an insect host adds one extra element to the evolutionary scenario that explains the reductive evolution of endosymbiont genomes, but there is no need to invoke any supplementary reductive factor. Subsequently, all three components of the association will coevolve, and the evolutionary process of genome shrinkage will now affect both bacteria. New genes will become unnecessary due to redundancy, but which one of the two bacterial genomes loses them will be a matter of chance. Depending on which genome is affected by the loss of genes needed for the synthesis of essential molecules, either both bacteria will become indispensable to keep a healthy consortium (complementation) or one of them can enter an extreme degenerative process, which may end with its extinction (replacement), and the retained bacteria will continue the degenerative process alone (Moya et al. 2009).

5.1 Complementation and the Establishment of Microbial Consortia

Many symbiotic consortia have been reported and sequenced in recent years, thanks to the use of new massive sequencing technologies. In these cases, both bacteria lose part of the gene complement necessary for their host fitness, so that both of them become indispensable and a stable consortium is established, leading to highly reduced genomes (up to only 112 kb in the case of *Nasuia deltocephalinicola*, endosymbiont of deltocephaline leafhoppers; Bennett and Moran 2013). In fact, the tiny genomes of some of the partners in endosymbiotic consortia have even lost genes needed for the maintenance of a living cell.

One of the first described consortia involves *B. aphidicola* BCc and *S. symbiotica* SCc living inside the cedar aphid *Cinara cedri* (Pérez-Brocal et al. 2006; Lamelas et al. 2011). Contrary to other sequenced *B. aphidicola* strains, BCc has partially lost its symbiotic role, as it cannot synthesize tryptophan. In fact, it was found that the pathway to synthesize tryptophan is distributed between both genomes: *B. aphidicola* BCc produces a metabolic intermediate that is then provided to *S. symbiotica* SCc to synthesize the final product (Gosalbes et al. 2008). Therefore, coexistence of both bacteria is needed to keep a healthy consortium due to metabolic complementation, and both of them keep an intracellular obligatory mutualistic association with their host. This is not an isolated case in aphids. In fact, the obligate

consortium between *B. aphidicola* and *S. symbiotica* has been proven in other members of the subfamily Lachninae to which *C. cedri* belongs, triggered by the ancient loss of the pathway for the biosynthesis of riboflavin in an ancestral *B. aphidicola* of this aphid lineage (Manzano-Marín et al. 2017).

Metabolic complementation has been described, at least, in three species of psyllids that harbor a second endosymbiont that compensates for the loss of enzymatic functions in different metabolic pathways due to the ongoing reductive process in the common P-endosymbiont *C. ruddii*. *Ctenarytaina eucalypti* and *Heteropsylla cubana* present a *Sodalis*-like gammaproteobacterium with a genome in an early stage of genome reduction (1.44 and 1.12 Mb, respectively; Sloan and Moran 2012), while the Asian citrus psyllid *Diaphorina citri* carries also the defensive symbiont *P. armatura* (465 kb; Nakabachi et al. 2013).

The establishment of an endosymbiotic bacterium consortium can be in the origin of big evolutionary changes in host lifestyle. This is the case of the consortium found in xylem-feeding Auchenorrhyncha formed by *Sulcia muelleri* and different co-resident Betaproteobacteria, *Nasuia*, *Zinderia*, and *Vidania*, collectively named as *BetaSymb* clade (Bennett and Moran 2013). Genomic and phylogenetic studies suggest that a common ancestor of all three beta-endosymbionts was present in the ancestor of this suborder before the split of the fulgoroid and cicadomorph lineages about 260 million years ago. Their genome analysis revealed that they have complementary sets of biosynthetic capabilities needed to provide to their host the nutrients that are lacking in the xylem sap.

An exceptional consortium is the one established on mealybugs of the subfamily Pseudococcinae, where a nested endosymbiotic system has been described, in which the betaproteobacterium *T. princeps* harbors a gammaproteobacterium. Several endosymbiotic consortia from different pseudococcinae mealybugs have been completely sequenced (McCutcheon and von Dohlen 2011; López-Madrigal et al. 2011, 2013; Husnik and McCutcheon 2016; Szabó et al. 2017). *T. princeps* display some unusual genomic features for a long-term P-endosymbiont, including high GC-content, low coding density, and presence of repeated sequences evolving under concerted evolution. With tiny genomes (137–144 kb), all sequenced strains depend on its inner gamma-endosymbiont to fulfill most metabolic functions (including ATP production) but also informational processes (i.e., replication, transcription, and translation). Also unusual is the presence of an almost complete machinery for DNA recombination in the gamma-endosymbiont, which appears to be involved in the concerted evolution of duplicated loci noticed in *T. princeps* (López-Madrigal et al. 2015). An even more surprising endosymbiotic system was detected in the bougainvillea mealybug *Phenacoccus peruvianus*. As in other members of the subfamily Phenacoccinae, *P. peruvianus* possess a single P-endosymbiont, whose 16S rRNA gene places it as a Betaproteobacteria of the *Tremblaya phenacola* clade. However, nearly half of their protein-coding sequences are taxonomically affiliated to Gammaproteobacteria (Gil et al. 2018). Additionally, the presence of abundant repeated sequences hampered genome assembly, and different possible genome organizations apparently coexist in a single host. It seems that, after a gammaproteobacterium entered the symbiotic consortium in the lineage leading to

this endosymbiont, genomic fusion and subsequent gene shuffling took place, likely involving homologous recombination genes encoded by the gammaproteobacterial donor, leading to an amazing physical and metabolic integration between two co-primary endosymbionts and the formation of an unprecedented chimeric organism. Whether a pseudococcinae-like nested consortium preceded the genomic fusion remains unclear. The phenomenon of genome fusion, followed by subsequent rearrangements mediated by homologous recombination systems, has been previously detected in some flowering plants' mitochondrial genomes (Maréchal and Brisson 2010; Rice et al. 2013).

5.2 Replacement

In some endosymbiotic systems, an ancient endosymbiont can be substituted by a new and healthier symbiont able to fulfill all functions needed for host fitness or even to allow the adaptation to a new niche. The first reported case of replacement, based on molecular evidences, corresponds to grain weevils of the family Dryophthoridae, where a former endosymbiont *Nardonella* was replaced by the ancestor of the *Sitophilus* P-endosymbionts, *S. pierantonius* (Lefèvre et al. 2004), as mentioned in Sect. 3.2. Since then, many more examples have been described, including the intricate examples corresponding to the dual symbiosis of Pseudococcinae and Auchenorrhyncha.

In the Pseudococcinae, a complex evolutionary history involving independent acquisition and replacements of different gamma-endosymbionts by the ancestor of the extant *T. princeps* in different mealybug lineages has been described (López-Madrigal et al. 2014; Husnik and McCutcheon 2016; Szabó et al. 2017). Most of the newly acquired inner endosymbionts belong to the *Sodalis*-like clade, although they do not form a monophyletic group revealing that, even in these highly integrated symbiotic systems, an extensive symbiont turnover is possible. The new symbionts may provide both ecological opportunity and evolutionary reinvigoration (Husnik and McCutcheon 2016).

