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Intestinal Microorganisms of Termites and Other Invertebrates

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Intestinal Microorganisms of Termites and Other Invertebrates

With 86 Figures, 7 in Color

Springer

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Foreword

Insects and other invertebrates ranging from nematodes to earthworms, isopods and millipeds usually host many microorganisms in both their intestinal tracts and tissues. Outside of the gut these organisms are usually found in the hemolymph or are restricted to specialized bacteriocytes (mycetocytes) and mycetoms. Many microorganisms have been characterized morphologically (for example, compilation by Edward A. Steinhaus 1967) and their transfer to other developmental stages has been studied thoroughly (Paul Buchner 1953). In the gut this intra- and extracellular microbial diversity is often reflected by culturable forms but due to molecular techniques more and more species can be identified taxonomically. It also became evident that arthropod digestive tracts – with their outpocketings such as foregut caeca, or malpighian tubules and their unidirectional bulk flow of ingested food – represent primary habitats for microorganisms including bacteria, fungi, yeasts and protozoa. These organisms may occur intraepithelially, adhere to the gut wall or are free floating in the lumen of the digestive tract. Number and composition of microorganisms can vary considerably, depending on longitudinal gut position, temperature, pH, anaerobic or aerobic conditions and various natural or laboratory diets.

These digestive tracts are characterized by an excess supply of more or less degradable food, controlled water activities and often rather stable pH and temperature conditions. On the other hand guts may represent unstable environments because food composition may change in time and cuticle linings of fore- and hindguts are lost with each molt. This reestablishing of microbiota is especially interesting in holometabolous insects where larvae and adults are found in different habitats.

The interaction between microorganisms and their host and the host guts respectively is so far unknown. They may pass through the gut with food, stay there for a longer period of time (as compared with the transportation of food) or represent true symbionts. In addition intracellular symbionts from outside the gut must be transferred through the digestive tract at some point in order to settle within the body cavity. The "symbiosis"-concept can be seen from a broad or narrow angle. Today symbiosis has strong connotations of strict mutualism and completely excludes parasitism (as symbiosis was defined in 1891 by Antom de Bary (1831–1888)). Paul Buchner (1886–

1978), who dedicated most of his life to the study of endosymbiosis, defined it as cohabitation between two partners of different species, where one is taken up in the body of the other, usually more highly organised, partner.

The biological role of these microorganisms may vary considerably: gut microorganisms of exotic hosts such as larvae of the oil fly *Helaeomyia petrolei* might be interesting due to their pronounced solvent tolerance or may be a future source of industrially useful solvent-tolerant enzymes. In addition these microbiota may detoxify allelochemicals or may be responsible for the biosynthesis of essential compounds such as vitamins, sterols, or nitrogen-containing constituents. Generally these microorganisms may be the source of novel metabolic capabilities such as defense substances and antibiotics or may fixate atmospheric nitrogen. It has been known for many years that enzymes in the gut which originate from ingested fungal tissue or gut microbes may mediate cellulolysis. A large number of insects and invertebrates which usually utilize internal or external microbes have evolved beneficial associations with these microorganisms.

The editors Helmut König and Ajit Varma deserve credit for compiling articles on these interesting phenomena. With *Intestinal Microorganisms of Termites and Other Invertebrates* they – together with the authors – present a fascinating and stimulating collection of articles focusing on intestinal microorganisms of soil invertebrates and especially of termites, which have been studied extensively. Finally, this book in the series *Soil Biology* is highly innovative in covering both molecular and micromethods.

I am sure that the recently founded working group of the German Society for General and Applied Entomology (DGaaE) entitled "Microbiology and Arthropods" together with this promising and comprehensive book will truly inspire this fascinating area of interdisciplinary research and I thank both editors and Springer for realizing this project.

Bayreuth, July 2005

Konrad Dettner,
President
of the German Society
for General and Applied
Entomology (DGaaE)

Preface

The soil is an important natural habitat for a large number of taxonomic groups such as bacteria, archaea, protists, fungi, algae as well as plants and animals, which have adapted to the environmental conditions in soil. It represents not only a steady state, but rather a constantly changing environment, which is influenced by natural environmental factors and human activities.

Soil biology describes the systematics, complex activities and interactions of the soil inhabitants. A special discipline, the soil microbiology, characterizes the microbial community in the substratum. It plays a major role in the degradation and recycling of organic material. Microbes are involved in the first step of the soil food web, soil fertility, degradation of xenobiotics as well as in plant pathology. In particular, the degradation of lignocellulose, the most important renewable natural material, is mainly a domain of microorganisms. It is therefore imperative to study ecological and agricultural aspects of soil microbes. In the past, attention was mainly directed towards the free-living or particle-bound microorganisms, and the role of intestinal microbes occurring in gut systems of soil animals has been neglected.

The primary decomposers of organic soil litter are the lumbricids, the diplopods, the isopods, and dipteran larvae as well as termites in subtropical and tropical regions. Only a small percent of the soil microbes have been screened axenically. The same is true for the intestinal microbes of soil invertebrates. However, with the aid of molecular methods (e.g. total 16S rDNA sequence analysis), it was possible to get a rough estimate of the total population in a given environment. In the last few years, the intestinal microbial communities (microbiota) of some soil invertebrates such as collembola, earthworms, nematodes, isopods, millipedes and termites have been studied in more detail. The most intensively studied group is that of the termites because of their interesting microflora and global role in the decomposition of organic material.

The intestinal microbiota has only been investigated from a few soil invertebrates. For the first time, the authors highlight this aspect and give an overview on the intestinal soil community in order to underline their role in the soil food web. The goal of this book is to bridge a gap and to add a new

mosaic stone to our knowledge of soil biology. The book is divided into three sections. The first describes the intestinal microbiota of the investigated soil invertebrates, while the second deals with termites, the best investigated soil invertebrates with respect to their gut microbiota. The third part presents novel techniques introduced in ecological microbiology, which have been successfully applied to studies of the intestinal microbiota. The book covers several novel facets and presents insights into the intestinal microbiota of soil invertebrates. The text is mainly directed towards graduate students and professional scientists with a general interest in the soil or intestinal microbiota or in ecological microbiology in general.

We are grateful to all authors for providing their expertises and contributions, making this special edition possible. We also express our thanks to Springer-Verlag for accepting this unconventional and less investigated, but ecologically important, subject for publication in this prestigious book. We appreciate the efforts of Dr. Dieter Czeschlik and Dr. Jutta Lindenborn from Springer Life Sciences Editorial, who managed this special book.

Mainz and Noida, April 2005 *Helmut König and Ajit Varma*

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Part I
Soil Invertebrates

1 Biology of Soil Invertebrates

G. Eisenbeis

1.1 Introduction

The soil is an important interface in nature which is influenced by different environmental spheres: the hydrosphere, the lithosphere and the atmosphere. The soil compartment has developed over eons, and passed through many stages of development and maturation. Soil is thus often described as an archive of earth history. All the spheres determine soil characteristics, but the influence of man and modern technology has increased significantly since the last ice age. It is therefore reasonable to add a fourth sphere influencing soil characteristics: the anthropo- or technosphere. This is a multifactorial web of human influences, the importance of which has increased greatly since the beginning of industrialization. Soil degradation and desertification are catchwords describing fundamental changes in natural soil functions which have led to an irreversible loss of high-quality soils worldwide. The key variables for the development of soils, the rocky subground, the climate and the vegetation have become more and more biased by modern soil tillage practices and inputs of contaminants and pollutants. Human impacts to soils have contributed much to the modification of the soil as a component of global change.

In this area of conflict and change we find a realm of organisms which have adapted to the specific conditions of life in the soil: the soil organisms. These are primarily microorganisms and invertebrates which play major roles in the soil community known as the edaphon and which are known to have specific adaptations to soil conditions. A wide range of life forms from euedaphic or endogeic species living within the soil profile up to epedaphic or epigeic species living mainly on the soil surface makes up the soil fauna. Between these main groups we can distinguish some intermediate life forms which are called hemiedaphic. Some authors also divide the edaphon into two major groups, the aquatic and the air-breathing organisms.

The French ecophysiologist Guy Vannier has characterized the soil habitat as a porosphere. It is composed of a hierarchy of micropores (fine,

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medium and large), of visually detectable macropores, and of megapore systems (i.e. larger cavities like corridors, tubes, crevices, clefts, and galleries). In this porosphere, Vannier differentiates three basic conditions depending on the water status of the soil: an 'aquatic system', an 'edaphic system' and an 'aerial system' as shown in Fig. 1.1. The best living conditions for soil organisms prevail in the 'edaphic system' with sufficiently high humidity within the pore spaces and capillary bound, residual water around the soil particles. The aquatic system, with its water filled pores, is unfavourable for all members of the air-breathing soil fauna, but all life forms which depend on free water or which are able to swim in water films will find good conditions there. Such organisms are the naked protozoans, the rotifers, and the nematodes often considered to be inhabitants of water films. According to Dunger (1983), most soil protozoans are the same species as occur in ponds and lakes, and only a small contingent can be classified as being endemic soil species. Many soil dwelling species differ from their free-living relatives only in their reduced body size. The aerial soil system, with its lower intrapore humidity is lethal for any soil organism with a highly permeable integument, and which lose body water rapidly. Generally, the atmosphere filling the soil pores differs from the free atmosphere above ground by its elevated humidity, relatively low temperature and increased concentration of CO_2.

The community of soil organisms forms the soil food web (Fig. 1.2), consisting of two subwebs (Beck 1993), which are composed of different trophic levels reflecting multiple relations and interactions between the soil fauna and micro-organisms. The main function of the food web is the processing of a variety of complex polymers, e.g. proteins and carbohydrates, to oligomers and monomers and finally to CO_2, H_2O and minerals.

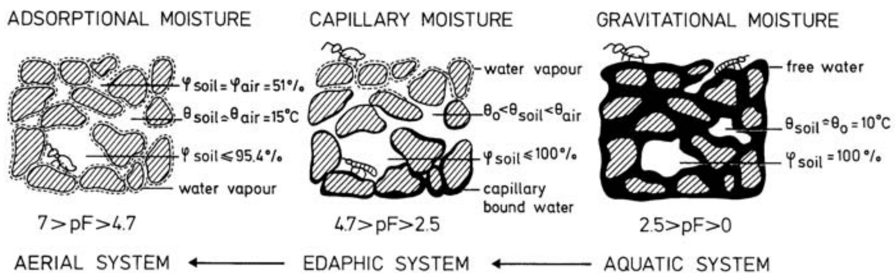

Fig. 1.1. The soil pore system (called the porosphere by Vannier 1983). It is classified into three main stages with relation to the soil water regime. *pF* Logarithm of the height in centimetres of a column of water corresponding to the water suction power of the soil, φ relative humidity, θ temperature (e.g. θ_{air} dry temperature, θ_0 wet temperature due to the cooling effect of evaporation of a water-saturated soil)

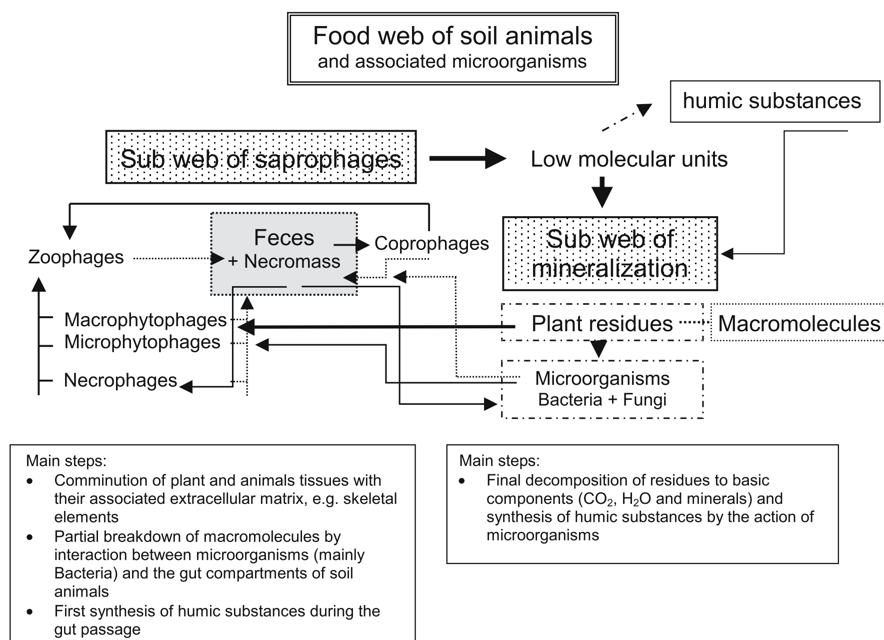

Fig. 1.2. Components of a dual soil food web (food web of saprophages and food web of mineralizers) derived from Beck (1983) and different authors

Only the indigestible fraction of carbon (20% in carbohydrates and 75% in lignins, tannins, aromatic amino acids, fats, and waxes) enters into the formation of humic matter.

For pragmatic reasons the classification of the soil fauna is based on the body length of the animals resulting in the following groups: the microfauna (< 0.2 mm), the mesofauna (0.2–4 mm), the macrofauna (4–80 mm), and the megafauna (> 80 mm). Whereas most soil invertebrates can be ranked among the smaller groups (micro- to macrofauna), the larger soil animals (> 80 mm), e.g. insectivores, rodents and hamsters, are mostly vertebrates. It must be noted that there is no correlation between the size of a soil animal and its trophic position in the food web. To carry out such an assessment, the life style and feeding habits must be known. A new method for detecting lines of food processing along food chains by primary and secondary consumers or by predators, has recently been introduced: the analysis of stable isotopes (Ehleringer et al. 1986; Eggers and Jones 2000; Scheu and Falca 2000). The ratio of a heavy to a light isotope of a given element (e.g. $^{14}N/^{15}N$ or C^{13}/C^{14}) within a sample is compared with a known standard sample, giving a difference (δ) in ‰. As a rule, the heavier isotope component is enriched as the organic material pass from a lower to a higher trophic level. Thus, if plant biomass (e.g. litter material) is transferred

into the body mass of a plant (litter) feeder, then the δ-value for $^{14}N/^{15}N$ commonly is about 3.4 ‰ (Eggers and Jones 2000).

According to Anderson (2000) the smaller soil animals contribute disproportionately to the metabolic activity of the edaphon, and there are many interactions between them and the microflora, e.g. by grazing. Conversely the larger soil animals are more important in the modification of the physical properties of soils, e.g. by geophagy, comminution of dead plant biomass and by promoting both vertical and horizontal translocation by the process of bioturbation or biomixis. Therefore the larger soil animals are often called 'soil engineers' because they have a strong input to such overall soil parameters as infiltration capacity and texture.

The main function of the soil food web (Fig. 1.2) is the processing and recycling of the dead biomass (litter) which is added annually to the soil. There are two main pathways of input. I: Above ground (exogenous) by (1) all sorts of vegetation products (e.g. leaves, remnants of sprouts, twigs and logs), (2) remnants of dead animals, large and small, (3) remnants of micro-organisms, e.g. the dead fruiting bodies of some fungi, and (4) animal excrement, e.g. the faeces of herbivores (phytophages) and carnivores (zoophages), and II: within the soil (endogenous) by (1) dead microbial biomass, e.g. bacteria and fungal hyphae, (2) dead roots, (3) root exudates, (4) animal carrion, and (5) the excrement of edaphic and hemiedaphic animals. The process of decomposition in a wider sense includes a cascade of steps resulting in both mechanical and chemical breakdown (comminution and reduction/mineralization) of complex structures and molecules (polymers) to basic units which are added again (recycled) to the pool of usable materials (CO_2, H_2O and minerals). The basic role of the animals in this context is the initial comminution of the litter by their feeding, but some of them are also involved in regulating the density of microorganisms, e.g. by grazing bacterial colonies and mycelia, and by cleaning up dead micro-organisms. It is known that parts of the microbial populations in a soil survive in a dormant stage; such dormant stages constitute an enormous pool of species. They are able to survive hard times and may be reactivated by better conditions and/or new substrates. Often they need a strong change within their milieu. Such hot spots of microbial biodiversity are considered to occur, e.g., during the gut passage of the biomass or within carrion and faecal deposits.

The basic ecological function of saprophytophagous soil animals is comminution of the dead plant biomass followed by incubation during gut passage. There under specific and constant conditions in succeeding gut compartments, the fine grained food particles are attacked by enzymes at high pH. It is assumed that most of the soil animals cannot digest the litter material using their own enzymes but need the help of the gut microflora. Therefore the incubation of food material in the gut takes place

in a close contact with micro-organisms which provide some of the enzymes for digestion, especially those for the breakdown of polymers like cellulose, lignin and their derivatives. But micro-organisms are themselves a further source of food for soil animals, both those which have colonized the food material before entering the gut and those which live permanently as endosymbionts within the gut.

In the case of non-specific soil feeders which ingest a mixture of organic material and mineral particles, both the gut milieu and the close contact of components insure that faeces leave the body enriched with organo-mineral (clay-humus-) complexes. These are regarded as valuable constituents of a healthy soil texture which minimizes soil erosion and improves the porosity and stability of the soil matrix.

Soil animals which are involved in the first steps of comminution of litter are classified as primary decomposers or saprophytophages. The key members of this group are the lumbricids, the diplopods, the isopods and the larger dipteran larvae. They produce large numbers of faecal pellets with a high proportion of undecomposed material. Such deposits in the soil which are more or less colonized by gut microorganisms are submitted to further colonization by free living micro-organisms. This stage, the material is ready to enter the next stages of decomposition. These are mainly the task of those soil animals which are classified as secondary decomposers. Here the key groups are mites, collembolans and enchytraeids, which feed on the pellet material. Isopods are also known to feed on their own faeces. This type of nutrition is called coprophagy. This transformation of litter into a fine grained stage increases the relative surface area of the material and improves the colonization and penetration of micro-organisms. Soil litter in a recalcitrant stage has to pass through the gut of soil animals several times, which increases the chance that different functional types of micro-organisms can colonize it. During these gut passages, first steps of humification are performed by micro-organisms which can synthesize cyclic (aromatic) compounds.

Other soil animals are less dependent on their own gut micro-organisms as the main food source. Their strategy of foraging is to graze bacterial colonies and fungal hyphae directly from soil substrates. This grazing of micro-organisms is thought to stimulate the metabolic activity and the renewal of microbial populations. Therefore it must be noted that all the activities of both soil animals and the micro-flora increase the overall activity of a soil resulting in high decomposition rates and rapid turnover of matter.

The spectrum of soil the fauna includes many groups of soil dwellers from Protozoa up to the Vertebrata. Vertebrates like rodents and hamsters are well-known for digging in soil and storing large amounts of food in subsoil cavities. This improves both soil aeration and soil mixing, but generally

they are regarded to be only locally active. Soil invertebrates, on the other hand, are more active over the whole soil area and their main task is litter processing and the promotion of basic cycling of elements like carbon and nitrogen. In the following the focus is turned to selected groups of soil invertebrates which are known to be very active in the soil or to share the soil life with interesting biological features.

1.2 The Microfauna

The most important microfaunal groups (< 0.2 mm) are the Protozoa and the smaller Nematoda. Other semiaquatic soil dwellers include the Rotatoria and the Tardigrada, which have evolved sophisticated mechanisms of anabiosis, which enable them to resist extreme conditions of drought and cold. They have commonly been neglected in soil studies because their role in the soil food web is considered to be less important.

1.2.1 Protozoa

Size: 5 to 500 µm in diameter; species number: $> 15,000$, about 10% are restricted to soil habitats; abundance: $\approx$ 10,000 per gram soil; key literature: Lousier and Bamforth 1990; Foissner 1994

Since the 1970s, the soil protozoans have received greater attention from soil biologists in the context of large-scale ecosystem studies (e.g. the Solling project in Germany) even though their study requires the experience of a well-versed expert (Weigmann 1998). For a good introduction into their systematics, biology and ecology with a fantastic visualization of their cell bodies, see Foissner (1994) and Darbyshire (1994).

Most relevant are the rhizopods including both testate (Testacea) and naked forms (Amoebina), the flagellates, the ciliates and parasitic sporozoans (Sporozoa). Foissner (1994) gives some good tips for the extraction, the fixation and the staining of protists. Further chapters treat their use as bioindicators and discuss a special phenomenon known from ciliates, and which has been termed the stasis phenomenon. It refers to extreme competition among species and the production of inhibitors by micro-organisms which result in a low population density of ciliates in a normal moistened soil, termed by Foissner ciliatostasis. Indeed, many ciliates are encysted in a dormant stage, especially in deeper soil layers. However, after a soil sample is dried and then rewetted, there is an explosion of the ciliate population as many of the cysts are activated. Foissner (1994) recommended this

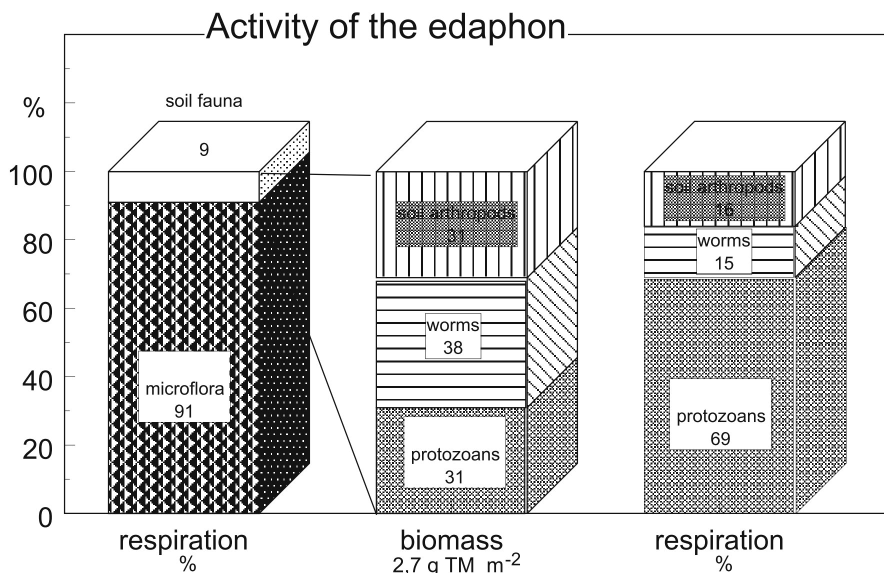

Fig. 1.3. The respiratory activity of: soil fauna, soil microorganisms, and of the major soil fauna groups according to Meyer et al. (1989)

procedure to survey the species pool of a soil sample. Analysis of 14 ecosystem studies revealed that the protozoa of a forest soil, regardless of their apparently low level of activity, make up nearly one-third of the biomass of the soil fauna (Fig. 1.3; Meyer et al. 1989). This high proportion doubles once again if their contribution to soil respiration is considered. More than two-thirds of soil respiration by animals is contributed by protozoans, and the share of worms and arthropods is overshadowed.

Protozoans feed mainly on bacteria, and thus accumulate in the rhizosphere. Foster and Dormaar (1991) used transmission electron microscopy to show the ingestion of bacteria by pseudopods of naked amoebae which were grazing close to the root surface called rhizoplane. Amoebae have been estimated to ingest about 10,000 bacteria per day. A further spectacular interaction between ciliates and fungal hyphae was described by Foissner (1994). The protoplasm of fungal hyphae is sucked out via a channel created by the ciliate, which penetrates the chitinous cell wall of a hypha. The same behaviour has been observed in amoebae of the family Vampyrellidae, which use enzymes to penetrate the cell wall and to suck out the nutritious protoplasm.

In summary, some important functions are attributed to protozoans in the soil food web, primarily within the inner zone of the rhizosphere, including:

- Regulation of bacterial and fungal populations by grazing.
- Mobilization of nutrients, especially of N (because the C/N ratio of their body is more extended (10:1) than that of bacteria (3:1 to 10:1). In turn, if they share a high biomass then nutrients are also immobilized by themselves.
- Protozoans do exert suppressive effects by downregulation of pathogenic microbial populations.
- They serve as an important food source for other soil organisms, e.g. nematodes.

1.2.2 Nematoda – Roundworms, Eelworms

Size: typically 50 µm in diameter/2 mm in length; species number: described 20,000, estimated half a million; abundance: $1–100 \times 10^6\ m^{-2}$, 10–1,000 g^{-1} soil; body mass: 1.6 µg (0.03 to 15 µg); key literature: Freckman 1982; Freckman and Baldwin 1990; Lee 2002

Nematode worms are some of the most common and widely distributed invertebrates on earth. They inhabit the soils in huge numbers and are often associated with plants and other animals or attack them as parasites. They are worms with a simple body construction consisting mainly of a monolayer muscle tube allowing them to move like a snake. Despite their simple body construction they exert key functions in the food web due to their high density and short life cycles. They take part in litter decomposition and colonize the rhizosphere or decaying animals in large numbers. Consequently, they are involved in nutrient cycling (e.g. release of NH_4^+), which stimulates microbial populations and plant growth. They contribute to the dispersal of microbes, and they attack and penetrate the root tissues. According to structural and behavioural differences they show a high diversity in feeding types (Yeates et al. 1993). These are classified mainly according to the external micro-morphological structures lining the mouth region, but the internal construction of the pharyngeal region is also very important distinguishing the type of nutrition. Ideally, the feeding-group-specific structures should reflect the type of food (e.g. spines, papillae, stylets). The following types of feeding have so far been described:

- Bacterial feeders
- Fungal feeders
- Plant feeders
- Predatory nematodes

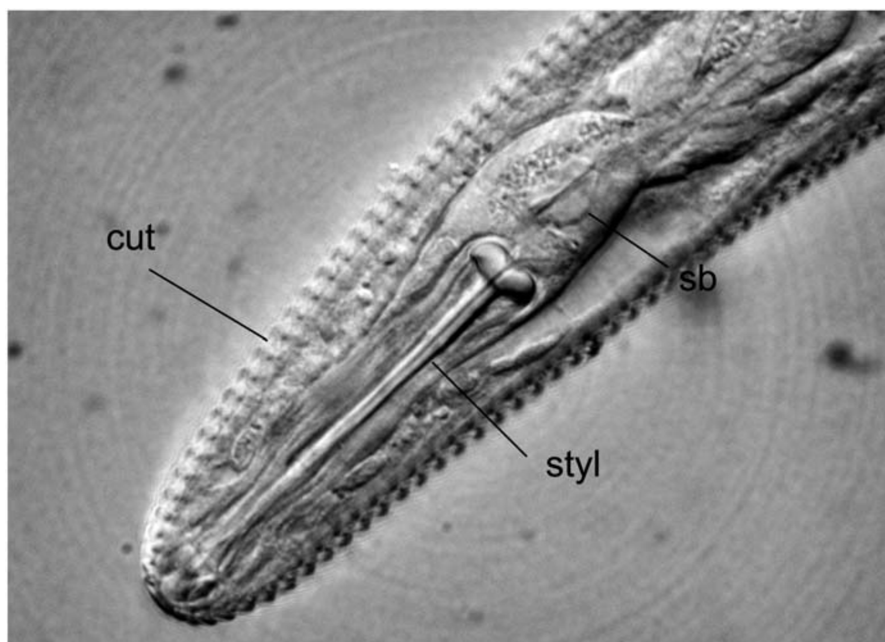

Fig. 1.4. Anterior body of a nematode with ribbed cuticle (*cut*), pharyngeal stylet (*styl*) and sucking bulb (*sb*)

- Omnivorous nematodes
- Plant associated nematodes
- Entomopathogenic nematodes

Those nematodes which penetrate plant tissue are provided with a stylet which is retracted with its base into the pharyngeal pocket (Fig. 1.4). To attack a target, it is protruded to open a channel in the food source. Predators which feed on nematodes include not only other predatory nematode species but also fungi which are specialized to catch nematodes, as well as predatory groups of mites, collembolans, protozoans, insect larvae and some bacteria. The fungi trap the nematodes in adhesive loops of their hyphae, which are exposed as sticky traps.

Since nematodes are indicators of soil changes in soil environments, due to their high density and high reproductive rate, they are used as bioindicators. For this purpose, the Maturity Index (MI) was introduced by Bongers (1990). It is primarily applied to the free-living forms, but later on it was modified to serve as a Plant Parasite Index (PPI) to characterize better the specific communities of parasitic species. The MI attempts to reflect the strategy of nutrition and reproduction of nematodes. It is

based on a so-called 'colonizer-persister-scale' (c-p). The colonizers are ranked as nematode species which are able to increase their population rapidly and to disperse, if soil conditions and food supply improve. They are able to colonize new substrates very quickly (r-strategists) and they tolerate greater disturbances of soil environments, such as mechanical and chemical stresses, e.g. tillage, eutrophication and anoxybiosis. On the other hand, there are the so-called persisters, which are less able to adapt when subjected to changing conditions. Their reproductive rate is low and their ability to disperse is reduced. They need a relative stable environment and their species are classified as K-strategists. The ranking along the c-p-scale starts with the value of 1 for the colonizers and ends with the value of 5 for the most specialized persisters. The intermediate values are assigned to intermediate forms. The classification is mostly based on the taxonomic level of families and genera and the MI emerges as the weighted mean of the specific c-p values according to the following formula:

$$MI = \sum_{i=1}^{n} v(i) * f(i) \tag{1.1}$$

where $v(i)$ =c-p value of the taxon i, $f(i)$ =relative frequency of the taxon i.

The calculation includes either the free-living or the parasitic taxa, although sometimes a mix from both groups has been used. In the literature one can find some modifications of the MI, e.g. Yeates (1994), Bongers and Ferris (1999) and Neher (2001). There is abundant literature available about the MI which can be downloaded via http://www.dpw.wageningen-ur.nl/nema.

1.3 The Mesofauna

The mesofauna of a soil includes invertebrates with a body diameter under 2 mm and a length under 4 mm. Important mesofaunal soil dwellers are the smaller Enchytraeidae, the Pseudoscorpionida, the Acari, the Symphyla, the Pauropoda, the Collembola, the Protura, the Diplura as well as some smaller groups of pterygote insects, e.g. Psocoptera, Hymenoptera (not included in this chapter, just the smaller Araneida). Collectively, the arthropods within this group are often called micro-arthropods. The populations of the mesofauna exceed those of the macrofauna by several orders of magnitude. However, if a soil environment becomes extreme, e.g. in the course of increasing soil acidification, mesofaunal groups become more and more dominant, resulting in a so-called 'mesofauna soil'.

1.3.1 Pseudoscorpionida – False Scorpions, Book Scorpions

Size: 1 to 5 mm, min 0.89, max 12 mm; species number: 2,400; abundance: 50 m^{-2}; body mass: 1–5 mg; key literature: Weygoldt 1966, 1969; Muchmore 1990a; Moritz 1993a

The small pseudoscorpions of the order *Pseudoscorpionida* are tiny arachnids which are rarely longer than 8 mm. They are clearly different from true scorpions because they do not terminate in a long segmented tail tipped with a robust venomous stinger. Biologically they are very interesting because they skilfully hunt other micro-arthropods and nematodes. Eyeless, or equipped only with one or two pairs of eyes (ocelli), they move very quickly forwards, backwards or sideways, and changing their direction very quickly. The most conspicuous appendages of their body are the long chelate pedipalps which are equipped with superlong sensory hairs (trichobothria) (Eisenbeis and Wichard 1987; Fig. 1.5). These are used to detect the location of prey, which is attacked and rapidly caught. The prey is grasped with the help of the long chelate pedipalps and subdued by venom from glands which open at the sharp tip of one or both fingers. The prey is then turned over to the chelicerae and crushed to small pieces which enter the mouth.

In a German beech forest soil, Beck (1983) found a correlation between the numbers of the pseudoscorpion *Neobisium* and the density of

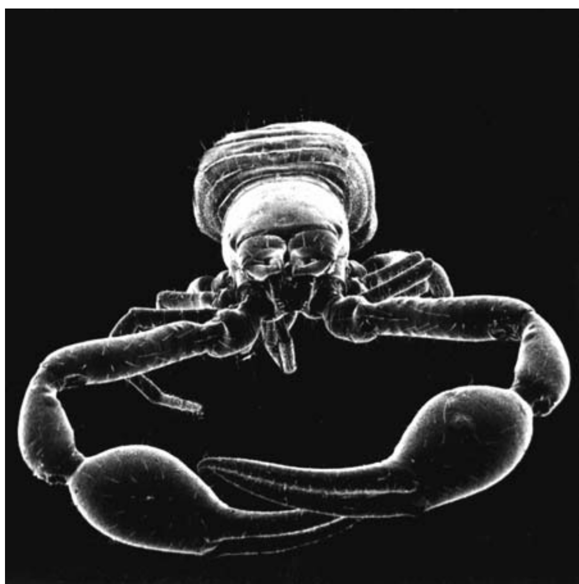

Fig. 1.5. SEM photograph of a pseudoscorpion, in frontal view. Magnification: 22×

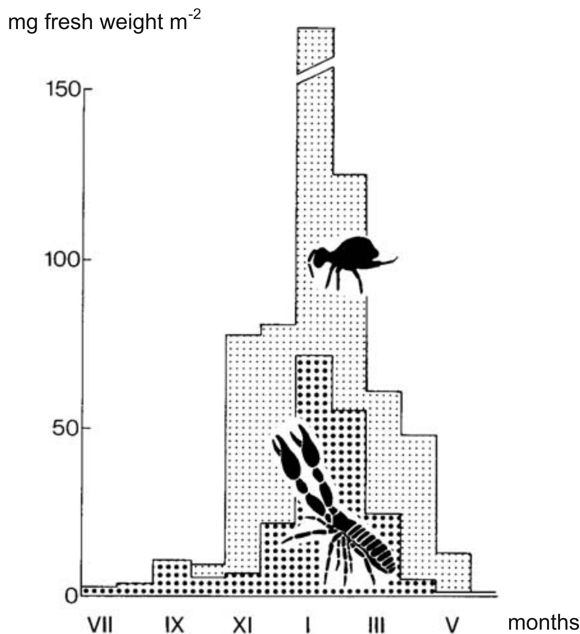

Fig. 1.6. Pseudoscorpions as predators of Collembola. (According to Beck 1983)

Collembola suggesting a trophic relation between them (Fig. 1.6). However, it must be noted that the importance of pseudoscorpions in the soil food web should not be overestimated since their population density is low.

1.3.2 Acari – Mites

Size: 200 nm to 2 mm; species number: > 40,000; abundance: 80,000 to > 1 million m^{-2}; life body mass: 8–600 μg; key literature: Wallwork 1983; Kethley 1990; Krantz and Ainscough 1990; Norton 1990; Philips 1990, Moritz 1993b; Karg 1994, Karg and Freier 1995; Alberti and Coons 1999; Maraun and Scheu 2000

The Acari (mites) are an extremely diverse group of arachnids which have successfully adapted to a wide range of habitats. They are divided into two main lines: the Parasitiformes and the Acariformes, which are distributed through the soil and the different layers of vegetation. Well known groups include the Gamasina and Uropodina (predatory mites, turtle mites; Fig. 1.7), and the Oribatida (cryptostigmate mites, moss mites; Fig. 1.8), which are very important in soil environments. Karg (1994) assessed the predatory mites as valuable regulators of the natural environment and

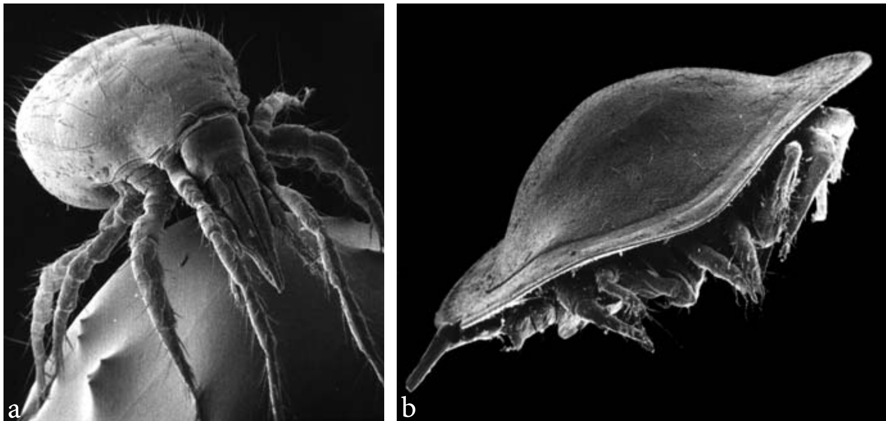

Fig. 1.7. SEM photographs of **a** a gamasid mite in frontal view with extended chelicerae; **b** a turtle mite with helm-shaped soma in frontal view. Magnification: 80 × , 120 ×

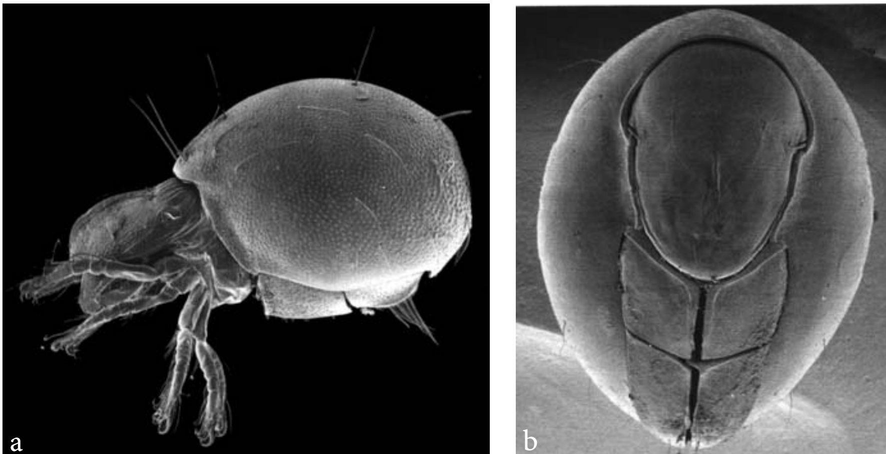

Fig. 1.8. SEM photographs of an oribatid mite – Phthiracaridae with **a** exposed prosoma; **b** closed prosoma, flexed like a jack-knife. Magnification: 80 × , 100 ×

Karg and Freier (1995) consider them to be bioindicators for the status of ecosystems.

Like other true soil animals, the predatory mites (Gamasina) lack eyes and commonly avoid light. To catch their prey they use a mechanism like a catapult. The chelicerae, normally held in a retracted position in a pocket of the gnathosome, are protruded very quickly and the prey, e.g. a nematode or another micro-arthropod, is very quickly grasped with the pincers of the chelicerae. Their main prey are nematodes and collembolans; the many species of mites have adapted to attack the different prey types living in

the different soil horizons. Karg (1994) has observed how the predatory mites use their pincer-like chelicerae very skilfully and are able to eat a large number of nematodes within minutes. The chelicerae of some of these mites have been transformed into stylets. They use the extraintestinal mode of digestion and suck in the liquid food with the help of a pharyngeal pump. Other predatory mites feed on particulate food in the form of minute fragments of the tissue of their prey. The uropodid mites are also predatory, particularly on nematodes. They are often used to monitor the progress of compost formation. The distribution of predatory mites in soil is believed to be more uniform than that of the oribatids, which are often found to have a tendency to aggregate. This corresponds more to the behaviour of predators which try to increase the spectrum of their prey by being more mobile. Both the biomass and metabolism of predatory mites have been found to be comparatively high in a forest. Consequently they play a major role in the energy flux of the soil fauna.

The oribatids, frequently termed moss mites (in German, *Hornmilben*), live in dense clusters in the decomposing litter within the upper soil layers. More than 6500 species are known worldwide, but in central Europe soil habitats are populated only by about 20 to 80 species. The more increased is their density with up to 5×10^5 counts per m^2 (Table 1.1). Key factors which determine the occurrence of the oribatids are the thickness of the organic horizons, the quality of the litter and the pH values of the soil. Both acidified soils and a recalcitrant litter material improve the dominance of this mesofaunal group. In contrast to the Gamasina, none of the oribatid species is parasitic. They feed on a variety of material, mainly of plant debris and carrion, but also on fungi, bacteria, lichens and living plants. Their metabolic rates are low reflecting more the consumption of low quality food, slow development and reduced fecundity. Of course this may be compensated for by the high population density of thousands of the mini-shredders. Therefore it is obvious that they must play a key role in the food web of mesofauna-dominated soils.

Table 1.1. Abundance and species numbers of oribatid mites in selected habitats of central Europe (adapted from different authors)

Habitats	Density m^{-2} ($\times 10^3$)	Species
Fields	20 ± 10	20
Meadows	25 ± 15	30
Fen	45 ± 20	50
Deciduous forest	56 ± 20	70
Mixed forest	123 ± 50	80
Spruce/fir forest	212 ± 100	40
Pine forest	425 ± 200	60

According to Rusek (1975), their faecal pellets are different from those of Collembola. Because oribatids do not take in mineral particles with their food the micro-pellets produced are round and with a smooth surface. In contrast, the pellets of Collembola have sharper edges in profile, indicating the presence of mineral particles.

Oribatids are eyeless, but have a pair of large, conspicuous sensilla (trichobothria), which are inserted into deep cups in the prosomal carapace. They should be able to detect vibrations of the air and to receive sensory inputs from their nearby surroundings. Their body is commonly covered with a thick cuticle giving a bizarre, armoured appearance. It has been found that the integument can be used as a store for minerals, e.g. calcium. Species of the family Phtiracaridae are able to shield their body against drought by rolling into a wall. All the free exposed appendages are then retracted and completely covered by the hood-like prosomal shield (Fig. 1.8).

1.3.3 Symphyla

Size: 2 to 9 mm; species number: 170; abundance: $< 100\,m^{-2}$ (forest soils), 1000 to 20,000 m^{-2} (cultivated soils); body mass: 0.5–1 mg; key literature: Edwards 1990; Dunger 1993b

Symphylans are elongated and unpigmented true soil animals divided into head and trunk, consisting of 14 segments and 12 pairs of legs (Fig. 1.9). Together with the pauropods and diplopods they form the Progoneata which have genital openings just behind the head region. The symphylan body terminates with two conical appendages called 'cerci' which contain spinning glands. Dorsally, the body is covered with overlapping smooth tergites. The lateral and ventral integument is covered with a felt of tiny microhairs above which project sensory hairs or scales.

Though the animals are eyeless they have thousands of sensory hairs. It is likely that most of them are mechanoreceptors, but some are thought to function as chemoreceptors. Most spectacular is the Tömösváry or post-antennal organ, which has been thought to be hygroreceptor. It is found near the antennal base in an open cavity of the head capsule (Eisenbeis and Wichard 1987). Symphylans require high humidity. Under completely dry conditions *Scutigerella immaculata* loses about 80% of its body water per hour (Eisenbeis, unpublished). To compensate for water loss by transpiration, the animals are provided on their ventral side with nine ventral, eversible pairs of coxal vesicles which are commonly used in arthropods to absorb water and ions from moist substrates.

Very remarkable in symphylans is their special mode of sperm transfer from an attached spermatophore to an egg. The female first opens the sper-

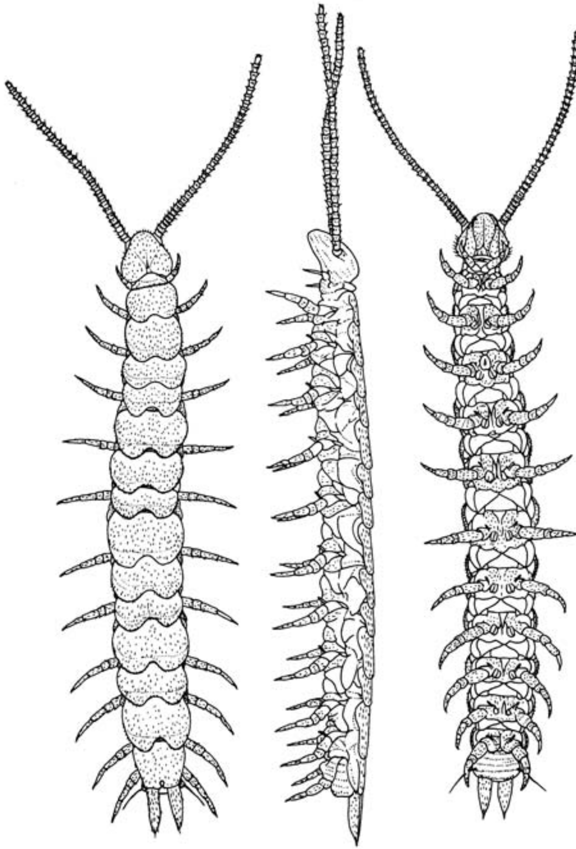

Fig. 1.9. The symphylan *Scutigerella immaculata* in dorsal, lateral and ventral view. (Modified according to Dunger 1993b)

matophore with its mouthparts. Then some of the sperm are carried into the buccal cavity and stored there. After attachment of an egg to a substrate insemination is accomplished by touching the mouth to the egg surface. The animals live between layers of moist litter but also move into deeper soil layers along crevices and root channels down to the subsoil (30–60 cm). Their food is includes algae, bacteria, and fungi, small fragments of litter and dead soil animals. Some species which prefer living roots are known to be pests in croplands if the population density is high. However, generally the impact of this group, with respect to the turnover of materials, especially in forest soils, is thought to be small. If the animals are excavated they immediately try to escape at speeds as high as $40\,\mathrm{mm\,s^{-1}}$. They are able to move extremely fast with undulating, swinging movements, but their normal speed is reported to be 5 to $20\,\mathrm{mm\,s^{-1}}$.

1.3.4 Pauropoda

Size: 0.5 to 0.7 mm, max 1.9 mm; species number: 500; abundance: 5 to $10\,l^{-1}$, max 200 to $300\,l^{-1}$; body mass: < 0.2 mg; key literature: Scheller 1990; Dunger 1993c

The eyeless pauropods are much softer and smaller than the symphylans; they are thus true euedaphic microforms. The trunk consists of 9–11 leg-bearing segments some of them partly fused as diplo-segments, showing a close relationship with diplopods. Because they are so tiny they are extremely susceptible to dessication. Consequently, pauropods are restricted to moist soils and woodland litter. Like the symphylans they have a postantennal organ, called the pseudoculus, which has been modified to form a sensory plate completely integrated into the surface of the head capsule (Eisenbeis and Wichard 1987). As in symphylans, it is thought to function as a hygroreceptor. Externally, the body of pauropods bears very long trichobothria some of them longer than half the body. Thus the animals have long distance mechanoreceptors giving the best to provide information about their surroundings. Further conspicuous structures are the branched antennae with globelike sensilla and secondary annulated segments. The animals are unpigmented and avoid light.

They are thought to feed on finely dispersed food particles and to suck out fungal hyphae using the peristaltic movements of their gut as a pump. Some animals have been observed to feed on dead bodies like other necrophages.

Pauropod density is low in soil indicating a negligible contribution to nutrient cycling. However, the possibility that they contribute to the propagation of micro-organisms cannot be excluded. Despite their size, pauropods can show escape responses and make short-runs of $4\,mm\,s^{-1}$. Because they are more susceptible to agrochemicals than other micro-arthropods (Edwards 1974), they could potentially serve as bioindicators for chemical loads in soil.

1.3.5 Collembola – Springtails

Size: 200 μm to 10 mm; species number: > 6500; abundance: 10,000 to $> 100{,}000\,m^{-2}$; body mass: 50 μg to 10 mg; key literature: Palissa 1964a; Schaller 1970; Christiansen 1990; Hopkin 1997; Fjellberg 1998; Dunger 1994–2004

Among soil micro-arthropods the Collembola are regarded as a supergroup in that they are distributed worldwide, with more than 6500 species and are found in nearly all terrestrial habitats. Systematically, 24 families

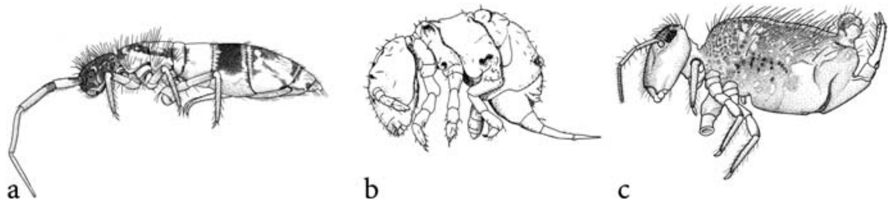

Fig. 1.10. Collembola, habitus of the main subclasses: **a** Arthropleona (*Orchesella cincta*); **b** Neelipleona (*Megalothorax minimus*) (redrawn from Börner 1906); **c** Symphypleona (*Dicyrtoma ornata*)

have been described which belong to the orders Arthropleona, Neelipleona and Symphypleona (Fig. 1.10). Although they occur in all climates, they are most numerous in the soils of the humid tropical and temperate zones, which reflects a close relationship to wet conditions. Highest densities can be found in forest soils, followed by grasslands and agricultural fields. Although most species are soil dwellers, some have been found which are not bound to soil. Some of them inhabit the most extreme habitats of the earth, e.g. the high alpine regions and the Antarctic. Very spectacular species in this context are the so-called glacier fleas, e.g. *Isotoma saltans*, which survives the hard winter deep in ice (about 30 cm; see also Eisenbeis and Meyer 1999). Some species like *Anurida maritima* inhabit the intertidal tidal line of rocky coasts, while others, like *Sminthurides aquaticus* shuttle between the moist border zones of ponds and puddles and the free water surface. A similar mode of life can be found within the vegetation, where the bark dwellers seek the moist surfaces of trees rich in algae and lichen.

Collembolans may move vertically in the soil as well as between the soil surface and the vegetation layer, as do some mites. They are often exposed to a gradient of biotic and abiotic factors. However, true edaphic species can only leave the soil under the shelter of darkness and when the humidity is high. This correlates with remarkable adaptations in structure, ecophysiology, and behaviour.

The collembolan body is composed of relatively few segments: head six, thorax three, and abdomen six. Their trunk length is thus usually small (< 5 mm), and only a few species reach dimensions around 10 mm, such as the longest European species *Tetrodontophora bielanensis*. Two main types of abdominal structure appear to have evolved within the Collembola: (1) the rod-shaped or cylindrical form of the Arthropleona with distinct abdominal segments, and (2) the globe-shaped body type of the Neelipleona and Symphypleona. This latter form has evolved both by segmental fusion and differential segmental growth (Fig. 1.10). In the Neelipleona the globular form is produced by enhanced growth of the thoracic segments, whereas in the Symphypleona the first abdominal segments become larger and lose

their segmental limits by fusion, giving the appearance of a 'big rounded abdomen'. In contrast, the last abdominal segments of the Symphypleona remain more or less small and distinct, producing the appearance of a 'small abdomen'.

Collembola bear three remarkable abdominal appendages: (1) the ventral tube (always present) on the first abdominal segment, with eversible vesicles or tubes (Fig. 1.11), (2) the furca (the collembolan jumping organ) on the forth abdominal segment (may be strongly reduced or absent in eudedaphic life forms), and (3) the retinaculum on the third abdominal segment, which is used to keep the furca in its flexed position ventrally.

The Collembolan integument is normally covered with a hexagonal pattern of cuticular micrograins (Fig. 1.12), which may be arranged in different patterns. These are thought to be responsible for the spectacular anti-wetting properties of the collembolan cuticle, which have been attributed to a lipid layer on the outer surface of the micrograins. Should the soil be flooded, the animals are protected from drowning for a limited period and have a chance to survive in a bubble of air. In contrast, the coxal vesicles of the ventral tube are covered with a smooth and wettable cuticle, permeable for water and ions. Inside the vesicles are lined with a highly specialized transporting epithelium (Eisenbeis 1982), which makes the ventral tube an effective water absorbing organ. It enables collembolans to rapidly absorb water and ions from moist surfaces into the body.

The life cycle of many collembolans is characterized by morphological changes with such different features, that early taxonomists often described distinctive developmental stages as different species. It is assumed that climatic influences (desiccation, warming), are responsible for

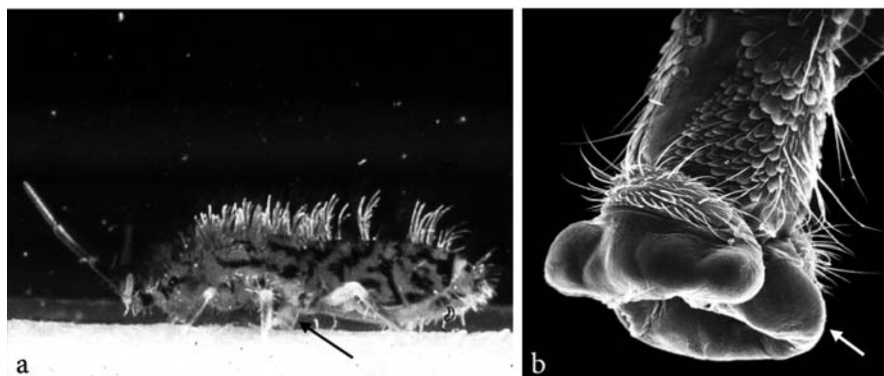

Fig. 1.11. **a** *Orchesella villosa* (Collembola) absorbing water from a moist paper using the ventral tube with its everted vesicles (*arrow*). **b** SEM photograph of the ventral tube of *Tomocerus flavescens* with everted vesicles (*arrow*). Magnification: 18 ×, 155 ×

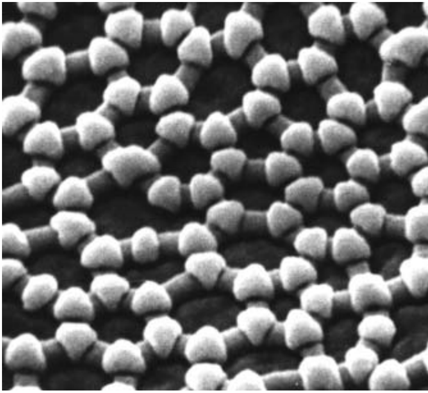

Fig. 1.12. Hexagonal pattern of a Collembolan cuticle with connected micro-tubercles. Magnification: 17,500 ×

inducing such changes, which are called ecomorphoses. They can also be induced artificially in the laboratory. Sometimes the animals stop feeding and change their appearance completely. However, in addition, some regular morphological changes do occur during ontogenesis, which are called epitoky, and a third phenomenon, called cyclomorphosis, occurs during the course of a year, if summer and winter forms regularly alternate (Hopkin 1997).

Another type of adaptation of soil animals occurs along the vertical gradient from the soil surface to the true soil life within the soil matrix (Schaller 1970). It refers to the evolution of peculiar morphological and physiological adaptations within the soil porosphere. Collembola are especially well-known as a model group in this regard, because a wide range of species occurs from the vegetation layer to the deep soil. The changes involve the shape and size of the body, which becomes more and more slender and smaller in soil and the shortening of legs, antennae, some other appendages, and bristles. As in the case of the furca, a complete reduction of an extremity can occur. Other changes include the sensory organs and the pigmentation, which are generally reduced in soil. Finally, the globe-like springtails demonstrate another path of body evolution becoming globular. This can be interpreted as a strategy to minimize the relative body surface area and, in combination with a reduced permeability of the cuticle, to give better protection against water loss. This could explain why the Symphypleona more often leave the shelter of the soil; they are obviously better protected against desiccation than most of the Arthropleona. Figure 1.13 shows some of the collembolan body types according to Fritsch (1994).

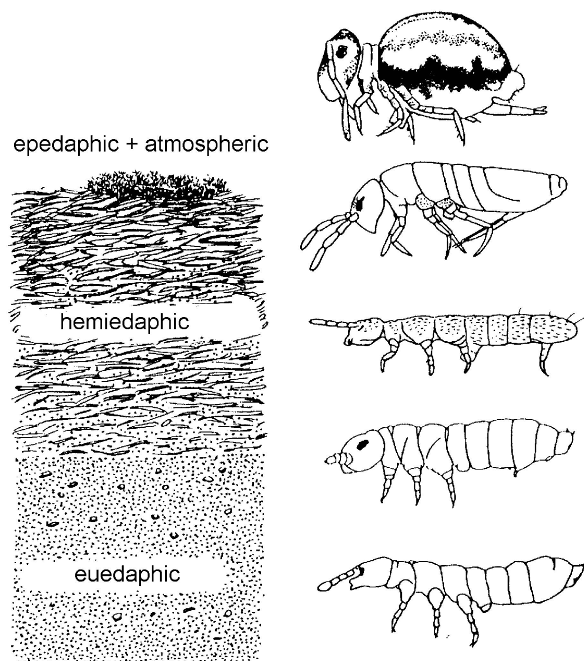

Fig. 1.13. Habitus of different species of Collembola within a soil profile. Note the gradual reduction of the furca. (According to Fritsch 1994, unpubl.)

1.3.6 Protura

Size: 0.5 to 2 mm; species number: > 500; abundance: 500 to > 2,000 m^{-2}; body mass: 50 to 200 µg (estimated); key literature: Palissa 1964b; Tuxen 1964; Nosek 1973; Tuxen 1986; Stumpp 1988; Copeland and Imadaté 1990

Proturans are a strongly derived group of apterygote hexapods with some of the attributes described above for euedaphic soil dwellers. They are nearly unpigmented, with a slender elongate body shape and shortened legs. The integument is covered with a non-sclerotized cuticle which is assumed to be highly permeable for water which would restrict the animals to the moistened layers of the soil.

The most spectacular feature of the Protura is the lack of antennae, which are functionally replaced by the forelegs, which are held in front of the body in an elevated position (Fig. 1.14). They bear some modified tactile hairs and chemical receptors. The animals are eyeless, but in the latero-dorsal wall of the head capsule there is a plate-like sensory organ, which is called the pseudoculus as in the Pauropoda. It appears to be a modified post-antennal organ, which probably functions as a hygroreceptor too.

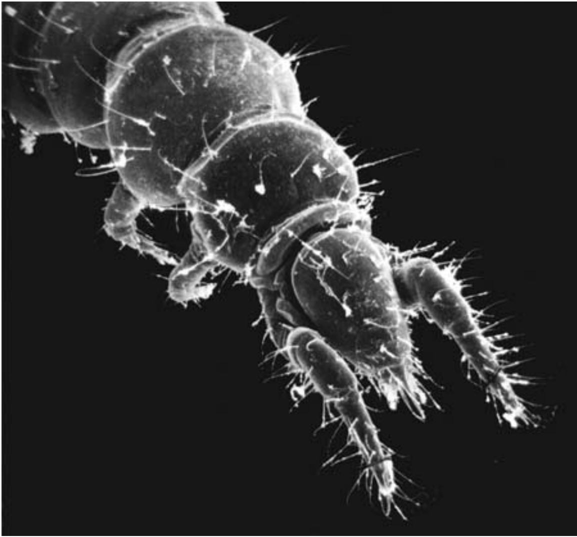

Fig. 1.14. Protura, SEM photograph of the anterior body with antenna-like prolegs. The head bears a stylet formed by the mouthparts. Magnification: 175 ×

In a first glance, the slender 12-segmented abdomen appears to have no distinct appendages, and to lack cerci. However, a closer look at the latero-ventral parts of the first three abdominal segments reveals short, articulated remnants of extremities bearing small coxal vesicles at their tip (Eisenbeis and Wichard 1987). Thus the animals should be able to take up water and ions like the Collembola and Diplura, provided that the vesicle epithelium consists of a transport epithelium.

Newly hatched proturans have only nine abdominal segments. However, each time they moult, a new segment is added near the end of the abdomen until they are fully grown (and sexually mature). This plesiomorphic mode of development is called anamorphosis. After maturity additional moults may occur.

As in the other entognathous hexapods, the basal parts of proturan mouthparts are retracted into the head capsule, from which they can be both protruded and retracted. The outer mouthparts themselves are modified in different ways. The maxillae and mandibles are elongate but distally they form finely toothed chisels (mandibles) or pincer-like structures (maxillae) enabling the animals to shred and to chew small food particles in a more usual way. However, in other species both the lobes of the maxillae (galea and lacinia) and the mandibles have been converted to mini-stylets which join together to form a complex 'hyper stylet' (Fig. 1.14) with the help of which the animals are able to pierce and to suck out their food. Indeed Sturm

(1959) has observed proturans using their stylet to suck on fungal hyphae. Based on this knowledge, studies were initiated to determine whether there is a correlation between the density of Protura within the root zone of a spruce forest soil and the density of intact ecto-mycorrhizae. In forests with a healthy root system the number of extracted proturans was always found to be exceptionally high (2,000–3,000 m^{-2}) whereas in such forests having acidified soils and a poor root system the density was significantly lower (< 1000). On the basis of these results some soil biologists tried to use proturans as valuable bioindicators indicating the health of a forest (Stumpp et al. 1986; Funke 1986; Funke et al. 1987).

1.3.7 Diplura – Double Tails

Size: 2 to 5 mm, max 58 mm; species number: 500 (central Europe 50); abundance: < 50 m^{-2}; body mass: 0.5 to 2 mg; key literature: Palissa 1964c; Ferguson 1990

The diplurans are unpigmented soil dwellers which prefer moist and dark habitats. They seek cavities near the soil surface (under stones and bark), but they also advance deep into the soil, much like the Symphyla. Readily visible characters are the multi-segmented, filiform antennae bearing thousands of sensory hairs and the cerci inserted at the end of the fully segmented (11) abdomen. Two types of cerci can be distinguished in the Diplura: (1) the Campodea-type (Fig. 1.15), with filiform cerci, very similar

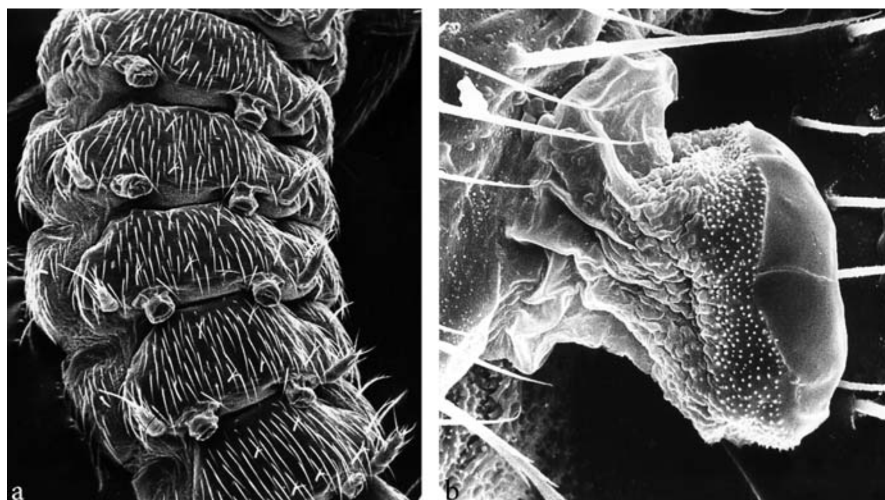

Fig. 1.15. *Campodea* (Diplura), SEM photographs with **a** ventral view with coxal vesicles and styli; **b** an everted coxal vesicle. Magnification: 95 ×, 950 ×

to the antennae, and (2) the Japyx-type, with cerci built as unsegmented forceps (Eisenbeis and Wichard 1987). Measurements of water loss in *Campodea* exposed in completely dry air (Eisenbeis, unpublished), indicate an enormous susceptibility to desiccation. They lose body water at a rate of about 80% h^{-1} (the same as for *Scutigerella*, Sect. 1.3.3) indicating a high permeability of the cuticle. This and the lack of any eyes or a protective pigmentation restrict the animals to the moist labyrinth of the soil. The feeding habits of campodeid diplurans are thought to be very similar to those of symphylans. The japygids, however, are hunters and use their forceps to grasp their victims. The low population density of diplurans indicates a small contribution to the bulk turnover of organic matter. It seems most probable that they stimulate microbial activity and propagate spores.

1.4 The Macrofauna

Size: 4 to 80 mm – Enchytraeidae, Lumbricidae, Araneida, Isopoda, Chilopoda, Diplopoda, Isoptera, Coleoptera, Diptera, insect larvae, and some other insect orders (not included)

The invertebrate macrofauna of the soil includes animals with (1) a short, compact body with a solid, semi-solid or soft trunk (e.g. isopods, glomerids, some dipteran larvae, termites), (2) an elongated, rod-like body with a solid trunk and reduced flexibility (e.g. some diplopods like julids and poydesmids, larvae of carabids), (3a) a soft wormlike body with a flexible trunk (e.g. earthworms, some larger dipteran larvae and snails), and (3b) a semi-solid wormlike body with a flexible trunk (e.g. chilopods). Some of them are able to change their body shape, e.g. if a glomerid changes from a stretched to a rolled up body.

The most important macrofauna group of the temperate zone are the earthworms, which are replaced in the warm and semi-arid zones by ants and termites. Soil biologists do classify some groups of the macrofauna as the “soil engineers” which actively form and modify the soil matrix. This relates to such issues as bioturbation, decomposition, and structuring the porosphere, e.g. by the burrowing and casting of earthworms.

Earthworm activity significantly changes the physico-chemical and biological status of the soil and may cause drastic shifts in soil biota. For this reason the soil zone in which they are active is often called the drilosphere. The same holds for the larger enchytraeids, which also actively modify the soil texture, e.g. by reprocessing earthworm castings to a finer quality humus. Lavelle et al. (1995) have broken down the spectrum of digestive relationships which have evolved between macro- and micro-organisms in

soil as follows. (1) Direct digestion: with little microbial participation is mainly found in predators. Only a few saprophages are candidates for using their own enzyme systems, e.g. higher termites, and probably isopods and earthworms. (2) An 'external rumen' type: digestion in which faeces enriched with active microorganisms are repeatedly ingested (coprophagy). Similar relationships occur in fungus cultivators. (3) Facultative mutualism: using the increased microbial activity (during the gut passage) of an unspecific microflora ingested with soil and litter. The animals provide the micro-organisms with an optimal gut milieu inducing an increased release of microbial metabolites (relevant in wood and leaf-litter feeding insect larvae (Dipera, Coleoptera), in termites, and in temperate and tropical earthworms). (4) Obligate symbiosis: a close relation between the animal and a highly specialized endemic gut microflora and/or microfauna (mainly bacteria and protozoa) in termites. The same authors consider the soil macrofauna to be the main regulators of microbial activity in soil. If the gut content is further enriched with mineral particles ingested with the food, then conditions become optimal for the creation of organo-mineral particles, which pass through the animal body to be integrated into the soil matrix.

It must also be noted that many animals are active in moving materials in the opposite direction, from the soil to the surface. Anecic earthworms, ants and termites move tons of casts and mineral soil up to the soil surface (see also Darwin 1871). The fascinating work of termites is visible as huge nest mounds hardened like concrete (Fig. 1.21). They are a mixture of triturated litter (visible as organic fibres), soil and faeces, which are cemented with secretions. If the mounds are abandoned by the termites, they collapse with time, and the material is recycled back into the soil pool. Besides animals mainly involved in the turnover of soil components, the soil food web needs an army of animals which exert regulative functions. These are the large predator groups like Araneida, Chilopoda and Coleoptera (and some other higher insects) as well as the large group of parasitoids.

1.4.1 Araneida – Spiders

Size: 1–20 mm; species number: ≈ 600 in central Europe; abundance: 50–850 m^{-2}; body mass: ≈ 1 to 25 mg; key literature: Moritz 1993d; Foelix 1996

In soil, spiders are most abundant near the soil surface and within the litter. Unlike many other soil fauna groups they do not participate in decomposition or grazing; they live exclusively as predators. To make available the food resources of their prey they have evolved a system of

extraintestinal digestion. After killing their prey by injection of a toxin with their cheliceral fangs, they regurgitate a gastric juice containing digestive enzymes into the body of the prey and suck out the liquefied body contents. To reach the widely ramified midgut gland within the opisthosoma the juice of nutrients has to pass (1) a filter apparatus made with long bristles, (2) an extremely narrow pharyngeal and oesophageal tube within the prosoma, and (3) a special muscular stomach acting as a pump.

The non-cursorial litter dwellers, which are often threatened with desiccation, have reduced the permeability of their cuticle to water significantly, allowing them to survive longer periods at low relative humidity. However, in some species of cursorial forest spiders, e.g. some lycosids, Baehr and Eisenbeis (1985) found comparatively high water loss rates. Therefore one must assume that such spiders can compensate for greater water loss with an increased mobility, enabling them to seek a shelter during drought.

The large agelenid spiders are able to catch prey like isopods, beetles and insect larvae, whereas the tiny soil spiders are more specialized for catching the smaller arthropods, e.g. collembolans or mites. Spiders use their large midgut gland as a central organ for digestion, metabolism, storage of nutrients, and excretion. Within the resorptive cells of the gland epithelium, Ludwig and Alberti (1988) described small cell organelles called spherites, which are able to accumulate minerals and heavy metals.

Spiders may hunt either by day or by night. Some spider groups – e.g. wolf spiders, jumping spiders, sac spiders and ground spiders – actively hunt for their food at the soil surface. Trapdoor spiders hide at the mouth of their burrows until an insect walks past, then rush out to grab it. Others spin webs and lie in wait for prey. Their webs are stretched between stones, leaves, pieces of wood, moss, and within the ground vegetation. Ground spiders often spin their webs across depressions of the soil surface as a sheet or as funnel-like constructions. Litter dwelling spider families are predominately minute web-building spiders.

Wagner et al. (2003) investigated the stratification of the litter-inhabiting spider community of a forest soil which encompassed 18 families. The web-spinning families were more abundant in the lower litter layers. In contrast, the non-web building cursorial spiders, which actively pursue their prey, were more abundant in the top litter layer. Cursorial spiders, on average, were larger than the web-building spiders found in the leaf litter. Web-building spiders from the top litter layer were also larger than the web-building spiders caught in the middle and the bottom litter layers. Well known spider families in the litter are the Agelenidae, Linyphiidae, Hahniidae, Micryphantidae, and Dictynidae.

In the Göttinger beech forest (Germany), known for its soil with mull humus over limestone, Sührig (1997) has found a spider community consisting of 156 species belonging to 89 genera in 21 families which 107 species

in 18 families are restricted to the soil zone. A soil spider community of nearly the same size (95 species/19 families) was found in a more acidified beech forest with rotted down humus in southern Germany (Dumpert and Platen 1985). The average abundance of spiders in a forest soil has been calculated to be 50–150 m^{-2}, but densities as high as $\gg$ 500 m^{-2} are not unusual (Eisenbeis and Feldmann 1991). These results suggest that that spiders play a key role in the regulation of the non-predatory fauna within a soil food web.

1.4.2 Opiliones – Harvestmen

Size: 2 to 10 mm, max 22 mm; species number: 3600; abundance: $< 30\,m^{-2}$; body mass: 1–10 mg; key literature: Berland 1949; Martens 1978; Edgar 1990; Moritz 1993c

The harvestmen are distinguished from spiders, their predatory relatives, by both structural and biological differences. The prosoma is joined broadly to the abdomen, with no visible stalk-like separation (petiole); the opisthosoma shows a clear segmental division and lacks spinerets. The pedipalps, which in many arachnid groups end as pincers, are of leg-like or palpal character. Opilionids lack venom glands. They grab their prey with the toothed pincers of the long three-segmented chelicerae. Then the prey is crushed and drawn to the mouth. Intruders or predators attacking harvestmen are repelled by malodorous secretions from prosomal glands at the anterior lateral margins of the carapace. They secrete or spray a mixture of repugnatorial substances which may contain quinones and phenols.

Most spectacular is the arrangement of the two eyes. Lying close together and with fields of view turned away from each other they are part of an 'eye-hill', called the tuber oculorum. It is raised above the dorsal prosoma, allowing the eyes to have an excellent field of view, enabling the animals to make quick and subtle movements.

Some harvestmen require a specific diet, but most of them are omnivorous. They are valuable scavengers, cleaning up dead insects and rotting vegetable matter, but do not hesitate to hunt for leafhoppers, aphids, snails, earthworms, flies, true spiders, other harvestmen and insect eggs. They also suck juices from soft berries and fruits, and will occasionally eat fungi. *Ischyropsalis hellwigi* is known to be a snail eater. Digestion is not external as in spiders; harvestmen ingest only fine particles of food using a pharyngeal pump.

Harvestmen are well known for their long and flexible legs, which often are used like a lasso. Therefore the preferred habitats of the long-legged species are above the ground, within the bush and grass layer, but they

climb also very skilfully on walls. Their body coloration is comparatively bright with definite patterns. In contrast the soil dwelling species move more slowly and have a smaller body with significantly shorter legs. True euedaphic life forms are found in the family Sironidae, living deep in the subsoil, but the Nemastomatidae, Trogulidae and Ischyropsalididae are also well adapted to soil life. They are limited to moist habitats and are abundant in forest soils, preferring the moist upper soil and litter layer as well as moss carpets. There the pigmentation tends to darker colours. Harvestmen are surely less abundant than spiders, but no data are available on their density in soil. Thus it is not possible to estimate their true impact on the soil food web.

1.4.3 Terrestrial Isopoda (Oniscoidea) – Woodlice

Size: 2 to 20 mm; species number: 3500; abundance: 30–2,000 m^{-2}; body mass: 1 to > 10 mg; key literature: Sutton 1972; Grünwald 1988; Muchmore 1990b; Gruner 1993; Zimmer 2002

Woodlice are the only crustaceans which pass their entire life cycle in terrestrial habitats. Their body is equipped with a number of types of appendages, like antennae, mouthparts, thoracopods, pleopods, and uropods. These provide a large surface area, so that they lose water rapidly. Furthermore their cuticle is not protected by a lipid layer forcing most terrestrial isopods to be hygrophilic. During the dry daylight hours, they seek the shelter of moist places, e.g. below pieces of bark and within the litter. There they try to minimise water loss by pressing their ventral side close to the moist substrate protecting completely their thin pleopods and the external water channel system (Fig. 1.16). Extremely susceptible to drought are the bog isopod *Ligidium hypnorum* and the small *Trichoniscus pusillus*, which lose up to 70% of their water per hour. Much better able to tolerate water loss are the common woodlice *Oniscus asellus* and *Porcellio scaber*, but only the pill bugs of the family Armadillidiidae, e.g. the cosmopolitan *Armadillidium vulgare*, are able to move freely at the soil surface in daytime. Besides the ability to roll the body into a ball, some of the better adapted woodlice have evolved both a tracheal system and short extremities.

An unusual feature of the woodlice is the so-called water channel system along the leg bases (Hoese 1981). One of its multiple functions is thought to be to keep the cuticle moist and to carry away ammonia from the body fluid. The argument is that the two longitudinal water channels beginning at the base of the first maxillipedes are continuously filled with an excretory fluid secreted from the paired maxillary glands in the head. The main flow occurs through the two ventral channels along the leg bases via the

Fig. 1.16. The woodlouse (Isopoda) *Onsicus asellus*, dorsal view. Magnification: 5 ×

pleo-ventral cavity underlying the pleopods and finally to the anus. On its way ammonia is released from the fluid and the remaining clean water may enter the hindgut again to be recycled. The longitudinal channels are connected dorsally by transversal channels, thus providing the scaled cuticle on the back of the animals with water too.

For long-distance information, woodlice use their second antennae which end in a two- or three-segmented flagellum (Fig. 1.16). At the end they bear the so-called paint-brush organ which consists of a bunch of long sensory hairs. The animals test the quality of the substrate in front of their head by keeping the tip of the organ on the ground. With the help of many mechanoreceptive and chemoreceptive sensilla, they are able to find suitable microhabitats for feeding and to aggregate in large groups of young and adults.

Terrestrial isopods feed on leaves (litter), old wood and nearly all kind of detritus, and are classified as saprophytophagous or primary decomposers. Their food includes freshly fallen leaves and pre-decomposed litter, but their choice of food depends widely on the quality of the leaves, the stage of decomposition and the moisture level. Woodlice also eat their own faeces and those from other soil animals so they are often regarded to be coprophagous. Bauer and Christian (1995) fed isopods maple leaves which had been contaminated both with faeces and secretion from enchytraeid worms. They found a significantly higher consumption rate of the preconditioned material. Kautz et al. (2002) have investigated the benefit of selective feeding on faecal pellets, if woodlice are coprophagous. Coprophagy was

revealed to be an advantage, if the quality of the available plant litter is comparatively low, such as oak leaves, which a lot of recalcitrant compounds. However, if the quality of the basic material is better from the start, e.g. alder leaves, then feeding on the faeces enriched with microorganisms is less important. Zimmer and Topp (2002) discuss the importance of woodlouse coprophagy for the release of nutrients like Ca^{2+}, Mg^{2+} and K^{+} if the faeces are produced by phytophagous insects, e.g. caterpillars of geometrid moths.

In a lowland alder forest Grünwald (1988) has found a woodlouse community of seven species which is extremely abundant, with nearly 2000 animals m^{-2}. Such high densities are thought to arise when an optimum for these isopod species, reflecting ideal food and high humidity, is present. In a beech forest near Mainz (Germany) growing on limestone, Eisenbeis and Feldmann (1991) have found an average density of about 700 m^{-2} of which 642 were distributed within the litter and 71 within the upper 5 cm of the mineral soil. At this site the dwarf isopod *Trichoniscus pusillus* was most dominant with several hundred animals m^{-2}. However, according to Dunger (1983), the normal abundance of woodlice is 30.

Most arthropods have evolved a long midgut tube for digestion, but woodlice have reduced this gut compartment to a very short connection between fore- and hindgut. Instead they have developed four long tubes (caeca) of the midgut gland (hepatopancreas), which diverge from the connection and float through the hemolymph space. Within the caecal lumen the resorption and final digestion of nutrients takes place. Further the midgut glands are used as a general storage compartment for lipids and minerals, e.g. heavy metals, and they harbour bacteria, which contribute their enzymes. The food of woodlice is comminuted by the mouthparts and maxillepedes and transported via the oesophagus to the stomach. There it is dispersed further and transmitted to the large sac-like dilation of the anterior hindgut. Passing the connection of the midgut glands enzymes from the caeca are added, which are thought to be responsible for the high cellulolytic activity in the anterior part of the hindgut (Zimmer and Topp 1998a,b) . After a definite period of incubation, the gut contents are transported back dorsally along the gutter of the typhlosole to the stomach, where a filtration occurs. Then the most valuable and smallest food particles are selected to pass into the midgut gland. According to Zimmer (2002), digestion is carried out by a wide spectrum of enzymes (carbohydrases, proteases, dehydrogenases, esterases, lipases, arylamidases and oxidases), which are contributed by microorganisms. So far all findings indicate that both microorganisms mixed with the food (exogenous) and bacterial gut endosymbionts contribute to the enzyme pool. Zimmer and Topp (1998b) believe that, contrary to the earlier assumption of Hartenstein (1964, 1982) and Zimmer and Topp (1998a), the cellulases found in woodlice are not

produced by the animals themselves, but more likely by bacteria within the hepatopancreas. They have called this kind of provision 'functionally endogenous'.

Nevertheless, whether the enzymes are exogenous or endogenous, the large anterior hindgut has the function of a rumen. Even phenoloxidases for the breakdown of lignocellulose are provided from endosymbiontic bacteria in the caeca, thus extending the pool of polymers (cellulose, lignin) which can be broken down. Besides breaking down litter, woodlice contribute many services within their gut compartments promoting the activation and cultivation of microorganisms. Therefore it is not surprising that the presence of woodlice in a soil accelerates the rate of decomposition. However, if the animals feed on leaves contaminated with a high concentration of heavy metals then only 3–14% is incorporated into the body (Alberti et al. 1996), and the remainder is lost by excretion. However, the main effect of such a contamination is that both the consumption and the assimilation rate decrease significantly. The animals feed less and grow less.

1.4.4 Chilopoda – Centipedes

Size: 5 to 60 mm, max 30 cm; species number: 3000; abundance: 30–2,000 m^{-2}; body mass: 1 to > 10 mg; key literature: Lewis1981; Mundel 1990; Dunger 1993a

The class of the Chilopoda is subdivided into five orders:

- Scutigeromorpha (house centipedes, mesic to xeric and thermophilic)
- Lithobiomorpha (stone centipedes, moisture-requiring and temperate)
- Craterostigmomorpha ('Tasmanian centipedes', moisture-requiring and temperate)
- Scolopendromorpha ('Skolopender', mesic to xeric and thermophilic)
- Geophilomorpha (subground centipedes, moisture-requiring and temperate)

The body of centipedes is divided into a compact head and a long multi-segmented trunk consisting of 15 to 181 segments, which may be either homonomous (all segments alike) or heteronomous (segments of more than one type).

The segments are often flattened, and some or all of them bear open spiracles for breathing. Each segment has one pair of short legs which are good for pushing the animals through the soil. Centipedes have no

waxy waterproofing layer on their cuticle, hence they tend to be limited to moist habitats. In temperate regions both the epedapic Lithobiomorpha and the euedaphic Geophilomorpha are the dominant groups. During the day, stone centipedes rest in crevices beneath stones, under tree logs or bark, or seek the shelter of moist stacks of leaves within the litter. However, in the evening and at night if the air humidity increases, the animals become active and start hunting. The Geophilomorpha, which have quite a slender and flexible body, allowing them to move skilfully through the narrow system of subground channels, are nearly independent of circadian rhythms. Neither group has a multilayered muscular body wall with a ring of circular and longitudinal muscles. Therefore they are not able to penetrate the mineral soil like an earthworm, which can move with the help of peristaltic contractions working against a hydroskeleton.

The well-known, common European centipede *Lithobius forficatus* has only 15 pairs of legs and dorsally the heteronomous trunk is covered with alternating short and long tergites which enable the animals to fold their body into narrow loops. The first trunk segment, which bears a pair of stout maxillipedes is strongly modified and functionally connected to the head. Caudally the body ends both with longer legs used for defense and the genital appendages. The maxillipedes are constructed as curved forceps which are used as poison fangs (Fig. 1.17). They are held horizontally and protrude their sharp tips always watching for a victim and to kill it with paralysing and toxic injections. Consequently most chilopods are carnivorous, searching for earthworms, enchytraeids, and insects. The coxal organs are another unusual feature of chilopods which deserve to be mentioned

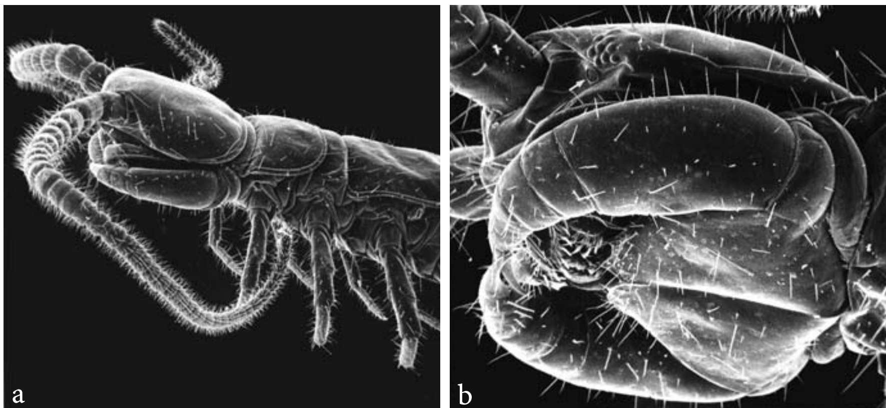

Fig. 1.17. SEM photographs of *Lithobius* sp.: **a** anterior body, in lateral view with segmented antennae; **b** head, oblique ventral, with maxillipedes. The *arrow* points to the plate-like 'post-antennal organ' below the eye. Magnification: 27 ×, 58 ×

(Rosenberg 1983). They are inserted into the last three coxae and consist of a pore canal and an underlying transporting tissue. They often are compared functionally with coxal vesicles and it is likely that they are involved in water and ion balance.

In central Europe the genera *Lithobius* and *Geophilus* are very common. *Lithobius* represents the epedaphic life form with eyes, strong body and longer extremities, whereas the eyeless *Geophilus* is more representative of the euedaphic type with thin body and short extremities. If active, all centipedes move restlessly while hunting, which is why they are regarded as voracious predators. They play a key role in soil food webs, especially in forests (Poser 1988). Their biomass may exceed those of carabids, staphylinids and araneids (Schaefer 1983), but their population density depends on the forest type. In monospecific spruce forests which are known for strongly acid soil the chilopod fauna is poor in species and less abundant (2–30 m^{-2}/2–4 species). In contrast, deciduous forests characterised by a better habitat diversity and soil status have a richer chilopod fauna (50–180 m^{-2}/7–14 species). It is likely that the reason must be the distinct food spectra of these two forest types. Spruce forests are well-known to be mesofauna soils with a low biomass, whereas deciduous forests commonly have a rich macrofauna with a high biomass, dominated mainly by earthworms.

1.4.5 Diplopoda - Millipedes

Size: average 20 to 40 mm, min. 3 mm, max 300 mm; species number: 10,000; abundance: 100 m^{-2}; body mass: < 1 mg to > 1 g; key literature: Hoffman 1990; Hopkin and Read 1992; Dunger 1993d

Diplopods are usually cylindrical, and heavily encased long-bodied animals, with two pairs of legs on most of the body segments (diplosomites). Each of the first four segments bears a single pair of legs, and the last somite is devoid of legs, but ends with a telson (pygidal shield). Most of the body somites carry a pair of openings of defence glands in a dorso-lateral position.

Diplopods have evolved several different body types:

- Rammer or bulldozer type (e.g. Iulidae)
- Globular type (e.g. Glomeridae)
- Borer type (e.g. Poyzonidae)
- Wedge type (e.g. Polydesmidae)
- Soft bark-dweller (e.g. Polyxenidae)

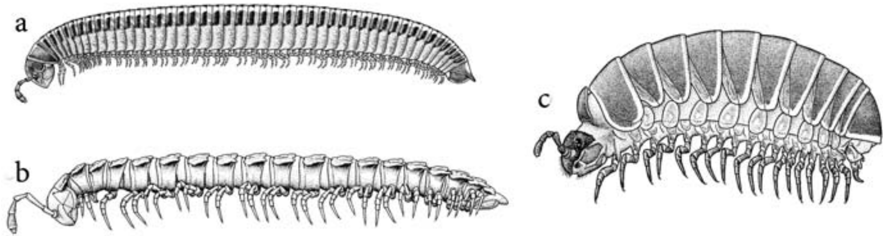

Fig. 1.18. Common diplopods from central Europe in lateral view: **a** *Ommatoiulus sabulosus*; **b** *Glomeris marginata*; **c** *Polydesmus angustus*

The most spectacular feature of some diplopod families is the box-like construction of the diplosomites, with a rigid calcareous body ring. This is best realised in the Iulidae (Fig. 1.18a) but it is also found in other families. In the polydesmids, the ring is modified by dorso-lateral lobes (Fig. 1.18c). Compared with the longbodied diplopods, the body of glomerids (and also of the giant tropical globular diplopods) is much shorter, because the animals are able to roll their body into a sealed ball (see also Eisenbeis and Wichard 1987). There the rigid ring is replaced by a system of movable sclerites, which overlap in the uncoiled stage and fit close together when the animal is rolled up. Millipedes lack poisonous fangs and do not bite; rather, to discourage predators they roll into a defensive ball and many emit poisonous or foul-smelling substances.

Despite the rigid construction of their body the animals are able to move skilfully through the soil and litter assisted by the powerstroke of the many legs. In addition the Iulidae have a notably solid head capsule followed by a collar shield (collum), allowing them to push aside the soil material like a bulldozer. As they are considered to be powerful burrowers, such diplopods are designated ‘soil engineers’, which have an essential role in mixing the soil. Finally they contribute passively to bioturbation if they deposit their faeces into deeper soil layers.

The nutrition of diplopods is very similar to that of woodlice. They are primary decomposers which consume considerable amounts of plant litter and produce large amounts of faeces. If some glomerids are enclosed in a box together with moist leaves or wood, then the material is transformed to faeces within few day (Fig. 1.19). Bonkowski et al. (1998) have documented an interaction between the earthworm *Octolasium lacteum* and the diplopod *Glomeris marginata*. The earthworms grew well only if the faeces of *Glomeris marginata* were available, and without feeding on pellets their biomass decreased. These authors have regarded this interaction as essential to the formation and preservation of a mull humus.

There is a remarkable difference between terrestrial isopods and diplopods concerning the construction of the gut system. In isopods, the

Fig. 1.19. The diplopod *Glomeris marginata* feeding on aged wood and producing a lot of faecal pellets. Magnification: 5 ×

midgut or intestine is completely replaced by the midgut gland caeca, which are responsible for the final processing of nutrients, but diplopods have a long midgut tube without elongate caecal appendages. A further diplopod specific feature concerns the renewal of the midgut epithelium. This is completely replaced during a moulting cycle by the so-called regenerative cells at the base of the gut tissue. The same procedure is known from Collembola, which use the gut renewal for excretion. Indeed in both groups the midgut cells contain clusters of spherites (sometimes called congrement vacuoles) which are released upon moulting of the old midgut epithelium. Spherites do accumulate minerals and they can also serve for the storage of heavy metals. Consequently the animals can use this procedure for the decontamination of waste material. In isopods and also in spiders minerals are stored in spherites within the midgut gland, but they do not completely replace the tissue.

If diplopods are contaminated with heavy metals, the resorptive cells of the midgut and the closely related hepatic cells react. Alberti et al. (1996) described ultrastructural changes in the plasmalemma, the cytoskeleton, the distribution of mitochondria, and the topography of the endoplasmic reticulum. The functional significance of these changes cannot yet be assessed.

1.4.6
Enchytraeidae – Whiteworms, Potworms

Size: 1 to 50 mm; species number: 600, ME 112, estimated 200 to 300; abundance: 5000 to 750,000 m^{-2}; body mass: 0.5 to 10 mg (estimated); key literature: Dash 1990

Enchytraeids are smaller relatives of earthworms and often inhabit soils with many species and a high population density. If only the body diameter of species is considered, most of them should be classified as mesofauna. However, many species are significantly longer than 4 mm, and these are often included in the macrofauna. All are hermaphrodites, but they have evolved different modes of reproduction: 1) amphimixis, 2) parthenogenesis, 3) asexual fragmentation (autotomy), and 4) self-fertilisation (autogamy). It is thought that such reproductive versatility has evolved with their ability to adapt to very different environmental conditions, and perhaps to stress. The ubiquitous species *Enchytraeus albidus*, for instance, is able to live in many different soil types, in compost, in organic manure, in the mud of polluted waters and in detritus along an ocean beach.

Enchytraeid worms utilize all kinds of food, and also ingest mineral particles. They are microphytophagous on bacteria and fungi, saprophytophagous on aged litter, coprophagous on faeces of primary and secondary decomposers, and necrophagous on carrion. It is thought that the most valuable nutrients besides those from carrion are recruited from microorganisms because their own content of digestive enzymes is estimated to be low. According to Zachariae (1965) they are active at different patches within a soil profile. One of their main tasks is feeding on earthworm castings deposited in the mineral humus layer and making them into a more valuable soil component, which is loosened and rich in plant nutrients. They also improve the palatability of litter as was shown in the case of maple leaves. If the leaves were contaminated with faeces and mucuous secretions from *Enchytraeus albidus* then their acceptance by the isopod *Porcellio scaber* was increased significantly (Bauer and Christian 1995).

According to Beylich et al. (1995) enchytraeids are important elements of the soil decomposer community which may be used to evaluate the soil state. But their population density is subject to large fluctuations which have not been correlated with definite soil conditions. Generally, they show a preference for acid soils with the highest densities in raw humus. *Cognettia sphagnetorum* is well-known, and is most abundant in such soils, as was found in Scandinavian coniferous forests. This species proliferates by fragmentation and can occur at several thousand animals per m^2. In soils with a high pH, enchytraeids are less abundant but rich in species. Therefore the liming of soils changes the enchytraeid community because acidophilic and acid-tolerant species decrease and other species replace them, and the diversity of such a site will be improved.

1.4.7
Oligochaeta: Lumbricidae – Earthworms

Size: 40 to > 80 mm (max 30 cm); species number: > 200; abundance: 50–500 m^{-2}; Body mass: 40–400 g m^{-2}; Key literature: Edwards and Lofty 1977; Lavelle 1988; Schwert 1990; Edwards and Bohlen 1996

In the humid temperate zone the earthworms are regarded as the most important soil fauna group with respect to biomass and function. According to Bouché (1977) the earthworms are divided into three main life style groups:

- Epigeic (epedaphic) species – surface dweller and litter species in forests like *Dendrobaena octaedra* with a small body. They often inhabit tube-like cocoons between leaves.
- Endogeic species – top soil species with a stronger body like *Aporrectodea caliginosa*. They are active both horizontally and vertically in the upper soil profile, burrowing temporary channels, which often are filled with castings.
- Anecic species – large species like *Lumcricus terrestris* inhabiting extended vertical burrow systems and moving between soil surface and deep soil (down to 2–3 m).