A similar scenario has been detected in Auchenorrhyncha. In this case, the *BetaSymb* endosymbiont that established a consortium with *Sulcia* in the common ancestor of the suborder has been replaced many times by different bacteria in the different insect lineages, i.e., *Baumannia cicadellinicola* (Gammaproteobacteria) in some leafhopper taxa (subfamily Cicadellinae), *Hodgkinia cicadicola* (Alphaproteobacteria) in cicadas (superfamily Cicadoidea), and *Sodalis*-like bacteria (Gammaproteobacteria) in spittlebugs of the tribe Philaenini (family Cercopidae; Bennett and Moran 2013). The case of *Hodgkinia* is extremely peculiar. When the first genome of this endosymbiont was sequenced from the glassy-winged sharpshooter *Diceroprocta semicincta* (McCutcheon et al. 2009), a reassignment of the UGA codon from a stop codon to tryptophan was found. The same change in the genetic code had been previously described for the small genomes of mycoplasmas (Yamao et al. 1985) and several mitochondrial lineages (Knight et al. 2001), and it

was later detected in the tiny genomes of *Zinderia* and *Nasuia* (McCutcheon and Moran 2010; Bennett and Moran 2013), all of which are AT-rich. *Hodgkinia*, however, has a GC-biased base composition genome (58.4%), ruling out the hypothesis that base composition is on the root of codon reassignment. It has been hypothesized that the reassignment is triggered by the loss of release factor RF2 (*prf*B), which recognizes the UGA stop codon (McCutcheon et al. 2009).

Furthermore, *Hodgkinia* strains endosymbiont of longest-lived cicada of genus *Magicicada* present an unprecedented genomic instability, leading to an impressive level of genome complexity. In each species of *Magicicada*, *Hodgkinia* genome is made of a sum of at least 20 circles of different size with an extremely low gene density (Campbell et al. 2015, 2017). The same gene can be present in different circles, and it is known that some of these circles are not present in all *Hodgkinia* cells within a single host insect. Thus, in the same host, several split *Hodgkinia* lineages with interdependent genotypes coexist. Contrary to previously sequenced *Hodgkinia* genomes, these strains have a low G+C content, similar to other highly reduced endosymbiont genomes. Once more, this pattern of genome instability, together with genome expansion due to the increase of "junk DNA," a diminished coding capacity and the presence of subgenomic molecules, resembles the evolution of mitochondria in some plants.

6 Archaeal Ectosymbionts and Their Reductive Genome Evolution

Only a few archaeal genomes corresponding to advanced symbiosis have been published. They belong to nanoarchaea that live as obligate ectosymbionts of several Crenarchaeota, attached to their hosts' external membrane, in thermophilic aquatic—marine and terrestrial—environments. The genomes of the two cultured representatives, *Nanoarchaeum equitans* (Waters et al. 2003; Podar et al. 2008) and *Nanopusillus acidilobi* (Wurch et al. 2016), have been fully sequenced, while those of *Nanobsidianus stetteri* (Podar et al. 2013) and two additional *Nanobsidianus* sp. (Munson-McGee et al. 2015) have different degrees of completeness, allowing comparative genomic analyses. The study of these associations, including the total or partial sequencing of the genome of both symbiont and host species, shows a highly specialized relationship, although no evident genomic-encoded complementation has been found. Additionally, no physiologically detectable benefits for the host have been detected in any of the cultured symbiotic systems. Therefore, considering that the nanoarchaea can only be cultured together with their hosts, while the hosts can live in isolation, it seems that these symbioses should be classified in the range between commensalism and parasitism (Wurch et al. 2016).

Nanoarchaeota genomes are, similar to those of insect endosymbionts, very reduced compared to other archaea genomes, indicating selective pressures in these thermophilic microorganisms to maintain compact and efficient genomes,

losing nonessential genes. Moreover, not only the small size but other features such as high A+T content, bias in codon usage, lack of repetitive elements, and evolutionary acceleration are common to those observed in bacterial endosymbionts. In both cases, there is a loss of metabolic genes to avoid redundancy between host and symbiont. Nevertheless, it is not clear if the observed similarities between the reduced genomes of symbiotic archaea and bacteria are an indication of the generality of the reductive mechanisms among prokaryotes. In fact, in the case of nanoarchaea, genes involved in DNA repair and recombination mechanisms have been maintained. In addition, their ectocellular location provides access to foreign DNA, diminishing the impact of genetic drift and allowing the occurrence of HGT events. Thus, it is difficult to explain genome reduction based on pseudogenization and subsequent loss, as it occurs in insect endosymbionts. Nicks and Rah-Lee (2017) suggested two different habitat-specific pathways driving genome reduction, isolated bacteriocytes in the case of insect endosymbionts, and thermophilic environments in the case of thermophilic obligate ectosymbionts. It has been observed that there is a negative correlation between growth temperature and genome size in thermophilic bacteria, suggesting that small genomes are adaptive at high temperatures (Sabath et al. 2013). Moreover, in addition to gene loss, genes tend to be shorter in thermophilic than in mesophilic archaea, indicating again selection for genome shortening. They proposed that an adaptive "thermal-symbiosis genome reduction" model—as opposed to the "isolation-symbiosis genome reduction" model that has been presented in previous sections of this chapter for insect endosymbionts—can better explain the reductive syndrome undergone by nanoarchaea. In the new thermophilic model, the reduction starts once two thermophilic archaea become associated and the redundant metabolic genes are lost in the ectosymbionts. Then, there is a continuous pressure toward genome reduction due to the thermophilic lifestyle, leading not only to the decrease in the intergenic space and gene length but also in cell size. Thus, the thermal-symbiosis model relies on metabolic redundancy and adaptation, rather than genetic drift. In any case, not all nanoarchaea described so far live in hyperthermophilic environments. Some mesophilic and halophilic nanoarchaea have been identified. Their genome sequencing would reveal if they are also affected by a reductive syndrome that, in this case, cannot be related with thermal considerations.

7 Concluding Remarks

Symbiosis between prokaryotes and eukaryotes is an expanding field, thanks to the extensive use of high-throughput sequencing technologies. Systems biology approaches are also allowing the exploration of metabolic interdependences among the members of symbiotic consortia. Now that endosymbiont genomes are accumulating, comparative analyses allow making predictions on the evolutionary paths followed by endosymbiotic bacteria in their adaptation to the intracellular environment provided by the host. For this reason, more clearly than ever before, the

association and functional interaction of genomes from different species observed during symbiosis can be viewed as a power to generate genetic variation, acting as a fuel for evolution. The action of forces such as natural selection and/or random drift will be responsible of transforming this variation in evolutionary novelties. As the number of the available genomes increases, we can better understand the evolutionary changes that lead to the establishment of obligate endosymbiotic relationship from free-living bacteria and even how new consortia are being established and replaced in different lineages. Yet, with the exploration of additional symbiotic models, a paramount of different genomic end products, as well as new genomic features, are appearing and open new questions that need to be experimentally solved. These newly discovered features are, in many cases, resembling the outcomes of some organelle features in specific lineages, which might indicate convergent evolution. Now that eukaryotic host genomes are becoming available, and the HGT of bacterial genes to the nuclear genomes are being detected, we will learn a lot more about the molecular mechanisms and evolutionary forces acting on these systems and the cross talk between host and endosymbionts.