Among earthworms, it is especially the mineral soil dwellers which contribute significantly to soil formation, soil structure and turnover of soil matter by their burrowing, mixing and depositing activity from the soil surface down to the subsoil. The most valuable function of earthworms is to supply the soil with aggregates of organo-mineral complexes which are formed during gut passage. The gut of earthworms can be compared with a production line functioning as a bioreactor. It begins with fragmentation of the ingested litter and secretion of mucous gland products by the gut epithelia and accessory glands, followed by mixing of the material, thus providing the best conditions for microorganisms to start the chemical breakdown of litter. The castings released from earthworms are rich in plant nutrients and pre-digested organic matter, which is available for further processing by secondary decomposers.

The evidence of earthworm activity in soils may be so significant that the soil zone which is affected by them is called 'drilosphere'. Earthworms improve hydraulic conductivity by loosening compressed soils (Joschko et al. 1989). Even a hardened "plough pan" is no barrier to the movement of anecic earthworms between top soil and subground. With the help of a very effective muscle system and the ventro-lateral rows of cuticular setae (hooks), they move and dig powerfully through the soil. Schrader and Joschko (1991) have visualized the impact of earthworms on the water

drainage capacity of soils using an infiltration method with dye markers, and Langmaack et al. (1999) tried to reconstruct the three-dimensional earthworm channel system by X-ray computer tomography. If a definite volume of water is added to the soil surface, it penetrates the soil much more quickly, when earthworms were active in the soil profile.

At first sight, the epidermis of earthworms seems to be unprotected. However, investigation of the fine structure of their integument revealed that they are covered with a multi-layered cuticle reinforced with collagen fibrils. Further, the epidermis contains many different gland cells (Fig. 1.20) which secrete a mucous layer to the outside of the animals which provides chemical protection and keeps the animals moist. Greven (1987) and Greven et al. (1987) have found that earthworms try to improve their integumentary protection by increasing the production of mucous cells if the substrate becomes more acid. However, if the pH of the soil goes below 4.2, most endogeic and anecic species disappear. Only the epigeic species like *Dendrobaena octaedra*, *Dendrodrilus rubidus* and *Lumbricus rubellus* have been found to be more resistant to acid conditions, but they too die if the pH falls below 3.5.

That earthworms are perfectly able to discriminate the quality of soil conditions was shown by Robinson et al. (1991). They exposed six species to both a loamy soil with pH7 and to acid litter material (F-layer, pH 3.8) from

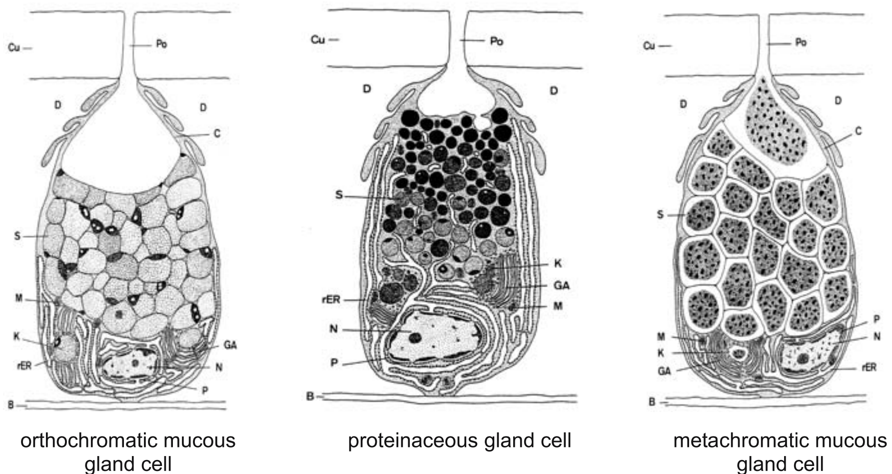

Fig. 1.20. Schematic drawings of epidermal gland cells of the epedaphic lumbricid *Lumbricus rubellus* (Eisenbeis, unpubl.). *B* Basal membrane, *C* apical cytoplasmic border of a secretory cell, *Cu* cuticle, *D* area of surrounding epidermal cells, *rER* rough endoplasmic reticulum, *G* dictyosome (Golgi apparatus), *K* dictyosomal formation of a secretory granule, *M* mitchondrion, *N* nucleus, *P* perinuclear cistern, - *Po* pore canal, *S* secretory granules

a spruce forest. After a few hours of incubation, a few specimens remained in the acid part of the microcosms, the others moved to the loamy soil. However, after liming of the F-material up to pH 7.3, the same treatment revealed a nearly equal distribution of the earthworms between the two soil compartments. In Europe the liming of acid forest soils is being employed more and more frequently as a standard treatment to stop soil acidification and to restore parts of the lost soil fauna. Indeed the epigeic earthworm fauna does recover to high abundances of up to several hundred specimens per square metre (Schauermann 1987; Wolters and Schauermann 1989; Makeschin 1991; Eisenbeis et al. 1997), but unfortunately none of the true endogeic or anecic species has yet been re-established in such soils. It is thought that old tree logs and stumps are the centres for the recolonisation by epigeic species. However, as was shown in a Swedish soil liming experiment, the earthworm population of extremely acid coniferous forests could not recover after liming (Rundgren 1994), even though some of the earthworms exposed within litter bags did survive 1–2 winters at these sites. There have been other field trials to re-establish the deeper soil dwellers among earthworms in forests sites. Beylich and Graefe (1996) and Judas et al. (1997) introduced an initial population of endogeic species on limed forest sites, but unfortunately it was not possible to monitor the site over a long term. It is now thought that not only the liming is a prerequisite for the amelioration of acid soils but that the recalcitrant coniferous litter must be replaced by litter from deciduous trees and a herbaceous plant layer, which can be more easily decomposed.

According to Lavelle et al. (1995) the earthworm community normally includes 8–11 species with lower numbers in extreme habitats. Surprisingly, the species pool of tropical habitats is not significantly higher. However, it was found that some of the tropical species have evolved such an effective digestion within their gut system that they are able to live within the deeper subgrounds of tropical soils which are known to be extremely poor in nutrients. In the colder zones of the northern hemisphere, the earthworms are more focused on exploiting the litter, because there the epedaphic species are the dominant group.

1.4.8 Terrestrial Gastropoda – Slugs and Snails

Size: 1.2 mm to 10–20 cm; species number: > 10; abundance: 5 to > 50 m^{-2}; body mass: 1 mg to > 10 g; key literature: Kerney et al. 1983; Bogon 1990; Burch and Pearce 1990

Within the upper soil and litter layer of damp habitats with calcareous subground there is established a rich gastropod community which

contributes to decomposition. It comprises small to tiny species of the Endodontidae, like the dwarf snail *Punctum pygmaeum* (1.2–1.5 mm) or the rounded snail *Discus rotundatus* (5.2–7 mm), further medium-sized species of the Hygromiidae, e.g. the hairy snail *Trichia villosa* (10–14 mm), and the larger slugs of the families Arionidae and Limacidae which grow to several cm. Altogether the literature on the role of snails and slugs in the soil food web is minimal, because this group has been considered in only a few studies (Franke and Greven 1990; Theenhaus 1997). In acid soils the calciophilic gastropods disappear completely, but it is likely that this group is underestimated in those habitats where suitable conditions favour a rich gastropod population. Gastropods are phytophages or saprophytophages with a digestion system comparable to that of isopods, which is based mainly on the function of a midgut gland.

1.4.9 Insecta – Pterygote Insects (Short Comments About the Role of Selected Groups of Higher Insects)

Many of the hemimetabolic and holometabolic orders of pterygote insects inhabit the soil, with a variety of life cycles:

- Both larval and adult stages are soil dwellers, e.g. cockroaches, gryllids, carabid and staphylinid beetles.
- Only larval stages are soil dwellers, the adults leave the soil, e.g. many dipterans and beetles.
- Insects which use the soil only as a shelter for oviposition, for periods of dormancy and diapause, and survival (drought, cold etc.); different orders.

Solitary individuals of both the wood-cockroach *Ectobius sylvestris* and the wood-cricket *Nemobius sylvestris* occur within dry litter and on the soil surface of warm and dry places, e.g. at the edge of woods or at clear cuts. Other crickets, like the field cricket *Gryllus campestris* live in their own burrows in warm, dry and porous soil, preferably on open sunny places of grassland. And finally the mole cricket *Gryllotalpa gryllotalpa* digs actively in the topsoil of warm sandy soils searching for roots. All these insects are known to be omnivorous, feeding mainly on plants and detritus, and both the larvae and the adults are soil dwellers.

Other insects start their life cycle as larvae in the soil stratum, but after pupation most of the adults leave the soil, seeking places for feeding or mating above ground within the vegetation. Many species of beetles and flies belong to this group, and their larvae can be attributed to nearly all

trophic levels of the soil food web. Many of the main dipteran families are well known litter feeders and contribute significantly to bioturbation, e.g. the huge tipulid larvae. Some beetle larvae are root feeders (e.g. elaterids), but most of them are predatory. Well known predators include the larvae of carabids and staphylinids, of which the adults often continue their soil life as ground beetles. Curiously, some adult beetles which are herbivores live in the canopy of the forest, e.g. the curculionid *Rhynachaenus fagi* which feed on beech leaves, indirectly contribute to the soil food web by the large amount of faeces which rain down. Greenslade (1985) describes the various effects of pterygote insects on soil, but she points to the fact that the identification of species is often difficult.

A fundamental role for the biology and ecology of soils is attributed to the nest-builders among pterygote insects which belong mainly to the order Isoptera (termites) (Krishna and Weesner 1969; Lee and Wood 1971; Wood 1988; Nutting 1990) and Hymenoptera – Formicoidea (ants) (Gösswald 1985; Hölldobler and Wilson 1990; Wheeler and Wheeler 1990). These are the so-called social insects which dwell in the soil in small to huge colonies. The nests can be organized either as (1) subterranean, (2) subterranean/above ground or (3) above ground units only. Because all these insects are often very abundant, they must be regarded as key groups within the related soil food webs. They are global players because they speed up the main nutrient cycles (C, N, minerals) within ecosystems and promote strong interactions within the soil biota and also between the soil and vegetation. They dig in the soil and transport the minerals above the ground and construct their nest mounds (Fig. 1.21). These are combined in a mixture of organic fibres, mineral soil and salivary secretions which resemble the organic-mineral complexes of the internal soil. In the higher Termites the nest part above ground can be compared with an iceberg, of which the visible part is only a small fraction of its total mass, which often extends over huge areas below ground. After a period of use the nests degrade and the material is added again to the soil. Thus the nest builders contribute to bioturbation. In the temperate zone, the role of termites is adopted by ants, but in North America termites can be found in Canada up to 47 ° northern latitude. In central Europe, the huge nests of the red ants are spectacular, but there are also many small nests distributed more or less evenly in soils which also contribute to soil activity and cycling of matter.

1.5 Conclusions

As mentioned above, some of the elementary steps in the breakdown of organic matter take place under the nearly constant conditions of the gut and

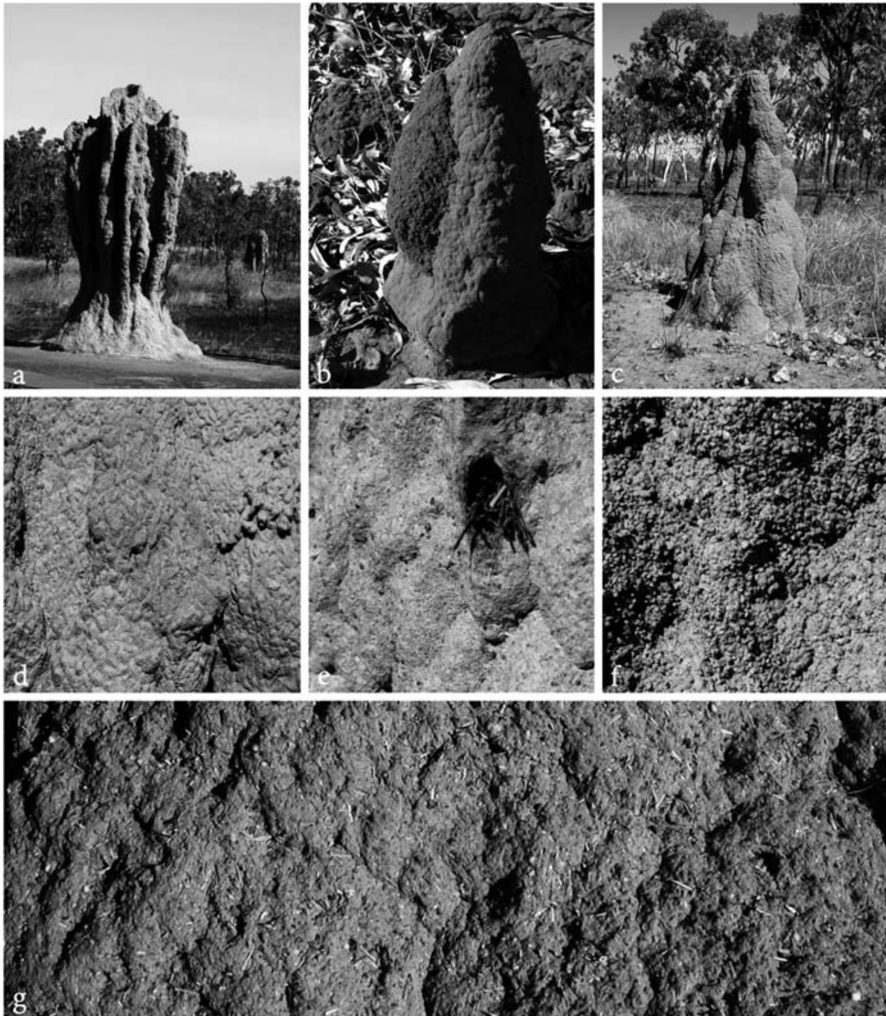

Fig. 1.21. Termite mounds in Australia: **a–c** different mound forms; **d** freshly deposited mound material; **e** mound entry with plant detritus; **f** globular mound material; **g** mound material mixed with plant fibres and soil

these are finally triggered and controlled by micro-organisms, especially bacteria. Even the earthworms have evolved a differentiated gut system (Lavelle et al. 1995) divided in foregut, midgut and hindgut, which is modified in the arthropod groups. It is present in a simple form in Collembola (Fig. 1.22), with both the foregut and the hindgut as thin tubes without any other functional subdivisions and lined only on the lumen side with a thin cuticle (intima). In between the wide open midgut, lined with a standard

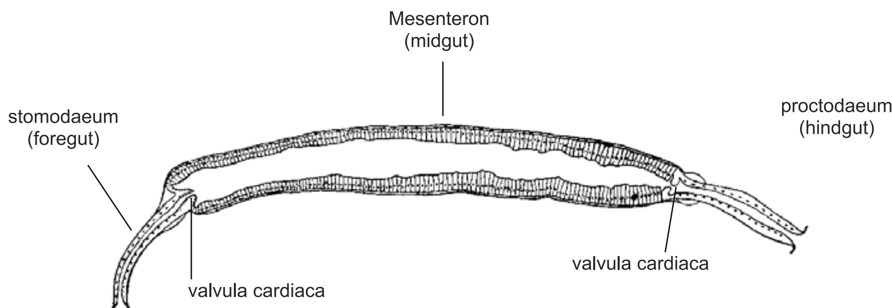

Fig. 1.22. Simple trimeric gut system of a collembolan without Malpighian tubules. (Modified according to Snodgrass 1935)

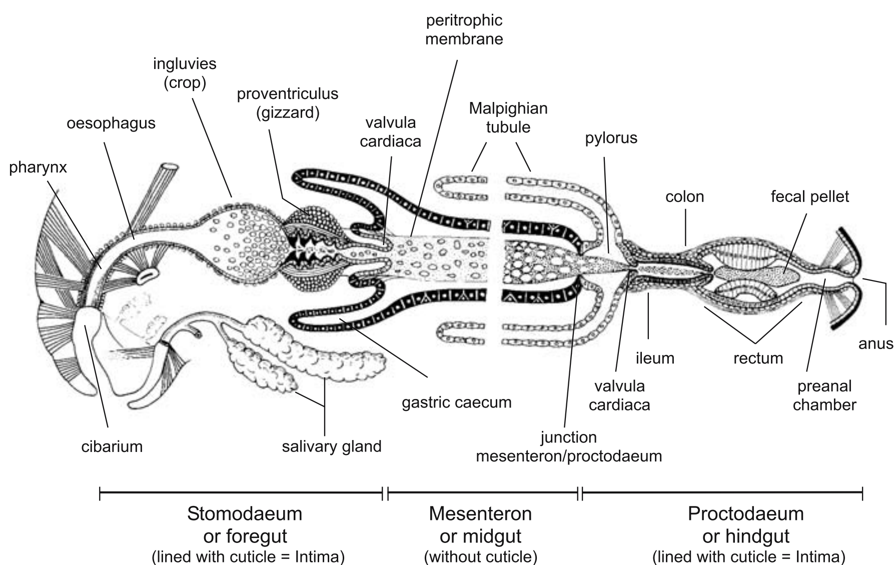

Fig. 1.23. Trimeric gut system of higher insects with many subdivisions. (Modified after Seifert 1995)

epithelium, extends with no zoning visible in the light microscope. Only in the higher insects are these main gut parts divided into functionally distinct compartments (Fig. 1.23). In the course of the foregut we can find the oesophagus, the crop (ingluvies) and the gizzard, which insure that the food is properly pretreated. In the midgut, the secretory caeca are added and along the main epithelium a functional zoning of cell types occurs. Finally, the most visible differentiation takes place in the hindgut, with pylorus, ileum, colon, and rectum, which undertake the conditioning of the faeces and which are involved in such basic functions as water balance and

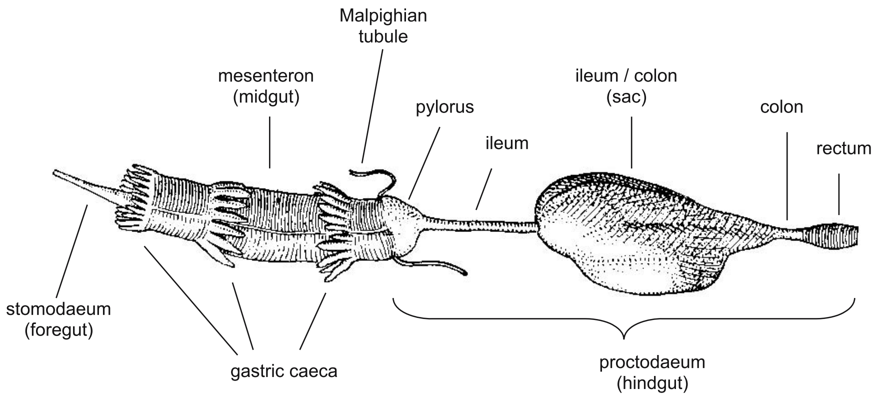

Fig. 1.24. Gut system of a beetle larva (*Oryctes nasicornis* – Coleoptera). Note the enlarged sac of the hindgut, which contains the endosymbionts

excretion. In some cases, further subdivisions are developed, such as ventricular extensions of the gut with special nutritive and digestive functions. For example, in termites the midgut is very short, although the food is composed of extremely recalcitrant material like lignin. However, the hindgut is enlarged by one single or up to five digestive chambers (sacs) which have adopted nearly all the functions of the midgut. With the help of endosymbionts (bacteria and protozoa) new modes of digestion have evolved in these insects which enable them to use the most recalcitrant polymers for nutrition (Noirot and Noirot-Thimothée 1969; König and Breunig 1997; Brune 1998). Similar modes of nutrition can be found in xylophagous beetle larvae, in which the area between ileum and colon is modified into an enlarged digestive sac (Fig. 1.24). The principle of shifting functions from the midgut to the hindgut has already been demonstrated in terrestrial isopods. There, the anterior hindgut is enlarged very strongly to a bursa, undertaking the predigestion of the chopped food until a finely ground fraction can be transported into the midgut gland for final digestion. A totally different strategy of digestion is displayed by those invertebrates in which digestion is extra-intestinal. In spiders the filtered liquid food is pumped into the branched canals of the midgut gland with the help of a special pump (suction stomach). There the final digestion, resorption and metabolic utilization of nutrients take place as well as excretion and detoxification. A hindgut is not present as in insects, instead the faeces enter a rectal bladder.

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2 Interactions Between Bacteria and Nematodes

David J. Clarke, Leo Eberl

2.1 Introduction

Bacteria can interact with nematodes in several ways: (1) they can serve as a food source and thus are merely prey, (2) they can be pathogenic for the nematode and (3) they can live in symbiosis with the nematode host. In this review, we will restrict our discussion to the pathogenic and symbiotic interactions between bacteria and nematodes.

2.2 Pathogenic Interactions

Many nematodes are free-soil organisms that feed on bacteria and it therefore may not be too surprising that various bacteria have developed defence mechanisms against nematode grazing. In fact, recent work has shown that several bacteria are capable of killing nematodes either by the production of toxic compounds or by establishing a fatal infection. This finding has led to the development of a facile model system of host–pathogen interactions to identify conserved pathways associated with microbial pathogenesis (for recent reviews see Kurz and Ewbank 2000; Aballay and Ausubel 2002). The model involves the killing of the soil nematode *Caenorhabditis elegans* by a variety of human pathogens. *Pseudomonas aeruginosa*, an opportunistic pathogen that causes chronic infections in cystic fibrosis patients, was the first bacterium whose pathogenic interaction with *C. elegans* was studied in great detail (Mahajan-Miklos et al. 1999; Tan et al. 1999a, b). Depending on the strain and the culture conditions, *P. aeruginosa* kills *C. elegans* by at least three distinct mechanisms. When *P. aeruginosa* strain PA14 is grown on nematode growth (NG) medium cells colonize the nematode intestine and killing occurs over the course of 2 to 3 days ("slow killing"). In contrast,

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PA14 grown on high-osmolarity medium (PGS) kills worms much faster (within 4 to 24 h – "fast killing") due to the production of low-molecular-weight toxins that are secreted into the culture medium. As a consequence, heat-killed bacteria are capable of fast but not slow killing. Likewise fast but not slow killing can be effected by PA14 bacterial supernatants with death of *C. elegans* occurring similarly as with bacteria. The toxic compounds were identified as phenazines a class of tricyclic pigments that includes pyocyanin. In the case of slow killing, a screen of transposon insertion mutants of *P. aeruginosa* strain PA14 revealed that the *lasR-lasI* quorum-sensing system the *lemA* and *gacA* two-component regulators and *toxA* (encoding exotoxin A) were involved in PA14 lethality. In all 19 of 23 genes identified in a screen as being involved in *C. elegans* fast or slow killing were also shown to be required for full virulence in a mouse thermal injury and infection model (Mahajan-Miklos et al. 1999; Tan and Ausubel 2000). These results provide strong evidence for a tight correlation of genes required for virulence across evolutionarily disparate hosts. More recently, it has been shown that another *P. aeruginosa* strain PAO1 kills the nematode by cyanide poisoning ("paralytic killing") when it is grown on brain-heart infusion (BHI) broth (Darby et al. 1999; Gallagher and Manoil 2001).

Over the past few years is has become evident that *C. elegans* is a highly valuable model for the study of pathogenicity of a large number of pathogens (Ewbank 2002; Mylonakis et al. 2002), including *Salmonella typhimurium* (Aballay et al. 2000; Labrousse et al. 2000) and *Serratia marcescens* (Kurz and Ewbank 2000). Several members of the genus *Burkholderia* (O'Quinn et al. 2001; Gan et al. 2002), and the Gram-positive bacteria *Enterococcus faecalis, Staphylococcus aureus, Streptococcus pneumoniae* and *Streptococcus pyogenes* kill *C. elegans* (Garsin et al. 2001; Jansen et al. 2002). Furthermore, in a recent study, it has been demonstrated that a large number of bacteria are pathogenic to L4 *C. elegans* when grown on brain heart infusion (BHI) medium (Couillault and Ewbank 2002). Among these are the plant pathogens *Erwinia chrysanthemi*, *Agrobacterium tumefaciens, Erwinia carotovora* pv. *carotovora*; the enterobacteria *Shewanella frigidimarina* and *Shewanella massilia,* the fish pathogen *Aeromonas hydrophila* as well as the insect pathogens *Photorhabdus luminescens* and *Xenorhabdus nematophila*. Of these, *A. tumefaciens*, *E. carotovora* pv. *carotovora*, *A. hydrophila* and *P. luminescens* require live bacteria for lethality, whereas *X. nematophila* does not. Recently, the *C. elegans* model has been further expanded to include fungal pathogens, particularly the model human pathogen *Cryptococcus neoformans* (Mylonakis et al. 2002).

It has also been reported that *C. elegans* can be chronically infected by *Microbacterium nematophilum* (Hodgkin et al. 2000). In this case, the bacteria adhere to the rectal and post-anal cuticle of susceptible nematodes and induce substantial local swelling of the underlying hypodermal tissue

known as the Dar (deformed anal region) phenotype. The swelling leads to constipation and slows growth in the infected worms but the infection is otherwise non-lethal.

2.3 Symbiotic Interactions

Bacteria can also live in symbiotic association with their nematode hosts. The majority of filarial nematodes including the major pathogenic species in humans *Wuchereria bancrofti, Brugia malayi* and *Onchocerca volvulus* (Taylor and Hoerauf 1999; Bandi et al. 2001) harbor bacterial endosymbionts that belong to the genus *Wolbachia*. Filarial nematodes are parasitic worms that cause some of the most devastating of all tropical diseases such as elephantiasis and river blindness. Studies on the inflammatory pathogenesis of filarial disease have shown that endotoxin-like activity derived from endosymbiotic *Wolbachia* bacteria is the major inflammatory stimulus of filarial nematodes. *Wolbachia* have so far been found in more than 20 species of filarial nematodes with only two species appearing to be uninfected (McGarry et al. 2003). Of those with *Wolbachia* the infection appears to be ubiquitous in all individuals developmental stages and populations throughout their global distribution (Taylor 2002). The bacteria reside in vacuoles and are restricted to the lateral cord cells and developmental stages within the female reproductive organs and intrauterine developmental stages as a consequence of their vertical transmission via the egg. Antibiotic depletion of bacteria shows that they are required for normal fertility and development of the worm and may even protect the parasites from host immunity. Given that filarial nematodes are major pathogens of humans throughout the tropics most research so far has focused on the contribution of *Wolbachia* to disease pathogenesis and as a novel target for antibiotic therapy (Taylor et al. 2000; Taylor and Hoerauf 2001).

Despite the obvious clinical importance of the symbiosis between *Wolbachia* and filarial nematodes, almost nothing is known about the underlying molecular mechanisms that are required for the interactions between the bacteria and the host nematode.

2.3.1 *Photorhabdus* and *Xenorhabdus*

Photorhabdus and *Xenorhabdus* are genera of Gram-negative bacteria that in addition to being pathogenic to insect larvae also have mutualistic interactions with nematodes from the families *Heterorhabditis* and *Steinernema*

respectively. The bacteria are found colonising the gut of a specialised free-living form of the nematode called the infective juvenile (IJ). The IJ migrates through the soil and infects susceptible insect larvae either by penetrating directly through the cuticle or through natural openings such as the mouth the anus and spiracles. Once inside the insect the IJ migrates to the insect circulatory system and releases the bacteria into the hemolymph. The bacteria proliferate and produce a wide range of toxins and hydrolytic enzymes that are responsible for the death and bioconversion of the insect larva into a nutrient soup that is ideal for nematode growth and development. The nematodes reproduce until the nutrient supply becomes limiting at which time they develop into IJs and are recolonised by the symbiotic bacterium.

This remarkable co-dependent reproductive cycle is the result of a highly evolved interaction between the bacterium and the nematode. The bacteria benefit from this interaction by being protected from the competitive environment of the soil and by being transported to the nutrient-rich hemolymph of an insect larva. In turn, the nematode takes advantage of the pathogenic potential of the bacteria to kill the insect host. The bacteria also supply the nutrient base for the growth and development of the nematode and suppress contamination of the insect cadaver by soil microorganisms by producing antibiotics. Recent studies in both *Xenorhabdus* and *Photorhabdus* have allowed us to identify genetic systems that play important roles in the tripartite association between the bacterium and its two eukaryotic hosts and these have been reviewed in recent publications (Forst and Nealson 1996; Forst et al. 1997; Forst and Clarke 2002; Ffrench-Constant et al. 2003). In this chapter, we will review our current understanding of the molecular mechanisms involved in the symbiosis between the bacteria and the nematode.

Although the *Photorhabdus-Heterorhabditis* and *Xenorhabdus-Steinernema* systems are remarkably similar in many ways, there is little doubt that these systems have emerged by convergent evolution. *P. luminescens* and *X. nematophilus* are the most extensively studied species of *Photorhabdus* and *Xenorhabdus* respectively and comparisons of major phenotypic traits of these bacteria reveal important differences (Forst and Clarke 2002). Several salient differences also exist between the nematode families *Heterorhabditidae* and *Steinernematidae* (Forst and Clarke 2002). The first generation adults of *Heterorhabitis* are hermaphroditic and the infective juveniles carry the symbiotic bacteria in the intestine below the basal bulb. In contrast the first generation adults of *Steinernema* exist as amphimictic males and females and the bacteria are carried in a specialised intestinal vesicle. While both nematodes belong to the Rhabditida family *Heterorhaditis* branch in the clade containing *Caenorhabditis elegans* and are most closely related to the *Strongylida* group, whilst *Steinernema* branch in a separate

clade and are more closely related to the *Strongyloides* group (Blaxter et al. 1998).

Significant differences also exist in the growth characteristics of the respective nematodes. *Steinernema* nematodes that do not contain the symbiotic bacteria (i.e. axenic nematodes) can grow on artificial medium while an artificial medium does not exist that supports axenic growth of *Heterorhabditis*. *Steinernema carpocapsae* can grow axenically and develop into infective juveniles when injected into *G. mellonella* whilst axenic *Heterorhabditis* develop into J1 progeny but these juveniles die, thus infective juveniles are not formed (Han and Ehlers 2001). Taken together, these observations support the idea that the congruence observed in the life cycles of these bacteria-nematode associations is the result of convergent evolution.

A remarkable feature of both *Xenorhabdus* and *Photorhabdus* is the occurrence of variant cell types that arise during prolonged culturing of the bacteria (Akhurst1980). The primary variant is characterised by the presence of numerous phenotypic traits that are generally greatly reduced or absent from the secondary variant (Boemare and Akhurst 1988; see Table 2.1). These traits include the formation of non-mucoidy colonies, the loss of dye-binding ability, a reduction in the amount of pigments antibiotics and siderophores produced by the bacteria. In addition, the production of proteases and lipases and in the case of *Photorhabdus* bioluminescence is also affected by phenotypic variation. Interestingly, the primary variant is always found associated with the infective juvenile and, in addition to being virulent, the primary variant supports nematode growth and development in vivo and in vitro. On the other hand, the secondary variant whilst remaining virulent to insect larvae shows a strikingly reduced ability to support nematode growth and development. The ability

Table 2.1. Phenotypes used to distinguish phenotypic variants of *Photorhabdus* and *Xenorhabdus*

Phenotypic trait	*Photorhabdus*	*Xenorhabdus*
Colony morphology	+	–
Dye uptake	+	+
Bioluminescence	+	–
Motility	+	+[a]
Pigment production	+	–
Antibiotic production	+	+
Crystal proteins	+	+
Protease production	+	–
Lipase production	+	–
Catalase	+	–

[a] Depending on strain

of *Heterorhabditis* and *Steinernema* nematodes to grow on the respective secondary variant cells is also strikingly different. *Steinernema* can grow to varying degrees on secondary cells of *Xenorhabdus* in vivo and in vitro, while *Heterorhabditis* is unable to grow on secondary cells of *Photorhabdus* (Volgyi et al. 1998; Han and Ehlers 2001; Joyce and Clarke 2003). Taken together, these findings indicate that primary-specific products and properties are involved in the symbiotic interaction between the nematode and the bacterium.

In *Photorhabdus* the primary-specific phenotypes are required for symbiosis with the nematode but do not appear to be necessary for pathogenicity, suggesting that different sets of genes (i.e. regulons) are required for each interaction (Forst and Clarke 2002; Joyce and Clarke 2003). Moreover, an understanding of how phenotypic variation is regulated could be extrapolated into an understanding of the regulatory pathways that control symbiosis. To this end, it has recently been reported that a single insertion in the genome of the secondary variant of *P. temperata* K122 that restores the production of many of the primary-specific phenotypes (Joyce and Clarke 2003). This insertion is within a gene with homology to *hexA*, a gene encoding a repressor of exoenzyme production in *Erwinia* (Mukherjee et al. 2000) and it has been shown that the *hexA*$^-$ secondary variant supports nematode growth and development, suggesting that *hexA* encodes a repressor of symbiosis. (Joyce and Clarke 2003).

Under standard laboratory conditions, it has been shown that the primary variant of *Photorhabdus* is motile whilst the secondary variant is non-motile (Akhurst 1980). We have shown that motility is important for the colonisation of the nematode gut (H.P.J. Bennettand D.J. Clarke DJ, unpubl. data), supporting the correlation between the production of primary-specific phenotypes and symbiosis. Interestingly, motility was one of the phenotypes unaffected in the *hexA*$^-$ mutant and, recently, it has been reported that motility in the secondary variant of *P. temperata* NC19 is derepressed when the bacteria are cultured under anoxic conditions (Hodgson et al. 2003). This suggests that there are least two independent signalling pathways HexA-dependent and oxygen-dependent controlling phenotypic variation and symbiosis in *Photorhabdus*. The molecular characterisation of these pathways will be important for a clear understanding of the bacterial commitment to the association with the nematode.

Steinernema can grow on secondary variants of *Xenorhabdus*, suggesting that phenotypic variation in this bacteria-nematode association is not as tightly correlated with symbiosis as it is in the *Photorhabdus*-*Heterorhabditis* system (Volgyi et al. 1998). Moreover, there is evidence suggesting that unlike *Photorhabdus* secondary variants of some *Xenorhabdus* strains are attenuated in their virulence to insect larvae (Volgyi et al. 1998). Nonetheless, genetic studies have identified loci in *Xenorhabdus* that

are involved in both phenotypic variation and symbiosis, suggesting that there is at least some degree of functional overlap (Volgyi et al. 2000). Indeed, as phenotypic variation (and thus symbiosis) in *Photorhabdus* is controlled by environmental signals, there is evidence that the expression of genes involved in the symbiotic interaction between *Xenorhabdus* and *Steinernema* is also controlled by the environment. The EnvZ-OmpR pathway has been extensively studied in *Escherichia coli* and *Salmonella*, where it has been shown to be involved in motility, biofilm formation, adaptation to acidic conditions and virulence (Pratt et al. 1996; Shin and Park 1995; Vidal et al. 1998; Lee et al. 2000). EnvZ is a membrane-spanning sensor kinase that responds to increasing osmolarity by phosphorylating the response regulator OmpR. OmpR-P then binds to DNA and alters the expression of certain genes. Functional copies of both *envZ* and *ompR* have been identified in *X. nematophila* and it has recently been shown that the *ompR* gene is required for the normal interaction between *Xenorhabdus* and *Steinernema* (Leisman et al. 1995; Tabatabai and Forst 1995; Forst and Tabatabai 1997; Boylan and Forst 2002; Kim et al. 2003). This suggests that *Xenorhabdus* is regulating the expression of genes required for symbiosis in response to uncharacterised environmental signals.

Recent studies into the association between *Xenorhabdus* and *Steinernema* have focused on the molecular mechanisms of the bacterial colonisation of the specialised vesicle in the nematode gut. It has been shown that the vesicle is initially colonised by a small number of bacteria and that these bacteria then grow inside the lumen of the vesicle (Martens et al. 2003). Moreover, growth of the bacteria in the gut appears to be limited by the nematode either through structural modification of the vesicle or nutrient availability in the gut. This highlights the active role played by the nematode in the association and suggests that there might be a cost to the nematode for supplying these nutrients, indicating that there is a strong selective pressure on the nematode to facilitate the colonisation of its gut by the bacteria. A recent genetic study identified 15 bacterial loci that were required for colonisation of the nematode by *Xenorhabdus* (Heungens et al. 2002). Several of these loci were identified as genes encoding proteins with homology to regulatory proteins in other bacteria, e.g. *rpoS rpoE* and *lrp.* These genes all encode proteins that are involved in controlling the bacterial response to environmental stresses such as starvation and membrane perturbations (Hengge-Aronis 1999; Raivio and Silhavy 2001; Helmann 2002). Other genes identified were predicted to encode proteins involved in amino acid and siderophore biosynthesis. This suggests that *Xenorhabdus* may be required to scavenge for the limited amounts of iron available in the nematode gut. Interestingly siderophore production is also required for *Heterorhabditis* growth and development when cultured with *P. temperata* NC19 (Ciche et al. 2001) and K122 (Watson and Clarke, unpublished

data) pointing to an important role for iron in controlling the mutualistic interactions in both of these systems.

2.4 Conclusions

In this review, we have described some examples of symbiotic and pathogenic interactions that occur between bacteria and soil-dwelling nematodes. Recent genetic analysis of these bacteria-nematode interactions has led to a significant increase in our understanding of the molecular mechanisms controlling how bacteria infect their hosts and, more importantly, the role of the host in determining the output of the infection, i.e. symbiotic or pathogenic. This research will continue to provide important information in our fight against pathogenic infections of humans and other mammals.

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3 Earthworm Gut Microbial Biomes: Their Importance to Soil Microorganisms, Denitrification, and the Terrestrial Production of the Greenhouse Gas N_2O

Harold L. Drake, Andreas Schramm, Marcus A. Horn

3.1 Introduction

Earthworms colonize many terrestrial ecosystems (Lee 1985; Edwards and Bohlen 1996). Although it is well established that the numerous species of earthworms contribute significantly to the cycling and physical restructuring of matter in soils and litter (Edwards and Bohlen 1996; Lavelle et al. 1997; Makeschin 1997), the gut microbial biome of this important invertebrate remains only partially resolved. The main objectives of this chapter are to (1) provide a brief overview of some of the microbial populations and processes that occur in the microbial biome of the earthworm gut and (2) address the potential importance of the in situ conditions of the earthworm gut to microorganisms that have been classically viewed as being members of soil microbial communities. Special emphasis is given to soil denitrifiers that appear to have enhanced activities in the earthworm gut and, thus, contribute to the terrestrial production the greenhouse gas N_2O via their in situ activities in the earthworm.

3.2 The Earthworm Gut as a Transient Microbial Habitat

Some earthworms are termed litter-feeding (e.g., *Lumbricus rubellus*) because they primarily inhabit and feed upon litter, while others are termed soil-feeding (e.g., *Octolasium lacteum*) because they mostly occur and feed in deeper soil layers (Edwards and Bohlen 1996; Curry 1998). Highly acidic soils do not favor the occurrence of certain earthworm species (Edwards and Bohlen 1996). As with all animals, the material ingested by the earthworm determines, in part, the chemical milieu to which the gut microbial biome is subjected. Thus, litter feeders theoretically offer a higher quality

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substrate to microorganisms in the gut than do soil feeders. However, the earthworm also contributes significantly to the chemical milieu of the gut, as will be noted elsewhere in this chapter.

It is general knowledge that many if not all gut ecosystems contain endemic microorganisms. Given the somewhat unique feeding habits of earthworms, the occurrence of endemic microbiota in their gut compartments might be thought to be of minimal importance, i.e., the ingestion of soil and litter would seem to favor the establishment of soil microorganisms as the primary gut inhabitants of the earthworm gut. Soil is arguably the most complex microbial habitat (Tate 1995) and harbors a large phylogenetic diversity of bacteria (Felske et al. 1999; Joseph et al. 2003), and it is therefore to be expected that the gut of an animal that feeds on this material will harbor a highly complex gut microbial biome. Although endemic microbes might occur in the earthworm gut, the microbial load of the earthworm gut appears to be determined primarily by the microbial load of the ingested material and, accordingly, is highly complex. However, the ecological fitness, in situ performance efficiency, and detectable diversity of ingested soil and litter microorganisms appear to be significantly influenced by the unique microenvironment of the earthworm gut during gut passage.

3.3 In Vivo and In Situ Emissions of the Greenhouse Gas N_2O by Earthworms

The gut environment of many animals is strictly anoxic or O_2 limited, and harbor anaerobic microbial biomes (e.g., see Wolin and Miller 1994; Mackie and Bryant 1994; Brune et al. 1995; Brune and Friedrich 2000; Johnson and Barbehenn 2000; Lemke et al. 2003). However, until recently, the O_2 content of the earthworm gut was unresolved (Horn et al. 2003; see the following section). Based on theoretical considerations, one might anticipate that the gut of the earthworm would be rich in anaerobic microbial processes, and that the earthworm might emit greenhouse gases indicative of such activities. For example, the emission of methane by the earthworm would be strong evidence of methanogens being active in the gut of this invertebrate. However, earthworms do not emit methane (Karsten and Drake 1997). Denitrification is another microbial process that occurs optimally under anoxic conditions and can yield the greeenhouse gas N_2O (Tiedje 1988). This gas is indeed emitted under in vivo conditions by earthworms, and the initial periods of emission are very close to linear when worms are collected and assayed for the production of N_2O (Fig. 3.1; Karsten and Drake 1997; Matthies et al. 1999). It is well known that soils are the main

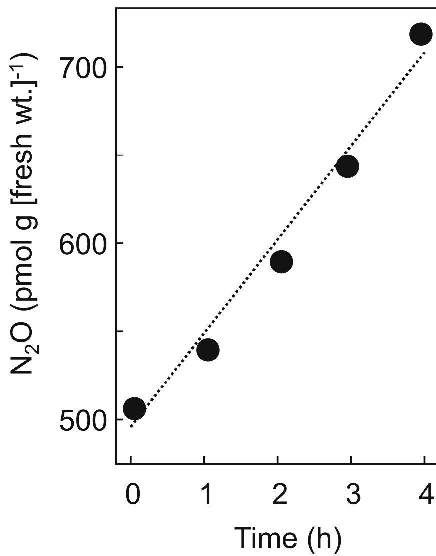

Fig. 3.1. In vivo emission of N_2O by the earthworm *Lumbricus rubellus* (collected from a beech forest soil). Data are indicative of an individual worm that has been washed with sterile water, placed in an aerated bottle that was then crimp sealed and incubated in the dark at 20 °C (Matthies and Drake, unpubl. data). The values at zero-time (t_0) for N_2O are due of the trace levels of N_2O in air

source of the global production of N_2O (Bouwman 1990; Davidson 1991; Conrad 1996), yet a direct involvement of an important soil invertebrate in this production went unnoticed until recently.

The initial rate at which N_2O is emitted by living earthworms is variable (Matthies et al. 1999). This stands to reason given the different feeding habits of earthworms, and the fact that this invertebrate enters periods of dormancy or low activity (Edwards and Bohlen 1996). Nonetheless, both soil and litter feeders can actively emit this gas. For example, specimens of *L. rubellus* (litter feeding) and *O. lacteum* (soil feeding) collected from gardens emit on average 0.65 and 0.16 nmol N_2O per hour per gram fresh weight, respectively (Matthies et al. 1999).

The discovery that living earthworms emit N_2O suggests that these invertebrates might contribute to the emission of N_2O in soils. Under experimental conditions, the rate at which N_2O is emitted from soil that contains earthworms is significantly greater than that of soil lacking earthworms (Fig. 3.2). In addition, the initial rates of emission by earthworms are similar to those calculated for eathworms in soil (Matthies et al. 1999). Thus, the N_2O emitted by earthworms is emitted from soils they inhabit. When such information is extrapolated to in situ conditions at the stand level, 16 and 33% of the N_2O emitted from forest and garden soils, respectively,

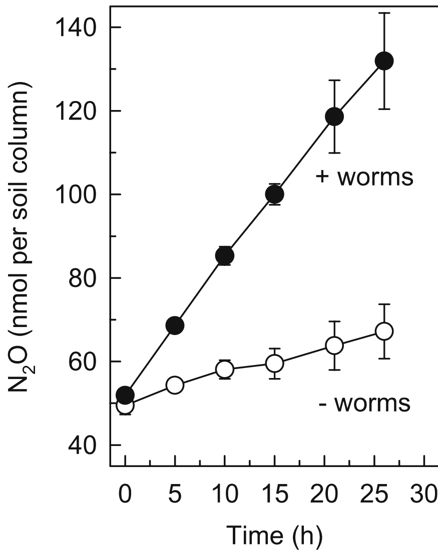

Fig. 3.2. Emission of N_2O by soil columns containing (*closed circles*) or lacking (*open circles*) earthworms. Incubation temperature was 15 °C (used with permission from Matthies et al. 1999)

might be derived from the earthworm (Karsten and Drake 1997; Matthies et al. 1999). Field studies corroborate this possibility, and the earthworm-dependent emission of N_2O can account for greater than 50% of the N_2O emitted from soils (Borken et al. 2000; Horn and Drake, unpublished data).

Earthworm biomass in soils they inhabit range from 10 to 300 g m^2 fresh weight (Waters 1955; Brady 1990). If one assumes that 20% of the terrestrial ecosystems of earth (which collectively have a surface area of approximately 1.5×10^{14} m^2 (Schlesinger 1997) is colonized by earthworms, and taking an average earthworm density of 30 g m^2 fresh weight, their total biomass in terrestrial habitats would approximate 10^{12} kg fresh weight. The rates at which earthworms emit N_2O depends on their in situ status and range from 5 to 225 ng N_2O h^{-1} per gram fresh weight (Karsten and Drake 1997; Matthies et al. 1999; Horn and Drake, unpublished data). Based on these estimations and an average emission rate of 30 ng N_2O h^{-1} per gram fresh weight earthworm, the annual global potential of earthworms to produce N_2O approximates 3×10^8 kg N_2O. Although this value is only a rough estimate, it illustrates the potential importance that just one megafauna in soil might have on the global emission of N_2O, which is estimated to be 3×10^{10} kg $year^{-1}$ (Kroeze et al. 1999). The capacity of other fauna in the global biosphere to emit N_2O due to their gut microbial biomes remains largely unknown.

The capacity of earthworms to emit N_2O decreases when they are kept for 3 days in soil treated with tetracycline and streptomycin (Matthies et al. 1999), suggesting that emission is linked to a microbiological process. Nitrate and nitrite are precursors of N_2O during denitrification (Tiedje 1988) and, surprisingly, traces of these anions on the exterior of the worm greatly stimulate the emission of N_2O by living earthworms (Matthies et al. 1999). Acetylene inhibits N_2O reductase (the enzyme that reductively converts N_2O to N_2 during denitrification (Yoshinari and Knowles 1976), and subjecting earthworms to acetylene (10 kPa) significantly increases the amount of N_2O emitted by living earthworms (Karsten and Drake 1997; Matthies et al. 1999). These observations indicate that the emission of N_2O by the earthworm is dependent on microbial processes in the gut of the earthworm, and that denitrification is important to this emission. As will be outlined in subsequent sections, the microenvironment of the earthworm gut significantly enhances the in situ activity of ingested denitrifiers during their passage through the worm.

3.4 Microenvironment of the Earthworm Gut

The gut of the earthworm constitutes a unique microenvironment in soils. Both the structure and physiochemical parameters of the digestive system of the earthworm contribute to the uniqueness of this transient habitat for soil microbes.

3.4.1 The Digestive System of the Earthworm

Most of the information on the digestive system of earthworms is derived from two species, *Lumbricus terrestris* and *Eisenia foetida*. The main components of the alimentary canal of earthworms are the mouth, esophagus,

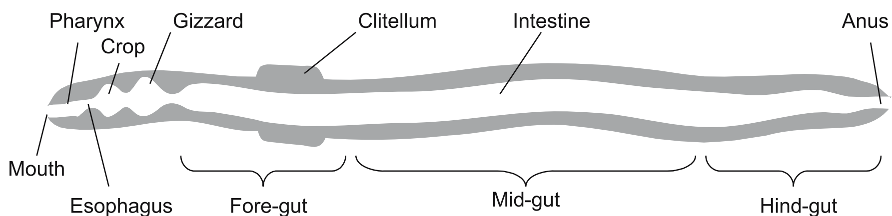

Fig. 3.3. Diagram of the digestive system of an earthworm (modified and used with permission from Horn et al. 2003)

crop, gizzard, intestine, and anus (Fig. 3.3; van Gansen 1963; Kükenthal and Renner 1982; Edwards and Bohlen 1996; Tillinghast et al. 2001). Soil and decaying organic matter (both of which contain nematodes, protozoa, and prokaryotes) enter the alimentary canal by the mouth and are amended with mucus that is derived from the pharyngeal glands. The mucus that is secreted by the pharyngeal glands contains an amylase and probably a protease. In the esophagus, calciferous, carbonic anhydrase-containing glands secrete $CaCO_3$-containing mucus into the alimentary canal, thus eliminating excess calcium and carbonates, and also regulating the pH of the coelomic fluid. The muscular gizzard is lined with a thick chitinous cuticula, and food is ground by the aid of ingested particles prior to passage into the intestine. Digestion and assimilation processes take place in the intestine. Organic matter is degraded by the collective activity of digestive enzymes (e.g., chitinases, xylanases, cellulases, proteases, amylases, and lipases) that are secreted by the worm and ingested microorganisms (Urbasek 1990; Urbasek and Pizl 1991; Lattaud et al. 1997a,b, 1998; Merino-Trigo et al. 1999; Garvin et al. 2000). Gut epithelial cells assimilate nutrients. In the hindgut, undigested components of ingested matter are enveloped by a peritrophic membrane that lines the intestine and are subsequently excreted.

3.4.2 Physicochemical Parameters of the Gut that Stimulate Ingested Microbes

The gut is rich in readily available nutrients. Some of the soil microorganisms that are ingested by earthworms constitute a part of their diet (Edwards and Bohlen 1996); the selective digestion of microbes in the gut (Sect. 3.6.1) influences the type of nutrients that are available for subsequent assimilation by both the earthworm and members of the gut microbial biome. Earthworm mucus contains large amounts of water-soluble organic carbon (Martin et al. 1987) that can be easily degraded by microorganisms; many of the organic molecules in gut contents appear to be derived from mucus (Horn et al. 2003). Thus, the earthworm gut environment is theoretically very favorable for enhancing the activity of soil microorganisms during gut passage, and microorganisms are hypothesized to become active in the gut and participate in the breakdown of complex organic matter by excreting degradative exoenzymes (Barois and Lavelle 1986; Lavelle et al. 1995). Because both ingested microorganisms and earthworms profit from the earthworm's secretion of mucus, the collective degradative processes in the gut can be viewed as a "mutualistic digestive system" (Barois and Lavelle 1986; Lavelle et al. 1995).

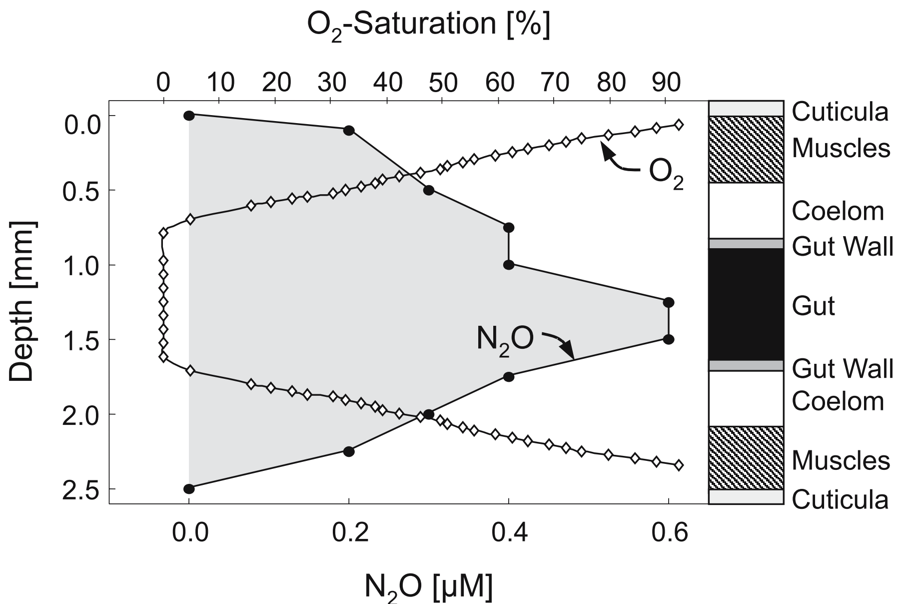

Fig. 3.4. N_2O (*closed circles*) and O_2 (*open squares*) profiles at the mid-gut region of an ethanol-sedated *Lumbricus rubellus*. The *right axis* identifies the anatomical regions of a crosscut through a worm (used with permission from Horn et al. 2003)

Microorganisms in the gut experience an anoxic environment with high concentrations of N_2O (Horn et al. 2003). Steep opposing gradients of O_2 and N_2O occur from the cuticula to the coelom, and the core of the anoxic gut is the main site of N_2O production in the earthworm (Fig. 3.4). In a longitudinal transect, *L. rubellus* had in situ N_2O concentrations in the gut of 2.7 μ mol l^{-1} behind the gizzard, 5.6 μmol l^{-1} in the midgut region, and 0.2 μmol l^{-1} near the anus (Horn et al. 2003). Thus, conditions in the fore- and mid-gut regions maximize the processes associated with the net production of N_2O.

The water content of gut content is 40 – 90% and is approximately twofold greater than that of soil (Barois and Lavelle 1986; Trigo and Lavelle 1993, 1995; Horn et al. 2003). In general, the water content decreases from anterior to posterior along the alimentary canal. The pH is near neutral throughout the intestine and less variable among earthworms than is the soil they inhabit, indicating that a pH-homeostasis exists in the earthworm gut (Horn et al. 2003). The pH increases slightly from the anterior to the middle part of the gut or remains essentially constant and subsequently decreases again towards the anus (Lee 1985; Barois and Lavelle 1986; Trigo and Lavelle 1993, 1995; Horn et al. 2003). High concentrations of total carbon, organic

carbon, and total nitrogen occur in the gut, resulting in a C/N-ratio of approximately 7 for gut contents, a value that is much lower than that of many soils (Piearce 1978; Trigo and Lavelle 1993, 1995; Karsten and Drake 1995; Michel and Matzner 2002; Horn et al. 2003).

High quality electron donors like glucose (up to 80 $mmol l^{-1}$) are abundant in the aqueous phase of gut contents (Karsten and Drake 1995; Horn et al. 2003). Many organic compounds including formate (average 5 $mmol\,l^{-1}$), acetate (average 5 $mmol\,l^{-1}$), lactate (average 2 $mmol\,l^{-1}$), and succinate (average 1 $mmol\,l^{-1}$) that are indicative of anaerobic metabolism are likewise found in the aqueous phase of gut contents. Sugars and organic acids in the aqueous phase of soil are usually below 0.1 $mmol\,l^{-1}$. Concentrations of free and total amino acids are 0.2 – 0.6 and 0.7–1.7 $mmol l^{-1}$, respectively, in the aqueous phase of earthworm gut contents, values that are approximately 40 times higher than those in soil (Horn et al. 2003). Ammonium concentrations in the aqueous phase of gut contents are approximately 10 $mmol\,l^{-1}$, which is markedly greater than that of soil (Tillinghast et al. 2001; Horn et al. 2003). Nitrate concentrations in soil are often higher than in the earthworm gut, whereas nitrite concentrations are higher in the gut, indicating that nitrate is consumed and nitrite is produced in the earthworm gut (Horn et al. 2003).

3.5 Microbial Processes in the Earthworm Gut

The availability of electron donors, a water content of 40 – 90%, the high concentrations of ammonium and amino acids, a near neutral pH, and anoxia favor anaerobic microbial activities in the gut of earthworms. Indeed, the abundant N_2O and fermentation products in the gut, as well as the comparatively low concentrations of nitrate in gut contents, indicate that nitrate-reducing and fermentative microorganisms are highly active in the gut of this invertebrate.

3.5.1 Processes Associated with the Production of N_2O

Most of the N_2O that is produced globally is derived from biotic processes. Denitrification, dissimilatory nitrate reduction to nitrite and/or ammonium, and nitrification are microbial processes that can lead to a significant production of this greenhouse gas (Vancleemput et al. 1976; Christianson and Cho 1983; Davidson 1992; Conrad 1995).

During complete denitrification, inorganic nitrogenous compounds like nitrate or nitrite are reduced to N_2 with N_2O as a concomitantly released

intermediate (Zumft 1997). Both earthworm gut homogenates and soil facilitate the conversion of nitrate to N_2 (Fig. 3.5). However, denitrification is much more rapid with gut-derived material than with soil. Nitrite and N_2O transiently accumulate in gut content microcosms but are essentially absent in soil microcosms. Additional electron donors do not affect the production of N_2O by nitrate-supplemented gut content microcosms (Ihssen et al. 2003), evidence that microbes in the gut are not limited in sources of readily utilizable reductant. Gut wall-associated microorganisms appear to be of minimal importance to the production of N_2O by gut homogenates, as evidenced by the inability of gut wall homogenates to produce N_2O when supplemented with glucose and nitrate at concentrations indicative of in situ conditions. N_2O-production rates by gut contents are consistently higher with supplemental nitrite than with supplemental nitrate. The ability of earthworms to emit N_2O in vivo is likewise more greatly stimulated by traces of nitrite on the exterior of the body than by traces of nitrate (Matthies et al. 1999; Sect. 3.3). Interestingly, earthworm casts also produce N_2O and display higher denitrification potentials than do adjacent soils (Scheu 1987; Mulongoy and Bedoret 1989; Elliott et al. 1990, 1991; Matthies et al. 1999). These collective observations indicate that denitrification in the gut content is the primary source of the N_2O that is emitted by earthworms.

Net release of N_2O during denitrification is regulated by many parameters, including pH, the availability of O_2, and the concentrations of electron donors, nitrate, and nitrite (Sahrawat and Keeney 1986; Ferguson 1994; Baumann et al. 1996; Kester et al. 1997a,b). The earthworm gut is an anoxic environment with a near neutral pH, high concentrations of ammonium, and water-soluble, high quality electron donors (Sect. 3.4.2). Thus, ingested soil microorganisms are subjected to conditions that increase the production rates of N_2O by pure cultures of denitrifying bacteria (Baumann et al. 1996, 1997; Otte et al. 1996). O_2 represses denitrification and the availability of O_2 is an important in situ parameter in the regulation of this process (Tiedje 1988). The production of N_2O by soil is greatly enhanced under gut-like conditions (i.e., conditions that simulate those that soils experience during passage through the earthworm gut), and anoxia appears to be the main in situ parameter regulating the production of N_2O in the gut (Horn et al. 2003).

Apart from denitrification, the dissimilatory reduction of nitrate to nitrite and/or ammonium might also contribute to the production of N_2O in the earthworm gut. N_2O is produced as a side product during the dissimilatory reduction of nitrate, presumably by the unspecific interaction of nitrate reductase with nitrite (Tiedje et al. 1982; Smith 1983). This reaction should be favored by the high concentrations of electron donors and nitrite in the earthworm gut. However, the dissimilatory reduction of nitrate to ammonium appears to be of minor importance for the production of

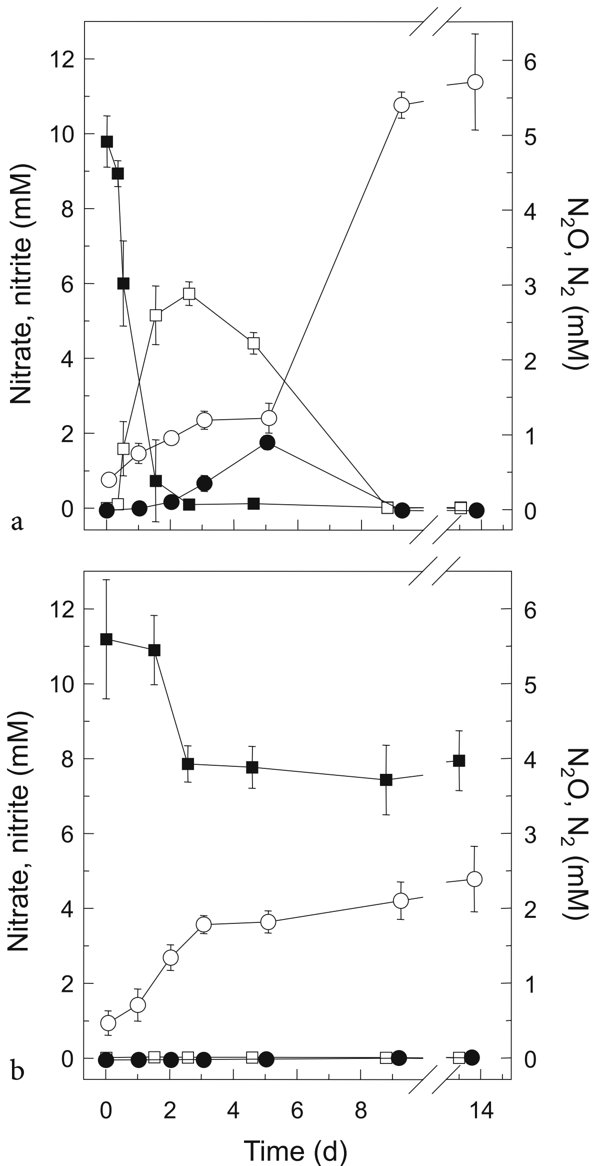

Fig. 3.5. Utilization of supplemental nitrate by: **a** earthworm (*Aporrectodea caliginosa*) gut content; **b** soil homogenates under anoxic conditions at 15 °C. Homogenates contained 24 and 161 mg (dry wt.) gut content or soil, respectively, per ml sodium phosphate buffer (15 $mmol l^{-1}$, pH 7). Organic carbon contents were 1.3 and 5.9 mg/ml gut content and soil homogenate, respectively. Symbols: *filled squares* nitrate; *open squares* nitrite; *filled circles* N_2O; *open circles* N_2 (used with permission from Ihssen et al. 2003)

N_2O in the gut, as evidenced by the apparent absence of this dissimilatory process in gut homogenate microcosms (Ihssen et al. 2003). Furthermore, initial production rates of N_2O by denitrifying isolates from the earthworm gut are approximately 30-fold higher than those of non-denitrifying, N_2O-producing isolates (Ihssen et al. 2003). Nitrite can accumulate as a transient product during the dissimilation of nitrate (Tiedje 1994). Non-denitrifying nitrate dissimilators in the earthworm gut might produce nitrite and contribute to the high nitrite concentrations in the earthworm gut, thereby enhancing the production of N_2O by nitrite-consuming denitrifiers.

Nitrification is an aerobic process by which ammonia is oxidized sequentially to nitrite and nitrate. N_2O is a side product when ammonia is oxidized to nitrite via the intermediate hydroxylamine (Ritchie et al. 1972; Webster and Hopkins 1996; Bollmann and Conrad 1998). The earthworm gut is anoxic, and nitrification would be impaired in the gut (though low amounts of this process could theoretically take place at the gut wall if O_2 leaked out of epithelial cells). Nitrification is presumably not a major source of N_2O in the gut of earthworms.

3.5.2
Fermentative and Other Microbial Processes

Anoxia and high quality electron donors should theoretically promote fermentative processes in the earthworm gut. Diluted earthworm gut homogenates produce ethanol, formate, butyrate, acetate, H_2, and succinate from glucose or cellobiose, evidence that the gut harbors a complex fermentative microbial biome (Fig. 3.6; Karsten and Drake 1995). The kinetics of fermentation is much faster for earthworm gut homogenates than for soil homogenates, indicating that the fermentative microbes in the earthworm gut are poised at a more active state than are those of soil. Many fermenters are known to produce exoenzymes that might aid the worm in digesting complex organic matter (Bergey et al. 1990). H_2 and acetate, both typical methanogenic precursors, are not consumed in anoxic incubations of earthworm gut homogenates, indicating the absence or inactivity of methanogenic microbial populations in the earthworm gut. Indeed, methane is essentially not produced by earthworms, their gut homogenates, or their casts (Hornor and Mitchell 1981; Karsten and Drake 1995). H_2-utilizing acetogens are likewise negligible in the earthworm gut (Karsten and Drake 1995), despite the fact that conditions in the gut are ideal for acetogenesis (Drake et al. 2004). Due to the absence of an H_2-scavenging processes, the partial pressure of H_2 in the earthworm gut is possibly too high for organic acid-utilizing secondary fermenters that rely on an efficient removal of H_2 (McInerney and Bryant1980). The assimilation of

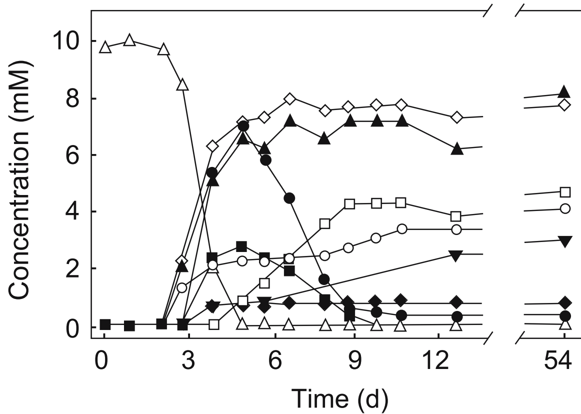

Fig. 3.6. Products formed from glucose under anoxic conditions by a gut section homogenate from *Lumbricus rubellus*. Symbols: *open triangles* glucose; *filled circles* lactate; *filled upright triangles* ethanol; *open diamonds* formate; *open circles* acetate; *open squares* butyrate; *filled diamonds* succinate; *filled squares* pyruvate; *filled inverted triangles* H_2. The amount of inoculum represented a 1 to 3000 dilution of gut material. Similar product patterns were obtained when cellobiose was used instead of glucose (used with permission from Karsten and Drake 1995)

microbial fermentation products by the earthworm is an important feature of the mutualistic digestion system of this invertebrate (Lee 1985; Lavelle et al. 1995; Trigo et al. 1999).

The consumption of aromatic compounds like the lignin derivative ferulate is more rapid in anoxic earthworm gut homogenates than in soil homogenates (Karsten and Drake 1995). Acetate, formate, and hydroferulate are produced from ferulate, and ring cleavage may occur in the earthworm gut under anoxic conditions (Karsten and Drake 1995). Microorganisms in the earthworm gut might thus enable the worm to obtain lignin-derived volatile fatty acids.

N_2-fixing organisms are found in the gut of earthworms (Citernesi et al. 1977; Striganova et al. 1993; Risal et al. 2002) and casts (Simek et al. 1991). However, the fixation of N_2 in the earthworm gut appears to be highly variable among individual earthworms and different earthworm species (Kaplan and Hartenstein 1977).

3.6 Microbial Populations in the Earthworm Gut

Most investigations of the microbial biome of the earthworm gut have centered on two questions: (i) how does gut passage influence the number and

community composition of the ingested soil microbes, and (ii) is there an endemic, earthworm-specific microbial population in the gut? This section highlights observed trends and recent developments that address these questions with respect to the special microenvironment of the earthworm gut (Sect. 3.4.2).

3.6.1 Quantitative Population Changes During Gut Passage

There seems to be general agreement that bacteria comprise only a minor constituent of the diet of earthworms (Edwards and Fletcher 1988; Edwards and Bohlen 1996). Thus, and in contrast to ingested protozoa (Bonkowski and Schaefer 1997; Cai et al. 2002), filamentous fungi (Schönholzer et al. 1999), and other eukaryotic microbes (von Aichberger 1914) that are mostly degraded during gut passage, ingested bacteria appear to increase in number when passed through the gut. However, the extent of that increase depends on the method used to detect it. With few exceptions (Dawson 1948; Day 1950; Kristufek et al. 1992), cultivation-based studies report 10- to 1000-fold greater numbers of different bacterial groups, e.g., aerobes (Bassalik 1913; Stöckli 1928; Kollmannsperger 1952; Ruschmann 1953; Parle 1963a,b; Edwards and Fletcher 1988; Pedersen and Hendriksen 1993; Fischer et al. 1995; Heijnen and Marinissen 1995; Karsten and Drake 1995), general anaerobes (Karsten and Drake 1995; Ihssen et al. 2003), and denitrifying and nitrate-dissimilating bacteria (Karsten and Drake 1997; Ihssen et al. 2003) in the earthworm gut when compared to soil from which the worms were obtained. Such differences in populations are also observed when hindgut contents are compared to foregut contents. Slight differences in populations can be attributed to different worm species, soils, food sources, or the fact that some studies compare soil and (fresh) casts rather than directly investigate the various gut compartments. However, the overall trend is clear and strongly suggests that there is a significant increase in the cultured bacteria during gut passage. It is thus in marked contrast that direct microscopic cell counts reveal only a minor difference in the total numbers of bacteria in soil and gut-derived materials (usually between 1.5- to 5-fold (Kristufek et al. 1992; Pedersen and Hendriksen 1993; Schönholzer et al. 1999; Wolter and Scheu 1999), and in some cases no difference at all (Kristufek et al. 1992; Heijnen and Marinissen 1995; Schönholzer et al. 2002). That bacterial biomass does not always increase during gut passage (Scheu 1987; Daniel and Anderson 1992; Devliegher and Verstraete 1995; Schönholzer et al. 2002) is likewise a paradox relative to information obtained with cultivation methods.

A comparison of cultured and total cell counts suggests that gut passage does not lead to massive multiplication of cells but rather renders bacteria more susceptible to cultivation, possibly by activation in the favorable conditions of the earthworm gut (Sect. 3.4.2), as has been proposed several times (Martin et al. 1987; Trigo and Lavelle 1993; Fischer et al. 1995; Karsten and Drake 1995; Lavelle et al. 1995; Ihssen et al. 2003). The few studies that have determined both total and live bacterial counts indeed indicate that the culturability of ingested microbes increase upon gut passage (Kristufek et al. 1992; Pedersen and Hendriksen 1993; Fischer et al. 1995). Gut passage also enhances the germination of *Bacillus* spores (Fischer et al. 1997) and increases the detectability of bacteria by fluorescence in situ hybridization (FISH) (Fischer et al. 1995; Schönholzer et al. 2002). Since the detection of cells by FISH depends on the cellular ribosome content, which in turn is often correlated with the metabolic activity of the cell (Amann et al. 1995), increased FISH detectability also indicates the activation of bacterial cells inside the earthworm gut.

In summary, the earthworm gut is a favorable habitat for numerous soil bacteria. In the gut, ingested microbes may switch from spores or dormant states to metabolically active cells that are more readily culturable and detectable by FISH. However, the time required for gut passage is too short (usually less than 24 h (Parle 1963b; Edwards and Bohlen 1996) for massive proliferation (Fischer et al. 1995; Ihssen et al. 2003); hence the total numbers of ingested bacteria increase only marginally.

3.6.2 Qualitative Population Changes Upon Gut Passage

Although most if not all bacteria of the earthworm gut are derived from ingested soil (Edwards and Bohlen 1996; Furlong et al. 2002; Singleton et al. 2003; Egert et al. 2004) and their total numbers increase only slightly during gut passage, major changes of the microbial community structure in the gut might still occur during gut passage. Large, metabolically active cells are preferentially digested in the digestive tract of the worm before they reach the foregut (Schönholzer et al. 2002), and different functional or phylogenetic groups of microorganisms respond differently to the gut environment. For example, nitrate dissimilating and denitrifying bacteria appear to be readily activated in the gut, as evidenced by their 300-fold increase in cultured numbers (Karsten and Drake 1997; Ihssen et al. 2003), and isolates from fresh casts are more likely to reduce nitrate than soil isolates (Furlong et al. 2002). In contrast, cultured numbers of ammonia-oxidizing bacteria and methanogens in gut contents and soil are similar (Ihssen et al. 2003), and enrichments for these microbial groups failed

(Khambata and Bhat 1957; Karsten and Drake 1997), indicating that the transient conditions of the gut do not stimulate these microbes. FISH analyses showed a shift from beta- and gamma-Proteobacteria to members of the *Cytophaga-Flavobacterium* group (Schönholzer et al. 2002); organisms from this group thrive on complex organic matter under anoxic conditions (Rosselló-Mora et al. 1999), i.e., conditions that are provided in the earthworm gut. Data from 16S rRNA gene clone libraries (Furlong et al. 2002) and terminal-restriction fragment length polymorphism (T-RFLP) of 16S rRNA gene fragments (Egert et al. 2004), though not truly quantitative information, also indicate that selective changes occur in the microbial community structure (e.g., increased abundance of some phylotypes) of the soil microbial biome during gut passage. The differential enhancement of the culturability of anaerobes versus aerobes in gut contents (Karsten and Drake 1995; Ihssen et al. 2003) is further evidence that the in situ conditions of the earthworm gut creates qualitative alterations in the status of ingested microorganisms.

3.6.3 The Quest for an Earthworm-Specific Microbial Population

The earthworm gut harbors a large diversity of bacteria (e.g., Bassalik 1913; Edwards and Bohlen 1996; Furlong et al. 2002; Ihssen et al. 2003). Virtually all the bacteria isolated from different species of earthworms are either identical, very similar, or at least very closely related to known soil organisms, including an *Aeromonas* species once thought to be indigenous to the earthworm *Eisenia foetida* (Toyota and Kimura 2000). To date, none of the isolates obtained from the earthworm gut meets the criteria for an earthworm-specific association (e.g., to occur in all worms of a certain species, irrespective of feeding conditions, but not in soil). Likewise, cultivation-independent analyses of 16S rRNA clone libraries (Furlong et al. 2002; Singleton et al. 2003) or T-RFLPs (Egert et al. 2004) do not reveal any signs of indigenous microorganisms in the gut of earthworms. Earthworm gut wall-attached microbes (filaments and rods) have been observed by electron microscopy (Jolly et al. 1993; Vinceslasakpa and Loquet 1995; Mendez et al. 2003). However, without an in situ identification of the attached organisms, and in the absence of additional molecular confirmation, the endemic nature of these microbes in the gut of earthworms remains unresolved.

Earthworms do contain endemic, species-specific microbial symbionts in the nephridia (Fig. 3.7). Originally described on the basis of microscopic observations (Knop 1926), the symbionts are members of a monophyletic branch of the genus *Acidovorax* (Schramm et al. 2003). They might be

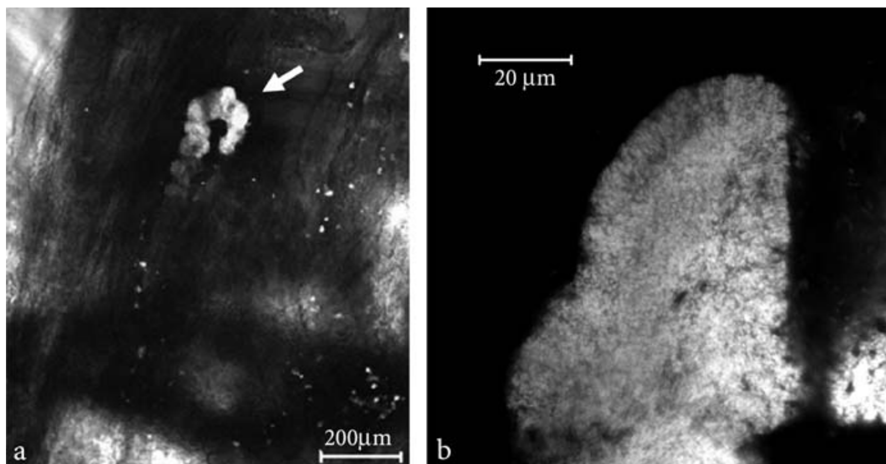

Fig. 3.7. In situ detection of bacterial symbionts in the nephridia of *Lumbricus terrestris* by hybridization with the *Acidovorax*-specific probe LSB145. **a** Ampulla (the hook-like structure, marked by *the arrow*) of the nephridium, densely packed with stained bacteria; **b** close-up of the ampulla, showing the biofilm-like arrangement of bacterial cells. See Schramm et al. 2003 for additional microscopic images

proteolytically active during excretion, facilitating the absorption of peptides and amino acids by the host (Pandazis 1931). Whether this association is a true mutualistic symbiosis, and to what extent it influences nitrogen excretion by earthworms and thus nitrogen cycling in soil remain open questions.

3.7 Conclusions

The feeding habits of the earthworm are important to the global turnover dynamics of terrestrial matter. The feeding habits and ecophysiological nature of the earthworm also create significant transient modifications of ingested matter. Thus, the gut of this animal provides a unique mobile microniche in certain terrestrial ecosystems for microbial processes. Many of these microbial processes (e.g., denitrification) appear to occur at highly accelerated rates during the passage of soil through the gut of the earthworm. The discovery that N_2O is emitted in vivo by earthworms and that this N_2O is derived from microbial processes in the gut is evidence that certain microbial processes linked to the production of this greenhouse gas do not occur exclusively in soil but can also occur in the gut of this soil-inhabiting animal (Fig. 3.8). Current evidence suggests that the contribution of earthworms to the emission of N_2O at the stand level is not

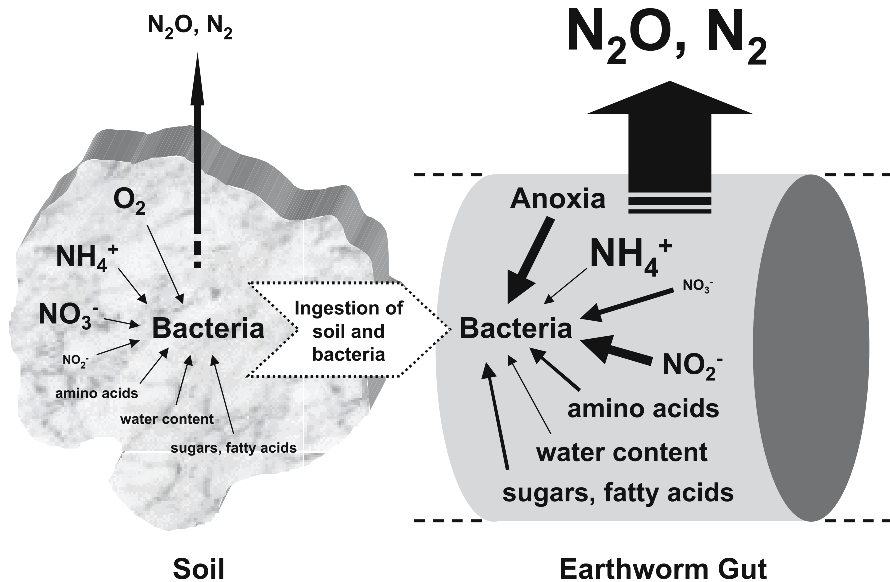

Fig. 3.8. Hypothetical model illustrating which factors stimulate the production of N_2O and N_2 by bacteria ingested into the gut of the earthworm. The relative concentrations of compounds are reflected in the font sizes, and the relative effect of each compound on the production of N_2O and N_2 in the gut is indicated by the thickness of *the arrows* (modified and used with permission from Horn et al. 2003)

trivial and can constitute the majority of this greenhouse gas emitted by soils they inhabit.

The apparent enhancement of denitrification by soil microbes during gut passage indicates that earthworms might be important to the emission of N_2 from certain terrestrial ecosystems (Fig. 3.8). Although this remains an unexplored possibility, the magnitude of earthworm-derived N_2O is enhanced three- to sevenfold when worms are exposed to acetylene (Karsten and Drake 1997; Matthies et al. 1999), and microbes in gut contents have the capacity to produce significant amounts of N_2 (Karsten and Drake 1997; Ihssen et al. 2003). Though speculative, it can thus be projected that N_2 rather than N_2O is the predominant nitrogenous gas emitted by earthworms. If this is true, earthworms may be an heretofore unknown important component of the terrestrial nitrogen cycle.

The unique microenvironment of the earthworm gut not only impacts on the catabolic activities of ingested soil microorganisms but also appears to contribute to their growth potentials. Indeed, ingested soil microorganisms that display limited activity and are difficult to culture become metabolically robust and culturable post-ingestion. Thus, it is very likely that both the viability and diversity of the prokaryotic community of

earthworm-colonized soils would decrease if the soil microbial biome did not periodically become the transient gut microbial biome of the earthworm. While there is much to learn about the microbiology of the earthworm, the observations outlined in this chapter demonstrate that this rather simple and largely unseen animal harbors a fascinating microbiological story. We look forward to future studies aimed at understanding the importance of this microniche to the ecological functions and in situ fitness of soil microorganisms.

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4 Intestinal Microbiota of Millipedes

Boris A. Byzov

4.1 Introduction

Soil millipedes (Diplopoda) possess a specific gut microbiota that differs from microbial communities in soil and leaf litter. A diverse microbiota has been found by the dilution plating in the gut with gamma proteobacteria, actinobacteria and yeasts most abundant. Microscope studies also revealed a variety of morphotypes of bacteria and yeasts attached to the gut walls. Evidence is discussed that the millipedes have symbiotic associations with microorganisms that have particular functional roles for the host animal. The possible functions of gut microorganisms are the participation in the digestive processes, the maintaining of microbial community in a steady state, in vivo production of methane. The “killing effect” (a lytic process) is discussed as one of the possible mechanism for the digestion of microorganisms by millipedes. It is most likely that the saprophagous millipedes do not harbour lignocellulose-degrading microorganisms; most likely, they feed on microorganisms and use microbial enzymes to digest recalcitrant molecules.

Scientific interest in millipedes is largely related to their participation in organic matter decomposition and nutrient cycling. These processes are mediated in soil by activities such as fragmentation of leaves, stimulation of microbial growth, and, subsequently, deposition of faecal pellets (Hanlon 1981). There are few habitats in which millipedes are responsible for ingesting more than 5–10% of the annual leaf litter fall; however, when earthworms are scarce, millipedes may occur at densities of several hundred/m^2 and consume 25% of the litter fall (Hopkin and Read 1992). Although millipedes (and other saprophages altogether) are directly responsible for less than 10% of chemical decomposition, their feeding activities are important in stimulating soil microorganisms, which carry out 90% of chemical breakdown (Anderson and Bignell 1980). This aspect

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of microbial-millipede interactions is vital in studying the importance of invertebrates in food web functioning.

The nature of the gut microbiota of millipedes is probably less important in terms of ecosystem related processes. However, the knowledge of species diversity of gut symbionts of soil invertebrates and their functional importance for the host animals are scare (except for termites) although millipedes, and many other soil invertebrates, have a specific digestive system that involves interaction with gut-associated microbes.

In this chapter, an attempt is made to present and discuss the data on morphological and taxonomic diversity of the millipede intestinal microorganisms and their possible functions.

4.2 Structure and Function of the Digestive Tract

The foregut of millipedes is poorly populated by microorganisms. The midgut represents the absorptive surface and continuously secretes the semipermeable peritrophic membrane. The peritrophic membrane has a highly ordered net-like structure composed of microfibres. In *Glomeris marginata* the fibres are arranged in uniformly sized squares of 0.1 μm (Martin and Kirkham 1989), in *Pachyiulus flavipes*, 0.05–0.1 μm (Byzov et al. 1993c), and in *Glomeris connexa,* 0.15–0.2 μm in size (Byzov et al. 1996). The membrane encloses the solid food materials preventing their direct contact with the midgut epithelium. There is no direct evidence that the peritrophic membrane represents a barrier to microorganisms; however, the small size of holes in the membrane would also prevent access to the midgut epithelium by the majority of microorganisms in the gut contents. The membrane is an envelope composed of chitin, protein and mucopolysaccharides. In electron microscopic studies, the membrane first appears amongst the microvilli or at their tips. Chitin appears at the bases of the microvilli along the whole length of the midgut. Protein is added to the chitin from the microvilli as the peritrophic membrane moves along the microvilli to the lumen. The completed peritrophic membrane extends around individual items in the gut contents as well as forming a multilayered envelope. The peritrophic membrane is continuously secreted, and moves towards the hindgut. More commonly, the membrane is broken up in the hindgut by muscular contractions or spiny projections of the cuticle. Disruption of the peritrophic membrane may be of importance in allowing ingested organisms access to colonization sites on the hindgut cuticle. It was also suggested that the disruption of the membrane permitted some parasites to reach the mesenteric epithelium by forward migration along

the ectoperitrophic space. The functions of the peritrophic membrane are not fully known, but its most obvious purpose is to protect the absorptive surfaces in the absence of mucus secretion (Bignell 1984; Hopkin and Read 1992). The semi-permeable structure of the membrane makes it probable that digestion is sequenced radially, with partial degradation of macromolecules only taking place in the endoperitrophic space. The hydrolysis to dimers and oligomers occurs in the ectoperitrophic space separating the membrane and the midgut epithelium, and some terminal digestion occurs intracellularly or mediated by enzymes bound to the apical cell membrane (Hopkin and Read 1992).

The cuticle-lined hindgut is strongly developed and bears both flat cuticular surfaces and ornaments such as spines of various shapes, which provide sites for microbial colonization. In *Orthoporus ornatus,* the cuticle of the hindgut is formed into numerous projections, each of which has central depression in which bacteria may reside (Crawford et al. 1983). Malpighian tubules open at a junction point between midgut and hindgut. Their function is to deliver to both the midgut and the hindgut a fluid containing many of the haemolymph constituents at concentrations proportional to their concentrations in the haemolymph. The secretion of the Malpighian tubules constitutes not only a nutrient broth of balanced composition but also an effective buffer solution. In physiological terms, this may imply that the hindgut of millipedes offers a suitable site for microbial colonization. The study made by scanning electron microscope confirmed that an abundant and diverse microflora occurred in this location. Additional fluid input to the gut takes place via salivary glands and accompanies ingestion of food (Bignell 1984).

The Malpighian tubules are also involved in excretion of nitrogenous wastes. In millipedes, two forms of nitrogenous wastes predominate: ammonia (which has to be excreted rapidly) and uric acid (the stored form). Urea is detected less frequently. The proportion of ammonia and uric acid varies between species. For example, in *Cylindroiulus londinensis,* only 20% of non-protein was ammonia whereas 70% was uric acid. Of the total non-protein nitrogen in the faeces of *Glomeris marginata*, 40% was ammonia and 33% was uric acid (Hopkin and Read 1992). However, Anderson and Ineson (1983) were unable to detect any uric acid in the faeces of *Glomeris marginata* although the animals themselves contained 2.5% uric acid by weight. The authors have suggested that uric acid might form a reserve of nitrogen for use when food contains insufficient amount of the element. The release of the uric acid in the hindgut may also provide source of nitrogen for gut microflora (Byzov et al. 1993b).

For more details related to the anatomic structure and functions of the millipede intestine, see Hopkin and Read (1992).

4.3 Physiological Conditions in the Gut

The redox potentials, determined with a platinum and calomel electrode combination, in the midgut of *Glomeris marginata* vary between +267 and +307 mV and in its hindgut between +167 and +277 mV. Mean redox potentials in the lumen of the digestive tract are +232 mV in the midgut and +204 mV in the hindgut (Bignell 1984). Therefore, the gut environment is not an anaerobic but has moderate reduced conditions (microaerophilic). The pH of the contents of the lumen of the midgut of millipedes is rarely more than one unit either side of neutrality (pH 6 to 8) (Bignell 1984). Byzov et al. (1998) found the mean pH of the midgut fluid of the diplopod *Pachyiulus flavipes* to be 5.6.

Concentrations of glucose were found to be 6.0 mmol/l (1.1 g/l) in the midgut fluid of the diplopod *Pachyiulus flavipes*, whereas in the hindgut extract it was 12.7 mmol/l (2.3 g/l). In the midgut fluid of *Rossiulus kessleri* the concentration of glucose was 7.1 (1.28 g/l) mmol/l (Byzov 2003). Such rather high concentrations of glucose are comparable to that of nutrient media and may indicate that active hydrolysis of carbohydrates takes place in the millipede digestive tracts.

Hopkin and Read (1992) have reviewed the data on enzymatic activities of the millipede digestive tracts. In many millipedes, enzymes have been found that are capable of digesting lipids, proteins, and simple carbohydrates (Nunez and Crawford 1976; Neuhauser and Hartenstein 1976; Marcuzzi and Turchetto-Lafisca 1977; Kaplan and Hartenstein 1978; Neuchauser et al. 1978). However, there is still no strong evidence that millipedes themselves are able to digest more refractory components of leaves. For example, Neuchauser et al. (1978) showed that *Oxidus gracilis* was unable to degrade [^{14}C]-lignin. In contrast, *Pseudopolydesmus serratus* was able to degrade components of ligneous compounds (Neuhauser and Hartenstein 1976). Similarly, there is conflicting evidence regarding cellulose breakdown. It was found that gut extracts of *Pachyiulus foetidissimus* hydrolysed cellulose (Striganova 1970) and those of *Polydesmus angustus* were able to break down cellulose, hemicelluloses, and pectin (Beck and Friebe 1981). However, no cellulase was found in millipedes from families Glomeridae and Polydesmidae (Nielsen 1962; Marcuzzi and Turchetto 1975).

Cellulases in these gut extracts were probably derived from gut inhabiting microorganisms (Hopkin and Read 1992) or from enzymes ingested with food (fungi), as known for the digestive process in insects. In the giant desert millipede *Orthoporus ornatus* and the slate millipede *Comanchelus* sp., enzyme assays indicated that most cellulose and hemicellulose degradation occurred in the midgut, whereas the hindgut was important site for pectin degradation. Hemicellulase and β-glucosidase in both species

and C_x-cellulase and pectinase in *O. ornatus* were of possible microbial origin. This was confirmed by the experiment with millipedes whose gut microbiota was reduced by antibiotic treatment and starvation. The treated animals assimilated less ^{14}C-cellulose and voided more ^{14}C in faeces (Taylor 1982). Finally, there is no convincing report of the production of C_1-cellulase by soil invertebrates, although β-gluconases (C_x-cellulase) and cellobiase of animal origin have been found. Most of the data on C_1-cellulase production by soil animals were obtained in studies where microbial activity (especially activity of symbionts) had not been excluded.

Chitinase activity was found in some millipedes suggesting that digestion of fungi could take place in the gut (Nielsen 1962; Marcuzzi and Turchetto 1975).

4.4 Microscope Studies of Intestinal Microbiota

4.4.1 Bacteria

In scanning electron microscopy studies by Bignell (1984), actinomycete-like filaments were found to be end-on attached to flat cuticular surfaces of *Cylindroiulus* sp. Actinomycete-like epibionts (diameter of hyphae 1.2–1.3 µm) were also found in the hindgut of *Tachypodiulus sp.* More recently, it was found that the inner surface of the intestinal walls of the millipedes *Chromatoiulus rossicus* and *Glomeris connexa* is sparsely colonized by bacteria of different morphotypes. Very small cocci (< 0.2 µm), rod-shaped bacteria, small rod-shaped slightly curved bacteria, rod shaped bacteria covered with the slime, V-type forms (presumably coryneforms), cocci or short rods forming short chains or mycelium-like nets. The latter type represents the most frequent bacterial form in all gut parts. On the endoperitrophic surface of the peritrophic membrane bacterial cells can also be seen. The surfaces of the gut content were colonized mainly by filamentous bacteria of two types, presumably belonging to coryneform bacteria and actinomycetes. The coryneform bacteria had a pseudomycelium with typical branching cells, which disintegrated into single cells. The actinomycetes had well branching filaments no more than 1.0 µm in diameter. The organic particles were also colonized by *Vibrio*-like bacteria and large rod shaped bacteria. In fresh excrement, rod-shaped bacteria of two types were observed. Some of them were partially lysed (Byzov et al. 1996a). In *Pachyiulus flavipes,* the actinomycete mycelium (0.5 µm in diameter) was not found on the midgut wall but was abundant on the hindgut wall. It occupied the inner surface of peritrophic membrane in the midgut, and

its remnants in the hindgut. Streptomycete-like hyphae produced spore chains indicating that the actinomycete can complete its life cycle in the gut. The numbers of bacteria in micro-colonies varied from several hundreds to thousands. The total number was higher in the hindgut (Byzov et al. 1993c).