Acknowledgments Financial support was provided by grants BFU2015-64322-C2-1-R and SAF2015-65878-R, co-financed by European Regional Development Fund (ERDF) and Ministerio de Economía y Competitividad (Spain) to At.M., A.L. and R.G., and PrometeoII/2014/065 (Conselleria d'Educació, Generalitat Valenciana, Spain) to A.M.

References

Andersson SG, Kurland CG (1998) Reductive evolution of resident genomes. Trends Microbiol 6:263–268

Aras RA, Kang J, Tschumi AI, Harasaki Y, Blaser MJ (2003) Extensive repetitive DNA facilitates prokaryotic genome plasticity. Proc Natl Acad Sci U S A 100:13579–13584

Baumann L, Baumann P (1994) Growth kinetics of the endosymbiont *Buchnera aphidicola* in the aphid schizaphis graminum. Appl Environ Microbiol 60:3440–3443

Baumann P (2005) Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol 59:155–189

Belda E, Moya A, Bentley S, Silva FJ (2010) Mobile genetic element proliferation and gene inactivation impact over the genome structure and metabolic capabilities of *Sodalis glossinidius*, the secondary endosymbiont of tsetse flies. BMC Genomics 11:449

Bennett GM, Moran NA (2013) Small, smaller, smallest: the origins and evolution of ancient dual symbioses in a phloem-feeding insect. Genome Biol Evol 5:1675–1688

Bordenstein SR, Reznikoff WS (2005) Mobile DNA in obligate intracellular bacteria. Nat Rev Microbiol 3:688–699

Bordenstein SR, Wernegreen JJ (2004) Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and recombination rates. Mol Biol Evol 21:1981–1991

Brune A, Dietrich C (2015) The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. Annu Rev Microbiol 69:145–166

Buchner P (1965) Endosymbiosis of animals with plant microorganisms. Interscience Publishers, New York

Burke GR, Moran NA (2011) Massive genomic decay in *Serratia symbiotica*, a recently evolved symbiont of aphids. Genome Biol Evol 3:195–208

Burke G, Fiehn O, Moran N (2010) Effects of facultative symbionts and heat stress on the metabolome of pea aphids. ISME J 4:242–252

Campbell MA, van Leuven JT, Meister RC, Careya KM, Simon C, McCutcheon JP (2015) Genome expansion via lineage splitting and genome reduction in the cicada endosymbiont *Hodgkinia*. Proc Natl Acad Sci U S A 112:10192–10199

Campbell MA, Lukasik P, Simon C, McCutcheon JP (2017) Idiosyncratic genome degradation in a bacterial endosymbiont of periodical cicadas. Curr Biol 27:3568–3575

Casjens S (1998) The diverse and dynamic structure of bacterial genomes. Annu Rev Genet 32:339–377

Clark MA, Baumann L, Munson MA, Baumann P, Campbell BC, Duffus JE, Osborne LS, Moran NA (1992) The eubacterial endosymbionts of whiteflies (Homoptera: Aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. Curr Microbiol 25:119–123

Clark MA, Moran NA, Baumann P (1999) Sequence evolution in bacterial endosymbionts having extreme base compositions. Mol Biol Evol 16:1586–1598

Clayton AL, Oakeson KF, Gutin M, Pontes A, Dunn DM, von Niederhausern AC, Weiss RB, Fisher M, Dale C (2012) A novel human-infection-derived bacterium provides insights into the evolutionary origins of mutualistic insect–bacterial symbioses. PLoS Genet 8:e1002990

Dale C, Maudlin I (1999) *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. Int J Syst Bacteriol 49 Pt 1:267–275

Dale C, Moran NA (2006) Molecular interactions between bacterial symbionts and their hosts. Cell 126:453–465

Dale C, Welburn SC (2001) The endosymbionts of tsetse flies: manipulating host-parasite interactions. Int J Parasitol 31:628–631

Dale C, Plague GR, Wang B, Ochman H, Moran NA (2002) Type III secretion systems and the evolution of mutualistic endosymbiosis. Proc Natl Acad Sci U S A 99:12397–12402

Dale C, Wang B, Moran N, Ochman H (2003) Loss of DNA recombinational repair enzymes in the initial stages of genome degeneration. Mol Biol Evol 20:1188–1194

De Clerck C, Fujiwara A, Joncour P, Leonard S, Felix ML, Francis F, Jijakli MH, Tsuchida T, Massart S (2015) A metagenomic approach from aphid's hemolymph sheds light on the potential roles of co-existing endosymbionts. Microbiome 3:63–76

Degnan PH, Moran NA (2008) Diverse phage-encoded toxins in a protective insect endosymbiont. Appl Environ Microbiol 74:6782–6791

Degnan PH, Yu Y, Sisneros N, Wing RA, Moran NA (2009) *Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. Proc Natl Acad Sci U S A 106:9063–9068

Degnan PH, Leonardo TE, Cass BN, Hurwitz B, Stern D, Gibbs RA, Richards S, Moran NA (2010) Dynamics of genome evolution in facultative symbionts of aphids. Environ Microbiol 12:2060–2069

Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu Rev Entomol 43:17–37

Dubilier N, Bergin C, Lott C (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. Nat Rev Microbiol 6:725–740

Ellegaard KM, Klasson L, Näslund K, Bourtzis K, Andersson SGE (2013) Comparative genomics of *Wolbachia* and the bacterial species concept. PLoS Genet 9:e1003381

Feldhaar H (2011) Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol Entomol 36:533–543

Foray V, Grigorescub AS, Sabrib A, Haubrugec E, Lognayd G, Francisc F, Fauconniere M-L, Hancea T, Thonartb P (2014) Whole-genome sequence of *Serratia symbiotica* strain CWBI-2.3 ^{T}, a free-living symbiont of the black bean aphid *Aphis fabae*. Genome Announc 2:e00767

Gil R (2014) The minimal gene-set machinery. In: Meyers RA (ed) Reviews in cell biology and molecular medicine. Wiley Online Library, pp 1–36

Gil R, Latorre A (2012) Factors behind junk DNA in bacteria. Genes 3:634–650

Gil R, Peretó J (2015) Small genomes and the difficulty to define minimal translation and metabolic machineries. Front Ecol Evol 3:123

Gil R, Sabater-Muñoz B, Latorre A, Silva FJ, Moya A (2002) Extreme genome reduction in *Buchnera* spp.: toward the minimal genome needed for symbiotic life. Proc Natl Acad Sci U S A 99:4454–4458