That the millipede guts represent a suitable place for actinomycete growth has been also shown by Polyanskaya et al. (1996). With the aid of luminescent microscopy, they found an orange luminescence emitted by the hyphae of *Streptomyces olivocinereus* after spores were fed to the millipede *Pachyiulus flavipes*.

The biometric analysis of 1953 microphotographs of bacterial cells associated with the guts or fresh faeces of millipedes *Glomeris connexa*, *Leptoiulus polonicus, Megaphyllum projectun* and *M. rossicum,* obtained by scanning electron microscope, revealed 24 morphotypes of bacteria of different size classes from very small cocci to long rods. It has been shown that their average size is 0.66 µm in diameter, 1.35 µm in length, and 0.6 μm^3 in volume. The average diameter, the length, and volume of the bacterial cells inhabiting native soil amended with organic substrates (glucose, starch, cellulose) (from 2645 micrographs) were found to be 32%, 16% and 36% larger, respectively. The authors have suggested that in the digestive tract of soil animals activation of the bacteria took place resulting in a higher metabolic activity of the cells and in a selection of smaller cells (Guzev and Zvyagintsev 2003).

Very limited data exist for the direct counts of the intestinal bacteria. With the aid of a luminescent microscopy, Polyanskaya et al. (1996) have found extremely high numbers of prokaryotic unicellular organisms in the midgut, hindgut and fresh faeces of the diplopod *Pachyiulus flavipes*, 1.0×10^{11}, 2.5×10^{11} and 1.0×10^{11} cells/g dry weight, respectively. The length of actinomycete hyphae was 5, 22 and 15 m/g, respectively. The animals were fed with sterile crystalline cellulose for 7 days, allowing appreciable voiding of the gut from transient microbiota. Thus, the counted bacteria were considered indigenous to the millipede. Similar results were obtained by Cazemier et al. (1997) who counted DAPI-stained bacteria in the gut preparations of *Chicobolus sp.* The bacterial counts were 1.7, 1.4 and 15×10^9 cells/ml gut in the foregut, the midgut and the hindgut, respectively, which is comparable to the numbers in *P. flavipes* (dry weight of its gut is about 10 mg) and the volume of the gut of *Chicobolus sp.* was found to be ca. 2 ml (Cazemier et al. 1997). However, the latter authors did not found actinomycete hyphae. Tret'yakova et al. (1996) have found 4.3×10^{10} bacterial cells per g dry gut of *Pachyiulus flavipes* fed broad leaves litter that corresponded to ca. 10^8 /gut.

Thus, the millipede guts are highly populated with diverse morphotypes of bacteria both unicellular and mycelial. The facts that they either were

isolated from the pre-washed gut tissue or they were visualized on the intact gut walls (end-on attached to the walls) let us to consider the bacteria indigenous to the animals.

4.4.2 Yeasts

Scanning electron microscopy of washed gut tissue of millipedes has been used to investigate the distribution of yeasts in *Glomeris connexa*, *Leptoiulus polonicus* and *Megaphyllum projectum*. It has been demonstrated that yeasts mainly colonize the hindgut of freshly collected diplopod with densities of about 10^3 cells/mm^2 (10^4 cells/gut). Only a few cells were found in the midgut (Byzov et al. 1993b).

4.4.3 Mycelial Fungi

No native fungal hyphae have been observed on the gut walls of the millipedes *Pachyiulus flavipes* (Byzov et al. 1993c) and *Glomeris connexa*, and *Chromatoiulus rossicus* (Byzov et al. 1996), suggesting that they are destroyed by the digestion.

4.5 Taxonomic Studies of Intestinal Microbiota

Hopkin and Read (1992) pointed out that, although it is generally accepted that microorganisms are of vital importance in digestion, there is no evidence that millipedes possess a permanent symbiotic microflora similar to that of termites. This review will demonstrate, however, that there are symbiotic interactions between microorganisms and millipedes. To prove this it is necessary to not only demonstrate the specificity of the gut microbes but also show their functional importance for the host as it is was shown for termites.

The basic context for this discussion is that the gut of millipedes provides an ideal environment for microorganisms. The lumen is protected from the vagaries of the outside environment, is permanently moist, is buffered to fairly constant pH and redox potential, and its contents are mixed thoroughly by muscular action (Bignell 1984).

A wide range of microorganisms has been isolated by the dilution plate method from the gut of millipedes. The taxonomic composition of bacteria and yeasts inhabiting the millipede guts are shown in Tables 4.1 and 4.2.

Table 4.1. Taxonomic composition of the dominant bacteria isolated from or detected[a] in the millipede guts and fresh faeces

Group/genus/species	Host	Source of isolation	Reference
Gamma proteobacteria: *Klebsiella* sp.	*Schizophyllum sabulosum* var. *rubripes*	Gut content	Baleux and Vivares (1974)
Actinobacteria: *Corynebacterium* sp.			
Clostridia: *Sarcina* sp.			
Bacilli: *Bacillus* sp.			
Actinobacteria: *Promicromonospora*-type bacteria	*Leptoilulus proximus, Chromatoiulus projectus, Cylindroiulus luridus. C. boleti, Unciger foetidus*	Fresh faeces	Dzingov et al. (1982)
Actinobacteria: *Promicromonospora enterophila*	*Chromatoiulus projectus*	Fresh faeces	Jager et al. (1983)
Actinobacteria: *Promicromonospora* sp.	*Leptoiulus proximus, Cylindroiulus luridus, C. boleti, Unciger foetidus*	Gut content	Szabó et al. (1983)
Gamma proteobacteria: *Klebsiella* spp., *Pseudomonas* spp.	*Chromatoiulus projectus*	Gut	Contreras (1985)
Actinobacteria: *Micrococcus* spp.			
Gamma proteobacteria: *Klebsiella* spp., *Enterobacter* sp.	*Chromatoiulus projectus, Cylindroiulus boleti*	Gut content	Marialigeti et al. (1985)
Actinobacteria: *Promicromonospora enterophila, Micrococcus* spp., *Nocardia* sp.			
Bacilli: *Bacillus* spp.			
Unidentified nocardioform actinomycetes	*Glomeris hexasticha*	Gut, fresh faeces	Szabó et al. (1985); Szabó et al. (1987); Chu et al. (1987)

Table 4.1. (continued)

Group/genus/species	Host	Source of isolation	Reference
Gamma proteobacteria: *Klebsiella pneumonia*, *Enterobacter cloacae*, *Plesiomonas* sp.	*Pachyiulus flavipes*	Intestinal walls	Byzov et al. (1993); Tret'yakova et al. (1996); Nguyen Duc TL et al. (1996)
Actinobacteria: *Promicromonospora citrea*, *Streptomyces chrysomallus*, *S. candidus*, *Streptoverticillium* spp., *Micromonospora* spp.			
Bacilli: *Bacillus* spp.			
Gamma proteobacteria: *Enterobacter cloacae*	*Pachyiulus flavipes*	Peritrophic membrane	Byzov et al. (1993); Tret'yakova et al. (1996); Nguyen Duc TL et al. (1996)
Actinobacteria: *Streptomyces badius*, *S. fluorescens*, *S. xanthocidicus*, *S. filamentosus*			
Gamma proteobacteria: *Pseudomonas* sp.	*Pachyiulus flavipes*	Gut content	Byzov et al. (1993); Tret'yakova et al. (1996); Nguyen Duc TL et al. (1996)
Actinobacteria: *Promicromonospora* sp., *Rhodococcus* sp., *Streptomyces carpaticus*, *S. griseolus*, *S. pseudogriseolus*, *S. paucidiastaticus*, *S. felleus*, *S. chrysomallus*, *S. aburaviensis*, *S. nitrosporeus*, *S. globisporus*, *S. pluricolorescens*, *S. longisporoflavus*, *S. echinatus*, *Streptosporangium albidum*, *Micromonospora* spp., *Actinomadura* spp.			

Table 4.1. (continued)

Group/genus/species	Host	Source of isolation	Reference
Bacilli: *Bacillus* spp.			
Flavobacteria: *Flavobacterium* sp.,			
Gamma proteobacteria: *Klebsiella* sp., *Enterobacter* sp., *Escherichia* sp., *Plesiomonas* sp., *Vibrio* sp., *Erwinia* sp., *Azotobacter* sp., *Pseudomonas* spp.	*Glomeris connexa*, *Chromatoiulus rossicus*	Intestinal walls	Byzov et al. (1996)
Alpha proteobacteria: *Beijerinckia* sp.			
Actinobacteria: *Mycobacterium* sp., *Rhodococcus* sp., *Promicromonospora-Oerskovia*			
Flavobacteria: *Flavobacterium* spp.			
Unidentified methanogens	*Chicobolus* sp.	Hindgut	Hackstein and Stumm (1994)
Free methanogenic bacteria; ciliates with intracellular methanogens	*Orthoporus* sp., *Rhapidostreptus virgator*; two unidentified species		
Free methanogenic bacteria	two unidentified species		
Gammaproteobacteria: *Erwinia* sp., *Pseudomonas* spp., *Azotobacter* sp.	*Glomeris connexa*, *Chromatoiulus rossicus*	Gut content	Byzov et al. (1996)
Alphaproteobacteria: *Beijerinckia* sp.			
Actinobacteria: *Streptomyces fimicarius*, *S. griseolus*, *S. wedmorensis*, *Promicromonospora-Oerskovia*			
Flavobacteria: *Flavobacterium* spp.			
Bacilli: *Bacillus megaterium*, *B. cereus*, *B. licheniformis*			
Flexibacteriaceae: *Cytophaga* sp.			

Table 4.1. (continued)

Group/genus/species	Host	Source of isolation	Reference
unidentified Myxobacteria			
Gammaproteobacteria: *Escherichia coli, Enterobacter agglomerans, Klebsiella* sp., *Pseudomonas fluorescens*	*Ommatoiulus sabulosus*	Gut content	Jarosz and Kania (2000)
Clostridia: *Sarcina* sp.			
Bacilli: *Bacillus* sp.			
Actinobacteria: *Micrococcus* sp.			
Six actinomycete and two fungal species			
Bacilli: *Bacillus brevis*	*Glomeris* sp.	Hindgut	Gebhardt et al. (2002)
Gammaproteobacteria: *Salmonella* subsp., *Pseudomonas fluorescens*[b]	*Pachyiulus flavipes*	Midgut fluid	Byzov (2003)
Betaproteobacteria: *Alcaligenes xylosooxidans, Burkholderia cepacia*[b]			

[a] Methanogenic bacteria were detected microscopically by their characteristic blue autofluorescence (Hackstein and Stumm 1994)
[b] Strains identified by BIOLOG

Table 4.2. Taxonomic composition of yeasts and mycelial fungi isolated from the millipede guts

Genus/species	Host	Source of isolation	Reference
Ascomycetous: *Debaryomyces hansenii, Torulaspora delbrueckii, Zygowilliopsis californicus* (=*Williopsis californica*), *Galactomyces geotrichum*	*Pachyiulus flavipes*	Midgut (intestinal walls + gut contents)	Byzov et al. (1993a)
Ascomycetous: *Debaryomyces hansenii, Torulaspora delbrueckii, Zygowilliopsis californicus* (=*Williopsis californica*), *Pichia membranaefaciens, Zygosaccharomyces rouxii, Saccharomyces paradoxus, Saccharomyces transvaalensis, Arthroascus schoenii, Kluyveromyces thermotolerans, Citeromyces matritensis, Pichia anomala, Galactomyces geotrichum, Candida valida, Candida ingens, Candida norvegica, Candida friedrichii, Kloeckera apis*	*Pachyiulus flavipes*	Hindgut (intestinal walls+gut contents) and fresh excrements	Byzov et al. (1993a); Vu Nguyen Thanh (1993)
Basidiomycetous: *Cryptococcus albidus, Trichosporon pullulans, Ttichosporon beigelii, Trichosporon cutaneum, Aureobasidium pullulans, Rhodotorula rubra*			
Ascomycetous: *Pichia guilliermondii*	*Glomeris connexa*	Hindgut (intestinal walls+gut contents)	Byzov et al. (1993a)
Trichomycetes	Diplopoda	Hindgut cuticule	Moss and Taylor (1996)
Trichomycetes: *Enterobryus* (Eccrinales)	Diplopoda	Hindgut cuticule	Alencar et al. (2003)
Unidentified yeast-like fungi	*Ommatoiulus sabulosus*	Gut content	Jarosz and Kania (2000)

4.5.1 Bacteria

Most studies of the bacterial diversity in the intestinal tracts of millipedes were made using the dilution plate method. In most cases, the identification of isolates was performed using phenotypic characteristics, morphological, physiological and biochemical features.

The number of aerobic, culturable bacteria was found by Ineson and Anderson (1985) to be 2.8×10^9 CFU/g dry weight in the whole gut of the diplopod *Glomeris marginata*. The ratio of viable bacterial counts to direct counts was lower in litter than in the gut and faeces of this diplopod, suggesting that the gut environment enhanced bacterial growth and viability (Anderson and Bignell 1980). In *Pachyiulus flavipes*, the total CFU counts of bacteria were found to be $1.0–7.8 \times 10^8$ CFU/g dry weight (Byzov et al. 1993c; Tret'yakova et al. 1996) with increasing counts from the foregut to the midgut, and to the hindgut, 0.15, 1.94 and 2.7×10^8, respectively (Tret'yakova et al. 1996). Lower bacterial counts have been recorded in the guts of the diplopods *Chromatoiulus rossicus* and *Glomeris connexa,* from 1.0×10^6 to 2.1×10^7 and from 8.0×10^6 to 2.7×10^7 CFU/g dry weight, respectively. The numbers of bacteria were quite similar in the midgut and hindgut. The numbers of bacteria isolated from the peritrophic membrane of *G. connexa* were similar to those for gut tissue, about 10^7 CFU/g dry membrane (Byzov et al. 1996a).

Most of bacterial strains isolated from the millipede guts and the gut contents belong to the gamma subclass of Proteobacteria and the phylum Actinobacteria, class Actinobacteria. The dominant bacteria found by many authors inhabiting the guts belong to facultative anaerobic bacteria of the family Enterobacteriaceae, genera *Klebsiella, Enterobacter, Plesiomonas, Salmonella, Erwinia, Escherichia,* and of the family Vibrionaceae, genus *Vibrio* (Table 4.1). It has been shown these two families of bacteria predominate on the washed intestinal walls of *Glomeris connexa, Leptoiulus polonicus* and *Pachyiulus flavipes*. They occupied all the gut parts but were most numerous in the hindgut. Their numbers ranged from 1.0×10^6 to 2.7×10^7 CFU/g dry gut weight; they represented 50–80% of all isolates. These bacteria were consistently isolated during several months of laboratory rearing of the animals; they were not isolated from the food (leaf litter); their populations remained relatively stable in the starving animal. These can be indirect evidence for their intestinal origin (Byzov et al. 1996a; Tret'yakova et al. 1996).

The second numerous group of bacteria, inhabiting the guts, is Actinobacteria. Among them were found representatives of the families Promicromonosporaceae, Cellulomonadaceae and Streptomycetaceae with *Promi-*

cromonoposra-Oerskovia group – nocardioform actinomycetes and *Streptomyces* predominated (Table 4.1).

Szabó and his group first isolated nocardioform actinomycetes from millipede guts (Dzingov et al. 1982; Szabó et al. 1983). The first species, the strain of which was isolated from the gut and faeces of *Chromatoiulus projectus* and formed large populations in the hindgut,was described as *Promicromonospora enterophila* (Jager et al. 1983). It was considered a true intestinal associate because it disappeared quickly from fresh excrement as it aged (Márialigeti et al. 1985). *Promicromonospora*-type bacteria were also found in the gut, the gut contents and fresh faeces of *Chromatoiulus rossicus, Leptoiulus proximus, Cylindroiulus luridus, C. boleti, Unciger foetidus* and *Pachyiulus flavipes* (Table 4.1). Another nocardioform monospore actinomycete has been isolated from the gut and faeces of *Glomeris hexasticha.* It was supposed that it represented an intermediate taxon between the genera *Oerskovia* and *Promicromonospora.* This indigenous intestinal microbe was completely absent from, or occurred sporadically, in the soil or litter of its host animal's feeding habitat. They do not multiply in soils in the presence of complex natural microflora. These data corroborated the conclusion of Anderson and Bignell (1980) that the specific gut symbionts do not proliferate in millipede faeces. This can be considered as indirect evidence of symbiotic origin of the gut actinomycetes. Direct evidence of the intestinal origin of nocardioforms can be drawn from the finding that these actinomycetes were isolated from the washed intestinal walls (Byzov et al. 1993c; Tret'yakova et al. 1996). It was concluded that nocardioform gut populations of different millipede species might belong not only to different species but also to various genera of actinomycetes (Chu et al. 1987).

Various streptomycete species have been isolated from both millipede gut walls and gut contents (Table 4.1). They were also found on the peritrophic membrane in the midgut (Byzov et al. 1993c; Nguyen Duc et al. 1996; Tret'yakova et al. 1996). The presence of actinomycete mycelium (less than 1 μm in diameter) on the intestinal wall in the hindgut of *Pachyiulus flavipes*, revealed by scanning electron microscopy (Byzov et al. 1993c), could be considered evidence that this actinomycetes belong to true intestinal microbiota. Numbers of streptomycetes reached 10^5 CFU/g gut tissue. Different species of *Micromonospora, Actinomadura* and *Streptosporangium* were also isolated, of which the numbers were less than 10^4 CFU/g. However, it was difficult to evaluate the real density of actinomycetes because of the limitation to count mycelial spore forming organisms by the dilution plate method (Byzov et al. 1993c; Nguyen Duc TL et al. 1996).

A strain of *Bacillus brevis,* which produced antifungal metabolites, was isolated from the millipede *Glomeris* sp. (Gebhard et al. 2002), but there was weak evidence than this was an endosymbiont.

Free methanogenic bacteria and ciliates (*Nyctotherus* type) with intracellular endosymbiotic methanogens have been detected microscopically by their characteristic autofluorescence in the hindguts of the tropical diplopods *Chicobolus* sp., *Orthoporus* sp., *Rhapidostreptus virgator* and two unidentified species. The ciliates carried > 4000 methanogens per ciliate cell. Methanogens were also found in their cysts. Unlike other arthropods, in which the methanogens were found in the hindgut pouch, the millipedes do not have such a pouch. The estimation of global methane production, based on laboratory measurements, shows that several species of millipedes (with and without intestinal protists) contribute a significant quantity of methane compared to that emitted by other arthropods. However, some species of diplopods did not emit methane, probably due to a secondary loss of the methanogens (Hackstein and Stumm 1994).

Among the bacterial isolates those with cellulase activity were not found with the exception of the study by Taylor (1982). Therefore, it is unlikely that millipedes possess cellulolytic symbionts as the termites and other insects.

4.5.2 Fungi

Trichomycetes (Zygomycota) are obligate symbionts that live in the digestive tract of various arthropods, including Diplopoda (White et al. 2000). The relationships of Trichomycetes to their hosts is generally commensalistic or pathogenesis, and, in some cases, mutualistic, depending on developmental and environmental conditions (Lichtwardt 1996). The Hindguts of many species of millipedes (Diplopoda) throughout the world are hosts to *Enterobryus* (Eccrinales) (Lichtwardt 1996), the first genus of Trichomycetes to be named. Alencar et al. (2003) reported one species of *Enterobryus* that is probably new, which was found in a small spirobolid millipede. The feature that makes this eccrinid unusual is the presence of extremely long holdfasts in mature thalli revealed by phase contrast microscopy of the hindgut. Holdfasts attach thalli to the hindgut cuticle, and are extruded through pores in the wall at the base of the thalli.

Most of yeast strains isolated from the millipede guts and the gut contents are ascomycetous. In *Pachyiulus flavipes*, the predominating species were *Debaryomyces hansenii, Torulaspora delbrueckii, Zygowilliopsis californicus* (=*Williopsis californica*) and *Pichia membranaefaciens*. Byzov et al. (1993a) determined counts up to 10^5 CFU/g in the hindgut, and less than 10^3 CFU/g in the midgut (Table 4.2). These yeasts proved to be obligate gut associates of millipedes. The yeasts dominated in the gut were not found in the animal food but survived gut passage. They are consistently present

in the gut of the diplopods reared under different external conditions such as long-term starvation, feeding of sterile substrates and artificial "wintering" of animals maintaining their composition and numbers at a relatively steady state. The fact that regardless of food quality and feeding regimens the composition of gut yeasts remained constant can be considered as indirect evidence of a very close association with the host organism (Byzov et al. 1993a,b). The gut yeasts are characterized by a fermentative metabolism and have a very narrow assimilation spectrum (Vu Nguyen Thanh 1993). These facts correspond to facultative anaerobic conditions (Bignell 1984) and high concentrations of glucose (Byzov 2003) in the hindgut of millipedes. Jarosz and Kania (2000) isolated yeast-like fungi and moulds from the gut content of *Ommatoiulus sabulosus* occurred at low population densities but species were not identified.

4.6 Functions of the Intestinal Microbiota

Functional importance of gut microorganisms for soil millipedes has not been generally investigated. There is indirect evidence that microbial associates participate in the digestion and can provide the animals with food. They can act as pathogenic agents or cause the colonization resistance. There is an indication that gut microbes produce methane (Table 4.3).

4.6.1 Digestive Functions of Gut Microorganisms

It was found that the efficiency of ^{14}C-cellulose breakdown and the assimilation by the desert millipedes *Orthoporus ornatus* and *Comanchelus* sp. were reduced if antibiotics are incorporated into the food. It was concluded that the millipede-bacterium association was mutualistic that enables millipedes to utilize otherwise unavailable plant polymers (Taylor 1982).

An in vitro study has demonstrated that gut actinobacteria isolated from the hindgut of the millipede *Pachyiulus flavipes* (coryneforms and streptomycetes) may kill and digest living yeast cells of *Debaryomyces hansenii* – one of the predominating gut species. It appears that this kind of symbiotic digestion may be important for the millipede (Byzov et al. 1993b). Similar antagonistic role of actinomycetes in the digestion of microorganisms was shown for soil feeding termites (Bignell et al. 1983).

In the hindgut of *P. flavipes*, unicellular bacteria and actinomycetes hyphae were found on the remnants of the peritrophic membrane, indicating that decomposition of this chitinous material takes place (Byzov et al. 1993b,

Table 4.3. Microbial associates and their possible functions in the digestive tract of millipedes

Microorganisms/ microhabitat	Function	Host	Reference
Unidentified bacteria/midgut, hindgut	**Digestion**: cellulose and hemicellulose, and pectin degradation	*Orthoporus ornatus, Comanchelus sp.*	Taylor (1982)
Klebsiella pneumonia, unidentified bacteria/faeces	**Digestion**: acetate as sole C-source	*Glomeris marginata*	Ineson and Anderson (1985)
Actinomycetes/hindgut	**Digestion**: destruction of peritrophic membrane	*Pachyiulus flavipes*	Byzov et al. (1993c)
Actinomycetes/hindgut	**Digestion**: yeast-lysing activity	*Pachyiulus flavipes*	Byzov et al. (1993b)
Oerskovia-Promicromonospora-Nocardioforms (OPNs)/hindgut	**Food for the host:** digestion of the gut OPNs	*Chromatoiulus projectus*	Szabó et al. (1985)
Yeasts of DTZ-group/hindgut	**Food for the host:** digestion of symbiotic yeasts	*Pachyiulus flavipes*	Byzov et al. (1993b)
Bacillus cereus/hindgut	**Antagonistic interaction:** heightened antibiotic activity against *Promicromonospora enterophila*	*Cylindroiulus boleti*	Contreras (1985)
Streptomyces chrysomalus, S. candidus/hindgut; *Streptomyces fluorescens, S. xanthoacidicus*/peritrophic membrane	**Resistance to colonization**: heightened Antibiotic activity against Gram-positive bacteria and yeasts	*Pachyiulus flavipes*	Nguyen Duc TL et al. (1996)
Enterobacter agglomerans, Escherichia coli, Klebsiella sp./gut content	**Resistance to colonization:** rapid colonization and overgrowth	*Ommatoiulus sabulosus*	Jarosz and Kania (2000)
Bacillus brevis/hindgut	**Resistance to colonization**: production of antifungal antibiotic bacillomycin D	*Glomeris* sp.	Gebhard et al. (2002)
Candida guilliermondii/hindgut	**Pathogenic agent:** overgrowth the whole gut	*Glomeris connexa*	Byzov et al. (1993b)

Table 4.3. (continued)

Microorganisms/ microhabitat	Function	Host	Reference
Unidentified yeasts/whole gut	**Pathogenic agent:** overgrowth the whole gut	*Leptoiulus polonicus, Megaphyllum projectum*	Byzov et al. (1993b)
Klebsiella pneumonia, unidentified bacteria/faeces	**Detoxification of nitrogenous wastes:** urease and uricase activities in vitro	*Glomeris marginata*	Ineson and Anderson (1985)
Yeasts: *Debaryomyces hansenii, Zygowilliopsis californicus, Torulaspora delbrueckii* (DTZ group), *Candida guilliermondii, Kloekera apis, Trichosporon cutaneum, T. pullulans, Geothrichum candidum*/midgut, hindgut	**Detoxification of nitrogenous wastes:** utilization of the waste end product uric acid as the sole N-source	*Pachyiulus flavipes*	Byzov et al. (1993b)
Methanogens/hindgut	**CH_4 and H_2** production	*Chicobolus* sp.	Hackstein and Stumm (1994)
Free methanogenic bacteria/hindgut; ciliates with intracellular methanogens/hindgut		*Orthoporus* sp., *Rhapidostreptus virgator*; two unidentified species	
Not determined		*Pycnotropis acuticollis*	
Free methanogenic bacteria/hindgut		two unidentified species	
No methanogens	**H_2** production	*Glomeris* sp.	

1996a). This may imply re-utilization of nitrogen-containing substrates and nitrogen balance in millipedes.

Another possible mechanism of nitrogen balance could be the utilization of the end products urea and uric acid by gut microorganisms. It has been demonstrated that bacteria isolated from the faeces of *Glomeris marginata* possessed urease and uricase that was less frequent for litter isolates (Ineson and Anderson 1985). The yeasts isolated from *P. flavipes* were able to grow on uric acid as a sole source of nitrogen (Byzov et al. 1993b). The urea and the uric acid are toxic and their detoxification must be important for the animals.

4.6.2 Intestinal Microbiota as a Food for Millipedes

Gut microbes can provide food for the host. It was hypothesized by Szabó et al. (1985) that the millipede *Chromatoiulus projectus* could utilize the cell materials of *Oerskovia-Promicromonospora-Nocardioforms*. It was found that the yeasts *Debaryomyces hansenii, Torulaspora delbrueckii, Zygowilliopsis californicus* that live in the hindgut of the millipede *Pachyiulus flavipes* did not occur in its food, but could be isolated at lower densities from the midgut. On the other hand, these microorganisms were sensitive to the digestive fluid of the host. This may indicate the yeast cells are transported to the digestive region of the gut by antiperistaltic movement and lysed there. Such an endogenous feeding might be important in starving animals (Byzov et al. 1993b).

It has been calculated that gut microorganisms might provide the millipedes with essential amino acids whose concentrations are low in plant litter (Pokarzhevskii et al. 1984).

Gut microorganisms may apparently directly supply the host with nutritive substrates. The hypothesis follows from the fact that ^{14}C-labelled yeasts *Debaryomyces hansenii* lost up to 80% of ^{14}C within 30 min incubation in the midgut fluid without loosing their viability (Byzov 2003).

These are mutualistic interactions when the host organism provides microorganisms with favourable place of residence, namely the hindgut. The hindgut of millipedes is a natural fermenter. The Malphigian tubules excrete mineral compounds, urea and uric acid that are transported into the hindgut space (Bignell 1984). Carbohydrates are also transported there as part of the food. Thus, there are all the essential nutrients in the hindgut to promote microbial growth.

4.6.3 Resistance to Colonization

One of the important functions of the intestinal microbiota is to protect the intestine from colonization by external microorganisms. Nguyen Duc TL et al. (1996) have found that actinomycetes isolated from the hindgut of *Pachyiulus flavipes* showed in vitro considerable higher antibiotic activity against bacteria as compared to litter isolates. Bacteria, isolated from the animal guts, were more sensitive than those isolated from litter. Moreover, Gram-positive bacteria were more sensitive than Gram-negative. It was concluded that the heightened antagonistic activity makes the actinomycetes more competitive with other Gram-positive bacteria. At the same time, they do not compete with Gram-negative bacteria that predominate in

the community. Similar results were obtained by Szabó (1974) who found that actinomycetes isolated from larval Bibionidae (Diptera) were more competitive with each other than with other gut bacteria.

The bacterium *Bacillus brevis* has been isolated from the hindgut of the millipede *Glomeris* sp.The culture filtrate extract of the bacterium exhibited strong antifungal activity (against standard strains for testing *Saccharomyces cerevisiae, Botrytis cinerea* and *Cladosporium cucumerinum*). The activity was explained by the presence of a member of the lipopeptide antibiotic of the iturin group. It was identified as bacillomycin D. It was also shown that this bacterial strain produced the lipopeptide antibiotics fengycin and surfactin (Gebhardt et al. 2002). These phenomena, however, were not demonstrated in vivo.

Jarosz and Kania (2000) have pointed out that the specific composition of bacterial flora in the millipede intestines cannot be explained simply by antibiotic inhibition of contaminating microflora. They have shown that the predominant types of gut enterobacteria of *Ommatoiulus sabulosus* were unable to produce in vitro any bacteriolytic activity of lysozyme-like enzymes, bacteriocins or other antimicrobial molecules. The lack of microbial contaminators could rather result from the unfavourable biochemical niche in the midgut enzymes and little or no competition for the enteric bacteria group predominant in the millipede alimentary tract. The rapid colonization and overgrowth of the intestines eliminate bacteria, yeasts and moulds ingested with food. Killing action of the midgut fluid against litter microorganisms was found earlier (Byzov et al. 1993b, 1998a)

4.6.4 Intestinal Microbiota as a Pathogenic Agent

These balanced interactions between the host and its intestinal microbiota can change into a pathogenic state if the natural protective ability of the midgut is disturbed. It can happen if unnaturally high amounts of gut microorganisms are introduced, following removal of natural microbiota. In the feeding experiment it was shown that the yeasts *Candida guilliermondii*, which normally inhabit the hindgut of diplopods *Glomeris connexa*, can cause the disease when their natural density is increased 100 times. To be killed by yeasts, a total colonization of the midgut epithelium is necessary. This can happen naturally during the long-term rearing of diplopods under laboratory conditions (Byzov et al. 1993b). The mechanism of the effect is unknown but it could be metabolic (yeast toxins, heightened CO_2 production due to yeast growth) and trophic (competition for nutrition, vitamins).

4.7
Digestion of Microorganisms by Millipedes

It is well known that soil millipedes not only regulate the numbers of microorganisms but they also modify microbial communities by eliminating some and enabling the growth of others (Anderson and Bignell 1980; Ineson and Anderson 1985, among others). The mechanism of such a selection is not fully understood; however, it is believed that the first barrier for those microorganisms that enter the gut is the killing activity of the midgut environment.

4.7.1
Killing Activity of the Midgut Fluid

The digestive midgut fluid of the millipede *Pachyiulus flavipes* has been shown to exhibit a selective biocidal killing activity against a variety of microorganisms: bacteria, actinomycetes, yeasts and filamentous fungi (Thanh et al. 1994). Assays using a range of microorganisms found their sensitivities to the fluid to be species and strain dependent. The effects ranged from those sensitive to resistant ones with the isolates of the millipede guts being the most resistant (Thanh et al. 1994; Byzov and Rabinovich 1997; Byzov et al. 1998a,b). The digestive fluid has also been found to kill some soil invertebrates, e.g. nematodes, enchytraeids, ants (B. Byzov, unpubl. data). A similar type of activity has been found in the digestive fluid of two other millipedes, *Rossiulus kessleri* and *Megaphyllum rossicum* (Byzov 2003). The killing activity was not found in the hindgut water extract (Byzov et al. 1996b).

4.7.2
Killing Effect

Those microorganisms that were found to be sensitive to the digestion were killed after only 1–2 min of incubation in the fluid. The longer incubation of the killed cells resulted in a subsequent total destruction of the cells. The effects were similar to both sterile filtered digestive fluid and with fluid obtained from the animals fed on sterile cellulose or cellulose enriched with streptomycin, thus pointing to an animal rather than a bacterial origin of the effect. The rapid death of the sensitive yeast *Saccharomyces cerevisiae* was accompanied by an immediate destruction of vacuoles, nuclei and membranes; however, the cell walls of the majority of the cells remained intact within 1–3 h of incubation (Byzov et al. 1998b).

4.7.3
Properties of the Killing Compound(s)

Byzov and Rabinovich (1997) separated the biocidal compounds from the native digestive fluid by a combination of chloroform-methanol extraction and LH-20 Sephadex chromatography. The concentration of the biocidal compounds was about 15 mg/ml. The biocidal activity was found to be associated with the non-protein fraction. The biocidal compounds can be dissolved in water, 50%-methanol and chloroform; they are thermostable (98 °C, 10 min), and act effectively down to a sixfold dilution of the native fluid.

Some purified biocidal fractions were water surface active. A decrease of surface tension of water from 72 to 42 mN/m caused by 1:100 or less diluted fraction is characteristic of a moderate surfactant. Elucidation of molecular structure of the killing compounds in the millipede *Pachyiulus flavipes* by mass spectrometry has revealed the presence of saturated fatty acids with different chain lengths carrying a hydrophilic group in the omega position. The preliminary structural and computer analysis showed that these compounds are not yet registered in the American Chemical Society Database, which documents all known chemical structures (Golyshin et al., unpubl. data).

4.7.4
Induced Autolysis

The killing compounds found in the digestive fluid of soil millipedes seem to play an important role in the digestive process in the animals. The microbiolytic activity of the midgut fluid most likely relates to its protein fraction. However, neither the protein nor the biocidal fractions caused the destruction of the cells, when applied separately. Complete destruction of the cells was only observed when a mixture of the two fractions or the native fluid was applied. The digestion process apparently involves two steps: microbial cells are initially killed by the non-protein substances and then hydrolysed by both midgut hydrolytic enzymes and autolytic enzymes of the microbial prey. Induced autolysis is suggested to play an important role in the digestion of microorganisms by the millipede (Byzov et al. 1998a,b).

4.7.5
Assimilation of Microorganisms

Wolters and Ekschmitt (1997) observed that the assimilation efficiency of microorganisms by millipedes is high and exceeds that of plant material

(which is generally < 20%). In feeding experiments with ^{14}C-labelled microbes, it was shown that in *Glomeris marginata* the assimilation efficiency reached 96.9% for *Erwinia herbicola,* 93.2% for *Pseudomonas syringae*, 82,6% for *Bacillus subtilis*, 72.2% for *Escherichia coli* and 73.5% for *Mucor hiemalis* (Bignell 1989). In *Pachyiulus flavipes,* it was 73% for *Rhodotorula graminis,* 80% for *Trichosporon pullulans,* 82% for *Debaryomyces hansenii*, 60% for hyphae of *Streptomyces pseudogriseolus* and 40% for *S. californicus* (Byzov et al. 1998).

4.8 Conclusions

Since the pioneer works in the 1980s, the intestinal microorganisms of soil millipedes have been mostly studied by routine isolation techniques and microscopy. Nevertheless, many interesting discoveries have been made. Almost 30 species of diplopods have been studied, most of which belonged to the family Julidae. A diverse microbiota has been found in the gut comprising facultative anaerobic enterobacteria, actinomycetes, nocardioforms, yeasts and fungi. It could be concluded that the intestinal microbiota of different diplopods has common features on generic and higher levels, but there is a high microbial diversity at the species level. A number of studies are indicative of intimate associations between microorganisms and the millipede gut. The most important are those that demonstrate possible functions of gut microbiota. Among them digestive activity, maintaining microbial community at steady state, in vivo production of methane. The discovery of killing mechanism can partially explain how diplopod digestive system is organized. It is most likely that these animals do not possess symbiotic microorganisms, capable of digesting lignocelluloses, as is the case in wood-feeding termites and other insects. It is believed that they feed on microorganisms and use microbial enzymes to digest recalcitrant molecules. Such a mechanism was suggested for isopods and soil-feeding termites and probably exists in other soil invertebrates, e.g. earthworms. Gut microbes are also important as the source of essential amino acids.

Fortunately, there are many starting points to continue more either detailed or extensive researches in the field of microbial-millipede interactions. Modern methods of isolation and identification of microorganisms and in situ studies open promising perspectives for enthusiasts to discover new symbiotic microorganisms and describe their ecological functions.

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5 Intestinal Microbiota of Terrestrial Isopods

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5.1 Introduction

Isopods are a cosmopolitan group of crustaceans, which inhabit marine environments ranging from deep-sea to intertidal areas, surface and underground freshwaters and terrestrial environments from high humidity to dry habitats, including deserts. More than one third of the described isopodan species (approximately 9000) belong to the terrestrial Oniscidea or woodlice (slaters, sowbugs and pillbugs; Schamlfuss 2003). The phylogeny and systematics of Oniscidea were analysed and presented by several authors (Schmalfuss 1989; Erhard 1998; Tabacaru and Danielopol 1999). In general, Oniscidea are assigned to five sections, namely Diplocheta, Tylida, Microcheta, Synocheta and Crinocheta, with 33 families altogether (Erhard 1998). Amphibious species of the family Ligiidae (slaters) and members of Mesoniscidae represent only approximately 50 of the described species, the rest belonging to the higher oniscideans, mostly to troglobiontic Synocheta and "truly terrestrial" Crinocheta.

Terrestrial isopods are effective herbivorous scavengers feeding predominantly on decayed plant material, fungi and algae, thus participating in decomposition and cycling of energy and organic matter in the terrestrial environments (Hopkin 1991; Zimmer and Topp 1997). Due to their significant ecological role and their ability to survive in polluted environments, a substantial amount of research was focused on these organisms, and as a result a comprehensive knowledge accumulated on their biology. Species like *Porcellio scaber* and *Oniscus asellus*, for example, are among the most studied organisms in terrestrial ecophysiology and ecotoxicology (Hopkin 1989; Drobne 1997). Terrestrial isopods have diverse feeding strategies including coprophagy and occasional cannibalism. The nutritional importance and significance of coprophagy in the field was not demonstrated

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(Carefoot 1993; Slavecz and Maiorana 1998). However, there is some evidence supporting the importance of coprophagy for development of juveniles (Wieser 1966; Hassall and Rushton 1984).

The food consumption of terrestrial isopods varies according to species (Warburg 1987) and is approximately 0.4 mg dry food per mg dry wt. of animal per day (own observ.). Food assimilation efficiency is between 30 and 50% in *P. scaber* (Nair et al. 1994; Drobne and Hopkin 1995). Food can remain in the gut for 4–17 h (Hartenstein 1964) and is digested and absorbed in a 24 h digestive cycle (Hames and Hopkin 1991). When the feeding rate is lower, food remains in the gut for a longer time. Animals produce 10–35 faecal pellets per day and one pellet is about 1.5 ± 0.2 mm long. About five to seven pellets fill the entire gut. Daily faecal production is independent of the animal's size (own observ.).

The role of isopods as decomposers in terrestrial environments is mainly indirect. They promote microbial activity by fragmentation of the substrate, by increasing the number of some of the ingested microbes in their gut and the distribution of microorganisms in the terrestrial ecosystem (Hanlon 1981a; Hassall et al. 1987; Neuhauser and Hartenstein 1978; Gunnarsson et al. 1988; van Wensem et al. 1993). At the same time, isopods utilise the ingested microorganisms as a source of nutrients, enzymes and vitamins (Neuhauser et al. 1974; Hassall and Jennings 1975; Neuhauser and Hartenstein 1976; Kozlovskaja and Striganova 1977; Kaplan and Hartenstein 1978; Carefoot 1984; Gunnarsson and Tunlid 1986; Kukor and Martin 1986; Ullrich et al. 1991).

5.2 Structure and Function of the Digestive System

The oniscidean digestive system has been extensively studied and well described in both slaters and sowbugs (Hames and Hopkin 1989; Štrus et al.1995). The tripartite digestive system of terrestrial isopods (Fig. 5.1) consists of a foregut comprising oesophagus and stomach (proventriculus), a midgut presented mainly by tubular midgut glands (hepatopancreas) and a hindgut. The latter consists of two anatomically and functionally different parts; an anterior chamber and a papillate region with rectum, which are innervated by separate nerves (Molnar et al. 1998).

Frequent moulting is an important characteristic of juvenile and adult oniscideans and is related to growth, renewal of the cuticle and reproduction in mature females. Adult sowbugs and pillbugs moult once monthly, slaters twice monthly. The cuticles of the foregut and hindgut are completely renewed during exuviation (Štrus and Blejec 2001). In contrast to

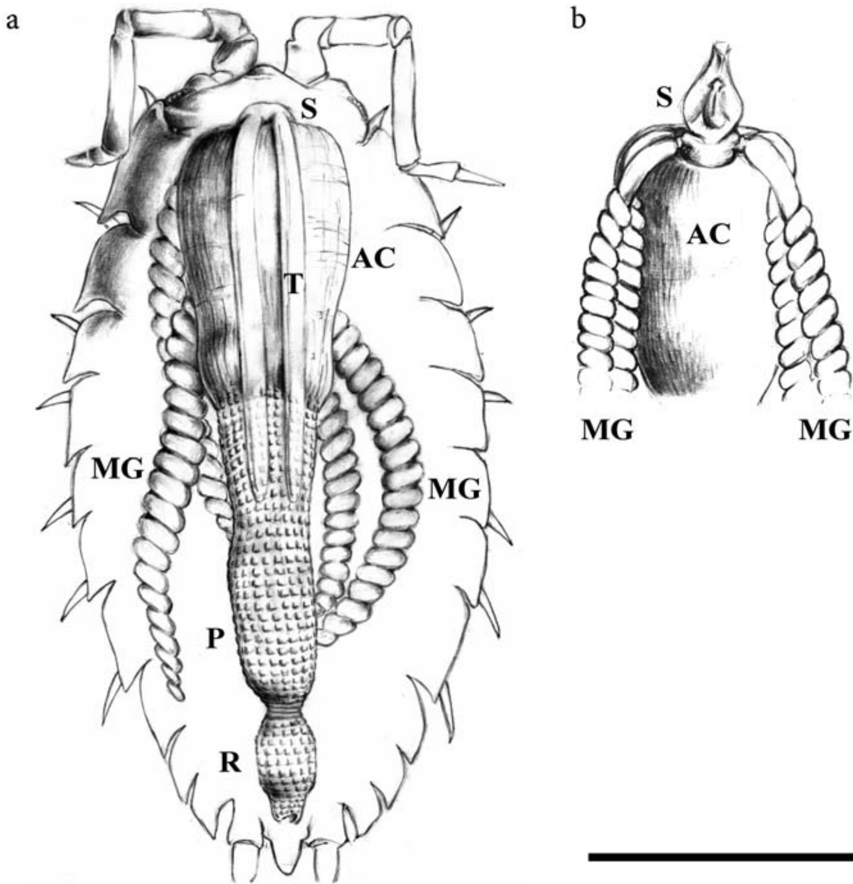

Fig. 5.1. Anatomy of the digestive system of *Porcellio scaber*. **a** Dorsal view of stomach (*S*), midgut glands (*MG*) and hindgut consisting of anterior chamber (*AC*) with typhlosole (*T*), papillate region (*P*) and rectum (*R*). **b** Ventral view of anterior chamber of the hindgut (*AC*) and the midgut glands (*MG*) connected to the stomach (*S*). *Scale bar* 5 mm

all other crustaceans, terrestrial isopods consume their old cuticle (external and gut) soon after exuviation.

The food is ground and taken up by mouthparts. Fragments of food pass via the oesophagus through the stomach where they are processed mechanically before passing into the anterior chamber of the hindgut. During this process the food is mixed with a secretion derived from the hepatopancreas. The anterior chamber of the hindgut has a pair of typhlosole channels on the dorsal part. When the hindgut is filled with food, contraction of the muscles surrounding the gut forces liquids and fine food particles back into the stomach via the typhlosole channels. This material

is filtered in the stomach and passed into the lumen of the hepatopancreas where the absorption of nutrients takes place (Hames and Hopkin 1989). The absorption of food in the hindgut is limited due to the cuticular lining. However, some absorption of nutrients was reported in the anterior chamber of the hindgut (Hryniewiecka-Szyfter and Storch 1986). Water and ions are absorbed in the papillate region and undigested food is passed into the rectum (Hames and Hopkin 1989). Dry faecal pellets are formed in the rectum and excreted through the anus.

Data on physicochemical conditions in the gut of woodlice were reviewed by Zimmer (2002). The pH levels in the digestive system vary from acidic in the midgut glands and anterior chamber of the hindgut to slightly acidic in the posterior part (reviewed in Zimmer 2002), which should be in accordance with conditions suitable for enzymatic degradation of litter in the anterior chamber and bacterial proliferation in the papillate region. The presence of Na^+/K^+-ATPases, known to regulate pH in the invertebrate gut (Dow 1992), was demonstrated in the apical part of the epithelial cells in the hindgut of *P. scaber* (Warburg and Rosenberg 1989; Štrus et al. 2002), although direct measurements of pH in the isopod gut were not performed in these studies.

The presence of an anaerobic zone in the radial centre of the hindgut, as a result of the oxygen-consuming metabolism of the ingested facultative anaerobes, was suggested by Zimmer (2002). However, the large surface to volume ratio of the gut, its tube-like anatomy, the presence of air pockets in ingested food (Hames and Hopkin 1989) and the redox potential between +50 mV and +10 mV (Hartenstein 1964) indicate the prevalence of aerobic conditions in the gut, but anaerobic microhabitats cannot be excluded (Savage 1978).

In terrestrial isopods digestive juices are produced in the midgut glands and possibly also in the short endodermal part of the midgut in amphibious species (Štrus et al. 1995). The midgut glands are the central metabolic organs with absorptive, secretive, storage and excretive functions. The enzymatic composition of digestive juices produced in digestive glands was reviewed by Zimmer (2002). Possible existence of endogenous cellulases was not studied in isopods. They were described in nematodes (Yan et al. 1998), termites (Watanabe et al. 1998) and crayfish (Byrne et al. 1999; Xue et al. 1999). It is assumed that microbial enzymes ingested with food (Kozlovskaja and Striganova 1977, Kukor and Martin 1986; Ullrich et al. 1991) or produced by endosymbiotic bacteria in the midgut glands (Zimmer and Topp 1998a, b) are utilised for decomposition of ingested lignocellulose. Since the origin of cellulases and the role of microbiota in the digestive system of terrestrial isopods were not proved indisputably, mainly due to the shortcomings of the methods applied, this still remains to be elucidated.

5.3 The Microbiota of the Digestive System

As in other arthropods, the foregut of terrestrial isopods is generally poorly inhabited by microorganisms, while rich and varied flora is present in the tube-like hindgut, especially in its posterior part (Bignell 1984; Drobne 1995; Kostanjšek et al. 2003). According to Zimmer and Topp (1998b), the total number of gut microbiota vary from 300 to 700 million cells/mg of gut content, among which only 0.01 to 1% are cultivable (Reyes and Tiedje 1976a; Coughtrey et al. 1980; Carefoot et al. 1984; Griffiths and Wood 1985; Zimmer and Topp 1998b; Drobne et al. 2002).

Since terrestrial isopods prefer to feed on food colonised by microorganisms (Soma and Saito 1983; Gunnarsson 1987; Zimmer et al. 1996) and lack the gut compartments in which abundant resident microflora would develop, their gut microbiota consists mainly of ingested microbes (Hassall and Jennings 1975; Reyes and Tiedje 1976a; Ineson and Anderson 1985). However, the presence of resident bacteria has been suggested in the hindgut (Kostanjšek et al. 2002, 2003) and midgut glands (Wood and Griffiths 1988; Zimmer 2002). Part of the ingested microbiota, mainly fungi and Gram negative bacteria, are digested during passage through the digestive system (Reyes and Tiedje 1976b; Coughtrey et al. 1980; Hanlon and Anderson 1980; Hanlon 1981b; Gunnarsson and Tunlid 1986; Clegg et al. 1994, 1996; Kayang et al. 1994; Zimmer and Topp 1998b; Kostanjšek 2002). Some of the undigested bacteria seem to be passive transients through the gut (Márialigeti et al. 1984). The others, mainly Gram positive bacteria, can proliferate in the hindgut and subsequently in faeces (summarised in Table 5.1) (Reyes and Tiedje 1976a; Coughtrey et al. 1980; Hanlon and Andreson 1980; Ineson and Anderson 1980; Hassall et al. 1987; Hanlon 1981b; Gunnarsson and Tunlid 1986; Kayang et al. 1994).

5.3.1 Bacteria in the Gut

Although it has been assumed that bacteria in the gut consist mainly of ingested bacteria, it would appear likely that isopods possess and exploit resident gut bacteria in digestion, like other lignocellulose-feeding animals. Due to the gut anatomy, frequent renewal of the gut cuticle and short retention time of food, the gut of terrestrial isopods seems at the first glance a rather unsuitable environment for the development of resident microbiota (Hassall and Jennings 1975). However, reports based on traditional microbiological techniques (Reyes and Tiedje 1976a; Griffiths and Wood 1985; Ineson and Anderson 1985; Gunnarsson and Tunlid 1986; Hassall et al. 1987;

Table 5.1. Bacterial taxa from the digestive system of terrestrial isopods

Bacterial taxa	G	MG	F	Isopod	References
Aeromonas sp.	–	+	–	*Porcellio scaber*	Ullrich et al. (1993)
Acinetobacter calcoaceticus	–	–	–	*Oniscus asellus*	Ullrich et al. (1991)
Arthrobacter sp.	+	–	+	*Oniscus asellus*	Ullrich et al. (1991)
Azotobacter agilis	+	–	–	*Oniscus asellus*	Beerstecher et al. (1954)
Bacillus sp.	+	–	–	*Porcellio scaber*	Kostanjšek et al. (2002)
Bacillus cereus	+	–	–	*Porcellio scaber*	Jorgensen et al. (1997)
	+			*Porcellio scaber*	Margulis et al. (1998)
Bacteroides sp.	+	–	–	*Porcellio scaber*	Kostanjšek et al. (2002)
Chrmombacterium violaceum	–	–	+	*Oniscus asellus*	Ullrich et al. (1991)
Citrobacter freundii	–	+	–	*Oniscus asellus*	Ullrich et al. (1993)
		+		*Porcellio scaber*	Ullrich et al. (1993)
Corynebacteriaceae	+	–	+	*Oniscus asellus*	Ullrich et al. (1991)
Corynebacterium sp.	+	–	–	*Oniscus asellus*	Ineson and Anderson (1985)
Cytophaga sp.	–	–	+	*Oniscus asellus*	Hassall et al. (1987)
			+	*Porcellio scaber*	Hassall et al. (1987)
			+	*Armadillidium vulgare*	Hassall et al. (1987)
Desulphotomaculum ruminis	+	–	–	*Porcellio scaber*	Lapanje et al. (2003); Kostanjšek et al. (2004a)
Enterobacteriaceae		+	–	*Oniscus asellus*	Ullrich et al. (1991)
Enterobacter agglomerans	+		–	*Oniscus asellus*	Griffiths and Wood (1985)
		+	–	*Porcellio scaber*	Ullrich et al. (1993)
Enterobacter intermedium	–	+	–	*Porcellio scaber*	Ullrich et al. (1993)
Enterocococcus faecium	+	–	–	*Porcellio scaber*	Kostanjšek et al. (2002)
Enterococcus sp.	+	–	–	*Porcellio scaber*	Kostanjšek et al. (2002)
Flavobacterium sp.	+	–	+	*Tracheoniscus rathkei*	Reyes and Tidje (1976a)
Klebsiella pneumoniae	+		+	*Oniscus asellus*	Ineson and Anderson (1985)
		+		*Porcellio scaber*	Ullrich et al. (1993)
Micrococcaceae	+	–	–	*Oniscus asellus*	Ullrich et al. (1991)
Mollicutes	+	+	+	*Porcellio scaber*	Kostanjšek (2002); Kostanjšek et al. (2004a)
Mycobacteriaceae	–	–	+	*Oniscus asellus*	Ullrich et al. (1991)
Neisseria sp.	+	–	–	*Porcellio scaber*	Kostanjšek et al. (2002)
Paracoccus sp.	+	–	–	*Porcellio scaber*	Own observation
Plesiomonas	+	–	–	*Oniscus asellus*	Griffiths and Wood (1985)

Table 5.1. (continued)

Bacterial taxa	G	MG	F	Isopod	References
Pseudomonadaceae	+	–	+	*Oniscus asellus*	Ullrich et al. (1991)
Pseudomonas fluorescens	+	–	–	Oniscus asellus	Griffiths and Wood (1985)
Pseudomonas sp.	+		–	*Porcellio scaber*	Kostanjšek et al. (2002)
	+			*Tracheoniscus rathkei*	Reyes and Tidje (1976a)
		+		*Porcellio scaber*	Kostanjšek (2002)
Rhabdochlamydia porcellionis		+		*Porcellio scaber*	Kostanjšek et al. (2004b)
Shevanella sp.	+	–	–	*Porcellio scaber*	Own observation
Spirilaceae	–	–	+	*Oniscus asellus*	Ullrich et al. (1991)
Streptomyces humidus	–	–	+	*Protracheoniscus amoneus*	Márialigeti et al. (1984)
Streptomyces moderatus	–	–	+	*Protracheoniscus amoneus*	Márialigeti et al. (1984)
Streptomyces nodosus	–	–	+	*Protracheoniscus amoneus*	Márialigeti et al. (1984)
Streptomyces pluricolorescens	–	–	+	*Protracheoniscus amoneus*	Márialigeti et al. (1984)
Streptomyces spadicis	–	–	+	*Protracheoniscus amoneus*	Márialigeti et al. (1984)

G, gut; *MG*, midgut glands; *F*, faeces

Ullrich et al. 1991) and molecular approaches applying 16S rRNA sequence analysis (Kostanjšek et al. 2002) indicated the possible presence of resident bacteria in the gut of terrestrial isopods. Moreover, long rod-like bacteria attached to the hindgut cuticle were observed in the posterior hindgut of *P. scaber* (Drobne 1995; Kostanjšek et al. 2003). Phylogenetic analysis based on 16S rRNA gene sequences grouped the latter in an independent and deeply branched cluster within *Mollicutes* (Kostanjšek et al. 2004a), while ultrastructural observations revealed a spherical attachment structure at the tip of these bacteria. Since such structures may be required for the specific attachment of bacteria to cuticular spines of the gut surface, thus revealing high adaptation to the digestive system, the attached bacteria might represent truly autochthonous gut bacteria of *P. scaber* (Kostanjšek et al. 2003).

Whereas a considerable part of resident gut bacteria colonises the gut via food (Reyes and Tiedje 1976a; Griffiths and Wood 1985; Ineson and Anderson 1985; Gunnarsson and Tunlid 1986; Ullrich et al. 1991), the presence of gut bacteria which cannot be detected in the soil or food (Hassall et al. 1987; Ullrich et al. 1991; Kostanjšek et al. 2002) indicates other possible ways of gut colonisation. This was supported by the discovery of attached rod-like bacteria on the newly-formed hindgut cuticle, still overlaid with

the old, intact cuticle (Kostanjšek 2002). Coprophagy and ingestion of the old hindgut cuticle as paths of gut recolonisation after exuviations may therefore not be the only and most important ways. It further indicates that rod-like bacteria somehow remain in the gut despite exuviation, enabling them to colonise the new gut cuticle soon after it has been formed (Kostanjšek 2002).

Although the environment in the hindgut of terrestrial isopods is thought to be generally oxic, the mucopolysaccharides present in large amounts on the surface of the gut could provide microniches for anaerobic bacteria. Apart from slowing down the diffusion of oxygen, these biopolymers also enable bacterial attachment (Hartenstein 1964; Savage 1978). Several unsuccessful attempts to cultivate isopod gut microbes under anaerobic conditions were described (Reyes and Tiedje 1976a; Ullrich et al. 1991), but the presence of anaerobic bacteria was indirectly indicated by the detection of mercury methylation in the gut of *P. scaber* (Jereb et al. 2003). Since mercury can be biomethylated by sulfate-reducing bacteria (SRB), further work was focused on these bacteria in the digestive system of isopods (Kostanjšek et al. 2004a). Termites were the only arthropods in which SRBs were reported previously (Trinkerl et al. 1990; Ohkuma and Kudo 1996).

The SRB utilise various low molecular weight acids in the presence of sulfate and are major decomposers of organic matter in anaerobic environments (Santegoeds et al. 1998; Vester and Ingvorsen 1998). One of the side effects of SRB metabolic pathways is the methylation of mercury, where up to 95% of available mercury is transformed into highly toxic methyl mercury (Compeau and Bartha 1984, 1985; Pak and Bartha 1998a, b). Anaerobic culturing techniques were used successfully to enrich SRB from the substrate and gut samples, but not from the hepatopancreas and faeces. According to phylogenetic analysis based on 16S rRNA gene sequence these, first strictly anaerobic bacteria isolated from the gut of a terrestrial isopod, were identified as *Desulfotomaculum ruminis* (Lapanje et al. 2003).

Although oxygen influx from the surrounding haemolymph through the gut epithelium keeps the peripheral ring of the hindgut oxic (Zimmer 2002), the presence of strictly anaerobic SRBs (Lapanje et al. 2003) and the discovery of ribosomal genes from other anaerobic bacteria (Kostanjšek et al. 2002) on the hindgut cuticle indicate anaerobic microniches in the hindgut of terrestrial isopods (Kostanjšek et al. 2004a).

5.3.2 Fungi and Protozoa in the Gut

The concentration of fungi that can be cultivated in terrestrial isopods varies from 10^4 to 10^5 cells/mg of gut content (Coughtrey et al. 1980;

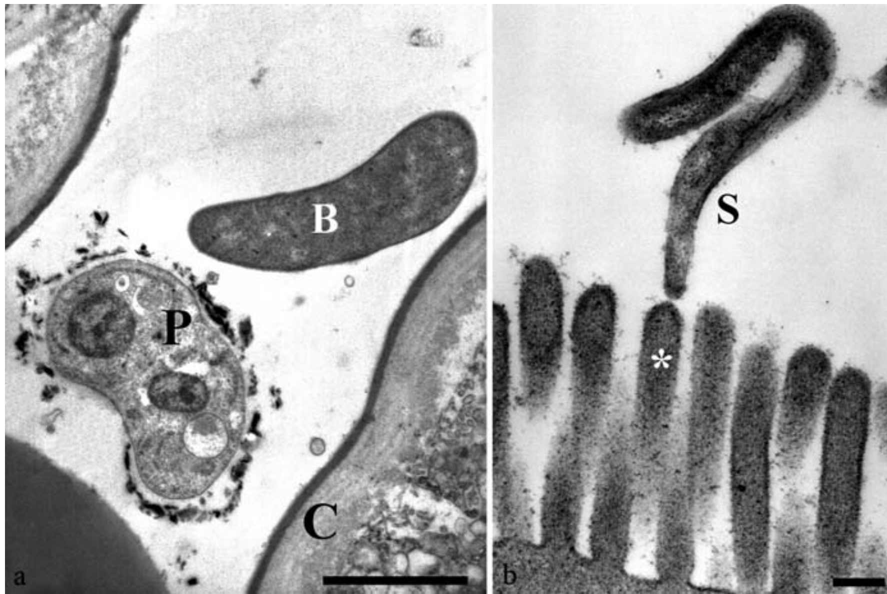

Fig. 5.2. Microorganisms in the digestive tract of terrestrial isopods. **a** Binucleate protist (*P*) and bacterium (*B*) above the hindgut cuticle (*C*) of *Ligia italica*. *Scale bar* 2 µm. **b** Spirochaeta (*S*) attached to microvillus (*asterisk*) of midgut gland cell of *Porcellio scaber*. *Scale bar* 200 m

Zimmer and Topp 1998b). However, the fungal flora of these animals is generally poorly known. Fungal genera observed in the isopod hindgut are *Penicillium*, *Trichoderma*, *Fusarium*, *Cladosporium*, *Aspergillus* and *Mucor* (Kayang et al. 1996). The symbiotic trichomycetes *Asellaria armadillidii, Parataeniella armadillidii, P. latrobi, P. dilatata, P. mercieri, P. scotonisci, Palavascia philosciae, Eccrinoides monticolae* and *E. helleriae* attached to the hindgut cuticle were reported in the gut as well (Lichtwardt et al. 2001). Although binucleate protist-like organisms were observed (Fig. 5.2) on the cuticular gut surface in the amphibious species *Ligia italica* and *Titanethes albus*, data on protozoa in the digestive system of isopods are scarce. However, some genera of trichomycetes described in the arthropod gut were found to be protists rather than fungi after detailed examination (Cafaro 2003).

5.3.3 Bacteria in the Midgut Glands

The entrance of microbes from the stomach into the digestive glands is supposed to be prevented by a system of chitinous filters (Hames and Hopkin 1989; Storch and Štrus 1989), allowing only fluids and particles smaller

than 40 nm to pass (Hames and Hopkin 1989). However, the presence of bacteria in the lumen of the digestive glands was observed in *Porcellio dilatatus* (Donadey and Besse 1972), *O. asellus* (Hopkin and Martin 1982; Wood and Griffiths 1988; Hames and Hopkin 1989, 1991; Ullrich et al. 1991; Clegg et al. 1996), *P. scaber* (Wood and Griffiths 1988; Hames and Hopkin 1989, 1991; Ullrich et al. 1991; Zimmer and Topp 1998a, b; Kostanjšek 2002) and *L. pallasii* (Zimmer et al. 2001) (Table 5.1). Ultrastructural investigations revealed the presence of various bacterial morphotypes in the digestive glands (Zimmer 2002). Among them rod-like hepatopancreatic bacteria described in *P. scaber* and *O. asellus* by Wood and Griffiths (1988) exhibit a high morphological resemblance to the *Mollicutes*-related bacteria attached to the hindgut cuticle of *P. scaber* (Kostanjšek 2002). Beside, the spirochetes attached to microvilli of gland cells and in the lumen of midgut glands were observed in *L. italica, T. albus* and *P. scaber* (Fig. 5.2).

In spite of abundant observations, the data on the occurrence of bacteria in the hepatopancreas may still be ambiguous. In some cases only the occasional presence of hepatopancreatic bacteria was observed in *P. scaber* and *O. asellus* (Wood and Griffiths 1988), contrary to observations from other authors, reporting their permanent presence and high bacterial counts in *P. scaber*, *O. asellus* and *L. pallasii* (summarised in Zimmer 2002). The latter led to the conclusion that the role of hepatopancreatic bacteria is crucial in the decomposition of complex organic compounds in the isopod diet (Zimmer 2002).

Our electron microscopic observations and results of the molecular detection of bacterial ribosomal genes (Kostanjšek 2002) supported the occasional presence of diverse bacteria in the digestive glands. Among hepatopancreatic bacteria genus *Pseudomonas* and rod-like *Mollicutes* commonly attached to the hindgut cuticle prevailed (Kostanjšek 2002). Since both these groups of bacteria are commonly found in the gut and are not always present in the hepatopancreas, it would be more likely that they occasionally invade the digestive glands from the gut, rather than being permanent hepatopancreatic endosymbionts (Wood and Griffiths 1988; Zimmer 2002).

5.3.4 Infections of the Digestive System

Tissues of terrestrial isopods are, like tissues of other crustaceans (Fryer and Lannan 1994), often invaded by intracellular bacteria (Federici 1984; Shay et al. 1985; Abd El-Aal and Holdich 1987; Bouchon et al. 1998; Drobne et al. 1999).

The first observed intracellular infection in terrestrial isopods was caused by *Rickettsiella grylli* (Weiss et al. 1984a; Roux et al. 1997). The infections were described in *Armadilidium vulgare* (Vago et al. 1970), *P. dilatatus* (Federici 1984), *Porcellio scaber* and *Oniscus asellus* (Abd El-Aal and Holdich 1987). The infection affected adipose tissue in haemocoel, muscular and connective tissue around the anal region, epidermis and tegumental glands (Fedrereici 1984; Abd El-Aal and Holdich 1987).

Another intracellular infection was described in the hepatopancreatic tissue of *P. scaber* (Shay et al. 1985; Drobne et al. 1999). In spite of certain similarities with *R. grilly*, the infection exhibits distinctive pathological changes in infected tissue and unique morphological features. In earlier descriptions different authors affiliated these bacteria with different bacterial taxa, despite the morphological and pathological similarities (Shay et al. 1985; Drobne et al. 1999). Phylogenetic analysis based on comparative 16S rRNA gene sequencing affiliated the infection agent to the ordo *Chlamydiales*, within which it forms an independent lineage, clearly distant from other known chlamydia. Due to the phylogenetic affiliation and distinctive morphology of the elementary bodies, the name '*Candidatus* Rhabdochlamydia porcellionis' was proposed (Kostanjšek et al. 2004b).

Beside the infections mentioned above, intracellular infection caused by bacteria from the genus *Wolbachia* also affects terrestrial isopods (Weiss et al. 1984b; O'Neill et al. 1997, Bouchon et al. 1998; Nyrö et al. 2002). The infection is transmitted maternally via egg cytoplasm and affects the host reproduction, enhancing the spread of the infectious agent (Rigaud and Rousset 1996; Bourtzis and O'Neill 1998). Although *Wolbachia* infection is generally focused on the gonads, it has also been detected in somatic tissues, including the digestive system of insects (Dobson et al. 1999) and terrestrial isopods (Martin et al. 1973; Rousset et al. 1992). The *Wolbachia* infection can alter the host's reproduction by male killing, parthenogenesis, cytoplasmic incompatibility and feminisation of genetic males (Bourtzis and O'Neill 1998). However, only the latter two alterations were detected in terrestrial isopods (Juchault et al. 1992; O'Neill et al. 1997).

5.4 Conclusions

Terrestrial isopods are decomposers of plant material in terrestrial environments. They promote microbial activity by trituration of plant material and distribution of microbes in the ecosystem. They preferentially feed on decayed plant material colonised by microorganisms, which are also utilised as source of nutrients and enzymes. Whereas some undigested mi-

croorganisms are passive transients, others proliferate in the hindgut and are distributed in the terrestrial environment with faeces.

The digestion of terrestrial isopods strongly depends on degradation of lignocellulose. The origin of cellulases in their digestive system still remains to be elucidated, however. Since the role of cellulolytic bacteria in the midgut glands is questionable, due to their only occasional presence in this organ, other sources of cellulases must be taken into account. The food preference of isopods indicates a potential role of ingested cellulolytic microbiota, but the presence of endogenous cellulases in the isopod digestive system cannot be excluded, either.

The gut of terrestrial isopods appeared for a long time to be an unsuitable environment for development of resident and anaerobic microbiota. The main arguments supporting this theory were its tube-like anatomy, the rapid passage of the food and frequent renewal of gut cuticle. However, the finding of bacteria attached to the hindgut surface with the ability to recolonise the gut cuticle after moulting indicates that some bacteria have adapted to this environment. The subsequent isolation of strictly anaerobic bacteria confirmed the presence of anaerobic microniches on the otherwise oxic hindgut surface. This shows the presence of microhabitats in the gut, which allow development of diverse resident microflora in the gut, in spite of its apparently unsuitable conditions.

In comparison to some other cellulose-feeding arthropods, data on microbiota in the digestive system of isopods are scarce, in spite of their importance as decomposers and reservoirs for various infections. However, previous work on this topic reveals that the digestive system of terrestrial isopods is a unique microbial environment, which remains an interesting field for further microbiological research.

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6 Collembola as a Habitat for Microorganisms

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6.1 Introduction – Diversity and Activity of Collembola

Collembola (springtails) are microarthropods that can range in size between 0.2 mm and 1 cm, with most having an average length of 1–5 mm. To date, approximately 7500 different species have been described, and there is no doubt that Collembola are among the most abundant groups of arthropods on Earth (www.tolweb.org). Traditionally, taxonomy has placed Collembola in the group of insects, but recent classification places them in a class alone under the Superclass Hexapoda. Collembola are regarded as a phylogenetically old group, with an age of almost 400 million years. They are probably the oldest Hexapoda alive. Their position in the phylogenetic tree is still under debate as there is recent evidence, based on molecular and morphological data, that Collembola are actually more closely related to crustaceans then to insects, which would mean that the Superclass Hexopoda is actually not monophyletic (Nardi et al. 2003; Bellinger et al. 2004). Within the class of Collembola, however, the systematic classification seems to be, at least roughly, in good accordance with new data from molecular phylogeny (Frati et al. 1997; Park 2002; D'Haese 2003). In a recent classification presented by Hopkin in his book about Collembola (Hopkin 1997), three orders can be differentiated: i.e., (1) the *Arthropleona*, with 15 families and more than 5,500 species, (2) the *Neelipleonea*, with only one family and 25 species, and (3) the *Symphyploeona*, with two families and almost 900 species.

Most Collembola live in soil or leaf litter covering the soil surface. Epedaphic species are adapted to living in the litter layer; hemiedaphic species colonise mainly the upper organic layers of soils; and euedaphic species have adapted to living in the soil matrix, but typically not deeper than 10 cm below the soil horizon (Hopkin 1997). Epedaphic Collembola tend to be pigmented, whereas the euedaphic Collembola are often non-pigmented. The geographical range of Collembola is enormous, as they

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are found in all imaginable climatic regions, from the Antarctic or Arctic environment to the tropical belt (Rusek 1998). In most terrestrial ecosystems, Collembola are found as an important part of the soil mesofauna, which also includes mites, nematodes, enchytraeids, millipedes, earthworms, ants, small gastropods, isopods, or larvae of insects. Densities of 10^4 to 10^5 specimens of Collembola per m^2 are not unusual for many soils and, in such soils, Collembola are an important part of the terrestrial food web. On the one hand, they feed on different organic materials and, on the other, they serve as prey, especially for predatory mites and spiders (Hunt et al. 1987; Bilde et al. 2000; Agusti et al. 2003; Bonte and Mertens 2003).

Together with mites, Collembola are often the major constituents of soil microarthropods. Microarthropods, as a functional group of ecological importance, initiate the degradation of organic material, e.g., that accumulates in the litter layer, and thereby ultimately enhance the cycling of carbon and nitrogen in soil (Filser 2002). By chewing on organic substrates, such as dead plant material, and subsequently passing the substrates through the gut, microarthropods restructure the organic material and facilitate microbial degradation (Rusek 1998). Collembola feed on fungal mycelia and some other organic substrates, among them animal remains (Hopkin 1997; Rusek 1998). *Folsomia candida* (Isotomidae, Entomobryoidea, Arthropleona), one of the most laboratory-reared Collembola species, was even shown to feed on nematodes (Lee and Widden 1996). Other Collembola can feed on living plants at certain environmental conditions and thereby cause some problems in agriculture (Sievers and Ulber 1990; Bishop et al. 2001). On the other hand, Collembola can consume plant pathogenic fungi or stimulate growth of mycorrhiza and thereby support plant health (Lussenhop 1996; Nakamura et al. 1992; Gange 2000; Sabatini and Innocenti 2000, 2001).

The feeding preferences of different Collembola species have been investigated in both laboratory studies and by analysing the gut of field-collected specimens. In the laboratory it could be demonstrated that different Collembola have different feeding preferences, e.g., for certain species of fungi or other microorganisms (Visser and Whittaker 1977; McMillan 1976; Bardgett et al. 1993; Chen et al. 1995; Kaneko et al. 1995; Thimm and Larink 1995). There are indications that Collembola can smell food or detect carbon dioxide gradients (Bengtsson et al. 1991; Hedlund et al. 1995) and both attributes probably serve to differentiate food in terms of palatability. In breeding stocks, e.g., with *F. candida*, the specimens can be fed with autoclaved baker's yeast or pea puree, but it can be observed that the animals also feed on their own faeces, on the exuvia they generate by moulting, and even on dead specimens, if the opportunity is given (Borkott and Insam 1990; own observations).

Analyses of the gut contents of field-collected Collembola frequently show a high diversity of different material, indicating that many Collembola

are actually omnivores and less selective than suggested by food preference assays in the laboratory. Numerous substrates have been detected in the gut contents from single specimen; e.g., fungal hyphae, collodial material, plant material, fungal spores, pollen, bacteria and animal remains (Chen et al. 1996). The composition of the contents of the gut material varies between species from the same habitat, but it also varies within a species. Seasonal changes, which affect the availability of different food sources, as well as environmental factors, including pollutants, account for these variations (McMillan 1975; Anderson and Healey 1972; Ponge 2000; Gillet and Ponge 2003). It should be noted that the uptake of different foods may not only affect processes associated with digestion and mineralisation, but also with dispersal. Dispersal of ingested substrates may be beneficial or harmful, depending on whether mycorrhizal spores, which eventually stimulate plant growth, or pathogenic fungi, which destroy plants, are transported to root surfaces (Williams et al. 1998; Dromph 2001). Collembola not only move actively by jumping – they can also be dispersed over long distances by wind (Hopkin 1997). Inevitably, the dispersal of Collembola also means that the gut contents (spores, pollen, microorganisms) are dispersed. Recently this aspect has drawn our attention with regard to the importance of Collembola in unintentionally disseminating genetically engineered microorganisms (Tebbe 2003) or plant pathogenic bacteria (Hildebrand et al. 2001; for more details, see Sects. 6.3, 6.4, and 6.5).

In this chapter we report on own experimental studies that were conducted to investigate the interactions between Collembola and soil bacteria. The objectives of the studies were to learn more about the specific conditions that bacteria face in the gut of Collembola and about the fate of ingested bacterial cells during the gut passage. Would certain bacteria be preferred as food sources and would the composition of the bacterial communities on substrates be affected during a gut passage, e.g., by differential digestion? One specific aspect of our studies also related to the importance of the gut of Collembola as a hot spot for gene transfer between bacteria; other studies were concerned with elucidating the diversity of indigenous bacteria that can be found in the gut or in other compartments of the collembolan body.

Most of our studies were conducted with the previously mentioned, euedaphic, non-pigmented collembole *F. candida* (Fig. 6.1), a species that is ubiquitous and that can easily be kept in laboratory breeding stocks. The species reproduces in the breeding stocks by parthenogenesis, all specimens were female. *F. candida* has a typical morphology for a collembole, with a ventral tube and a springing organ, the *furca*. The furca, a typical feature of Collembola, is held by the tentaculum (a catch) and it is used to make jumps, by snatching out of this tentaculum. In the breeding stocks, which can be maintained in jars with plaster of Paris and charcoal on the bottom

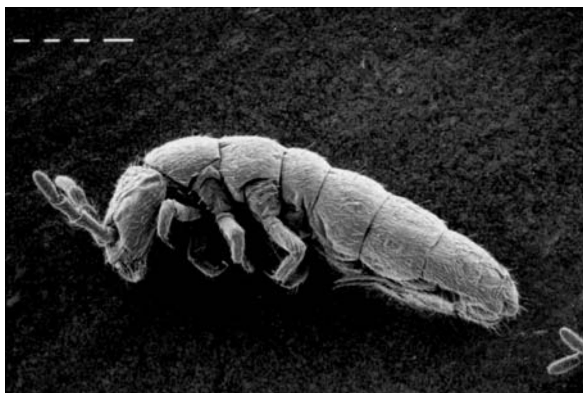

Fig. 6.1. *Folsomia candida*, a euedaphic parthenogenetic species that is can easily be reared in the laboratory, as seen by scanning electron microscopy (SEM) (length of the specimen approx. 2 mm)

(Goto 1960), the females lay their eggs and after 8 to 10 days, the young collemboles hatch. While growing, *F. candida* moults frequently, i.e., it peels off the old cuticula and replaces it by a new cuticula, which is generated under the old one by the epithelium. In contrast to insects, Collembola also regenerate their midgut epithelium during moulting (Humbert 1979). After hatching, *F. candida* moults at intervals of 3 to 10 days. The frequency of moulting decreases during the life of *F. candida* (Snider 1972). After only six moulting stages, the *F. candida* start laying eggs. In our breeding stocks, the specimens reach an age of approx. 10 months when they are kept at 18 °C.

6.2 The Gut of *Folsomia candida* – an Unusual Microbial Habitat That Is Affected by Moulting

The gut of *F. candida* is a rod-like tube, with a small foregut, a large midgut that can increase in size with the amount of food, and a short hindgut. Based on microscopic sections and the direct analyses of lactic acid-treated specimens under the microscope, the volumes of the gut were estimated and the small foregut was found to have a volume of 0.21 nl, the midgut of 6–12 nl, when it was filled, and only 1 nl when it was empty (Thimm et al. 1998). The faecal pellets that were deposited by *F. candida* in the breeding stocks had an approximate volume of 1 nl. With scanning electron microscopy (SEM), a biofilm-like dense layer of mainly rod-shaped bacterial cells could be detected in the region of the peritrophic mem-

brane (Fig. 6.2). The peritrophic membrane is a layer on the gut epithelium which is common in insects and collemboles (Wang and Granados 2001). It is composed of chitinous microfibrills embedded into a proteoglucan matrix (Terra 2001b), and its function is to facilitate the transport of the food bolus through the gut and possibly also to protect the gut epithelium, e.g., from the attack of microbial pathogens (Terra 2001a). Beside this biofilm-like region of the peritrophic membrane, bacterial cells could also be detected in the food bolus (Fig. 6.3), even when sterile food was

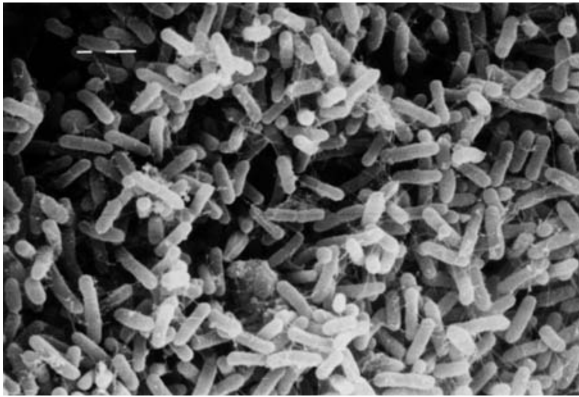

Fig. 6.2. Detection of bacterial cells forming a biofilm-like structure in the peritrophic membrane (PM) as seen with SEM. Note that the mucous layer of the PM is removed by dehydration of the samples, necessary for SEM (figure from Thimm et al. 1998, courtesy of ASM Press). Length of the *left bar*, 1 µm

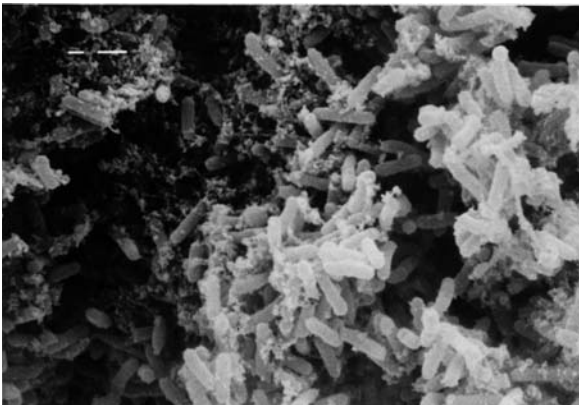

Fig. 6.3. Bacterial cells in food bolus taken from the midgut as detected by SEM. Note that the applied food was sterile and the bacteria originated from the region of the peritrophic membrane, shown in Fig. 6.3. Length of the *left bar*, 1 µm

fed. It is likely that the bacteria located in the region of the peritrophic membrane start to colonise the ingested food and utilise it for growth. Borkott and Insam (1990) detected a total of 4×10^{11} cfu g^{-1} in faeces of *F. candida*, which would correspond, considering the gut volumes determined in these studies, to about 10^6 cfu per specimen. This is about the upper limit of what we detected in our studies. During the moulting cycles, the midgut epithelium is regenerated, the old epithelium, including the peritrophic membrane, is excreted and replaced by a new epithelium that has developed underneath the old one. During the moulting process, the specimens stop feeding, probably because of a transiently non-functional gut. The new epithelium is then coated again by a peritrophic membrane which is excreted from specific epithelium cells in the foregut (Hopkin 1997). Since the peritrophic membrane is densely colonised by bacterial cells, the moulting process results in a dramatic change of the bacterial population in the gut. These changes in bacterial gut colonisation could be quantified by comparing the bacterial cell density in the gut of actively feeding *F. candida* to those that were not feeding (Thimm et al. 1998). Feeding specimens had 1.6×10^4 to 2.7×10^5 cfu per specimen in their gut, as determined by cultivation under aerobic conditions on yeast extract agar. In contrast, the bacterial cell densities in the non-feeding population ranged from 4.9×10^2 to 2.3×10^6 cells per specimen. The high cell numbers were found in specimens immediately before, and the low cell numbers immediately after, the excretion of the old gut epithelium and peritrophic membrane. It can be concluded that the moulting process generates high fluctuation rates and turbulences within the bacterial community and se-

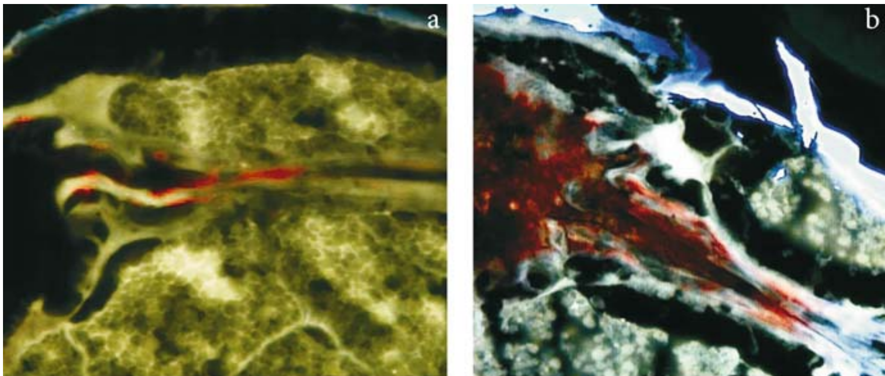

Fig. 6.4. Recolonisation of the gut by bacteria after moulting. **a** Pylorus region with remaining bacteria after the moulting process – a possible starting point for bacterial recolonisation of the gut; **b** growth of bacteria into the hindgut region. Bacterial cells (*red*) were detected by fluorescence in situ hybridisations (FISH) with the 16S rRNA gene probe EUB388, Aargetting all bacteria (Thimm and Tebbe, 2003)

lects for bacteria that are able to rapidly multiply in the gut. The question emerged, from where the bacteria actually recolonise the gut after moulting. Probably, a main pathway for inoculation is faeces, including exuvia, on which *F. candida* normally feeds. Another pathway, however, may come from the opposite direction. We have recently identified structures in the pylorus region, located between the midgut and hindgut, in which bacterial cells were detected even in specimens that had just moulted. Detection of bacterial cells by fluorescence in situ hybridization (FISH) in this region indicated that the bacterial recolonisation of the gut may also start from this point (Fig. 6.4).

6.3 Feeding Preferences of *Folsomia candida* and Fate of Ingested Bacterial Cells

Under natural conditions, *F. candida* seems to be an omnivore, as it feeds on dead organic material as well as on fungal mycelia, nematodes or bacteria. *F. candida*, in fact, is very adaptable to different food sources. To understand the impact of collembolan feeding on the bacterial community structure and diversity in soil, we conducted feeding preference studies with different bacterial species. At the outset of these investigations, it was unclear whether bacteria would be digested, not affected or even stimulated in growth during a gut passage. The feeding preferences for bacteria were tested with a total of twelve different bacterial strains, among them Gram-positive and –negative strains from type-culture collections. These strains were fed to *F. candida* specimens in petri dish-size microcosms offering pairs of choices (Thimm et al. 1998).

From a total of 66 tests, 22 showed significant preferences. Eight different preference classes could be differentiated. The most preferred class contained the type culture strain *Pseudomonas putida* PaW340 and a strain isolated from faeces of *F. candida*. As indicated by 16S rRNA gene sequencing, this strain was also a *P. putida* or a close relative (similarity to the 16S rRNA gene of *P. putida* PaW340, 99.1%). The second class contained two bacterial strains, both isolated from *F. candida* faeces: the Gram-positive isolate, *Arthrobacter citreus* and a close relative of the Gram-negative *Alcaligenes faecalis*. Interestingly, the *A. citreus* was an isolate from a different breeding stock. In fact, the bacteria were kept for nine years in a culture collection (by H. Borkott, Braunschweig) before they were fed to *F. candida* in our studies. The lower preference classes contained different type culture strains but no isolates from *F. candida*. The least preferred classes contained *Corynbacterium glutamicum* and *Bacillus subtilis*. Both species have a potential to live in soil. Also, *Escherichia coli* fell into a low preference class. We

were aware that these choice experiments were not very realistic in regard to natural food sources of *F. candida*, as it is unlikely that *F. candida* finds opportunities to choose between different bacterial species in its natural habitat. On the other hand, the obvious feeding preference of *F. candida* for its own gut bacteria indicated that these bacteria were recognised as beneficial and probably not as a well-digestible food source. Interestingly, the isolate *A. citreus* exhibited chitinase activity and, thus, could possibly act as a gut symbiont, as suggested by Borkott and Insam (1990).

The digestibility of different bacterial species was tested by feeding *F. candida* for one day with strains that had been genetically tagged with the luciferase gene (*luc*) or the gene encoding for the Green Fluorescence Protein (*gfp*). By this means, the bacteria could easily be differentiated from other, indigenous bacteria. The faeces of the specimens were analysed over a period of 56 days in which only sterile food was available for the specimens. *E. coli* cells could only be detected in faeces one day after feeding of the bacteria. The soil bacteria *Sinorhizobium meliloti* or *Pseudomonas stutzeri* were only detected during the first week. In contrast, *P. putida* cells were detected for 20 days and *A. faecalis* even until the end of the experiment (56 days). The data indicated that bacterial species can differ significantly in their capacity to survive or even colonise the gut of *F. candida*.

The "pulse-feed" studies were complemented by studies in which the effect of the gut passage on ingested bacteria was quantified in more detail. Based on feeding colorised food we first determined that the period between ingestion and excretion of food was only 35 min. When we fed *E. coli*, we only detected an average of 4.3 cfu in the gut of each specimen. In contrast, with *S. meliloti* and *A. faecalis*, the other two strains tested, cell numbers were approx. four orders (!) of magnitude higher, indicating these bacteria were not as efficiently digested as *E. coli*. Consequently, due to the efficient digestion, the numbers of *E. coli* cells in faeces were also very low (2 cfu per faecal pellet) and the number of *S. meliloti* and *A. faecalis* were one to two orders of magnitude higher. As estimated from uptake and release rates of the selected strains, *E. coli* populations were reduced 60,000-fold whereas *A. faecalis* was only reduced 500-fold (Thimm et al. 1998). The studies demonstrated that even though the period of time for a gut passage was relatively short, the species specific effects were quite dramatic.

We extended this type of study on the survival of ingested bacterial cells to another Collembola species, i.e., *Protaphorura fimata* (formerly *Onychiurus fimatus*; Onychiuridae, Poduromorpha, Arthropleona). *P. fimata* is a sexually reproducing, non-pigmented, euedaphic species that is less associated with the litter fraction of soils than *F. candida* and that can feed on mycorrhizal fungi as well as on the roots of living plants. In accordance with our studies on *F. candida*, *E. coli* was efficiently eliminated from the gut within two days after feeding, but in contrast to *F. candida*, the soil

bacterium *S. meliloti* was only detectable for two days instead of a full week (Hoffmann et al. 1999). Thus, it can be expected from these results that different Collembola will impose different selective pressures on ingested bacterial cells. Interestingly, we included a strain isolated from the gut of *F. candida*, closely related to *Stenotrophomonas maltophilia*, in the feeding experiments with *P. fimata*, and this strain persisted much longer in the gut after feeding (Hoffmann et al. 1999). We assume that some bacterial species, among them this relative of *S. maltophilia*, developed a certain capacity to survive or even grow in the gut of Collembola. It should be noted that despite some microhabitat preferences, *F. candida* and *P. fimata* can coexist in the same soils and may feed on the same substrates. Possibly, some soil bacteria may have evolved to utilise gut passages, as they occur in most soils with microarthropods, to grow and maintain their populations. *S. maltophilia* may be good example of such an ecological adaptation (see also Sect. 6.6).

6.4 The Gut of Collembola: a Hot Spot for Conjugative Gene Transfer Between Bacteria

Gene transfer between microorganisms in soil has become an issue of public interest in the context of the debate on the environmental risks associated with the deliberate or accidental release of genetically engineered microorganisms. Two mechanisms for gene transfer were considered to be potentially most important for soil: transformation and conjugation. Transformation is the process in which bacterial cells take up cell-free DNA and incorporate it by recombination into their own genome. Conjugation is a process by which self-transferable, mobile genetic elements (plasmids) which carry genes for transfer, replication and possibly other properties, are transferred from a donor to a recipient strain. The transfer can be of narrow-host range, between only closely related bacteria, or it can be of broad-host range, between more distantly related species. The detection of a gene transfer event normally requires the expression of the transferred gene in the new host organisms.

Early studies on gene transfer in soil revealed no or only low rates of conjugative plasmid transfer from a donor to a recipient in bulk soil (Ramos-Gonzalez et al. 1991; Smit et al. 1991). However, transfer rates in rhizospheres were much higher, probably because of the presence of metabolically active recipient cells (van Elsas 1992). In analogy to the rhizosphere, we suspected that the gut of invertebrates, and especially that of earthworms (Thimm et al. 2001) and Collembola could also be a “hot spot” for gene transfer. The high number of bacterial cells observed in the

gut of *F. candida* indicated that sufficient recipients were present in the gut, and both the population dynamics in response to the moulting cycles and the release of nutrients by digestion of food indicated that these gut bacteria were in fact metabolically active. In order to demonstrate bacterial gene transfer in the gut of Collembola under laboratory conditions, *E. coli* cells with different types of plasmids were fed to *F. candida* in microcosm experiments (Hoffmann et al. 1998).

We chose *E. coli* strains with self-transferable conjugative plasmids of broad or narrow-host range. In addition, *E. coli* strains with mobilisable plasmids were included. Mobilisable plasmids are only transferred to a recipient if another "mobilising" plasmid is present. These mobilising plasmids can either be in the donor cell itself, or it can be provided by a third partner, a mobilising strain, in a so-called triparental mating. Finally, we also included an *E. coli* strain with a plasmid (pUC18) that was not efficiently mobilisable. We suspected that this plasmid would possibly be transferred by transformation and not conjugation. A transfer of this type was demonstrated to occur under certain conditions in mineral water as a substrate (Baur et al. 1996) and thus it was not unlikely that it would also occur in the gut of *F. candida* . The experimental set-up for gene transfer studies was as follows. A total of 50 or 100 specimens of *F. candida* were fed in one arena (petri dish) with agar that was inoculated with the respective

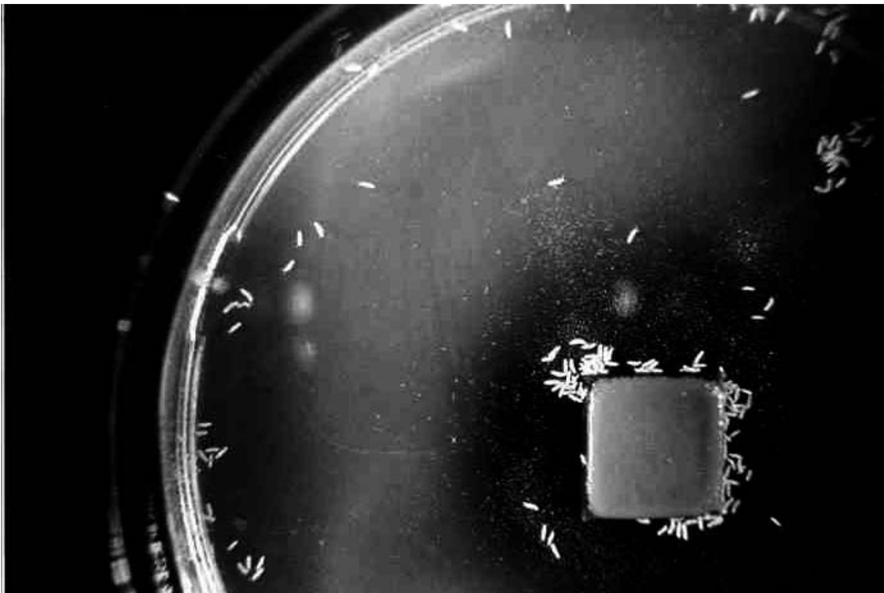

Fig. 6.5. Petri-dish microcosm with *F. candida* feeding on donor bacteria, placed on an agar cube. Note the faecal depositions (*white dots*) on the agar surface

donor strains placed on an agar cube (Fig. 6.5). After feeding for several hours, the specimens were transferred to a new arena without food, but with an agar-surface that contained the antibiotic nalidixic acid, an inhibitor of conjugative gene transfer (Hane 1971). The antibiotic was chosen to prevent conjugative gene transfer in the faeces to show whether gene transfer would take place in the gut. The faeces that were deposited within a period of up to 24 h was then collected and analysed for the presence of donor bacteria, recipients and transcipients (transconjugants or transformants). In order to follow the transfer of plasmids, we chose plasmids that carried a marker-gene (*luc* or *gfp*) and a gene encoding for an antibiotic resistance. The inhibition of the growth of donor cells was achieved by cultivating on an agar with benzoic acid as a sole source of carbon. In preparation for those studies we found that, in contrast to *E. coli*, most gut bacteria of *F. candida* could utilise benzoic acid for growth.

Despite their low survival rate in the gut, several *E. coli* cells could in fact transfer their plasmids to indigenous bacteria of *F. candida*. We expected transformation to be important, because the digestion of the donor cells would possibly result in the release of significant amounts of DNA which would have been available for transformation. However, we could not detect transfer of the non-mobilisable plasmid. In contrast, conjugative transfer of self-transferable narrow- and broad-host range plasmids to indigenous bacteria were detected. Mobilisable plasmids were only transferred when the mobilising genes were located in the donor cell, but not by triparental mating with mobilising genes or plasmids provided by the bacterial community in the gut. Such mobilisation by other bacteria had been shown to occur in soil amended with manure (Götz and Smalla 1997). In our studies, the transfer rates of the broad-host range plasmid RP4, expressed as the transconjugants-to-donor ratio in the faeces, were in the region of 1×10^{-1}. This was as high as rates can be measured under optimised laboratory conditions in filter-mating. The results of our studies underlined the importance of the feeding activity and gut microhabitat conditions of *F. candida* for promoting conjugative gene transfer.

In order to confirm that the Collembolan gut is a hot spot for conjugative gene transfer we conducted similar studies as described above for *F. candida* with another species, i.e., *P. fimata*. We were interested to see if a Collembola with somewhat different feeding preferences than *F. candida* would also provide suitable conditions of conjugative gene transfer in the gut – and in fact, this was the case (Hoffmann et al. 1999). In contrast to the studies with *F. candida*, however, transfer of narrow-host range plasmids could not be detected. On the other hand, in accordance to the results with *F. candida*, conjugative plasmids and also mobilisable plasmids, with the mobilising genes in the donor cells, were transferred to indigenous gut bacteria. Plasmid mobilisation by indigenous gut bacteria was not detected,

but possibly the threshold of detection was just too insensitive in our studies. We assume that plasmid mobilisation in the collembolan gut is possible, because it is likely that mobilising strains occur in the gut of Collembola, just as they occur in soil or other environments (Smalla et al. 2000).

6.5 Diversity of Microorganisms in the Gut of *F. candida* and Other Collembola

Only a very limited number of studies has looked at the microbial diversity associated with the gut of Collembola. It could be argued that the gut of Collembola is too small, its structure too simple, and the gut passage of ingested material too quick, to allow the development of a specifically adapted or even symbiotic microbial community. In fact, some evidence was collected by high resolution microscopy that at least some Collembola do not possess any intestinal microbial community (Kilbertus and Vannier 1981; Saur and Ponge 1988). An analysis of the gut contents of *F. candida*, fed with hornbeam (*Carpinus betulus*) leaves, however, showed a high abundance of fungal mycelia (Tochot et al. 1982) but (surprisingly) no bacteria were seen. First indications that the gut of Collembola also harbours bacteria and that these bacteria actually contribute to the digestion of food, were reported by Doeksen and Hitchen (cited by W.G. Hale 1967), who cultivated a *Bacillus* sp. from faeces with a capacity to degrade chitin. Chitin is an important substance in the gut as it is a major constituent of fungal mycelia and the cuticules of arthropods. Chitin is also a component of the exuvia (including old cuticules), that are released during the moulting of insects and Collembola, and that many Collembola may feed on. In later studies, Borkott and Insam (1990) found that one third of the culturable bacteria from gut and faeces of *F. candida* showed chitin degrading activity on agar plates. Two of these bacteria were identified; one was *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*; *Gammaproteobacteria*) and one *Curtobacterium* sp. (*Actinomycetes*, *Actinobacteria*).

In an initial attempt to characterise the diversity of bacteria found in the gut of *F. candida*, we isolated a total of 45 different bacterial pure cultures which had been kept in a breeding stock for several years (Thimm et al. 1998). These isolates could be grouped into 11 different groups according to their Gram-staining, fatty acid profiles, physiological tests and ARDRA (amplified ribosomal DNA restriction fragment length analysis). The abundance of each of these groups ranged from 4×10^2 to 1.2×10^5 cfu perspecimen. Only one group, with an estimated abundance

of 5.3×10^3 cfu per specimen, exhibited chitinase activity. The most abundant group was represented by an isolate most closely related to *Erwinia amylovora* (96.2% similarity of the almost complete 16S rRNA gene). The second and third most dominant groups were represented by isolates related to *Staphylococcus captitis* and to *Pantoea agglomerans*. *E. amylovora* is an important pathogen in orchards as it is the causative agent of the fire-blight disease and we were interested to know if *F. candida* could possibly act as a vector. Feeding experiments with a pathogenic strain of *E. amylovora*, expressing a recombinant *gfp*-marker gene, however, indicated that the pathogen was, in fact, efficiently digested in the gut of *F. candida* (Hildebrand et al. 2001). In the course of this cultivation-dependent detection of microorganisms from the gut of *F. candida*, we only isolated one fungus, which was identified as a cellulose-degrading *Acremonium charticola* (Ascomycetes) (Thimm et al. 1998). However, we did not determine whether this fungus was cultivated from a spore or a mycelium.

A number of different *Proteobacteria* were isolated as transconjugants receiving plasmids from *E. coli* cells (see previous paragraph) (Hoffmann et al. 1998). The *Proteobacteria* comprised one isolate from the Alpha-subclass, related to *Ochrobactrum anthropi* (99.8% similarity of the 16S rRNA gene) and several isolates from the Beta-subclass with isolates related to *Alcaligenes xylosoxidans*, *A. faecalis*, *Comamonas acidovorans* and *Comamonas testosteroni*. Other isolates, among them different *Pseudomonas* species, *S. maltophilia*, or *P. agglomerans*, belonged to the Gamma-subclass. It should be noted that the host-range of the plasmids was responsible for the fact that no bacteria outside of the class *Proteobacteria* could be found. On the other hand, the occurrence of certain bacteria like *S. maltophilia* or *P. agglomerans* in the gut of *F. candida* was confirmed.

Recently, a molecular approach independent of cultivation has been used to elucidate further the bacterial diversity found in *F. candida*. By means of PCR, 16S rRNA genes were directly amplified from DNA extracted from *F. candida* specimens (Czarnetzki and Tebbe, 2004a). In addition to the detection of intracellular bacteria (see next paragraph), a number of different 16S rRNA genes, which probably originated from bacteria of the gut, were identified. These 16S rRNA genes were related to *Proteobacteria* of the Alpha-subclass (closest relative: *Paracoccus denitrificans*), of the Gamma-subclass (100% similarity to *S. maltophilia*), of the *Firmicutes* (*Bacillus weihenstephaniensis*; from the *Bacillus cereus* group) and from the *Planctomycetales*, the latter only with as yet uncultured relatives. We assume that the diversity of gut bacteria is much higher than described to date and also that this diversity will be affected by the quality of the ingested food. However, the evidence is accumulating that certain bacteria from soil can utilise the gut passage through *F. candida* or other microarthropods to grow. We suspect that bacteria like *S. maltophilia* or *B. weihenstephaniensis*

are representatives of such a life-style. In a recent review it was suggested that bacteria of the *B. cereus* group utilise the gut of insects to grow and survive in terrestrial habitats (Jensen et al. 2003). Collembola should also be considered in this context.

In a screening experiment, we compared the diversity of 16S rRNA amplified partial sequences from other species than *F. candida*. The SSCP (single strand conformation polymorphism) technique was utilised to compare the amplified products by generating genetic profiles (Schwieger and Tebbe 1998). The profiles of seven different species indicated that different bacteria were dominant in each species(Czarnetzki and Tebbe, 2004a). Interestingly, the patterns of the two closely related species *Mesaphorura macrochaeta* and *Mesaphorura italica* were more similar to each other than to other species. A total of 24 partial sequences were recovered and identified from these profiles, indicating the presence of different members from the group of *Proteobacteria* (Alpha-, Gamma-, and Delta-subclass), *Firmicute*s, *Actinobacteria* and *Bacteroidetes*. The sequences however, were not long enough to allow a more detailed phylogenetic analysis. In several cases, closest relatives as indicated by database searches were 16S rRNA genes from uncultured bacteria from other environments, e.g., soil, wastewater, lake sediment, potato rhizosphere or, in one case, from a tissue of the honey bee (*Apis mellifera*). Again, a sequence related closely to *S. maltophilia* was detected in the DNA of *P. armata*, confirming this species as a common gut inhabitant.

6.6 Collembola Can Harbour the Reproduction Parasite *Wolbachia* and Other Intracellular Bacteria

The long coexistence of arthropods and bacteria for approx. 400 million years, has allowed the development of sophisticated interactions between both groups. Striking examples are the intracellular bacteria, e.g., the endosymbiont *Buchnera* in aphids (Douglas 1998) or the parasite *Wolbachia*, the latter affecting the sexual reproduction patterns of many insects and other arthropods (Stouthamer et al. 1999). In Collembola, intracellular bacteria were first detected by transmission electron microscopy (TEM) in the fat body and ovarial tissue of *F. candida* by Palévody in 1972 (Palevody 1972). In 1999, Vanderkerckhove et al., detected by PCR a 16S rRNA sequence that was closely related to group of *Wolbachia* (*Alphaproteobacteria*) (Vanderkerckhove et al. 1999). In the same study, the authors detected intracellular bacteria in fat bodies and intestinal cells by means of TEM. In our own laboratory, we used the fluorescence in situ hybridisation technique (FISH) with universal gene probes for *Bacteria* on microscopic sections of

Fig. 6.6. Detection of intracellular bacteria of *F. candida* in the furca and neighbouring regions. The hindgut region and anus are seen in the upper part of the figure, the furca below. Bacterial cells (*red*) were by detected by FISH using the probe EUB388 for *Bacteria*

whole *F. candida* specimens (and not specific tissues) and we found that in addition to the gut, several compartments of the body cavity were colonised by bacterial cells (Thimm and Tebbe 2003).

Intracellular bacteria were detected in fat bodies in different regions and tissues of the body, including the furca (Fig. 6.6), brain and ovaries (Thimm and Tebbe 2003). FISH with a specific gene probe for *Wolbachia*, however, only hybridised with bacteria that were located in the ovarial tissue or the brain region. We concluded that other bacterial species than *Wolbachia* must also be present in the body cavity of *F. candida* (Czarnetzki and Tebbe 2004b). And in fact, recently we detected a 16S rRNA gene related to the intracellular *Rickettsiella grylli* (gamma-Proteobacteria) of other arthropods. We found this sequence by generating clone libraries of 16S rRNA genes amplified from total DNA extracted from *F. candida*. Interestingly, when we compared clone libraries generated from specimens of two different breeding stocks, we found the *Rickettsiella* sequence to be dominant in one stock but completely absent in the other stock. The phenotype of *F. candida* did not seem to affected by the *Rickettsiella* infection. We therefore assume that *Rickettsiella* is a facultative coloniser of *F. candida* and probably a commensal or weak pathogen.

In contrast to *Rickettsiella*, we detected *Wolbachia* in all of five breeding stocks of *F. candida* analysed (Czarnetzki and Tebbe 2004b). In addition, we found *Wolbachia* by 16S rRNA specific PCR in other parthenogenetic Collembola, i.e., *M. macrochaeta*, *M. italica*, and *P. callipygos*, but we could not detect it in the sexually reproducing species *P. fimata* or *Isotoma viridis*. This indicated that *Wolbachia* may in fact induce parthenogenesis in Collembola. Parthenogenesis is a powerful option in the success of populations in the environment as it allows organisms to multiply more efficiently, e.g., when entering a new ecological niche (Koivisto and Braig 2003). It should be noted, however, that in the case of *Wolbachia* and Collembola, more experimental evidence needs to be collected to confirm this hypothesis. Our own studies, in which we tried to eliminate *Wolbachia* from *F. candida*, were so far unsuccessful (unpublished results). In order to understand better the *Wolbachia*-host relationships, we conducted a phylogenetic analysis of *Wolbachia* with both the 16S rRNA and *ftsZ* genes amplified from *F. candida* of the different breeding stocks and from the other parthenogenetic species. The *Wolbachia* 16S rRNA genes of all *F. candida* breeding stocks was identical and in fact they were also identical to the sequence reported by Vanderkerckhove (Vanderkerckhove et al. 1999). The 16S rRNA sequences of the other Collembola were much more closely related to the *F. candida* sequence than to any sequence from other arthropod hosts. In fact, a monophyletic branch for Collembola could be demonstrated for the phylogenetic tree of *Wolbachia*.

The monophyletic branch of *Wolbachia* from Collembola was also seen in phylogenetic analyses based on *ftsZ* genes (Czarnetzki and Tebbe 2004b). Here, the *Wolbachia* sequences from the different breeding stocks of *F. candida* showed some variations. In summary, our phylogenetic analyses indicated a new Supergroup E for *Wolbachia* in Collembola, Both studies based only on a single sequence from *F. candida* had already postulated that such a supergroup might exist (Vanderkerckhove et al. 1999; Lo et al. 2002). Compared to the approx. 7,500 species that are known in the class of Collembola it is much too early to conclude that supergroup E is an exclusive group for Collembola or that *Wolbachia* of other supergroups have no option to infect Collembola. In fact, we assume that the long coevolution of Collembola and *Wolbachia* make it probable that many such exceptions exist. Our phylogenetic analyses indicated that new supergroup E is a sister group of supergroup A. Supergroup A, like supergroup B, harbours *Wolbachia* from a high diversity of different hosts of the Class *Insecta* and there is no congruence between host and *Wolbachia* phylogeny. Interestingly, the other major group of the soil microarthropods, the mites, which can also be hosts for *Wolbachia*, have no own branch or supergroup in the *Wolbachia* tree. Instead, all yet detected *Wolbachia* sequences belonged to Supergroup B (Breeuwer and Jacobs 1996). In our own study,

a rough estimate of evolutionary rates of the different marker genes (rRNA genes, *ftsZ* genes) indicated that the differences between the Collembola supergroup E and A were much smaller than the phylogenetic distance between Collembola and Insecta (Czarnetzki and Tebbe 2004b). We therefore assume that *Wolbachia* infections took place long after Collembola had diversified. If *Wolbachia* really induces parthenogenesis in Collembola, this would indicate that parthenogenesis is a rather young development during the evolution of Collembola.

6.7 Conclusions

Collembola are a quantitatively and functionally important organisms in most terrestrial ecosystems. Together with mites and some other less abundant groups, they build up the group of microarthropods. Microarthropods enhance the mineralisation and restructuring of organic substrates in soil. Collembola can select for specific food sources, e.g., they prefer certain fungi to others in choice experiments, but many are also quite adaptable to different food sources, which probably explains their high adaptability and success in most soils. The size of the gut of Collembola is very small with a volume of less than 20 nl, as measured for the representative species *F. candida*. The gut passage of ingested food in *F. candida* can last less than 1 h. However, during this passage food is inoculated with bacteria which further enhances the degradation of these substrates. In contrast to some other studies, our own studies indicated that the Collembolan gut can be densely colonised with bacterial cells and that certain bacterial species, like relatives of the type culture strains *Stenotrophomonas maltophilia* or *Bacillus weihenstephaniensis*, have adapted to live in this microhabitat. A precondition for successfully colonising the collembolan gut is that the bacteria resist digestion by the host. Also, successful gut bacteria in Collembola need to grow quickly, since moulting cycles frequently change the total bacterial population by several orders of magnitude within less than a week. The feeding activities of Collembola in the terrestrial ecosystem thus clearly affect both the quality of the organic substrates and the composition of the microbial communities. The high densities of bacterial cells and the microhabitat conditions provide excellent conditions for conjugative gene transfer between bacteria, a factor that should be considered when evaluating rates of bacterial gene transfer in soil. Beside the gut, other compartments of the body of Collembola can also be colonised by bacteria. Two bacteria with an intracellular life-style have been detected so far: one relative of *Rickettsiella grylli* and one belonging to the group of *Wolbachia*. To our knowledge, all *Wolbachia* that have been detected in Collembola are more related to each other

than to *Wolbachia* from other arthropod hosts. The biological importance of *Wolbachia* infections are yet unknown, but as suggested by the hosts that have so far been analysed, such infections may induce parthenogenesis.

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7 Methane Production by Terrestrial Arthropods

Johannes H. P. Hackstein, Theo A. van Alen, Jörg Rosenberg

7.1 Introduction

Biopolymer degradation under anaerobic conditions is responsible for most of the atmospheric methane (Ferry 1997). Methanogenic archaea thriving in the gastro-intestinal tract of animals are a major source, and domestic ruminants such as cattle, goats and sheep, in particular, are known for their substantial contribution to global methane production, and hence to global change and global warming (Crutzen et al. 1986; Fraser et al. 1986; Khalil and Shearer 1993; Chynoweth 1996; Hansen et al. 2000, 2002). These methanogenic archaea are part of a highly complex, biopolymer-degrading microbial community that generates H_2 and CO_2, which provide the nutritional basis for most of the intestinal methanogens via the inter-species hydrogen transfer (Hobson 1988). However, the presence of methanogenic archaea in guts is far from being trivial, and not the inevitable consequence of the availability of H_2 and CO_2 in the intestine. For example, while large herbivorous mammals such as cattle, goats and sheep produce large amounts of methane, the strictly herbivorous panda, which also provides high concentrations of H_2 and CO_2 in his gut, does not (Hackstein and van Alen 1996). Also, all Old World monkeys and apes make methane, but many South American apes and many humans do not host significant numbers of methanogenic archaea in their guts, and, consequently, do not produce methane (Hackstein et al. 1995; Hackstein and van Alen 1996). Many hypotheses have been put forward to explain these oddities (see for example Hackstein et al. 1996, 1998), but, until now, there is no generally accepted theory that could explain why certain animals host methanogens in their gut while others do not. Thus, there is still neither a well supported hypothesis nor conclusive evidence for a molecular basis for the symbiosis between mammals and methanogenic archaea.

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Arthropods represent by far the largest global biodiversity of all multicellular animals, and many of them host a complex microbiota in their guts (for review see Hackstein 1997 and references therein; Cazemier et al. 1997). Notably, only a few taxa (i.e. millipedes, termites, cockroaches, and scarab beetles, see below) host methanogenic archaea (Hackstein and Stumm 1994). As in mammals, there is no strict correlation between having a plant-based (or detritus/soil-based, respectively) nutrition and methane production. For example, cockroaches make methane, but locusts and crickets do not (Hackstein and Stumm 1994). Obviously, a small gut volume is not limiting the symbiosis between arthropods and methanogens, since there are many species with similar sized (small) guts and similar diets, which differ just by their ability to host methanogens. Also the presence of oxygen which is the consequence of diffusion of O_2 into the tiny guts does not hamper the presence of symbiotic methanogens in partially oxygenated environments. Moreover, protozoa with epi- or endosymbiotic methanogens, similar to the situation in ruminants and hindgut fermenters such as horse and elephant (Vogels et al. 1980), are also found in arthropod guts. Notably, they occur only in methanogenic taxa. Thus, there is a clear taxonomic component, which governs the symbiosis between methanogens and arthropods, but, as in mammals, the molecular or physiological basis for these limitations in the occurrence of gut methanogens remained unknown so far (Hackstein and Stumm 1994).

In this chapter, we will discuss the available evidence with respect to the symbioses between methanogenic archaea and terrestrial arthropods. The compartmentalisation of the intestinal tracts of the hosts, the consequences of the small size of these compartments, and the evolution of specialised differentiations of the gut wall will be major issues. Attention will also be paid to the inter-compartment hydrogen transfer, the presence of anaerobic gut-protozoa, and lastly the biodiversity of symbiotic methanogens.

7.2 Symbiotic Methanogens and Terrestrial Arthropods

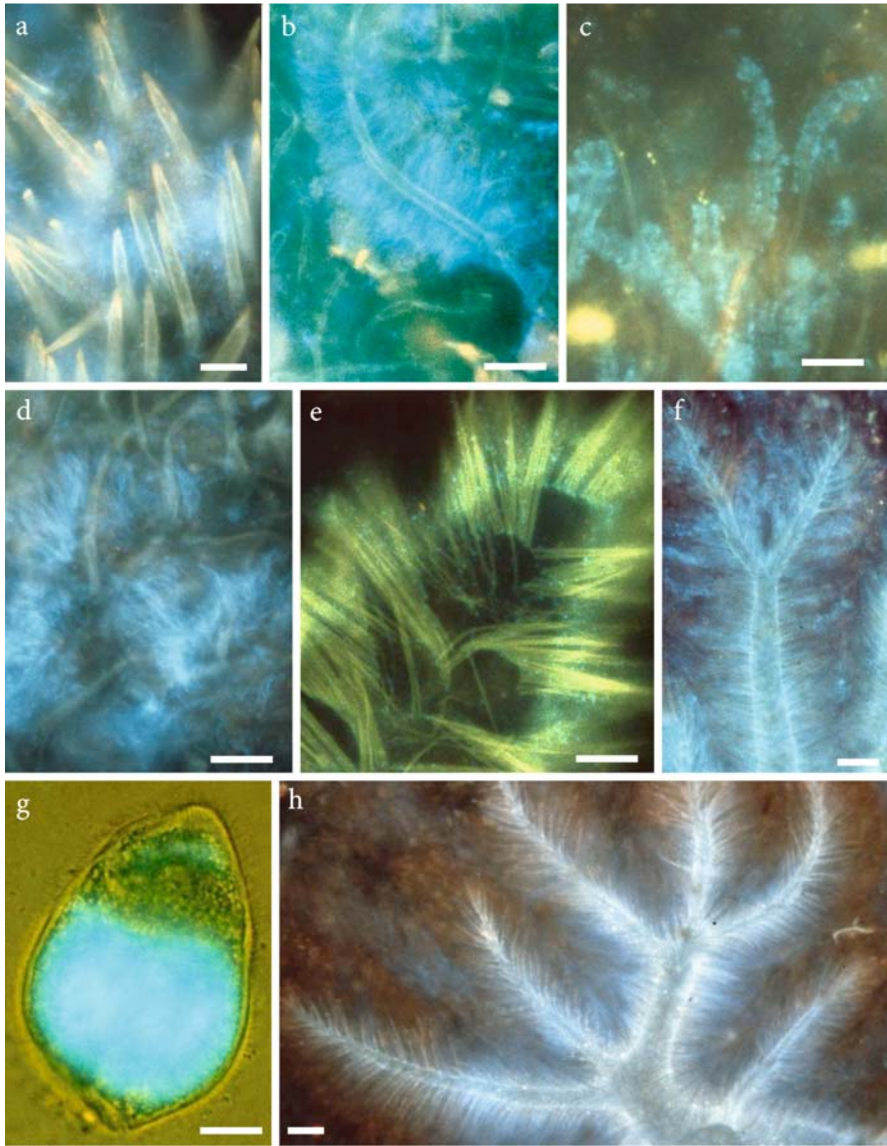
In his seminal monograph, Paul Buchner (1953) described the fascinating universe of symbiotic associations between animals and bacteria. He emphasised both the structures created by the various hosts facilitating the transmission and maintenance of bacterial symbionts, and the potential contributions of the symbionts to the host's nutrition. Only recently has the enormous progress in molecular biological techniques and bioinformatics allowed some aspects of the molecular basis for these symbiotic associations to be unravelled (Moran 2003; Moran and Baumann 2000; Hofmeister and Martin 2003; Dillon and Dillon 2004; Canback et al. 2004),

and to get first insight into the fascinating effects that symbiotic bacteria eventually exert on animal (host) development (McFall-Ngai 2002).

Buchner and all the early investigators of bacterial-animal symbioses were not aware of the existence of symbiotic methanogenic archaea, which were not recognised as representatives of a separate kingdom until 1990 (Woese et al. 1990). Therefore, certain symbioses described by the early authors as involving "bacteria", eventually turned out as symbiotic associations with methanogenic archaea. Even in the decade before the identification of the methanogenic archaea as representatives of a novel kingdom, symbiotic methanogenic "bacteria", and protists hosting methanogenic endosymbionts had been described in termite and cockroach guts (Breznak 1982). However, many of the early studies focused on dietary aspects, and the challenging observation that some arthropods make methane, while others do not produce methane did not receive particular attention.

In comparison with "ordinary" bacteria, methanogenic archaea exhibit a number of unique properties that facilitate a systematic analysis of the distribution of symbioses between methanogens and arthropods. First of all, a single methanogen can produce approximately 1×10^{-15} mol methane/h (Fenchel and Finlay 1992), a gas, which can be measured easily and with high sensitivity using either gas chromatography (GC) or more advanced detection methods such as laser-photo acoustics (Bijnen et al. 1996) or mass spectrometry (Beckmann and Lloyd 2001). Although the methanogens are found exclusively in the gastro-intestinal tract of their hosts, methane measurements can be performed with intact specimen because the methane derived from the intestinal fermentations is released together with the exhaled breath of their host animals. Therefore, the presence of methanogenic archaea in the gut of an individual arthropod can be traced easily (and non-invasively) by measuring methane concentrations in its breath, provided that the animals host more than 10^6 methanogens. Using photo acoustic laser techniques methane release can be monitored even below ppb level (Bijnen et al. 1996).

Second, methane formation requires a set of unique enzymes and cofactors (Ferry 1993, 1999; Shima et al. 2002). One of these cofactors, F_{420}, exhibits a characteristic autofluorescence. Because it is present in large amounts in most of the methanogens, methanogenic archaea exhibit a strong blue autofluorescence when irradiated with UV-light of a wavelength of 420 nm (Fig. 7.1). Therefore, it is possible to detect even a single (fluorescing) methanogen directly among thousands of (non-fluorescing) eubacteria using epifluorescence microscopy (Doddema and Vogels 1978). The application of this technique led to the identification of the first endosymbiotic and episymbiotic methanogens of anaerobic protozoa (Vogels et al. 1980; van Bruggen et al.1983; Fenchel and Finlay 1995).



Third, the polymerase chain reaction (PCR) and the fluorescent in situ hybridisation (FISH) using methanogen-specific primers and probes, respectively, allow the identification of specific methanogens in their natural environments without the need for culturing (Amann et al. 1995, Amann and Ludwig 2000). It was not until 1992 when Embley and his coworkers identified the first methanogenic endosymbionts of ciliates by rDNA

◀ **Fig. 7.1.** Methanogenic archaea in the hindguts of the various arthropods. 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 autofluorescence*) covering the hindgut of the cockroach *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

sequencing and in situ hybridisation (Embley et al. 1992a,b; Dyal et al. 1995). Eight years later, the complex correlations between the phylogenies of endosymbiotic methanogens and their hosts could be explained by a principally "vertical inheritance" of the methanogenic endosymbionts from one ciliate generation to the next. However, the phylogenetic analysis can be blurred by rare evolutionary endosymbiont replacements accompanying the adaptations of the ciliate host to novel environments (van Hoek et al. 2000).

Lastly, recently developed profiling techniques such as 16S rRNA community analysis, DGGE (denaturing gradient gel electrophoresis) or T-RFLP (terminally labelled restriction fragment length polymorphism) allow the analysis of complex archaeal symbiotic associations (Tokura et al. 2000; Brauman et al. 2001; Friedrich et al. 2001; Egert et al. 2003; Wright and Pimm 2003; Donovan et al. 2004; Regensbogenova et al. 2004a,b). Real-time PCR, finally, allows a quantitative assessment of particular methanogenic taxa in complex environments (Tang et al. 2004; Sawayama et al. 2004).

7.3 Why Do Certain Arthropods Make Methane and Others Not?

From 1992 on, we started a systematic search for "methanogenic" arthropods in order to gain insights into the problem of why certain arthropods make methane and others do not. In a first experiment, 110 representatives

of 35 higher taxa of terrestrial arthropods were screened for methane and hydrogen emissions with the aid of a gas chromatograph. Then the animal was sacrificed, dissected and analysed by epifluorescence-, phase contrast- and differential interference contrast (DIC) microscopy for the presence or absence of methanogens, and their localization in the various compartments of the gut (Hackstein and Stumm 1994). In a second screen some 70 strains of cockroaches representing 44 different species were analysed in the same way (Hackstein 1997; Hackstein and van Alen, unpubl.).

Summarising the results of these screens briefly, it has to be concluded:

1. Only representatives of millipedes, cockroaches, termites and scarab beetles produce methane. Representatives of other taxa do not produce methane – irrespective of whether these arthropods have diets very similar to those of methanogenic species. This suggests a taxonomic,

Table 7.1. Methane production in cockroaches

Species	Methane emissions	Hindgut	Protists
Blattoidea			
Blattinae			
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	+	+	M
Blattellinae			
Blattella germanica	+/–	+/–	–
Ischnoptera sp.		–	–
Loboptera decipiens		–	–
Parcoblatta lata	+	nd	nd
Shawella couloniana	–	–	–
Symplece pallens	–	nd	nd

Table 7.1. (continued)

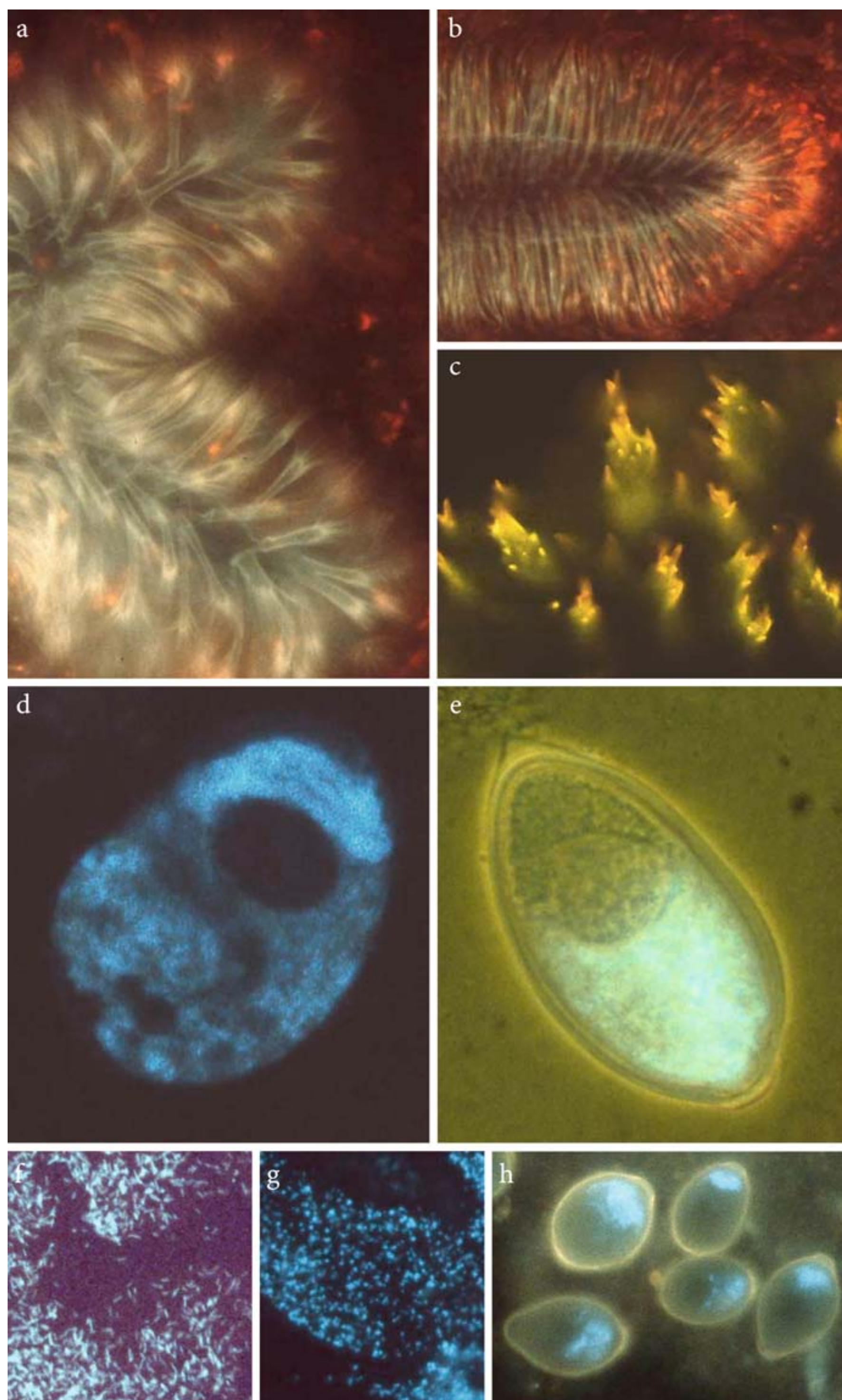
Species	Methane emissions	Hindgut	Protists
Ectobiinae			
Ectobius sylvestris	–	–	–
Ectobius sp.	–	–	–
Nyctiborinae			
Nyctibora sp. (Costa Rica)	+	+	–
Blaberidae			
Blaberoid complex			
Zetoborinae			
Schultesia lampyridiformis	+	+	–
Blaberinae			
Archimandrita sp.	+	+	–
Blaberus craniifer	+	nd	nd
Blaberus fuscus	+	+	C
Blaberus discoidalis	+	+	C
Blaberus giganteus	+	+	C
Blaberus sp. CR	+	nd	nd
Byrsotria fumigata	+	+	(C)
Eublaberus distanti	+	+	–
Eublaberus posticus	+	+	–
Blaptica sp.	+	+	C+M
Panchloroid complex			
Pycnoscelinae			
Pycnoscelus surinamensis	+	+	(C+M)
Diplopterinae			
Diploptera punctata	+	+	C
Panchlorinae			
Panchlora nivea	–	–	–
Oxyhaloinae			
Gromphodorhina chopardi	+	+	C
Gromphodorhina portentosa	+	+	C
Leucophaea maderae	+	+	–
Nauphoeta cinerea	+	+	M
Gen. near *Griffiniella*	+	+	C
Epilamproid complex			
Epilamprinae			
Rhabdoblatta sp.	+	+	–

Abbreviations:
C: cilates,
M: mastigotes (flagellates),
nd: not determined,
(c) low numbers of ciliates

and, consequently, hereditary basis for methanogenesis. The screen for methanogenic cockroaches strongly supports this assumption since nearly all non-methanogenic species are restricted to the taxon Blattellinae, whereas most of the Blaberidae and Blattinae tested were methanogenic (Table 7.1).

2. Microscope inspection confirmed the presence of methanogenic archaea in the methane-emitting animals, and, likewise, their absence in non-methanogenic animals. Thus all methanogenic species studied hosted more than 10^6 methanogens (because of the detection threshold of the GC), and the absence of methanogenesis correlated strictly with the *complete* absence of methanogens as judged by epifluorescence microscopy. Notably, in all methanogenic species, the occurrence of methanogens was restricted to the hindgut. Methanogens were never found in the foregut or the midgut of any methanogenic species.
3. Methanogens in hindguts are found free-floating in the gut lumen, attached to food particles, or adhering to the gut wall. In certain insects, these gut walls possess elaborated cuticular differentiations, which eventually enlarge the inner surface of the hindgut by several orders of magnitude (see below). Moreover, many methanogenic arthropods host protozoa with endosymbiotic methanogens (Figs. 7.1, 7.2d–h, 7.3, 7.4, Table 7.1).
4. In general, the hindguts of methanogenic arthropods exhibit a remarkable complex longitudinal compartmentalization and radial differentiation, i.e. cuticular structures protruding into the hind gut lumen, and/or

► **Fig. 7.2.** Differentiations of the hindgut epithelium of short-horned grasshoppers and crickets. Note the complete absence of blue-fluorescing methanogens. Neither taxa 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. 7.1g (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, g** Endosymbiotic methanogens from ciliates thriving in the hindgut of the cockroach *Periplaneta americana* (strain Amsterdam) (**f**), and the cockroach *Blaberus* sp. (strain 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 (Gen. near Griffiniella) containing endosymbiotic methanogens (*blue autofluorescence*). (*Bar* 10 μm)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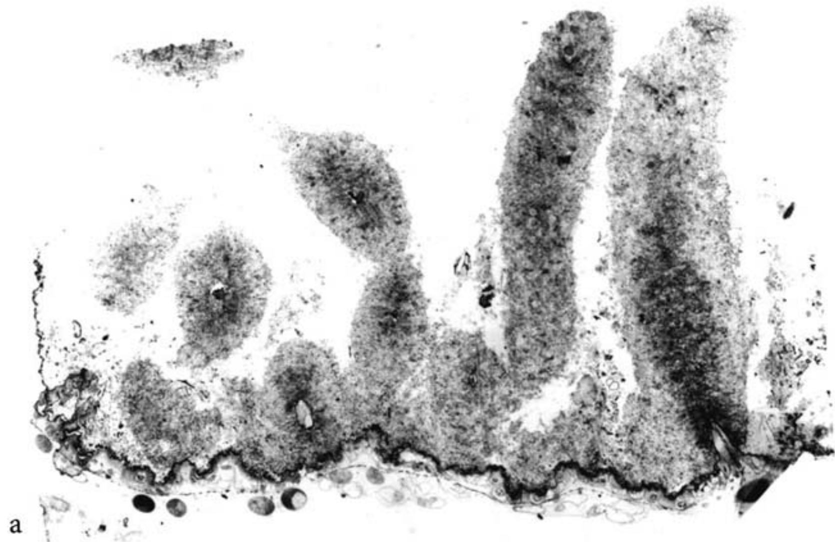
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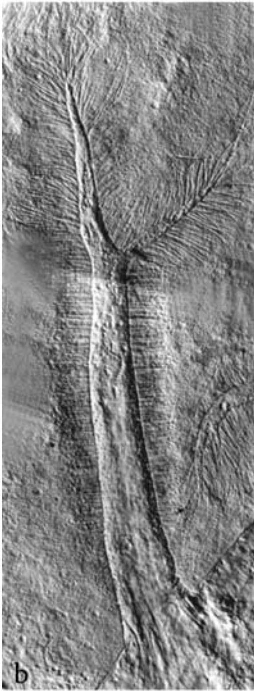
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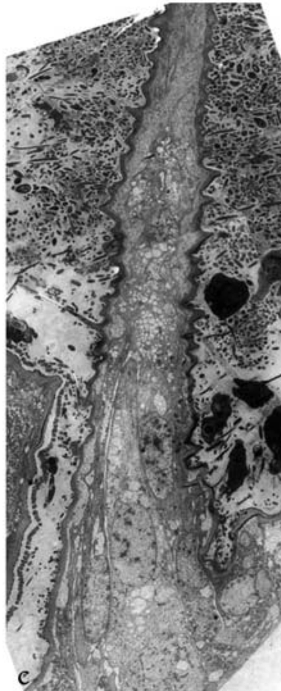
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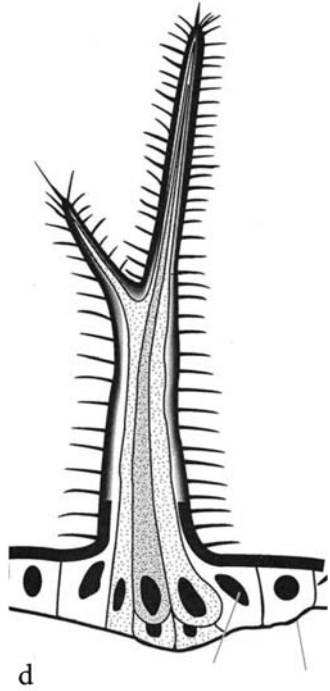
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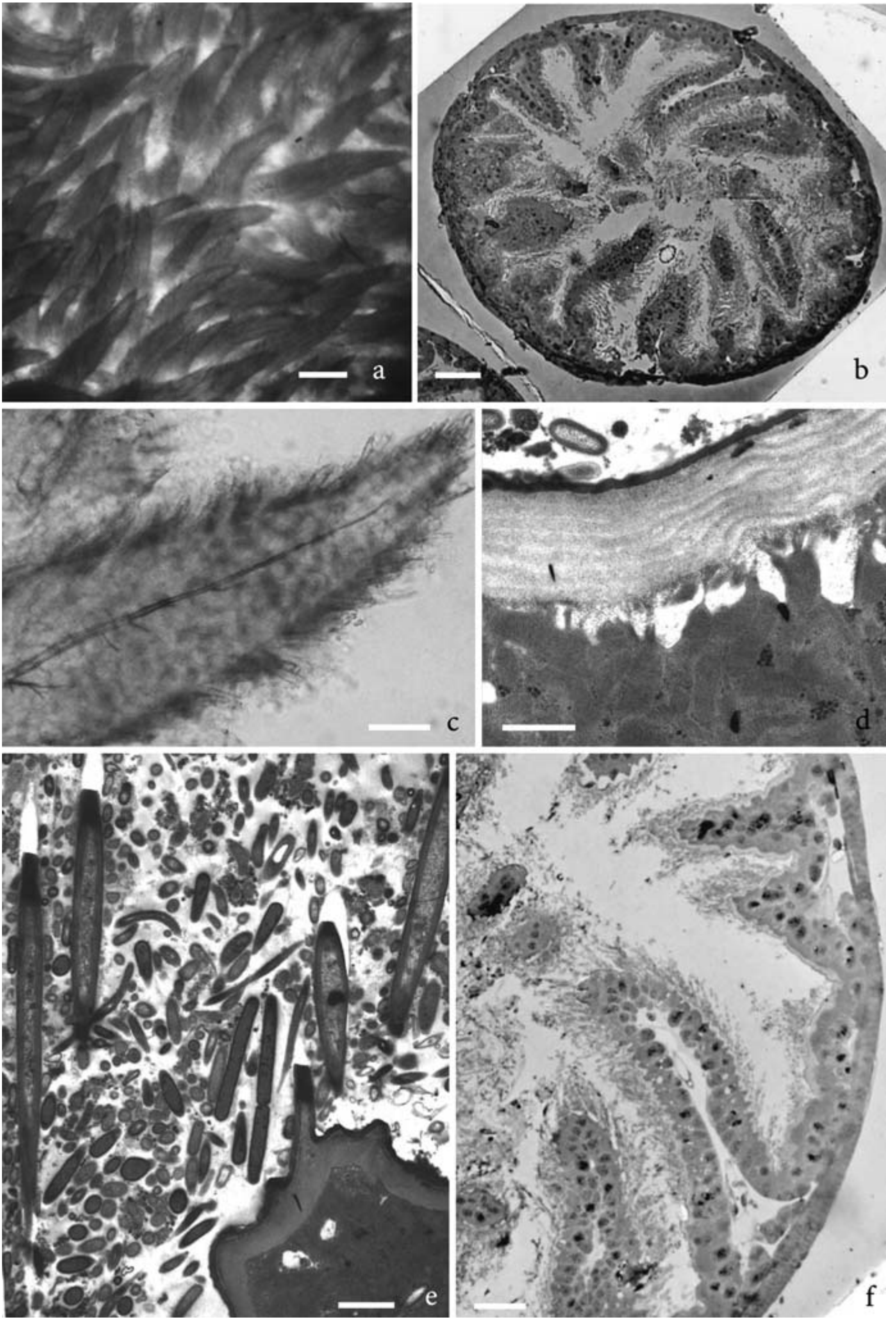
◀ **Fig. 7.3.** Light micrograph (semi-thin section) of the hindgut epithelium of a larva of *Dynastes hercules* (Scarabaeidae). Villus-like structures, measuring between 200 and 500 μm in length protrude into the lumen of the hindgut. These structures, which we have named "pseudosetae", are composed of several, elongated cells of the hindgut epithelium and covered by a complex prokaryotic microbiota, including methanogens (cf. Fig. 7.1f,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, 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 pseudoseta. 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 hemolymph. *Bar* 5 μm. *Black ovals* in **d** indicate the nuclei of the hindgut epithelium and the pseudoseta.

a characteristic radial distribution of intestinal protozoa (Figs. 7.3–7.6). However, the presence of bristles and pseudosetae for the attachment of bacteria is not restricted to methanogenic hosts (Fig. 7.2a,b).

5. Substantial numbers of (anaerobic) symbiotic protozoa were only found in the hindgut of methanogenic species. Parasitic protozoa such as Gregarines, for example, thrive in the midgut, whereas parasitic worms are found frequently in the hindgut compartment.
6. Terrestrial arthropods thriving in boreal climates seem to lack intestinal methanogens, even if they belong to a methanogenic host-taxon. The potential influences of the ambient temperatures on the occurrence of methanogens in the guts of arthropods require additional studies.

In conclusion, symbioses between arthropods and methanogenic archaea are not ubiquitous, and a plant-based diet of an arthropod does not necessarily imply the presence of methanogenic archaea in the gut. Symbioses between arthropods and methanogenic archaea are restricted to certain taxonomic groups. Thus, symbioses between arthropods and methanogenic archaea depend primarily on the evolutionary descent and, consequently, on hereditary factors rather than on dietary habits. Symbiotic methanogens are always restricted to the hindgut. They occur free-floating in the gut lumen, attached to digesta, attached to the hindgut wall, or as endosymbionts in anaerobic protists thriving in the same gut compartment (see below). There is, until now, no explanation for the observed restriction of such symbioses to a few higher taxa – neither at the physiological nor at the molecular level. Moreover, the small size of the arthropod hosts raises a number of questions concerning the survival of strictly anaerobic methanogens in tiny gut compartments that are exposed to atmospheric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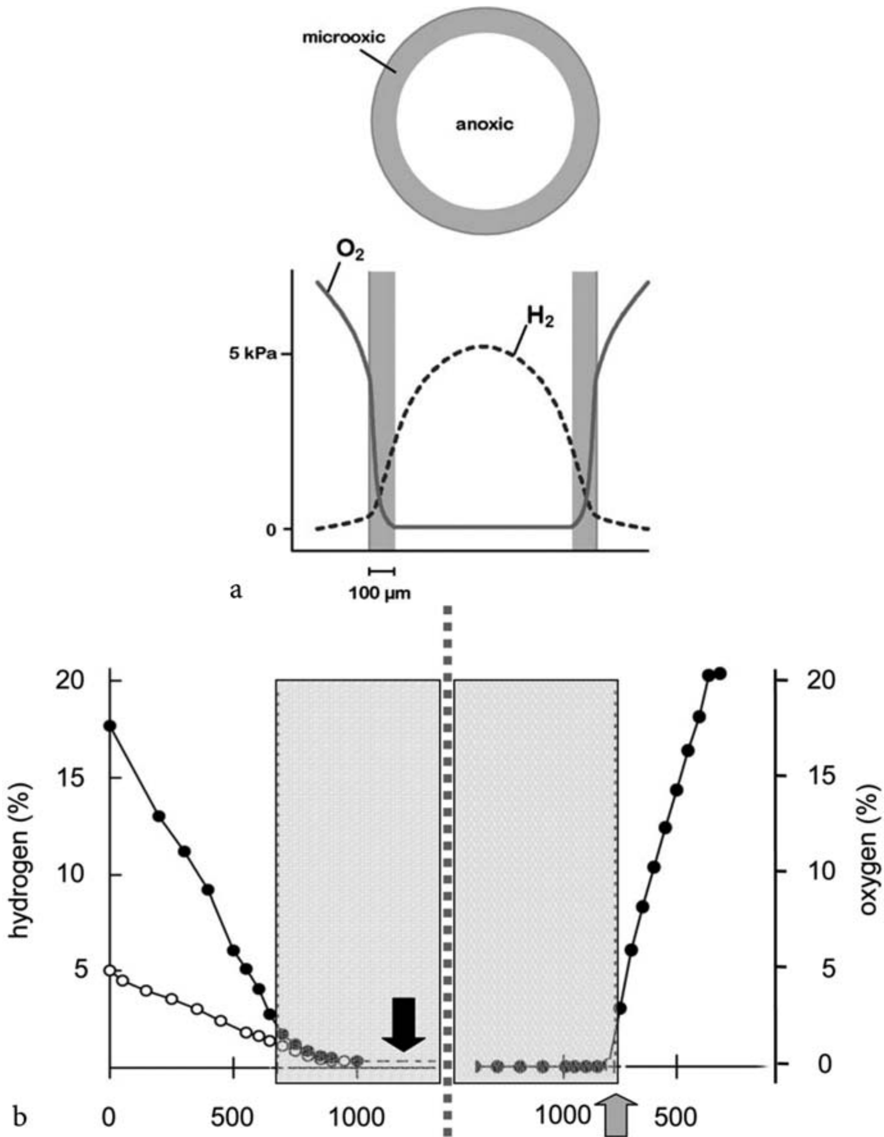
◀ **Fig. 7.4.** Various aspects of the hindgut epithelium of the cockroach *Nyctibora* sp. **a** Low magnification light microscopy reveals that the inner surface of the hindgut is covered by villus-like protrusions of the hindgut epithelium (*bar* 200 µm). **b** A cross section of the hindgut shows that these villi fill nearly the whole volume of the gut (*bar* 200 µm). **f** The same aspect at higher magnification (*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

oxygen, which is transported through the tracheae directly into the gut epithelia. Several of these issues are discussed in some detail below.

7.4 "Small Is Beautiful": The Elusive Co-Existence of Aerobes and Anaerobes in Arthropod Guts

The rumen of a cow has a volume between 100 and 200 l and a macroscopic surface of about 1 m^2. Consequently, the surface/volume ratio of this "fermenter" is about 10 m^2/1 m^3. Due to the large volume and the relatively low surface to volume ration, the diffusion of oxygen into the rumen is rather limited. The rumen is largely anoxic due to the metabolic activities of facultative aerobic rumen bacteria, and therefore, comparable to a laboratory-scale anaerobic fermenter. Insect guts, on the other hand, have volumes at the microlitre scale, i.e. from about 1 µl in termites and small cockroach larvae to not more than a few thousand microlitres in the case of adult giant cockroaches or of the giant larvae of cetonid beetles (Fig. 7.6a,c). These dimensions implicate a surface area of these guts in the order of some mm^2, and consequently, a surface/volume ratio of some thousand m^2/m^3 (Brune 1998) . This raises the question as to whether under these conditions anoxic niches, which are required for the strictly anaerobic methanogens can exist at all. The introduction of microsensor (O_2, H_2, and pH electrodes) techniques into insect physiology by Andreas Brune allowed these questions to be answered (Brune et al. 1995). It could be shown that, despite of the small size, insect guts can be completely anoxic just a few micrometers below the hindgut wall (Figs. 7.4, 7.5). Because of the metabolic activities of aerobic and facultative anaerobic bacteria adhering to the gut wall (or positioned closely to it) very steep oxygen gradients can be established, which render the majority of the hindgut volume anoxic (Brune and Friedrich 2000).

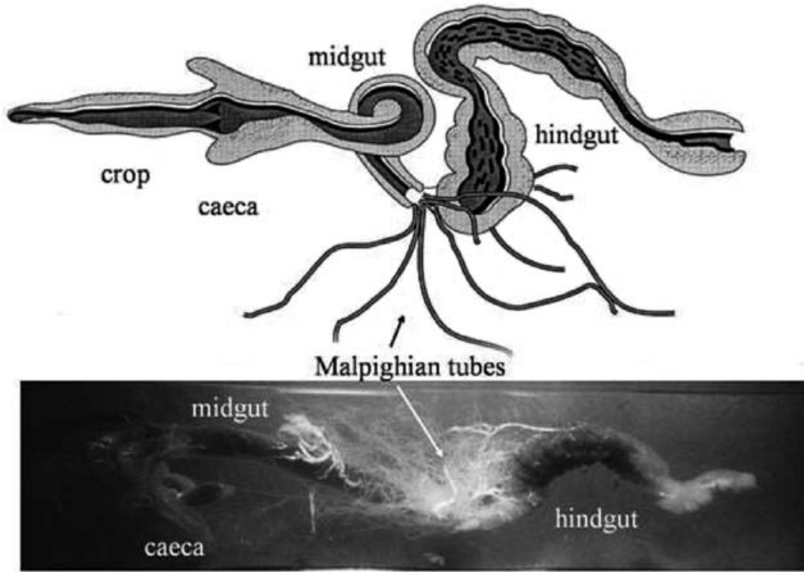


The H_2 accumulation in these particular termite hindguts is believed to be due to the presence of an extremely dense population of flagellates (Figs. 7.5, 7.6b). The flagellates are restricted to the "paunch", the anterior, dilated part of the termite hindgut. They occupy nearly the whole lumen of this gut compartment. These flagellates are supposed to possess hydrogenosomes, although the evidence for this is still rather circumstantial. Histochemical tests to demonstrate the presence of hydrogenase activity (cf.

◀ **Fig. 7.5.** Cartoons illustrating the radial profiles of H_2 and O_2 partial pressures in termite (**a**) and cockroach hindgut (**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 setup, see Fig. 7.6a,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 colonising 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 in 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 micrometers. **a** reproduced with permission from Brune and Friedrich (2000)

Zwart et al. 1988) have not been performed, and methanogenic endosymbionts are lacking in most of the flagellates, but some contain endosymbiotic methanogens (Fröhlich and König 1999). However, H_2 microsensor measurements indicate a hydrogen release by isolated flagellates (Brune, pers. comm.). Microscope inspection does indeed reveal the presence of numerous methanogens adhering to the hindgut wall, and the absence of significant amounts of methanogens adhering to the gut contents.

Cockroach guts are very similar to that of termites with respect to the oxygen gradients. Very steep O_2 gradients reveal anoxic conditions a few micrometers below the hindgut wall (Fig. 7.5b). The H_2 gradients, however, behave completely different. Electrode measurements in the hindgut of several species of cockroaches reveal that the H_2 partial pressure in the centre of the gut is at detection limit of the electrode. Also, increasing the ambient H_2 partial pressure to 18% does not lead to an increase in the H_2 partial pressure in the central region of the hindgut – in clear contrast to the situation in the termite hindgut. These data indicate the presence of H_2 sinks throughout the lumen of the hindgut, and there is no evidence for a particular concentration of hydrogen sinks near the gut wall. Microscope inspection of the particular guts shows that only a few methanogens adhere to the hindgut wall; the majority are more or less evenly distributed through whole gut lumen, or adhere to cuticular structures, such as spines and hairs, which protrude into the gut lumen (Figs. 7.1, 7.3; see below). Notably, gut ciliates are not concentrated in the centre of the hindgut, and in contrast to the flagellates from the termite gut, they possess methanogenic endosymbionts, which convert intracellularly formed hydrogen into methane inside the ciliate cells (Figs. 7.1g, 7.2d–h). Therefore, ciliates do not release detectable amounts of hydrogen, and the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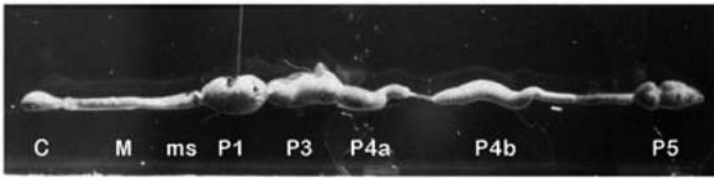
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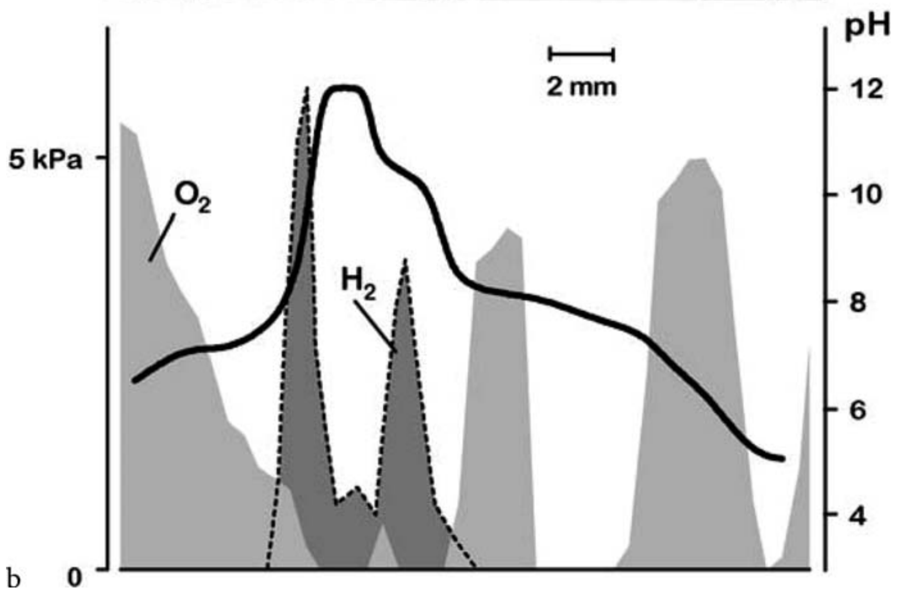
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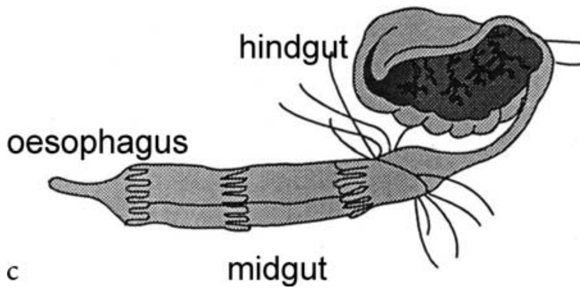

Fig. 7.6. Macroscopic views of the gut of cockroaches, termites and larvae of scarab beetles. **a** *Top*, a cartoon of the intestinal tract of a cockroach (*Periplaneta americana*). *Below*, a picture of a gut of *Periplaneta americana,* which has been embedded into agarose for microsensor measurements (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, *P1–5* proctodeal regions. **b** reproduced with permission from Brune and Friedrich (2000). **c** A cartoon demonstrating the gross organisation of the intestinal tract of the larva of a 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*).

hydrogen in the hindgut, which fuels the ubiquitous methanogens must be generated by bacteria throughout the hindgut (Fig. 7.5b). Interestingly, a substantial fraction of the hydrogen, which is consumed in the hindgut of cockroaches by the methanogenic archaea stems from hydrogen formed in the midgut and is acquired by inter-compartment hydrogen transfer (Lemke et al. 2001; see below).

The metabolic activities of a facultatively aerobic microbial biota in the guts are responsible for the formation of steep oxygen gradients, which eventually cause the formation of a narrow microoxic zone in the periphery of the gut. These oxygen gradients are characteristic for the intestinal tract of termites, cockroaches, beetle larvae, and, most likely of all insects, which host a complex facultatively aerobic microbial biota in their guts (Brune et al. 2000). The cockroach *Nyctibora* sp. possesses an extremely enlarged hindgut wall. Finger-like protrusions of the gut epithelium stick out into the lumen until the very centre of the hindgut (Fig. 7.4a–f). The epithelium of these protrusions is supplied with oxygen by tracheae and tracheoles, which extend intracellularly just below the cuticle lining the site of the epithelium facing the gut lumen. One wonders if any part of the gut lumen can be completely anoxic, but methanogens are found adhering to the inner surface of the gut wall, at a micrometer distance to tracheoles and mitochondria, which are frequent in the hindgut epithelium (Figs. 7.1e, 7.4d).

7.5 Longitudinal Differentiation of the Intestinal Tract of Methanogenic Arthropods

Arthropod guts are clearly compartmentalised 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 cockroaches is structured relatively simply (Fig. 7.6a). The gut of termites, especially of humivorous species, can be rather complex (Fig. 7.6b), whereas the intestinal tract of cetonid and scarabeid larvae is dominated by a giant midgut and hindgut (Fig. 7.6c). Notably, in all methanogenic arthropods studied so far, methanogens are restricted to the hindgut (Hackstein and Stumm 1994). This compartment has a pH around neutral, whereas certain parts of the guts of humivorous insects can be strongly alkaline, which might prevent a colonisation by methanogenic archaea (Fig. 7.6b,c). In cockroaches, however, all gut compartments have a similar, near neutral pH. Nevertheless, there are no methanogens found in other compartments of the intestinal tract besides the hindgut. Interestingly, in all methanogenic arthropods studied, the peritrophic membrane (Dettner and Peters 2003), which wraps the gut contents during its passage through the midgut, is disintegrated at the junction between midgut and hindgut. In many cockroaches, cuticular structures located at the junction between midgut and hindgut, are involved in the mechanical disruption of the peritrophic membrane (Fig. 7.2c).

7.6 Intercompartment Hydrogen Transfer

Indirectly, the midgut, which does not host methanogenic archaea in any of the arthropods studied so far, contributes to the methane emissions. In the midgut of many species of terrestrial arthropods dense populations of bacteria were identified, which produce hydrogen. This also holds true for midguts, which possess an extremely high pH in this compartment (Cazemier et al. 1997; Schmitt-Wagner et al. 2003a,b). These bacterial microbiota allow a net production of hydrogen, which is released into the hemolymph. This hydrogen fuels the methane production in the hindgut (Lemke et al. 2001). In both cockroaches and larvae of scarab beetles, but also in certain termites, the anatomy of the gut facilitates a close apposition of hydrogen-producing and hydrogen-consuming, i.e. methane-producing compartments (Fig. 7.7; cf. Hackstein and Stumm 1994; Brune and Friedrich 2000; Hackstein et al., unpubl.). The intercompartment hydrogen transfer between hydrogen-producing and hydrogen-consuming compartments is very effective. Although significant amounts of hydrogen

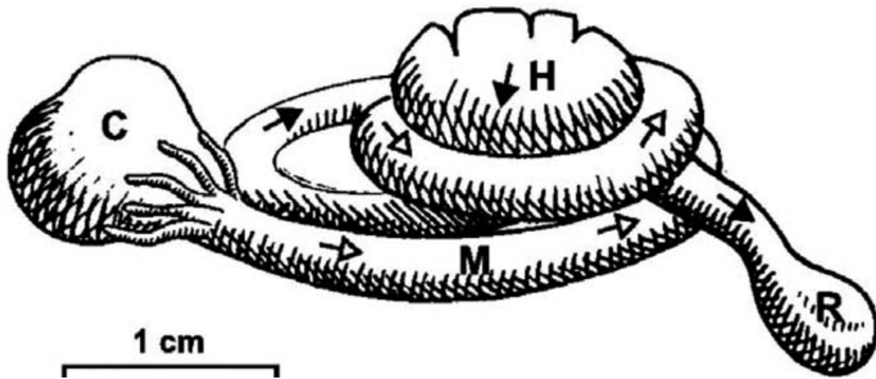

Fig. 7.7. Cartoon of the intestinal tract of the cockroach *Blaberus* sp. Ingested food is stored in the crop (*C*) before it is passed into the midgut (*M*), where it is fermented by a complex microbiota, which generates hydrogen. The midgut is wrapped around the hindgut (*H*), which provides a strong hydrogen-sink because of the presence of methanogens. The close anatomical association between midgut and hindgut facilitates an efficient intercompartment hydrogen transfer, which has been confirmed by microsensor measurements (cf. Lemke et al. 2001). Reproduced with permission from Lemke et al. (2001).

are produced by the isolated midguts of quite a number of insects (measured with the aid of a GC in vitro using isolated, ligated midguts, which were incubated anaerobically for several hours), only rarely traces of H_2 emissions could be measured in the breath of methanogenic insects (Hackstein and Stumm 1994). Consequently, the surplus of H_2 produced in the midgut must be very efficiently metabolised in the hindgut to methane. We estimated that about 25–30% of the total methane production might be due to hydrogen, which stems from intercompartment hydrogen transfer. There are indications that also methanol (and potentially also formate) might be transferred from the midgut to the hindgut of cockroaches and *Pachnoda* sp. larvae with the consequence of an increased methane production in the hindgut (Fig. 7.8; Hackstein et al. 2000; Lemke et al. 2003). We have shown earlier by the isolation of *Methanomicrococcus blatticola* that certain methanogens in the hindgut of cockroaches use methanol and H_2 as substrates for methanogenesis instead of CO_2 and H_2 (Sprenger et al. 2000).

7.7 Differentiations of the Intestinal Tract to Host Methanogenic Archaea (and Other Prokaryotes)

Millipedes, certain termites, many cockroaches, and larvae of cetonid and scarab beetles evolved cuticular projections of the hindgut epithelium,

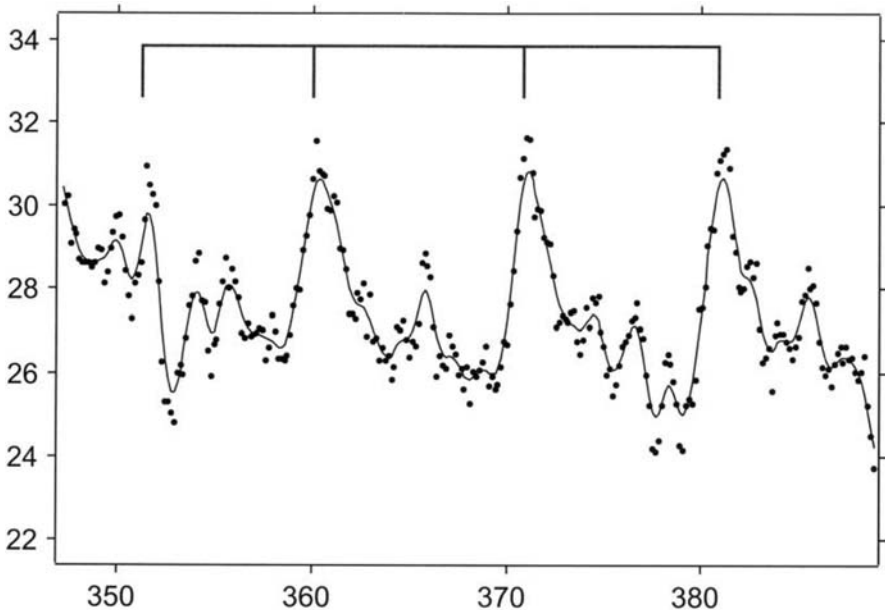

Fig. 7.8. Methanol release via breathing by a cockroach (*Blaberus* sp.). Methanol is produced by the fermentations in the intestinal tract of the cockroach (in both the midgut- and hindgut compartments). It is released periodically via the tracheae exhibiting the characteristic fluctuations in concentration known from the resting respiration of cockroaches (Bijnen et al. 1996). Methanol is detected using proton-transfer mass-spectrometry (Boschetti et al. 1999). Incubations of isolated gut compartments revealed a release of methanol by the midgut, and a concomitant stimulation of methanogenesis in the hindgut. *Ordinate* Methanol concentration in ppb. *Abscissa* Time in minutes. The scale above the recording marks four complete respiration cycles

which increase the surface providing attachment sites for the microbiota in the gut. In methanogenic species, methanogens represent a major fraction of the prokaryotic community attaching to the cuticular hairs (trichomes). However, such differentiations are not specific for methanogenic hosts, since similar structures are found also in non-methanogenic insects such as for example crickets and locusts (Fig. 7.2a, b). Thus, these differentiations are not specific attachment sites for methanogens. In cockroaches, the cuticular differentiations are in general simple cuticular hairs (trichomes, see Fig. 7.1a–e). In the larvae of cetonids, complex differentiations of the hindgut epithelium evolved consisting of several highly differentiated, elongated epithelial cells that together form a "pseudoseta" (Figs. 7.1f,h, 7.3). Macroscopically, the inner surface of the hindgut of the larvae of the various scarabaeids resembles the "villi" known from mammalian guts. These structures not only serve as attachment for eubacteria and methanogenic

archaea, rather they are also engaged in the resorption of the fermentation products. The presence of large bundles of microtubules, which are closely associated with potentially fatty acids-containing vacuoles, strongly suggests that the pseudosetae of *Pachnoda* sp. larvae absorb fermentation products in the gut lumen and transport them to the hindgut epithelium, which contains numerous mitochondria. Mitochondria and tracheae are lacking in the distal parts of the pseudosetae, in clear contrast to the hindgut structures of *Nyctibora* spec. described above (Figs. 7.1e,f,h, 7.3, 7.4).

7.8 Biodiversity of Intestinal and Endosymbiotic Methanogens

Most gut bacteria and, in particular, most of the intestinal and endosymbiotic methanogenic archaea cannot be cultured yet. The few exceptions of cultured methanogens from insect guts (Leadbetter and Breznak 1996; Leadbetter et al. 1998; Sprenger et al. 2000) reveal significant differences in both the taxonomic position and the metabolic properties. Culture-independent methods such as PCR amplification of the 16S rRNA genes of intestinal methanogens provided a first overview over the biodiversity of methanogens in the arthropod guts. The methanogenic endosymbionts of gut-dwelling ciliates have been identified by FISH and PCR based amplification of the methanogenic 16S rRNA genes (van Hoek et al. 2000). Community analysis using archaeal 16S rRNA libraries and profiling techniques such as T-RFLP analysis of methanogenic archaea from arthropod guts (Ohkuma et al. 1999; Tokura et al. 2000; Brauman et al. 2001; Friedrich et al. 2001; Egert et al. 2003) revealed an enormous biodiversity. The analyses of termite guts reveals a surprisingly stable gut community, which might be host-specific. It could be shown that the gut microbiota is clearly distinct from the microbial communities in different soils, and also characteristic for the particular compartments of the termite gut (Schmitt-Wagner et al. 2003a,b; Donovan et al. 2004).

Moreover, many cockroach species host anaerobic ciliates with endosymbiotic methanogens (Table 7.1). By an analysis of the 16S rRNA genes of the methanogenic endosymbionts *and* the 18S rRNA genes of their particular ciliate hosts, it could be shown (1) that nearly every strain of a (methanogenic) cockroach species hosts a specific ciliate, and (2) that every ciliate species seems to carry a specific endosymbiont (Embley et al. 1992a,b; Dyal et al. 1995; Fenchel and Finlay 1995; van Hoek et al. 1998, 2000; see Hackstein et al. 2002 for a review). In contrast to cockroaches, only a few flagellates from the termite gut host endosymbiotic methanogens. Nevertheless, the phylogenetic analysis of the flagellate-associated methanogens

reveals that they are clearly different from those methanogens, which adhere to gut wall (Fröhlich et al. 1999; Tokura et al. 2000).

A broad, systematic analysis of the biodiversity of arthropod-borne methanogens has not been published until now. However, given the presumed host-specificity and the evidence in favour of a long-lasting coevolution between the arthropod hosts and their methanogenic symbionts one might predict that arthropod-methanogen symbiosis will provide one of the biggest sources of biodiversity of methanogenic archaea. Given that there are some 2200 described species of termites (Brauman et al. 2001), and that there is a remarkable biodiversity of Euryarchaeota in the various gut segments, then one might expect some 20,000–200,000 different methanogens only in termite guts. Comparable studies have not been published for cockroaches, but we have shown that about 70% of the (estimated) 5000 species of cockroaches have the potential to host methanogens *and* ciliates with endosymbiotic methanogens (Table 7.1; Hackstein 1997). If the Euryarchaeota in cockroach guts exhibit a diversity comparable to the one observed in termite guts, we must at least double the estimate for methanogens in termite guts. Since it has also been shown that most of the local populations of cockroaches host their "own" specific ciliate (each with its own endosymbiont) we are envisaging previously unrecognised, gargantuan biodiversity of symbiotic methanogens (Hackstein 1997).

7.9 Conclusions

Little is known about the function of the methanogenic archaea in the guts of arthropods, besides their role in lowering the H_2 partial pressure by producing methane. This lowering of the H_2 partial pressure might have similar consequences for the fermentation patterns of the gut microbiota similar to the situation known from mammalian guts and rumen (Hobson 1988). In addition, it may be speculated as to whether intestinal methanogens can contribute also to the nitrogen-carbon balance in the hindgut by the fixation of atmospheric nitrogen, since methanogenic archaea possess the whole set of genes required for nitrogen fixation (Raymond et al. 2004). Recently, it has been shown that the detrivorous and humivorous insects or their larvae have a much greater importance in the mineralization of organic compounds and the supplementation of soil with nitrogen than anticipated by earlier investigators (Nardi et al. 2002; Lemke et al. 2003; Ndiaye et al. 2004; Zhang and Brune 2004). Thus, the role of methanogenic arthropods exceeds their role as methane-emitters and potential contributors to global warming. Future studies will provide further information about their complex role in terrestrial ecosystems.

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Part II
Termites as Model Organisms

8 Termites as Soil Engineers and Soil Processors

David E. Bignell

8.1 Introduction

Termites have a highly significant impact on pedogenesis, soil properties and soil functions over large areas of the tropics and sub-tropics. The impact varies with temperature, rainfall, altitude, seasonality, latitude, longitude and parent geology; but generally arises from frequent high abundance and biomass, combined with the habit of creating extensive gallery systems in soil and the use of excavated mineral material to build mounds both above and below ground and runways above ground. This manipulation affects the mineral material both physically and chemically; broadly, soils which are well populated by termites are better drained, more stable and likely to have a higher retained organic content than counterpart soils which are depauperate, either for natural reasons or because of anthropogenic land-use change. Additionally, termites can digest a wide range of types of organic detritus, ranging (in different taxa) from freshly dead wood and dried grass through to highly humified organic-rich whole soil. This arises from a number of highly evolved mutualisms with microbes and appears to give them a role in decomposition processes out of proportion to their diversity and taxonomic identity as a single invertebrate order, though perhaps not to their biomass. There is also evidence of a role in the N-cycle, especially symbiotic nitrogen fixation, which may be the primary contribution of some of the associated microbes, rather than the degradation of lignocellulose.

All termites are eusocial, that is, the ability to reproduce can be suppressed in individuals (usually the great majority), which then reversibly or irreversibly specialise behaviourally and morphologically for other activities such as defence, foraging, construction or nurturing of the young. The result is a colony, ranging (in different cases) from a few individuals to many millions, where the members have a relatively high degree of relatedness to one another, as well as an absolute dependence on the continuation of the colony and its social order. The impact of termites in

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soils in not a direct consequence of their social organisation (for example, earthworms can have a comparable or greater impact without being eusocial), but inasmuch as the mutualisms with microbes have developed in a social context (Nalepa et al. 2001), sociality has been strongly selected in termite evolution and remains essential for their continued existence and niche exploitations.

Termites are frequently seen as actual or potential pests. A small number of relatively primitive species, loosely termed "drywood termites", "dampwood termites" and "subterranean termites" can infest timber used for buildings, poles, furniture and fencing. This is often to spectacular effect, and such species are inadvertently distributed by man, becoming pantropical or even more widely distributed. Other types of termite can become agricultural or silvicultural pests, but this is normally in the context of the destruction or modification of their natural habitats by the introduction of non-indigenous crop plants (including trees) or the clearance or burning off of natural mulches (Wood 1996). The large majority of termite species are not pests under any circumstances, but instead carry out wholly beneficial activities. These include not only the facilitation of drainage through the maintenance of macropores, but also the mixing of organic and mineral materials, the translocation of soil and microbial propagules, the stimulation of microbial metabolism and the promotion of hydraulic conductivity. The extent of the benefits of termites is only just gaining recognition and has scarcely been valued by any economic calculation. In the tropics as a whole, termites are thought to constitute 10% of all animal biomass (up to 95% of soil insect biomass), and to impact C mineralisation (decomposition) to roughly the same extent as all mammalian herbivores and natural fires. Overall, they mediate somewhere between 2 and 5% of the CO_2 flux to the atmosphere from all terrestrial sources (the variation in estimate depending on the assumptions made in scaling-up calculations), a large contribution for such a discrete taxon. Earlier concerns about the possible role of CH_4 production by termites in global warming have now been shown to be unfounded (Sugimoto et al. 2000) and will not be considered in this review, despite the relatively large recent literature on the subject.

The chapter will summarise the basic biology of termites relevant to their impacts on soils, but with a more extensive discussion of gut processes, before reviewing the empirical evidence for the role of termites in pedogenesis and soil processes. We conclude with an assessment of whether manipulation of termite populations can promote soil fertility and stability.

For a modern synthesis of termite biology readers are advised to see the following book and reviews for details (Lal 1987; Lobry de Bruyn and Conacher 1990; Wood 1996; Black and Okwakol 1997; Pearce 1997; Lavelle et al. 1997; Abe et al. 2000; Holt and Lepage 2000; Lavelle and Spain 2001; Bignell and Holt 2002). The classic book "Termites and Soils" (Lee and

Wood 1971) is still valuable as a source of much original data, as is Chapter 8 of Grassé (1986, in French). Wood and Sands (1978) remains probably the most influential review of termite ecology and includes an evaluation of the physical and chemical modifications of soils caused by the insects. Wood (1988) and Jones and Nutting (1989) are also useful as introductions to the topic.

8.2 Current State of Termite Science

Termites have been studied scientifically since the late eighteenth century. However, local expertise with termites is usually orientated towards pest control, which is a billion-dollar industry worldwide, and relatively few academic termite scientists are tenured in universities or other public institutions. Taxonomic expertise and capacity is particularly deficient in least developed countries and therefore the inventory of naturally occurring species in incomplete. Even in 2005, the main global centres for termite taxonomy (and often the only places where some specimens can be identified unequivocally) are in the UK, the USA, Australia, South Africa, France and Japan. Excepting Australia and South Africa, these are all outside the main geographical zones where termites are abundant. However the situation is changing: taxonomic training is assuming greater importance in bilateral development/co-operation agreements between Western countries and those of the humid tropics, such that Brazil, Kenya, Pakistan, India, China and Malaysia, which have established local taxonomic expertise and fully curated national collections. Unfortunately, taxonomic expertise is not so easily exchanged between countries. This is because termites show high endemicity, i.e. most species are confined to a defined area, usually a part of one of the world's major biogeographical regions, and do not occur elsewhere. There are also imbalances between historical taxonomical work and actual diversities; for example, the termite faunas of N America and SE Asia are well described, but relatively depauperate, compared to those of Africa and S America (Eggleton 2000; Eggleton and Tayasu 2001; Davies et al. 2003). Other anomalies exist: the termite literature is notable for the high proportion of African studies, a legacy of the colonial process, and studies of savannah systems are more frequent than those of forests, despite most tropical forest systems supporting a higher diversity and abundance of termites overall. These problems notwithstanding, more than 2600 species of termites have been described in 270 genera and 6 families: an educated guess would be that this represents about 70% of the species that actually exist, and that most of the undescribed fauna is located in African and South American lowland humid tropical forests (Bignell et al. 2004).

If termites are sampled with reasonable thoroughness in any one place, a range of species can usually be found which are characteristic for the locality, the land-use and land-cover in question. Termites are sensitive to the disturbance of their environment by man, and will generally respond by diminishing in diversity (usually with particular trophic functional groups showing the greatest response), abundance (fewer individuals and smaller colonies) and biomass (less impact on natural environmental processes). Therefore, termites may be indicator species and monitoring their assemblages may be a surrogate for ecosystem health as a whole (Jones and Eggleton 2000; Bignell and Eggleton 2000). In practice, however, there is no universally agreed protocol for sampling in the field, which makes comparisons of studies by different authors difficult and suggests that some of the existing published information on termite diversity and abundance may be inaccurate, most likely underestimates (Eggleton and Bignell 1995; Bignell and Eggleton 2000). However, efforts to standardise sampling methodology are providing useful comparative data (Anderson and Ingram 1993; Jones and Eggleton 2000; Davies et al. 2003), and such methods may be more widely adopted in the future (Swift and Bignell 2001). Paradoxically, it is easier to standardise sampling under forest canopies, where a thorough examination of a small area, ca. 0.1 ha and including the top 20 cm of the soil, would reveal most of the species present, than in savannahs, where more widely separated nests rather than galleries contain the majority of individuals and where dry seasons drive termites to considerable depths.

Fields to benefit from recent research in termite science include chemical ecology (production of pheromones and their influence on termite behaviour, especially foraging), sociobiology (how and why termite societies have evolved), evolution (how and why termites evolved from cockroach ancestors) and microbiology (what is the true diversity of archaea, eubacteria, actinobacteria, protists and fungi associated with termites, and what are their individual roles?). Modern molecular methods have also made an impact: these permit the phylogenies of termites and their symbionts (including even intracellular parasites) to be reconstructed when other evidences are conflicting, and have confirmed the close relationship between termites, cockroaches and mantids. In addition, there have been major advances in understanding the physiological regimes within the alimentary canal, defining the metabolic capabilities of the termite host and some of the main groups of microbial mutualists, and, by the use of natural stable isotope ratios, defining the balance of C and N sources used in different taxa and trophic functional groups. Most pleasing of all, Lepage and Darlington (2000) were recently able to write a comprehensive review devoted entirely to termite population ecology, documenting our accumulated knowledge of the growth and dynamics of individual colonies, and how work is divided between castes.

Keen interest in the terrestrial C cycle has been stimulated by attempts to fully quantify it, over the whole land surface of the earth, and more recently by concern about global change and, most notably, greenhouse gas emissions. As a consequence, there are now a large number of quantitative studies of termite assemblages, often accompanied by attempts to estimate their contribution to the C fluxes to the atmosphere (as CO_2 and CH_4) that result from organic decomposition. Comprehensive recent reviews of these data appeared (Martius 1994a; Sanderson 1996; Bignell and Eggleton 2000; Sugimoto et al. 2000). Termite abundance varies from less than 50 individuals m^{-2} in arid savannahs to more than 7,000 m^{-2} in some African forests. The corresponding biomass densities range from negligible to more than 100 gm^{-2}, in exceptional cases. In general, abundance and biomass increase with rainfall (at the same altitude) and decrease with altitude (at the same level of rainfall). Decreasing rainfall tends to favour wood-feeding and grass-cutting over soil-feeding trophic groups, but there are many subtleties related to biogeography and geology. Some soils do not support termites at all, especially those which are excessively sandy, contain heavy metals or are subject to severe cracking (Wild 1975; Holt et al. 1980; Bignell and Eggleton 2000). Seasonal flooding tends to reduce overall diversity and abundance, but in relative terms favours arboreal termites and those able to build epigeal mounds over those that are subterranean (Collins 1979; Martius 1994b, 1997). For details of more than 60 published estimates of termite abundance and biomass see Tables 8.1 and 8.2 in Bignell and Eggleton (2000).

Table 8.1. Definitions of putative (heuristic) termite feeding groups, based on inspection and knowledge of natural history (after Eggleton et al. 1997)

Group	Descriptions
Soil	Termites distributed in the soil profile, surface litter (leaves and twigs), and/or epigeal mounds, feeding on mineral soil; workers dark-bodied
Soil/wood	Termites feeding only or predominantly within soil under or plastered within logs, or feeding within highly decayed wood that has become friable and soil-like; workers dark-bodied (=intermediate feeders sensu de Souza and Brown 1994)
Wood	Termites feeding on wood and excavating galleries in large items of woody litter, which in some cases become colony centres. This group may also include species with arboreal (carton) nests, epigeal (soil or carton) nests or subterranean nests; also includes some Macrotermitinae cultivating fungus gardens
Litter	Termites that forage for leaf litter and small woody litter; includes some mound-building and subterranean Macrotermitinae, also epigeal mound builders and arboreal nesters of the Nasutitermitinae (nasute soldiers), which forage on the surface of the litter layer
Lichen	Termites that forage for lichen, mosses and algae on the bark of trees

Table 8.2. Feeding group classification of Donovan et al. (2001a). The gut contents of worker caste termites representing 46 species or morphospecies, taxonomically diverse and collected throughout the tropics, were analysed microscopically for the relative proportions of silica, plant tissue fragments, fungal mycelia, arthropod parts, plant root hairs and non-plant cells. Principal component analysis showed that an axis (axis 1) mainly derived from plant tissue fragments and silica, and defining a humification gradient, could explain the largest proportion of the variation in the data. A redundancy analysis, with Monte-Carlo permutation tests, showed that three gut morphological characters (out of 22 significantly associated with gut content variation) permitted an unambiguous classification of the specimens into four feeding groups, with generally consistent placement along the humification axis (except group II). This allows unknown specimens to be allocated to an objectively-defined feeding group without resort to gut content analysis and without subjective assessment of natural history and abdominal colour. Clades follow Kambhampati and Eggleton (2000)

Group	Diagnostic gut morphological characters			Typical gut contents		Examples
	Malpighian tubules	Rt. mandible molar plate ridges	Enteric valve	Silica	Plant fragments	
I	Eight or more			Low	High	Lower termites
II	Four or less, attached at mesenteron/Pone junction only	Prominent	No armature between ridges	Low or moderate	High, but with variable degrees of humification	Macrotermitinae, and representatives of: *Amitermes* clade, *Cornitermes* clade, *Nasutitermes* clade
III	Four or less, attached as above or otherwise	Vestigial	Unsclerotised ridges	Moderate or high	Moderate	Representatives of: *Anoplotermes* clade
		Absent	Some sclerotisation	Moderate or high	Moderate	*Termes* clade, *Cornitermes* clade, *Nasutitermes* clade
IV	Four or less, attached as above or otherwise	Absent	At least half the ridges 50% sclerotised	High	Low	*Apicotermes* clade, *Cubitermes* clade and representatives of: *Anoplotermes* clade, *Amitermes* clade, *Termes* clade, *Cornitermes* clade, *Nasutitermes* clade

Scaling-up calculations by Sanderson (1996), Bignell et al. (1997) and Sugimoto et al. (2000) all agree that CO_2 fluxes by termites are in the range 2–5% of the total from all terrestrial sources, despite the different assumptions these authors made in their calculations. For a single insect order with about 0.01% of the global terrestrial species richness, 2% is an impressive figure, but is nevertheless only a minor source in the context of the whole C budget. With CH_4 emission, it now appears that the net contribution by termites is very small, despite the fact that some species (more correctly some trophic groups) produce this potent greenhouse gas in large amounts. Studies of static chambers and natural stable isotope ratios show that mound walls and undisturbed soil between mounds have a strong sink capacity, presumably due to the presence of methylothrophic archaea (Sugimoto et al. 1998; MacDonald et al. 1999). Intuitively, this is disappointing for termite scientists, but there has been a large concomitant gain of new data. In addition to better estimates of abundance and biomass, many studies have tried to estimate the consumption of organic matter by termites (not necessarily the same thing as C mineralisation) and C cycling through their populations (e.g. Lepage 1973, 1974; Peakin and Josens 1978; Wood 1976, 1978, 1996; Wood and Sands 1978; Collins 1981; Holt 1987, 1988; Waller and Le Fage 1987; Jones and Nutting 1989; Jones 1990; Whitford et al. 1991; Martius 1994a,b, 1997). The published estimates of termite wood consumption rates range from 6 to 270 mg per termite per day (dry weight basis; Wood 1978; Wood and Sands 1978), a difference of more than one order of magnitude. Estimates of population respiration rates in moist savannah systems suggest that roughly 20% of C mineralisation could be attributed to termites (Wood and Sands 1978; Collins 1981), but this is equivalent to the consumption of 55% of surface litter, including about a quarter of the standing crop of dry grass. In semi-arid systems, the proportion of litter removed by termites may be even higher, although the process is slow (Bodine and Uekert 1979; Buxton 1981). In forests, C fluxes are dominated by the metabolism of the trees, and the relative contribution of termites is small, although abundance and biomass may be higher than in savannahs (Bignell et al. 1997). However, it was still possible for Matsumoto and Abe (1979) to estimate that the termites of Pasoh Forest (Malaysia) consumed more than 30% of tree leaf litter. There is thus a high visibility to termite feeding activity, although its importance in terms of C mineralisation, is rather variable. Termite biomass is quite often exceeded by that of earthworms (Lavelle et al. 1997), so the notion that termites always dominate tropical macrofaunas and are the primary agents of decomposition is untrue. Productive tropical forest and savannah systems in which termites are relatively less abundant and less diverse (e.g. in SE Asia) do not have noticeably less vigorous decomposition processes. In such cases decomposition of organic litter is presumably shared with

other animals (such as cockroaches, millipedes, various types of insect larvae, collembolans and arachnids) and microorganisms. These observations help us to the conclusion that the main impact of termites in the biosphere is on soil formation and conditioning, rather than decomposition per se.

Heuristic feeding classifications for termites have been proposed by Eggleton et al. (1995, 1996; see Table 8.1). These are useful in the field up to a point, but suffer the disadvantage of depending on circumstantial evidence, especially the site of discovery of the specimens concerned, without confirmation from an inspection of the gut contents. A new system, based on the relative proportions of silica and plant tissue fragments in the posterior hindgut has been put forward by Donovan et al. (2001a; see Table 8.2). This has the additional advantage that unknown specimens can be allocated to feeding group by reference to relatively straightforward morphological character states, which strongly correlate to feeding group, and no gut content analysis is required. It is still necessary to dissect the intestine; however this can be done after preservation in alcohol and should be within the competence of well-trained parataxonomists.

The success of termites (there are estimated to be more than 1×10^{18} in the biosphere, making them more numerous than the human population by about six orders of magnitude) is still the cause of debate amongst scientists. Broadly, the key evolutionary advantages that termites have are (1) social organisation and (2) a well-designed digestive system, including very effective mouthparts, combined with an obligate symbiosis with microorganisms which makes it possible to gain energy and nutrients efficiently from abundant but refractory food substrates (Bignell 1994, 2000). Until quite recently, these twin features of termite biology have been investigated quite separately, such that the evolutionary connection between the two has been unclear, but some synthesis is now emerging (Nalepa and Bandi 2000; Nalepa et al. 2001).

8.3 Termite Biology and Evolution

Termites are eusocial, polymorphic insects (this means the adult stage is represented by up to five morphologically distinct types or castes, only one of which has functional gonads) and live in large family groups comprised of reproductive forms (sometimes winged) together with numerous sterile soldiers and workers. Unlike ants, the termite soldier is a distinct caste with no role other than to defend the colony, although its abundance relative to workers varies greatly from species to species, and some of the more highly evolved cryptic termites are soldierless. Eusocial organisation allows the

majority of individuals (the workers) to forage for food co-operatively, while at the same time they are expendable without immediate cost to the colony, as the reproductives are protected within the central part of the mound or gallery system until they fly. Cost/benefit calculations based on energy expenditure (in finding food and building mound and gallery systems) and energy gain (from the food secured) show that insect social systems are efficient (Brian 1978). However, because of the selfish behaviour of DNA, they can only evolve mechanistically (i.e. without a learned culture) where the genes of the sterile castes are sufficiently similar to those of the reproductives for the best chance of the worker genes being represented in the next generation to reside in supporting the reproductives rather than investing in their own gonads (Higashi et al. 2000). The nest is a characteristic of all eusocial insects; in termites there is a notable diversity of nests (mounds if wholly or partly above ground), sometimes with complex architecture and in one or two genera reaching a huge size (Noirot and Darlington 2000). The nest is a closed system with few connections with the outside world (in some cases none). This permits the cryptic behaviour of termites, restricting attacks by predators, and avoidance of air currents, which reduces water loss and allows them to reduce nutrient investment in a thick, impermeable cuticle.