Gil R, Silva FJ, Zientz E, Delmotte F, Gonzalez-Candelas F, Latorre A, Rausell C, Kamerbeek J, Gadau J, Hölldobler B, van Ham RCHJ, Gross R, Moya A (2003) The genome sequence of *Blochmannia floridanus*: comparative analysis of reduced genomes. Proc Natl Acad Sci U S A 100:9388–9393

Gil R, Latorre A, Moya A (2004a) Bacterial endosymbionts of insects: insights from comparative genomics. Environ Microbiol 6:1109–1122

Gil R, Silva FJ, Pereto J, Moya A (2004b) Determination of the core of a minimal bacterial gene set. Microbiol Mol Biol Rev 68:518–537

Gil R, Vargas C, López-Madrigal S, Santos-García D, Latorre A, Moya A (2018) *Tremblaya phenacola* PPER: an evolutionary beta-gammaproteobacterium collage. ISME J 12:124–135

Goebel W, Gross R (2001) Intracellular survival strategies of mutualistic and parasitic prokaryotes. Trends Microbiol 9:267–273

Gómez-Valero L, Latorre A, Silva FJ (2004) The evolutionary fate of nonfunctional DNA in the bacterial endosymbiont *Buchnera aphidicola*. Mol Biol Evol 21:2172–2181

Gosalbes MJ, Lamelas A, Moya A, Latorre A (2008) The striking case of tryptophan provision in the cedar aphid *Cinara cedri*. J Bacteriol 190:6026–6029

Grote A, Voronin D, Ding T, Twaddle A, Unnasch TR, Lustigman S, Ghedin E (2017) Defining *Brugia malayi* and *Wolbachia* symbiosis by stage-specific dual RNA-seq. PLoS Negl Trop Dis 11:e0005357

Hackstein JHP, van Hoek AHAM, Leunissen JAM, Huynen M (2002) Anaerobic ciliates and their methanogenic endosymbionts. In: Seckbach J (ed) Symbiosis: mechanisms and model systems. Kluwer Academic, Dordrecht, pp 451–464. ISBN 1-4020-0189-4

Hackstein JHP, van Alen TA, Rosenberg J (2006) Methane production by terrestrial arthropods. In: König H, Varma A (eds) Intestinal microorganisms of termites and other invertebrates. Springer, Berlin, pp 155–182

Hansen AK, Vorburger C, Moran NA (2012) Genomic basis of endosymbiont-conferred protection against an insect parasitoid. Genome Res 22:106–114

Henry LM, Peccoud J, Simon JC, Hadfield JD, Maiden MJC, Ferrari J, Godfray HCJ (2013) Horizontally transmitted symbionts and host colonization of ecological niches. Curr Biol 23:1713–1717

Husnik F, McCutcheon JP (2016) Repeated replacement of an intrabacterial symbiont in the tripartite nested mealybug symbiosis. Proc Natl Acad Sci U S A 113:E5416–E5424

Jin Q, Yuan Z, Xu J, Wang Y, Shen Y, Lu W, Wang J, Liu H, Yang J, Yang F, Zhang X, Zhang J, Yang G, Wu H, Qu D, Dong J, Sun L, Xue Y, Zhao A, Gao Y, Zhu J, Kan B, Ding K, Chen S, Cheng H, Yao Z, He B, Chen R, Ma D, Qiang B, Wen Y, Hou Y, Yu J (2002) Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157. Nucleic Acids Res 30:4432–4441

Klasson L, Walker T, Sebaihia M, Sanders MJ, Quail MA, Lord A, Sanders S, Earl J, O'Neill SL, Thomson N, Sinkins SP, Parkhill J (2008) Genome evolution of *Wolbachia* strain wPip from the *Culex pipiens* group. Mol Biol Evol 25:1877–1887

Klasson L, Westberg J, Sapountzis P, Naslund K, Lutnaes Y, Darby AC, Veneti Z, Chen L, Braig HR, Garrett R, Bourtzis K, Andersson SG (2009) The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. Proc Natl Acad Sci U S A 106:5725–5730

Knight RD, Landweber LF, Yarus M (2001) How mitochondria redefine the code. J Mol Evol 53:299–313

Koga R, Tsuchida T, Fukatsu T (2003) Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. Proc Biol Sci 270:2543–2550

Lamelas A, Gosalbes MJ, Manzano-Marín A, Peretó J, Moya A, Latorre A (2011) *Serratia symbiotica* from the aphid *Cinara cedri*: a missing link from facultative to obligate insect endosymbiont. PLoS Genet 7:e1002357

Latorre A, Manzano-Marín A (2017) Dissecting genome reduction and trait loss in insect endosymbionts. Ann NY Acad Sci 1389:52–75

Latorre A, Gil R, Silva FJ, Moya A (2005) Chromosomal stasis versus plasmid plasticity in aphid endosymbiont *Buchnera aphidicola*. Heredity 95:339–347

Lefèvre C, Charles H, Vallier A, Delobel B, Farrell B, Heddi A (2004) Endosymbiont phylogenesis in the Dryophthoridae weevils: evidence for bacterial replacement. Mol Biol Evol 21:965–973

Lindsey ARI, Werren JH, Richards S, Stouthamer R (2016) Comparative genomics of a parthenogenesis-inducing *Wolbachia* symbiont. Genes Genom Genet 6:2113–2123

Lo WS, Huang YY, Kuo CH (2016) Winding paths to simplicity: genome evolution in facultative insect symbionts. FEMS Microbiol Rev 40:855–874

López-Madrigal S, Gil R (2017) Et tu, Brute? Not even intracellular mutualistic symbionts escape horizontal gene transfer. Genes 8:247

López-Madrigal S, Latorre A, Porcar M, Moya A, Gil R (2011) Complete genome sequence of "*Candidatus* Tremblaya princeps" strain PCVAL, an intriguing translational machine below the living-cell status. J Bacteriol 193:5587–5588

López-Madrigal S, Latorre A, Porcar M, Moya A, Gil R (2013) Mealybugs nested endosymbiosis: going into the "matryoshka" system in *Planococcus citri* in depth. BMC Microbiol 13:74

López-Madrigal S, Beltrà A, Resurrección S, Soto A, Latorre A, Moya A, Gil R (2014) Molecular evidence for ongoing complementarity and horizontal gene transfer in endosymbiotic systems of mealybugs. Front Microbiol 5:449

López-Madrigal S, Latorre A, Moya A, Gil R (2015) The link between independent acquisition of intracellular gamma-endosymbionts and concerted evolution in *Tremblaya princeps*. Front Microbiol 6:642

López-Sánchez MJ, Neef A, Peretó J, Patiño-Navarrete R, Pignatelli M, Latorre A, Moya A (2009) Evolutionary convergence and nitrogen metabolism in *Blattabacterium* strain Bge, primary endosymbiont of the cockroach *Blattella germanica*. PLoS Genet 5:e1000721

Luan J-B, Chen W, Hasegawa DK, Simmons AM, Wintermantel WM, Ling K-S, Fei Z, Liu SS, Douglas AE (2015) Metabolic coevolution in the bacterial symbiosis of whiteflies and related plant sap-feeding insects. Genome Biol Evol 7:2635–2647