Termites are derived forms of cockroaches (this means that termites and cockroaches share the same basic body plan and that cockroaches are ancestral, termites having evolved relatively rapidly, and more recently, with a subset of specific specialist adaptations). Termites depend on mutualistic intestinal microbes, mostly archaea and bacteria and, in some higher forms, externally cultivated basidiomycete fungi, for assistance with energy metabolism and the provision of nitrogen. Some termites (generally the more primitive forms) also contain populations of flagellate protists in the hindgut, a number of which share with the termite host the ability to degrade cellulose and other plant structural polysaccharides. Where flagellates have been dispensed with (i.e. in higher termites), the ability to degrade cellulose is not necessarily impaired, but the site of expression and secretion of the termite's own cellulases is moved from the salivary glands (typical of the more primitive termites with flagellates) to the midgut epithelium (Slaytor 2000). No explanation of this unprecedented phenomenon has yet been offered and it remains one of the mysteries of termite biology.

Although the presence of dense microbial populations in termite guts has been known for more than 75 years, and termite-microbe interactions are often used in textbooks to illustrate biological symbiosis, their role is yet to be specified exactly. Most termites that feed on wood and other relatively intact plant residues seem able to produce appropriate digestive enzymes for degrading cellulose; hence the main duty for termite-associated microbes may be to meet some other need.

Natural termite assemblages (an assemblage is a set of species living in the same place, though not necessarily with the same niche) normally contain a range of species which feed, individually, on materials of differing provenance (wood, grass, leaf litter, animal dung and living lichens), and at different stages in the decomposition process (living tissues, freshly dead plant tissues, decayed wood and organic-rich soil; see Noirot 1992; Bignell and Eggleton 1995). Those species feeding on more humified food (extensively decayed wood and organic-rich soil) seem to have a more limited digestive capacity of their own, so the intestinal microbes here are, presumably, the primary agents of nutrient acquisition by degrading lignocellulose and soil organic matter in some manner yet to be fully elucidated (see below). A further complication arises from the existence of termites that have obligate mutualism(s) with fungi. In these species, the fungal mycelium is grown on a structurally complex substrate, derived from primary forage (mostly wood and leaf litter) and furnished by the termites, who manipulate the culture to exclude competing fungi and then consume, at different stages of their development, the spores and the senescent mycelium. This relationship seems to cater for different needs in different circumstances: in some species fungal products assist with digestion, while in others the fungus accumulates nitrogenous compounds (a kind of composting), which are eventually passed to the termite when the mycelium is consumed (Rouland-Lefèvre and Bignell 2001). Taxonomic characterisation of termite-associated microbes is still in its infancy, but the available evidence suggests that some are quite commonplace organisms, while others have co-evolved with their termite hosts and are not found elsewhere in nature.

The available fossil evidence suggests the main adaptive radiation of termites (and their trajectory towards ecological dominance in the lowland tropics) was a mid-Tertiary event, and occurred after the start of the general continental break-up in the Cretaceous (Thorne et al. 2000). The major event in the evolution of eusocial termites seems to have been the use of soil, initially for construction of mounds and galleries, and subsequently as a food (Donovan et al. 2000). This apparently coincides with the loss of protist intestinal symbionts, and the acquisition (or co-evolution), in some clades, of gut bacteria which can degrade soil organic matter sufficiently to provide the termite host with adequate nutrition. The advantage of soil-feeding is (1) that this resource is available in infinite amounts and (2) that it can be easily accessed by the construction of relatively simple subterranean gallery systems around the colony centre. The energetic cost of feeding is therefore low, competition for resources with other colonies or other types of termite is minimal and defence of the colony can be achieved largely by the avoidance of predators, rather than the production of large numbers of specialised soldiers and/or foraging workers protected by

thick cuticles (Bignell 1994). Such considerations weigh heavily in eusocial systems, where it can be argued that the optimum evolutionary position is to provide the maximum supply of nutrients to the functional reproductive castes (queen and king) at the least cost in terms of the replacement of expended workers and soldiers. It is tempting to see a connection between the adoption of soil as a building material and the loss of the (relatively fragile) flagellate mutualists from the gut (Donovan et al. 2001a).

Constructions made by termites include: numerous galleries tunnelled through wood and soil; underground chambers containing the reproductives and developing nymphs (and in some cases symbiotic fungus gardens); runways attached to the sides of trees, decaying wood and buildings; soil sheeting covering the surface of the ground and items of dead wood; and arboreal or epigeal (i.e. emerging from the surface) mound-nests with a complex internal structure. Materials utilised, manufactured or translocated by termites include surface soil, sub-soil, compacted faeces and carton (a lightweight organic-rich mixture of partially digested cellulose, saliva and soil); such manipulations contribute to the role of termites as conditioners of soils (Holt and Lepage 2000). The colour of termite mounds gives some clue to the identity of their builders or occupants. A lighter orange or brown colour indicates the use of sub-soil in construction and such soil is unlikely to have been passed through the termite gut, being carried instead in the mandibles of teams of workers without ingestion. Dark mound material is organic-rich soil foraged from the surface layers and probably having first passed through the guts of the workers of soil-feeding species, to be deposited as faeces. However, one must also remember that termite mounds can change ownership (technically this is called secondary occupancy or inquilinism), so the occupants discovered during sampling may not be the constructors. A large number of species are not primary mound builders, either living entirely in diffuse subterranean gallery networks or becoming established in pockets within the mounds of other species as secondary occupants (Eggleton and Bignell 1997; Noirot and Darlington 2000).

8.4 Soil Ecosystem Engineers: Is This a Valid Concept?

Termites, ants and earthworms are considered to be "soil ecosystem engineers". This term is now attributed to Jones et al. (1994), although the concepts can be found in earlier contributions (e.g. Lee and Wood 1971; Stork and Eggleton 1992; Anderson 1993). There are several definitions, but the essence of the idea is of larger organisms that directly or indirectly affect the availability of resources to others, generally smaller, through

modifications and turbations of the physical environment. At the heart of the engineer concept is the ability to move through soil and to create organo-mineral complexes (biogenic structures), directly as a consequence of feeding on the main medium and mixing organic and mineral materials within the gut, or indirectly by the secretion of organic substances or other types of mixing during constructions. Soil engineers are usually thought of as invertebrates, but the definition does not exclude either plant roots or vertebrates, provided their numerical and biomass densities are sufficient to exert a predominate influence and some identified pedogenic process is the outcome of their growth and activity (Lavelle et al. 1997). It is important to distinguish between the engineers and other soil macrofauna, for example millipedes and woodlice, as well as epigeic earthworms producing holorganic faecal pellets, whose contribution is that of litter transformation (physical and, to a limited extent, chemical) but without the creation of new structures. Litter transformers and smaller invertebrates (mesofauna and microfauna, see Fig. 8.1) are said to be dependent on engineers to

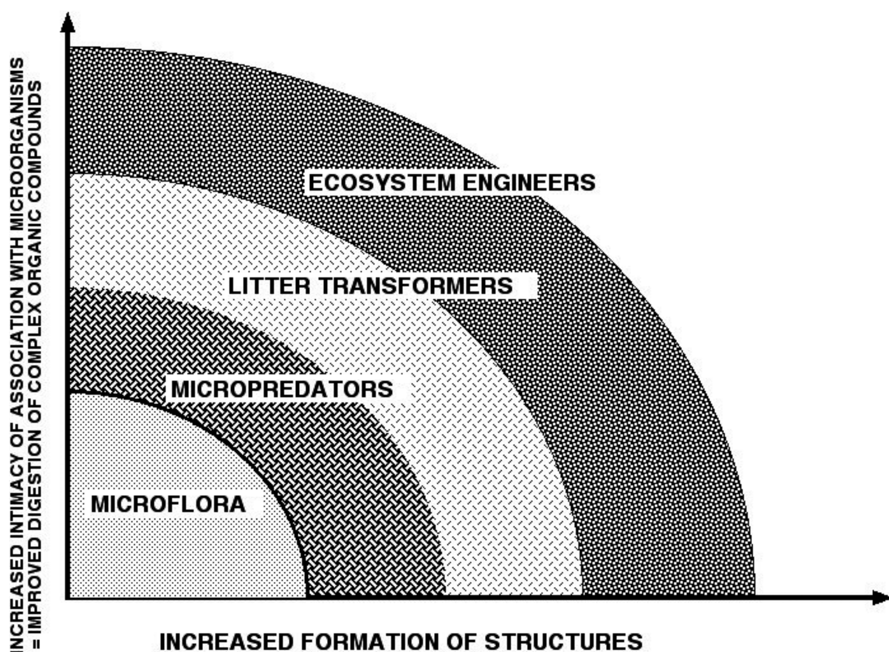

Fig. 8.1. Functional classification of soil organisms proposed by Lavelle et al. (1997). In this scheme, relative influence over soil functions through the formation of biogenic structures increases with the intimacy of association with microorganisms in the digestion of complex organic compounds

generate and maintain a soil structure in which there are pore spaces of appropriate size. Neither soil engineers nor litter transformers exert fundamental transformations of inorganic and organic materials (for example lignin degradation, nitrification, denitrification and sulphate reduction), at the heart of the C, N and S cycles. These remain the prerogative of microorganisms, even if such microorganisms are housed within the guts of soil engineers, as is the case with termites and, apparently, also some earthworms (Barois and Lavelle 1986; Barois 1987; Lavelle et al. 1994). Microbes in soil generally have limited abilities to move and it is hypothesised that they are dependent on much larger invertebrates and living roots to activate and transport them to new substrates after periods of inactivity, as well as providing those substrates if the microbes are internalised. Lavelle (1996) takes the argument further by suggesting that each variety of engineer delimits its own three-dimensional physical sphere of influence within a soil system, with a distinctive population of client organisms in smaller size categories. Thus there would be a termitosphere regulated by termites, a drilosphere regulated by earthworms and a myrmecosphere regulated by ants. Such hypotheses could explain the empirical evidence (see below) that removal or addition of soil engineers has strong effects on plant communities and that engineer diversity is, to some extent, a surrogate for the diversity of primary producers. However, at the present time the speculation exceeds the clear evidence. Nevertheless, litter transformers can have their own relationships with microorganisms: their pellets are known sites of enhanced microbial activity (Anderson and Bignell 1980), and the initial bacterial community in the pellets resembles the gut community (Ineson and Anderson 1985) and at a later time (days or weeks) the same invertebrate or another quite different in nature may re-ingest the pellets (refection, a form of coprophagy) and assimilate nutrients made available by the microbes (external rumen principle: Hassall and Rushton 1984; Szlàvecz and Pobozsny 1992). Growth of fungi on faecal pellets is the basis of the mutualism between host and microorganisms in the termite subfamily Macrotermitinae, but the termites still qualify as soil engineers because salivary secretions are used to bind inorganic sub-soil particles in mound and gallery construction, and indeed the resulting structures may have a life of years or decades (Wood 1996). The micromorphology of biogenic structures produced by termites is reviewed by Brussaard and Juma (1996), Lavelle et al. (1997) and by Holt and Lepage (2000). Within broad limits, their production determines porosity, aggregation and soil organic matter dynamics and the effects persist after the life of the engineer concerned, for months, years and decades. On a longer scale of centuries and millennia, termite burrowing and construction can determine soil profile development (Lal 1987; Stoops 1989; Quedraogo and Lepage 1997). Ultimately, soil structure is strongly influenced by engineers, and this defines

the microsites where microorganisms perform primary nutrient transformations.

Lavelle et al. (1997) proposed that a gradient of interaction with microorganisms can be seen in the digestive processes of soil animals as they increase in size and, consequently, assume different functional roles, contributing progressively more to the formation and maintenance of structure (Fig. 8.1). In litter transformers, the refection process provides access to more labile products temporarily accumulated in the organic matrix after the microbial succession, but there seems only a limited ability to digest plant cell wall components, including lignocellulose, directly (Bignell 1989). As an alternative strategy, litter transformers may be able to digest microbial tissues accumulated during preconditioning of their food or in the external rumen process. In termites and earthworms, by contrast, it can be construed that microorganisms are directly harnessed by the intestinal machinery as mutualists to degrade recalcitrant materials, either in the gut lumen or via an external rumen, at a rate which is consistent with the metabolic demands of the host (Bignell 1984, 1994). Consequently, the huge resources represented by lignocellulose in litters and humified organic matter within the mineral soil matrix become available as nutrients, and the engineers evolve to a dominant position in soil faunas (Nalepa et al. 2001). With termites, it is necessary to qualify this argument to make it consistent with the facts of the mutualisms, as we presently understand them. All termites which consume plant litter, whether composted or uncomposted, can express an indigenous cellulase (or in some cases a hemicellulase). The enzyme is relatively inefficient but can be secreted copiously (Slaytor 1992, 2000; Slaytor et al. 1997); similar enzymes (both in terms of biochemical properties and parent gene sequence) are secreted in wood-feeding cockroaches, the putative ancestors of modern-day termites. There is therefore some disagreement about the real purpose of the mutualisms, with a strong counter-hypothesis that the main role of microbial associates is in the provision of organically combined nitrogen for the host, either by direct fixation from the air (by prokaryotes) or by reduction of the C/N ratio of harvested forage (by fungi). Many wood-feeding termites and some wood-feeding cockroaches select litter items to consume which are already well degraded by fungi (Rouland-Lefèvre 2000); the distinction between litter transformers and grass- and wood-eating engineers may therefore be less distinct than the hypothesis of Lavelle et al. proposes. Ipso facto, soil-feeding is the best example of engineering, as soil is not only used to construct mounds and galleries, but is also ingested in large amounts and passed through a highly differentiated alimentary canal supporting a very diverse prokaryotic microbiota (Bignell 1994, 2000; Breznak and Brune 1994; Breznak 2000). Soil is a “free” resource, in the sense of being in infinite supply and having only small foraging costs, but is of

poor quality as a C source and therefore requires extensive chemical and microbial processing in the gut (Eggleton et al. 1998; Bignell 2000) (see Fig. 8.2). Faeces seem to be a major construction material and are used to line existing galleries, but further metabolism occurs by microbes and the linings are re-ingested, more in the fashion of an external rumen (Brauman 2000). Recent evidence suggests that some soil-feeders can lyse and assimilate ingested microorganisms (Fujita and Abe 2002), as is thought to be the mode for litter transformers, so perhaps there are different versions of soil-feeding or else soil engineers are really only litter transformers under another guise. Assays of soil-feeder guts for cellulase and xylanase activities have proved negative (Rouland et al. 1986, 1989). Estimates of the biomasses of microorganisms in different regions of the alimentary canal of a typical *Cubitermes*-clade soil-feeding termite are given in Table 8.3. Such data are difficult to interpret, as the microbiota is composed in part of organisms temporarily or permanently attached to the gut wall and in part of unattached forms which pass through with the food (Bignell et al. 1980, 1983; Bignell 2000).

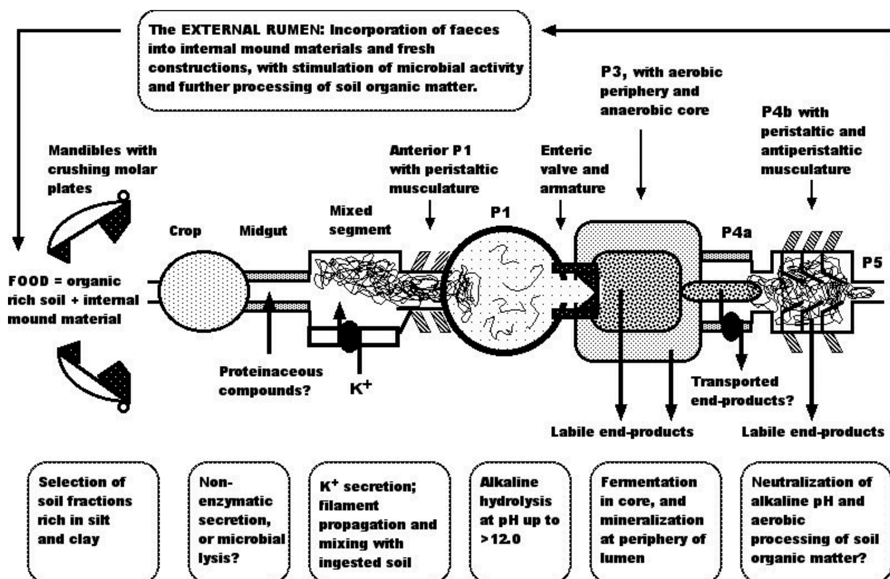

Fig. 8.2. Hypothesis of gut organisation and sequential processing in soil-feeder termites of the *Cubitermes* clade (sensu Kambhampati and Eggleton 2000). The model emphasises the contributions of (separated) microbial and chemical processing of soil organic matter, the principal nutritional resource. Not to scale. (Brauman et al. 2000)

Table 8.3. Biomasses of non-filamentous and filamentous prokaryotes (as mg g^{-1} gut contents) in the alimentary canal of *Cubitermes severus* by direct microscope observation. (Brauman et al. 2000)

Group	Foregut (crop)	Midgut	Mixed segment	P1	P3 (paunch)	P4a (ant. colon)	P4b (post. colon)	P5 (rectum)
Non-filaments	28.4	30.8	21.4	8.2	33.6	93.0	86.4	28.6
Filaments	8.4	9.8	41.9	3.6	10.7	23.8	19.4	10.9
Stat. sig.	abcd	efgh	abejlmno	fjqrs	lptu	cgmqtv	dhnruw	osvw

Means of five observations (20 guts per observation) are shown. Stat. sig., statistical significance: gut regions with the same letter are significantly different overall (non-filaments and filaments combined), by STP. Mixed segment includes the extreme anterior P1

8.5 Microbial Processing During Gut Transit

The microbiology of the termite gut is reviewed by O'Brien and Slaytor (1982), Breznak (1982, 2000), Breznak and Brune (1994), Inoue et al. (2000) and Koenig et al. (2002). Useful interpretations of the available data and hypotheses in the context of bioreactor theories have appeared (Bignell 1994, 2000; Brune 1998; Brune and Friedrich 2002). Morphological diversity of the intestine and the attendant phylogenetic implications are considered by Noirot (1995, 2001), Donovan et al. (2000) and Bitsch and Noirot (2002). Details of new descriptions and the discoveries of novel types amongst termite associated prokaryotic microbiotas are given by Brauman et al. (2000), Breznak (2000), Lilburn et al. (2001) and in a series of elegant papers from Japanese laboratories describing the application of modern molecular biological techniques to assess the heterogeneity of termite microbiotas and their possible phylogenies (Ohkuma and Kudo 1996, 1998; Ohkuma et al. 1995, 1996). Knowledge of the physiological environment within the termite gut has been recently revolutionised by the use of oxygen- and proton-sensitive microelectrodes (Brune et al. 1995; Brune and Kühl 1996; Schmitt-Wagner and Brune 1999), such that the availability of oxygen and electron donors, and the subsequent diversity of metabolic niches for prokaryotes are much better understood (Leadbetter et al. 1999; Tholen and Brune 1999, 2000; and for the Macrotermitinae see Aanen et al. 2002). The new data permit some hypotheses to be advanced concerning the difference in intestinal processes between wood-feeding and soil-feeding termites (e.g. Kappler and Brune 1999; Ji et al. 2000; Brune and Friedrich 2000), but many unanswered questions remain, notably the nature of the carbon sources utilised by chemoorganotrophic bacteria in the midgut and the hindgut (including those that fix atmospheric nitrogen). Our ignorance on

this matter is surprising, not only because of the long-established textbook dogma that termites can degrade cellulose only by virtue of their symbioses with mutualistic microbes, but also because of the overwhelming and long-standing evidence now available that all termites feeding on predominantly cellulosic foods can produce their own cellulases (reviewed in Slaytor 2000; Watanabe and Tokuda 2001; see also Watanabe et al. 1998; Tokuda et al. 1999; Lo et al. 2000). The best summaries of this dilemma are given by Slaytor et al. (1997) and Slaytor (2000). The somewhat separate case of the fungus-growing Macrotermitinae is summarised by Hyodo et al. (2000), Rouland-Lefèvre and Bignell (2001) and Hyodo et al. (2003). In this group, competent cellulases (and in some cases hemicellulases) are produced both by the termite host and the associated fungus. It is by no means clear that degradation of lignocellulose is the raison d'être of the mutualisms in any termite; nevertheless no termite can exist without its microbial symbionts (Bignell 2000), and theoretical arguments concerning the evolution of the termite-symbiont system, and its reinforcement by social organisation, still turn on the advantages to be gained by harnessing microbes to digest recalcitrant food components (Nalepa et al. 2001).

The termite gut is divided into three regions: foregut, midgut and hindgut (Noirot 1995, 2001; Bignell 1984, 2000). All termites have a prominent hindgut, which contains the great majority of microorganism present in the intestinal system as a whole and which may contribute as much as 40% of the animal's weight (Schulz et al. 1986; Slaytor et al. 1997). Hindgut shape, size and the degree of differentiation all vary considerably between taxa. Bignell (1994) and Bignell and Eggleton (1995) argue that the degree of compartmentalisation increases with the humification of the food consumed, such that the most recalcitrant materials receive the most extensive processing. A large proportion of the microorganisms are semi-permanent and attached either to the cuticular wall of the hindgut or to chitinous spines protruding into the lumen (Bignell 2000; Noirot 2001); the remainder are suspended in the gut contents, but a series of valves and muscular constrictions between the main gut compartments regulate the movements of the contents, so that each is, in effect, a continuous culture chemostat (Fig. 8.2). Archaea, eubacteria, actinobacteria and spirochaetes have all been identified as elements of the permanent prokaryotic microbiota, although it is still unclear whether representatives of all four groups are present in every case. There is dense packing of cells in some places and microbial population densities up to 1×10^{12} organisms ml^{-1} have been described (Bignell et al. 1980). Where flagellate protists are present (in lower termites), they may reach a population of 1×10^{5} per individual, representing about 60% of total hindgut weight, closely packed, but still motile to some extent, in the lumen of the hindgut. It is now clear that the flagellates are differentiated into cellulose and xylan utilisers (Inoue et al. 1997), and that much of their

digestion involves the internalisation of fragmented food. Bacteria can also be consumed and in both cases the process involved is endocytosis (Inoue et al. 2000). Both the flagellates and their termite hosts produce polysaccharidiases, so the digestive process is shared (Watanabe and Tokuda 2001). Earlier views that cellulolytic microbiota could not be isolated from termite guts (O'Brien and Slaytor 1982; Slaytor 2000) might now be revised in the light of new evidence (e.g. Rouland and Lenoir-Labé 1998; Wenzel et al. 2002), but the principle that termites do not have an absolute dependence on their microbial associates for cellulose and hemicellulose digestion remains secure.

Rouland-Lefèvre and Bignell (2001) suggested that the following activities could be ascribed to the intestinal microbial community, including protists where present:

1. Dissimilatory carbohydrate metabolism (including cross-feeding reactions), either from plant cell wall polysaccharides or their depolymerisation products, or from the products of glycolysis, yielding short chain fatty acids which are energy sources for other intestinal microbes and/or the termite host.
2. Oxygen consumption (as an electron acceptor), generally at the periphery of the gut lumen, rendering the centre of the lumen microaerobic or anaerobic. This permits fermentation of polymerised and labile carbohydrates, and also possibly proteins and amino acids, again yielding short chain fatty acids. Low redox may also facilitate symbiotic N_2 fixation.
3. Dissimilatory and assimilatory N metabolism, providing for the conservation of excretory N (produced as uric acid) by the termite host as new microbial biomass, plus assimilation of the primary products of N_2 fixation in organic form and (probably) transamination, balancing the amino acid spectrum available to the host. Interventions in N metabolism may be unnecessary where the C:N ratio of the food is favourable, for example in soil-feeding termites (Tayasu et al. 1997) and in Mactrotermitinae after processing by the fungal mutualist (Rouland-Leféyre and Bignell 2001).
4. Electron (or hydrogen) consumption by reductive acetogenesis or methanogenesis, assisting energy conservation by the system as a whole and preserving redox balances.
5. N_2 fixation on a facultative basis.
6. Demethylation, deacetylation and decarboxylation of aromatic polymers, possibly accompanied by limited aromatic ring cleavage.
7. Humification, or further humification, of complex organic material passing through the alimentary canal.

Available evidence suggests that lignin is not modified during gut passage (Hopkins et al. 1998; Hyodo et al. 1999), but significant mineralisation of carbon from the proteinaceous component of a model humic acid was demonstrated in a soil-feeding species (Ji et al. 2000). This accords with previous evidence from pyrolysis/mass spectrometry and other analyses that peptides are depleted in soil-feeder faeces, compared with their parent soil (Bignell 1994; Garnier-Sillam and Harry 1995). Physical changes to soil polysaccharides during gut passage by humivores, affecting structural stability, are described by Garnier-Sillam and Toutain (1995).

It remains unclear whether some or all of the organisms mediating the above functions are specialist forms confined to termites, or are recruited from inocula available in their immediate environments. The list of novel organisms isolated from termites continues to grow (Breznak 2000; Brauman et al. 2000; Koenig et al. 2002), while molecular genetic studies suggest that mutualist communities evolve with their hosts (Ohkuma et al. 1995, 1996; Aanen et al. 2002).

8.6 The Special Case of Fungus-Growing Termites

Rouland-Lefèvre and Bignell (2001) proposed that filamentous fungi cannot become established in termite guts because of the relatively large size of the cells and fragile nature of the mycelium, in a highly contractile system. However, mutualism with fungi should have a high value, owing to their ability to produce potent enzymes degrading cellulose, hemicellulose, lignin and lignocellulose. In the nesting systems of the higher termite subfamily Macrotermitinae, fungus-combs of the basidiomycete genus *Termitomyces* are cultivated in purpose-built chambers (within the mound or entirely subterranean), then harvested and consumed by the termite host. This subfamily is distributed in the tropics from Africa (where diversity is highest) through the Middle East, South and South East Asia, but is absent from Central and South America, and from Australia (Eggleton 2000). Like other higher termites (family Termitidae), they lack intestinal flagellates, but in contrast to the remaining three subfamilies of higher termites, the Macrotermitinae have not developed soil-feeding, sensu stricto. This is surprising, as the group is noted for the complex nature of their mound/nest constructions and for the ability to manipulate and translocate large quantities of soil (especially sub-soil) during mound building and repair, gallery maintenance and foraging (Wood 1996). Phylogenetic analyses show the Macrotermitinae in a basal position within the higher termites, suggesting that they may currently show some characteristics of the common ancestor of all four subfamilies of higher termites (Donovan et al. 2000; Bitsch and

Noirot 2002). Donovan et al. (2001a) and Nalepa et al. (2001) suggest that the use of soil for nest-building can account both for the elimination of intestinal flagellates and the early use of soil as a feeding substrate. Crucially, modern Macrotermitinae workers do accumulate soil in their guts, though it is clearly not a nutrient, presumably as a consequence of the working and carrying of mineral materials, in which the mandibles and buccal cavity are principally involved (Badertscher et al. 1983). Soil is a good source of fungal inoculum, so the specific association with basidomycete fungi, and the complex behaviour patterns needed to support extensive construction activities and to propagate the fungus are, presumably, derived characters. This reconciles the complex biology of the Macrotermitinae with their basal phylogenetic position. The Macrotermitinae are also physiologically advanced, showing a notable efficiency in moisture acquisition and conservation, which often allows them to dominate the termite faunas of arid and semi-arid environments (Desmukh 1989; Noirot and Darlington 2000; Traniello and Leuthold 2000). Foraging by Macrotermitinae is very intensive per unit of termite biomass, presumably as the strongly aerobic metabolism of the fungus, less constrained by physical space than intestinal mutualists, is correspondingly vigorous (Wood and Sands 1978; Collins 1981; Lepage 1983). Consumption of litter and C fluxes mediated by termites appear to increase when Macrotermitinae are well represented in the assemblage. Estimates of litter consumption by Macrotermitinae range up to 1,500 kg (dry weight) ha^{-1} $year^{-1}$ in moist savannahs, giving them a role in C mineralisation comparable to (or exceeding, see Buxton 1981; Deshmukh 1989) that of bush fires and/or mammalian herbivores. Organic matter turnover by other types of termite in such dry-land systems would be less than 10% of this amount. Visual evidence of foraging by Macrotermitinae is provided by red soil sheeting, covering stems, lying dead wood of all descriptions and the surface of the ground. Soil utilised by Macrotermitinae for building seems to come from deeper layers with a relatively low inherent organic content. Most other termites collect soil from the top 15 cm of the mineral profile.

The nature of the interaction between termite and fungus is controversial (see Rouland-Lefèvre and Bignell 2001). Dry grass, dead wood and leaf litter (most species specialise in one of these foodstuffs) are brought into the colony and consumed by a subset of the worker termites, together with existing conidia from the fungal mutualist. New fungus-comb is made from the resulting faeces, in which the forage appears to be only lightly digested. As the fungus grows it composts the forage, degrading cellulose, hemicellulose and lignin (though apparently with different absolute and relative efficiencies in different host species/fungus species combinations), and at the same time increasing the relative N content of the substrate than remains. The mycelium then fruits and subsequently becomes senescent, when it is

eaten by an additional subset of the termite workers. The second digestion is more thorough and little final faecal material is produced. Among termites, the Macrotermitinae are by far the most effective in degrading plant polymers, especially polysaccharides (Rouland et al. 1988) and polyaromatic compounds (Mora et al 1998; Hyodo et al. 2000). However, the relative contributions of termite-derived and fungus-derived enzymes are unclear, and may well vary between termite genera and species (Rouland et al. 1991). The worker castes of Macrotermitinae have prokaryotic gut microbiotas, which are broadly comparable to those of other higher termite taxa, in terms of diversity, abundance and biomass, but their functions, if any, are unknown. As in other termites, a number of short chain fatty acids (but mainly acetate) accumulate in the gut (Anklin-Muhlemann et al. 1995). This, and the observation that small amounts of hydrogen and methane are evolved, suggests that some anaerobic microsites exist.

8.7 The Fate of Termite Faeces

Although termite feeding and metabolism removes a substantial amount of organic matter by mineralisation as CO_2, material is also redistributed and chemically modified, largely as a result of gut transit and the incorporation of faeces into nest/mound materials and gallery linings (Lee and Foster 1991; Wood 1988, 1996). Lignin is not substantially degraded, though it may be modified by marginal demethylation and decarboxylation; consequently, the faeces of wood-feeding species are characteristically enriched in lignin (Hopkins et al. 1998). In soil-feeders, the precise nature of the change of organic matter during gut transit remains unclear. In the *Cubitermes* clade (sensu Eggleton 2000), soil organic matter is increased up to three times, but this is thought to be a consequence of selective feeding (Garnier-Sillam et al. 1988; Garnier-Sillam 1991; see also Donovan et al. 2001b; Jouquet et al. 2000). Nitrogen content also increases up to five-fold (see Table 8.4, with C/N ratio falling by 40%), but again the explanation is selective feeding on organic-rich fractions. Some of the nitrogen in these fractions is derived from plant and animal biomass and is presumably released in organic form from combination with tannins by alkaline hydrolysis in the anterior hindgut (Kappler and Brune 1999; Ji et al. 2000). What happens subsequently is a matter of conjecture, but the dense microbiota of the posterior hindgut is ideally placed to ferment or otherwise degrade amine-N. Changes in the balance of fulvic and humic acids in faeces are interpreted to imply that the organic matter in the termite faeces is less polymerised, although the same effect would be achieved if feeding was selective for more recently formed soil organic matter (Brauman 2000). Intense mixing of soil

Table 8.4. Analysis of organic matter in the faeces and components of the termitosphere of *Thoracotermes macrothorax*. (After Garnier-Sillam and Harry 1995)

Fraction analysed	Organic content (%)	Total C (%)	Fulvic acid as % of C extracted	Humic acid as % of C extracted	Fulvic acid/ humic acid	Total N (%)	Hydrol. N, as g/100g	Resid. N, as g/100g	Amino- N as % of hydrol. N	Comb. N as % of hydrol. N
Parent soil*	6.2	2.8	63	38	1.66	0.21	0.16	0.03	52	20
Worked-over soil**	9.4	4.7	44	57	0.76	0.45	0.34	0.11	43	22
Mound	8.3	4.0	44	56	0.79	0.38	0.27	0.11	34	23
Faeces	17.2	7.9	65	35	1.87	1.08	0.85	0.23	44	28

Abbreviations: hydrol., hydrolysable; resid., residual; comb., combined

accompanies gut transit (Bignell 2000), creating organo-mineral complexes which are more stable than the initial ingested humus (Garnier-Sillam and Harry 1995). The stability is in part attributable to increases in the levels of exchangeable bases, especially Ca, Mg, K and Na (Brauman 2000). NH_4–N and available P are also increased (Anderson and Wood 1984; Garnier-Sillam and Harry 1995), which may explain the use of soil-feeder mounds as a crude fertiliser in some subsistence agricultural practices.

The difficulty of transporting soil-feeding termites and keeping them in viable cultures has inhibited direct experimental work on the physical effects of gut transit. However, Donovan et al. (2001b), working with live mounds of *Cubitermes fungifaber* temporarily placed in containers of fresh, unworked forest soil, showed that the soil increased in pH, organic carbon, water and relative kaolinite content after being worked by the termites ("worked" soil includes material used or moved during constructions, as well as faeces), while at the same time relative quartz content fell.

In the Macrotermitinae, mounds are made from mineral sub-soil cemented with organic-rich saliva (Wood 1996). In other termites using soil for mound construction, it is widely assumed that the mounds are assembled with a mixture of faeces and uningested soil (Wood, 1988). In soil-feeders, there is some evidence that mound materials are richer in silt and clay fractions than the parent soil (e.g. Brauman 2000). This could be the result of selective feeding or active sorting of mineral fractions in the gut (Donovan et al. 2001b), although the organic matter content of mounds generally tracks that of the parent soil, with a relative enrichment from whatever is the base level. It is becoming clear that termite mound walls are sites of intensive microbial growth and metabolism Brauman 2000; Holt and Lepage 2000). Some of these organisms are presumably derived from those initially contained in the termite faeces. Garnier-Sillam (1987) and Brauman (2000) have suggested an external rumen mechanism, in which faeces are used to line existing galleries; microorganisms in the linings continue to metabolise polyaromatic materials and the linings are then reingested by the termite hosts. Consumption of gallery walls is a known behaviour of soil-feeding termites (Brauman et al. 2000). Evidence that bacteria in gallery linings are metabolically active is given in Table 8.5.

8.8 Evidence of the Role of Termites in Pedogenesis and Soil Properties

The specific termite literature in this area is reviewed by Lee and Wood (1971), Wood (1988, 1996), Lobry de Bruyn and Conacher (1990, 1995) and by Holt and Lepage (2000). Comparison of the effects of termites with other

Table 8.5. Microbial biomass and bacteria-specific 16S rRNA content in different components of the termitosphere of *Cubitermes fungifaber*. (Brauman et al. 2000)

Fraction analysed	Microbial biomass as % of total C[a]	Bacterial RNA (ng/mg)[b]	Water content (%)	Oxygen consumption ($\mu l\, g^{-1}\, h^{-1}$)	Carbon dioxide emission ($\mu l\, g^{-1}\, h^{-1}$)
Faeces	nd	177.7 ± 9.7	nd	nd	nd
Fresh constructions	nd	94.1 ± 14.2	20.0	14.95 ± 1.97	20.97 ± 2.46
Internal chamber walls	16.0 (*n*=1)	20.3 ± 3.0	14.7	9,12 ± 1.61	13.96 ± 0.55
External wall (old)	0.4	6.2 ± 1.5	11.2	7.55 ± 0.14	10.51 ± 1.61
Soil under the mound	2.2	3.4 ± 0.5	20.9	18.48 ± 2.79	20.77 ± 1.46
Surrounding soil 0–5 cm	0.3	1.3 ± 0.0	18.0	12.18 ± 2.84	1.84 ± 0.54
Surrounding soil 5–10 cm	ndet	ndet	20.0	4.21 ± 0.58	5.14 ± 0.54

Mean ± SE (*n*=3, except where stated); nd, not determined; ndet, not detected
[a] Microbial biomass determined by a fumigation/extraction method
[b] Determined by a probe for conserved eubacterial 16S rRNA oligonucleotide sequences

soil-forming processes is made by Lal (1987). Here, the five most important impacts of termites on the soil (see Bignell and Holt 2002) are highlighted.

8.8.1 Soil Profile Development

The construction activities of termites have a significant influence on profile development through soil translocation, microped formation and the creation of subsurface galleries. Large amounts of soil are collected, either from the superficial horizons or further down the profile (depending on the termite concerned), for incorporation into mounds, runways and surface galleries. Runways are purely temporary structures (Jouquet et al. 2002) and even mounds have, on average, a life of months to a few years (Dejean and Ruelle 1995), so translocated soil begins almost at once to be returned to the profile surface by erosion. As much as 4.7 tonnes ha^{-1} $year^{-1}$ may be turned over in this way in savannah systems (the calculation is based on estimates of mound erosion and may be inaccurate; see Lobry de Bruyn and Conacher 1990; also Badawi et al. 1982; Kooymand and Onck 1987; Mackay and Whitford 1988). When the purely subterranean translocations of soil-feeders are taken into account, estimates of turnover can rise by an

order of magnitude (Bignell and Eggleton 2000). A more useful way of placing these estimates in context is the calculation by Aloni and Soyer (1987) for an African humid forest that the top 37 cm of the mineral profile would be completely turned over by termites every 1000 years. Eroded runway and mound material may retain a relatively complex microped structure, reflecting its origin as faecal aggregates or cemented uningested particles. As much as 20% of the soil matrix may be biogenic in this way, with a significant influence on microporosity, moisture storage capacity and infiltration rates (Kooyman and Onck 1987).

Relict features of tropical soils, such as certain microaggregates and buried stone lines are also attributed to bioturbation by termites, combined with the selection of finer fractions for their constructions (e.g. Folster 1964; Soyer 1987). Tardy and Roquin (1992) summarise the evidence for the role of termites in creating lateritic landscapes. They note that in addition to bringing finer materials up the profile, termite galleries and mounds permit laterite particles to move both downwards and sideways in the soil profile. Termite galleries and chambers are themselves a component of the profile, and can extend as much as 50 m downwards to make contact with the water table in semi-arid systems (Lepage et al. 1974). In the humid tropics, termite activity is generally concentrated in the top 20 cm, which may be riddled with galleries. It is estimated that termite chambers occupy 2% of the total soil volume (Whitford et al. 1991), but perhaps a more striking demonstration of the impact of termites is the observation that almost any core, monolith or even spade-full of soil dug in large areas of the tropics (below 2000 m altitude) will reveal some evidence of termite activity.

8.8.2 Bulk Density and Structural Stability of Mound Materials

It is a matter of common observation that termites pack soil to form hard outer layerings for their mounds, with bulk densities ranging (in different species) from 1.0–2.0 $Mg\,m^{-3}$, in almost all cases significantly higher than any part of the soil profile from which the mounds are constructed. The hardness of mounds inhibits plant growth, as well as excluding invertebrate predators and rainwater, but in some larger mounds a mound-specific flora may eventually become established (Wood 1996). Inner materials may be softer, or even wholly organic (e.g. *Coptotermes acinaciformis*), such that once the hard outer layer is breached, erosion may proceed more rapidly. In soils, stability (i.e. resistance to dispersion by wetting) is usually a function of the organic (largely polysaccharide and glycoprotein) content of the micro- and macro-aggregates, and of the availability of multivalent cations. Both these stability-promoting agents are generally enhanced in termite

mound materials, even where faeces are not used to any great extent in the construction. In the latter case, salivary secretions are the probable source of the organic cement (Gillman et al. 1972). The selection of clay sub-soils probably accounts for the elevated cations (Wood 1996). It is not known whether stability-promoting ingredients of mound materials retain this function in the soil-column after erosion. The more widely accepted view is that termites contribute to the stability of non-mound soil through their role as engineers sensu stricto, i.e. by mixing organic and mineral material in the course of active subterranean feeding and construction (Lavelle et al. 1997; see above).

8.8.3 Permeability to Water

Experimental evidence from exclusion plots suggests that removing termites (by insecticide application) reduces infiltration rates significantly, but the effect takes some years to appear (Elkins et al. 1986; Holt et al. 1996). Hydraulic conductivity is also reduced. The most obvious explanation is that the loss of termite galleries reduces bulk flow. This is supported by the demonstration that the recolonisation of bare ground by termites, following mulching, is accompanied by an increase in water infiltration rates by up to six times (Mando et al. 1996; Mando 1997; Mando and Miedema 1997). In contrast, hard mound materials have very low ponded infiltration rates, but resistance of mound materials to water ingress is not universal. Some termite mounds, especially those of soil feeders or highly decayed abandoned mounds, are porous and show higher hydraulic conductivity than adjacent surface soil (Garnier-Sillam et al. 1988; Martius 1994b). This is hard to rationalise for occupied mounds: it may be that one of the soil-feeding species in question, *Thoracotermes macrothorax*, has an unusual biology and is polycalic, with a tendency to abandon their characteristic pillar-shaped mounds and move elsewhere whenever disturbed (D. Bignell and P. Eggleton, unpublished observations). In this case, a hard mound, which must be energetically expensive to produce in comparison with a relatively uncompacted structure, may not be necessary. Soil-feeders seem the most fragile and cryptic of creatures: this should make us more than ever zealous for their conservation. Occasionally, in African lowland humid forests, one observes large columns of soil-feeders on the surface of the ground, apparently leaving one mound to seek another (this author has seen the phenomenon twice in 20 years). The attempted migration can also be seen in colonies forced into laboratory culture, even when appropriate temperatures and moisture conditions are supplied. The behaviour may be an important, but as yet under-appreciated, facet of soil-feeder biology.

8.8.4 Soil Chemistry

Changes in the composition of mound materials, compared with parent soils, have been outlined above, and are reviewed in Holt and Lepage (2000). To recap, the notable contribution of termites is the incorporation of cation-rich clay subsoils, together with organic-rich faecal and salivary secretions, into nutrient-rich constructions which are subsequently eroded, placing relatively beneficial material at the soil surface. However, it is also worth noting that differences in the $\delta\,^{13}C$ signatures of mound soil in different litter-feeding species are consistent with the known diet, i.e. whether predominantly C3 (wood), C4 (grass) or mixed feeders (Spain and Redell 1986). This supports the contention that termites are not only important agents of direct C mineralisation, but also generate the long-lived pools of C in soil organic matter.

Data from Australian savannah systems strongly suggest that nitrogen in termite mound materials is an important input into the ecosystem N budget (Holt and Lepage 2000). Part of the N (the larger part) is organic, and part is inorganic, as NH_4^+ and NO_3^-. The inorganic component is the more significant, as this is immediately available to plant roots. Budgets for savannah woodlands suggested that the annual rate of leaching of inorganic N from termite mounds to the soil was approximately 16% of the standing stock of soil inorganic nitrogen (Congdon et al. 1993). This N may come from the slow microbially mediated degradation of lignoproteins in termite excreta, but it might also be enhanced by direct fixation by the gut microbiota (Anderson 1994). The termite mound can therefore be seen as a bottleneck for N release, as the redistribution of N to the non-mound soil is dependent on mound erosion rates and leaching (Bonell et al. 1986). By contrast, the available nutrients stored in temporary termite runways and sheetings may be reapplied to the soil in days (Mackay and Whitford 1988). Overall, the evidence that significant amounts of available N can be leached from termite mounds is an interesting counterpoint to the argument that the growth of termites is N-limited, and that N-fixation is therefore the sine qua non of their mutualisms with microorganisms.

8.8.5 Organic Matter Decomposition

Free-living bacteria and fungi are abundant in termite mound materials (reviewed by Holt and Lepage 2000; see also Brauman et al. 2000 and Brauman 2001). Their growth can be attributed to higher moisture levels and substrate availability, the latter derived from faeces and salivary secretions, with possible additions from partially digested plant structural

polysaccharides added as cements (see above). The microbiotas isolated from mound materials include lignin, cellulose and xylan decomposers, denitrifiers, ammonifiers, nitrifiers and methylotrophs. Further, they appear to have greater biomass density than in adjacent parent soils (Arshad 1981). This assemblage embraces most of the functional groups believed to be required for efficient mineralisation of C and N, and termite mounds may therefore be important foci for accelerated organic decomposition.

The recent literature proposes a critical role for termites in decomposition processes in the tropics (e.g. Wood and Sands 1978; Bignell et al. 1997; Bignell and Eggleton 2000), but most of the relevant quantitative evidence has been obtained either in African savannahs dominated by Macrotermitinae or in Australian parklands, where mounds and subterranean nests are the major population centres, and foraging of organic detritus, which is subsequently composted within the colony system, is the most obvious feature of termite biology. In these systems, severe dry seasons are a major constraint on all soil biotas, and drought-adapted termites can perhaps be seen as gate-keepers in the overall mineralisation process. By contrast, the wetter humid forest systems support a wide range of independent decomposer organisms, notably including free-living fungi, isopods, diplopods, earthworms and a variety of mesofaunal groups, in addition to termites (Burghouts et al. 1992). This may intensify competition for organic detritus (Bignell 1994), with selection pressures favouring termite species which can feed lower down the humification gradient, ultimately accounting for the dominance of soil-feeding forms, which incur the least competition in obtaining access to their nutrients.

8.9 Conclusions

Although the main stimulus for field work with termites over the last decade has been the imperative to define and quantify their role in the terrestrial C cycle, the greatest dividend from the research has been the realisation of their beneficial impact of soil physics and chemistry. The conservation of termite populations under conditions where land-use changes are accelerating and natural habitats increasingly disturbed and reduced, is therefore clearly linked to soil fertility and sustainable subsistence agriculture. Growing evidence indicates that termite assemblages are vulnerable at forest margins. For example, along an intensification gradient in Cameroon, the diversity of soil-feeding termites was strongly reduced due to disturbance associated with increasing intensity of cultivation, whereas the diversity of wood-feeding termites was little affected and slightly increased (Eggleton et al. 2002). Amongst the macrofaunal groups monitored

across seven land uses from forest to *Imperata* savannah or cassava fields in eastern Sumatra, termites showed the greatest sensitivity to increasing intensification, their diversity diminishing with the ratio of (aboveground) plant species richness to plant functional types (a measure of the morphological complexity of the plant community, and with woody basal area of trees; Gillison et al. 2003). There was an attendant strong switch from dominance of endogeic to epigeic macrofauna across this disturbance gradient and the bulk density of soil increased, indicating decreases in soil porosity and permeability.

Loss of soil macrofauna biodiversity on conversion of rainforest to pasture (from > 160 species to < 40 species) coupled with the invasion of a compacting earthworm species (*Pontoscolex corethrus*) resulted in extreme problems of degradation in an Amazonian system by preventing water infiltration and plant growth (Chauvel et al. 1999). Barros et al. (2001) showed that the situation is reversible in less than one year through an elegant experiment in which compacted soil monoliths were translocated from the pasture to the forest, and vice versa. In compacted monoliths translocated to the forest, decompacting species of ants and termites dug holes in the casts of the earthworm to maintain their own galleries and accesses to surface. In the Sahelian system, mulching was shown to restore termites to degraded, semi-arid landscapes, with a resulting re-establishment of good water infiltration and increased soil water content and stability (Mando 1997; Holt and Lepage 2000). Termite activity was also found to increase soil porosity, saturated hydraulic conductivity and to reduce bulk density and resistance to cone penetration. While the differences between termite and non-termite treatments was less than 10% in the major parameters, there was a critical improvement in the ability of plant roots to extend through the soil in the rooting zone.

The clearest opportunities that exist for management of below-ground biodiversity are through the manipulation of organic inputs, or through manipulation of the soil physico-chemical environment as indicated above. Macroinvertebrate communities of the well-drained savannahs (Decaëns et al. 2001b) and Andean hillsides (Feijoo et al. 2001) of Colombia were shown to be very sensitive to environmental changes associated with agricultural intensification. In extensively grazed native pastures, earthworms are favoured by grazing but traditional management by burning has the opposite effect on termites. This suggests that the earthworm/termite ratio may be a sensitive indicator of soil health. Whereas introduced forage grasses and legumes and increasing animal production caused a ten-fold increase in soil macrofauna, annual cropping showed a dramatic impact on earthworms and arthropod populations, with marked decreases in biomass, population density and taxonomic richness. We stand on the verge of beginning to understand how termites can be harnessed to the task of promoting

the use of tropical soils to producer staple foodstuffs, while conserving their diversity and abundance as our duty to the future viability of the tropical biosphere.

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9 Cellulose Digestion in the Termite Gut

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9.1 Introduction

Termites are among the most important lignocellulose-digesting insects and possess a great variety of symbiotic microorganisms in their hindguts, including Bacteria, Archaea and Eukarya, i.e. Archaezoa and yeasts (König et al. 2002). In the digestive tract of lower termites cellulose seems to be synergistically degraded by flagellates, bacteria and yeasts (Breznak and Brune 1994; Varma et al. 1994; König et al. 2002) as well as by the termite's own cellulases (Watanabe et al. 1998; Tokuda et al. 1999; Tokuda et al. 2002). Cellulases of termite origin belong to glycosyl hydrolase family 9. Flagellated Archaezoa are unique symbionts in the phylogenetically lower termites, belonging to the Archaezoa of the orders Trichomonadida, Hypermastigida, and Oxymonadida. They are cellulolytic and produce acetate from cellulose for the benefit of their host (Odelson and Breznak 1985). Only three species of the flagellate flora have been obtained in culture: *Trichomitopsis termopsidis* (Yamin 1978, 1980) and *Trichomympha sphaerica* (Yamin 1981) from *Zootermopsis* sp., and *Trichomitus trypanoides* from *Reticulitermes santonensis* (Berchtold et al. 1995) and from *Reticulitermes flavipes* (Huntenburg et al. 1986). The problem of pure cultures restricted the identification and characterization of cellulases of Archaezoan origin, until Ohtoko et al. (2000) obtained diverse genes of protist cellulases of glycosyl hydrolase family 45 in the termite *Reticulitermes speratus* by means of a culture-independent PCR approach.

Cellulose is one of the most abundant renewable biomass on the earth. It is a polymerized form of glucose molecules with β-1,4-linkage, consisting of composite forms of highly crystallized microfibrils among amorphous matrices, therefore creating the very complex and recalcitrant physical structure of cellulose found in plant cell walls (Delmer and Amor 1995). Many cellulolytic systems from a wide array of organisms, including higher flowering plants, Archaezoa, fungi and bacteria, have been studied. In the biological conversion of cellulose to glucose at least three distinct types

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of glycolytic enzymes are involved (Fig. 9.1). Endoglucanases (EG, endo-1,4-β-glucanase, EC 3.2.1.4) randomly hydrolyse 1,4-β bonds of the cellulose chains. Cellobiohydrolases (CBH, exo-1,4-β-glucanase, EC 3.2.1.91) cleave cellobiosyl units from non-reducing ends of the cellulose chains. β-Glucosidases (EC 3.2.1.21) cleave glucosyl units from non-reducing ends of cello-oligosaccharides. Two different systems have been proposed for the action of these components. One is the synergistic action of free components (EG, CBH, and β-Glucosidase), while the other involves cellulosomes

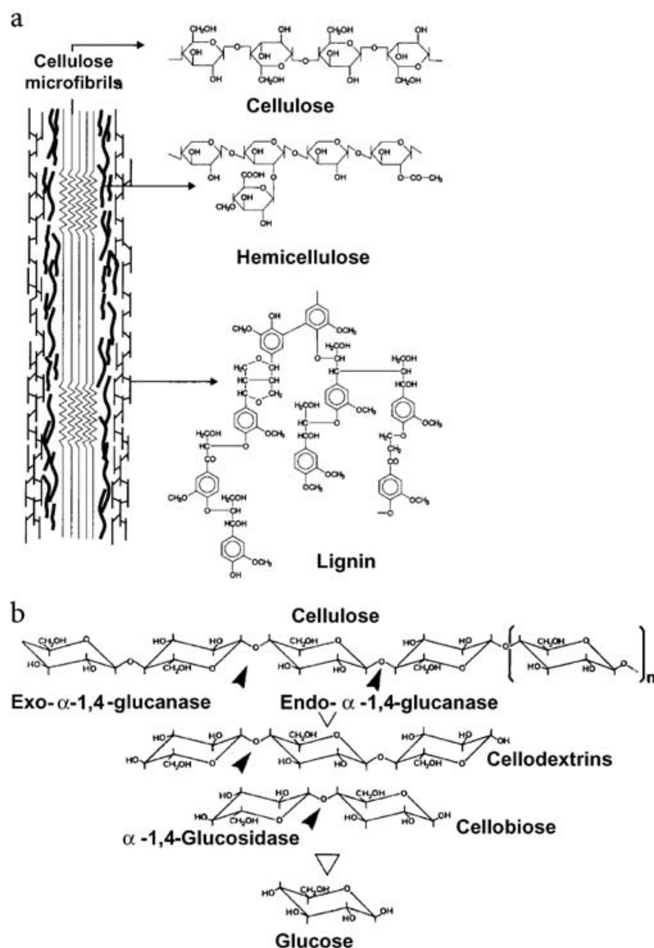

Fig. 9.1. a Major components of woods (modified; Fritsche 1998; copyright with permission from Gustav Fisher Verlag Jena, Jena, and Dr. Frische). **b** Enzymatic cellulose digestion (modified; Malburg et al. 1992; copyright with permission from Wiley-VCH Verlagsgesellschaft mbH, Weinheim and Dr. Malburg)

binding different components into a large structure (Tomme et al. 1995; Béguin and Lemaire 1996; Bayer et al. 1998). Classification of cellulolytic enzymes into EG, CBH, and β-Glucosidase does not reflect the structural features of the enzyme molecules and, thus, conceals their phylogenetic relationships. Therefore, Henrissat et al. (1989) advocated the classification of cellulolytic enzymes by hydrophobic cluster analysis of amino acid sequences, which reflects structural similarities in the grouping. The diverse spectra of cellulases are classified into 12 of the 67 glycosyl hydrolase families based on catalytic domain sequence similarities (Henrissat and Davies 1997; http://www.expasy.ch/cgi-bin/lists?glycosid.txt).

The majority of cellulases so far classified have a catalytic domain linked to a N-terminal or a C-terminal cellulose-binding domain (CBD) by a glycosylated, Pro/Thr/Ser-rich spacer sequence as a common structure. The presence of a CBD apparently enhances the enzymatic activity of the catalytic core against crystalline cellulose (Tomme et al. 1995). All known plant and animal cellulases, except for one from higher plants (Brummell et al. 1997) and two from plant-parasitic nematodes (Smant et al. 1998), do not have linker and CBDs and consist only of a single catalytic domain.

9.2 Termite's Cellulases

Early studies indicated that cellulose-degrading ability was restricted to microbes and plants, and that animals which fed on cellulose, i.e. lower termites were able to digest it only because they harbor cellulolytic bacteria, fungi or Archaezoa in their guts. Later, the presence of both endogenous and archaezoal cellulolytic activities were demonstrated in several termite species (Yokoe 1964; Yamaoka and Nagatani 1975; Botha and Hewitt 1979; O'Brien et al. 1979; Veivers et al. 1982; Hogan et al. 1988). The endogenous secretion of cellulases by termites themselves was first suggested by Yokoe (1964). Yamaoka and Nagatani (1975) demonstrated the presence of endogenous carboxymethylcellulose hydrolysing activity (endo-1,4-β-glucanase) in the salivary glands of *Reticulitermes speratus*, and also showed that the hindgut Archaezoa of this species has the ability to hydrolyse crystalline cellulose (filter paper and cotton). Hogan et al. (1988) reported that the Archaezoan and endogenous cellulolytic components of *Coptotermes lacteus* are chromatographically distinct from each other. Furthermore, two endoglucanase components (41 and 42 kDa) were isolated from the lower termite *Reticulitermes speratus* (Watanabe et al. 1997), and one more (47 kDa) from the Japanese arboreal higher termite *Nasutitermes takasagoensis* (Tokuda et al. 1997). A β-glucosidase component was also eluted from the whole body extract of the termite *Reticulitermes speratus*, which was chromatographically different from the β-glucosidase

components of the hindgut extract (Watanabe et al. 1997). Subsequently, several endogenous endoglucanase genes were cloned and identified in termites (Watanabe et al. 1998; Tokuda et al. 1999). Moreover, cellulolytic proteins have also been reported in preparations from the digestive organs of other insects in particular cockroaches (Scrivener and Slaytor 1994), woodlice (Zimmer and Topp 1998) as well as molluscs (Stark and Walker 1983) and gastropods (James et al. 1997). It appears now that cellulase is an invertebrate enzyme, as proposed by Yokoe and Yasumasu (1964), and that the distribution of cellulase among animals is closely linked to their phylogenetic relationships.

Molecular analyses revealed that the endogenous endoglucanases are members of the glycosyl hydrolase family 9 (GHF9) (Watanabe et al. 1998; Tokuda et al. 1999; Nakashima et al. 2002a), which is the most taxonomically diverse family, containing cellulases from plants, bacteria, slime molds, but not from fungi. Other GHF9 members related to those of termites have been found in cockroaches and crayfishes, suggesting the presence of an ancestral enzyme before the symbiotic relationship with gut protists was established in termites (Lo et al. 2000). Indeed, wood-feeding species of higher termites decompose cellulose efficiently despite the absence of gut protists. All known termite cellulases appear to be single domain enzymes. Such a structure is found in most plant cellulases characterized to date, but is less common among microbial cellulases, which usually contain linker and CBDs.

The three-dimensioned structure of the endoglucanase NtEg1 from the higher termite *Nasutitermes takasagoensis* was investigated (Khademi et al. 2002). NtEg1 has the general folding of an $(\alpha/\alpha)_6$ barrel, which is a common folding pattern of GHF9. Glycosyl hydrolases cleave the sugar chain with inversion of configuration at the anomeric C-atom. The mechanism requires an acid to protonate the glycosidic O atom and a base to extract a proton from a nucleophilic water molecule that subsequently attacks the anomeric C-atom. Three-dimensional structural analysis showed that the conserved Glu412 is the catalytic acid residue and the conserved Asp54 or Asp57 is the base. The enzyme has a Ca^{2+}-binding site near its substrate-binding cleft.

By RT-PCR and in situ hybridization, cellulase expression in termites has been localized. Endoglucanase expression in higher termites (*Nasutitermes takasagoensis* and *Nasutitermes walkeri*) has so far only been detected in the midgut, while EGase cDNAs from the lower termites (*Reticulitermes speratus*, *Neotermes koshunensis*, *Hodotermopsis japonica* and *Mastotermes darwiniensis*) were amplified only from salivary gland tissue and not from midgut tissue (Watanabe et al. 1998; Tokuda et al. 1999; Lo et al. 2000; Li et al. 2003). Since these lower termites are known to be relatively primitive lineages of termites, it appears that EGase expression primarily in the salivary glands of termites is the ancestral condition.

9.3 Microbial Cellulases in the Hindgut

As well as the study of endogenous termite cellulase, much research has also been devoted to demonstrating symbiotic cellulase production in particular by Archaezoa, in addition to bacteria, yeasts and fungi, which are harboured in the digestive systems of termites. These findings arise a question as to the relative contribution of specific gut organisms versus the termite's own enzymes to the digestion process. Higher termites decompose cellulose efficiently without gut protists. It is believed that their gut bacteria may play only a little role in the breakdown of cellulose in the termite hindgut and there is no direct evidence that they are of quantitative importance in cellulose digestion (Breznak 1982; O'Brien and Slaytor 1982; Slaytor 1992). After a critical evaluation of the obtained data on cellulose digestion in termites and cockroaches, Slaytor (1992) came to the conclusion that cellulose is mainly hydrolysed in these insects by endogenous cellulases. Watanabe et al. (1997) reported that the eluted peaks of endoglucanases from salivary glands of the termite *Reticulitermes speratus* and from hindgut extracts were chromatographically identical. The cellulase activity from hindgut extracts is likely to have originated from upper regions of the intestinal lumen. On the other hand, many cellulolytic microorganisms (Bacteria, Archaezoa, yeasts, and filamentous fungi) were isolated from termites (cf. König et al. 2002; Wenzel et al. 2002; Bakalidou et al. 2002). Moreover, in lower termites, substantial activity particularly against crystalline cellulose is found in the hindgut (Yamaoka and Nagatani 1975; Inoue et al. 1997; Itakura et al. 1997). Recently, several cellulase genes of Archaezoan origin were also isolated and identified (Ohtoko et al. 2000; Nakashima et al. 2002b; Watanabe et al. 2002). Because of the lack of pure cultures of the gut symbionts in particular flagellated protists, it is difficult to distinguish qualitatively or quantitatively between endogenous and symbiotic cellulolytic activities. So, the extent to which these microorganisms are actually involved in cellulose degradation in situ remains to be elucidated. Nevertheless, up to date it is widely believed that a dual system exists in lower termites to achieve the assimilation of wood glucan to an extent greater than 90% (Breznak and Brune 1994; Watanabe et al. 1998; Nakashima et al. 2002a).

The unique flagellated protists in the hindgut of lower termites are the main target microorganisms in the study on cellulose digestion in termites, because they ingest wood particles and exhibit significant cellulase activity (Table 9.1). However, cellulases of protist origin have rarely been analysed at the molecular level due to the difficulty of getting pure cultures. Consequently, there have been relatively few biochemical investigations. Due to the development of culture-independent approaches involving PCR

Table 9.1. Glycolytic and laccase activities of *Mixotricha paradoxa* and the hypermastigids (µU per cell) from the termite *Mastotermes darwiniensis*. (Berchtold and König, unpubl.)

Enzyme	*M. paradoxa*	Hypermastigids
α–L-Arabinosidase	0.3	0.2
β–L-Arabinosidase	–	–
β–D-Cellobiosidase	1.2	0.7
α–D-Galactosidase	0.2	0.2
β–D-Galactosidase	–	–
β–D-Glucosidase	4.0	5.9
β–D-Glucuronidase	–	–
α–D-Mannosidase	–	–
β–D-Mannosidase	0.2	0.4
β–D-Xylosidase	0.5	0.3
Cellulase	169.0	164.0
Xylanase	135.0	98.0
Laccase (ABTS)[a]	0.001	0.001

–, Not found
[a] 2,2-Azinobis-3-ethylbenzthiazolinesulfonic acid

amplification of a certain gene and identification of the origin of the gene by in situ hybridization, studying the Archaezoan cellulases has recently become possible. Moreover, according to the hydrophobic cluster analysis-based cellulase family classification of Henrissat (1989), it was possible to design degenerate primers that encoded amino acid sequences conserved in an intra-family (but not inter-family) manner for PCR amplification of the genes coding for specific cellulase family members (Sheppard et al. 1994). Based on this strategy, genes encoding cellulase homologs belonging to the GHF45 family were firstly isolated from gut protists of the termite *Reticulitermes speratus* (Fig. 9.2; Ohtoko et al. 2000). Some of these were identified as originating from the hypermastigotes *Teranympha mirabilis* and *Trichonympha agilis*.

After direct N′-terminal sequencing of an active band of cellulase exhibited in the hindgut extract, which was loaded on native polyacrylamide gel followed by Congo red staining (activity analysis), a degenerate primer was designed to clone successfully a cellulase gene PgCBH-homo from the hypermastigote *Pseudotrichonympha grassii* in the termite *Coptotermes formosanus*, which was considered to belong to the CBH group of GHF7 based on structure analysis and phylogenetic analysis (Nakashima et al. 2002b). All GHF7 members previously reported originated from fungi. Meanwhile, an N′-terminal sequence was obtained from a cellulase component with CMC activity crudely isolated from the hindgut extract of

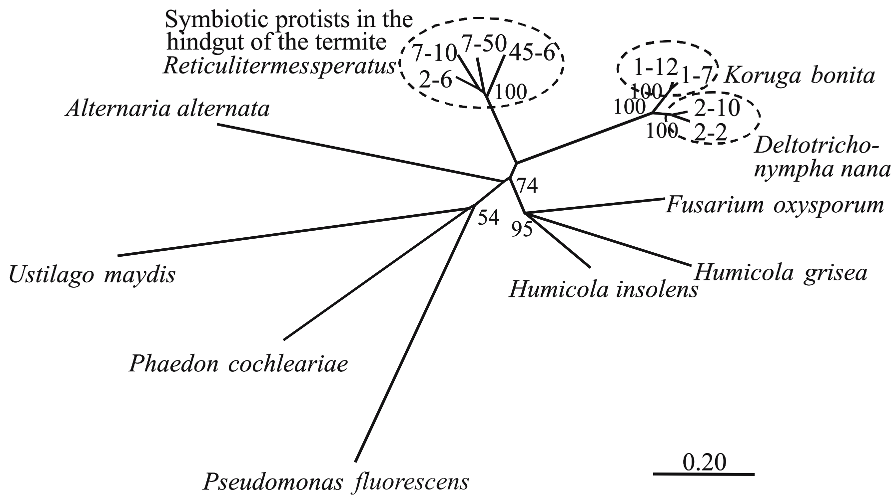

Fig. 9.2. Phylogenetic relationship among members of GHF45. (Li et al. 2003)

the termite *Coptotermes lacteus* by gel filtration. The designed degenerate primer based on the sequence resulted in cellulase genes belonging to GHF7, which were isolated from the hypermastigotes *Pseudotrichonympha grassii* and *Holomastigotoides mirabile* from the guts of the termites *Coptotermes formosanus* and *Coptotermes lacteus.* Phylogenetic analysis of these endoglucanases, fungal EGs and CBHs form distinctive clades (Watanabe et al. 2002). GHF 45 members or their cDNAs could not be found in the hindgut of either C. *lacteus* or C. *formosanus.* Heterologous expression of the cellulase genes of GHF7 in *Escherichia coli* showed CMC activity (Nakashima et al. 2002b; Watanabe et al. 2002).

The cellulases of both termite origin (belonging to GHF 9) and protist origin (belonging to GHF 7 or GHF 45) consist of a single catalytic domain and lack the ancillary domains such as cellulose-binding domains found in most microbial cellulases. As discussed by Watanabe and Tokuda (2001), termites grind and crunch their ingested material, which may enhance digestion by increasing the amount of surface that can be accessed by the cellulolytic enzymes. Heterologous expression of the cellulase genes, particularly those of protist origin, may help to characterize their encoded enzymatic properties. Whether any synergistic cooperation among the termite and microbial cellulases is present or not should be addressed in order to understand efficient cellulose decomposition by termite symbiotic systems.

9.4 Cellulose Digestion in the Termite *Mastotermes darwiniensis*

9.4.1 Termites' and Flagellates' Cellulases

Up to now it has been believed that the hindgut flagellates produce nutrients using their own cellulolytic enzymes for the benefit of their termite host. However, two endoglucanases Cel I and Cel II, with the molecular mass of approx. 48 kD, were isolated from the not yet culturable symbiotic Archaezoa living in the hindgut of the most primitive Australian termite *Mastotermes darwiniensis* (Li et al. 2003). The N-terminal sequences of these cellulases exhibited significant homology to cellulases of termite origin, which belong to glycosyl hydrolase family 9. The corresponding genes were detected not in the mRNA pool of the flagellates, but in the salivary glands of *Mastotermes darwiniensis*. A protein with the molecular mass of approx. 48 kD was also detected in crude extract of these Archaezoa, by Western blot analysis using a polyclonal antiserum against the cellulase of the termite *Mastotermes darwiniensis*. The results gave evidence that cellulases isolated from the nutritive vacuole of the flagellates originated from the termite host. Probably, the cellulases are secreted from the salivary glands of *Mastotermes darwiniensis*. During the mechanical grinding of the wood particles by the termites, the cellulases are attached to wood particles or mixed with them, then the attached cellulases or the mixture move to the hindgut where they are most probably endocytosed by the flagellates.

It has been shown for *Coptotermes formosanus* that the endoglucanases of this termite are restricted to the salivary glands, the foregut, and the midgut (Nakashima et al. 2002a). According to our work, the main endoglucanase activity found in cells of the hindgut flagellates of *Mastotermes darwiniensis* is likely to originate from the termite's cellulases. It has also been found that 40% of the endoglucanase activity of *Mastotermes darwiniensis* is present in the hindgut and most (ca. 84%) of the cellulase activity of the whole hindgut is present in the flagellate extract (Veivers et al. 1982). This implies that a certain amount of termite cellulases, secreted from the salivary glands, moves into the hindgut and enters the flagellate cells. They may be involved in the digestion of cellulose in the flagellate cells.

Using a PCR-based approach DNA encoding cellulases belonging to glycosyl hydrolase family 45 were obtained from micromanipulated nuclei of the flagellates *Koruga bonita* (clone1–7 and 1–12) and *Deltotrichonympha nana* (clone2–2 and 2–10). The cellulase sequences of the termite sym-

biotic protists were phylogenetically monophyletic, showing more than 84% amino acid identity with each other (Fig. 9.2). The deduced cellulase sequences of termite origin and flagellate origin consist of a single catalytic domain, lacking a cellulose-binding domain (CBD) and a spacer sequence found in most microbial cellulases.

Although flagellate endoglucanase genes were even expressed in vivo, significant cellulase activity of flagellate origin was not found in the nutritive vacuole by SDS-PAGE. The flagellate cellulase proteins were not detected by western blot analysis. This implied that the native endoglucanase of flagellate origin has very low or even no CMC activity and the translation efficiency could also be very low.

It is conceivable that in the course of 200∼300 million years, the symbiosis between termite and initially free-living Archaezoa mutually affected the enzymatic equipment of the other, such that the excess of termite cellulases led to a disuse of the flagellates' own enzymes. The lack of a selection pressure in the hindgut possibly directed low level translation, mutation, and inactivation of cellulolytic enzymes, from which the corresponding genes are still expressed. The production of an inactive enzyme may result in a complete loss of the corresponding genes. This means that the symbiotic Archaezoa are progressing to a state void of their own cellulolytic activities as was probably the case before the existence of cellulose-containing plants (though some cellulose-producing microorganisms, e.g. *Acetobacter*, appeared earlier than plants, the amount of the microbial cellulose should be much less than that of plants). Presently, the symbiotic gut Archaezoa of the primitive Australian termite *Mastotermes darwiniensis* owe their endocellulolytic activity to their host.

9.4.2 Comparison of Termite's Cellulases

Five full-length cDNAs of termite endoglucanases were cloned from the salivary glands of the termite *Mastotermes darwiniensis* (Li et al. 2003). The first group (MD5 and MD13) and the second one (MD1, MD6, and MD11) shared 87% amino acid similarity on average. These results indicated that the termite *Mastotermes darwiniensis* contained at least two endoglucanase components, which was also demonstrated by western blotting analysis. Many endoglucanases consist of multiple components, such as YEG1 and YEG2 from the termite *Reticulitermes speratus* (Watanabe et al. 1997), EG1 and EG2 from the cockroach *Panesthia cribrata* (Scrivener and Slaytor 1994), three components from the fungus *Aspergilllus japonicus* (Kundu et al. 1988), and three (Okada 1975, 1976) or four components (Kim et al. 1994) from the fungus *Trichoderma viride*.

The cDNAs of endogenous endoglucanases have been amplified from the salivary glands of the termite *Reticulitermes speratus* and the termite *Coptotermes formosanus*. Two cDNAs (*RsEG1* and *RsEG2*) from the termite *Reticulitermes speratus* (BAA34050 and BAA31326, respectively) showed 98% similarity at the amino acid level. Four cDNAs from the termite *Coptotermes formosanus* (BAB40693, BAB40695, BAB40696, BAB40697) shared more than 99% identity at the amino acid level. However, the cDNAs from the termite *Mastotermes darwiniensis*, i.e. MD6 and MD13 (CAD54727 and CAD54729, respectively), had 84% amino acid similarity, which could cause obvious changes in catalytic function of the respective enzymes (Li et al. 2003).

The cDNAs from the termite *Reticulitermes speratus* or from the termite *Coptotermes formosanus* with high amino acid similarity could be the result of a recent gene duplication or be different versions of alleles at the same locus (Tokuda et al. 1999). Clone MD5 and MD13 or clone MD1 and MD11 appeared possibly due to such a mechanisms. However, 84% amino acid similarity between MD13 and MD6 could not be explained. Similarly, the EG1 and EG2 cDNAs from the cockroach *Panesthia cribrata* (AAF80584 and AAF 80585) shared 76% amino acid identity with each other.

The deduced endoglucanases of the termite *Mastotermes darwiniensis* consist only of a single catalytic domain, do not have linker and cellulase binding domain (CBD), which is the common feature of termite cellulases. The presence of a CBD in cellulase is thought to enhance activity towards crystalline cellulose. Moreover, no endogenous cellobiohydrolase of termite's origin has been reported until now, the activity of which is known to attack the crystalline region of natural cellulose and produce cellobiose. The absence of CBH and CBD region in EG might be explained by the presence of mandibles that physically grind wood into small particles, i.e. into less than 50 µm in size in *Coptotermes formosanus* (Itakura et al. 1995) or into the size ranges (10–30 and 100–300 µm) in *Nasutitermes takasagoensis* (Yoshimura et al. 1996). This may aid digestion by widening the surface areas of the food debris into substrates that the cellulase compounds can then easily attack.

The evolutionary origin of cellulases in termite is intriguing. Although termite cellulase genes exhibit marked similarity to bacterial and protist members of GHF9, the fact that termite cellulase genes contain introns (Watanabe et al. 1998; Tokuda et al. 1999) makes the prospect of recent horizontal gene transfer from these species unlikely. Therefore, based on the high identity between termite and cockroach endoglucanases, Watanabe et al. (1998) supposed that an ancestral GHF9-like cellulase gene was present before the divergence of cockroaches and termites, an estimated 250 million years ago. Afterwards, with the aim of settling the debate over the evolutionary origin of termite, Lo et al. (2000) determined the sequences

of genes encoding 18S ribosomal RNA, mitochondrial cytochrome oxidase subunit II (COII) and endogenous endo-β-1,4-glucanase (EG) from a diverse range of dictyopterans including termites, cockroaches and praying mantids. Maximum parsimony and likelihood analysis of these sequences revealed strong support for a clade consisting of termites and subsocial wood-feeding cockroaches of the genus *Cryptocercus*. This clade is nested within a larger cockroach clade. Their work indicated that the endoglucanases of termite could evolve from those of wood-feeding cockroaches (Fig. 9.3).

For characterization of the salivary endoglucanases of *Mastotermes darwiniensis*, one of the endoglucanases cDNAs cloned in our study, MD13 was expressed in *E. coli* and purified. Up to date, three endoglucanase components have been purified from termites and characterized, two (YEG1 and YEG2, denoted as RsEGs in the following discussion) from the lower termites *Reticulitermes speratus* (Watanabe et al. 1997) and one more (47 kD, denoted as NtEG) from the Japanese arboreal higher termite *Nasutitermes takasagoensis* (Tokuda et al. 1997).

The properties of MD13 were similar to the endoglucanases components from *Reticulitermes speratus* and *Nasutitermes takasagoensis* (Table 9.2). The analysis of temperature effect indicated that MD13 was more stable than

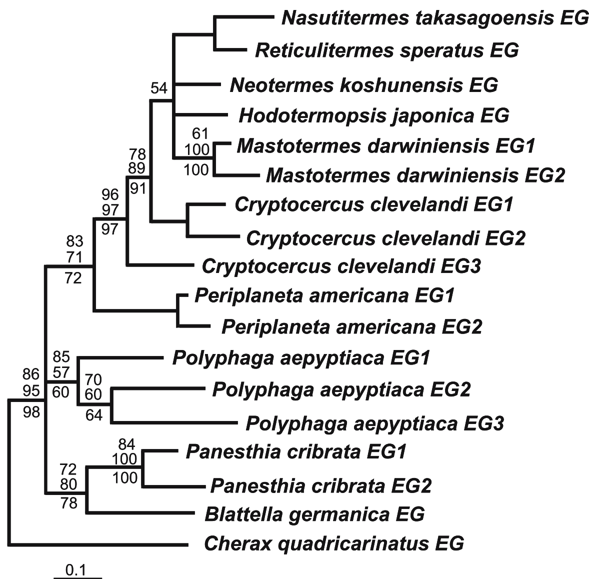

Fig. 9.3. Phylogeny of endo-β-1,4-glucanase genes from termites, cockroaches and a crustacean. Trees were rooted with non-arthropod glycosyl hydrolase family 9 sequences (modified; Lo et al. 2000; copyright with permission from Elsevier, Oxford, UK, and Dr. Lo)

Table 9.2. Comparison of enzymatic properties among purified endo-β-1, 4-glucanase components from various organisms

Species	MW (kDa)	pH^{a}_{opt}	T^{b}_{opt} (°C)	T^{c}_{max} (°C)	K_m (mg/ml)	V_{max} (U/mg)	DS[d]
Higher termites							
Nasutitermes takasagoensis[e]	47	5.80	65	~60	8.70	2222	0.70
Macrotermes mülleri[f]							
Cellulase I_T	34	4.40	55	~55	7.50	n.d.	0.71
Cellulase II	52	4.40	37	~42	1.00	n.d.	0.71
Lower termites							
Mastotermes darwiniensis[t]							
MD13		6.00	55	~50	43.40	950	0.70
Reticulitermes speratus[g]							
YEG1	42	6.0	50	~40	1.83	527	0.55–0.65
YEG2	41	6.0	50	~40	1.48	540	0.55–0.65
Cockroach							
Panesthia cribrata[h]							
EG1	53.6	n.d.	n.d.	n.d.	9.40	123.20	n.d.
EG2	48.8	n.d.	n.d.	n.d.	6.80	490.10	n.d.
Longicorn beetle							
Ergates taber[i]							
Cellulase A	25	4.00–4.70	n.d.	~ 60	20	n.d.	0.71
Abalone							
Haliotis discus hannai[j]							
HdEG66	66	6.3	38	30	n.d.	n.d.	n.d.
Blue mussel							
Mytilus edulis[k]	20	4.6	30–50	100 (10 min)	n.d.	n.d.	n.d.
Fungi							
Aspergillus niger[l]	31	4.0	45–50	n.d.	0.86	n.d.	0.62–0.64
Tricoderma viride[m]							
Cellulase IIA	30	5.0	60	~ 60	0.81	n.d.	0.62–0.64
Cellulase IIB	43	5.0	50	~ 50	0.96	n.d.	0.62–0.64
Cellulase III	45	5.0	50	n.d.	0.54	n.d.	0.62–0.64
Humicola grisea[n]							
EGL3	n.d.	5.0	60	80 (10 min)	n.d.	n.d.	n.d.
EGL4	n.d.	6.0	75	80 (10 min)	n.d.	n.d.	n.d.
Robillarda sp.$Y-20$[o]							
CMCase I	56	5.0	60	~50	0.60	n.d.	0.51
CMCase II	59	4.0–5.0	55	~50	n.d.	n.d.	n.d.

Table 9.2. (continued)

Species	MW (kDa)	pH^{a}_{opt}	T^{b}_{opt} (°C)	T^{c}_{max} (°C)	K_m (mg/ml)	V_{max} (U/mg)	DS[d]
Bacteria							
Neisseria sicca SB[p]	41	5.0	60	40	12.8	22.8	0.6–0.7
Thermomonospora fusca[q]							
E1	94	6.0	74	n.d.	0.36	n.d.	n.d.
E2	46	6.0	58	n.d.	0.12	n.d.	n.d.
Clostridium thermocellum[r]	83–94	5.2	62	n.d.	n.d.	n.d.	n.d.
51-kDa subunit of cellulosome of *C. thermocellum*[s]	51	5.0	60	n.d.	n.d.	n.d.	n.d.

MD13 from *Mastotermes darwiniensis* and EGL3/EGL4 from *Humicola grisea* are heterologous expressed endoglucanases. n.d. = Not determined. One unit is the amount of enzyme which produced 1 μmol of reducing sugar (glucose equivalents)/min. Each endo-β-1,4-glucanase activity was measured at 37 °C unless otherwise indicated

[a] Optimal pH value
[b] Optimal temperature
[c] Maximal temperature for enzyme stability
[d] Degree of substitution for used CMC
[e] Tokuda et al. (1997)
[f] Rouland et al. (1988). CellulaseI_T was purified from whole termites but was supposed to be originated from fungus
[g] Watanabe et al. (1997)
[h] Scrivener and Slaytor (1994). Activity was measured at 40 °C. Original values were 1850 and 3440 units/mg (units were mg reducing sugar/h) for specific activities of EG1 and EG2, respectively. V_{max} values were 22.2 and 88.3 units/mg (units were mg reducing sugar/h) for EG1 and EG2, respectively
[i] Chararas et al. (1983)
[j] Suzuki et al. (2003). Activity was measured at 30 °C
[k] Xu et al. (2000). Activity was measured at 40 °C
[l] Okada (1985). Activity was measured at 30 °C
[m] Okada (1975, 1976). Activity was measured at 30 °C
[n] Takashima et al. (1999)
[o] Yoshigi et al. (1988)
[p] Moriyoshi et al. (2002). Activity was measured at 30 °C
[q] Calza et al. (1985). Activity was measured at 56 °C
[r] Ng and Zeikus (1981)
[s] Mori (1992). Activity was measured at 60 °C
[t] Activity was measured at 55 °C

RsEGs, NtEG more stable than MD13. The properties of termite EGs were also similar to the EG components of most fungi and bacteria. The weakly acidic optimal pH is therefore comparable to that of EG components from the microorganisms listed in Table 9.2. The optimal and stable temperatures were also comparable to fungal endoglucanases, like those from *Trichodema viride* (Okada 1975; Kundu et al. 1988; Yoshigi et al. 1988).

In kinetic analysis, due to the different CMC sources and the different conditions in activity determination (i.e. temperature, pH), the data of K_m and V_{max} from different termites are not comparable. Since CMC has varied in degree of polymerization and substitution among lots of supplies, enzyme affinity and reaction velocity are affected by the degree of them (Tokuda et al. 1997). As the K_m value decreases with higher polymerization degree of substrate, the purified endoglucanase is considered to increase affinity to the substrate when the polymerization degree becomes higher. The V_{max} values indicate that specification also becomes higher when the polymerization degree increases. That means that endo-1,4-glucanases activity depends on the length of cellulase chains (Tokuda et al. 1997). RsEGs showed K_m values of 1.83/1.48 mg/ml with CMC from Kokusan Chemical (degree of polymerization: 500–700; degree of carboxymethyl substitution: 0.55–0.65; average molecular weight ca. 100,000, used concentration 1–19 mg/ml). The K_m value for NtEG was 8.7 mg/ml with CMC from Aldrich (degree of carboxymethyl substitution: 0.7; standard molecular weight: 250,000, used concentration 1−40 mg/ml). Cellulase MD13 had an apparent K_m value of 43.4 mg/ml with CMC from Aldrich (degree of carboxymethyl substitution: 0.7, average molecular mass ca. 90,000, used concentration 10–40 mg/ml). One possible reason is that the CMC from Aldrich with molecular mass ca. 90,000 could not be the optimal substrate for MD13 because the length of the cellulase chain used for kinetic analysis of cellulase MD13 was smaller than those for NtEG. Secondly, the expressed endocellulase MD13 (with N′-terminal heterogeneous 21 amino acids) had a different 2D or 3D structure compared to native protein leading to a lower affinity to CMC than native protein. Kwon et al. (1999) had reported that an endoglucanase EGI expressed in *E. coli* formed inclusion bodies and after refolding the expressed enzyme had 67.8% specific activity compared to native EGI.

Termite endoglucanases hydrolysed not only CMC but also crystalline cellulose. But the activity against crystalline cellulose (Sigmacell type 20) was very low (Veivers et al. 1982; Schulz et al. 1986; Watanabe et al. 1997; Nakashima et al. 2002a). The hydrolysed products of Sigmacell type 20 from different termites were also similar. According to previous study, the salivary endoglucanase from *Mastotermes darwiniensis* produced cellodextrin, cellobiose and a very small amount of glucose (Veivers et al. 1982). RsEGs hydrolysed crystalline cellulose to cellobiose and a trace amount of

glucose. The hydrolytic products from crystalline cellulose by *Nasutitermes walkeri* (Schulz et al. 1986) were cellobiose, cellotriose and a small amount of glucose. EG1 and EG2 from cockroach *Panesthia cribrata* produced also cellobiose and cellotriose from Sigmacell type 20, but no glucose (Scrivener and Slayter 1994). It seems that these cellulase components mentioned above are typical endo-β-1,4-glucanases which are less active against crystalline cellulose.

9.4.3 Comparison of Archaezoan Cellulases

cDNAs of Archaezoan cellulases GHF45 were amplified from the hindguts of the termite *Mastotermes darwiniensis* and were proved to originate from large hypermastigotes *Koruga bonita* and *Deltotrichonympha nana* (Li et al. 2003). Diverse genes (cDNAs) encoding cellulase homologues belonging to GHF45 were also identified from the symbiotic protists in the hindgut of the termite *Reticulitermes speratus*, some of which were attributed to the large hypermastigote protists *Teranympha mirabilis* and *Trichonympha agilis* (Ohtoko et al. 2000). Recently, several Archaezoan endoglucanase cDNAs of GHF7 have been reported (Watanabe et al. 2002). They were amplified from the large hypermastigotes *Pseudotrichonympha grassii* and *Holomastigotoides mirabile* from two species of *Coptotermes* (*C. formosanus and C. lacteus*). The cDNAs of GHF45 members could not be found in the hindgut of either *C. lacteus* or *C. formosanus*. It might be explained by the difference in Archaezoan fauna between termites. *Teranympha mirabilis* and *Trichonympha agilis* are absent in the hindgut of *Coptotermes* species.

The cDNA of Archaezoan cellulase isolated from the flagellates of the termite *Mastotermes darwiniensis*, clone 2–2, was expressed in *E. coli* in a soluble form but without enzyme activity (Li et al. 2003). Several endoglucanases of GHF45 have been successfully characterized. These enzymes were either purified from the organisms to homogeneity or expressed in eukaryotic systems (fungi or yeasts) in an active form. For example, two endogluanases EGL3 and EGL4 from *Humicola grisea* were expressed in *Aspergillus orzyae* with relatively high activity toward CMC (20.7 and 8.06 U/mg, respectively; Takashima et al. 1999). An endoglucanases gene (*eg1*) from *Scopulariopsis brevicaulis* was expressed in yeast *Saccharomyces cerevisiae*. The activity against CMC in the culture supernatant (109 U/l) was about 163-fold higher than the activity in the culture supernatant of *Scopulariopsis brevicaulis* (Nakatani et al. 2000).

In the known members of GHF45, EGL4 from *Humicola grisea*, EGI from *Scopulariopsis brevicaulis* and an endoglucanase from blue mussel *Mytilus edulis* have no CBD and spacer like the Archaezoan endoglucanases in ter-

mites. This coincides with the fact that they showed activity towards CMC and nearly no activity towards Avicel (Takashima et al. 1999; Nakatani et al. 2000; Xu et al. 2001), while the GHF45 members containing CBD, i.e. Kfam1 of the fungus *Fusarium oxysporum*, EGV of the fungus *Humicola insolens*, EGI of the fungus *Ustilago maydis* and EGB of the bacterium *Pseudomonas fluorescens*, showed activity towards insoluble cellulose substrates, crystalline cellulose such as Avicel, and amorphous cellulose such as phosphate-swollen cellulose (Nakatani et al. 2000).

Several reported GHF45 members showed high temperature stability. EGL3 and EGL4 from *Humicola grisea* retained more than 75% of the relative activity against heating up to 80 °C for 10 min (Takashima et al. 1999). Their optimal temperatures were also relatively high, 60 and 75 °C, respectively. One more endoglucanase from blue mussel *Mytilus edulis*, can withstand 10 min at 100 °C without irreversible loss of enzymatic activity (Xu et al. 2001), although it displayed a low optimal temperature range 30–55 °C, which was explained by the six disulfide bridges of the enzyme (deduced from X-ray crystallography data). It showed no obvious similarity to other members of GHF45, except for a 38% identity in amino acid level to an endoglucanase of the fungus *Trichoderma reesei*.

The optimal pH values of the EGI from *Scopulariopsis brevicaulis* and EGV from *Humicola insolens* were pH 7.0 and 7.5, and especially EGV had a pH optimum in the broad range of 7.0–9.0 (Schulein et al. 1993; Nakatani et al. 2000). Alkaline cellulases belonging to GHF5 and GHF6, the genes of which were cloned and sequenced, from alkalophilic *Bacillus* and *Streptomyces* genera have been hitherto known (Tomme et al. 1995). Although very little is known about alkaline cellulase produced by filamentous fungi, EGI and EGV, both displayed their activity at alkaline pH. This feature could not be explained by the amino acid sequence because the catalytic domain of EGV showed 98.4% identity with that of EGL3, with optimal pH values of 7.5 and 5.0, respectively (Takashima et al. 1999). The structural feature formed by catalytic and cellulose-binding domains jointed with linker segment may play an important role in determining the activity of cellulases under alkaline condition.

9.5 Conclusions

Despite the difficulty in getting pure cultures of flagellates, there have been many efforts to determine the participation of termites and their symbiotic flagellates in cellulose digestion. The common assumption is that there is a dual cellulose-digesting system in lower termites (Watanabe et al. 1998, 2002; Nakashima et al. 2002a): one originating from hindgut Archaezoa and

the other endogenously from the termite itself. Furthermore, research on cellulose digestion in *Coptotermes* termites suggested that the two cellulase systems work independently. Neither termite's EG (GHF9) inflow from the midgut into the hindgut nor Archaezoan EG (GHF7) reflux (back flow) from the hindgut into the midgut were observed (Nakashima et al. 2002a; Watanabe et al. 2002). This finding was supported by the previous study on *Coptotermes lacteus*. After removal of the Archaezoa in *Coptotermes lacteus* by oxygen treatment, midgut cellulase activity was not significantly affected, but the cellulase activity in the hindgut was completely lost after 48 h (McEwen et al. 1980). Hogan et al. (1988) demonstrated that the Archaezoa and endogenous cellulolytic components of *Coptotermes lacteus* are chromatographically distinct from each other and most of the cellulase of termite origin does not enter the paunch. According to Nakashima et al. (2002a), the demand of glucose metabolism in termites may explain the dual system. The glucose production rate of the endogenous cellulolytic system (foregut and midgut) is not enough to support the metabolic needs of *Coptotermes formosames*. The symbiotic cellulolytic system in the hindgut has the ability to compensate for the metabolic requirements.

After defaunation of the termite *Mastotermes darwiniensis* by starch-diet and cultivation on a diet of wood again (Veivers et al. 1983), the cellulase activity in the hindgut increased to a level similar to that in salivary glands and the midgut, implying that the increase in endoglucanase activity in the hindgut may be due to carrying over of the termite cellulase from the salivary gland. The inflow of termite cellulase was also demonstrated into the hindgut. Moreover, the flagellate cellulases of GHF45 did not exhibit the obvious activity in vivo and in vitro. Thus, it suggested that the inflow of termite cellulase into the hindgut could compensate for the metabolic requirement of *Mastotermes darwiniensis* and result in the loss of the activity of flagellate endoglucanase. Therefore, the dual cellulose-degradation system in *Mastotermes darwiniensis* is not independent. Despite this result, defaunation led to death of *Mastotermes darwiniensis* after two to three weeks.

To date, there has been no further study on the dual system in the termite *Reticulitermes speratus*. It is known that the endoglucanase components from the whole termite bodies and that from the hindgut are chromatographically identical (Watanabe et al. 1997) and the flagellates in *Reticulitermes speratus* contain the endoglucanase genes of GHF45 (Ohtoko et al. 2000).

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10 Symbiotic Protozoa of Termites

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10.1 Introduction

Lower termites and the roach *Cryptocercus* harbour in their hindgut a dense fauna of flagellate protozoa mixed with other microorganisms, such as bacteria, archaebacteria and yeasts. The termite hindgut and its symbionts form a microaerophilic ecosystem that acts in the digestion of plant material ingested by these insects. This chapter presents the diversity of the flagellate protozoa, their cytological features and phylogeny (Part 1) and several aspects of the biology and adaptations of these eukaryotes to this particular ecosystem (Part 2).

10.2 Diversity, Cytology and Phylogeny of Symbiotic Protozoa in Lower Termites

This chapter is too short to include an extensive study on the relationships between symbionts and the termite host, which are reviewed elsewhere (Honigberg 1970; Inoue et al. 2000; König et al. 2002). Flagellate protozoa of termites live exclusively in the hindgut, being abundant in the dilated paunch and colon. They occur only in lower termites, namely: Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae and Serritermitidae. Higher termites, the Termitidae, host no or very few protozoa. The wood-feeding roach *Cryptocercus* harbours a faunule of flagellates similar to that of lower termites (Cleveland et al. 1934); xylophagous cockroaches harbour a limited and non-original protozoan fauna (Brugerolle et al. 2003). In his review, Yamin (1979) listed 205 termite species known to harbour intestinal flagellates; but among the 800 lower termites species

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many have not been examined for symbionts or their symbionts have not been identified.

The symbiotic flagellates of termites belong to two lineages of protozoa: the Oxymonadida and the Parabasalia. Most of the species were identified before 1960 by light microscopy, and Yamin (1979) listed 434 species of flagellates. From 1960, electron microscopy (EM) has helped greatly in characterising the features of the genera and families but this task is not yet complete (Brugerolle and Lee 2001a,b and references herein). The use of molecular methods for the identification of species and genera is still in its infancy and has been mainly used for phylogenetic studies (Dacks et al. 2001; Keeling 2002).

10.2.1 Cell Organisation in Oxymonad and Parabasalid Termite Flagellates

A common feature among these flagellates is the lack of conventional mitochondria in their cell, which is related to their anaerobic mode of life. Oxymonads have no remaining mitochondrial structure and this property is shared with other protozoa, the diplomonads, retortamonads and pelobionts (Brugerolle and Müller 2000). Parabasalids have organelles named hydrogenosomes that are structurally and functionally different from mitochondria; their role in energy metabolism has been studied in cultured trichomonads, which have close relatives in termites (Part 2). Although many termite flagellates have not been studied by electron microscopy, the ultrastructure and organisation of a large majority of genera and families are known. It is now possible to present the cytological features of these two lineages and of the main subgroups, giving a comprehensive view of the cytological diversity and phylogenetic relationships in these lineages.

Flagellar Apparatus and Cytoskeleton in Oxymonads. Oxymonads live mostly in insects; among the 12 genera recorded, 8 have been found in termites: *Monocercomonoides, Dinenympha, Pyrsonympha, Streblomastix, Oxymonas, Microrhopalodina, Barroella, Sauromonas*, and 3 in the roach *Cryptocercus*: *Notila, Paranotila, Saccinobaculus* (Yamin 1979) (Fig. 10.1a). The genus *Monocercomonoides* is not specific to termites and occurs in the intestine of a wide variety of vertebrates and invertebrates, and *Polymastix* has been described in other xylophagous insects such as cockroaches (Brugerolle and Lee 2001a; Brugerolle et al. 2003).

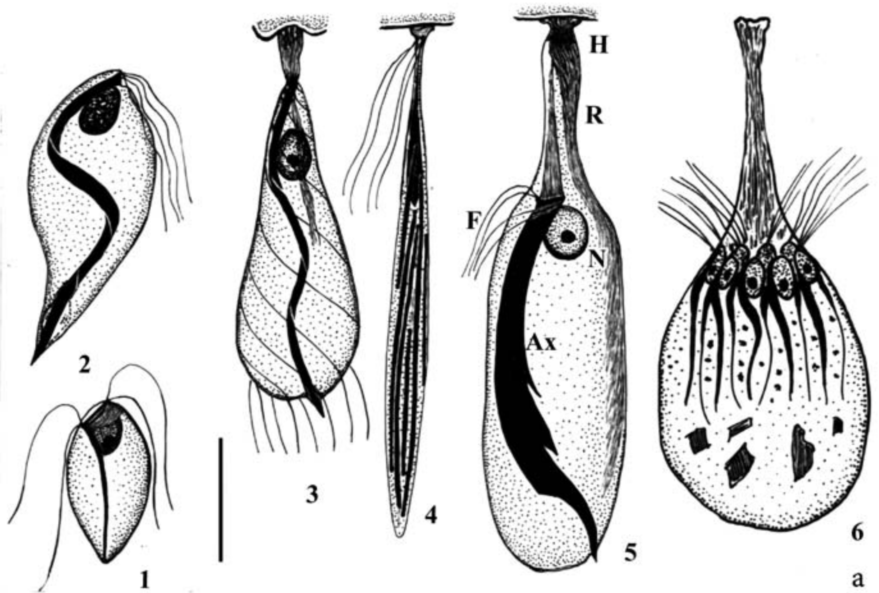
As in other flagellated protist cells, the basal bodies of the flagella and their connected roots constitute the flagellar apparatus typical of each lineage of protists (Brugerolle 1991; Radek 1994). In oxymonads there are two pairs of flagella/basal bodies distantly separated and linked by a fi-

bre, the preaxostyle (Fig. 10.1a–c). From the preaxostyle arise the microtubules of the axostyle, which traverses the cell axially. A ribbon of microtubules named the pelta covers the anterior part of the cell and the nucleus (Fig. 10.1b). At the base of the recurrent flagellum, there is a microtubular fibre, also named the funis, and several fibrous structures considered as the remnants of a groove. The axostyle is composed of staggered rows of microtubules interlinked by bridges giving a paracrystalline structure that is contractile in several genera such as *Saccinobaculus*, *Pyrsonympha,* and *Oxymonas* (Fig. 10.1d). Some genera such as *Dinenympha* and *Pyrsonympha* have recurrent flagella adhering to the cell body and presenting para-axonemal and desmosomal structures (Hollande and Carruette-Valentin 1970). Several genera have acquired a holdfast that helps the flagellate to adhere to the cuticle of the gut (Fig. 10.1a). In *Pyrsonympha* and *Streblomastix*, the rhizoids of the holdfast are filled with actin microfilaments (Fig. 10.1e). In *Oxymonas* the holdfast comprises an extensile rostellum with several rows of microtubules (Fig. 10.1f) and a terminal set of rhizoids with microfilaments (Brugerolle and König 1999; Hollande and Carruette-Valentin 1970; Radek 1994; Rother et al. 1999). These flagellates do not have a Golgi body and the nuclear division is a closed mitosis with an internal spindle (Grassé 1952a).

Phylogenetic Relationships. Ultrastructure comparison has shown that oxymonads share several features with *Trimastix*, jacobids and heterolobosean amoeboflagellates, such as the preaxostyle fibre and the remnant structures of a groove present in their free-living ancestors (Simpson et al. 2002). Ribosomal RNA sequence analysis has shown that oxymonads are a sister group of *Trimastix* (Dacks et al. 2001; Moriya et al. 2003).

Flagellar Apparatus and Cytoskeleton in Parabasalids The parabasalids form an important lineage of about 80 genera, most of which live in termites and roaches (Brugerolle and Lee 2001b; Yamin 1979). They were classically split into two major groups, the small, simple trichomonads and the large, more complex hypermastigids, but this division is no longer phylogenetically valid (Brugerolle and Patterson 2001; Keeling 2002). Broadly, they belong to four major subgroups distinguishable in both ultrastructure and molecular phylogeny: the trichomonadids, the cristamonads, the trichonymphids and the spirotrichonymphids. Their common features are the typical organisation of the flagellar apparatus, the presence of hydrogenosomes and the pleuromitotic type of mitosis (Brugerolle 1991).

Trichomonadids. This subgroup comprises small trichomonads such as *Tricercomitus* and *Hexamastix* which have no undulating membrane and a costa, formerly classified in the Monocercomonadidae, a family that is now split. It also contains genera that have a lamellar undulating mem-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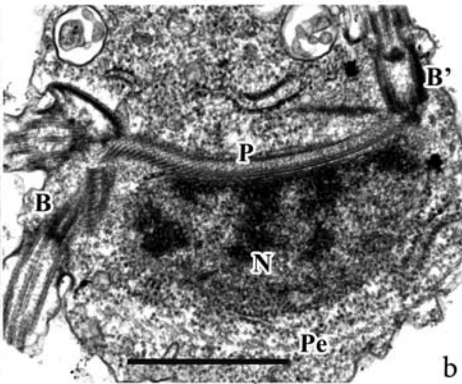
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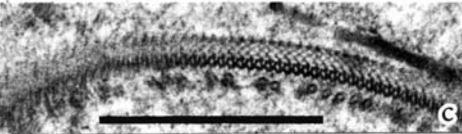
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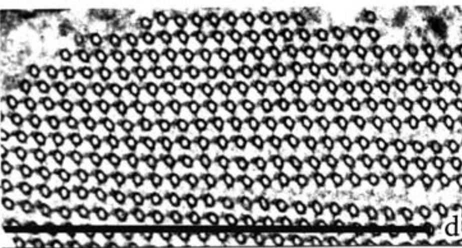
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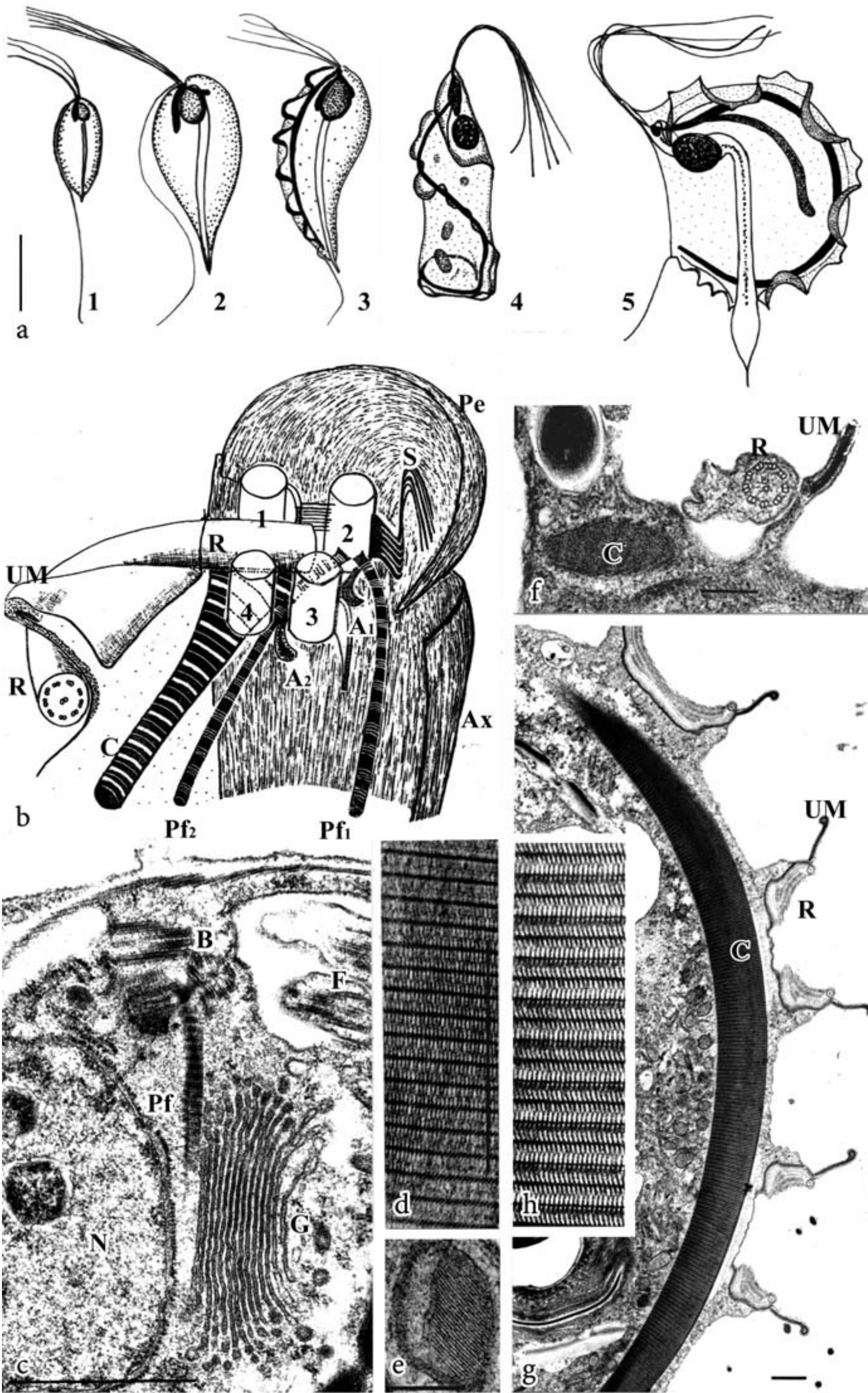
◀ **Fig. 10.1.** Oxymonads. **a** Oxymonad genera: *Monocercomonoides* (1), *Saccinobaculus* (2), *Pyrsonympha* (3), *Streblomastix* (4), *Oxymonas* (5), *Microrhopalodina* (6). **b** In *Monocercomonoides,* two pairs of basal bodies (*B*, *B′*) are distantly separated by the preaxostyle (*P*) close to the nucleus (*N*) covered by the pelta (*Pe*). **c** Composite structure of the preaxostyle in *Pyrsonympha*. **d** Organisation of the paracrystalline axostyle in *Oxymonas* showing intra- and inter-row bridges connecting the microtubules. **e** Rhizoids with microfilaments in the holdfast of *Pyrsonympha*, attached to the cuticle (*Cu*) of the hindgut. **f** Microtubular ribbons in the rostellum of *Oxymonas*. *Bar drawings*=10 µm, EM=1 µm. (Photographs from G. Brugerolle)

brane and a costa, such as *Trichomonoides* with four anterior flagella, *Pentatrichomonoides*, *Trichomitopsis* and *Pseudotrypanosoma* (Fig. 10.2a) and classified in the Trichomonadinae and Trichomitopsiinae (Brugerolle and Lee 2001b).

The flagellar apparatus is typical, the basal body (R) of the recurrent flagellum being orthogonal to the basal bodies of the anteriorly directed flagella #1, #2, #3, #4 or #5 (Fig. 10.2b). Basal body #2 is the most important; it bears the sigmoid fibres and the two striated parabasal roots. The enrolled microtubular ribbon of the axostyle crosses the cell and protrudes posteriorly. It is surmounted by the microtubular ribbon of the pelta, which partially surrounds the flagellar area. Sigmoid fibres are in contact with the pelta-axostyle origin. The two parabasal roots attached to basal body #2 are striated (A-type striations) and support a Golgi body, the whole forming the parabasal apparatus (Fig. 10.2a–c). The costa root is attached to the basal body of the recurrent flagellum and located under the undulating membrane (Fig. 10.2b). It is striated (B-type) and shows a lattice substructure in *Trichomonoides* and *Pentatrichomonoides* (Fig. 10.2d,e). In the large *Trichomitopsis* and *Pseudotrypanosoma*, the lattice structure is different and this costa is contractile (Fig. 10.2g,h). The undulating membrane has a lamellar shape and contains a dense structure (Fig. 10.2f). The recurrent flagellum adheres by the cell coat to the undulating membrane and contains amorphous para-axonemal material in *Pentatrichomonoides, Trichomitopsis* (Fig. 10.2f,g) (Brugerolle 1999; Brugerolle and Bordereau 2004; Brugerolle et al. 1994). Two organelles referred to as "batachii" or "atractophores" are suspended under the basal bodies and polarise the mitotic spindle (Fig. 10.2b) (Brugerolle 1991).

Cristamonads. A second subgroup has been discerned by both ultrastructure and molecular studies. The ultrastructure study has shown a series of closely related genera/families: *Devescovina* (Devescovinidae, Calonymphidae), and *Projoenia, Joenina* (Joeniidae). Additional families such as Lophomonadidae, Deltotrichonymphidae, Rhizonymphidae, and Kofoidiidae have been tentatively assembled in this subgroup, named the cristamonads (Brugerolle and Patterson 2001).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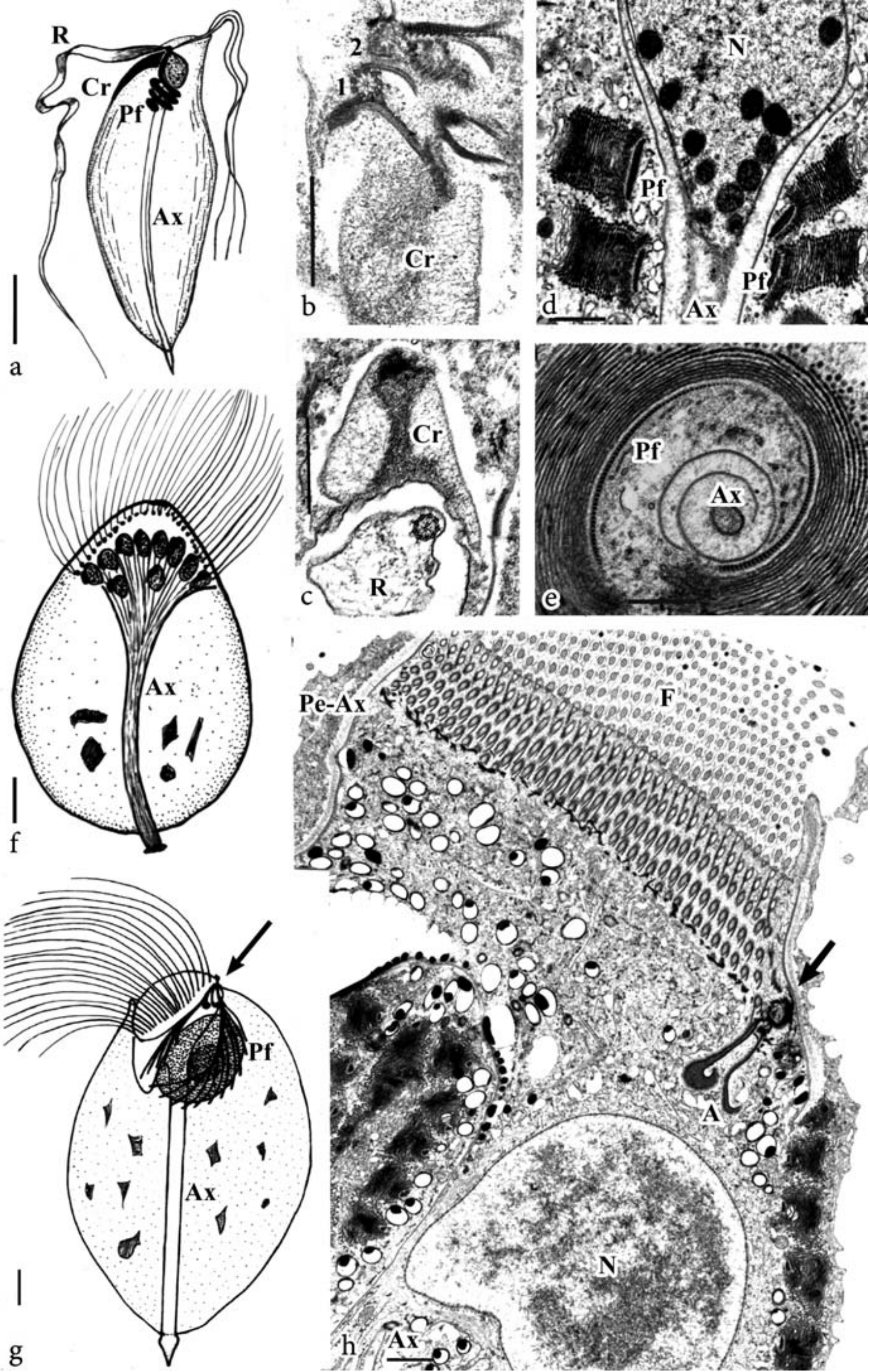
◀ **Fig. 10.2.** Trichomonadids. **a** Genera *Tricercomitus* (1), *Hexamastix* (2), *Trichomonoides* (3), *Pentatrichomonoides* (4), *Trichomitopsis* (5). **b** Organisation of the flagellar apparatus in a trichomonad, basal body of recurrent flagellum (*R*) orthogonal to basal bodies #1, #2, #3, #4, sigmoid fibres (*S*), pelta-axostyle microtubular rows (*Pe*, *Ax*), parabasal fibres (*Pf1*, *Pf2*), atractophores (*A*1, *A*2), costa fibre (*C*) under the undulating membrane (*UM*). **c** Parabasal striated fibre (*Pf*) supporting Golgi body (*G*) in the parabasal apparatus; basal body (*B*), flagellum (*F*), nucleus (*N*). **d, e** Striated costa of *Pentatrichomonoides* in longitudinal (**d**) and cross section (**e**). **f** Recurrent flagellum (*R*) adhering to the lamellar undulating membrane (*UM*) close to the costa (*C*) in *Pentatrichomonoides.* **g** Undulations of the recurrent flagellum (*R*) and the undulating membrane (*UM*) with the underlying costa (*C*) in *Trichomitopsis.* **h** Striated costa of *Trichomitopsis* in higher magnification. *Bar drawings*=10 µm, EM=1 µm, 0.5 µm in **d**, **h**. (Photographs from G. Brugerolle)

Devescovinids, like trichomonads, have three anteriorly directed basal bodies/flagella and a recurrent flagellum with a cord-like or ribbon-like structure. There is no costa and undulating membrane, but a microfibrillar structure named a cresta in front of the recurrent flagellum (Fig. 10.3a–c). The cresta extends along the length of the cell in the genus *Foaina* and is limited to a falx-shaped structure in other devescovinids such as *Devescovina* (Fig. 10.3a; Brugerolle 2000). The long branch of the parabasal apparatus is frequently twisted around the enrolled microtubular ribbon of the axostyle (Fig. 10.3d,e). The second type in the series is the genus *Projoenia*, which has the devescovinid structures but also an additional flagellar area of about 400 basal bodies close to the four privileged basal bodies. The third type in the series is *Joenina* and joeniids, which have the privileged basal bodies and attached roots, and a flagellar area of 1000 to 2000 basal bodies/flagella; the cresta and the recurrent flagellum have been lost (Fig. 10.3g,h; Brugerolle and Patterson 2001). In this series, *Projoenia* represents the missing link between devescovinids and joeniids. Ultrastructure studies have demonstrated that calonymphids are polymastigote devescovinids that have their axostyles assembled in a common trunk (Fig. 10.3f; Dolan et al. 2000a; Rösel et al. 1996). Lophomonads that live in roaches have privileged basal bodies and an additional flagellar area (Hollande and Carruette-Valentin 1972). Deltotrichonymphids such as *Koruga* from *Mastotermes* also have privileged basal bodies and a dome-shaped flagellar area that is more complex than in the joeniids (Tamm and Tamm 1973). Rhizonymphids and Kofoidiids probably have the same organisation but have not been studied by electron microscopy (Grassé 1952b). Division morphogenesis distinguishes cristamonads from trichonymphids (see below); at division the flagellar area regresses, the cell divides like a trichomonad and each daughter cell reforms a new flagellar area (Grassé 1952b). Molecular studies based on rRNA analysis of devescovinids, calonymphids, and deltotrichonymphids indicate that they form a subgroup (Gerbod et al. 2000; Gunderson et al. 1995; Fröhlich and König 1999; Keeling 2002).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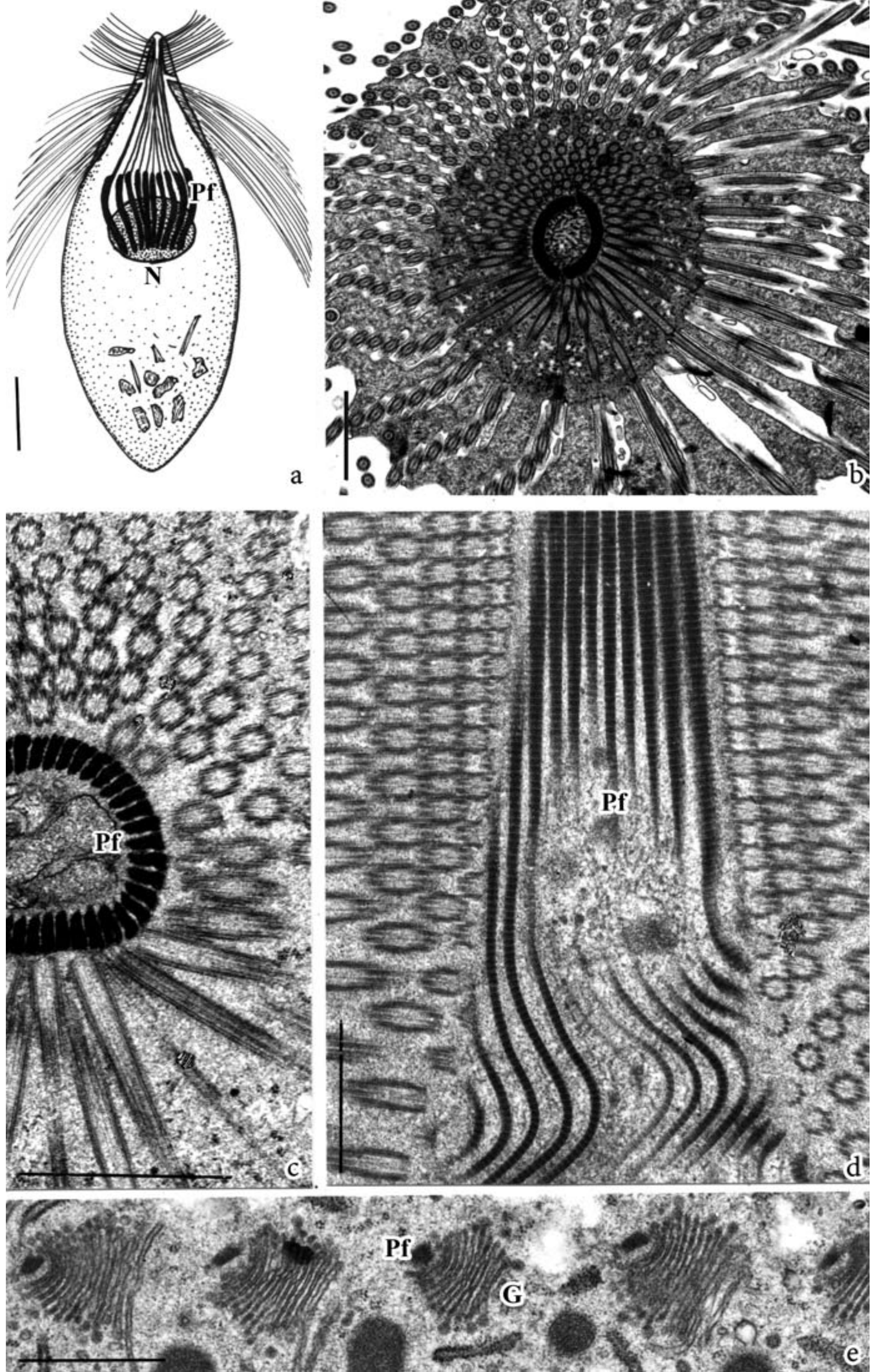
◀ **Fig. 10.3.** Cristamonad families: devescovinids, calonymphids, and joeniids. **a** In *Devescovina*, ribbon-like recurrent flagellum (*R*), falx-like cresta (*Cr*), twisted parabasal fibres (*Pf*) around the axostylar trunk (*Ax*). **b, c** Cresta (*Cr*) attached to basal bodies (#1, #2) and modified recurrent flagellum (*R*); **d, e** Parabasal fibres (*Pf*) twisted around the axostylar trunk (*Ax*); nucleus (N). **f** Polymastigote *Calonympha* with several karyomastigonts (nucleus + flagellar apparatus) and assembly of axostyles (*Ax*); **g, h** In *Joenina*, privileged basal bodies (*arrow*), two ramified parabasal fibres (*Pf*) and two atractophores (*A*) close to the flagellar area (*F*) surrounded by the pelta-axostyle (*Pe-Ax*); axostylar trunk (*Ax*), nucleus (*N*). *Bar drawings*=10 μm, EM=1 μm. (Photographs from G. Brugerolle)

Trichonymphids. They are large cells, 100 μm or more, bearing several hundred to several thousand flagella; they live in termites and in the roach *Cryptocercus*. They comprise six families: Hoplonymphidae, Staurojoeninidae, Trichonymphidae, Eucomonymphidae, Teranymphidae, and Spirotrichosomidae (Brugerolle and Lee 2001b). They share the same general organisation, an anterior rostrum divided into two hemi-rostra each of which bears a flagellar area in bilateral symmetry. At the top of each flagellar area there is the privileged basal body #2 bearing the sigmoid fibres associated with the reduced pelta-axostyle. The basal bodies of the rostrum are positioned radially on a plaque of merged parabasal fibres (Fig. 10.4a,b); to the rear of the rostrum, the parabasal fibres individualise and at the nucleus level they support Golgi bodies (Fig. 10.4c–e). In Staurojoeniidae the two flagellar areas are subdivided, giving four flagellar areas; in Trichonymphidae the two hemi-rostra are juxtaposed (Fig. 10.4b), in Teranymphidae and Spirotrichosomidae the post-rostral lines of flagella are spiralled around the cell. At division the spindle is stretched between the two atractophores depending on each hemi-rostrum. Hemi-rostra separate and migrate in each daughter cell reconstituting a complete rostrum (Cleveland et al. 1934; Hollande and Carruette-Valentin 1971). Molecular studies placed the trichonymphids at the base of the parabasalid tree (Dacks and Redfield 1998).

Spirotrichonymphids. They are small to large flagellates, all living in termites; the best-known genera are *Spirotrichonympha, Holomastigotoides, Spirotrichonymphella* and *Holomastigotes* (Brugerolle and Lee 2001b). They are characterised by several flagellar lines arising at the top of the cell and spiraling around the cell body (Fig. 10.5a). Each line of basal body/flagella begins with the basal body #2, which bears the sigmoid fibres associated with an anterior pelta-axostyle microtubular row (Fig. 10.5b). In the flagellar lines, basal bodies are interlinked by bridges or by a continuous centrin microfibrillar layer (Fig. 10.5b; Lingle and Salisbury 1997). Basal body lines are accompanied by a parabasal lamina along which Golgi bodies are generally positioned (Fig. 10.5c–e). Axostylar rows of microtubules concentrate around the nucleus and converge to form a central axostylar trunk in certain genera such as *Spirotrichonympha* and *Holomastigotoides* (Fig. 10.5a,c;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◀ **Fig. 10.4.** Organisation of trichonymphids. **a** *Trichonympha* with rostral and subrostral flagella, parabasal fibres (*Pf*) bearing Golgi bodies at the nucleus level (*N*). **b–e** Merged parabasal fibres (*Pf*) forming two juxtaposed plaques in the rostrum (**b**), they individualise to the rear of the rostrum (**c**, **d**) and support a Golgi body (*G*) at the nucleus level (**e**). *Bar drawing*=50 µm, EM=1 µm. (Photographs from G. Brugerolle)

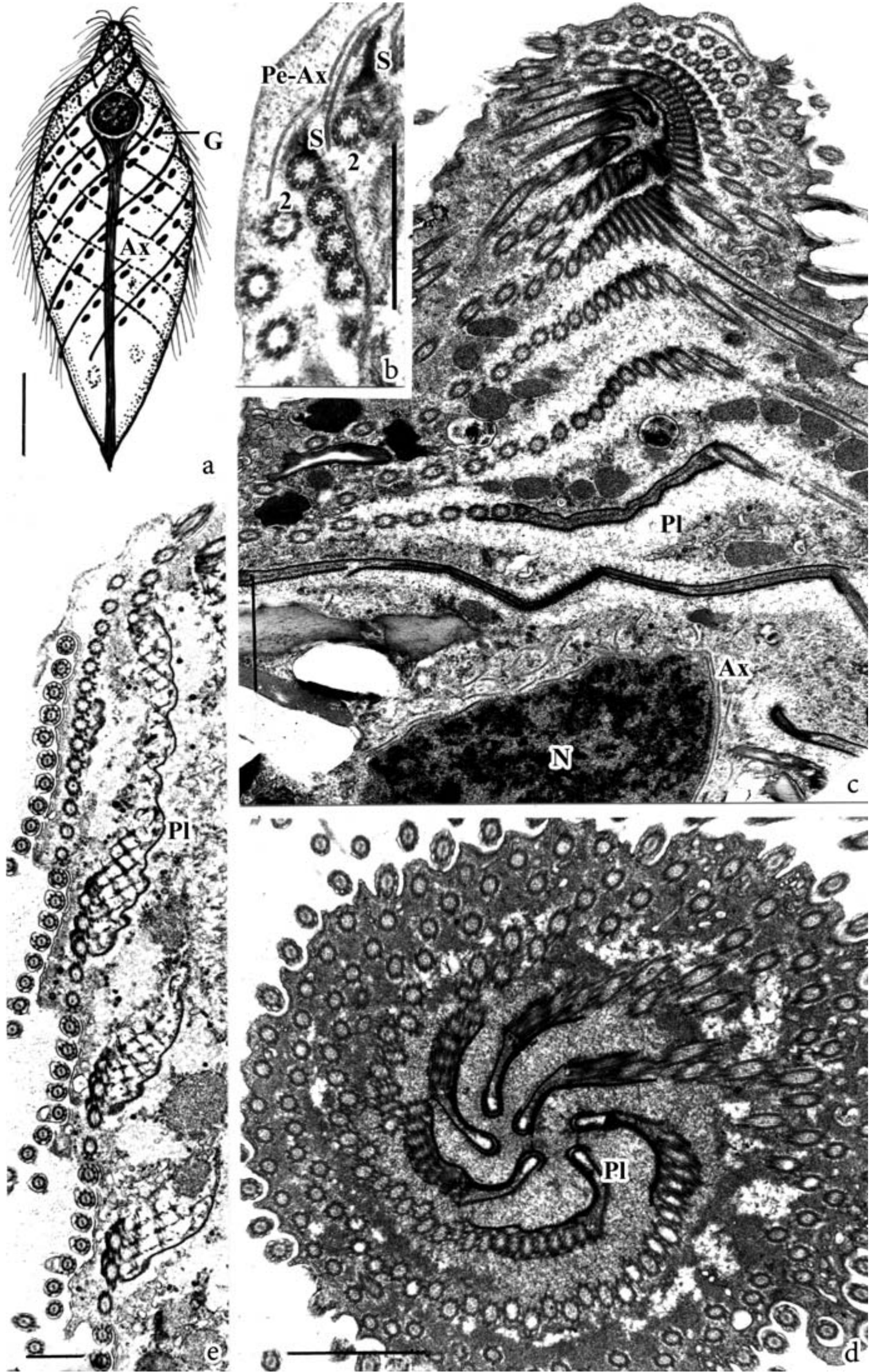
Brugerolle 2001; Brugerolle and Bordereau 2004; Hollande and Carruette-Valentin 1971; Radek 1997). At division the spindle forms between two neighboring parabasal laminae close to the nucleus. Mitosis leads to the separation into two groups of flagellar lines which migrate in each daughter cell. Molecular studies indicate that spirotrichonymphids form a subgroup situated between trichonymphids and cristamonads (Ohkuma et al. 2000).

10.3 Biology of Termite Flagellates

10.3.1 Relationships Between Flagellates and Host

Lower termites depend on their intestinal symbionts for nutrition, and these symbionts profit from their hosts in several respects. They are regularly supplied with food particles, are protected from enemies and are kept in a constant environment. However, life in the gut is not always safe. Although an enteric valve prevents overflow of the paunch into the midgut, the contents of the paunch can get lost through the anus by two events: during moulting, and by supplying young larvae and moulted nest mates with symbionts by proctodeal feeding. Some of the consumed flagellates may be damaged or digested during the passage through the gut, but sufficient numbers reach the paunch and multiply.

This direct transmission of flagellates renders it superfluous to produce true, resistant cysts (Grassé and Noirot 1945; Honigberg 1963). However, morphological changes to the flagellates such as reduction in size or rounding up do occur during the moult of the termites (May 1941), possibly evoked by the moulting hormone ecdysone. Pseudocysts which are limited by a normal cell membrane and internalise their flagella may be formed (Kulda et al. 1986). Induction of sexual cycles was reported for symbionts of different termites, as for example the Australian termite *Mastotermes darwiniensis* (*Mixotricha paradoxa*, Cleveland 1966a; *Deltotrichonympha*, Cleveland 1966b; *Koruga*, Cleveland 1966c). Generally, unequal divisions during gametogenesis lead to the production of small (male) and large (female) gametes which differ in their behaviour (Cleveland 1966b). De-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◀ **Fig. 10.5.** Organisation of spirotrichonymphids. **a** *Spirotrichonympha* with spiralled flagellar lines associated with Golgi bodies (*G*); central axostylar trunk (*Ax*). **b** Each flagellar line begins at the basal body #2 bearing sigmoid fibres (*S*) close to the pelta-axostyle rows (*Pe-Ax*); **c–e** Spiralled flagellar lines lined by parabasal lamina (*Pl*) in *Spirotrichonympha* (**c**, **d**) and in *Holomastigotoides* (**e**); axostylar rows (*Ax*) around the nucleus (*N*) (**c**). *Bar drawing*=50 µm, EM=1 µm. (Photographs from G. Brugerolle)

pending on the genus, the male gamete joins the female one at a special region of the termite body (e.g. in *Trichonympha* and *Mixotricha*) or anywhere (e.g. in *Deltotrichonympha*). During fusion, the flagella, parabasal bodies and the axostyle of the male gamete degenerate. Instead of complete destruction, the male rostral region and its flagella may be pinched off from the zygote by a cytoplasmic constriction (e.g. in *Deltotrichonympha* and *Trichonympha*). The male pronucleus then migrates to the stationary female pronucleus and fuses with it.

Also well studied are the gut flagellates of the wood roach *Cryptocercus*, which harbours a set of (about 30) symbiotic flagellates (Cleveland et al. 1934). The reactions of the parabasalids and oxymonads inhabiting *Cryptocercus* moults are different from those of termite flagellates and more diverse. Several flagellate species form thick, two layered cyst walls that make them capable of surviving outside their host's body for a considerable time. All cystic specimens lose motility by discarding their flagella and lose most of their cytoplasmic organelles such as parabasal bodies and axostyles. There are several types of sexual cycles in the different flagellate genera involving formation of iso- or anisogametes, gametic nuclei, or only gametic daughter chromosomes (Cleveland 1949). The gamonts are haplonts or diplonts. Meiosis may be zygotic, as in *Barbulanympha*, or gametic, as in *Rhynchonympha*, *Urinympha*, and *Macrospironympha* (Cleveland 1966b). Under the influence of ecdysone, the flagellates transform to gamonts or pre-gamonts with or without morphological changes. Gamonts of *Trichonympha*, a hypermastigote genus that also occurs in termites, round up, form a cyst wall, destroy all extranuclear organelles and divide into a male and a female gamete which leave the cyst. The smaller male gamete may then attach to the posterior cell pole of a female gamete and penetrate (Fig. 10.6).

During vegetative reproduction, the flagellates generally divide by binary fission, but budding has also been reported for multinucleate trichomonads (Dolan et al. 2000b). The nuclear membrane persists during mitosis. The spindle apparatus is intranuclear in oxymonads and extranuclear in the other taxa.

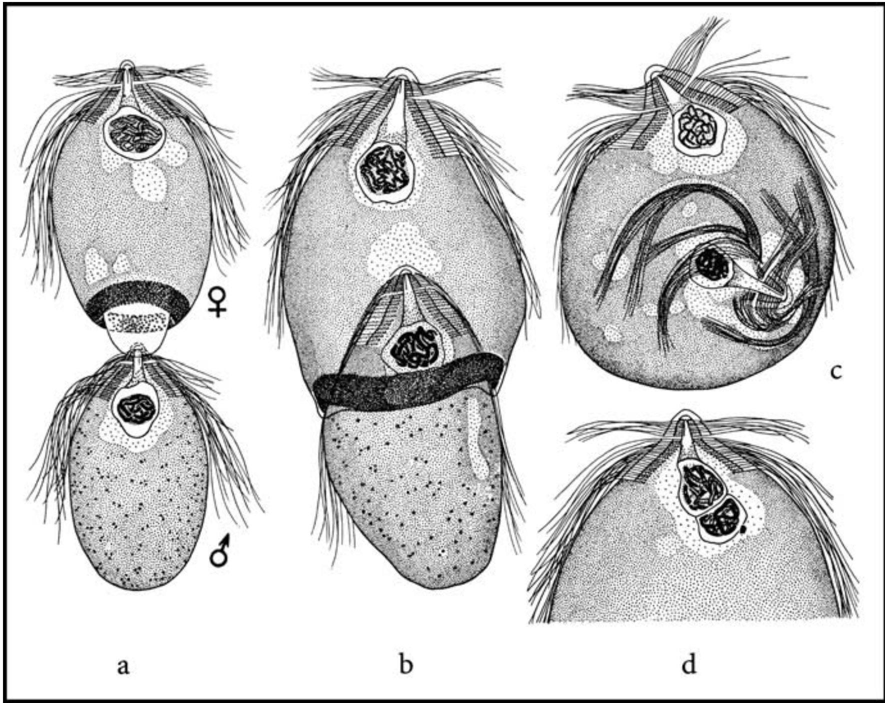

Fig. 10.6. Fertilisation of *Trichonympha*. **a** Male gamete attaches itself with its rostrum to the posterior fertilisation cone of a female gamete. **b** Male gamete penetrates the female one. **c** Fusion of the gametes proceed; the male gamete looses its extranuclear organelles. **d** The two pronuclei begin to fuse. (After Cleveland 1949)

10.3.2 Populations of Flagellates

Species composition of flagellates is generally specific to a termite species or a regional population (Honigberg 1970; Kitade and Matsumoto 1993). Transmission between nest mates seems to be responsible for the fact that protist assemblage composition is often mirrored by termite phylogeny (Inoue et al. 2000). A divergent consortium might be explained by transfaunation between different termite genera.

The smaller flagellate species often outnumber the larger ones (Yoshimura 1995). In field colonies, the abundance of some species is dependant on the season. The highest numbers of *Holomastigotoides* in *Coptotermes formosanus*, for example, occur in autumn and are positively correlated with wood consuming activity of the termites, suggesting an important role in digestion (Yoshimura 1995). An effect of season, geographical location and colony age on species composition and population dynamics of

the flagellates was also described for other termites (Mannesmann 1974; Grosovky and Margulis 1980). Furthermore, the type of wood that termites feed on is also a factor which influences variation in the composition of gut flagellates (Cook and Gold 2000; Mannesmann 1972; Mauldin et al. 1981). The flagellates are either differentially susceptible to certain components in the various woods or they have different nutritional requirements.

10.3.3 Nutrition

Many lower termites feed exclusively on wood, although this food is difficult to digest and poor in nutrients, particularly nitrogen. Some of the enzymes necessary for the degradation of lignocellulose are produced by the termites themselves, i.e. endoglucanases and cellobiases, which are released in the salivary glands and midgut. Cellulose and hemicellulose have thus already been partially broken down by the time they reach the paunch (see review Breznak and Brune 1994). Furthermore, diverse microorganisms with lignocellulosic activities were found in the gut (see also Chap. 11). However, generally lower termites can not survive without their symbiotic flagellates. Specimens that have been deprived of their flagellates by experimental defaunation die within few weeks despite continued feeding. Thus, the flagellates are the major agents of cellulose hydrolysis in lower termites (Honigberg 1970; Breznak 1982, 1983; O'Brien and Slaytor 1982; Odelson and Breznak 1985a). Hypothesis assigning enzyme production to endo- or epibiotic bacteria of the flagellates have not been confirmed. For example, bacteria-free, axenically cultivated flagellates such as *Trichonympha sphaerica* (Yamin 1981) and *Trichomitopsis termopsidis* (Yamin 1978; Yamin and Trager 1979; Odelson and Breznak 1985a) were capable of decomposing cellulose on their own. It is presumed that most of the exo-cellobiohydrolase (which is generally indispensable for degrading the crystalline cellulose such as wood cellulose) originates from the flagellates (Yoshimura 1995).

Feeding termites (pseudergates) grind the wood with their mandibles and the cuticular spines of the proventriculus to small particles (ca. 20–100 μm). These may then be phagocytosed by the hindgut flagellates (Fig. 10.7). Ingestion can take place at any point on the body surface except in flagellated regions; special feeding apparatuses or cytostomes do not exist. The ingested lignocellulose is gradually decomposed within the food vacuoles, independent of bacterial activity (Yoshimura et al. 1994, 1996). In addition to cellulosic material, food is complemented by uptake of bacteria living in the hindgut fluid (Fig. 10.7, upper inset) and occasionally small gut flagellates are ingested by large ones. Bacteria attached to the surface of the

flagellates may be ingested as well (Radek et al. 1992) when the flagellates' membrane pinches off to form a food vacuole. Dissolved substances may be incorporated by pinocytosis (Fig. 10.7, lower inset; Hollande and Valentin 1969).

The roles that different flagellate species of a certain host termite play in digestion seem to differ (Yoshimura et al. 1993, 1994; Yoshimura 1995). For example, Yoshimura (1995) showed that in the termite *Coptotermes formosanus* (force-fed with celluloses of different degrees of polymerisation), the large species *Pseudotrichonympha grassii* is involved in the decomposition of highly polymerised cellulose, while the small cells of *Holomastigotoides hartmannii* and *Spirotrichonympha leidyi* can only use cellulose of low molecular weight. In addition, the distribution of the flagellate species in the gut differs. Each species prefers a specific region: *P. grassii* is most abundant in the anterior and *S. leidyi* in the posterior portion, and *H. hartmannii* is homogenously distributed. Distribution can be correlated with the functions in digestion (Yoshimura 1995). Interestingly, *S. leidyi* does not ingest particular food at all and may play a role in later stages of cellulose metabolism (e.g. methanogenesis); it possesses intracellular methanogenic bacteria (Yoshimura et al. 1996).

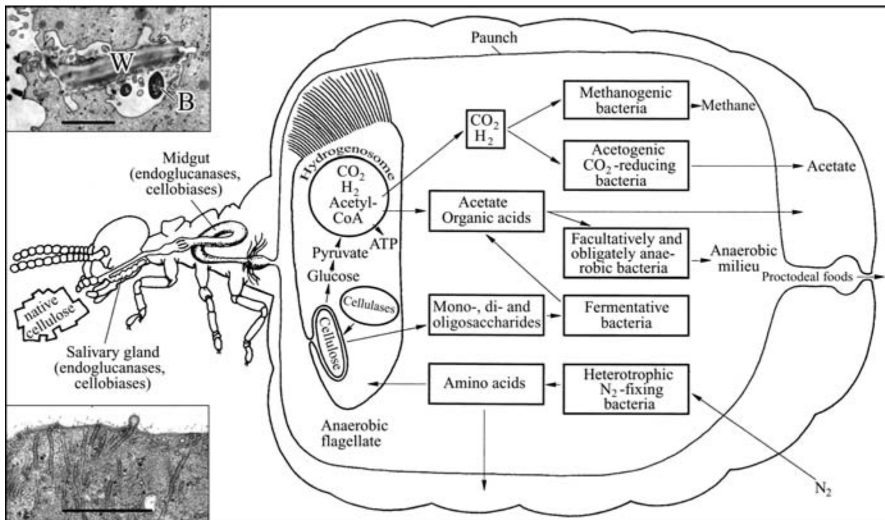

Fig. 10.7. Simplified scheme of the digestion processes taking place in the paunch of a lower termite. *Upper inset* Phagocytosis of a wood particle (*W*) and bacteria (*B*); *bar*2 µm. *Lower inset* Pinocytosis at the plasma membrane; *bar*=1 µm. (Photographs from R. Radek)

10.3.4
Energy Metabolism/Hydrogenosomes

During the digestion of wood particles, the flagellates release glucose and other small sugar oligomers into the cytoplasm (Fig. 10.7). Excess glucose can be stored in the cytoplasm as glycogen. For energy production in parabasalids, glucose is glycolytically transformed to pyruvate, which then enters a special organelle, the hydrogenosome (Müller 1993). Hydrogenosomes also substitute for mitochondria in other unicellular organisms living in anoxic environments, e.g. rumen- or sediment-dwelling ciliates and chytridiomycetes. Parabasalid hydrogenosomes do not contain DNA, however their nuclear genome encodes proteins (e.g. heat shock proteins HSP11, HSP70 and chaperonin) characteristic of organelles of endosymbiotic origin (Müller 1997). Since in phylogenetic reconstructions these sequences unambiguously group within the mitochondrial clade, a common ancestor with mitochondria is evident. In parabasalids, hydrogenosomes are spherical bodies measuring 0.5–1 μm in diameter (Kulda et al. 1986). They are generally delineated by two closely adjacent membranes which may be separated in some regions (Fig. 10.8a). The resulting spaces were shown to contain calcium ions suggesting an involvement of hydrogenosomes in intracellular Ca^{2+} regulation (Chapman et al. 1985). The granular matrix of hydrogenosomes may contain amorphous or paracrystalline in-

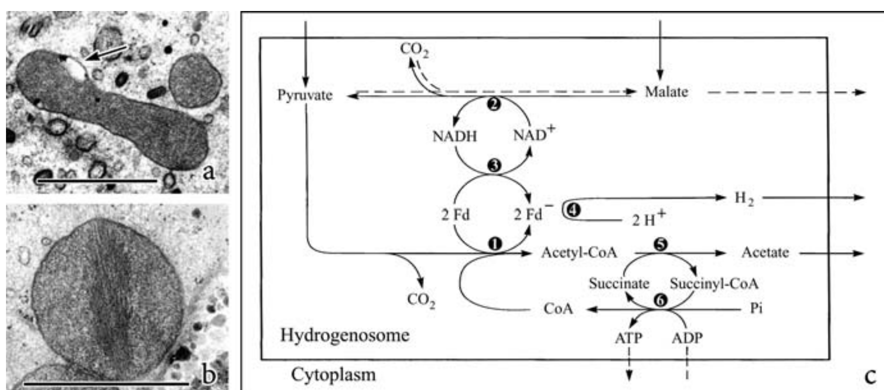

Fig. 10.8. Hydrogenosomes of *Deltotrichonympha* showing Ca-containing spaces between the two delineating membranes (*arrow*); the elongated hydrogenosome may be in a stage of division. **b** Hydrogenosome of *Placojoenia* with paracrystalline inclusions. **c** Schematic metabolic map of trichomonad hydrogenosomes. *Arrows* indicate assumed physiological directions in vivo, *dashed arrows* indicate detected formation of malate in vitro. Enzymes: 1 pyruvate:ferredoxin oxidoreductase, 2 malate dehydrogenase (decarboxylating) (NAD), 3 NAD:ferredoxin oxidoreductase, 4 H_2:ferredoxin oxidoreductase, 5 acetate:succinate CoA-transferase (after Müller 1993). *Bar* TEM=1 μm. (Photographs from R. Radek)

clusions (Fig. 10.8b). Hydrogenosomes multiply by binary division (Kulda et al. 1986).

In the hydrogenosomes pyruvate is oxidatively decarboxylated (Fig. 10.8c) (Brul and Stumm 1994; Müller 1993). The acetyl group is linked to coenzyme A, thus forming acetyl-CoA; molecular CO_2 and H_2 are released. The CoA moiety of acetyl-CoA is subsequently transferred to succinate (acetate is released as an end product); succinyl-CoA then serves as substrate for a substrate-level phosphorylation reaction producing ATP. Typical enzymes involved in the function of the hydrogenosome are for example pyruvate:ferredoxin oxidoreductase and dehydrogenase (H_2:ferredoxin oxidoreductase). The acetate released is used as the major oxidisable energy source and as an important biosynthetic precursor of the termites (Odelson and Breznak 1985b).

Eukaryotic organisms which contain no organelles of energy metabolism (e.g. oxymonads) generally perform an extended anaerobic glycolysis with the generation of additional ATP (Müller 1988). Their energy metabolism takes place entirely within the cytoplasm.

10.3.5 Motility

Movement of most gut flagellates is achieved by the action of several or numerous flagella, although some species may also make use of a contractile axostyle or costa, or of attached motile bacteria. Due to the crowded and restricted habitat within the paunch, it seems probable that motility is not used for long-distance travel, but rather for gathering food particles. It may also play an important role in mixing gut contents.

Most small parabasalids and oxymonads possess four flagella (Figs. 10.9a–c). The recurrent flagella may be attached to the cell surface, forming undulating flagella/membranes (Fig. 10.9b). When the flagella are attached to the body surface in a spiral, the cell rotates as it moves forward (e.g. *Trichomonas* and *Pyrsonympha*; Cleveland and Cleveland 1966). When cells have multiple flagella, these beat in a special co-ordinated manner depending on their arrangement. For example, in *Koruga* and *Deltotrichonympha*, about 30,000 flagella arranged in longitudinal rows cover the body except at the posterior end (Tamm 1999). Metachronic flagellar waves with definite amplitudes and wave lengths move from anterior to posterior and evoke corresponding body waves of the non-rigid cell surface (Fig. 10.9a–c; Cleveland and Cleveland 1966; Machemer 1974; Tamm 1999). Metachronism has a symplectic pattern, since the power stroke of the flagella is directed in the same direction (posteriorly) as wave propagation (Tamm 1999).

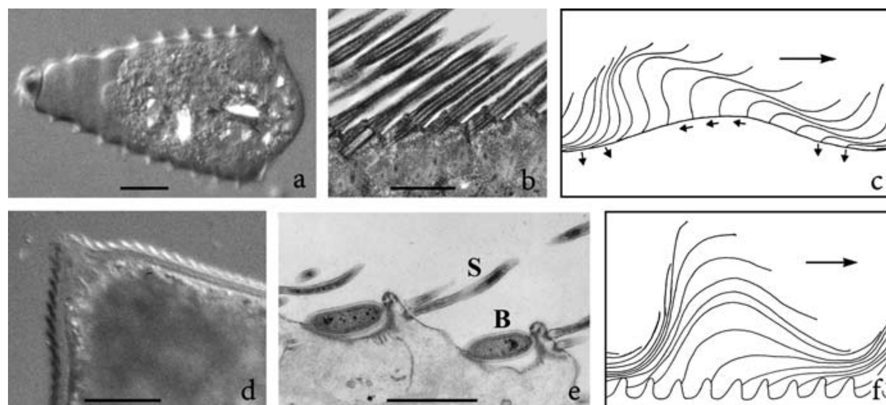

Fig. 10.9. *Koruga* and *Mixotricha*. **a** Metachronal flagellar waves of *Koruga*, interference contrast; **b** *Koruga* – row of flagella in ultrathin section; **c** *Koruga* – model showing how the metachronal flagellar waves cause body waves. Beating flagella produce forces of pressure against the cell membrane (*short arrows*). Phase shifting of flagella reorients the forces periodically. *Long arrow* shows direction of metachronal and body waves (after Machemer 1974); **d** metachronal spirochetal waves of *Mixotricha*, interference contrast; **e** *Mixotricha* – ultrathin section depicting spirochetes (*S*) and bacterial rods (*B*) attached to brackets; **f** *Mixotricha* – the scheme shows how the regularly attached spirochetes propel their host by synchronous undulations. *Arrow*: direction of spirochetal waves (after Cleveland and Cleveland 1966). *Bar* LM=50 µm, EM=1 µm. (Photographs from R. Radek)

Synchronous waves of attached spirochetes may also cause undulations of the surface, e.g. of a devescovinid flagellate from *Cryptotermes hilli* (Tamm 1999). Even more spectacular is the association of spirochetes with the trichomonad *Mixotricha paradoxa* (Fig. 10.9d–f; Cleveland and Grimstone 1964; Cleveland and Cleveland 1966). The four flagella are unable to propel the large cell. Instead, spirochetes which are attached to regularly spaced rows of brackets on *Mixotricha*'s plasma membrane evoke locomotion of their host by a coordinated undulating movement (Fig. 10.9d–f). Recent investigations on the molecular identification of spirochetes from *Mixotricha* have revealed an astonishing diversity of the associated spirochetes. At least three species belonging to the *Treponema* cluster attach to the brackets (Wenzel et al. 2003). Spirochetes that randomly attach to the body surface of a flagellate may also influence the movement of their host but do not synchronise (e.g. on *Caduceia* and *Deltotrichonympha*, Cleveland and Cleveland 1966).

A motile costa may also support locomotion of trichomonads, such as the large species *Pseudotrypanosoma giganteum* (max. 230 µm) and the smaller *Trichomitopsis termopsidis* (Fig. 10.2g; Amos et al. 1979; Brugerolle 1999). Their cell bodies display rapid changes in shape, such as bend-

ing or twisting or local deformations of the cell surface. The contractions of the costa are independent of the action of the undulating membrane (Brugerolle 1999). Contraction might be produced by alternate tilting of longitudinal protein filaments (Amos et al. 1979). Immunological and biochemical studies showed that costal proteins may represent a novel class of striated root proteins in addition to centrin, giardin, and assemblin (Viscogliosi and Brugerolle 1993, 1994).

Axostyles with bending motion are only known from oxymonads, for example *Notila*, *Saccinobaculus*, and *Pyrsonympha*. Similar to the situation found for motile costas, a wave of bending propagates from its anterior to its posterior end with a speed of up to 100 µm/s (Grimstone and Cleveland 1965; McIntosh 1973). The axostyle of *Saccinobaculus* (from *Cryptocercus*) also performs a repetitive coiling and extending of the whole axostyle (Woodrum and Linck 1980). The semicrystalline array of microtubules in the axostyle is held by two sets of linkages: intrarow cross-bridges connecting the microtubules into rows (sheets) and interrow projections making contact to the adjacent inner row (Wodrum and Linck 1980; cf. Fig. 10.1d). There is evidence for a dynein-like motor molecule that may actively shift the microtubules of the axostyles and thus generate motion (Bloodgood 1975; Mooseker and Tilney 1973).

The axostyles of some trichomonads such as *Devescovina versatilis* or *Hyperdevescovina balteata* are capable of a rotary movement that leads to a uni-directional rotation of the anterior cell pole in relation to the cell body (d'Ambrosio et al. 1999; Tamm 1976, 1978, 1979). Since the plasma membrane at the shear zone is continuous, this clearly demonstrates the fluid nature of cell membranes. The motor for rotation is unknown; the axostyle of *D. versatilis* is surrounded by an extra sheet of 5–7 nm filaments and a differentiated girdle of cytoplasm, structures that might be involved.

10.3.6 Associations with Bacteria

In addition to the few described interactions of motile ectosymbionts with flagellates, immotile, rod-like, Gram-negative bacteria are often found on some species (Dolan 2001). They adhere by their tips or by their sides and colonise all or part of the non-flagellated regions of the cell body (Fig. 10.10a–c). Their roles are less clear. Substances such as enzymes or nutrients may be exchanged. Since they are phagocytosed they may also supplement the nitrogen-poor wood diet. The pseudomonad-like rods of *Streblomastix strix* have been demonstrated to be sensory symbionts, enabling their host to orient itself in nutrient (sodium acetate) rich gradients (Dyer and Khalsa 1993). Special junctional zones are developed at the

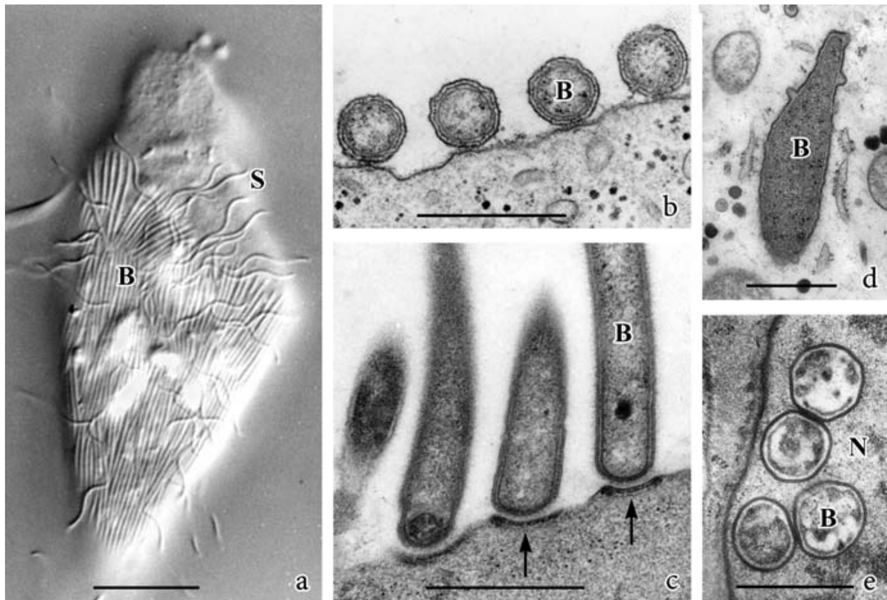

Fig. 10.10. Ecto- and endosymbiotic bacteria. **a** Except at the anterior cell pole, *Devescovina glabra* is covered with rod-like bacteria (*B*) that adhere by their sides and scattered spirochetes (*S*). **b** Ectobiotic rods (*B*) of *D. glabra* in ultrathin cross section. **c** Rod-like bacteria (*B*) attached to the surface of *Joenia annectens* with their tips; *arrows* hint at electron-dense material supporting the cell junction. **d** Intracellular bacterium (*B*) within the cytoplasm of *Mixotricha paradoxa*. **e** Endonuclear coccoid bacteria (*B*) in the nucleus (*N*) of *Joenia annectens*. *Bar* LM=10 µm, EM (**b**, **c**, **e**)=1 µm, **d**=0.5 µm. (Photographs from R. Radek)

prokaryotic/eukaryotic contact region. They involve filamentous extracellular material bridging the space between the two contacting partners; cytoplasmic electron-dense material may also support the plasma membrane of the flagellate (Fig. 10.10b,c; Radek et al. 1992; Tamm 1980). Not one but several mechanisms for bacterial attachment seem to be realised, e.g. adhesion via lectins and sugars or hydrophobic proteins (Radek et al. 1996; Radek and Tischendorf 1999).

Frequently, endobiotic bacteria colonise the cytoplasm of termite flagellates, either enclosed in vacuoles or lying in direct contact with the host cytoplasm (Fig. 10.10d). They may contribute cellulolytic enzymes for digestion (Honigberg 1970) or fixing nitrogen (Smith and Arnott 1974); some were shown to be methanogens (Radek 1997). The nucleus sometimes contains coccoid bacteria (Fig. 10.10e). None of the bacterial infections seem to be pathogenic.

10.4 Conclusion

Despite important progress made with the use of modern techniques, biodiversity of termite protozoa is far from being completed. From the 800 known lower termites, about 200 have been examined for their protozoa. The characterisation of the genera has been forged ahead by using electron microscopes with high resolution in the last 30 years. Species identification is a more difficult task that needs the combination of morphological features and molecular phylogeny. Molecular identification and molecular phylogeny of termite protozoa are at the beginning and many sequences have not been assigned to certain species. As biodiversity, cell biology of these protozoa is greatly impaired by the difficulty to cultivate them. Considering their number and their biomass, they might play an important role in the ecosystem of the smallest bioreactor represented by the termite hindgut. Certainly the diversity/evolution of the flagellar apparatus or cytoskeleton of these protozoa is fascinating, as it is also the case with the centrosome-like atractophore. The pleuromitotic type of nuclear division is worth studying to understand the mechanism of mitosis and its evolution. The demonstration of a sexual cycle mediated by the host ecdysone in the 1950s by Cleveland is another facet of the relationships between the symbionts and the host.

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11 Diversity and Lignocellulolytic Activities of Cultured Microorganisms

Helmut König, Jürgen Fröhlich, Horst Hertel

11.1 Introduction

Termites thrive between the 47° latitude north and 47° latitude south, which accounts for 68% of the Earth's landscape. Their main area of distribution is in tropical and subtropical regions (Krishna 1970; Myles 1999). The total population is estimated to be 2.4×10^{17} individual termites (Zimmermann et al. 1982). Termites are assigned to 282 genera. The intestinal microbiota of the higher termites including 71% of 2761 species lack flagellates. Termites (Isoptera) and ants (Formicidae) represent 80% of the individual insects and 30% of the total animal biomass near Manaus in Brazil (Fittkau and Klinge 1973). They process 28% of the Earth's net primary production. Termites release large amounts of gases and they are estimated to produce about 1.5×10^8 tonnes methane (total emission: up to 12.1×10^8 tonnes), 2×10^8 tonnes hydrogen, 4.6×10^{10} tonnes of carbon dioxide, 1×10^7 tonnes of carbon monoxide and 7×10^5 tonnes of dimethyl disulfide per year (Zimmermann et al. 1982; Hackstein and Stumm 1994; Hackstein et al. 1996).

Because of their intestinal flora termites are among the most important wood- and litter-feeding insects (Wood and Sands 1978). In amber containing the Miocene termite *Mastotermes electrodominicus,* a 20 million-year-old fossil microbial community consisting of protists, spirochetes and other bacteria has been observed (Wier et al. 2002). The gut microbes play an indispensable role in the digestion of food. The dense gut microbiota can include a variety of microorganisms from the domains Bacteria, Archaea, flagellates (formerly named Archaezoa) and also yeasts and fungi (cf. König et al. 2002). Because of the interesting microbial symbionts and their ecological importance in the global carbon cycle, termites have attracted the interest of many scientists from different disciplines. The microbial flora is not distributed randomly in the gut, but plays certain roles in the degradation of lignocellulose and occupies distinct microhabitats. The degradation of lignocellulose in the oxygen-limited hindgut paunch

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formally occurs in three steps: a hydrolytic step, an oxidative/fermentative step and methanogenic/acetogenic step.

In recent years, a large portion of the involved microorganisms was characterized and their phylogenetic position was determined. Although, several hundred microbial strains have been obtained in pure culture from the termite gut, still a major portion of the microbial species has not been isolated yet. The cultured species will be described in this chapter, while the non-cultured microbial clones identified by SSU rRNA sequence analysis is dealt with in Chapter 12.

11.2 Flagellates

Unique groups of flagellates (formerly named Archaezoa), which have branched off very early in the evolution of the eukaryotes, are found in the termites gut. These species seem not to occur elsewhere in nature except in wood-eating roaches of the genus *Cryptocercus*. All protozoa of the termite gut belong to the oxymonads, trichomonads and the hypermastigotes (Honigberg 1970; Yamin 1979; Radek and Hausmann 1993; Brugerrolle et al. 1994; Viscogliosi et al. 1993; Keeling et al. 1998; Kitade and Matsumoto 1998; Moriya et al. 1998; Brugerolle 2000; Brugerolle and König 1995; Dacks and Redfield 1998; Ohkuma et al. 1998; Brugerolle and Lee 2000a,b; Delgado-Viscoliosi et al. 2000). From 205 examined termite species 434 species of flagellates were described up to 1979. The flagellates occur in high number in the paunch (10^3–10^7) and they can occupy more than 90% of the paunch volume (cf. König et al. 2002). Only three species of the flagellate flora have been obtained in culture: *Trichomitopsis termopsidis* (Yamin 1978, 1980), *Trichonympha sphaerica* (Yamin 1981) from *Zootermopsis* sp. and *Trichomitus trypanoides* from *Reticulitermes santonensis* (Berchtold et al. 1995). Whether lower termites obligatory depend on the flagellates is a matter of debate. We observed one colony of *Zootermopsis angusticollis* and one colony of *Kalotermes flavicollis*, which lived without flagellates.

11.3 Bacteria

The total number of prokaryotes in the hindgut of termites lies within the range of 10^7–10^{11} ml^{-1} (Krasil'nikov and Satdykov 1969; Bignell et al. 1980b; To et al. 1980; Tholen et al. 1997; Berchtold et al. 1999). The bacterial groups from the termite gut identified by the 16S rDNA approach

mainly belong to the spirochetes, proteobacteria, Gram positives and *Bacteroides/Flavobacterium* branch (Berchtold et al. 1994, 1999; Berchtold and König 1996; Paster et al. 1996; Kudo et al. 1998; Ohkuma et al. 1999a–c; Tokuda et al. 2000; Watanabe et al. 2003). Differences in domain-level profiles were correlated with the termite diet, e.g. methanogenic bacteria were more abundant in soil-feeding termites compared to wood-feeding species (Braumann et al. 2001). The predominant bacterial flora in the hindgut contents is represented by spirochetes (Berchtold et al. 1994; Berchtold and König 1996; Ohkuma et al. 1999b; Lilburn et al. 2000). Up to now three species have been obtained in pure culture (Leadbetter et al. 1999; Graber et al. 2004). Most isolates obtained in pure culture belong to the branch of Gram positive bacteria and proteobacteria, but also isolates from the *Bacteroides/Fusobacterium* branch were obtained (Table 11.1). Intestinal bacteria are involved in the degradation of cellulose, hemicellulose and aromatic compounds as well as nitrogen fixation. They also contribute to the redox status of the gut.

11.4 Archaea

All Archaea identified in the termite gut belong to the families Methanobacteriaceae, Methanosarcinaceae and Methanomicrobiales and most identified species were assigned to the genus *Methanobrevibacter* (Braumann et al. 2001; Fröhlich and König 1999b; Tokura et al. 2000). Three species of the genus *Methanobrevibacter* have been obtained in pure culture from the termite *Reticulitermes flavipes*: *Mbr. cuticularis*, *Mbr. curvatus* and *Mbr. filiformis* (Leadbetter and Breznak 1996; Leadbetter et al. 1998).

11.5 Yeasts and Fungi

A variety of yeasts were isolated from the gut of the lower termites *Mastotermes darwiniensis*, *Zootermopsis angusticollis*, *Zootermopsis nevadensis*, *Neotermes jouteli*-related termite, *Reticulitermes santonensis*, *Heterotermes indicola* and the roach *Cryptocercus punctulatus* (Prillinger et al. 1996). Between 10^7 and 5×10^8 yeast cells were found per ml gut contents in *Zootermopsis angusticollis* and *Neotermes castaneus*. The isolates were assigned to 13 different species, which belonged to the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Pichia* and *Sporothrix*. Some isolates were able to hydrolyse xylan or cellulose (Schäfer et al. 1996; Wenzel et al. 2002). Some filamentous fungi (*Alternaria alternater*, *Aspergillus awamuri*, *As-*

Table 11.1. Bacterial isolates from the termite gut

Species	Termites	References
Branch: Gram-positive Bacteria		
Subbranch: Gram positive bacteria with high G + C content		
Order: Actinomycetales		
Arthrobacter sp.	*Nasutitermes nigriceps* (h) *Reticulitermes hesperus* (l) *Reticulitermes santonensis* (l)	Kuhnigk et al. (1994)
Aureobacterium sp.	*Nasutitermes nigriceps* (h) *Reticulitermes santonensis* (l)	Kuhnigk et al. (1994)
Cellulosimicrobium variabile	*Mastotermes darwiniensis* (l)	Bakalidou et al. (2002)
Cellulomonas cellulans	*Zootermopsis angusticollis* (l) *Nasutitermes nigriceps* (h)	Kuhnigk (1996)
Cellulomonas sp.	*Neotermes castaneus* (l) *Schedorhinotermes intermedius* (l) *Heterotermes indicola* (l) *Mastotermes darwiniensis* (l) *Zootermopsis angusticollis* (l)	Kuhnigk (1996); Wenzel et al. (2002)
Kocuria varians	*Zootermopsis angusticollis* (l)	Wenzel et al. (2002)
Microbacterium sp.	*Zootermopsis angusticollis* (l)	Wenzel et al. (2002)
Micrococcus luteus	*Reticulitermes santonensis*(l)	Kuhnigk (1996)
"*Micromonospora acetoformici*"	*Reticulitermes lucifugus*	Sebald and Prévot (1962)
Micromonospora propionici	*Amitermes minimus* (h)	Hungate (1946)
Rhodococcus equi	*Zootermopsis angusticollis* (l)	Kuhnigk (1996)
Nocardia sp.	*Reticulitermes santonensis* (l)	Kuhnigk and König (1997); Schäfer et al. (1996)
Streptomyces sp.	*Nasutitermes nigriceps* (h) *Cubitermes severus* (h) *Heterotermes indicola* (l) *Mastotermes darwiniensis* (l) *Procubitermes aburiensis* (h) *Zootermopsis angusticollis* (l)	Bignell et al. (1980b); Kuhnigk and König (1997); Mannesmann and Piechowski (1989); Schäfer et al. (1996)
Subbranch: Gram-positive bacteria with low G + C content		
Order: Bacillales		
Bacillus brevis	*Anacanthotermes ahngerianus* (h) *Zootermopsis angusticollis* (l)	Krasil'nikov and Satdykov (1969); Wenzel et al. (2002)

Table 11.1. (continued)

Species	Termites	References
Bacillus cereu	*Anacanthotermes ahngerianus* (h) *Nasutitermes nigriceps* (h) *Neotermes castaneus* (l) *Reticulitermes hesperus* (l) *Reticulitermes santonensis* (l)	Krasil'nikov and Satdykov (1969); Kuhnigk and König (1997); Thayer (1976); Schäfer et al. (1996)
Bacillus cereus-related isolate	*Zootermopsis angusticollis* (l)	Wenzel et al. (2002)
Bacillus circulans-related isolate	*Zootermopsis angusticollis* (l)	Wenzel et al. (2002)
Bacillus coagulans	*Mastotermes darwiniensis* (l)	Schäfer et al. (1996)
Bacillus firmus	*Reticulitermes santonensis* (l)	Kuhnigk (1996)
Bacillus licheniformis	*Reticulitermes santonensis* (l)	Kuhnigk and König (1997); Kuhnigk et al. (1994); Schäfer et al. (1996)
Bacillus megaterium	*Anacanthotermes ahngerianus* (h) *Mastotermes darwiniensis* (l) *Zootermopsis angusticollis* (l)	Krasil'nikov and Satdykov (1969); Kuhnigk (1996); Mannesmann and Piechowski (1989); Wenzel et al. (2002)
Bacillus mycoides	*Anacanthotermes ahngerianus* (h)	Krasil'nikov and Satdykov (1969)
Bacillus oleronius	*Reticulitermes santonensis* (l)	Kuhnigk et al. (1995)
Bacillus sp.	*Schedorhinotermes intermedius* (l) *Coptotermes acinaciformis* (l) *Coptotermes formosanus* (l) *Heterotermes indicola* (l)	Eutick et al. (1978); Kuhnigk (1996); Mannesmann and Piechowski (1989)
Bacillus sphaericus	*Odontotermes distans* (h) *Odontotermes obesus* (h) *Reticulitermes santonensis* (l) *Zootermopsis angusticollis* (l)	Kuhnigk (1996); Kuhnigk and König (1997); Schäfer et al. (1996)
Bacillus subtilis	*Anacanthotermes ahngerianus* (h) *Reticulitermes santonensis* (l)	Krasil'nikov and Satdykov (1969); Kuhnigk and König (1997); Schäfer et al. (1996)
Brevibacillus brevis	*Zootermopsis angusticollis* (l)	Wenzel et al. (2002)
Listeria sp.	*Reticulitermes santonensis* (l) *Zootermopsis angusticollis* (l)	Kuhnigk et al. (1994)
Paenibacillus macerans	*Mastotermes darwiniensis* (l)	Schäfer et al. (1996)
Paenibacillus sp.	*Zootermopsis angusticollis* (l)	Wenzel et al. (2002)

Table 11.1. (continued)

Species	Termites	References
Order Clostridiales		
Acetonema longum	*Pterotermes occidentis* (l)	Kane and Breznak (1991)
Clostridium mayombei	*Cubitermes speciosus* (h)	Kane et al. (1991)
Clostridium sp.	*Mastotermes darwiniensis* (l) *Nasutitermes nigriceps* (h)	Kuhnigk et al. (1994)
Clostridium termitidis	*Nasitutermes lujae* (h)	Hethener et al. (1992)
Clostridium beijerinckii	*Coptotermes formosanus* (l)	Taguchi et al.(1993)
Sporomusa aerivorans	*Thoracotermes macrothorax*	Boga et al. 2003
Sporomusa termitida	*Nasutitermes nigriceps* (h)	Breznak et al. (1988)
Order Lactobacillales		
Enterococcus avium	*Mastotermes darwiniensis* (l)	Kuhnigk (1996)
Enterococcus faecalis	*Anacanthotermes ahngerianus* (h) *Mastotermes darwiniensis* (l) *Reticulitermes flavipes* (l)	Bauer et al. (2000); Krasil'nikov and Satdykov (1969); Kuhnigk (1996)
Enterococcus faecium	*Mastotermes darwiniensis* (l)	Schäfer et al. (1996)
Enterococcus sp.	*Reticulitermes flavipes* (l)	Tholen et al. (1997)
Lactobacillus sp.	*Reticulitermes flavipes* (l)	Schultz and Breznak (1978)
Lactococcus cremoris	*Reticulitermes flavipes* (l)	Schultz and Breznak (1978)
Lactococcus lactis	*Reticulitermes santonensis* (l) *Reticulitermes flavipes* (l) *Thoracotermes macrothorax*(h)	Bauer et al. (2000); Kuhnigk (1996); Schultz and Breznak (1978)
Staphylococcus saprophyticus	*Reticulitermes santonensis* (l)	Kuhnigk (1996)
Staphylococcus sp.	*Nasutitermes exitiosus* (h) *Nasutitermes graveolus* (h) *Nasutitermes walkeri* (h)	Eutick et al. (1978)
Streptococcus sp.	*Nasutitermes exitiosus* (h) *Coptotermes lacteus* (l) *Cryptotermes primus* (l) *Heterotermes ferox* (l) *Mastotermes darwiniensis* (l) *Schedorhinotermes intermedius* (l) *Reticulitermes flavipes* (l)	Eutick et al. (1978); Kuhnigk (1996); Potrikus and Breznak (1980)

Table 11.1. (continued)

Species	Termites	References
Branch: Proteobacteria		
Division: α-Proteobacteria		
Order: Rhizobiales		
Ochrobactrum anthropi	*Heterotermes indicola* (l) *Nasutitermes nigriceps* (h) *Neotermes castaneus* (l) *Mastotermes darwiniensis* (l) *Reticulitermes santonensis* (l)	Kuhnigk (1996); Kuhnigk et al. (1994); Schäfer et al. (1996)
Rhizobium-related isolate	*Nasutitermes nigriceps* (h)	Kuhnigk et al. (1994)
Rhizobium etli-related isolate	*Zootermopsis angusticollis* (l)	Wenzel et al. (2002)
Order: Sphingomonadales		
Sphingomonas sp.	*Zootermopsis angusticollis* (l) *Mastotermes darwiniensis* (l)	Fröhlich and König (1999b); Kuhnigk (1996); Wenzel et al. (2002)
Division: β-Proteobacteria		
Order: Burkholderiales		
Alcaligenes faecalis	*Reticulitermes santonensis* (l)	Kuhnigk and König (1997)
Alcaligenes sp.	*Reticulitermes hesperus* (l)	Thayer (1976)
Alcaligenes xylosoxidans	*Mastotermes darwiniensis* (l)	Kuhnigk (1996)
Burkholderia cepacia	*Coptotermes formosanus* (l) *Nasutitermes nigriceps* (h) *Zootermopsis angusticollis* (l) *Reticulitermes santonensis* (l)	Mannesmann and Piechowski (1989); Schäfer et al. (1996)
Burkholderia sp. strain VE22	*Coptotermes formosanus*	Harazono et al. (2003)
Burkholderia sp.	*Heterotermes indicola* (l)	Kuhnigk (1996)
Comamonas acidovorans	*Nasutitermes nigriceps* (h)	Kuhnigk and König (1997); Kuhnigk et al. (1994)
Division: γ-Proteobacteria		
Order: Pseudomonadales		
Acinetobacter baumanii	*Mastotermes darwiniensis* (l) *Nasutitermes nigriceps* (h)	Kuhnigk and König (1997); Kuhnigk et al. (1994); Schäfer et al. (1996)
Acinetobacter sp.	*Nasutitermes nigriceps* (h)	Kuhnigk and König (1997)

Table 11.1. (continued)

Species	Termites	References
Pseudomonas aeruginosa	*Kalotermes flavicollis* (l) *Mastotermes darwiniensis* (l) *Nasutitermes nigriceps* (h) *Reticulitermes santonensis* (l)	Kuhnigk and König (1997); Kuhnigk et al. (1994); Mannesmann and Piechowski; (1989) Schäfer et al. (1996)
Pseudomonas cepacia	*Reticulitermes santonensis* (l)	Kuhnigk and König (1997)
Pseudomonas citronellolis	*Mastotermes darwiniensis* (l)	Kuhnigk and König (1997)
Pseudomonas fluorescens	*Kalotermes flavicollis* (l)	Mannesmann and Piechowski (1989)
Pseudomonas luteola	*Reticulitermes lucifugus* (l)	Mannesmann and Piechowski (1989)
Pseudomonas putida	*Nasutitermes nigriceps* (h) *Reticulitermes lucifugus* (l)	Kuhnigk et al. (1994); Mannesmann and Piechowski (1989)
Pseudomonas sp.	*Coptotermes formosanus* (l) *Kalotermes flavicollis* (l) *Nasutitermes nigriceps* (h) *Reticulitermes lucifugus* (l) *Odontotermes obesus* (h) *Odontotermes distans* (h)	Kuhnigk (1996); Mannesmann and Piechowski (1989)
Order: Aeromonadales		
Aeromonas sp.	*Kalotermes flavicollis* (l) *Nasutitermes nigriceps* (h)	Mannesmann and Piechowski (1989)
Order: Enterobacteriales		
Citrobacter freundii	*Coptotermes formosanus* (l) *Neotermes jouteli*-related (l)	Kuhnigk (1996); Mannesmann and Piechowski (1989)
Citrobacter sp.	*Nasutitermes nigriceps* (h) *Mastotermes darwiniensis* (l) *Neotermes castaneus* (l) *Odontotermes obesus* (h) *Odontotermes distans* (h) *Reticulitermes flavipes* (l) *Reticulitermes santonensis* (l)	Kuhnigk (1996); Mannesmann and Piechowski (1989); Potrikus and Breznak (1980); Schultz and Breznak (1978)
Citrobacter amalonaticus	*Neotermes castaneus* (l)	Mannesmann and Piechowski (1989)

Table 11.1. (continued)

Species	Termites	References
Citrobacter diversus	*Coptotermes formosanus* (l) *Neotermes castaneus* (l) *Neotermes jouteli*-related (l) *Reticulitermes lucifugus* (l)	Mannesmann and Piechowski (1989); Schäfer et al. (1996)
Citrobacter sp.	*Coptotermes formosanus* (l)	Harazono et al. (2003)
Enterobacter aerogenes	*Mastotermes darwiniensis* (l) *Coptotermes formosanus* (l) *Anacanthotermes ahngerianus* (h)	Krasil'nikov and Satdykov (1969); Kuhnigk et al. (1994); Mannesmann and Piechowski (1989); Schäfer et al. (1996)
Enterobacter agglomerans	*Coptotermes formosanus* (l)	Mannesmann and Piechowski (1989); Potrikus and Breznak (1977)
Enterobacter cloacae	*Anacanthotermes ahngerianus* (h) *Coptotermes formosanus* (l) *Mastotermes darwiniensis* (l) *Nasutitermes nigriceps* (h) *Odontotermes obesus* (h) *Odontotermes distans* (h) *Reticulitermes lucifugus* (l) *Reticulitermes santonensis* (l)	Krasil'nikov and Satdykov (1969); Kuhnigk (1996); Mannesmann and Piechowski (1989)
Enterobacter sp.	*Coptotermes acinaciformis* (l) *Coptotermes lacteus* (l) *Heterotermes ferox* (l) *Mastotermes darwiniensis* (l) *Nasutitermes graveolus* (h) *Reticulitermes flavipes* (l) *Reticulitermes santonensis* (l) *Schedorhinotermes intermedius* (l)	Eutick et al. (1978); Krasil'nikov and Satdykov (1969); Kuhnigk et al. (1994); Schultz and Breznak (1978)
Enterobacter sakazaki	*Reticulitermes santonensis* (l)	Schäfer et al. (1996)
Escherichia coli	*Anacanthotermes ahngerianus* (h)	Krasil'nikov and Satdykov (1969)
Hafnia alvei	*Coptotermes formosanus* (l)	Mannesmann and Piechowski (1989)
Hafnia-related sp.	*Odontotermes distans* (h) *Odontotermes obesus* (h)	Kuhnigk (1996)
Klebsiella oxytoca	*Coptotermes formosanus* (l)	Mannesmann and Piechowski (1989)

Table 11.1. (continued)

Species	Termites	References
Klebsiella pneumoniae	*Mastotermes darwiniensis* (l)	Kuhnigk et al. (1994); Mannesmann and Piechowski (1989); Schäfer et al. (1996)
Klebsiella sp.	*Coptotermes formosanus* (l)	Mannesmann and Piechowski (1989)
Serratia ficaria	*Reticulitermes santonensis* (l)	Kuhnigk et al. (1994); Schäfer et al. (1996)
Serratia marcescens	*Coptotermes formosanus* (l) *Mastotermes darwiniensis* (l) *Neotermes jouteli*-related (l) *Reticulitermes hesperus* (l)	Kuhnigk et al. (1994); Mannesmann and Piechowski (1989); Thayer (1976)
Serratia sp.	*Reticulitermes lucifugus* (l)	Mannesmann and Piechowski (1989)
<u>Order</u>: Xanthomonadales		
Xanthomonas maltophilia	*Nasutitermes nigriceps* (h) *Kalotermes flavicollis* (l) *Reticulitermes lucifugus* (l) *Odontotermes obesus* (h)	Kuhnigk (1996); Mannesmann and Piechowski (1989)
Xanthomonas sp.	*Zootermopsis angusticollis* (l)	Kuhnigk (1996)
<u>Division</u>: δ-Proteobacteria		
Desulfovibrio termitidis	*Heterotermes indicola* (l) *Cryptocercus punctulatus* (roach)	Fröhlich et al. (1999); Trinkerl et al. (1990)
Desulfovibrio intestinalis	*Mastotermes darwiniensis* (l)	Fröhlich et al. (1999)
Desulfovibrio desulfuricans	*Cubitermes speciosus* (h) *Reticulitermes santonensis* (l)	Braumann et al. (1990); Kuhnigk et al. (1996)
Desulfovibrio giganteus	*Cubitermes speciosus* (h)	Braumann et al. (1990)
<u>Branch</u>: *Bacteroides/Flavobacterium*		
<u>Order</u>: Bacteroidales		
Bacteroides termitidis	*Reticulitermes flavipes* (l)	Potrikus and Breznak (1980)
<u>Order:</u> Flavobacteriales		
Flavobacterium menigosepticum	*Reticulitermes lucifugus* (l)	Mannesmann and Piechowski (1989)

Table 11.1. (continued)

Species	Termites	References
Flavobacterium sp.	*Mastotermes darwiniensis* (l) *Reticulitermes santonensis* (l)	Eutick et al. (1978); Schäfer et al. (1996)
Order: Fusobacteriales		
Fusobacterium sp.	*Reticulitermes flavipes* (l)	Schultz and Breznak (1978)
Order: Sphingobacteriales		
Spirosoma-related sp.	*Zootermopsis angusticollis* (l)	Wenzel et al. (2002)
Branch: Spirochetes		
Treponema primitia	*Zootermopsis angusticollis* (l)	Graber et al. (2004)
Treponema azotonutricium	*Zootermopsis angusticollis* (l)	Graber et al. (2004)

Designation of the branches according to Woese et al. (1990). Designation of the orders according to Madigan et al. (2001): l, lower termite; h, higher termite

pergillus clavatus, *Aspergillus flavus*, *Aspergillus nidulans*, *Cladosporium* sp., *Paecilomyces fusiporus*, *Rhizopus stolonifer*) have also been isolated from gut fluids of termites (Rajagopal et al. 1979, 1981). Whether they grow with mycelia in the termite gut has yet to be demonstrated.

11.6 Microhabitats

The digestive system of termites consists of the foregut with the crop and the gizzard, the midgut and the hindgut (Fig. 11.1; Noirot 1995; Noirot and Noirot-Thimotheé 1969). Despite their small volumes of about 0.5–10 µl, the hindguts of termites are morphologically complex systems. The hindgut consists of five segments (P1–P5): the proctodeal segment, the enteric valve, the paunch, the colon and the rectum. The paunch is the microbial fermentation chamber, but the midgut and colon also contain microorganisms.

The termite gut can be described as an anaerobic gradient system, which is constantly supplied with oxygen via the epithelium. Examinations using microelectrodes have shown a pronounced spatial differentiation of termite hindguts with respect to pH (Noirot and Noirot-Timotheé 1969; Brune and Kühl 1996) and axial or radial gradients of oxygen or hydrogen concentrations (Veivers et al. 1980, 1982; Brune 1998; Brune et al. 1995a,b; Ebert and Brune 1997; Schmitt-Wagner and Brune 1999). In the case of *Mastotermes darwiniensis* oxygen diffusion gradients could be detected up to 100 µm below the epithelium (Berchtold et al. 1999).

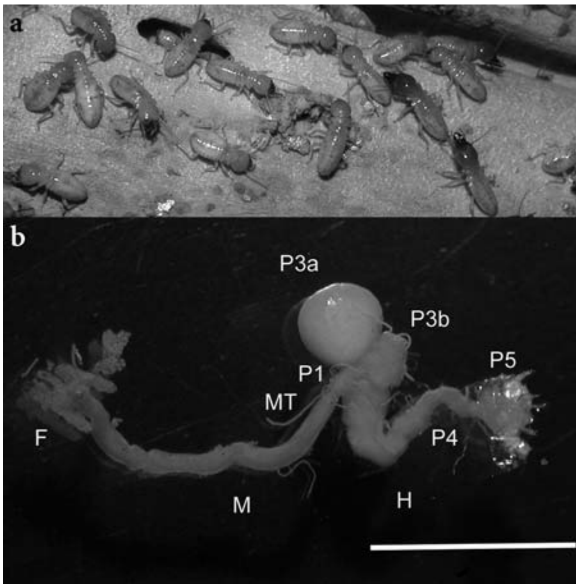

Fig. 11.1. The intestinal system of the Australian termite *Mastotermes darwiniensis*. **a** Workers and soldiers of *Mastotermes darwiniensis*. **b** Intestine. *F*=foregut with salivary glands, esophagus, crop and gizzard; *M* midgut; *MT* Malpighian tubules; *H* hindgut; *P*1 proctodeal segment of the hindgut followed by the enteric valve; *P3a* thin-walled part of the paunch; *P3b* thick-walled part of the paunch; *P*4 colon; *P*5 rectum; *bar* 5 mm

The pH of the midgut is about neutral and that of the hindgut is in the range between 6.0 and 7.5. In higher termites such as *Odontotermes obesus* parts of the gut have an alkaline pH up to 10.4 (Noirot and Noirot-Timotheé 1969; Bignell and Anderson 1980; Paul et al. 1986). The soil-feeding termites in particular possess more elongated and compartmentalized hindguts than wood-eating termites. Microscale pH measurements with microelectrodes demonstrated alkaline pH values of up to 12 in parts of the gut (Brune and Kühl 1996).

The paunch of termites is about 10^8 times smaller than a rumen resulting in a 500 times larger oxygen influx per unit volume (Brune 1998). Because of the small size the passage time of the food through the intestinal tract is only about 24 h (Breznak 1984). These facts have significant influences on the composition of the microbial flora. In the hindgut of termites strictly aerobic and microaerophilic bacteria could be isolated (Kuhnigk et al. 1994; Kuhnigk and König 1997; Wenzel et al. 2002).

In the termite gut four distinct microhabitats can be distinguished: the gut lumen, the surface and the cytoplasm of the flagellates and the gut epithelium. Electron microscopy studies of termite guts have shown that

prokaryotes occur either suspended in the contents, located within or on the surface of flagellates, or they are attached to the gut wall (Breznak and Pankratz 1977; Czolij et al. 1984–1986). Electron microscopic examinations of the gut of *Reticulitermes flavipes* and *Coptotermes formosanus* revealed that a heterogenous assemblage of bacteria colonize the gut (Breznak and Pankratz 1977; Schultz and Breznak 1978). Most morphotypes were located close to the epithelium. The midgut was scarcely colonized, but did possess distinctive cuboid-shaped, endospore-forming bacteria between the microvilli of the epithelium. Similar observations were made with other termites (Bignell and Anderson 1980; Bignell et al. 1980a; Czolij et al. 1986). Direct microscopic counts of bacteria ranged from 10^6 to 10^7 per gut and that of protozoa up to 4×10^4 per gut of *Reticulitermes flavipes*, while about 10^6 colony forming units per gut were obtained (Breznak 1982; O'Brien and Slaytor 1982).