Luisi PL, Oberholzer T, Lazcano A (2002) The notion of a DNA minimal cell: a general discourse and some guidelines for an experimental approach. Helv Chim Acta 85:1759–1777

Manzano-Marín A, Latorre A (2014) Settling down: the genome of *Serratia symbiotica* from the aphid *Cinara tujafilina* zooms in on the process of accommodation to a cooperative intracellular life. Genome Biol Evol 6:1683–1698

Manzano-Marín A, Latorre A (2016) Snapshots of a shrinking partner: genome reduction in *Serratia symbiotica*. Sci Rep 6:32590

Manzano-Marín A, Szabó G, Simon JC, Horn M, Latorre A (2017) Happens in the best of subfamilies: establishment and repeated replacements of co-obligate secondary endosymbionts within Lachninae aphids. Environ Microbiol 19:393–408

Maréchal A, Brisson N (2010) Recombination and the maintenance of plant organelle genome stability. New Phytol 186:299–317

Margulis L (1993) Symbiosis in cell evolution. Microbial communities in the archaean and proterozoic eons. W. H. Freeman, New York

Martinez AJ, Ritter SG, Doremus MR, Russell JA, Oliver KM (2014a) Aphid-encoded variability in susceptibility to a parasitoid. BMC Evol Biol 14:127

Martinez AJ, Weldon SR, Oliver KM (2014b) Effects of parasitism on aphid nutritional and protective symbioses. Mol Ecol 23:1594–1607

McCutcheon JP (2010) The bacterial essence of tiny symbiont genomes. Curr Opin Microbiol 13:73–78

McCutcheon JP, Moran NA (2010) Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. Genome Biol Evol 2:708–718
McCutcheon JP, von Dohlen CD (2011) An interdependent metabolic patchwork in the nested symbiosis of mealybugs. Curr Biol 21:1366–1372
McCutcheon JP, McDonald BR, Moran NA (2009) Origin of an alternative genetic code in the extremely small and GC-rich genome of a bacterial symbiont. PLoS Genet 5:e1000565
McGraw EA, O'Neill SL (2004) *Wolbachia pipientis*: intracellular infection and pathogenesis in *Drosophila*. Curr Opin Microbiol 7:67–70
Metcalf JA, Jo M, Bordenstein SR, Jaenike J, Bordenstein SR (2014) Recent genome reduction of *Wolbachia* in *Drosophila recens* targets phage WO and narrows candidates for reproductive parasitism. Peer J 2:e529
Montllor CB, Maxmen A, Purcell AH (2002) Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecol Entomol 27:189–195
Moran NA, Bennett GM (2014) The tiniest tiny genomes. Annu Rev Microbiol 68:195–215
Moran NA, Mira A (2001) The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola*. Genome Biol 2:research0054
Moran NA, Plague GR (2004) Genomic changes following host restriction in bacteria. Curr Opin Genet Dev 14:627–633
Moran NA, Telang A (1998) The evolution of bacteriocyte-associated endosymbionts in insects. Bioscience 48:295–304
Moran NA, Russell JA, Koga R, Fukatsu T (2005) Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. Appl Environ Microbiol 71:3302–3310
Moya A, Latorre A, Sabater-Munoz B, Silva FJ (2002) Comparative molecular evolution of primary (*Buchnera*) and secondary symbionts of aphids based on two protein-coding genes. J Mol Evol 55:127–137
Moya A, Pereto J, Gil R, Latorre A (2008) Learning how to live together: genomic insights into prokaryote-animal symbioses. Nat Rev Genet 9:218–229
Moya A, Gil R, Latorre A (2009) The evolutionary history of symbiotic associations among bacteria and their animal hosts: a model. Clin Microbiol Infect 15:11–13
Munson MA, Baumann P, Moran NA (1992) Phylogenetic relationships of the endosymbionts of mealybugs (Homoptera: Pseudococcidae) based on 16S rDNA sequences. Mol Phylogenet Evol 1:26–30
Munson-McGee JH, Field EK, Bateson M, Rooney C, Stepanauskas R, Young J (2015) Nanoarchaeota, their Sulfolobales host, and Nanoarchaeota virus distribution across Yellowstone National Park Hot Springs. Appl Environ Microbiol 81:7860–7868
Nakabachi A, Yamashita A, Toh H, Ishikawa H, Dunbar HE, Moran NA, Hattori M (2006) The 160-kilobase genome of the bacterial endosymbiont *Carsonella*. Science 314:267
Nakabachi A, Ueoka R, Oshima K, Teta R, Mangoni A, Gurgui M, Oldham NJ, van Echten-Deckert G, Okamura K, Yamamoto K, Inoue H, Ohkuma M, Hongoh Y, Miyagishima S, Hattori M, Piel J, Fukatsu T (2013) Defensive bacteriome symbiont with a drastically reduced genome. Curr Biol 23:1478–1484
Nicks T, Rahn-Lee L (2017) Inside out: archaeal ectosymbionts suggest a second model of reduced-genome evolution. Front Microbiol 8:384
Nikoh N, Hosokawa T, Moriyama M, Oshima K, Hattori M, Fukatsu T (2014) Evolutionary origin of insect-*Wolbachia* nutritional mutualism. Proc Natl Acad Sci U S A 111:10257–10262
Oakeson KF, Gil R, Clayton AL, Dunn DM, von Niederhausern AC, Hamil C, Aoyagi A, Duval B, Baca A, Silva FJ, Vallier A, Jackson DG, Latorre A, Weiss RB, Heddi A, Moya A, Dale C (2014) Genome degeneration and adaptation in a nascent stage of symbiosis. Genome Biol Evol 6:76–93
Ochman H, Moran NA (2001) Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. Science 292:1096–1099

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. Science 325:992–994

Oliver KM, Smith AH, Russell JA (2014) Defensive symbiosis in the real world – advancing ecological studies of heritable, protective bacteria in aphids and beyond. Funct Ecol 28:341–355

Parks DH, Rinke C, Chuvochina M, Chaumeil P-A, Woodcroft BJ, Evans PN, Hugenholtz P, Tyson GW (2017) Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. Nat Microbiol 2:1533–1542

Pérez-Brocal V, Gil R, Ramos S, Lamelas A, Postigo M, Michelena JM, Silva FJ, Moya A, Latorre A (2006) A small microbial genome: the end of a long symbiotic relationship? Science 314:312–313

Podar M, Anderson I, Makarova KS, Elkins JG, Ivanova N, Wall MA, Lykidis A, Mavromatis K, Sun H, Hudson ME, Chen W, Deciu C, Hutchison D, Eads JR, Anderson A, Fernandes F, Szeto E, Lapidus A, Kyrpides NC, Saier MH Jr, Richardson PM, Rachel R, Huber H, Eisen JA, Koonin EV, Keller M, Stetter KO (2008) A genomic analysis of the archaeal system *Ignicoccus hospitalis-Nanoarchaeum equitans*. Genome Biol 9:R158