Combining the rRNA approach with confocal laser scanning microscopy and oxygen microelectrode measurements, the gut microbial community within the hindgut of the wood-feeding Australian termite *Mastotermes darwiniensis* was examined (Berchtold et al. 1999). The anterior part of the paunch (P3a region) of *Mastotermes darwiniensis* is tightly packed with large flagellates (1285 ± 244 cells of *Deltotrichonympha operculata*, *Deltotrichonympha nana*, *Koruga bonita*, and *Mixotricha paradoxa* per termite) (Berchtold et al. 1999). From the combined volume of the larger flagellates it can be estimated that 95% of the anterior part of the paunch (P3a region) is occupied by flagellate protozoa. In *Mastotermes darwiniensis* approximately 90% of the DAPI-stained cells were associated with the protozoa in the P3a region, only 2% were attached to the gut wall and the rest were found in the residual liquid volume of the lumen fraction. In contrast, the flagellate population in the P3b/P4 region was much smaller. The flagellate cells represented only 10% of the total volume. The potentially colonizable surface area provided by the flagellates in the P3a region exceeds that of the wall by a factor of 18. In contrast to the P3a region about 85% of the prokaryotes of the P3b region are attached to the wall. The prokaryotic cell density on the P3b/P4 epithelium (2×10^6 mm^{-2}) is considerably higher than that on the P3a surface (3×10^4 mm^{-2}). The concentration of non-attached cells in the residual volume is higher in the P3a region (7×10^9 cells ml^{-1}) than in the P3b/P4 region (1×10^9 cells ml^{-1}). The flagellates preferentially colonize the paunch, while low numbers are found in the colon (P4 region) (Breznak and Pankratz 1977; Yoshimura et al. 1992; Berchtold et al. 1999).

It is surprising that strictly anaerobic bacteria such as methanogens (Leadbetter and Breznak 1996; Leadbetter et al. 1998), sulfate reducers (Berchtold et al. 1999) and clostridia (Tokuda et al. 2000) occur also attached to the aerated gut epithelium. Beside the gut epithelium (Leadbetter

and Breznak 1996; Leadbetter et al. 1998) methanogens occur as ecto- and endosymbionts of flagellates (Fröhlich and König 1999a,b,c; Tokura et al. 2000). Ectosymbiotic bacteria of flagellates can easily be detected by electron microscopy (Radek et al. 1992, 1996; Dyer and Khalsa 1993; Radek and Tischendorf 1999) or after staining the cells with ethidium bromide (Fröhlich and König 1999a).

Ectosymbiotic spirochetes have been identified on the surface of flagellates (Iida et al. 2000; Noda et al. 2003). *Mixotricha paradoxa,* a trichomonad from the hindgut of the Australian termite *Mastotermes darwiniensis* Froggatt, is a rare example of a movement symbiosis between eukaryotic and prokaryotic microorganisms (Cleveland and Grimstone 1964). The surface of *Mixotricha paradoxa* is covered with spirochetes and a rod-shaped bacterium. The four flagella at the anterior end seem only to alter the direction of movement, while the ectosymbiotic spirochetes propel the flagellate cells. Based on a 16S rDNA sequence analysis after a semi-specific PCR and subsequent fluorescence in situ hybridization applying helper oligonucleotides and a denaturing step of the 16S rRNA, three different spirochete clones could be clearly identified on the surface of the protozoal cells (Wenzel et al. 2003). They belonged to the *Treponema* cluster. The rod shaped bacterium showed highest 16S rDNA sequence similarity to *Bacteroides*-related species. Due to its low phylogenetic relationship to its next relatives in the data base it should represent a so far undescribed species.

11.7 Lignocellulose Degradation

The microbiology of the gut ecosystem of termites has been summarized in several review articles (Breznak 1982; O'Brien and Slaytor 1982; Breznak and Brune 1994; Varma et al. 1994). Formally, the microbial degradation of lignocellulose can be divided into three stages (Table 11.2; cf. König et al. 2002); a hydrolytic stage, a fermentative/oxidative stage and a methanogenic/acetogenic stage. These stages have been proved by microbial isolates (cf. König et al. 2002). The molecular aspect of cellulose degradation in the termite gut is described in Chapter 9.

11.7.1 The Hydrolytic Stage of Lignocellulose Degradation

Termites have different feeding habits. They can feed on wood, grass, dung, humus and soil. The passage of the food through the digestive tract needs 24 h (Breznak 1984). Therefore, this symbiotic system degrades wood much

more efficiently than fungi. The symbiosis between termites and the gut flora can be described as a synergistic interaction of a mechanical rasp provided by the termites and of microbial enzymes located in the gut. The fermentation products are acetic acid, propionic acid and butyric acid (mol%; 94, 3.3, 2.3 in *Reticulitermes flavipes*; 98.6, 1.3, 0.0 in *Zootermopsis angusticollis*) and the gases CO_2, H_2 and CH_4 (Odelson and Breznak 1983).

Wood consists of cellulose, hemicellulose, and lignin (Fengel and Wegener 1984). Since the 1920s it has been known that termites hydrolyse cellulose (Cleveland 1924; Beckwith and Rose 1929; Dickmann 1931) The extent of cellulose degradation is between 59 and 99% (Seifert and Becker 1965; Esenther and Kirk 1974; Mishra 1979). In lower termites wood particles are endocytosed by the archaezoa (Honigberg 1970). The isolated flagellates *Trichomitopsis termopsidis* and *Trichonympha sphaerica* from *Zootermopsis* need cellulose for growth (Yamin 1980, 1981; Odelson and Breznak 1985). These protozoa have significant cellulose and hemicellulose-degrading activities. Cellulase genes in flagellates have been identified (Othoko et al. 2001; Li et al. 2003). Cellulose is fermented to acetate, CO_2, and H_2. Acetate is mainly used by the termites as an energy source and is oxidized to CO_2.

A different situation was found in the case of the flagellates of the most primitive Australian termite *Mastotermes darwiniensis* (Li et al. 2003). Two endoglucanases with similar apparent molecular mass of approx. 36 kD have been isolated. Surprisingly, the N-terminal sequences of these cellulases exhibited significant homology to cellulases of termite origin, which belong to glycosyl hydrolase family 9. The corresponding genes were detected not in the mRNA pool of the flagellates, but in the salivary glands of *Mastotermes darwiniensis*. This showed that cellulases isolated from the flagellate cells originated from the termite host. Using a PCR-based approach DNA encoding cellulases belonging to glycosyl hydrolase family 45 were obtained from micromanipulated nuclei (Fröhlich and König 2000) of the flagellates *Koruga bonita* and *Deltotrichonympha nana*. These results indicated that the intestinal flagellates of *Mastotermes darwiniensis* take up the termite's cellulases from gut contents, probably attached to wood particles. *Koruga bonita* and *Deltotrichonympha nana* possess at least their own endoglucanase genes, which are still expressed, but without significant enzyme activity in the nutritive vacuole. These findings give the impression that the gut flagellates of *Mastotermes darwiniensis* are heading towards a secondary-loss of their own endoglucanases and they use exclusively termite cellulases.

Cellulose digestion by bacteria was discussed controversially (Thayer 1978; Slaytor 1992). Several cellulolytic bacteria were isolated from termites (Wenzel et al. 2002; cf. König et al. 2002). In contrast to the rumen, where strictly anaerobic cellulolytic bacteria belonging to the genera

Table 11.2. Intestinal isolates involved in the different stages of lignocellulose degradation in the termite gut

	Substrate	I. Stage Hydrolytic microorganisms	II. Stage Oxidative/fermentative microorganisms	III. Stage Acetogenic, methanogenic, sulfate-reducing bacteria	
Wood mechanical degradation to µm-particles by termites	**Cellulose** (34–62%)	**Cellulase** Flagellates: *Trichomitopsis termopsidis*, *Trichonympha sphaerica*	β–Glucosidase		
		Bacteria: *Alcaligenes*, *Azospirillum*, *Bacillus*, *Brevibacillus*, *Cellulomonas*-related spp., *Clavibacter*, *Clostridium*, *Corynebacterium*, *Klebsiella*, *Kocuria*, *Paenibacillus*, *Microbacterium*, *Micromonospora*, *Nocardioforme*, Rhizobia, *Ochrobactrum*, *Paenibacillus*, *Sphingomonas*, *Spiroso-ma*-related spp., *Streptomyces*	*Actinobacteria*, *Arthrobacter*, *Au-reobacterium*, *Bacillus*, *Citrobacter*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactococcus*, *Nocardia*, *Ochrobactrum*, *Pseudomonas*, Rhizobia, *Rhodococcus*, *Serratia*, *Streptomyces*		**Food source of termites: acetate, propionate, butyrate, microbial cells**
		Yeasts (*Cryptococcus*, *Filobasidium*)	Yeasts		
	Hemicellulose (14–32%)	1.4-β-Xylanase 1.3-β-Galactanase	β–D-Galactosidase α–L-Arabinosidase β–L-Xylosidase	2 H_2+O_2 $\Rightarrow$ H_2O Lactate $\Rightarrow$ Acetate+CO_2 *Desulfovibrio*	**Excreted end products:**

		Actinobacteria, *Acinetobacter*, *Bacillus*, *Flavobacterium*-related spp., *Ochrobactrum*, *Paenibacillus*, *Pseudomonas*, *Streptomyces*	*Actinobacteria*, *Acinetobacter*, *Arthrobacter*, *Aureobacterium*, *Bacillus*, *Burkholderia*, *Citrobacter*, *Cellulomonas*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Nocardia*, *Ochrobactrum*, *Pseudomonas*, *Paenibacillus*, *Rhizobium*, *Serratia*, *Staphylococcus*, *Streptomyces*	$4H_2+2CO_2 \Rightarrow$Acetate$+2\ H_2O$ *Acetonema*, *Clostridium*, *Sporomusa*, Spirochetes $4H_2+CO_2 \Rightarrow CH_4+2H_2O$ *Methanobrevibacter*	**CO_2, H_2, CH_4** **Microbial cells**
		Yeasts (*Candida*, *Debaryomyces*, *Pichia*, *Sporothrix*)	Yeasts		
	Aromatic compounds (5%)		*Acinetobacter*, *Arthrobacter*, *Alcaligenes*, *Aureobacterium*, *Bacillus*, *Burkholderia*, *Citrobacter*, *Comamonas*, *Enterobacter*, *Klebsiella*, *Listeria*, *Nocardia*, *Ochrobactrum*, *Pseudomonas*, Rhizobia, *Serratia*, *Streptomyces*		
	Lignin (18–39%)	*Streptomyces*			**Excreted: modified lignin**
N_2-fixation			*Enterobacter*, *Desulfovibrio*, *Citrobacter*, Rhizobia, *Klebsiella*, Spirochetes		

Ruminococcus, Butyrivibrio and *Bacteroides* are present, the cellulolytic bacteria in the termite gut are facultatively anaerobic or microaerophilic bacteria. Species of the genera *Bacillus* are predominant with titers of up to $10^7\,ml^{-1}$ gut contents (Wenzel et al. 2002). Cellulolytic yeasts have also been isolated from *Zootermopsis angusticollis* and *Mastotermes darwiniensis* (Wenzel et al. 2002). The isolates from *Zootermopsis angusticollis* belong to the genus *Sporothrix*. Fungus-growing termites of the subfamily Macrotermitinae culture the fungus *Termitomyces* sp. in their nests. Together with the fungal nodules they probably consume fungal cellulolytic enzymes (Martin 1991; Rouland et al. 1991; cf. Chap. 17). Termites themselves produce also cellulases (Yokoe 1964). Recently the corresponding gene was sequenced (Watanabe et al. 1998; Tokuda et al. 1997, 1999; Li et al. 2003). In *Reticulitermes speratus* the main portion of the cellulolytic activities (cellulase, glucosidase) were found in the salivary glands, while the xylanolytic activities (xylanase, xylosidase) were found in the anterior part of the hindgut (Inoue et al. 1997).

Hemicelluloses are chemically complex and include different heteropolysaccharides such as arabinans, galactans, glucans, mannans, and xylans. The most common polyose is xylan. The complete degradation of xylan requires a synergistically acting set of enzymes: endo-β-1.4-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinosidase, acetylesterase and feruloyl- or p-coumaroyl esterase (Fengel and Wegener 1984; Varma et al. 1994). The full set of enzymes required for release of all constituents of xylan has not been found in a single bacterial isolate from the termite gut (Saxena et al. 1993; Schäfer et al. 1996). Hemicellulose is degraded through synergistic activities of the glycolytic enzymes. An effective hemicellulose-degrading community of microbes is found in the termite gut (Breznak and Brune 1994; Varma et al. 1994; Schäfer et al. 1996). Hemicelluloses are degraded between 49 and 78% (Mishra 1979). The xylan degrading bacterial isolates mainly belong to the actinomycete- and *Clostridium*-branch of Gram positive bacteria as well as enterobacteria and *Pseudomonas*, *Acinetobacter* and *Ochrobactrum* species (Schäfer et al. 1996).

Based on the analysis of termite faeces some authors reported between 5% and 83% of lignin degradation (Seifert and Becker 1965; Butler and Buckerfield 1979; Cookson 1988). The involvement of gut microbes was not determined. In order to approach the problem of microbial lignin degradation in the gut the degradation of lignin monomers, other aromatic compounds, and dimeric lignin model compounds by the intestinal flora was studied (Kuhnigk et al. 1994; Brune et al 1995a; Kuhnigk and König 1997). From the lower termites *Mastotermes darwiniensis* and *Reticulitermes santonensis* and from the higher termite *Nasutitermes nigriceps* anaerobic, facultatively anaerobic and aerobic bacterial strains were isolated in media containing lignin monomers or other aromatic compounds. Most of the

monomeric aromatic compounds and dimeric model compounds were degraded in the presence of oxygen by the mixed gut flora and pure cultures. Under anaerobic conditions the side chains of the aromatic compounds were only modified, but the aromatic ring was not split. Decarboxylation and reduction of the double bond in the side chain of phenyl propane derivatives were obtained in the absence of oxygen. Synthetic dehydrogenative lignin was not degraded under anaerobic or aerobic conditions. The results suggested that in the termite hindgut the breakdown of the aromatic ring systems requires oxygen (Kuhnigk et al. 1994), which is most probably supplied via the aerated paunch epithelium.

Besides stomodeal and proctodeal food exchange termites feed also on their own faeces. During passage of wood particles through the digestive tract they are inoculated with microorganisms. Members of bacterial genera, especially of Gram positive bacteria, belonging to the actinomycete branch, such as *Streptomyces* sp. (Pasti and Belli 1985; Pasti et al. 1990; Schäfer et al. 1996) which are believed to attack lignin, also were isolated. However, the breakdown activities of the gut isolates seems to be negligible, except in *Nasutitermes takasagoensis*. In this termite lignin components were found to be degraded in significant amounts (28%; Kato et al. 1998). The microorganisms may start to attack lignin near the aerated gut epithelium and they continue their activity outside of the gut. The faecal pellets may also be inoculated by fungi in the nest. Therefore, repeated recycling of faecal material may increase the efficiency of the digestion of wood particles by a termite colony. This procedure may be a kind of extracorporal digestion. In fungus combs of the fungus-growing termite *Macrotermes gilvus* the mutualistic fungus *Termitomyces* sp. was shown to progressively degrade lignin and thus enhance the digestibility of cellulose for the termites (Hyodo et al. 2000). Laccase activity acquired probably from fungi, was only found in fungus-growing termites of the genera *Macrotermes, Odontotermes* and *Pseudacanthotermes* (Mora and Lattaud 1999). Wood contains a large number of extractable low-molecular weight compounds such as phenols, tannins and terpenoid (Fengel and Wegener 1984), which may be used by aromatics-degrading bacteria. The degradation of these extractives may not only provide fatty acids as carbon sources for the host, but represents also a detoxification, since these compounds are known to be harmful to insects.

The majority of termite species are considered to be humivorous. In feeding experiments with ^{14}C-labeled humic model compounds labeled either in their proteinaceous or aromatic building blocks as substrates, it was shown that the peptide moiety were selectively digested, but the aromatic components seemed not to be an important food source of humivorous termites such as *Cubitermes orthognathus*, *Cubitermes umbratus* and *Thoracotermes macrothorax* (Ji et al. 2000).

11.7.2
The Oxidative/Fermentative Stage of Lignocellulose Degradation

A diverse variety of bacterial strains, belonging mainly to the Gram positive bacteria and proteobacteria have been isolated. They are able to degrade monosaccharides and oligosaccharides, which are produced during the hydrolysis of cellulose and hemicellulose. Oligosaccharide-hydrolyzing enzyme activities of the isolates have been determined (Schäfer et al. 1996).

11.7.3
The Methanogenic/Acetogenic Stage of the Lignocellulose Degradation

In the third step of lignocellulose degradation three physiological prokaryotic groups are involved: methanogenic bacteria, homoacetogenic bacteria and sulfate reducing bacteria. Symbiosis between animals and intestinal methanogens is widespread (Hackstein und Stumm 1994; Hackstein et al. 1996). High amounts of methane are released into the atmosphere by termites (Zimmermann et al. 1982; Martius et al. 1993; Rouland et al. 1993). Acetogenesis and methanogenesis were considered to be the most important terminal electron-accepting processes in the hindgut of termites (Braumann et al. 1992). Methanogens detected in the termite gut belong to the families Methanobacteriaceae, Methanosarcinaceae and Methanomicrobiales (Braumann et al. 2001; Ohkuma et al. 1999c). At least for xylophagous termites, acetate is the major end product of microbial fermentation in the gut (Breznak and Switzer 1986). Acetate can be used by the termites as an energy and a carbon source. Relatively high concentrations of acetate (58–81 mmol l^{-1}) have been found (Odelson and Breznak 1983). The homoacetogens belong to the genera *Acetonema*, *Clostridium*, and *Sporomusa*. Spirochetes occur free living and attached to the surface of protozoa. One homoacetogenic isolate has been described (Leadbetter et al. 1999).

Until now, all sulfate-reducing bacteria isolated from the termite gut belong to the genus *Desulfovibrio* (Braumann et al. 1990; Trinkerl et al. 1990; Fröhlich et al. 1999b; Kuhnigk et al. 1996) and have been assigned to four species (*D. desulfuricans*, *D. giganteus*, *D. intestinalis*, *D. termitidis*). Up to 10^7 living cells per ml paunch content were counted in *Mastotermes darwiniensis* and *Reticulitermes santonensis* (Kuhnigk et al. 1996). When termites were fed with cellulose powder moistened with a Na_2SO_4 solution, the total number of sulfate reducers in the gut contents increased up to 10^8 ml^{-1} in *Reticulitermes santonensis* and *Heterotermes indicola*. Typical features of *Desulfovibrio* are the incomplete oxidation of electron donors to acetate, and the capacity of hydrogen consumption (Cypionka 2000). This genus is also able to reduce oxygen with hydrogen (Knallgas reaction).

Microaerophilic conditions, with a constant inflow of oxygen via the epithelium, are found near the gut epithelium (Brune et al. 1995b). Sulfate reducing bacteria, alike methanogens, have been found attached to the aerated gut epithelium (Berchtold et al. 1999). The sulfate concentration in the termite gut (0.3–0.7 mmol l^{-1}) is higher than in fresh water sediments (Kuhnigk et al. 1996). Under hydrogen limitation sulfate reducers may outcompete acetogenic and methanogenic bacteria. The nutritional versatility of acetogens may be one of the reasons, why they outcompete methanogens in xylophagous termites (Breznak and Blum 1991). Experiments with *Cubitermes* spp. showed a difference in the spacial activity of methanogens and acetogens (Tholen and Brune 1999) depending on the hydrogen concentrations. The anterior regions of the gut of *Cubitermes* spp. accumulated hydrogen, whereas the hydrogen concentration in the posterior hindgut was below the detection limit (Schmitt-Wagner and Brune 1999). All three groups may principally co-exist, although differences in the number of methanogens and acetogens have been found in wood-feeding and soil-feeding termites (Braumann et al. 1992).

Sulfate-reducing *Desulfovibrio* species are thus able to catalyze all reactions of a complete sulfur cycle (Cypionka 2000). *Desulfovibrio termitidis* has been found in the termite *Heterotermes indicola* and in the wood-feeding roach *Cryptocercus punctulatus*, which live in Asia and North America (Fröhlich et al. 1999). Likewise, *Desulfovibrio intestinalis* exists in geographically remote hosts, *Mastotermes darwiniensis* from Australia and *Odontotermes obesus* from India. Two species, *Desulfovibrio desulfuricans and Desulfovibrio giganteus,* were found in the termite *Cubitermes speciosus* (Braumann et al. 1990). Based on the fact that members of the same *Desulfovibrio* species can be found in phylogenetically and geographically remote hosts, it may be speculated that *Desulfovibrio* species have repeatedly and independently colonized the intestine or were already present in termites before the separation of the continents. Belonging to the physiological group of sulfate reducers, members of the genus *Desulfovibrio* are anaerobes, although some exhibit strong oxygen tolerance (Cypionka 2000). Termites benefit from the *Desulfovibrio* species, which produce acetate, and thus provide nutrition for their hosts. In co-culture *Desulfovibrio desulfuricans*, from the gut used the fermentation products of other fermentative gut bacteria and produced mainly acetate. They also contribute to the anoxic milieu of the gut by producing hydrogen sulfide and removing oxygen with hydrogen or low molecular weight organic or reduced sulfur compounds. The isolates were able to oxidize sulfur compounds such as sulfide, thiosulfate and sulfite. Sulfide was oxidized to sulfate as indicated by the amount of sulfide to oxygen consumed (molar ratio: 1:2). Performing a complete sulfur cycle the desulfovibrios can balance the redox status of the gut contents. This activity would allow sulfate recovery and keep

a complete sulfur cycle running. Sulfide reoxidation could prevent toxic effects of H_2S. Only substrates that are of no direct use for the termites (H_2, formate) were oxidized completely, while others were transformed to acetate. Rates of oxygen consumption were as high as 1,570 nmol O_2 min^{-1} (mg protein)$^{-1}$ with electron donors in excess, which is also the case in the termite gut. These are the highest respiration rates of sulfate-reducing bacteria ever measured. These rates exceed by far the respiration rates of typical aerobes or facultative anaerobes such as *Escherichia coli* (300 nmol O_2 min^{-1} (mg protein)$^{-1}$) (Kuhnigk et al. 1996).

A sulfate-reducing bacterium was also enriched from the gut of the rose-chafer *Pachnoda marginata*, which showed the highest 16S rDNA sequence identity (93%) to *Desulfovibrio intestinalis* and *Desulfovibrio* strain STL1 (Dröge et al. 2005). Compared to *Mastotermes darwiniensis* (1×10^7 cells of SRB per ml gut contents), sulfate reducing bacteria occur in higher numbers in the gut contents of *Pachnoda marginata* reaching cell titers of up to 2×10^8 cells per ml gut contents. In vitro sulfate reduction rates were determined with SRB from the gut contents of the termite *Mastotermes darwiniensis* and the beetle *Pachnoda marginata*. Due to the higher cell titer, the sulfate reduction rate of *Pachnoda marginata* was 104 nmol h^{-1} ml^{-1} and, therefore, 21 times higher than that of *Mastotermes darwiniensis*. In addition, in vivo sulfate reduction was detected in *Mastotermes darwiniensis*, which indicates that sulfate reducers play an active role in the sulfur metabolism in the termite gut.

11.8 Nitrogen Fixing Bacteria

Wood is deficient in nitrogen (Fengel and Wegener 1984). Nitrogen fixation has been demonstrated in all families of termites, and atmospheric nitrogen is a considerable nitrogen source for xylophagous termites (Tayasu et al. 1994; Golichenkov et al. 2002). Several bacteria have been isolated with the capability to fix molecular nitrogen. They belong to the genera *Enterobacter* (Potrikus and Breznak 1977), and *Desulfovibrio* (Kuhnigk et al. 1996) as well as *Rhizobium-/Sinorhizobium*-related (Wenzel et al. 2002) and *Treponema*-related strains (Lilburn et al. 2000). The nitrogen-fixing bacteria improve the nitrogen balance of their hosts. In the gut of the termite *Neotermes koshunensis* expression of nitrogen fixation genes was detected by direct amplification of the nifH cDNA from mRNA (Noda et al. 1999; Ohkuma et al. 1999a). Seasonal patterns of nitrogen fixation in termites was found. The nitrogenase activity was found to be highest in autumn and spring, and lowest in winter and summer (Curtis and Waller 1998).

11.9 Intracellular Symbiosis

While all examined species of cockroaches have been shown to harbour intracellular bacteria in specialized cells (mycetocytes, bacteriocytes) of the fat body, in termites bacteriocytes are restricted to *Mastotermes darwiniensis*. All of these bacteria have been assigned to the same eubacterial lineage, with the intracellular bacteria of *Mastotermes darwiniensis* as the sister group to the cockroach bacteria. (Bandi et al. 1997). They belong to the *Blattabacterium* group of the *Bacteroides/Flavobacterium* branch. These symbionts are thought to have originated from a bacterium that infected an ancestor common to cockroaches and termites. Furthermore, non-mycetocyte intracellular bacteria are widespread in termites, which belong to the *Wolbachia* group (Bandi et al. 1995, 1997).

11.10 Conclusions

The symbiotic ecosystem "termite gut" can be described as a mechanical rasp provided by the host, which grinds wood down to a micron scale and a fermentation chamber of microbial and termite's own hydrolytic enzymes. Microbial genera containing species with the ability to produce polysaccharide- or oligosaccharide-degrading enzymes are combined in Table 11.2 (stages 1 and 2). The goal of this symbiotic system is to convert lignocellulose mainly to acetate, propionate and butyrate (Odelson and Breznak 1983) and microbial cells. The advantage for the host is the capability to use hardly degradable compounds as a food source, while the microflora has a constant substrate supply from the host. The low chain fatty acids produced by the microbiota are used by the termites as an energy and a carbon source. The gases hydrogen, methane and carbon dioxide are released in significant amounts into the atmosphere. Lignin seems not to be degraded during the passage through the gut, but is excreted. The ecosystem "termite gut" is small, but the microbial flora and the interactions between the microbiota and their host are very complex. Termites play a significant ecological role in the mineralization of organic material, especially of lignocellulose. Further studies of this exciting ecosystem may also lead to novel biotechnological applications of the wood-degrading flora.

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12 Diversity and Molecular Analyses of Yet-Uncultivated Microorganisms

Moriya Ohkuma, Yuichi Hongoh, Toshiaki Kudo

12.1 Introduction

Termites harbor a dense and diverse population of microorganisms consisting of both eukaryotes and prokaryotes in their gut. The relationship between termites and the gut microorganisms is a well-known example of symbiosis, and it is established that termites are totally dependent on the microbes for the utilization of their food. The metabolism in the gut and the roles of the symbionts in host nutrition have been extensively studied (see other chapters in this volume and recent reviews: Bignell 2000; Breznak 2000; Brune and Friedrich 2000; Inoue et al. 2000; König et al. 2002; Ohkuma 2003). A large number of studies involving pure cultures of symbionts have contributed to the remarkable progress. However, our understanding of biology of the gut microbiota is still poor because many of dominant species within the community are yet-uncultivated in laboratories. An estimation of culturability of the gut microbiota is at most 10% of the direct microscopic count. Such a situation is not unusual in microbial ecology study in which culture-based methods enable to sample only a limited fraction of inhabitants in natural environments.

The application of molecular methodology to ecological studies in the past decade has enhanced our ability to assess naturally occurring biodiversity. Culture-independent molecular approaches are advantageous to estimate real microbial assemblages. In those approaches, small-subunit rRNA genes in nucleic acid extracted from natural microbial communities are usually analyzed. Such approaches have been applied to the analysis of the gut community of termites, to demonstrate that a majority of the gut community consists of a diverse array of yet-uncultivated and novel species (Ohkuma 2002). In this chapter, we outline the recent advances in the culture-independent molecular studies of the gut microbial community, particularly that of lower termites which harbor flagellated protists (single cell eukaryotes) in the gut.

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12.2
Phylogenetic Identification of Symbiotic Protists

Molecular phylogeny of the symbiotic flagellate protists in the termite gut has been studied in most cases based on the 16S-like rRNA genes. These genes are obtained from a mixed-population of gut protists by PCR-based methods, and the origins of the sequences are identified by whole-cell in situ hybridization using sequence-specific probes (Berchtold and König 1995; Ohkuma et al. 1998, 2000; Dacks et al. 2001; Gerbod et al. 2002; Keeling and Leander 2003; Moriya et al. 2003; Stringl and Brune 2003). Alternatively, a careful collection of specific cells is subjected to PCR (Dacks and Redfield 1998; Keeling et al. 1998; Fröhlich and König 1999; Keeling 2002). The genes encoding phylogenetic indicators such as elongation factor-1α, and α-tubulin are also studied (Moriya et al. 1998, 2001).

The gut flagellates have been classified by their morphology and they belong to the orders Hypermastigida, Trichomonadida, and Oxymonadida. The former two are classified into the phylum Parabasalia. Hypermastigotes and oxymonads are unique in nature in that their occurrence has been documented only in termites and wood-eating cockroaches. A sister group relationship between trichomonads and hypermastigotes is evident, supporting the classification of Parabasalia. Parabasalids together with diplomonads represent earlier extant lineages of eukaryotes. Parabasalids, diplomonads, and oxymonads lack mitochondria, while parabasalids contain instead an anaerobic metabolic organelle called a hydrogenosome. Oxymonads and diplomonads seem to have neither mitochondria nor hydrogenosomes, and thus a close evolutionary relationship between them was proposed. However, a specific sister relationship between oxymonads and diplomonads is ruled out. The 16S-like rDNA studies reveal that oxymonads are related to an excavating group of protists (the genus *Trimastix*) which have small membrane-bound organelles resembling hydrogenosomes in the cell, suggesting that oxymonads are not primitively amitochondriate but that they lost mitochondria secondarily (Dacks et al. 2001). Although oxymonads and parabasalids share a similar cytoskeletal structure of huge bundled microtubules, called an axostyle, they are distantly related, which is also evident by the analysis of microtubule using α-tubulin sequences (Moriya et al. 2001).

16S-like rDNA sequences of a number of parabasalids including yet-unidentified taxa have been investigated (Berchtold and König 1995; Gunderson et al. 1995; Dacks and Redfield 1998; Keeling et al. 1998; Ohkuma et al. 1998, 2000; Fröhlich and König 1999; Gerbod et al. 2000, 2002; Keeling 2002; Keeling and Leander 2003). Trichomonads seem to form a monophyletic group while hypermastigotes are paraphyletic, and trichomonads have emerged within a lineage of the hypermastigotes. Hypermastigotes are

distinguished from trichomonads by having a large number of flagella and they show a more complex cellular morphology than trichomonads. The molecular studies imply that loss of cytoskeletal structures and reduction of the number of flagella may have occurred during parabasalian evolution (Viscogliosi et al. 1999; Ohkuma et al. 2000). A number of oxymonad taxa have also been studied with 16S-like rDNA sequences and they are found to be monophyletic (Keeling and Leander 2003; Moriya et al. 2003; Stringl and Brune 2003). One of the outstanding cytoskeletal features of oxymonads is the holdfast or attachment apparatus used to adhere to the gut wall of the host. It is postulated that this apparatus may have occurred once during their evolution and then lost in some lineages (Moriya et al. 2003).

12.3 Methanogenic Archaea

Methane emission from termites has often been claimed as a significant contribution to global atmospheric methane. Methanogenic archaea in the gut of termites have been characterized by culture-independent analyses of archaeal 16S rDNA (Ohkuma et al. 1995, 1999b; Fröhlich and König 1999; Shinzato et al. 1999, 2001; Tokura et al. 2000; Brauman et al. 2001; Friedrich et al. 2001). Methanogens in the gut of lower termites are mostly related to the genus *Methanobrevibacter* in the family Methanobacteriaceae, whereas those in the gut of higher termites belong to the family Methanosarcinaceae or the order Methanomicrobiales in addition to *Methanobrevibacter*. The former is related to the genus *Methanomicrococcus* and the latter represents a not-yet-cultivated genus. Archaeal 16S rDNAs affiliated with the Termoplasmales and with the Crenoarchaeota were also identified from the gut of some termites (Shinzato et al. 1999; Friedrich et al. 2001). In general, soil-feeding higher termites emit more methane than wood-feeders, and a correlation of relative archaeal abundance with the methane emission rates is observed (Brauman et al. 2001).

12.4 Diversity of Eubacteria

The gut eubacterial population is extensively investigated in the termite *Reticulitermes speratus* by the 16S rDNA sequences. In order to avoid some biases in PCR, several combinations of different primers and several PCR conditions are examined. A total of 1923 clones in the 16 clone libraries of different PCR conditions are analyzed (Hongoh et al. 2003a,b). The clones are sorted into phylotypes using the criterion of 97% identity of nearly

entire sequences of 16S rDNA region, to obtain up to 312 phylotypes. There are few numerically dominant and many very rare phylotypes, which is different from a species abundance deduced theoretically to approximate a log-normal distribution, namely, few dominant and few rare species but many species of intermediate abundance. This difference implies that the number of sequenced clones is still insufficient to reflect the diversity of all bacteria in the termite gut, though the coverage of eubacterial population by the clone analysis is expected higher than 90%. The eubacterial diversity is roughly estimated as about 6000 phylotypes per ml and 700 phylotypes per gut. It is noted that some dominant phylotypes represent the bacteria associated physically with the gut protists (see below), thus the accurate estimation of the microbial diversity in the gut must take much their localization into account.

Table 12.1 shows phylogenetic affiliation of the eubacterial 16S rDNA phylotypes found in the gut community of *R. speratus*. Spirochetes are the most abundant eubacterial population in the gut accounting for about a half of the analyzed clones and sorted to 61 phylotypes. Most of the spirochete phylotypes are affiliated to the genus *Treponema* (57 phylotypes) and divided into two phylogenetic clusters (Fig. 12.1). The cluster I contains diverse phylotypes of gut spirochetes and includes strains isolated recently as pure cultures from the termite gut (Leadbetter et al. 1999), whereas cluster II is also abundant in clone numbers but the fewer phylotypes. Similar diversity in spirochete population is shown in *Reticulitermes flavipes* and *Neotermes koshunensis* (Lilburn et al. 1999; Noda et al. 2003) by using a spirochete-specific PCR primer.

The clones affiliated with the low G+C Gram-positive bacteria particularly those with clostridia were very diverse and sorted into 134 phylotypes, though the clone abundance of about 10% was less than that of spirochetes. Other predominant groups of 5–15% clone abundance were *Bacteroides*-related and Termite group I clones. The termite group I of bacteria are distantly related to any known eubacterial division (Ohkuma and Kudo 1996), and only five phylotypes within the range of more than 95% sequence similarity are found from the termite so far. The rest are comprised of Proterobacteria, Actinobacteria, *Mycoplasma*, and so on (see Table 12.1).

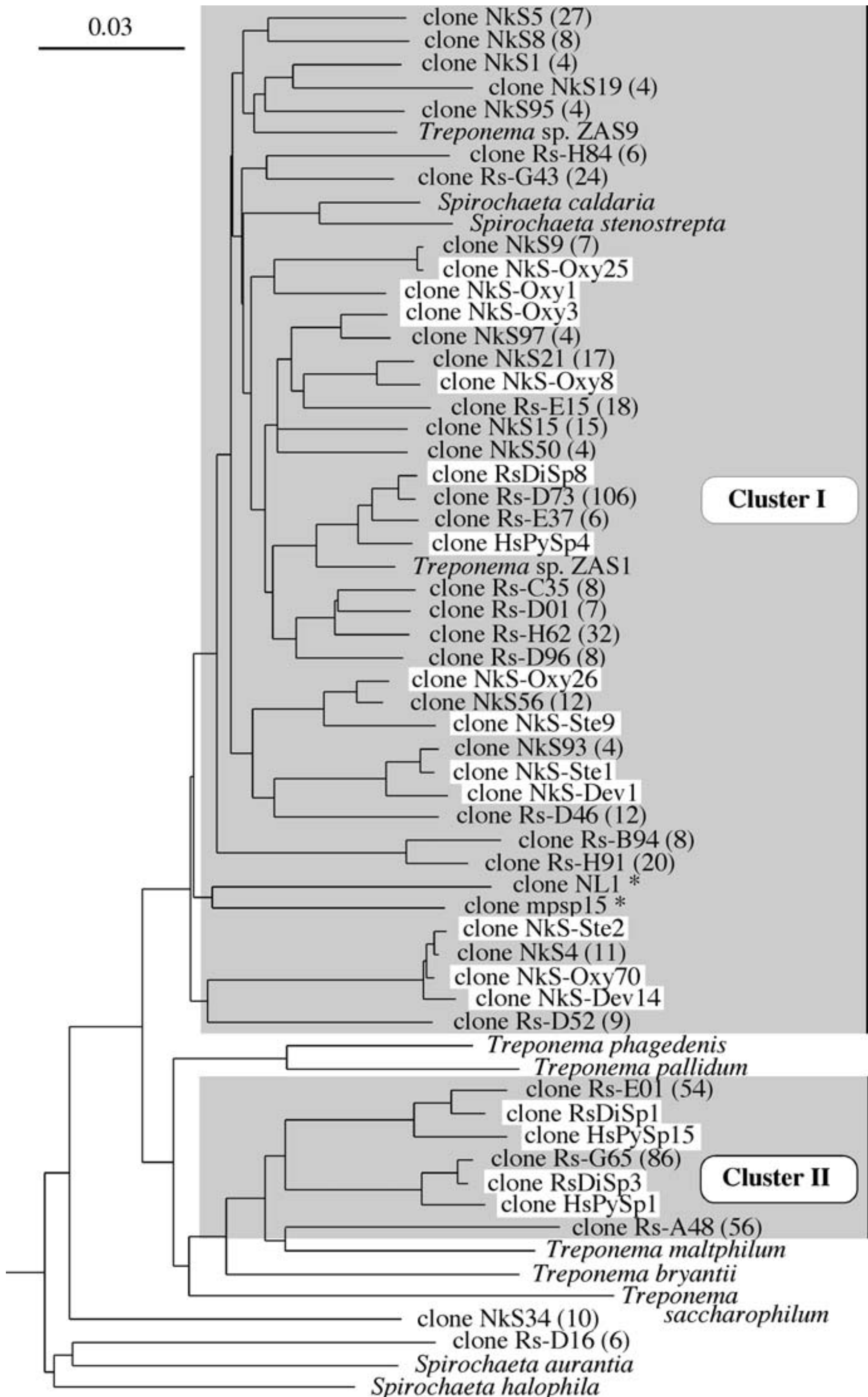
Comparisons with the database sequences reveal that almost all phylotypes found in the gut are new sequences at the phylotype-level criterion (97% nucleotide identity). Some phylotypes are clustered with each other and/or with sequences derived from other termite species, implying the existence of bacterial lineages unique in the termite guts. These results indicate that the termite gut community is a rich reservoir of novel and yet-uncultivated bacterial diversity. When spirochetes are compared between two *Reticulitermes* species, several phylotypes are closely

Table 12.1. Classification and number of eubacterial 16S rDNA phylotypes found in the gut community of *Reticulitermes speratus*

Taxon	RDP-code	Number of phylotypes
Spirochaetes		
Treponema	[2.27.3.2]	57
Spirochaeta	[2.27.3.1, 3]	3
Undescribed cluster	None	1
CFB group		
Bacteroides and relatives	[2.15.1]	36
Low G+C Gram-positives		
Clostridium/Eubacterium	[2.30.4, 5, 9]	134
Mycoplasma	[2.30.8.4]	7
Lacto-, *Enterococcus*	[2.30.7.20, 21]	4
Desulfosporosinus	[2.30.3.2]	1
Termite group I	[2.19]	5
Actinobacteria	[2.30.1]	14
Proteobacteria		
Alpha-	[2.28.1]	17
Beta-	[2.28.2]	4
Delta-	[2.28.4.1, 10]	6
Epsilon-	[2.28.5]	3
Gamma-	[2.28.3]	2
Planctomycetes	[2.20]	4
Anaerobaculum	[2.11]	3
Verrucomicrobia	[2.10]	2
Cyanobacteria	[2.21]	1
Acidobacteria	[2.25.3.6]	1
OP11	[2.28.4.2]	2
TM7	[2.21.5]	1
Unidentified cluster	None	3
Unidentified bacterium	None	1
Total number of phylotypes		312

Table is modified from Hongoh et al. (2003a). See the reference for the taxa not defined by RDP code. The data include the results obtained in Hongoh et al. (2003b)

related to each other. However, phylotypes of distinct phylogenetic positions are usually obtained from different termite genera when comparing both spirochetes and *Bacteroides*-related clones (Ohkuma and Kudo 1998; Ohkuma et al. 1999a, 2002). In terms of co-evolution with the host termites, the gut symbionts shows complex evolutionary history due to their occurrence in numerous phylogenetic positions. Probably, phylogenetically distinct species have been acquired as symbionts at many times by diverse termite species and then evolved within the gut to the present diversity.


0.03
clone NkS5 (27)
clone NkS8 (8)
clone NkS1 (4)
clone NkS19 (4)
clone NkS95 (4)
Treponema sp. ZAS9
clone Rs-H84 (6)
clone Rs-G43 (24)
Spirochaeta caldaria
Spirochaeta stenostrepta
clone NkS9 (7)
clone NkS-Oxy25
clone NkS-Oxy1
clone NkS-Oxy3
clone NkS97 (4)
clone NkS21 (17)
clone NkS-Oxy8
clone Rs-E15 (18)
clone NkS15 (15)
clone NkS50 (4)
clone RsDiSp8
clone Rs-D73 (106)
clone Rs-E37 (6)
clone HsPySp4
Treponema sp. ZAS1
clone Rs-C35 (8)
clone Rs-D01 (7)
clone Rs-H62 (32)
clone Rs-D96 (8)
clone NkS-Oxy26
clone NkS56 (12)
clone NkS-Ste9
clone NkS93 (4)
clone NkS-Ste1
clone NkS-Dev1
clone Rs-D46 (12)
clone Rs-B94 (8)
clone Rs-H91 (20)
clone NL1 *
clone mpsp15 *
clone NkS-Ste2
clone NkS4 (11)
clone NkS-Oxy70
clone NkS-Dev14
clone Rs-D52 (9)
Cluster I
Treponema phagedenis
Treponema pallidum
clone Rs-E01 (54)
clone RsDiSp1
clone HsPySp15
clone Rs-G65 (86)
clone RsDiSp3
clone HsPySp1
clone Rs-A48 (56)
Cluster II
Treponema maltphilum
Treponema bryantii
Treponema saccharophilum
clone NkS34 (10)
clone Rs-D16 (6)
Spirochaeta aurantia
Spirochaeta halophila

◀ **Fig. 12.1.** Phylogenetic relationship of gut spirochetes of the termites *Reticulitermes speratus*, *Neotermes koshunensis*, and *Hodotermopsis sjoestedti*. Clone names are tagged with the initials of the host termites (*Rs*, *Nk* and *Hs*, respectively). The number of clones belonging to each phylotype is indicated in parentheses. Only the phylotypes that represent more than six clones from *R. speratus* and four from *N. koshunensis* are shown. The two clusters are *highlighted on the gray background*. The clones *on the white background* are obtained from ectosymbiotic spirochetes of gut protists. The clones tagged with *RsDiSp* and *HsPySp* are from the ectosymbionts of the oxymonads *Dinenympha* and *Pyrsonympha*, respectively (Iida et al. 2000). The clones tagged with *NkS-Dev*, *NkS-Ste*, and *NkS-Oxy* are from the ectosymbionts of the trichomonads, *Devescovina* and *Stephanonympha*, and the oxymonad *Oxymonas*, respectively (Noda et al. 2003). The clones marked with *asterisks* denote that origins of the sequences are identified as free-swimming spirochetes by in situ hybridizations (Berchtold and König 1996; Paster et al. 1996). Tree was constructed by the neighbor-joining method and rooted with other spirochete genera (not shown). The *scale bar* represents 0.03 substitutions per nucleotide position

12.5 Spatial Organization of Gut Community

The gut microbial community is not evenly dispersed. A variety of associations of prokaryotes with gut protists are observed (Radek 1999; Dolan 2001; Dyer 2002; Ohkuma 2002, 2003; Wenzel et al. 2003). Dense populations of endosymbiotic bacteria are frequently occurred inside the cells of protists. On the cell surfaces of the protists, the attachment of prokaryotes (ectosymbiosis) is also frequently observed. A typical case for the ectosymbiosis is the attachment of spirochete-like bacteria on the protist cells. In addition, there are significant amount of both free-swimming populations and populations associated directly or indirectly with gut epithelium. In order to investigate the distribution of respective microbial population in the gut, several approaches have been applied. In situ hybridization targeting rRNA of individual cells is a powerful approach to visualize localization and morphology of the cells (Paster et al. 1996; Berchtold et al. 1999; Berchtold and König 1996; Iida et al. 2000; Tokuda et al. 2000; Noda et al. 2003). Micro-scale fractionation is also advantageous. A micromanipulator-aided microcapillary has been successfully applied to collect a single cell or a pool of the cells under microscopy (Fröhlich and König 1999; Iida et al. 2000; Tokura et al. 2000; Noda et al. 2003).

Methanogens, which are easily detected by their autofluorescense, often occur on and within the cells of the gut protists. Associations of methanogens with the gut wall are also known. Methanogens free in the gut fluid are rarely observed at least in lower termites. After the micro-scale fractionation of the gut protist species, the endosymbiotic methanogens are phylogenetically identified as yet-uncultivated species of

Methanobrevibacter (Fröhlich and König 1999; Tokura et al. 2000). Some closely related methanogens are shared between different protist species. The phylogenetic relationships of the endosymbiotic methanogens tend to be closer among those harbored by different protists within a single termite species than among those harbored by the respective protists in different termite species. The methanogens may have the nature to infect or to be transferred to the protists and it is possible that the protists have acquired their endosymbionts within the gut of each termite species. After careful fractionation of the gut epithelium, the resident methanogens are also investigated phylogenetically (Tokura et al. 2000). The sequences identified from the gut wall are related to the *Methanobrevibacter* species isolated from the termite gut (Leadbetter and Breznak 1996; Leadbetter et al. 1998). These cultivated species are morphologically similar to the cells residing on or near the gut epithelium. The protist-associated methanogens are phylogenetically distinct from those attached to the gut epithelium. Thus, a single termite species harbors several methanogen species and they show a distinct spatial distribution within the gut.

The ectosymbiotic spirochetes attached onto the cell surfaces of the gut protist species have been identified phylogenetically by the clone analyses after micro-fractionations and in situ hybridizations (Iida et al. 2000; Noda et al. 2003). Spirochetes belonging to both clusters I and II are identified as the ectosymbionts in the termites *R. speratus* and *Hodotermopsis sjoestedti* (Fig. 12.1). A group-specific probe for cluster II enables to detect a large population of ectosymbionts.Two 16S rDNA phylotypes in cluster II are identified in each protist species. A probe for cluster I also detects a population of the ectosymbionts in each protist. Thus, at least three spirochete species attach to a single protist cell, though the composition of each spirochete species is different among the protist species. The cluster I probe recognizes free-swimming spirochetes as well as the ectosymbionts whereas the cluster II probe rarely detects free-swimming ones.

In the case of the termite *N. koshunensis*, the cluster II probe gives no positive signal in the gut community while the cluster I probe detects both ectosymbiotic and free-swimming spirochetes. The absence of the cluster II spirochete population is supported by the analyses of 16S rDNA clones from the whole gut community (Noda et al. 2003). In the gut of *N. koshunensis*, three protist species harbor ectosymbiotic spirochetes, and at least two distinct phylotypes of the cluster I spirochetes are detected as ectosymbionts in each protist. The results indicate that attachment of multiple spirochete species to a single protist cell is a general feature. Common phylotypes are often shared among the protist species. Since the ectosymbiotic spirochetes are dispersed in the phylogenetic tree (Fig. 12.1), there seem to be multiple independent origins of spirochete attachment to gut protists. Some spirochetes distribute randomly over the surface of the host protist cells

whereas others locates on a restricted portion of the protist cells, which is especially the case with *Mixotricha paradoxa*, a rare example of moving symbiosis between protists and spirochetes (Wenzel et al. 2003). The latter observation evokes a specific relation between the cellular organization of the protists and the role of the spirochetes. Most of the ectosymbiotic spirochete species correspond to the abundant phylotypes in the 16S rDNA clone libraries from the whole gut community, implying the ectosymbionts represent dominant populations in the gut.

The studies of methanogens and spirochetes reveal that gut microbes are not evenly dispersed but occupy distinct micro-niches within the termite guts. The various associations involving diverse microbial species are observed with gut protists and with gut wall. Not only the micro-scale fractionation and in situ hybridization but also sectioning of gut is advantageous as the structural integrity of gut is retained (Berchtold et al. 1999; Tokuda et al. 2000). It is noted that the relationships between the gut protists and their associated prokaryotes are attractive to investigate in terms of the symbiosis-accelerated evolution of eukaryotic cells, since the gut protists represent early emerging groups of eukaryotes. Meanwhile, studies using microelectrodes have shown the presence of steep gradients of oxygen and hydrogen within the gut, and these physicochemical conditions have a significant impact on the microbial activities (Brune and Friedrich 2000). Since the gut community is highly structured, a link between microbial activities and spatial organization of respective populations is important in order to understand real nature of the gut symbiotic system.

12.6 Toward the Function of Gut Symbionts

Analysis of rRNA sequences has opened a window to investigate the diversity and composition of natural microbial communities, avoiding the largely unrepresentative nature of microbial cultivation. In some instances, metabolic functions of microbes enable to be inferred within specific rRNA phylogenetic groups such as methanogens. However, in general, physiological properties of individual microbial populations in a community cannot be predicted on the basis of only the rRNA sequences. Particularly, yet-uncultivated groups of microbes are hard to predict their functions (cultured microorganisms from the termite gut: cf. Chap. 14) Under such circumstances, genes encoding metabolically important enzymes can be useful to investigate the microbial functions in the community.

The gut protists are essential for decomposition of the ingested cellulose as well as hemicellulose in lower termites. It is considered that the ingested cellulose can be partially degraded by the cellulases of termite origin, which

are excreted from the salivary glands or the mid-gut, and the cellulose not hydrolyzed in the anterior portion of the gut then travels to the hindgut, where it can be endocytosed and fermented by the symbiotic protists. The existence of this dual system explains the capacity of termites to assimilate cellulose almost completely. Cellulases (endo-β-1,4-glucanases) of termite origin have been identified and characterized (Watanabe and Tokuda 2001). However, phylogenetic studies tell nothing about the cellulolytic system of the gut protists. Through the analyses of cDNA library constructed with the mixed-population of gut protists, their cellulase genes have been identified (Nakashima et al. 2002; Ohtoko et al. 2000; Watanabe et al. 2002; Li et al. 2003). The cellulases of the gut protists identified so far are classified into glycosyl hydrolase families (GHF) 7 and 45, whereas those of the termite origin to GHF9. A diverse array of cellulase genes are detected, suggesting a cooperation of multiple cellulases within a single protist cell to attain their efficient decomposition.

Nitrogen fixation by the gut symbionts is critical since termites thrive on a nitrogen-poor diet. Identification based on the cultivation as well as by the 16S rDNA analysis, however, provides limited information on the diversity and types of organisms that fix nitrogen in termites. Indeed, culture-independent analyses of nitrogen fixation gene *nifH*, which is PCR-amplified and characterized from the gut microbial community as in the case of 16S rDNA clones, have provided evidence for a previously unexpected diversity of nitrogen fixing microbes in diverse termites (Ohkuma et al. 1996, 1999c; cultured nitrogen fixing bacteria: cf. Chap. 14). The gene *nifH* encoding dinitrogenase reductase is evolutionarily conserved and has often been used to detect nitrogen-fixing microorganisms in natural samples. Most of the *nifH* clones analyzed from the gut communities so far are affiliated to the anaerobic *nif*, the alternative *nif*, and pseudo-*nif* groups, the latter of which probably functions in some process other than nitrogen fixation. The anaerobic *nif* group includes *nifH* from anaerobic microorganisms such as clostridia and sulfur reducers. The alternative *nif* group consists of nitrogenases carrying no molybdenum (Mo) as a cofactor and those from some Archaea. The detected *nifH* groups are similar within each termite family but different among termite families, suggesting an evolutionary trend of diazotrophic inhabitants of the gut community. Most of the sequences from the termites form lineages distinct from those previously recognized in studies of cultivated nitrogen fixers, although some lineages are related to the *nifH* sequences identified from spirochetes that includes isolates from the termite gut (Lilburn et al. 2001). The results indicate the presence of diverse potentially nitrogen-fixing microbial assemblages in the guts of termites, and the majority of them are as yet uncharacterized.

Since the expression of nitrogenase genes is strictly regulated, it must be carefully addressed that the existence of *nifH* sequences does not always

mean that nitrogen fixing activity is being expressed by the corresponding microbes. In fact, the analysis of the *nifH* mRNA constituents of the gut microbial community in *N. koshunensis* reveals that only a few among the diverse *nifH* sequences found in the gut community are preferentially expressed in the gut (Noda et al. 1999). The preferentially expressed gene encodes a member of the alternative *nif* group. The quantitative analysis of its mRNA under several feeding conditions of the termite indicates that the expression level of this gene is critical for nitrogen fixation activity of the termite. The expression is completely repressed with the presence of nitrogen sources in the termite food, whereas no significant decrease in the expression level is observed when the diet contains Mo, which ordinarily represses alternative *nif* genes. In contrast, the *nifH* mRNA analysis of the gut community in *Coptotermes formosanus*, in which no alternative *nif* member is found, reveals that members of the anaerobe group of *nif* are preferentially detected (Noda et al. 2002). The factors involved in the choice of diazotrophic symbionts by termites need to be clarified in order to understand the nature of symbiotic nitrogen fixation in termites.

As shown in the analyses of nitrogen fixation genes, the marker genes directly linked to the microbial activities are useful to characterize the responsible populations. It is emphasized that not only the presence of the genes but their expression should be studied to know real contribution of the respective microbial populations. This points to an important concept in microbial ecology in general. Even if the cultivation in laboratory reveals a microbial function, the pure cultures in any artificial condition could not represent their natural habitats. Therefore, it is very important to know the real contribution of respective microbial populations to a certain activity in natural environments. Studies on diverse microbial functions with more genes are anticipated, and for this purpose, the development of PCR primers or probes for targeting functions is important. As shown in the studies of the protist cellulases, genome or cDNA surveys may also be advantageous, though exhaustive, to search for a novel function of microbes, which is particularly attractive because the gut community consists of a numerous number of novel and yet-uncharacterized microbes.

12.7 Conclusions

The application of culture-independent molecular approaches provides a new way to characterize the microbial populations in the gut community of termites. Beyond the mere description of phylogenetic diversity, the future studies will be directed to the characterization of the in situ localization of individual populations, and to the direct link of the identity

of individual cells to their functions. We must make much account of the various interactions among the symbionts and with the host termites. Cultivation and characterization of some specific microbes is greatly anticipated, which may be helpful if aided by molecular probes. However, now that the gut symbiotic community of termites has been shown to consist of yet-uncharacterized novel microbes, culture-independent approaches will be of more and more significance.

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13 The Intestinal Yeasts

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13.1 Introduction

Yeast isolates were obtained from the hindgut of the lower termites *Mastotermis darwiniensis* (Mastotermitidae), *Zootermopsis angusticollis*, *Zootermopsis nevadensis* (Hodotermitidae), *Neotermes jouteli* (Kalotermitidae), *Reticulitermes santonensis*, *Heterotermes indicola* (Rhinotermitidae) and the roach *Cryptocercus punctulatus* (Prillinger et al. 1996). The monosaccharide composition of the cell wall, the ubiquinone system, partial sequencing of 18S ribosomal DNA and the ultrastructure of septal pores indicate that most yeast species belonged to the Endomycetales. They were assigned to the genera *Candida*, *Debaryomyces*, *Pichia* and *Sporothrix*. Other species showed affinities to the Basidiomycetes in particular to the genera *Trichosporon*. Between 10^7 and 5×10^8 yeastcells were found per ml gut contents in *Zootermopsis angusticollis* and *Neotermes castaneus*. Some isolates were able to hydrolyse cellulose or xylan (Schäfer et al. 1996; Wenzel et al. 2002). The yeasts isolated from the hindgut can be considered symbionts.

13.2 Morphological Characterization

Table 13.1 contains the yeast isolates obtained from the gut of lower termites and the roach genus *Cryptocercus*. Species 1–3 and 5–13 revealed a uniform cell wall morphology. Walls were electron-transparent and non-lamellate (Prillinger et al. 1996). Usually, a small outer electron-dense layer was additionally present. An electron-dense multilayered lamellar cell wall and an enteroblastic mode of budding was characteristic for species 4. Hyphal septa were found in species 1, 3, 4, and 7–12 only. Among these species, a central micropore-like channel was observed in species 1, 3, 8–12. Distinct

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Table 13.1. Species assignment of the yeast isolates after RAPD-PCR analysis and phenotypic features

Species no.	Host	Strain	Cell wall composition (mol%)						UREA	DBB	EAC	FERM	UB	Closest relative[a]
			Glc	Man	Gal	Xyl	Rha	Fuc						
1	*M. d.*	MM1	30	56	14	–	–	–	–	–	–	–	9	*C. b.*
	M. d.	MS2	40	48	12	–	–	–	–	–	–	–	9	*C. b.*
	M. d.	MS3	42	46	12	–	–	–	–	–	–	–	9	*C. b.*
	M. d.	MS4	39	48	13	–	–	–	–	–	–	–	9	*C. b.*
	M. d.	MYG4	51	38	11	–	–	–	–	–	–	–	9	*C. b.*
	M. d.	MYG9	41	48	11	–	–	–	–	–	–	–	9	*C. b.*
2	*M. d.*	MYG1	52	48	–	–	–	–	–	–	–	+	9	*D. h.f.*
	M. d.	MYG2	50	50	–	–		–	–	–	–	+	9	*D. h.f.*
3	*M. d.*	MYG3	60	48	–	–	–	–	–	–	–	+	9	*D. h.f.*
4	*M. d.*	MYG6	59	16	–	20	–	–	+	+	–	–	9	*T.* sp.
5	*Z. a.*	ZAG1	55	45	–	–	–	–	–	–	–	+	9	*D. h.f.*
	Z. a.	ZAG6	51	49	–	–	–	–	–	–	–	+	9	*D. h.f.*
6	*Z. a.*	ZAG8	47	53	–	–	–	–	–	–	–	+	9	*D. h.f.*
7	*Z. a.*	ZAG10	52	25	2	–	24	–	+	–	–	–	$10H_2$	*S. s.*
	Z. n.	ZM1	38	33	2	–	21	–	+	–	–	–	$10H_2$	*S. s.*
	Z. n.	ZM2	39	33	2	–	26	–	+	–	–	–	$10H_2$	*S. s.*
	Z. n.	ZM3	41	34	2	–	23	–	+	–	–	–	$10H_2$	*S. s.*
8	*Z. n.*	ZM5	42	52	–	–	–	–	–	–	–	+	9	*D. h.f.*
	Z. n.	ZS5	52	48	–	–	–	–	–	–	–	+	9	*D. h.f.*
9	*H. i*	HG1	42	39	19	–	–	–	–	–	–	+	9	*D. h.f.*

Table 13.1. (continued)

Species no.	Host	Strain	Cell wall composition (mol%)						UREA	DBB	EAC	FERM	UB	Closest relative[a]
			Glc	Man	Gal	Xyl	Rha	Fuc						
10	*N. j.*	NM1	30	56	14	–	–	–	–	–	–	–	9	*C. e.*
	N. j.	NS1	34	51	15	–	–	–	–	–	–	–	9	*C. e.*
	R. s.	RM7	36	47	17	–	–	–	–	–	–	–	9	*C. e.*
11	*R. s.*	RM1	40	60	–	–	–	–	–	–	–	+	9	*D. h.f./P. g.*
	R. s.	RM6	46	54	–	–	–	–	–	–	–	+	9	*D. h.f./P. g.*
12	*R. s.*	RS2	37	49	14	–	–	–	–	–	–	–	9	*P. p.*
	R. s.	RM3	36	59	5	–	–	–	–	–	–	–	9	*P. p.*
13	*C. p.*	CG1	38	62	–	–	–	–	–	–	–	+	9	*C. s.*
	C. p.	CG3	58	42	–	–	–	–	–	–	–	+	9	*C. s.*

C. b.=*Candida blankii*, *C. e.*=*Candida edax*, *C. s.*=*Candida sake*, *D. h. f.*=*Debaryomyces hansenii* var. *fabryii*, *H. i.*=*Heterotermes indicola*, *M. d.*=*Mastotermes darwiniensis*, *N. j.*=*Neotermes jouteli*, *P. g.*=*Pichia guilliermondii*, *P. p.*=*Pichia pini*, *R. s.*=*Reticulitermes santonensis*, *S. s.*=*Sporothrix schenkii*, T=*Trichosporon*, *Z. a.*=*Zootermopsis angusticollis*, *Z. n.*=*Zootermopsis nevadensis*

[a] Preliminary assignment

UREA=hydrolysis by urease, DBB=diazonium blue B test, EAC=formation of extracellular amyloid compounds, FERM=fermentation of glucose, UBI=major ubiquinone system

simple pores with Woronin bodies were only present in species 7. Dolipores associated with a number of more or less sacculate bodies were characteristic for species 4. Each saccule consisted of an outer limiting membrane which was continuous with the endoplasmic reticulum at adseptal base and an inner curved electron-dense layer at the abseptal base. Thus cell wall ultrastructure indicates that species 4 is a basidiomycete and species 1–3 and 5–13 are ascomycetous. The species 1, 3, 8–12 share the narrow micropores with members of the Endomycetales (van der Walt and von Arx 1985). The presence of distinct simple pores with Woronin bodies confirms the affinities to filamentous Ascomycetes for species 7 (Smith and Batenburg van der Vegte 1985, 1986). The septal pore apparatus of species 4 is essential similar to that of the Tremellales s. str. (Filobasidiaceae included; Oberwinkler 1985). It is known that the pore-associated saccules vary considerably in length and shape among the Tremellales (Berbee and Wells 1988). The saccules of species 4 are similar to those reported for the Tremellales s. str., but they are in contrast to, e.g., *Sirobasidium magnum* (Moore 1978) and *Tremella globospora* (Berbee and Wells 1988) rather shallow and appear to represent somehow an archaic form of the typical *Tremella* saccules. Nearly all species with this septal pore type are mycoparasites (Bauer and Oberwinkler 1990). Similar doliporous septa were shown recently in the genus *Trichosporon* by Gueho et al. (1992). Although the genus *Trichosporon* is remarkable homogeneous on the basis of partial 26S rRNA sequences, except *T. pullulans*, various septal types are known in different species. The parenthesome could be nearly absent (*T. sporotrichoides*), irregularly vesicular (*T. asahii*, *T. laibachii*) or consists of tubuli which in cross section were seen as small round vesicles with double membranes (*T. asahii*, *T. coremiforme*).

13.3
Phenotypic and Genotypic Characterization

Based on RAPD-PCR analysis, yeasts isolated from the hindgut of the lower termites *Mastotermis darwiniensis* (four species) (Mastotermitidae), *Zootermopsis angusticollis* (three species), *Zootermopsis nevadensis* (two species) (Hodotermitidae), *Neotermes jouteli* (one species) (Kalotermitidae), *Heterotermes indicola* (one species), *Reticulitermes santonensis* (two species) (Rhinotermitidae), and the roach *Cryptocercus punctulatus* (one species) were assigned to 13 different species (Table 13.1; Prillinger et al. 1996).

To characterize the yeast isolates from lower termites and a wood-feeding roach on the molecular level the qualitative and quantitative monosaccharide patterns of purified and hydrolyzed yeast cell walls, the ubiquinone

system, partial18S rDNA sequences (positions 948 to 1273 homologous to the *Saccharomyces cerevisiae* gene) were used. Based on this polyphasic approach it becomes obvious that the majority of yeast species of lower termites and the roach comprise a phylogenetically homogeneous group belonging to the Endomycetales (species 1, 2, 3, 5, 6, 8–13; Table 13.1; Prillinger et al. 1996). These species are characterized by an ubiquinone Q-9. From the cell wall monosaccharide pattern two distinct groups could be separated. The presence of mannose and glucose ("*Saccharomyces* type") is characteristic for species 2, 3, 5, 6, 8, 11, and 13. A glucose-mannose-galactose pattern was found in species 1, 9, 10, and 12. Based on yeast cell wall sugars, the ubiquinone Q-9 and ribosomal DNA sequence homology of 100% (region 948–1273), species 3 and 11 belong to the genus *Debaryomyces* represented by the type species *D. hansenii* (CBS 789). Species 6 and 8 came close to the genus *Debaryomyces* but need further investigations. Species 2 and 5 are congeneric with each other. Similar to species 13 these isolates were not certainly identified at the genus level so far. Although the galactose containing group of yeast species 1, 9, 10, and 12 exhibited a closer relationship to the Lipomycetaceae in comparison with species of the Dipodascaceae it was not possible to establish conspecificity with an already known yeast species of the Endomycetales. Species 4 is a heterobasidiomycetous yeast with high sequence homology to the genus *Trichosporon*. *Cryptococcus humicolus* suggested by the PC identification program (Version 3; Barnett 1994) could be excluded. This is in agreement with the presence of arthroconidia. Species 7 showed a cell wall monosaccharide pattern and ubiquinone system characteristic for filamentous Ascomycetes of the genus *Ophiostoma* s. str. or anamorphic species of the genus *Ophiostoma* (De Hoog 1993) and *Hyphozyma roseonigra*. The genus *Hyphozyma* was heterogeneous with respect to the ubiquinone system. Whereas the cell wall monosaccharide pattern exhibiting glucose-mannose-galactose-rhamnose was similar in *H. lignicola* (UAMH 7002T), *H. roseonigra* (CBS 514.83T), *H. sanguinea* (CBS 406.52T), and *H. variabilis* var. *odora* (CBS 328.80T) only *H. roseonigra* showed the ubiquinone Q-10 H_2. The remaining *Hyphozyma* species are characterized by a ubiquinone Q-10.

13.4 Cellulose and Hemicellulose-Degrading Yeasts

Termites play a major role in the recycling of photosynthetically fixed carbon. With the aid of their symbiotic intestinal flora they degrade to a high extent the wood constituents cellulose and hemicellulose. Nevertheless, the microbial species involved in the degradation of cellulose and hemicelluloses have been yet poorly defined before the investigations of Schäfer et

al. (1996) and Wenzel et al. (2002). They identified different yeast strains with cellulose- or xylan-degrading capability.

Cellulose (34–62%), hemicellulose (14–36%) and lignin (18–39%) are the three major constituents of plant cell walls (Fengel and Wegener 1984). An effectively cellulose and hemicellulose-degrading natural community of microbes is found in the termite gut (Cook 1943; Mishra 1979; Breznak and Brune 1994; Varma et al. 1994, König et al. 2002). Cellulose and hemicellulose are digested to a high extend during the passage of food through the digestive tract. Mishra (1979, 1980) determined values between 49 and 78% and he also found xylanase activity in *Neotermes bosei*. In termites the digestion of cellulose is maintained by flagellates, bacteria (Wenzel et al. 2002) and termite's own cellulases (see Li et al., Chap. 9). Intrinsic hemicellulases have not been found, but in fungus-eating termites they can be ingested with the feed (see Rouland-Lefevre et al., Chap. 14). The higher termite *Macrotermes mülleri* has a symbiotic relationship with a fungus from the genus *Termitomyces*. Comparison of a xylanase found in the gut and the enzyme produced by the fungus led to the conclusion that the gut enzyme originated from the fungus. In other termites hemicellulose seemed to be digested with the help of enzymes secreted by symbiotic bacteria (Saxena et al. 1993; Schäfer et al. 1996). Information about xylan-decomposing microbes is scarce and only a few xylan-decomposing bacteria have been obtained in pure culture from the termite gut (Saxena et al. 1993).

In addition to the bacterial strains a series of different hemicellulose-degrading yeasts were isolated for the first time from the termite gut (Schäfer et al. 1996; Table 13.2). Twelve yeast isolates out of 35 produced clear zones on agar plates containing insoluble xylan (oat spelt) or dye-linked xylan or solubilized xylan (oat spelt) in liquid media. The isolates from the lower termites belonged to the ascomycetes, while the isolates from the higher termite *Odontotermes obesus* belonged to the Basidomycetes (not shown; Prillinger et al. in prep.).

Reports on cellulase production by yeast wild-type strains are rare (Oikawa et al. 1999). Some of the yeast isolates from termites produced hemicellu1ases (Schäfer et al. 1996) and some were also able to degrade cellulose (Wenzel et al. 2002). The cellulolytic yeasts (strains ZM1, ZM2 and ZM3) from *Zootermopsis nevadensis* are related to *Sporothrix schenkii*. Authors have also isolated cellulolytic yeasts from *Mastotermes darwiniensis* (strains MYG6 and MYG7). Strain MYG6 belongs to the genus *Trichosporon*.

Table 13.2. Glycosidase spectrum of yeast isolates from the termite gut

Strain	Termites	Enzyme activities											
		α–l-Arabinosidase		β–d-Galactosidase		β–d-Glucuronidase		Xylanase		β–d-Xylosidase			
		A	B	A	B	A	B	A	B	A	B	CZ	SX
MM1[a]	*M. d.*	–	–	–	–	–	–	3.23	–	–	–	+	+
MS2[c]	*M. d.*	–	0.34	–	0.34	–	0.34	0.17	–	0.17	0.51	+	+
MS3[c]	*M. d.*	–	0.34	–	0.85	–	0.34	0.34	–	–	0.51	+	+
MS4[c]	*M. d.*	–	–	–	–	–	1.02	0.17	–	0.17	1.02	+	+
NM1[a]	*N. j.*	–	–	–	–	–	–	0.51	7.14	0.17	–	+	+
NS1[c]	*N. j.*	–	–	–	–	–	–	0.17	9.01	–	–	+	+
RM3[a]	*R. s.*	0.17	–	–	–	–	–	–	–	0.68	0.17	–	–
RM7[a]	*R. s.*	–	–	–	–	0.17	1.36	0.17	–	0.17	4.08	+	+
RS2[c]	*R. s.*	0.34	–	0.17	–	–	–	0.51	–	0.51	–	–	–
ZAG10[b]	*Z. a.*	–	–	–	0.51	–	0.51	1.02	–	–	0.85	–	+
ZM1[a]	*Z. n.*	–	–	–	–	–	–	0.51	5.61	–	–	+	+
ZM2[a]	*Z. n.*	–	0.85	–	0.85	–	0.85	0.51	–	–	0.85	+	–
ZM3[a]	*Z. n.*	0.85	–	–	–	–	–	0.34	–	0.34	–	+	–
ZS5[c]	*Z. n.*	–	–	–	–	–	–	–	23.80	0.17	–	+	+

For identification of the yeast strains termite species cf. Table 13.1;
A = cell extract (nkat/mg protein); B = culture medium (nkat/ml); – = not detected
[a] Isolated on malt agar
[b] Isolated on GYP agar
[c] Isolated on Sabouraud agar

13.5 Evolutionary Considerations

Roaches and termites are well known for their specific prokaryotic and eukaryotic gut symbionts (Douglas 1992; Breznak and Brune 1994; Varma et al. 1994; König et al. 2002). The greatest density of microorganisms in the alimentary tract of termites is in the proximal portion of the hindgut, known as the paunch (Douglas 1992). In all termites, the paunch harbors bacteria at 10^9 to 10^{10} cells per ml gut contents. It is widely accepted that the symbiotic flagellates (Yamin 1981; Breznak and Brune 1994; König et al. 2002) in *Cryptocercus* and lower termites have been inherited from the common ancestor of the Isoptera and Blattaria, possibly in the Carboniferous or Permian periods (Thorne and Carpenter 1992). In lower termites the association with fungi was studied by Hendee (1935). Representatives of 31 genera of Zygo-, Asco-, Deutero-, and Basidiomycetes and 20 non-sporulating cultures of fungi were isolated from the nests and fecal pellets of termites belonging to the genera *Kalotermes*, *Reticulitermes*, and *Zootermopsis*. There was no evidence of any specific relation between a given species of termite and any genus of fungus. *Penicillium* and *Trichoderma* were the genera of fungi most frequently isolated from colonies of all three species of lower termites. A comparatively small number of Basidiomycetes was detected. The fungi most commonly associated with the termites showed no apparent correlation with the species of wood in which the termite colonies occurred. Zoberi and Grace (1990) added same additional genera of Dictyosteliales, Stemonitales, Mucorales, and Hyphomycetes recently for the subterranean termite *Reticulitermes flavipes*. Parasitic interactions between lower termites and fungi are known for *Absidia coerulea* Bainier and same *Entomophthora* species (Sands 1969).

The species of lower termites from which yeasts have been isolated (Prillinger et al. 1996) represent the three important habitat types: *Zootermopsis angusticollis* and *Zootermopsis nevadensis*, the damp wood termites which live in rotting wet wood in warm temperate regions; *Neotermes jouteli* represents a dry wood termite, which excavates logs and maintains colonies strictly in wood without necessary soil connections and *Reticulitermes santonensis* and *Heterotermes indicola* are the subterranean termites. According to the traditional systematics, the Mastotermitidae are most primitive and the closest relatives to the roaches. They had a worldwide distribution in the tertiary period. Today, they are restricted to Australia with a single species *M. darwiniensis*. *Reticulitermes santonensis* is a termite species representative for the palearctic region (e.g. France; Krishna and Weesner 1969). *Heterotermes indicola* is found throughout northern India (oriental region; Krishna and Weesner 1969). The two species of *Zootermopsis* and *Neotermes jouteli* are termites from the nearctic region. The *Zootermopsis*

species occur in the forested areas along the Pacific coast, in the Cascade and Sierra Nevada Mountains and in same of the inland ranges west of the Continental Divide (Krishna and Weesner 1969). *N. jouteli* is a termite of the coastal areas of Florida, Cuba, the Bahamas, and both the east and west coasts of Mexico, as well as Socorro Island (Krishna and Weesner 1969). Despite their wide distribution of the investigated termites in different continents the majority of yeast isolates from lower termites and the roach *Cryptocercus punctulatus* comprise a homogeneous assemblage of representatives from the Endomycetales (Prillinger al. 1996). Based on a genotypic approach species 4 was the only basidiomycetous yeast isolated from lower termites. This species belongs to the genus *Trichosporon*. The phenotypic characteristics of this species come close to *Cryptococcus humicolus*. It is noteworthy that *C. humicolus* was the only *Cryptococcus* species found with the *Trichosporon* cluster on the basis of partial large subunit rRNA, sequences by Guého et al. (1993). Byzov et al. (1993a,b) detected a highly symbiotic yeast community in the hindgut of the diplopod *Pachyiulus flavipes* (see Byzov, Chap. 4). Terrestrial arthropods belonging to the Diplopoda are well known from the Paleozoic (Shear and Kukalová-Peck 1990). The yeast communities consist almost exclusively of Ascomycetes phenotypically identified to be *Debaryomyces hansenii*, *Torulaspora delbrueckii*, and *Zygowilliopsis californica* (DTZ-group; Byzov et al. 1993a,b). Typical litter yeasts like *Aureobasidum pullulans*, *Rhodotorula rubra*, and *Candida* sp. found in the food of diplopods were eliminated completely during the passage through the digestive tract. Beside the DTZ-group *Trichosporon cutaneum*, *Geotrichum candidum*, and *Pichia membranaefaciens* were minor components of the yeast community in the excrement. The cell walls of these yeasts probably resist the enzymatic attack in the digestive tract. In contrast to lower termites, basidiomycetous yeasts were commonly found in the hindgut of the higher termite *Odontodermes obesus*. From *O. obesus* only, four different species of basidiomycetous yeasts were isolated (Prillinger et al., unpublished). Based on yeast cell wall sugars and partial ribosomal DNA sequences all species come close to *Rhodosporidium toruloides* as a representative of the *Microbotryum* type (Prillinger et al. 1991a, 1993b). Three other species contained xylose in their cell walls and showed affinities to the genera *Filobasidium* and *Tremella* (Prillinger et al. 1991b, 1993b).

The assimilation of NO_3^- from species 6, 8, and 11, however, does not agree with the general genus description of *Debaryomyces* (Barnett et al. 1990). Ribosomal DNA sequences from Kurtzman and Robnett (1991) and Yamada et al. (1991, 1992) as well as cell wall neutral sugars (Prillinger and Lopandic, unpublished results) suggest that the genus *Debaryomyces* is presently heterogeneous. Therefore it was decided to concentrate in species identification to species 7 which is a dimorphic ascomycete. Species 7 was

identified genotypically at the species level. Based on a negative DBB reaction, hydrolysis by urease, an absence of fermentation, and the broad assimilation pattern of carbohydrates, especially an oxidative degradation of inositol, starch, and erythritol *Hyphozyma sanguinea* (De Hoog and Smith 1981; Hutchison et al. 1993) and *Sporothrix schenckii* (De Hoog et al. 1985) became the favorites for species identification. Although rhamnose was detected in the cell wall of all *Hyphozyma* species type strains, *H. sanguinea* could be excluded based on a ubiquinone Q-10. RAPD-PCR data demonstrate convincingly that the yeast isolates from *Zootermopsis angusticollis* and *Z. nevadensis* identified as species 7 are conspecific with the type strain of *Sporothrix albicans* S.B. Saksena only. *S. albicans* was isolated from soil in England and described by Saksena (1965). Later de Hoog (1974) synonymized *S. albicans* with *S. schenckii* var. *schenckii*. Summerbell et al. (1993) noticed in cases where *S. schenckii* was isolated from natural materials, nonpathogenic, non-perithecial isolates very similar to *S. schenckii* are also frequently obtained (Mackinnan et al. 1969; Mariat 1975). Like *S. albicans* these isolates lack pigmented secondary conidia. In addition, their vitamin requirements may differ from *S. schenckii*'s characteristic requirement for thiamine (Mariat 1975; Mariat et al. 1962). Using type strains and RAPD-PCR analysis conspecificity of *O. stenoceras* with *S. schenckii* (Summerbell et al. 1993) was excluded. Studies from Ryter and Fromentin (1985) revealed that *S. schenckii*, but not *O. stenoceras*, is able to resist digestion by macrophage cells of the mouse immune system.

With respect to symbiotic association and coevolution the phylogenetic homogeneity of at least 11 yeast species isolated from the hindgut of lower termites from different habitats and geographical areas (e.g. Australian, Nearctic, Oriental, and Palearctic Region) signals that the yeasts can be considered symbionts that remain in a steady state. A similar symbiotic yeast population consisting of *Debaryomyces hansenii*, *Torulaspora delbrueckii*, and *Zygowilliopsis californica*, detected in the hindgut of the diplopod *Pachyiulus flavipes* (Byzov et al. 1993a,b) corroborates this interpretation. Similar to the roaches there is a very rich fauna of carboniferous diplopods known from Central Bohemia (Kraus 1974). The first Diplopoda are known from Late Silurian and Early Devonian deposits (Almond 1985; Ross and Briggs 1993).

Based on the fossil record and 18S rDNA sequences, Berbee and Taylor (1993) regarded the Endomycetales as a sister group from the filamentous Ascomycetes. Considering a new concept of sexuality and mating type evolution (Prillinger 1987; Prillinger et al. 1989, 1993a) and the carbohydrate pattern of purified yeast cells, Prillinger et al. (1993b) interpreted the Endomycetales as primitive fungi which have lost their filamentous stage based on the extinction of the hosts (Messner et al. 1995). Kurtzman (1993) added further arguments to the second interpretation. There is a general

consensus that roaches, mantids, and termites represent a monophyletic grouping (Hennig 1969; Thorne and Carpenter 1992; DeSalle et al. 1992).

According to Shear and Kukalová-Peck (1990), nothing suggests that the ecological role of millipeds (Diplopoda) has changed in the more than 400 million years since they first may have appeared. *Kluyveromyces blattae* is an other yeast so far only known from the intestinal tract of the cockroach *Blatta orientalis* (Henninger and Windisch 1976). *K. blattae* shows the Man-Glc monosaccharide pattern and a ubiquinone Q-6 system. Phylogenetically *K. blattae* is very close to *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* (Messner et al. 1995). Neither in lower termites and roaches nor in diplopods was there evidence for yeast isolates representing the *Ustilago* type pattern (Prillinger et al. 1990, 1993b). Berbee and Taylor (1993) considered *Ustilago* and *Tilletia* ancestral to the Endomycetales and filamentous Ascomycetes. The presence of a symbiotic Endomycetes yeast community representing the Man-Glc carbohydrate pattern in diplopods and lower termites documents that the "*Saccharomyces* type" is most probably ancestral to the *Protomyces*, *Microbotryum*, *Ustilago*, and *Tremella* type as suggested by Prillinger et al. (1993b). Messner et al. (1995) have shown a tight phylogenetic relationship between the classical yeasts *Saccharomyces* and *Kluyveromyces* and primitive filamentous fungi from the borderline between polykaryotic sporangia and uninucleate asci based on ribosomal DNA-sequencing.

The roaches are well documented from the Carboniferous (Hennig 1969). According to Carpenter (1934), cockroaches appeared to be the most important group of insects during the Carboniferous. Although fossil reports of termites exist only from the Cretaceous (Emerson 1967; Jarzembowski 1981, 1984; Lacasa-Ruiz and Martinez-Delclos 1986; Krishna 1990) the oldest, *Meiatermes bertrani*, was found in a limestone deposit in Spain dating approximately 130 million years. Martynov (1937) considered the Isoptera with homonymous wings the ancestral condition in winged insects. He proposed a phylogeny and evolutionary time frame in which roaches and termites diverged in the late Devonian or early Carboniferous, and mantids radiated from roaches in the mid-Carboniferous (Thorne and Carpenter 1992). Although intimate associations are known between Agaricales (Termitomyces) or filamentous Ascomycetes (Laboulbeniales: *Laboulbenia*; Pyrenomycetes: *Cordycepioideus*) and higher termites (Termitidae) (Sands 1969; Becker 1976; Amburgey 1979; Oertel and Schäller 1984; Breznak and Brune 1994), there is no information with respect to the yeast mycoflora in termites and its usefulness to trace the phylogeny of Asco-and Basidiomycetes. If Martynov's (1937) speculation about the evolution of roaches and termites is correct, coevolution between yeasts and termites may be extremely useful to trace the phylogeny of filamentous Asco- and Basidiomycetes.

Based on the preliminary study of yeast communities in the hindgut of roaches and termites (Prillinger et al. 1996) it may be concluded that an amalgamation of information obtained from obligate parasitic or symbiotic associations, yeast cell wall sugars, the ubiquinone system, fermentation and a broad or rather small assimilation pattern with ribosomal DNA sequencing and the fossil record might be a more reliable indicator for fungal phylogeny than evolutionary trees based on ribosomal DNA sequencing alone to trace fungal phylogeny back to early Paleozoic periods. Genotypically similar yeasts in roaches and termites are in favor of the hypothesis from Martynov (1937). Martynov (1937) proposed an evolutionary time frame in which roaches and termites diverged in the late Devonian or early Carboniferous. This, however, antedates the first fossil record of termites known from 120 million years considerably (250 million years; Thorne and Carpenter 1992).

13.6 Conclusions

The data on yeasts in the termite gut are scarce. Nevertheless, the first investigations showed that the yeast isolates occur at high numbers in the intestine of lower and higher termites of different systematic positions. Since they have been constantly isolated they belong most probably to the autochthonous symbiontic microbiota of the paunch. They produce cellulolytic and hemicellulolytic enzymes and may play a significant role in the degradation of lignocellulose beside the flagellates, which thrive only in lower termites, and bacteria.

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Notes added in proof. Species 4 was described as new Trichosporon species by Molnár, O., Schatzmayr, G., Fuchs, E. and H. Prillinger. Trichosporon mycotoxinivorans sp.nov., a new yeast species useful in biological detoxification of various mycotoxins. System.Appl.Microbiol. 27:661–671 (2004).

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14 Termitomyces/Termite Interactions

Corinne Rouland-Lefèvre, Tetsushi Inoue, Toru Johjima

14.1 Introduction

The first observation of brain like formations inside termite nests were made by the German naturalist, J. D. König (1799), in the East Indies. These formations were several centimeters in diameter and were later given the name of fungus combs. A fungus was developing on these plant structures in the form of mycelium with small white nodules (mycotetes) which are characteristic of the group. More than half a century later, the English cryptogamist, G. Gardner (cited by Petch 1913), collected a large carpophore-bearing fungus of the Agaricales order from a termite nest in Ceylon, However, it was only in 1906 that Petch (1906) pointed out the relationship between the mycotetes and carpophore fungal formations and showed that these large mushrooms were cultivated by the termites inside their nests. Heim (1941) created a new genus for these fungi, *Termitomyces,* which contains all the "termitophilic Agaricales".

Termitomyces have since been studied by entomologists seeking to clarify the relationship between termites and fungi. Grassé (1959) was the first to show that *Termitomyces* were associated with only a single subfamily of termites, the Macrotermitinae, and that this is a real symbiosis as neither of the two symbionts can exist without the other. Other more recent works have shown the details of this symbiosis: on the one hand, because termite nests are both temperature and humidity regulated (Matsumoto 1977; Johnson 1981; Thomas 1987a), they provide ideal conditions for the growth of these *Termitomyces* and, on the other hand, the fungus helps the termites to degrade the plant matter (e.g. wood, dry grass, leaf litter) on which they live. It grows on a special structure in the nest, the fungus comb, which is actively maintained by the termites, by continuously adding plant matter and consuming the comb.

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The precise nature of the relationship between termites and the associated fungus, as well as its specificity, have provoked considerable controversy which has been largely resolved by new biochemical and molecular biology techniques. In this chapter, we shall present the results obtained using molecular biology to study termite/*Termitomyces* co-evolution. We shall then look at recent scientific advances regarding the contribution of the termite and its symbiotic fungus to the degradation of major plant components (cellulose, hemicellulose, lignin).

14.2 Phylogeny and Co-Evolution of Fungus-Growing Termites and *Termitomyces*

Symbiotic relationships have played an essential role in termite evolution, involving a range of symbionts including protists, methanogenic archaea and bacteria (Bignell 2000). Only one Termitidae subfamily, the Macrotermitinae, has evolved an external mutualistic symbiosis with fungi of the *Termitomyces* genus.

14.2.1 The Symbionts

Macrotermitinae or "Fungus-Growing" Termites. Macrotermitinae belong to the family of higher termites (Termitidae), which accounts for 70% of known species of termite. They are organized in complex societies with a number of well-defined casts and do not have symbiotic protists in their digestive tract. These characteristics are specific to the Termitidae family which encompasses 235 of the 281 currently recognized genera in the order Isoptera. Termitidae are divided into four subfamilies, one of which is the Macrotermitinae.

Macrotermitinae are only found on the African and Asian continents where they are the dominant group in tropical areas (Abe and Watanabe 1983; Wood and Thomas 1989). The Macrotermitinae have been divided into 11 taxonomically distinct genera and approximately 330 species (Khambhampati and Eggleton 2000). Most of the diversity occurs in Africa, where 10 of the 11 genera are found. Genera are also found in Asia and Madagascar, and one genus is exclusively Asian (Khambhampati and Eggleton 2000).

Termitomyces Fungi or "Termitophilic" Fungi. *Termitomyces* are Basidiomycotina fungi from the order Agaricales, the Tricholomatacea Roze family and

the Termitomyceteae (Jülich) Singer tribe. Approximately 40 *Termitomyces* species have been described. However, their taxonomy was based on the fruit structures (Heim1977) and, as some *Termitomyces* never fruit, their taxonomy is thus difficult to determine. However, since Heim, no other mycologist has been interested in the composition of the *Termitomyces* genus, even though it contains fungi of extremely diverse form, origin and biology. The common denominator of the *Termitomyces* fungi is that all species in the genus are cultivated by termites in their nests. Furthermore, all the fungi cultivated by Macrotermitinae termites belong to this genus.

Although additional varieties have been identified using molecular biology (Rouland et al. 2002; Aanen et al. 2002), the small number of fungal species compared with the number of Macrotermitinae species (40/330) suggests that many of these fungi are shared by different termite species.

14.2.2 Evolution of Fungus-Growing Termites and *Termitomyces*

There has recently been a resurgence in interest in phylogeny of *Termitomyces,* phylogeny of Macrotermitinae and co-evolutionary dynamics. The first study by Rouland et al. (2002) on 15 African *Termitomyces* species sequenced their internal transcriber spacer region (ITS1–5.8S-ITS2) and showed that *Termitomyces* was clearly a monophyletic group belonging to the family Tricholomataceae. The hypothesis that "each species of termite is associated with a separate species of fungal symbiont" (Heim 1942; Grassé 1982) seems unlikely since identical ITS sequences were obtained from fungi cultivated by three species of termites in different genera. On the other hand, Katoh et al. (2002) demonstrated, by sequencing ITS and 18S rDNA, that the symbiotic fungi cultivated by several colonies of the same termite species, *Odontotermes formosanus,* belonged to two different *Termitomyces* species. The work of Taprab et al. (2002) on the molecular phylogeny of symbiotic *Termitomyces* of several species of Asian Macrotermitinae also indicates that the relationships of symbiotic fungi with host termites and their locality were apparently complex.

The most comprehensive study on termite/*Termitomyces* evolution was conducted by Aanen et al. (2002) who created phylogenetic trees of the two symbionts in this mutualism across several genera and reconstructed both the origin and a number of key aspects of the co-evolutionary dynamics of the symbiosis. They studied 38 colonies of 32 species, covering 9 of the 11 genera of fungus-growing termites from three locations in Africa (Cameroon, Senegal and Gabon) and 3 in Asia (Sumatra, Kalimantan (Indonesia) and Sri Lanka), spanning most of the distribution of the Macrotermitinae. Maximum likelihood and Bayesian analyses of DNA

sequences were used to reconstruct the phylogenic trees of both symbionts. This study confirmed that the fungus-growing termites are monophyletic within the Isoptera order and the fungi cultivated by these fungus-growing termites are monophyletic within the Basidiomycotina order, as has been described in other papers (Rouland et al. 2002; Monçalvo et al. 2002). Moreover, these authors demonstrated that the symbiosis has a single African origin and that the secondary domestication of other fungi or reversion of mutualistic fungi to a free-living state has not occurred. According to the papers mentioned above (Taprab et al. 2002; Katoh et al. 2002), host switching has been frequent especially at lower taxonomic levels, and different nests of a particular termite species can have different symbionts. These observations are consistent with horizontal transmission of fungal symbionts. But, in spite of frequent host switching, there is a very high correlation between the termite and fungal phylogenic trees because the mutualistic interactions at higher taxonomic levels are highly specific. If horizontal transmission is the main transmission mode, the symbiont specificity at higher taxonomic levels is surprising. The question arises how this specificity has evolved. One possibility is that termites select their fungal symbionts indirectly (i.e. by supplying substrates suitable only for particular groups of fungi). If this is the case, we must consider that not all *Termitomyces* have the same capacity to degrade plant matter and therefore their importance in the digestive symbiosis with termites varies. This will be discussed in the following section.

14.3 The Role of Termitomyces in Mutualistic Symbiosis

14.3.1 Nature, Structure and Dynamics of the Fungus Comb

Fungus combs are sponge-like structures enclosed in specially constructed chambers inside a termite mound and the soil. The structure provides a large surface area, allowing air circulation and access by the termites. The fungus comb is the culture medium for *Termitomyces* as well as food for the termites and it is constructed with the undigested feces of termite workers (Josens 1991; Grassé 1978; Sieber and Leuthold 1981; Johjima et al. 2003). In most *Macrotermes*, the fecal material is deposited on the top rim of the fungus comb. There is an age gradient within the fungus comb, such that the upper part of the fungus comb is newer than the lower part. The termites eat the fungus comb from the lower rim (Josens 1971; Sieber and Leuthold 1981). The termites, therefore, eat the mature part of the fungus comb which creates a highly efficient method of digesting plant matter. The fungus

comb has a cycle time of approximately 40 days (Traniello and Leuthold 2000). An exception has been reported for *Macrotermes carbonarius* where gradient exists among individual combs (Hyodo et al. 1999). Other fungus comb structural forms have been observed in Macrotermitinae other than *Macrotermes*, and were described in detail in the review by Rouland (2000). Some species of fungus-growing termites have a food store in addition to the fungus comb. This comprises small particles cut from plant litter and looks like moist sawdust (Wood 1978; Darlington 1994). This food store is also ingested by the termites and then excreted on the fungus comb without obvious digestion. The function of this food store is not well understood. Lower concentrations of plant secondary metabolites have been detected in *Macrotermes gilvus* food stores than in the leaf litter around the mound (Johjima et al. 2003). The food store may well help in detoxification or improve the palatability of the litter.