Podar M, Makarova KS, Graham DE, Wolf YI, Koonin EV, Reysenbach AL (2013) Insights into archaeal evolution and symbiosis from the genomes of a nanoarchaeon and its inferred crenarchaeal host from Obsidian Pool, Yellowstone National Park. Biol Direct 8:9

Price DRG, Wilson ACC (2014) A substrate ambiguous enzyme facilitates genome reduction in an intracellular symbiont. BMC Biol 12:110

Rao Q, Wang S, Su YL, Bing XL, Liu SS, Wang XW (2012) Draft genome sequence of "*Candidatus* Hamiltonella defensa," an endosymbiont of the whitefly *Bemisia tabaci*. J Bacteriol 194:3558

Reyes-Prieto M, Latorre A, Moya A (2014) Scanty microbes, the "symbionelle" concept. Environ Microbiol 16:335–338

Rice DW, Alverson AJ, Richardson AO, Young GJ, Sanchez-Puerta MV, Munzinger J, Barry K, Boore JL, Zhang Y, de Pamphilis CW, Knox EB, Palmer JD (2013) Horizontal transfer of entire genomes via mitochondrial fusion in the angiosperm *Amborella*. Science 342:1468–1473

Rispe C, Delmotte F, van Ham RCHJ, Moya A (2004) Mutational and selective pressures on codon and amino acid usage in *Buchnera*, endosymbiotic bacteria of aphids. Genome Res 14:44–53

Russell JA, Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc Biol Sci 273:603–610

Russell JA, Latorre A, Sabater-Munoz B, Moya A, Moran NA (2003) Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. Mol Ecol 12:1061–1075

Sabater-Munoz B, van Ham RC, Moya A, Silva FJ, Latorre A (2004) Evolution of the leucine gene cluster in *Buchnera aphidicola*: insights from chromosomal versions of the cluster. J Bacteriol 186:2646–2654

Sabath N, Ferrada E, Barve A, Wagner A (2013) Growth temperature and genome size in bacteria are negatively correlated, suggesting genomic streamlining during thermal adaptation. Genome Biol Evol 5:966–977

Scarborough CL, Ferrari J, Godfray HC (2005) Aphid protected from pathogen by endosymbiont. Science 310:1781

Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. Nature 407:81–86

Silva FJ, Latorre A, Moya A (2001) Genome size reduction through multiple events of gene disintegration in *Buchnera* APS. Trends Genet 17:615–618

Silva FJ, Latorre A, Moya A (2003) Why are the genomes of endosymbiotic bacteria so stable? Trends Genet 19:176–180

Sloan DB, Moran NA (2012) Genome reduction and co-evolution between the primary and secondary bacterial symbionts of psyllids. Mol Biol Evol 29:3781–3792

Sloan DB, Nakabachi A, Richards S, Qu J, Murali SC, Gibbs RA, Moran NA (2014) Parallel histories of horizontal gene transfer facilitated extreme reduction of endosymbiont genomes in sap-feeding insects. Mol Biol Evol 31:857–871

Su Q, Zhou X, Zhang Y (2013) Symbiont-mediated functions in insect hosts. Commun Integr Biol 6:e23804

Sudakaran S, Kost C, Kaltenpoth M (2017) Symbiont acquisition and replacement as a source of ecological innovation. Trends Microbiol 25:375–390

Sutton E, Harris S, Parkhill J, Sinkins S (2014) Comparative genome analysis of *Wolbachia* strain wAu. BMC Genomics 15:928

Szabó G, Schulz F, Toenshoff ER, Volland J-M, Finkel OM, Belkin S, Horn M (2017) Convergent patterns in the evolution of mealybug symbioses involving different intrabacterial symbionts. ISME J 11:715–726

Tamames J, Gil R, Latorre A, Pereto J, Silva FJ, Moya A (2007) The frontier between cell and organelle: genome analysis of Candidatus *Carsonella ruddii*. BMC Evol Biol 7:181

Taylor M, Mediannikov O, Raoult D, Greub G (2012) Endosymbiotic bacteria associated with nematodes, ticks and amoebae. FEMS Immunol Med Microbiol 64:21–31

Toft C, Andersson SGE (2010) Evolutionary microbial genomics: insights into bacterial host adaptation. Nat Rev Genet 11:465–475

Toh H, Weiss BL, Perkin SA, Yamashita A, Oshima K, Hattori M, Aksoy S (2006) Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. Genome Res 16:149–156

Touchon M, Rocha EP (2007) Causes of insertion sequences abundance in prokaryotic genomes. Mol Biol Evol 24:969–981

van der Wilk F, Dullemans AM, Verbeek M, van den Heuvel JFJ (1999) Isolation and characterization of APSE-1, a bacteriophage infecting the secondary endosymbiont of *Acyrthosiphon pisum*. Virology 262:104–113

van Hoek AH, van Alen TA, Sprakel VS, Leunissen JA, Brigge T, Vogels GD, Hackstein JH (2000) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol Biol Evol 17:251–258

van Leuven JT, Meister RC, Simon C, McCutcheon JP (2014) Sympatric speciation in a bacterial endosymbiont results in two genomes with the functionality of one. Cell 158:1270–1280

Waters E, Hohn MJ, Ahel I, Graham DE, Adams MD, Barnstead M, Beeson KY, Bibbs L, Bolanos R, Keller M, Kretz K, Lin X, Mathur E, Ni J, Podar M, Richardson T, Sutton GG, Simon M, Soll D, Stetter KO, Short JM, Noordewier M (2003) The genome of *Nanoarchaeum equitans*: insights into early archaeal evolution and derived parasitism. Proc Natl Acad Sci U S A 100:12984–12988

Wei J, Goldberg MB, Burland V, Venkatesan MM, Deng W, Fournier G, Mayhew GF, Plunkett G III, Rose DJ, Darling A, Mau B, Perna NT, Payne SM, Runyen-Janecky LJ, Zhou S, Schwartz DC, Blattner FR (2003) Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. Infect Immun 71:2775–2786

Wernegreen JJ (2015) Endosymbiont evolution: predictions from theory and surprises from genomes. Ann NY Acad Sci 1360:16–35

Wilson AC, Duncan RP (2015) Signatures of host/symbiont genome coevolution in insect nutritional endosymbioses. Proc Natl Acad Sci U S A 112:10255–10261

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci U S A 87:4576–4579

Wrede C, Dreier A, Kokoschka S, Hoppert M (2012) Archaea in symbioses. Archaea 2012:596846

Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N, Wiegand C, Madupu R, Beanan MJ, Brinkac LM, Daugherty SC, Durkin AS, Kolonay JF, Nelson WC, Mohamoud Y, Lee P, Berry K, Young MB, Utterback T, Weidman J, Nierman WC, Paulsen IT, Nelson KE, Tettelin H, O'Neill SL, Eisen JA (2004) Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biol 2:E69

Wurch L, Giannone RJ, Belisle BS, Swift C, Utturkar S, Hettich RL, Reysenbach AL, Podar M (2016) Genomics-informed isolation and characterization of a symbiotic Nanoarchaeota system from a terrestrial geothermal environment. Nat Commun 7:12115

Yamao F, Muto A, Kawauchi Y, Iwami M, Iwagami S, Azumi Y, Osawa S (1985) UGA is read as tryptophan in Mycoplasma capricolum. Proc Natl Acad Sci U S A 82:2306–2230

Zug R, Hammerstein P (2015) Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. Biol Rev 90:89–111

Index

A
Acetaldehyde, 159
Acetate, 156, 157
Acetogens, 108
Acetylating acetaldehyde dehydrogenase, 160
Acetyl-CoA, 159, 161, 170
Acetyl phosphate, 161
Acidaminobacter hydrogenoformans, 173
Acidaminococcus fermentans, 172
Acquired chaperonopathy, 107
Activated carbon, 175
ADP/ATP carriers, 206
Alanine, 172
Alcohols, 154
Amination, 169
Amino acids, 154, 171
Amino acid synthesis, 169
Aminobacterium colombiense, 173
Aminomonas paucivorans, 173
Ammonia, 168, 171
Anaerobic chytridiomycete fungi, 196
Anaerobic ciliate, 13–21
Anaerobic oxidation of methane, 177
Anaerobic protozoa, 154
Anaeromusa acidaminophila, 172
ANME, 177
Anoxic, 37
Anthraquinone disulfonate, 175
Archaea, 107, 153
Archaea diversity, 113
Aromatic compounds, 154, 169
Arthropods, 132–147
Asgard archaea, 114
ATP, 154
ATPases, 159
Autoimmune condition, 107

B
Bacillus
B. jeotgali, 171
B. stamsii, 170
B. subtilis, 171
Bacillus PeC11, 171
Bacteria, 107, 153
Bacterial endosymbionts of insects, 230
Bacteroidales, 65–67, 69, 71
Bcd/EtfAB complex, 164
B_{12}-dependent, 169
Beer breweries, 171
Benzoate, 169, 176
Bifurcating [FeFe]-hydrogenase, 161
Biochar, 175
Biogas, 181
Biopolymers, 156
Bioreactors, 156
Blastocystis sp., 196
Blue F_{420} autofluorescence, 132
Brachonella sp., 15
Branched-chain fatty acids, 168
Bromoethanesulfonic acid, 176
Butyrate, 157, 161, 163, 174

C
Caenomorpha, 2
Caenomorpha-like, 15, 16
Canonical aerobic mitochondria, 194

© Springer Nature Switzerland AG 2018

J. H. P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, https://doi.org/10.1007/978-3-319-98836-8

Chaperoning system, 106
Chaperonopathies, 106
Chlorobium limicola, 174
CH_4 production, 110
Chronic inflammatory pathologies, 105
Chytridiomycete fungi, 200
Ciliates, 37
Cloacimonas acidaminovorans, 174, 176
Cloacimonetes, 174
Clostridia, 164
Clostridium, 172
  C. coolhaasii, 173
  C. proteoclasticus, 173
  C. tetanomorphum, 173
CO_2, 154
Cockroaches, 132
CO-dehydrogenase, 168
Coenzyme A, 159
Coenzyme M, 176, 179
Colobid monkeys, 130
Commensalism, 246
Communities, 153
Comparative genomics, 234
Complementation, 243
Comproportionating [FeFe]-hydrogenase, 161, 167
Comproportionating hydrogenase, 163
Consortium, 243
Crotonate, 163, 176
Cultivability, 175
Culture chip, 177
Culturing approaches, 175
Cyclidium, 5
Cyclohexadiene, 169
Cysteine, 163
Cytochromes, 178, 179

D
Dairy, 171
Deamination, 172
Desulfovibrio, 65
  D. vulgaris, 161
Desulfuromonas acetoxidans, 174
Diet, 108
Diplodinium dentatum, 28–30
Disaccharides, 170
Doubling times, 168

E
Electron flow, 157
Embden-Meyerhof-Parnas pathway, 170
Embden-Meyerhof pathway, 161
Endomicrobia, 67, 69
Endosymbiont replacement, 13, 19–21
Endosymbiosis, 224
Endosymbiotic, 122
Energy efficiency, 154
Entodinium furca, 28–30
Environmental factors, 107
Epifluorescence microscopy, 132
Ethanol, 159
Eubacterium acidaminophilum, 163, 174
Eudiplodinium maggii, 28
Eukaryotic genes, 114
Eutrophic lakes, 154

F
$FADH_2$, 167
Fatty acids, 154
F_{20} autofluorescence, 14
Fe(III), 154
FeFe hydrogenase, 163, 214
Ferredoxin, 160, 164, 167, 172
Firmicutes, 165
Flagellate, 55–74
Flavoenzymes, 163
Foregut and hindgut microbiota, 130
Formate, 163, 174
Formate dehydrogenases, 160, 163, 164, 166, 168
Fossil fuels, 181
Fructose, 170
Fumarate respiration, 167

G
Gastrointestinal tracts, 107, 122, 156
Gelria glutamica, 173
Genome, 114
  rearrangements, 238
  remodeling, 114
  size reduction, 232
Geobacter
  G. metallireducens, 169
  G. sulfurreducens, 175
Giant panda, 130
Gibbs' free energy, 157
Global methane emissions, 95
Glucose, 170
Glutamate, 172, 173
Glycine reductase, 171
Glycolysis, 170
Guilds, 154, 181

Gut compartmentation, 94
Gut microbiota, 108

H
H_2-consuming microorganisms, 110
High-throughput screening, 177
High-throughput sequencing, 230
Homoacetogenic, 168
Horizontal gene transfer, 232
Hsp60, 105
Human gut, 107
Human microbiology, 106
Human microbiota, 107
Humic acid, 175
H_2-utilizing microbes, 108
Hydrogen, 37, 174
  turnover rates, 84
Hydrogenases, 160, 164, 168
Hydrogen emission, 85
Hydrogenosomes, 2, 13, 14, 16, 19, 21, 37, 122, 195, 207
Hydrogenotrophic processes
  spatial separation, 90
Hydrogen partial pressure, 157
Hydrogen pressure, 157
Hydrogen-producing mitochondria, 195
Hydrogen transfer
  between compartments, 94, 95
Hydroxyglutarate pathway, 172

I
Immune cross-reactivity, 105
Immune response, 105
Immune system, 105
Insects, 156
Insertion sequences (IS), 235
Interactions, 115
Intercompartment hydrogen transfer, 146
Interspecies electron transfer, 113
Interspecies formate transfer, 166
Interspecies hydrogen transfer, 92
Interspecies interactions, 175
Intestinal ecosystem, 112
Intestinal inflammation, 110
Intracellular mutualistic symbioses, 224
Iron-only hydrogenase, 173
Iron oxides, 154, 175
Isobutyrate, 168
Isoleucine, 172
Isotricha intestinalis, 28–30, 33
Isovalerate, 168

L
Lacrymaria, 4
Lactate, 170
Landfills, 156
Leucine, 172
Life domains, 114
Lignocellulose, 62, 71, 73
Lokiarchaeum, 114
Low G+C Gram-positive bacteria, 164
Lung, 113

M
Malate, 167
Marine sediments, 154
Mastigella, 4
Medical applications, 115
Menaquinol, 164
Menaquinone, 160, 164
Metabolite transfer, 180
Metadinium medium, 28–30
Metagenome, 176, 179
Metaproteome, 179
Metatranscriptome, 179
Methane, 45, 107
  in the breath, 123
  emission rates, 83, 95
Methane-producing subjects, 110
Methanobacteriales, 13, 16, 21, 108, 156
Methanobacteriales
  termite gut, 85
Methanobrevibacter, 57, 60–62
  M. boviskoreani, 112
  M. oralis, 110
  M. ruminantium, 28, 29, 33
  M. smithii, 29, 33, 107
Methanocorpusculum labreanum, 112
Methanogen, 13–16, 18–21
Methanogenesis, 109
  aceticlastic, 83
  history, 83
  hydrogenotrophic, 83
Methanogenic Archaea, 104
Methanogenic endosymbiont, 13–21
Methanogenic symbionts, 229
Methanogens, 37, 104, 122
  association with anaerobic protists, 91
  benefit for host, 89
  coexistence with homoacetogens, 89–91
  community structure, 87–88
  diversity, 85–87
  higher termites, 87–88
  lower termites, 87

Methanogens (*cont.*)
populations size, 88–89
surface colonization, 94
termite guts, 88–89
Methanomassiliicoccales, 108, 156
termites, 87
Methanomassiliicoccus
M. intestinalis, 109
M. luminyensis, 108
Methanomethylophilus alvus, 109
Methanomicrobiales, 13, 16, 21, 156
Methanomicrobiales
termites, 87
Methanomicrobium mobile, 28, 30
Methanosaeta, 156
Methanosarcina, 156
M. barkeri, 27
Methanosarcinales
cockroach, 85
termites, 87
Methanosphaera stadtmanae, 29, 33, 107
Methanospirillum hungatei, 170
Methanothermobacter thermautotrophicus, 175
3-Methylaspartate, 172
β-Methylaspartate, 172
Methylene-tetrahydromethanopterin, 179
Methylmalonyl-CoA, 167, 173
Methylomirabilis oxyfera, 179
Metopus, 2, 4, 5, 7
M. palaeformis, 18
Metopus sp., 15, 18
Microbiota, 104, 122
Micro-Petri dishes, 177
Millipedes, 132
Mitochondrial complex I, 199, 211
Mitochondrial proteins, 209
Mitochondrion related organelles (MROs), 194
Mitosomes, 195
Mixed acid fermentation, 202
Mn(IV), 154
Mobile elements, 236–239
Modular structure, 181
Molecular chaperones, 105
Monozygotic *vs.* dizygotic twins, 131
Mosaicism, 114
Mutualism, 232
Myelostoma, 4

N
NADH-ferredoxin oxidoreductase, 173
Nanowires, 174, 175, 178
Neocallimastix, 202
Neovalerate, 168
Nests, 181
Nitrate, 154
Nitrate-reducing bacteria, 169
Nitrate reduction, 179
Non-acetylating acetaldehyde dehydrogenase, 160
Nose, 113
Nyctotherus ovalis, 14, 196

O
Obligate intracellular mutualistic symbiosis, 231
Omics, 177
Ophryoscolex caudatum, 28–30, 33
Oral mucosa, 114
Outside-directed hydrogen gradient, 144
β-Oxidation, 163

P
Pangenome, 108
Parasitism, 232
PCR-based methods, 113
Pelobacter
P. acetylenicus, 159
P. carbinolicus, 159
Pelomyxa, 4
Pelotomaculum
P. schinkii, 165
P. thermopropionicum, 175
3-Pentenoate, 176
Peptides, 171
Peptostreptococcus asaccharolyticus, 172
Periplaneta americana, 14
PFL, 204, 205
Phenol, 169
Phylogenetic tree, 113
Pili, 174
Piromyces, 202
Plagiopyla, 4, 5, 7
Plant-based diet, 126
Polyplastron multivesiculatum, 26–30, 33
Polysaccharide, 170
Primary (P-) endosymbiont, 229
Primary fermenting bacteria, 154
Pro-inflammatory cytokines, 109
Prokaryotes, 114
Propionate, 157, 174
Proteins, 171
Proteolytic, 171
Proton gradient, 164

Psalteriomonas, 4
Pseudosetae, 140
Pyruvate, 167, 170, 171
  dehydrogenase, 209
Pyruvate:ferredoxin oxidoreductase (PFO), 199

Q
Quinones, 164

R
Reductive acetogenesis
  spirochetes, 90
Regulation, 181
Repetitive DNA, 238
Replacements, 122, 243
Reverse electron transport, 154, 159, 163, 167
Rice paddies, 156
Rnf complex, 164, 167
Rumen, 25–33, 157
  microbiota, 112
  protozoa, 26–28
Ruminal methanogenesis, 26
Ruminants, 156, 171
Ruminococcus albus, 170

S
Saprodinium, 4
Scarab beetles, 132
Secondary fermenting bacteria, 155
Secondary (S-) symbiont, 229
Sediments, 154
Selenium, 171
Sequence exchanges, 114
Serine ammonia lyase, 172
Setae (bristles), 146
Skin, 113
Slaughterhouses, 171
Smithella propionica, 165
Sodium ions, 164
Sonderia, 4
S organism, 159
Spirochete, 66
SSU rDNA, 14, 18
Standard free energy changes, 158
Stickland reaction, 171
Substrate-level phosphorylation, 154, 161, 168
Succinate, 167, 170
Succinyl-CoA, 173
Sugars, 170–171
Sulfate, 154
Sulfur, 174
Sustainable, 181
Symbiosis, 38, 224
Syntrophic communities, 122
Syntrophism, 157
Syntrophobacter, 165
  S. fumaroxidans, 165
Syntrophomonas wolfei, 164, 174
Syntrophospora
  S. bryantii, 176
  S. fumaroxidans, 176
Syntrophus, 169
  S. aciditrophicus, 164, 169
  S. buswellii, 169
  S. gentianae, 169

T
Termites, 55–74, 132
  gut flagellates, 92
  guts, 157
Tetrahydrofolate, 168
Thermanaerovibrio acidaminovorans, 173
Thermophilic, 168
Thermotoga maritima, 161, 167
Transport paths, 180
Trichomonadina, 198–200
Trimethylamine, 111
Trimethylaminuria, 111
Trimyema, 7
Tungsten, 166

U
UASB bioreactor, 174

V
Vagina, 114
Valine, 172
Vertebrates, 122–131
Virulence factors, 235

W
Waste, 181
Woesearchaeota, 113
Wolinella succinogenes, 167
Wood-Ljungdahl pathway, 168