Termitomyces have only been found in association with fungus-growing termites in the form of mycelium with aggregations of asexual spores which have been called various names such as mycotetes, synnemata, conidia, nodules. Mycotetes are also consumed by the termites. The sexual stage of the fungus, the basidiocarps with a long pseudorhiza extended from fungus comb (Singer 1986), is developed at specific times in the rainy seasons. However, as they last no more than few days, they are easily overlooked. *Termitomyces* is absent everywhere else in the nest structure including the food store and the internal walls of the nest. Outside the fungus comb it only occurs in the guts of workers (Thomas 1987a,b). Although the fungus comb seems to have a pure culture of *Termitomyces* when it is in the termite nest, other fungi such as *Xylaria* proliferate on the fungus comb within a few days if the comb is removed from the nest. It is not fully understood how the growth of contaminants is controlled in the termite nest. If there are still live termites on the comb after it has been removed from the nest, the growth of non-symbiotic fungi is suppressed for a few days (Grassé and Noirot 1958; Thomas 1987b). In a study of *Macrotermes bellicosus*, very little evidence of germination of fungal spores in the food store was observed despite the presence of various fungal spores (Thomas 1987b). The food store consists of comminuted food mixed with saliva. Furthermore, extracts of the food store, whole termites and the termite gut showed fungistatic activity, suggesting that termite secretions inhibit fungal spore germination and growth. Recently, antimicrobial peptides were identified in the fungus-growing termite *Pseudacanthotermes spiniger* (Lamberty et al. 2001). Termicin is present in hemocyte granules and salivary glands of the termite which has an activity against several fungi. However, the antimicrobial activity against *Termitomyces* and the typical contaminant fungus *Xylaria* has not been studied. The fungistatic activity of termite secretions, especially saliva, appears to be a major part of fungus comb

maintenance. Other possible factors that control fungal activities have also been discussed by Thomas (1987b).

14.3.2 Role of *Termitomyces* in the Digestive Metabolism of Termites

Sands (1956) showed that *Odontotermes badius* workers supplied with fresh fungus comb survived much longer than workers supplied with wood chips and filter paper containing starch or sugars. Workers supplied with fungus comb from which the mycotetes had been removed survived only slightly longer than starved workers. These observations indicate that fungus comb, especially mycotetes, is important for termite nutrition. Four roles in the symbiotic relationship with host termites have been proposed for *Temitomyces*: (1) enrichment in nitrogen (Matsumoto 1976; Collins 1983); (2) a source of metabolic water and heat (Lüscher 1951); (3) provision of glycosyl hydrolases which work synergistically with enzymes from the host termites (Martin and Martin 1978; Rouland et al. 1988b,c); (4) the degradation of lignin to improve cellulose digestibility (Grassé and Noirot 1958). Termites feed mainly on plant debris that is poor in nitrogen. The nitrogen content of the mycotetes was reported to be much higher (6–8%) than in the fungus comb (0.8–2.0%) and, therefore, the mycotetes are a nitrogen-rich food for the termites (Matsumoto 1976; Rohrmann 1978, Wood and Thomas 1989). The roles suggested in (1) and (2) will be more or less significant for all fungus-growing termites, while the roles suggested in (3) and (4) are controversial and would depend on the host as discussed below.

Provision of Polysaccharide-Degrading Enzymes. The role of *Termitomyces* in plant polysaccharide degradation was first clarified by determining the enzyme activities of the various parts of termite workers digestive tract as well as of the fungal nodules (Abo-Khatwa 1978; Martin and Martin 1979; Rouland et al. 1988a–d, 1991; Sengupta and Sengupta 1990). To define the respective role of each partner of the symbiosis, a multi-variance analysis of 19 enzymatic activities detected in 5 termites and fungal species was made. The first two axes of the PCA (Fig. 14.1a) accounted for 74% of the total variance (41% on axis 1, 33% on axis 2). Discriminant analysis carried out on 10,000 permutations showed that the PCA was highly significant ($P < 0.00001$). On axis 1, the correlation circle showed the separation of samples having polysaccharidase activity and those with oligosaccharidase activity. On axis 2, samples with β-xylosidase activity were separated from those with heterosidase activity. The projection of sample classes (termites and associated fungal species) on the first two PCA axes (Fig. 14.1b), on the one hand, clearly differentiate the low level of oligosaccharidasic and heterosidasic activity of the fungi from the higher level of activity of the

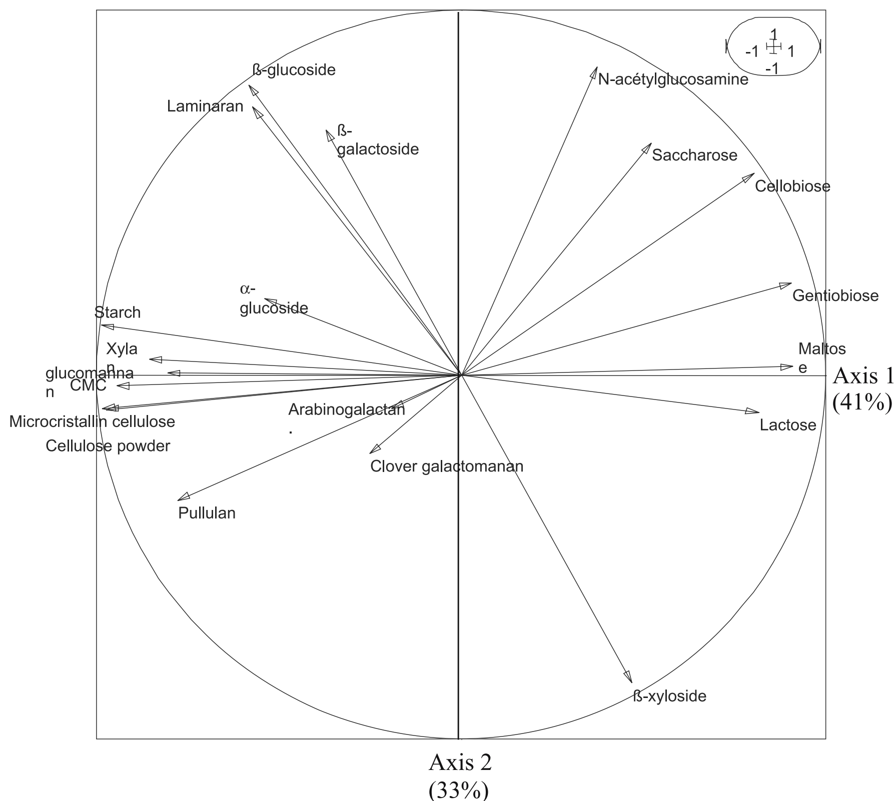

Fig. 14.1. Results of principal components analysis (PCA). **a** Correlation circle; Results of principal components analysis (PCA).

termites and, on the other hand, clearly differentiate the fungi/termite associations of the species *A. cavithorax* and *M. toumodiensis* from the other species of termites. The differentiation between these termite/fungus associations is principally due to a greater degradation of polysaccharides by *A. cavithorax* and *M. toumodiensis* workers than by their associated fungi whereas, in the other species studied, the two symbionts (termite/fungus) degrade polysaccharides to the same extent. Furthermore, for some associations, the substrate most degraded by the termites' digestive tract is also the substrate most degraded by the symbiotic fungus. In this case, the enzymes produced by the symbionts are not complementary unlike the enzymes produced by fungus-cultivating ants (D'Ettore et al. 2002). To explain this anomaly, several authors have proposed an acquired enzyme hypothesis (Martin 1991; Martin and Martin 1978, 1979; Kukor and Martin 1983). This hypothesis suggests that some fungi can produce polysaccharidases which act synergistically with the enzymes produced by the termites.

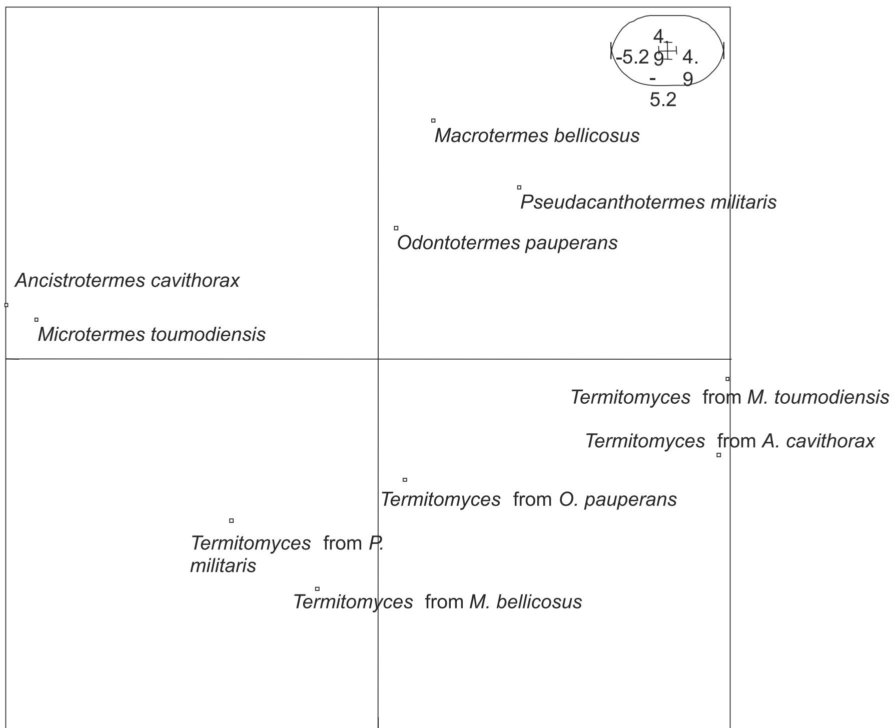

Fig. 14.1. (continued) **b** ordination of the samples in the plane defined by axes 1 (41%) and 2 (33%) of the PCA

Degradation of polysaccharides could, therefore, be achieved in the worker termite guts by the combined action of fungal and termite enzymes. Fungal cellulases and xylanases have been found in the digestive tract of two species of *Macrotermes* (Rouland et al. 1988a–c; Matoub and Rouland 1995), but without any equivalent recent work on other species, this hypothesis is still contested by some authors (see Rouland 2000).

For polysaccharide degradation, symbiotic fungi exhibit different enzyme activities. For example, *T. eurhizus*, associated with the termite *P. spiniger*, is highly amylolytic, whereas *Termitomyces*, associated with *Macrotermes bellicosus*, is both xylanolytic and cellulolytic (Rouland et al. 1991; Ikhouane 1995). The differences in enzyme activities could be due to different genetic patterns in the fungi or to different environmental conditions. In order to distinguish between these factors, the enzyme production of several species of *Termitomyces* cultivated under the same conditions has been examined (Table 14.1). It is, therefore, possible to distinguish two groups of *Termitomyces*: in the first group (*Termitomyces*. sp., *T. eurhizus* and *T. medius*), the fungi can produce enzymes which vary ac-

Table 14.1. Production of several osidasic enzymes on fungus comb extracts from *Ancistrotermes guineensis*, *Macrotermes bellicosus*, *Microtermes subhyalinus* and *Pseudacanthotermes spiniger*

	Cellobiase	CMCase	Maltase	Amylase	β–Xylosidase	Xylanase
T. eurhizus from *P. spiniger*						
A. guineensis comb	12 ± 3	84 ± 8	12 ± 2.5	18 ± 4	24 ± 3	112 ± 5
M. bellicosus comb	0	270 ± 36	11 ± 1.3	0	48 ± 5	1385 ± 59
P. spiniger comb	14.6 ± 1.3	5.2 ± 0.3	2.3 ± 0.1	10.6 ± 0.5	12.8 ± 1.2	18.2 ± 2.4
T. medius from *A. guineensis*						
A. guineensis comb	15.4 ± 1.1	76.05 ± 8.6	12.4 ± 2	278 ± 25	25.3 ± 1.6	824 ± 56
M. bellicosus comb	0	0	0	17.9 ± 2.4	22.7 ± 3.6	821 ± 47
P. spiniger comb	12.3 ± 0.6	76 ± 5.2	74.8 ± 8.3	75.2 ± 6	18.4 ± 2	992 ± 36
T. sp bellicosus from *M. bellicosus*						
A. guineensis comb	0	0	0	0	14.5 ± 2.3	672 ± 42
M. bellicosus comb	0	198 ± 36	28 ± 6.4	0	48 ± 9	986 ± 51
P. spiniger comb	0	0	0	0	10.9 ± 3	550 ± 37
T. sp subhyalinus from *M. subhyalinus*						
M. subhyalinus comb	4.5 ± 0.3	2.05 ± 0.2	5.3 ± 0.8	3.4 ± 0.6	14.6 ± 1.2	14.6 ± 1.6
P. spiniger comb	4.1 ± 0.5	2.0 ± 0.6	3.8 ± 0.8	2.5 ± 0.4	3.7 ± 0.9	12.3 ± 1

Activities are expressed in µmol permn per mg protein (Mora and Rouland 1994; Ikhouane 1995)

cording to the substrate and thus enzymatic production is correlated with environmental factors. These fungi are generalists and can be cultivated by several species of termite. In the second group, however, the variety of substrates that induce enzyme production is more limited. The enzymes produced in culture media are constitutive. This type of fungus appears to be more specialized in that it is found only in association with a single termite species. The group includes the symbiotic fungus associated with *M. bellicosus* (Ikhouane 1995) and *T. clypeatus* which produces several constitutive enzymes in vitro (Ghosh and Sengupta 1987; Sengupta and Sengupta 1990; Roy et al. 1994; Sinha and Sengupta 1995; Mukherjee et al.

1995). This classification of *Termitomyces* according to their metabolism seems to be correlate strongly with the co-evolution conclusions discussed above. *T. medius* has been found as a cultivated fungus associated with three species of *Microtermes*, two species of *Ancistrotermes* and with *Synacanthotermes heterodon*, whereas the symbiotic fungi associated with *M. bellicosus* have only been cultivated in the nests of this species (Aanen et al. 2002; Rouland et al. 2002).

Lignin Degradation in the Fungus Comb. Lignin is a heterogeneous random phenylpropanoid polymer that constitutes 15–30% of woody plant cell walls. Free-radical condensation of lignin precursors (coniferyl, sinapyl and *p*-coumaryl alcohols) results in the formation of an amorphous and highly branched polymer with at least 12 different types of linkage (Monties and Fukushima 2001). Since lignin forms a matrix surrounding the cellulose microfibrils, cellulose-depolymerizing enzymes are locked out and the decomposition of the cellulose will be retarded. Experimental evidence of lignin degradation in fungus combs was reported by Rohrmann (1978), and later by Hyodo et al. (2000). They determined proximate fractions for parts of the fungus comb differing in age, showing that lignin concentrations in the older parts of the combs were lower than those in the newer parts (Table 14.2). The structural changes of the lignin as the comb aged were analyzed using solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy of the molecules (Hyodo et al. 2000). Compared with the NMR spectrum of newer comb, the relative peak intensities at 56 and 154 ppm were clearly lower in the older comb. The peaks at 56 and 154 ppm were assigned to methoxyl and aromatic carbon in lignin, respectively. Further

Table 14.2. Chemical composition of various fungus combs (as dry weight %)

	Part of comb	Cellulose	Lignin	Ash
M. ukuxii[a]	Newer	20.82[c]	14.30	8.00
	Older	27.18[c]	2.43	7.45
M. natalensis[a]	Newer	17.78[c]	9.80	16.22
	Older	21.82[c]	5.67	15.47
M. gilvus[b] colony 1	Newer	17.1[d]	32.6	14.0
	Older	26.3[d]	15.0	15.5
M. gilvus[b] colony 2	Newer	21.5[d]	34.6	5.7
	Older	33.8[d]	53.4	11.2

[a] Data from Rohrmann (1987)
[b] Data from Hyodo (2000)
[c] Cellulose + chitin
[d] Calculated from glucose content

studies for precise peak assignment will be required because fungus comb consists of a variety of plant material so that the spectrum must be very complex.

There have been extensive biochemical studies of lignin degradation by basidiomycetes which have revealed that extracellular peroxidases are responsible for the depolymerization of lignin (see reviews by Kirk and Farrell 1987; Gold and Alic 1993; Hatakka 2001). Laccase is one of main components of ligninolytic enzymes in some basidiomycete (e.g. *Pycnoporus cinnabarinus*, Eggert et al. 1997). However, many fungi that are not able to degrade lignin also produce laccase (e.g. *Neurospora crassa*). Lignin peroxidase (EC 1.11.1.14) catalyzes the oxidation of various aromatic compounds to form aryl cation radicals that are unstable and undergo a variety of nonenzymatic (chemical) reactions such as ring-opening and side-chain cleavages (Kirk and Farrell 1987). The substrate of manganese peroxidase (EC 1.11.1.13) is Mn^{II}. The enzyme oxidizes Mn^{II} to Mn^{III}, which diffuses from the enzyme surface and oxidizes a variety of phenolic compounds (Gold and Alic 1993). Thus Mn^{III} acts as a diffusible oxidant and attacks macromolecular lignin. In the case of lignin peroxidase, substrate oxidation site is located on the surface of the enzyme (Doyle et al. 1998). Direct interaction and electron transfer between lignin peroxidase and lignin were observed (Johjima et al. 1999). Both enzymes show broad substrate specificities and are suitable for degradation of lignin because lignin structure is a random, highly branched polymer with various linkages.

The understanding of the lignin degradation mechanism in fungus comb is limited. Rohrmann and Rossman (1980) detected pigmentation in *Macrotermes ukuzii* and *Termitomyces* combs grown on comb agar in the presence of syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde). This suggests that *Termitomyces* produces extracellular phenol oxidase(s) such as peroxidase and laccase in the fungus comb. However, this does not show that *Termitomyces* can degrade lignin because laccase and peroxidase are secreted by non-ligninolytic fungi.

The work discussed above was based on *Macrotermes*. Hyodo et al. (2003) analyzed chemical composition of the fungus combs from *Odontotermes*, *Hypotermes makhamensis*, *Ancistrotermes pakistanicus* and *Pseudacanthotermes militaris* in addition to four species of *Macrotermes*. In all *Macrotermes* species, the carbohydrate:lignin ratio increased progressively with comb age, whereas it decreased or remained constant in three species of *Odontotermes*, *H. makhamensis*, *A. pakistanicus* and *P. militaris*. This indicates that, in *Macrotermes*, lignin is preferentially degraded in the combs, whereas in the other four genera carbohydrate is preferentially degraded. The authors also conducted a stable carbon isotope analysis and suggested that *Macrotermes* depend more on plant matter for nutrition than the other four genera. It is uncertain whether the differences are accounted for by the

different sources of the fungus combs or by the phylogenetic differences between the symbiotic fungi. The authors mentioned that the *Macrotermes* species examined in the study, where lignin content in fungus combs decreased with age, seem to tend to feed on leaf litter, while the other genera appear to feed predominantly on wood with the exception of *P. militaris*. *M. gilvus*, in which the lignin degradation was analyzed by solid-state NMR as described above, also mainly uses leaf, grass and stalks as a source of the fungus comb (Roonwall 1970). It has been observed that grass lignin is more easily degraded by fungi than wood lignin (Rodriguez et al. 1996). It is possible that *Termitomyces* can degrade grass and leaf lignin but not wood lignin. Molecular biological studies covering the cloning of ligninolytic enzymes and the distribution of the genes within the symbiotic fungi from various host termites will resolve the question.

The existence of plant secondary metabolites as well as lignin will interfere with carbohydrate utilization by termites because these compounds are apparently key components of plant defence against pests and pathogens and are, therefore, considered to be an important controlling factor for litter decomposition in soils (Swift et al. 1979; Bennett and Wallsgrove 1994). Phenolics such as tannins and flavonoids are one of major classes of plant secondary metabolites. The concentration of phenolics in the fungus comb of *M. gilvus* is much lower than in the fallen leaves around the termite mound (Johjima 2003). The concentration of water-soluble phenolics in extractable compounds from older comb is lower (40%) than that in newer comb, suggesting that phenolics are degraded in the fungus comb. Phenol oxidase activity has been detected in the fungus comb as mentioned above. This enzyme might be responsible for the degradation of phenolics in the fungus comb.

Chemical analyses of fungus comb at molecular level will provide further insights into the function of fungus comb. However, it is nearly impossible to determine the fungus comb compositions in molecular level using degradative chemical methods since the fungus comb consists of a variety of plant debris and microorganisms. For example, the Klason lignin method has been shown to overestimate lignin content in litter, where the residue contains cutin, suberin and tannins as well as lignin (Kögel 2003 and references therein). The results obtained by Rohrmann (1978) and Hyodo et al. (2000) may be due to degradation of cutin, suberin and tannins rather than lignin being degraded in the fungus comb. New analytical approaches such as solid-state NMR spectroscopy and analytical pyrolysis/gas chromatography/mass spectrometry combined with conventional methods will allow a detailed analysis of fungus comb components and help the understanding of the detailed mechanism of lignin degradation and the tracing the fate of lignin and plant secondary metabolites such as tannin in Macrotermitinae colonies.

14.4 Conclusions

The efficiency of degradation of lignocellulose by fungus-growing termites is largely attributed to their symbiotic *Termitomyces*. These fungi provide a variety of functions that termites do not possess. Recently, the application of new technologies and molecular methods has greatly improved our knowledge of lignin degradation by both Macrotermitinae termites and *Termitomyces* fungi and on the co-evolution of the symbionts. However, there are still gaps in our knowledge on the adoption of a particular species of fungus by termites. As described above, the same fungus can be cultivated by several different termite species. A crucial and as-yet-unanswered question is: to what extent do the Macrotermitinae manage to reduce the genetic diversity of horizontally acquired symbionts to a single strain to prevent the evolution and appearance of non-cooperative symbiont traits?

The other important unsolved questions concern the acquired enzymes: do these enzymes exist in genera of Macrotermitinae other than *Macrotermes*? Can lignin-degrading enzymes also be acquired by termites? New biochemical and molecular studies are required to answer these questions.

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15 Microbiology of Termite Hill (Mound) and Soil

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15.1 Introduction

Termites thrive in great abundance in terrestrial ecosystems and play important roles in biorecycling of lignocellulose. Together with their microbial symbionts, they efficiently decompose lignocellulose (Varma et al. 1994). Termites (order Isoptera) comprise a complex assemblage of diverse species, divided into so-called lower and higher termites (Abe et al. 2000). Lower termites, a dual decomposing system, consist of the termite's own cellulose and those of its gut protists, i.e., diverse population of prokaryotes and flagellated protists (single cell eukaryotes). Termites have developed a unique hindgut flora consisting of bacteria, archaea, archaezoa, yeasts and probably fungi. This specialized flora enables the termites to feed on hardly degradable polymers such as lignocellulose. The degradation of lignocelluose in the oxygen-limited hindgut paunch occurs in three steps: first a hydrolytic, second an oxidative/fermentative and, third, a methanogenic/acetogenic step. Cellulose and hemicellulose are degraded by bacteria, yeasts, and some members of flagellates. Higher termites compromise only one apical family (Termitidae), and they degrade cellulose apparently using only their own enzymes, because of the absence of symbiotic prostists. Higher termites show considerable variation in their feeding behavior, which is not limited to xylophagy.

In contrast to cellulose, lignin degradation does not appear to be important in the gut of wood-feeding termites. Soil-feeding termites decompose humic substances in soil at least partly, but little is known about their decomposition. Fungus growing termites are successful in the almost

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complete decomposition of lignocellulose in a sophisticated cooperation with Basidiomycetes fungi cultivated in their nest.

Termites are one of the most important soil insects that efficiently decompose lignocellulose with the aid of their associated microbial symbionts. Termites are said to dissimilate a significant proportion of the cellulose (74–99%) and hemicellulose (65–87%) components of lignocellulose they ingest. Due to their digestive ability and their huge abundance, termites have a tremendous ecological impact on the biorecycling of lignocellulose. They also greatly contribute to the physical and chemical modification of habitats particularly soils.

15.2 Features Distinguishing Termites from Other Insects

Termite, often misnamed white ants, are small to medium-sized, soft-bodied insects which range in color from dull white to light and dark brown, and belong to the order Isoptera (Krishna and Weenser 1969, 1970; Varma et al. 1994). Termites are social insects and live in small to large colonies. They also exhibit polymorphism, and a well-defined grouping of individuals into different functional castes, namely, larvae, workers, pseudergates, soldiers, nymphs and reproductives. Of these, the first four are wingless and incapable of reproduction. Pseudergates retain their ability to differentiate and nymphs develop to alated reproductives. Nymphs already have wing buds. Larvae, soldiers, workers and nymphs are sometimes dimorphic and even polymorphic. The castes are morphologically distinguishable and have different functions. The soldiers, which have a strongly chitinized head, usually with large mandibles, take an active part in the defense of the colony. The majority of individuals in a colony are workers; there are few soldiers (up to 10%) and usually only a pair of reproductives. As a rule, a colony has only a pair of primary reproductives (a male and a female often termed king and queen) which are generally imprisoned by the workers in a royal cell and their sole function is to reproduce. Fertility is high and the queen (e.g., *Odontotermus obesus*) can lay almost one egg per second. The eggs hatch in a few days into whitish larvae, which develop into workers, soldiers or winged reproductives as the case may be.

15.3 Current Taxonomic Status

The termite belongs to the insect order Isoptera. It refers to the adult primary reproductive, which possesses two pairs of equally long wings. Their

morphology is rather simple, but they are advanced in social behavior. The vast majority of termite species occur in the tropics (Thompson et al. 2004). Only one species is resident in Ontario, Canada, *Reticulitermes flavipes*. Unlike most insects, which have only one linear developmental pathway, they are polymorphic. A substantially modified taxonomy, along with newly identified synapomorphies includes 2 suborders, 7 families, 21 subfamilies and 16 tribes. The accepted families are: Termopsidae, Hodotermitidae, Mastotermitidae, Kalotermitidae, Heterotermitidae, Rhinotermitidae and Termitidae. Figure 15.1. shows the current phylogenic tree of termites.

Termites are subdivided into seven families, all of which comprise wholly social species. They are most primitive and the closest relatives to the cockroaches and the Mastotermitidae. They are distributed worldwide but are restricted in Australia as *Mastotermes darwiniensis*. Kalotermitidae is another primitive, but widespread family, maintaining their colonies in wood without any soil connections. Hodotermitidae, which has fossils dating

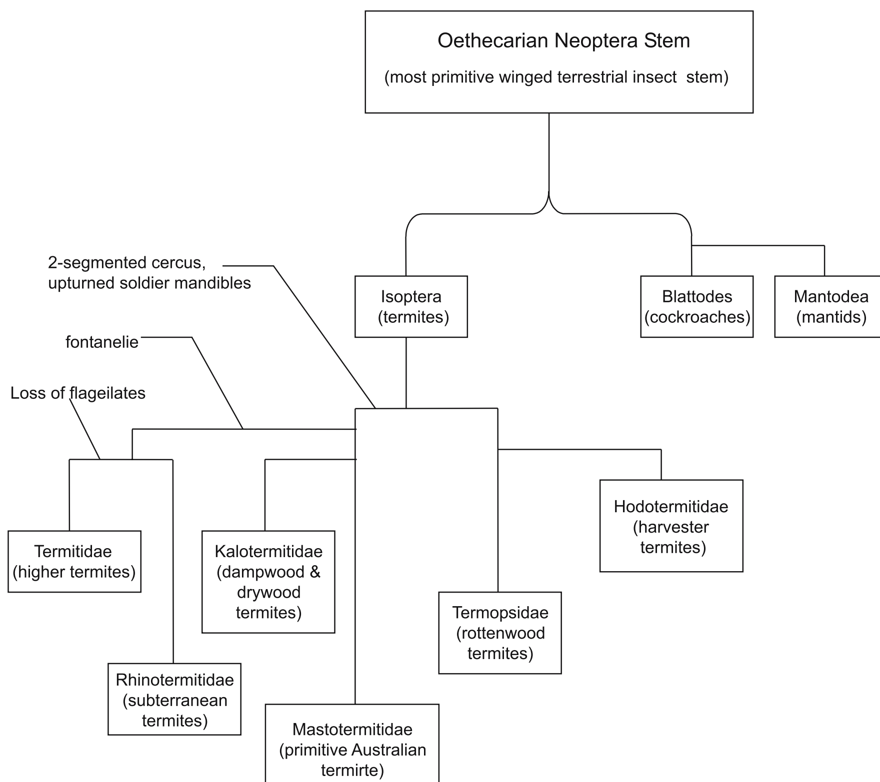

Fig. 15.1. Isopteran phylogenetic tree.
Source: http://www.utoronto.ca/forest/termite/termite.htm

back to the mid-Cretaceous in Labrador, has maintained some primitive characteristics such as mandible structures. Out of four subfamilies, Termopsinae (damp wood termites) live under warm temperate conditions and Hodotermitinae are found in desert areas and store pieces of grass in their nests. The Rhinotermitidae are so named because they have evolved small soldiers with snouts for the ejection of repellents. The Seritermitidae possess mandibles with serrations along the entire inner margin. There is only one species of this family that is found in Brazil. The most highly evolved family, the Termitidae, include 75% of the more than 1800 known termite species. This family of termites has some remarkable soldiers with snap-closing mandibles. Within Termitidae, Amitermitinae is the simplest subfamily. The Macrotermitinae culture a fungus (*Tremitomyces* sp.) or fungal combs and the Nasutitermitinae have soldiers that shoot jets of toxic and sticky material.

15.4 Ecophysiological Distribution

Termites are distributed all over the world. Termitidae or higher termites originated from Rhinotermitidae The members of Termitidae are heavily concentrated in the tropics. About 85% of the species known in 1955 were present in the three principal tropical zoological regions (Oriental, Ethiopian, and Neotropical), while only 13–17% of the species were recorded from Palaeoarctic and Neoarctic regions. Africa, south of the Sahara, is reported to contain 66 genera (Garcia et al. 2002; Goodisman and Crozier 2002). *Odontotermes obesus* (Rambur) is one of the most widely distributed species of termites and has been reported for most parts of India, e.g., Andhra Pradesh, West Bengal, Bihar, Maharashtra, Gujrat, Delhi, Kerela, Tamil Nadu, Karnataka, Madhya Pradesh, Orissa, Punjab, Rajasthan, and Uttar Pradesh (Myles 1999). The morphological appearance of representative group of termites is shown in Fig. 15.2.

15.5 Termite Colonies and Castes

Termite colonies are composed of three castes; the reproductives (king and queen), the soldiers, and the workers. The king and queen are sexually mature termites, with compound eyes and fully developed wings. The workers and soldiers lack wings and compound eyes. Sexually mature termites, or reproductives, are produced in large numbers during certain

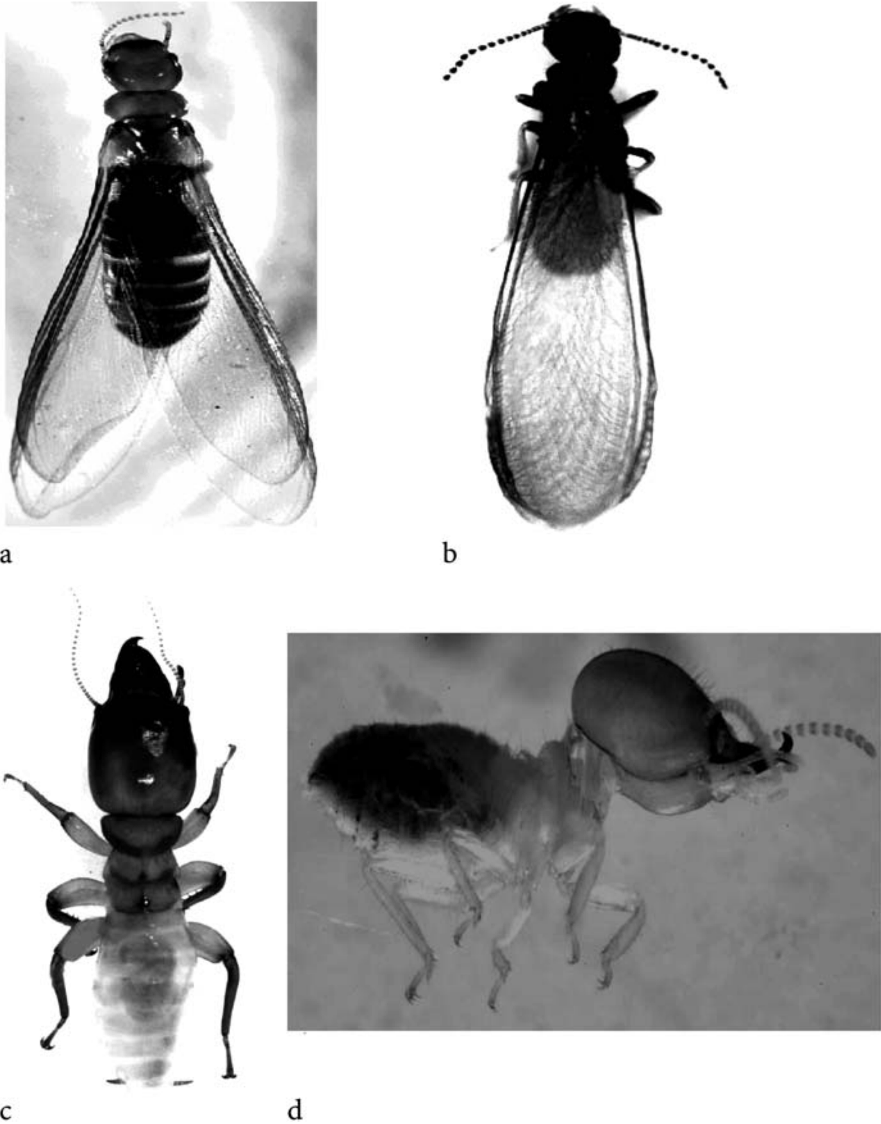

Fig. 15.2. Different termite families. **a** Kalotermitidae; **b** Rhinotermitidae; **c** Termopsidae; **d** Termitida (higher termites). Source: www.termitrsurvey.com/distribution/images

seasons and leave the colony in a swarm. They are poor fliers, and birds and other animals eat them (Fig. 15.3).

When the surviving termites settle, their wings break off along a weakened seam at the base. They then form pairs, each of which establishes a new colony. A couple excavates a chamber in wood or soil, in which they mate;

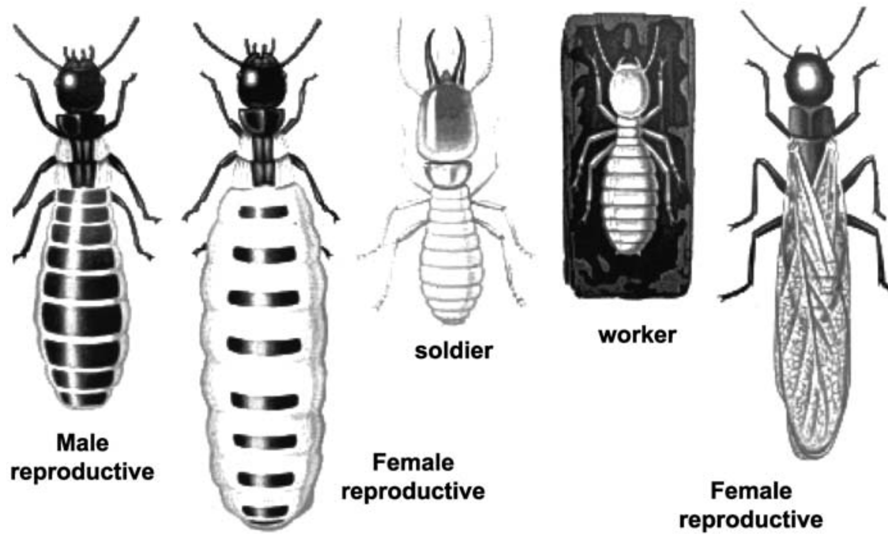

Fig. 15.3. Termite castes. Source: www.encyclopedia.com/htm/t1/termite.asp

they remain permanently paired, and the queen eventually produces as many as 30,000 eggs per day. Two or 3 weeks after mating, the young nymphs hatch and are fed on liquid secreted by the parents and on fecal wastes, from which they obtain the protozoan or bacterial symbionts essential for life.

The caste into which the young termite, or nymph, develops depends upon the amount of growth-inhibiting substance (a pheromone) passed to it during feeding and grooming. The pheromone is secreted by the reproductives and, when present in a high concentration, prevents the development of nymphs into reproductives. A large colony may have several pairs of reproductive pairs. As more workers and soldiers are added, since they do not produce the pheromone, its concentration in the colony is correspondingly decreased. Therefore, when the colony reaches a certain size, some of the nymphs begin to develop into reproductives, which then produce pheromones. This phenomenon also occurs if the original reproductive pair dies. The increase in the pheromone level prevents the maturation of additional nymphs into reproductives; these remaining nymphs then become workers. In a similar way, the appearance of soldiers appears to inhibit the production of more soldiers.

In some families, no workers develop, and the nymphs perform worker functions, which include feeding the royal couple, the soldiers, and the very young nymphs; caring for the eggs; grooming the queen; constructing and repairing the nest; and foraging for food. The soldiers have heads as large as the rest of the body and equipped with strong mandibles used in defense

of the colony (Parmentier and Roisin 2003). They attack any intruders to the colony and stand guard at the entrances, in some species closing the entrances by putting their heads in the holes. Soldiers of certain species squirt a sticky, poisonous secretion at enemies. Description of important castes are given below:

Reproductives One king and one queen begin each colony and reside there until killed. The king works to produce sperm to fertilize the queen (she produces up to 3000 eggs/day). The queen releases hormones that signal differentiation among workers, soldiers, etc. With many species, the life of the mound depends on the life of the reproductives; both the mound and the queen can often be more than 25 years of age. In some species, reproductives are replaced when they die, allowing the colony to live on, thereby sometimes exceeding a century.

Winged alates This most often sighted (and eaten) class of termite functions chiefly in the finding of new colonies. They possess well-developed compound eyes, are capable of flight and reproduction, and can replace the king and queen if they are killed. Winged alates are the most common class of termite used for classification due to variations in shape and size of wings. The name Isoptera means "equal-winged" and is given to termites because the two pairs of wings of the winged alates are the same size and similar in appearance. Alates emerge at night to disperse and mate, but they never return to the nest.

Workers This grouping of termites maintains perhaps the most important role in the colony. Blind, deaf, and sterile, the workers are the smallest active termites (sometimes only a millimeter or two in length). As the most abundant form of termite, they function in nest building, regurgitating food for soldiers and parents, exploring food and water sources, caring for eggs and younger siblings, and chemically guarding against fungi and other micro-organisms. One of the most important responsibilities of a colony is the gathering of food and water. This important task is carried out by the worker caste.

Soldiers Soldiers are sterile like workers, yet they are not blind. They function in protection of the nest, often becoming suicidal for the good of the colony. The members of this caste have large mandibles (size and shape differ with species) used to slash and lacerate enemies. Soldiers have large heads so they can block entrances from intruders. They also possess frontal projections used to eject defensive chemicals that entangle enemies (Grandcolas and D'Haese 2004). When there is a breach in a mound's wall or a hole in a mud tube many soldiers will rush outside to defend the aperture while workers repair the damage. Frequently

the guards are sealed outside the nest and later die of starvation or desiccation.

Nymphs Spawned from eggs, nymphs are inactive and undeveloped termites awaiting chemical signals from the queen that lead to differentiation. A relatively small number of nymphs become winged alates or reproductives; a moderate number become soldiers (10–15% of population) while the majority of nymphs develop into workers. For details readers are advised to check the website: www.cwc.ca/publication/building_performance/termite/termites.php.

15.6 Life Cycle

Life cycles of termites vary from species to species. Periodically, the colony produces numerous winged imagoes (alates) which swarm out in considerable numbers usually once or twice a year, most often in the rainy season. After a short flight lasting a few minutes to an hour or so, the alates descend to the ground, cast-off their wings, and segregate into pairs, a male chasing a female, tandem fashion. The cast-off wings litter the ground where the swarming individuals have descended and provide tell-tale evidence of swarming. The pair settles down in some hole or crevice and starts breeding. The queen gradually becomes much larger in size due to enlargement of the abdomen (physogastry), but the king remains unchanged. The eggs, which are small, white and longish-oval with rounded ends hatch in a few days and the young larvae undergo a number of molts. They produce mostly workers and soldiers. Periodically, they produce fully winged or primary reproductives, which swarm out and repeat the cycle. If the primary reproductives die or are removed, some of the larvae may develop into supplementary reproductives which are not fully winged but are still capable of reproduction: this happens commonly in the 'lower' or more primitive termites, and less frequently in the 'higher' (Roonwal 1979).

15.7 Topography of the Termite Hill (Mound) and Nest

Termite nests, called termitaries, vary widely. The nests of certain tropical species are huge mound-like structures, often 6 m (20 ft.) in height. These mounds have extremely hard walls, constructed from bits of soil cemented with saliva and baked by the sun. Inside the walls are numerous chambers and galleries, interconnected by a complex network of passageways. Ventilation and drainage are provided, and the heat required for

hatching the eggs is obtained from the fermentation of organic matter, which is stored in the chambers serving as nurseries. The most spectacular mounds are made by the Macrotermitinae. They may be round, 20–30 m in diameter, reaching a height of 8–9 m (Varma et al. 1994). For details readers are advised to check the website: www.worldalmanacforkids.com/explore/animals/termite.html.

The nests of termite societies exhibit large variations. Some species form only simple galleries in wood or the ground, while others construct most complex edifices. They are the wood and ground dwellers. The wood dwellers are confined throughout their lives to wood in which they make tunnels and also nest. The termite-infested wood may be dead or alive. The wood dwellers, based on their requirement of the wood's content, are further divided into two categories, damp wood termites (e.g., Termopsinae) and dry wood termites (e.g., Kalotermitidae).

The ground dwellers (e.g., Rhinotermitidae) live exclusively, or at least partly, in soil. The ground connection is necessary for their normal life and breeding. From the ground, the workers come out for foraging either in the open or into wood, having a direct ground connection or earthen runways and shelter tubes made earlier by the workers on the ground, wall surface or tree trunk. Termites that excavate hard wood or soft wet wood cut out tunnels and then plaster them with faeces which is heavily fibrous with plastic texture (Evans 2002). They often make partitions by placing a grain of sand and cementing it with faeces to create a lenticular fabric up to 2 mm thick.

Many live underground and make chambers connected by interwoven tunnels. The chambers are either for reproductive or for fungus combs or nursery purposes. Chambers with fungal combs are generally a little bigger and are plastered with clay but have a concave floor which gives access to the soil beneath and also allows air to circulate.

15.8 Microorganism from the Termite Soil

Termite soils provide a very distinct ecological environment which harbors and promotes very specialized cellulolytic and hemicellulolytic microorganisms. These bacteria provide a distinct environment for isolation of several new and more potentially active lignocellulose-degrading bacteria producing extracellular hydrolytic enzymes with far-reaching consequence in maintaining nutrient and microbial balance in nature, especially in arid and semi-arid soils. In addition, the congregation of large numbers of specialized microorganisms in an ecosystem plays a vital role directly or indirectly in maintaining parasitic and symbiotic balances and regulates

the ecology of microorganisms. In most semi-arid ecosystems, the polysaccharide cellulose constitutes the major component of the flow of carbon and thus most studies on microbial polysaccharide decomposition were followed on cellulolytic microorganisms. Termites play a key role in the carbon cycle of native soil ecosystems. The diet of termites is rich in cellulose and hemicellulose. The microflora inhabiting termite soil and their gut play a significant role in the dissimilatory activity. Microbial population estimates in termite-infested soil mounds in Kenya (Africa) showed that bacteria and actinomycetes are the most abundant during the wet season (Keya et al. 1982). The highest density of bacteria recorded was 10^6 and that of actinomycetes 10^5 per gram of dry soil. In contrast, fungi, which dominate only during dry periods, numbered 10^4 and declined to 10^2 cells per gram of dry soil during the wet period (van Borm et al. 2002). Fungi, actinomycetes, bacteria and protozoa occur in higher numbers in dead than in live mounds. However, during the wet period, bacteria were dominant in dead mounds. Cellulose decomposers were more numerous than all other groups of bacteria examined and were more abundant in mound soils than in adjacent soils (O'Brein et al. 1982).

The nutritional strategy of soil feeding termites is still quite puzzling. How do they manage to assimilate nutrients in tropical soils which are often impoverished, devoid of sugars or other easily decomposed energy-source substrates?

Polysaccharide-depolymerizing microorganisms isolated from termite habits are compiled in Table 15.1. Some bacterial cellulolytic isolates such as *Cellulomonas* and *Bacillus* species from termite-infested soils have been characterized in more detail (Rajaram and Varma 1990; Sarkar et al. 1988; Krishna and Varma 1990; Paul et al. 1985; Paul and Varma 1990). Among the cellulose degraders, a mesophilic *Bacillus* sp. (Paul and Varma 1993a) was found to contain a complete set of cellulolytic as well as hemicellulolytic enzymes. Similarly, Saxena et al. (1991) also isolated a *Micrococcus* sp. which is able to produce all three enzymes. Another cellulose degrader, *Bacillus thermialkalophilus*, a unique bacterium which grows and metabolizes in a wide range of pH (7–9) and temperature (37–60 °C), was isolated from termite soils (Sarkar et al. 1988). Another unique feature of these isolates was that cellulose degradation was invariably accompanied by change in pH of incubation medium from neutrality to alkalinity.

Molecular microbial ecology has revealed remarkable biodiversity – prokaryotic and eukaryotic – in numerous soil environments. However, no culture-independent surveys of the termitosphere exists, although termites dominate tropical rainforests. Here, we focused on soil feeders, building nests with their soilborne faeces, enriched with clay-organic complexes, thus contributing to the improvement of soil fertility. In order to assess the fungal community composition of these termitaries compared with soils

Table 15.1. Cellulose- and xylan-decomposing microorganisms isolated from termite hills and nests

Organisms	Associated termite	Habitat
A. Cellulose-decomposing microorganisms		
I. Fungi		
Formes lividus (white rot)	*Nasutitermes exitiosus*	Infested wood
Acremonium sp.	*Reticulitermes flavipes*	Termite surface
Aspergillus sp.		
Alternaria sp.		
Cladosporium sp.		
Fusarium sp.		
Stachybotrys sp.		
Penicillium sp.		
Trichoderma sp.		
II. Bacteria		
Bacillus sp.	*Odontotermes obesus*	Mound soil
Bacillus thermoalkalophillus	*Odontotermes obesus*	Mound soil
Cellulomonas galba 'Cellvibrio flavifaciens'	*Odontotermes obesus*	Mound soil
Cellulomonas sp.	*Odontotermes obesus*	Infested soil
Cellulomonas sp.	*Odontotermes obesus*	Mound soil
B. Xylan-decomposing microorganisms		
Bacillus sp.	*Odontotermes obesus*	Mound soil
Bacillus thermoalkalophillus	*Odontotermes obesus*	Mound soil
Cellulomonas sp.	*Odontotermes obesus*	Mound soil

Sarkar (1991); Paul and Varma (1990, 1993a,b); Saxena et al. (1993); Paul et al. (1985); Zoberi and Grace 1990)

not foraged by termites, samples of the two types were collected in the Lopé rainforest, Gabon, and processed for generation of fungal internal transcribed spacer (ITS) clone libraries. Although primers were universal, most of the recovered sequences represented Ascomycete that were previously uncharacterized, the proportions of which reached 72.5% in soils and 80% in termitaries. Their affiliation with identified fungi was analyzed in performing a phylogenetic tree based on 5.8S rDNA. Furthermore, the ascomycete communities of soil-feeding termitaries and soils shared only 6.3% of sequences. This discrepancy of composition between soil and nest may result from the building behavior of termites, as the organic matter in the nest is chemically modified, and some vacant ecological microniches are available for more specialized fungi.

Querying databases with these full-length ITS sequences defined 21 phylotypes similar to known sequences (> 94% identity), 11 originating from soils and 10 from termite nests. Although primers were universal, within

these identified sequences, apart from one affiliated with Basidiomycota, all were Ascomycota, from the classes Dothideomycete (two phylotypes), Eurotiomycete (three phylotypes), Sordariomycete (14 phylotypes) and an *Incertae sedis* class (one phylotype). So the following results focused on Ascomycete diversity. Four of the five numerically dominant phylotypes belonged to these identified sequences and were related to *Penicillium*, *Eurotium*, *Clonostachys* and *Trichoderma* sp. Many sequences, without close ITS blast matches, could not be assigned to any known taxa and were designated as unidentified. They could originate from identified fungi with as yet undetermined ITS sequences, from fungi not yet isolated or be of chimeric origin.

Bidochka et al. (1999) performed phylogenetic analyses that aimed at representing the overall molecular ascomycete diversity of soil samples and designing putative fungal phylogroups. Owing to the high variability of ITS, we restricted our analysis to the 5.8S rDNA, which provides enough information to check gross linkages of unidentified sequences to established taxa. In order to have a representative set of phylotypes, we included 44 of the retrieved ascomycete phylotypes (those identified and those represented at least twice) plus the 21 matched known sequences (Bridge and Spooner 2001). The phylograms constructed by the neighbor-joining distance analysis and by the maximum parsimony method were congruent except for the relative position of AY273300, AY273337 and AY273331. Twelve ascomycete phylogroups were identified and included 35 phylotypes. Five other phylogroups remained unidentified (I–V) and were extremely diverse, as revealed by their spreading across the phylogram. The *Fusarium-Verticillium* and *Aspergillus-Penicillium* groups showed the highest diversity (eight and five phylotypes respectively). Moreover, the *Fusarium-Verticillium* group was divided into two distinct clusters underlining its problematical taxonomy (Guadet et al. 1989). In the studied samples, we demonstrated that a high proportion of the biodiversity remained undescribed and that, by modifying the environment, soil-feeding termites drastically affect the soil ascomycete community structure. The observed gap most probably results from the building behavior of the termites because, in the nest, the organic matter is chemically transformed, and some vacant ecological microniches are available for more specialized environmental fungi. As a consequence, soil-feeding termites could be considered, according to Waid (1999), as true metabionts, because they create habitats and supply resources to dependent organisms such as fungi that may adapt, evolve and hence diversify. Nonetheless, we should take care about the fact that the sampling of the microbial community was not exhaustive in this study. Further analyses are required to assess whether such ascomycete diversity modifications in nests of *Cubitermes*, the most abundant genus in some African forests may affect some biogeochemical

cycles, and this leads us to question the putative link between molecular diversity and functional redundancy. Furthermore, in such environmental biotopes, tropical rainforest soils are among the best candidates able to shelter the highest fungal diversity (Christensen 1989; Hawksworth and Rossman 1997; Bridge and Spooner 2001) and termite nests could confer resilience to ecosystem function facing putative environmental changes.

The seasonal variation obtained in the population of microorganisms from termite mounds revealed the dominance of cellulose degraders as well as other bacterial populations to be the highest during the monsoon and minimum in summer (45–50 °C). In winter months (5–15 °C), the population was moderate (Sarkar 1991).

15.9 Soil-Feeding Termites

Out of 2300 species of termite recorded in the world, only a small number can be considered as devastators of wood. These insects are in reality highly diversified in their feeding and nutritional regimes, which allows them to assimilate plant material in various forms: whereas some species derive nutrition from dry wood (the xylophages) or plant materials (fungus growing termites) and can be harmful in this way, over half the known species (humivores or soil-feeders) feed on decomposed plant substances associated with soil particles (humus). Soil feeders constitute approximately 50% of all known species of termites and dominate the termite assemblages in tropical forest (Abe et al. 2000). They are highly influenced by cycling of organic matter and nutrients, and influence both structural and physicochemical properties of soils. Microbial diversity is enhanced by the passage of soil (tunnel), probably contributing further processing of organic matter. However, soil-feeding termites have been studied very rarely due to short life span and difficulty in sampling. The substrates degraded by soil-feeders are still obscure, presumably polysaccharides, polyaromatic compounds and polypeptides immobilized by tannins of plants origin. In contrast to wood-feeding termites, the diet of soil feeders appears to be low in carbohydrates and highly enriched with phenolic and humic compounds. Feeding experiments with synthetic humic acids showed that peptidic components of humic substrates are selectively digested, whereas aromatic components are not (Ji et al. 2000). Stable-isotope analysis (Tayasu et al. 1998) may provide further information. The gut of soil-feeding termites is highly compartmentalized, and there are extremely alkaline compartments, exceeding pH 12 in the anterior hindgut (Bignell and Eggleton 1995; Brune and Kühl 1996). The high alkalinity enhances chemical oxidation and solubilization of soil organic matter (Kappler and Braune 1999).

15.10
Fungus-Growing Termites

Inside the termite mound, insects form fungus for consumption. This fungus must be kept at an optimum temperature. By carefully adjusted convection currents air is sucked in at the lower part of the mound, down into enclosures with muddy walls and up through a channel to the peak of the termite mound. Termites constantly dig new vents and plug old ones to regulate the temperature (Aanen et al. 2002; Taprab et al. 2002). So, if the air from outside the termite mound is warmer it should warm up the inside of the termite mound. On the other hand, if the air is cooler it cools the termite mound. This way, the fungus in the mound should be kept at an optimum temperature and the insects in the mound will not starve.

The so-called fungus-growing termites belong to an evolutionary-related group of higher termites (Termitidae, Macrotermitinae) and are abundant in the tropics of Asia and Africa. They consume more than 90% of dry wood in some arid tropical areas and directly mineralize up to 20% of the net primary production in wetter savannas (Abe et al. 2000). The extensive decomposition of lignocellulose by fungus-growing termites is due in large part to their specific symbiosis with Basidiomycetes fungi of the genus *Termitomyces* (Agricales, Tricholomataceae). In addition to symbioses with gut microorganisms as in other guilds of termites, they cultivate symbiotic fungi within their nests (Cornelius et al. 2002). In some types of fungus-growing termites, the "termite mushroom", the fruiting body of *Termitomyces*, blooms seasonally from termite nests. The termite mushrooms are unique in nature, growing from only the termite nests, and are commercially interesting due to their prized edibility.

There have been several suggestions for the role of the symbiotic fungi in termite nutrition: (1) decomposition of lignin and (2) supply of cellulose and xylanase to act synergistically with the enzymes produced by the termite. Since endogenous cellulases have been recognized in termites, it is difficult to make generalization as to the significance of the acquired fungal cellulase in cellulose digestion in fungus-growing termites.

The well-coordinated cooperation between the termites and the fungi enables efficient utilization of lignocellulose (Hinze et al. 2002). So-called old workers forage outside the nest and collect plant litter. In the nest, young workers masticate and ingest the collected plant litter, which passes rapidly through the termite gut without digestion. The resulting fecal pellets (primary faeces) are pressed together to form a sponge-like structure, the fungus comb, whose matrices support the growth of the symbiotic fungi. The fungi form mycelia, and white, round asexual conidial structures called "fungus nodules". The termite nest is a favorable environment for the growth of *Termitomyces* as its humidity and temperature is controlled. It has

been reported that the lignin content progressively decreases as the fungus comb matures (Hyodo et al. 2000). It has also been shown that in vitro digestibility of cellulose in a mature fungus comb is approximately threefold higher than in a newly formed one. Young workers usually consume "fungus nodules", whereas old workers consume old senescent combs to produce final faeces. However, final faeces are rarely found in the nest of fungus growing termites, suggesting the highly efficient decomposition and the complete mineralization of plant litter. These observations support the finding that symbiotic fungi have the ability to degrade the lignin, which makes cellulose more easily degraded by the cellulose produced by the termite.

Basidiomycetes, which cause white-rot decay and thus are called white-rot fungi, are able to degrade lignin in wood efficiently. *Termitomyces* are classified into white-rot fungi. Lignin degradation by white-rot fungi has been extensively studied, and results revealed three kinds of extracellular phenoloxidases, namely, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac). These are responsible for initiating the depolymerization of lignin (Pham et al. 2004; Varma 1999). In addition to lignin, white-rot fungi are able to degrade a variety of environmentally persistent pollutants, and thus their application is of interest not only in industrial processes but also in bioremediation (Pointing 2001; Singh and Ward 2004).

Molecular phylogenetic relationship of *Termitomyces* species has been examined based on DNA sequences of nuclear rRNA, the internal transcribed spacer region ITS1 5.8S...ITS2, a partial large-subunit rRNA (Rouland-Lefevre et al. 2002; Taprab et al. 2002). In congruence with the taxonomy based on morphology, *Termitomyces* appears to be monophyletic in members of the order Agricales. For certain genera of termites, such as African *Macrotermes* and *Pseudacanthotermes*, the hypothesis of a termite-fungus co-evolution is seemingly acceptable.

15.11 Chemical Nature of Lignocellulose

15.11.1 Cellulose

In the biosphere there is more carbohydrate than all other organic matter. Cellulose is major extracellular structural constituent of rigid cell wall of fibrous and woody tissues of higher plants. Apart from its biological significance in the carbon cycle, it is an alternative source of fuel. As a general rule, plant products are cycled through animals before man can obtain the necessary nutritive proteins from his environment. During

this active cycling process, the efficiency of conversion of plant products to animal proteins is dependent upon the digestibility of cellulosic products by animals (Nakashima et al. 2002).

Seed-bearing plants are major synthesizers of cellulose, but algae, fungi and certain bacteria are also active producers. Animals are poorly equipped for the digestion of such polysaccharides and the main agents of decomposition are microorganisms. It is degraded as polysaccharides occurring in close association with other substances such as hemicellulose, lignin and pectin. High polymeric substances and their occurrences in the nature have been studied by Fan et al. (1980) and Varma et al. (1985).

Cellulose is a linear glucose polymer, composed of anhydrous-d-glucose units with 1–4, β–d-glucosidase linkages, although the latest theory regards disaccharide cellobiose as the functional unit of cellulose – up to 10,000 β-d-glucose residues are linked in long chains; this gives a molecular weight of over one million and molecules about 5 mm long. The chain length of cellulose varies from source to source.

The smallest structural units of cellulose are elementary fibrils in which a small number of polymer chain are obtained in a parallel arrangement and strongly bound together by many hydrogen bonds. In the elementary fibril, regions of complete order of the crystallite alternate with less ordered amorphous regions. However, there is no sharp boundary between these regions. Microfibrils are slender bundles of elementary fibrils and can be the units from which cellulose fibers are formed.

15.11.2 Hemicellulose

Hemicellulose, usually the second most abundant polymer, accounts for up to 20–40 wt% of the total dry weight of lignocellulosic plants (Timell 1967). They differ in their chemical composition depending upon their origin. Mannans constitute the main part of hemicellulose of softwood and galactans are present in larch woods. The xylans are most commonly found in hardwoods (15–30 w/w) and are also a minor constituent (7–12 w/w) of softwoods (Table 15.2). The main structural element of xylan is a backbone consisting of 1–4 linked β-d-xylanopyranose units. Depending on the source, this is substituted with mainly acetyl arabinosyl and glucuronosyl residues (Timell 1967; Aspinall 1980). The major constituents of straw and grasses are single arabinose units which are attached α-glycosidically up to 12% of the C_3 position of the d-xylose residues on the main xylose chain (Biely 1985). The majority of the hemicellulosic polysaccharides are derived from cell wall lamella. Some of non-starch, non-cellulose polysaccharides, excluding pectic materials, known as cereal pentoses, are sometimes

Table 15.2. Percentage content of hemicellulose in different natural products

Natural product	(wt%)
Hardwood	26
Softwood	22
Various agricultural crop residues	30

The hemicellulose sugar content varies greatly with the plant species. In addition, the individual sugars may be methylated or acetylated (Knull and Inglett 1980; Gordon et al. 1983)

Table 15.3. Percentage of sugars in hemicellulose from different sources

Sources	Percentage dry weight of sugar				
	Xylan	Araban	Galactan	Mannan	Glucan
Hardwood	17.4	0.5	0.8	2.5	50.1
Softwood	5	1	1.4	11.2	46.3
Straw	16.2	2.5	1.2	1.1	36.5

also considered as hemicelluloses (Timell 1965). Hemicelluloses are composed of neutral sugars, uronic acid and acetyl groups, all present as their respective anhydrides, i.e., xylan, araban, glucan, galactan and mannan (Table 15.3).

Hemicellulose chains are simple or mixed polysaccharides of smaller dimensions than cellulose. The interior chain of hemicelluloses consists of polysaccharides that are attached to a variety of sugar residues that are same or different from the sugars that form the side chains. Except for galactose-based hemicellulose with a β-1–3-linkage, most hemicellulose are based on β-1–4-linkage of their constituents sugars. d-Xylose backbone with l-arabinose side chains is the most abundant form (Rydholm 1965).

15.11.3 Lignin

Lignin is the most variable and recalcitrant component of lignocellulose and comprises 20–30% of the dry weight of soft and hard woods. Lignins are the diverse group of three-dimensional polymers of aromatic alcohols that provide the structural rigidity (Higuchi 1990) with aryl ether linkages (Crawford and Crawford 1984).

In addition to the structural heterogeneity of lignocellulose, the interactive nature of these three polymers within the cell wall significantly increases its recalcitrant nature. The cellulose microfibrils are deeply embedded within a matrix of hemicellulose and are consequently well pro-

tected from enzymatic or chemical attack. The hemicellulose branch points can also form cross links to the lignin content (Chesson et al. 1983; Das et al. 1984); ferulic acid may be present which binds the hemicellulose component to lignin via the arabinose substituent on xylan (Smith and Hartley 1983). Further, the phenolic substituents of lignin can form ester linkages via their carboxyl groups to both the xylans and other structural polysaccharides such as pectins (Hartley and Jones 1976; Markwalder and Neukom 1976) which enhance the structural integrity of lignocellulose.

15.12 Biodegradation of Biomass

Cellulose possesses a catalytic domain, the sequence of which determines the family to which a given enzyme belongs. In some cases an individual cellulase may bear more than one catalytic domain. Cellulase is not a single enzyme but a unique system in which several enzymes act simultaneously to carry out the hydrolysis of cellulose. There are three major components in cellulase complex which act on different sites of β-glucosidic linkages (Bisaria and Ghosh 1981). The first is endo β-1,4-glucanases which randomly split off the β-1,4-glucosidic linkage somewhere in middle of the cellulose chain (Watanabe et al. 2002). Second is the exo-β-1,4-glucosidic linkage at the end of chain which produces cellobiose and/or glucose and the third is β-glucosidases or cellobiose (also called β-glucohydrolase) which hydrolyze the β-1,4-glucosidic linkage of cellobiose and produce glucose.

Hemicelluloses are digested to a high degree by termites (Cook 1943). Xylan constitutes the main portion of hemicelluloses and in *Neoteres bosei* xylanase activity was observed (Mishra 1980). In addition, termites can possess different kinds of carbohydrases, such as sucrose, maltase, trehalase and raffinase (Malaka et al. 1986; Mishra 1980). Only a few xylan-decomposing bacteria have been obtained from the termite gut (Saxena et al. 1993; Schäfer 1993, 1996).

Many isolates that aerobically degrade lignin monomers and dimers have been obtained from the guts of various termites. Since oxygen penetrates gut epithelium, these aromatic-compound-degrading bacteria presumably reside on or near the gut periphery (Rouland-Lefevre et al. 2002). Microbial activity of metabolizing lignin and its components is one of the plausible evolutionary origins for the degrading pathway of aromatic xenobiotics and/or environmental pollutants, such as polychlorinated biphenyl (PCB). On the other hand, analysis of termite faeces by some authors reported around 5% and 83% lignin degradation.

15.13 Conclusions

Social insects have always been of interest because of their seemingly well-ordered societies. The accepted families are Termopsidae, Hodotermitidae, Mastotermitidae, Kalotermitidae, Heterotermitidae, Rhinotermitidae and Termitidae. Different kinds of termites are found. They live all over the world and create mounds of various morphology, height and diameter. These mounds are made using very clever architecture through which the insects not only freely move but also bring the chewed biomass and deposit deep into the soil. In some of these social insects, the mound-building termites and the leaf-cutting ants, there has evolved a rather unique strategy in the consumption of cellulolytic plant materials. These insects cultivate cellulolytic fungi in underground gardens. They establish pure cultures of their fungus. That is, they grow only one fungus in their garden, which is not easily done, since there are so many sources of contamination that can occur. However, these insects are able to keep their gardens pure by constantly weeding out foreign fungi. They also care for their garden by providing suitable a food source, i.e., plant material, and moisture. So the fungi obviously benefit from this arrangement, but the ants and termites also benefit from the relationship. These insects are exclusively mycophagous, i.e., they eat fungi. The fungi that they cultivate break down the wood and leaves brought in by the termites and ants, respectively, and provide them with digestible and nutritious mycelium. Biodegradation by diverse group of microorganisms gives rise to the "Fungus Garden".

The so-called fungus-growing termites belong to an evolutionary related group of higher termites (Termitidae, Macrotermitinae) and are abundant in the Asian and African tropics. They consume more than 90% of dry wood in some arid tropical areas and directly mineralize up to 20% of the net primary production in wetter savannas. The extensive decomposition of lignocellulose by fungus-growing termites is due in large part to their specific symbiosis with Basidiomycetes fungi of the genus *Termitomyces* (Agricales, Tricholomataceae).

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16 The Termite Gut Habitat: Its Evolution and Co-Evolution

Paul Eggleton

16.1 Introduction

We usually think of habitats as rather large entities: they tend to be things like forests, grasslands, lakes. However, this isn't always so; the only requirements for a habitat is that it should be a definable bounded space containing a number of persistently co-existing organisms. Some habitats are large and obvious, some are small and hidden. The inside of a termite gut is at the small and cryptic end of the habitat spectrum, but it is still a habitat, as it contains a rich community of micro-organisms.

This chapter looks at the environment within the termites gut, how it has evolved and what has happened to the gut community during that evolution. I concentrate, especially, on those microbial groups where there is an interesting evolutionary narrative to tell, with the caveat that in nearly all cases the story is incomplete. I also discuss the few published examples that hint strongly at co-evolution (usually that actually means co-cladogenesis) within the termite gut.

16.2 Background: Some Definitions

16.2.1 Termite Biology

Termites are eusocial cockroaches that have a complex social structure with four clearly differentiated castes. These castes are generally workers, soldiers, queens and kings. Workers are the foraging, nest building, maintenance, and nursemaid caste. The soldiers' only job is to defend the colony, and in some groups (e.g. Apicotermitinae) this caste is absent. Kings and queens start off their adult lives as winged reproductives within their parents' nests. After a short nuptial flight, males and females

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pair up and begin new colonies. Their most important role is therefore dispersal and reproduction. Kings and queens generally mate for life and are the only reproductively active members of a colony. Colony centres can be, among other places, within arboreal carton nests, epigeal soil mounds or in amorphous tunnels underground. Termites almost all feed on dead plant material at different stages of decomposition. These feeding substrates include dead wood, dry grass, humus and soil organic matter. For a majority of studied termites, cellulose is their main energy source. Most termites are probably nitrogen-limited, as their feeding substrates have high C:N ratios. Termites are classified into seven families: Mastotermitidae, Hodotermitidae, Termopsidae, Kalotermitidae, Serritermitidae, Rhinotermitidae and Termitidae. The Termitidae ('higher termites') make up ca. 60% of the species: they are particularly diverse in tropical forests and savannahs. They also have the widest range of feeding preferences. The other eight families contain species that tend to live in more marginal termite habitats (deserts, temperate forests, tropical rain forest canopies) and feed on wood or dry grass. I will follow tradition and refer to these eight families collectively as 'lower termites', even though they are paraphyletic, because they have important similarities in their symbiont assemblages (see below). Within the Termitidae, the only phylogenetic distinction important for this chapter (predominantly due to lack of comprehensive data) is between the fungus-growing termites (the Macrotermitinae) and their plesiomorphically soil-feeding sister group (I will call this group the *Foraminitermes* major clade, after the most basal subfamily, the Foraminitermitinae; Engel and Krishna 2004). Within this latter clade there have been numerous reversions to wood- and grass-feeding, which, although of potentially great ecological and biogeographical importance (e.g. see Eggleton and Tayasu 2001), have not been sampled widely enough for any major evolutionary differences to be seen in the gut symbionts.

16.2.2 Which Environments?

I include here all the order Isoptera and the woodroach *Cryptocercus*. My assumption throughout is that these two taxa form a monophyletic group (e.g. see evidence in Lo et al. 2000; Nalepa and Lenz 2000). I realise that this placement is still controversial (Eggleton 2001). However, it makes the story easier to tell: if *Cryptocercus* is not the sister group of the termites then a hypothetical ancestor, rather like *Cryptocercus*, would have to be postulated. The present 'best bet' model is that termites are eusocial wood-feeding cockroaches (Grimaldi 2001).

Worker (or pseudergate) guts represent the environment in this case. Workers do the main work of the colony: foraging, brood care, building etc. They are also the primary energy providers in the colony and so they directly process feeding substrates in their guts – this means that for physiological and biochemical reasons they need gut symbionts. Alate and soldier guts are fed by the workers and so they have simplified versions of worker guts. Their symbionts are not well known and have generally been assumed to be depauperated versions of worker symbionts.

Worker guts are complex systems, consisting of a series of chambers connected by tubes, running from mouth to anus, arranged clearly into standard foregut, midgut and hindgut region, with greatest variability in the hindgut region. Detailed descriptions of termite guts are given in Noirot (1995, 2001). Perhaps most importantly from a microbial perspective, the largest chambers are anoxic at their centres, but most parts of the gut are aerobic or microaerobic (Brune et al. 1995). There appears to be a great deal of variation in microbial community structure between different parts of the gut (Bignell et al. 1980a, b), partly because of this gradient in oxygen availability. This has allowed aerobic (e.g. heterotrophic bacteria; Schultz and Breznak 1978), aerotolerant (e.g. lactic acid bacteria; Bauer et al. 2000) and anaerobic (e.g. methanogens; Breznak 2000) microbes to colonise the gut. However, detailed data showing these patterns have only been presented for one species of termite, *Cubitermes orthoganthus,* an East African soil-feeder (Friedrich et al. 2001; Schmitt-Wagner et al. 2003). This means that here I have no choice but to 'black-box' the gut and treat symbiont data in the literature as if it came from a single gut compartment representing the entire gut.

16.2.3 Community Ecology Definitions

Each termite worker gut is a habitatblock, the entire microbiota makes up a *community,* and any subset of that community is an *assemblage.* An assemblage may be *monophyletic*, containing all descendants of a common ancestor, or it may not. Most taxa within a termite gut are not monophyletic: there are other species in other termite habitats and in other non-termite environments. When considering the evolutionary ecology of such groups I have tried to include what is known about the whole clade of which the termite symbionts are a subset. Questions concerning how organisms colonise the termite gut habitat are of particular interest, but the data is mostly too incomplete to give clear answers.

Which Assemblages? I recognise four types of associations within the termite gut: (1) tourists, with no intimate association with host, (2) parasites, not

always associated but causing a reduction in colony fitness when present, (3) generalists, in a broadly mutualistic (or phoretic) relationship with the host but found in a range of hosts, (4) specialists, found only in termite guts, often only in subset of termite habitats. It is group (4) that is of most interest here, as specialists are the most likely to tell us something useful and definitive about the evolution of termite gut habitats. I generally deal only with (3) and (4) in this chapter. Except where a mutualistic relationship is very well established (e.g. for *Termitomyces*), I refer to gut microbes as 'symbionts', using the term in its strict sense of an organism living habitually with another organism. This seemed sensible to me because a number of termite gut symbionts (e.g. flagellate protists) have ecological relationships with their hosts which are scientifically unresolved.

Termite Life Types. Termite life types can be classified within a matrix of nesting x feeding types (for full details see Eggleton and Tayasu 2001):

Nesting types: Some termites nest and feed in the same single item of substrate (*single-piece nesters*, e.g. all Termopsidae, most Kalotermitidae), some nest in one or more items of feeding substrate and forage out onto other items of substrate (*intermediate nesters*, e.g. many species of *Microcerotermes*), some nest in one substrate and forage out onto a different substrate (*separate-piece nesters*, e.g. mound-building *Macrotermes*).

Feeding type: Termites feed on dead plant material at different stages of decomposition ('humification'). Humification is the process by which plant material is slowly degraded until only the most refractory parts of it are left. Termites feed on substrates along this humification gradient and have been classified into feeding groups accordingly. This broadly corresponds to the following substrates and groups: epiphytic bryophytes and lichens (**II**), dry grass and leaf litter (**I, II**), newly fallen dead wood (**I, II**), highly decayed dead wood with plastered soil (**II, III**) humus (**III**), soil organic matter attached (primarily) to clay particles in sub-humic horizons (**IV**). The feeding groups were defined by gut contents and morphological criteria (Donovan et al. 2001), so there is some overlap between the categories. However, generally the higher the number of the feeding group the more humified the feeding substrate. I find it useful to split group II feeders into two evolutionarily separate sub-groups, IIf, which contains the Macrotermitinae, and IIr that do not farm fungi and appear to have passed through an ancestral soil feeding state before reverting to non-soil-feeding.

Phylogenetics of Assemblages. In some cases, the published molecular phylogenetic trees (nearly always based on small subunit [16S] rDNA data); see Wuyts et al. 2002) for assemblages are not complete or are methodologically

flawed. In those few cases I have downloaded all available sequences from Genbank and reanalysed the data using Bayesian methods. The resulting trees are not always given in full in the text but full details of sequences, alignments and analyses are available from me (pe@nhm.ac.uk). The simplified trees that I present are only intended to be heuristically useful – I fully expect additional taxon sampling, sequencing and analyses to change the topology of the trees. In addition, I have presented Bayesian trees here not for any particularly sound methodological reasons but because they have produced results with the most evolutionarily interesting hypotheses.

Co-evolution. Termite:symbiont relationships represent excellent opportunities to examine co-evolution. However, there are very few cases where taxon sampling of both symbiont and host are adequate to address co-evolutionary questions. The few that exist are discussed below.

16.3 Biodiversity of Termite Guts

The diversity of termite gut communities is extraordinarily high, and is drawn from all three domains of life (see list in König et al. 2002). The functions of each group of symbionts is poorly known, although these probably include lignocellulose degradation, nitrogen fixation, methanogenesis and acetogenesis, recycling of nitrogen from uric acid, and the maintenance of a low redox potential (Breznak 2000). We have direct fossil evidence that the relationship between gut microbes and termites is at least 20 million years old (Wier et al. 2002).

Termite gut symbionts have been studied for more than 100 years, yet we have been able to culture only a small proportion of them (Breznak 2000). This accurately reflects the general picture for prokaryotes, where $> 90\%$ of known phylotypes have never been cultured. For this reason, culture-independent studies of micro-organisms, predominantly using small subunit (16S) RNA, have re-invigorated our knowledge of microbial diversity. From 1987 to 1998, using these methods, the number of estimated major groups of bacteria rose from ca. 12 to ca. 45 (Hugenholz et al. 1998; Hugenholtz 2002). It is these approaches that have also revolutionised our knowledge of the biota of the termite gut. However, although the list of, for example, bacterial groups found in termite guts is now long we have good inventory data for only two termite species, *Reticulitermes speratus* [Rhinotermitidae] (Ohkuma and Kudo 1996) and *Cubitermes orthognathus* [Termitidae] (Schmitt-Wagner et al. 2003). There are a range of other termite species from which we have a variable number of symbiont sequences (Table 16.1).

Table 16.1. Prokaryote 16S sequences associated with termite guts as recorded in Genbank as of 7 July 2004

Genus	Bacteria	Archaea	Total	Species studied	Region[a]
Reticulitermes	496	15	511	*speratus*[b], *flavipes*[b]	Jap, NAm
Cubitermes	262	164	426	*orthognathus, fungifaber*	E.Af, W.Af
Neotermes	234	0	234	*koshunensis*, [four others]	Jap
Coptotermes	90	0	90	*formosanus*[b]	PT
Mastotermes	17	16	33	*darwiniensis*[b]	NAus
Nasutitermes	26	2	28	*takasagoensis, nigriceps, lujae*	Jap, CAm, WAf
Zootermopsis	27	0	27	*angusticollis*	NAm
Pericapritermes	21	3	24	*latignathus, nitobei*	Thai, Jap
Kalotermes	22	0	22	*flavicollis*[b]	Eur
Hodotermes	19	0	19	*mossambicus*[b]	SAf
Termes	18	0	18	*comis*	Thai
Hodotermopsis	15	2	17	*sjoestedti*	Jap
Microcerotermes	13	0	13	*crassus*	Thai
Speculitemes	12	0	12	sp.	Thai
Incisitermes	10	0	10	*marginipennis*	NAm
Cryptotermes	8	1	9	*domesticus*[b]	PT
Glyptotermes	6	0	6	*fuscus*	Jap
Amitermes	3	0	3	*longignathus*	Thai
Pterotermes	2	0	2	*occidentis*	NAm
Macrotermes	2	0	2	*subhyalinus*[b], *convulsunareium*	Af,. IND
Thoracotermes	2	0	2	*macrothorax*	WAf
Heterotermes	1	0	1	*indicola*[b]	IND
Microtermes	1	0	1	sp.	WAf
Odontotermes	1	0	1	*obesus*	IND
Sinocapritermes	1	0	1	*mushae*	Jap
Total 25	1309	203	1512	35	

[a] Regions: *Jap* Japan, *NAm* North America, *EAf* East Africa, *WAf* West Africa, *PT* pantropical, *NAus* north Australia, *CAm* central America, *Thai* Thailand, *Eur* Europe, *IND* India, *SAf* South Africa

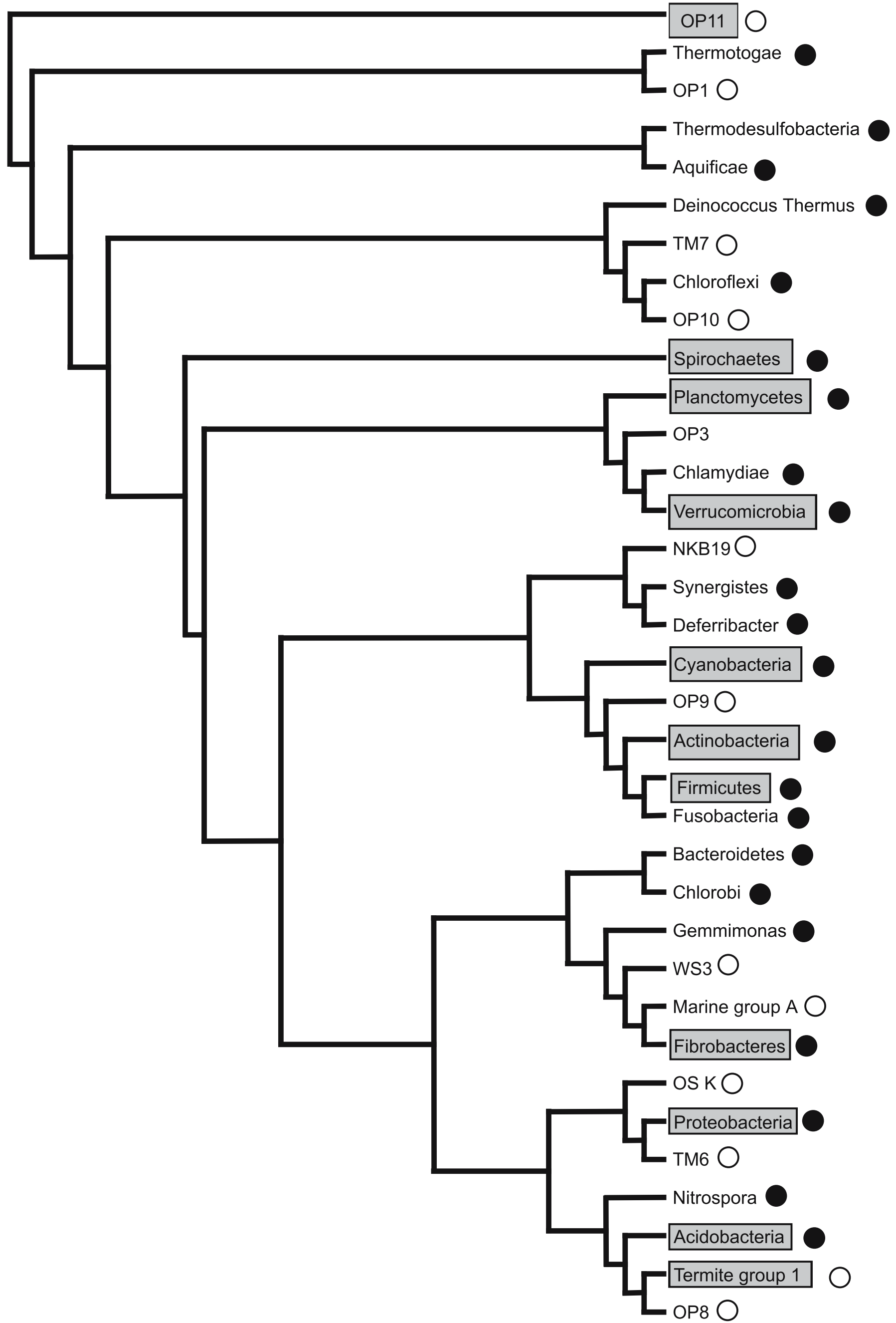
[b] Known pest species

Culture-free methods of surveying the termite gut habitat are not without their own set of problems (see Hugenholtz et al. 1998). One of the most important of these is the presence within Genbank of a larger number of relatively short sequences (<500 bp). These sequences are generally hard to place in their correct branching order on a phylogenetic tree when sequence similarities drop below ca. 85%. This greatly complicates the problem of describing the evolution of these groups within the gut. In this chapter, for this reason, I generally discuss separate clades with internal sequence similarities above this level.

Bacteria. Bacteria are abundant and diverse in all termite guts and we know a relatively large amount about them due to the inventory work referenced above. Some are found attached to the gut wall, some in the middle of the gut and some associated with flagellates (especially Spirochaetes) (Breznak 2002). The most important groups (Garrity et al. 2001), based on numbers of phylotypes recorded in existing molecular cloning studies, are the spirochaetes, the Firmicutes (= low G+C Gram-positive bacteria), the Proteobacteria, the Bacteriodetes (= *Cytophaga-Flexibacter-Bacteroides* group) and Actinobacteria (see Fig. 16.1). However, there is clearly a long tail of rarer groups, such that by 2004 ca.1300 16S rDNA sequences from 11 out of the ca. 45 recognised major groups of bacteria have been recovered from termite guts (Table 16.1). Some of these rarer groups represent bacterial clades that have never, or have only rarely, been cultured. Two of the most interesting of these are the Acidobacteria and 'candidate group OP11'. The *Acidobacterium* group has been sampled from soils, peat bogs, contaminated aquifers and acid mines, and has only a handful of cultured representatives. Group OP11, recorded from soils, freshwater sediments, the deep subsurface and hot springs (Hugenholtz et al. 1998), has never been cultured. Both these groups are also found apparently abundantly outside the termite gut, but there is one group that has so far been sampled almost entirely within the termite gut – the so-called termite group I. The termite representatives within this group appear to form a monophyletic cluster, while other putative representatives of the wider clade (recorded from activated sludge, the bovine rumen, wine vinasse and activated sludge) are not very closely related. However, this group has only so far been recorded from *Reticulitermes*; it was not recorded in the detailed inventory study of *Cubitermes orthognathus* (Schmitt-Wagner et al. 2003).

Although of considerable interest, these inventory-type data have to be treated with considerable caution; we cannot know exactly what the ecological relationship between termite and recovered bacterial group might be. Usually, in fact, we know almost nothing about the biology of the sampled group. When we do, we cannot always safely assume a close relationship. For example, among the recorded bacteria from termites are a number of very general taxa found very commonly in environmental samples. These groups include *Bacillus* spp., a number of species of which have been recorded from completely artificial habitats (e.g. inside the Kennedy Space Center; La Duc et al. 2003).

In addition, these bacterial inventories are not only from a very limited number of termite taxa, but also are severely limited in the number of clones they have studied. Rarefaction curves calculated in Schmitt-Wagner et al. (2003) show no tendency for the rate of accumulation of new phylotypes to level off at either 97% ('species') or 95% sequence similarity level. Only at the 90% level ('genus' or 'group') was any levelling-off seen. This implies


OP11
Thermotogae
OP1
Thermodesulfobacteria
Aquificae
Deinococcus Thermus
TM7
Chloroflexi
OP10
Spirochaetes
Planctomycetes
OP3
Chlamydiae
Verrucomicrobia
NKB19
Synergistes
Deferribacter
Cyanobacteria
OP9
Actinobacteria
Firmicutes
Fusobacteria
Bacteroidetes
Chlorobi
Gemmimonas
WS3
Marine group A
Fibrobacteres
OS K
Proteobacteria
TM6
Nitrospora
Acidobacteria
Termite group 1
OP8

◀ **Fig. 16.1.** 16S rDNA phylogenetic tree of bacterial phyla showing groups recovered from termite guts (*grey boxes*). *Closed circles* Phyla with at least one species that has been cultivated; *open circles* phyla with no cultivated species. Redrawn from Hugenholtz (2002), branch lengths are set as arbitrary and equal for all sister groups (ultrametric tree). Note that at present much of the topology of the tree is not well supported. (Hugenholtz 2002)

that their inventories were incomplete, and indeed new major clades are still being discovered (e.g. within the Bacteriodetes; Stingl et al. 2004). This does not mean, however, that the basic information on the relative importance of phylotypes within the gut is invalidated, just that we may be missing a number of rare groups.

Archaea. Archaea are a group of prokaryote organisms thought to form a third domain of life, separate from the Bacteria and Eukaryote. They have comparable abundances within termite guts as bacteria, although they are perhaps present at lower diversities, and again a reasonable amount of inventory work has been conducted for the group, especially within *Cubitermes* (Friedrich et al. 2001; Donovan et al. 2004). Termite Archaea are predominantly drawn from the following methanogenic clades: Methanosarcina, Methanomicrococcus, Methanomicrobiales and *Methanobrevibacter* (discussed in more detail below). These groups tend to cluster close to organisms extracted from activated sludge, waterlogged soils, and invertebrate and vertebrate guts. Methanogens have been recorded from a variety of termite groups. The detailed community of ecology of termite guts is in its infancy, but complex axial differences have already been observed in the guts of *Cubitermes* species (Friedrich et al. 2001; Schmitt-Wagner et al. 2003). A reasonably broad set of termite taxa have also been examined using RNA probe techniques (Brauman et al. 2001). However, these gave rather coarse-grain family-level data across the termite tree.

Non-methanogenic Archaea have been found in clone libraries from both *C. orthoganthus* (Friedrich et al. 2001) and *C. fungifaber* (Donovan et al. 2004), although for technical reasons they did not recover the same groups. The Donovan et al. study recovered the halophilic alkaliphile *Natronoccocus*, while the Schmitt-Wagner et al. (2003) study recovered Thermoplasmales and Crenarcheota. These non-methanogen groups are not thought to be functionally important members of the gut biota (Donovan et al. 2004).

Protists. All termite families save the Termitidae have specialised flagellate protists as symbionts in their guts. The two main groups found in the gut are Parabasalids (trichomonads and hypermastigotes, dealt with in more detail below) and Oxymonads (Stingl and Brune 2003), both amitochondriate flagellate groups. These cellulolytic organisms, often described as a vital component of termite nutrient assimilation processes, can be present in the

guts at very high abundances (Inoue et al. 2000). Much more is known about the biology and evolution of parabasalids than is known about oxymonads. Lesser components of termite guts include Retortomonads (Martínez-Díaz et al. 2001). Of particular evolutionary interest is the discovery of cellulolytic amoebae in the guts of species of the Termitidae (Slaytor 2000), although these have not yet been well characterised.

Fungi. The most evolutionarily important relationship between termites and fungi is that between the Macrotermitinae and their mutualistic partners, species of the basidiomycete fungus, *Termitomyces*. I will discuss this in more detail later in the chapter. Within guts, yeasts have been reported, all within lower termite guts (Prillinger et al. 1996). In addition, filamentous fungi have also been detected in termite guts (Rajagopal et al. 1979), although it remains uncertain whether they actually grow there under normal conditions.

16.4 The Termite Gut Habitat and its Evolution

Termite guts are all small and, as mentioned above, they all have aerobic, microaerobic and anoxic zones. They differ greatly, however, in their complexity, and this is related most directly to the nature of their feeding substrates. I propose four main habitat types based on community composition, evolutionary history and gut structure (Fig. 16.2). I have named them after the phylogenetic range of termites within which they are found (e.g. see Donovan et al. 2000).

1. The *Cryptocercus-Mastotermes* habitat. These are simple 'cockroach' guts (Noirot 1995), with a single large hindgut chamber, known as the *paunch*. They also share other cockroach characteristics plesiomorphically (Nalepa and Lenz 2000). The gut community is very similar to that found the habitat 2 (below), but the fat bodies contain the endosymbiont *Blattobacterium*. This habitat is found only in *Cryptocercus* and *Mastotermes*, the earliest branching 'termite' families, as well as in other studied cockroaches. All these hosts are group I wood-feeders. They are thought to represent the closest approximation to the ancestral symbiont community that are extant.
2. The *Hodotermes-Coptotermes* habitat. These are the standard 'text book' Group I termites, predominantly single-piece nesters in dead wood (all Termopsidae, most Kalotermitidae). However, some are separate-piece grass-feeders (Hodotermitidae), and some intermediate or separate-piece wood-feeders (Coptotermes/ Reticulitermes), sometimes forming

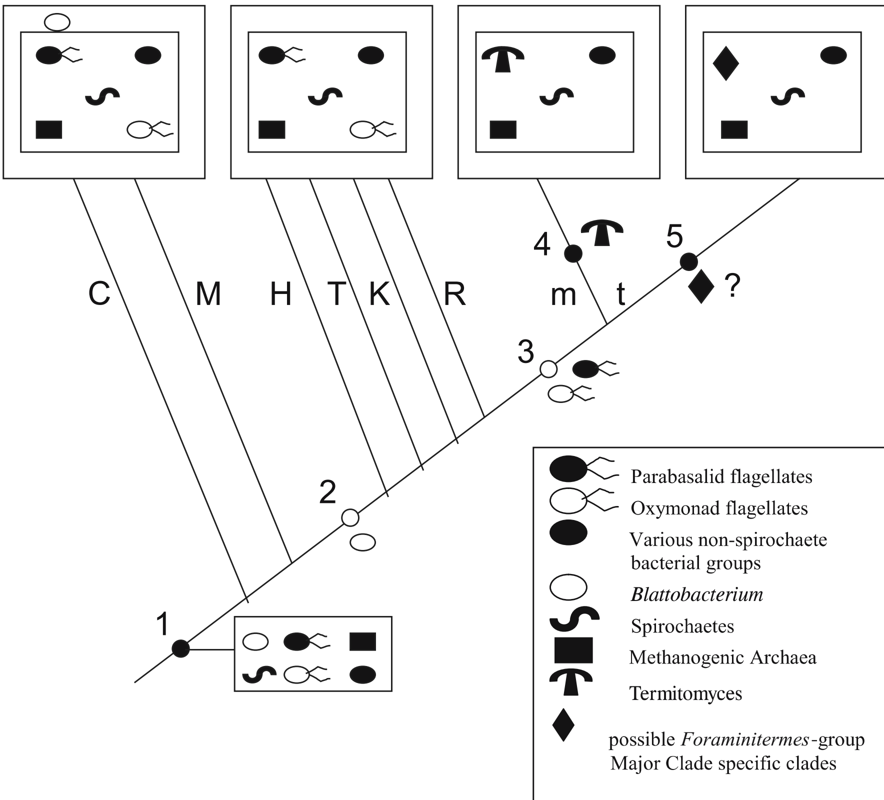


Fig. 16.2. Schematic diagram showing evolution of gut habitats with key symbiont changes. *Numbers* refer to major evolutionary events as indicated by reconstructions of termite phylogeny (Donovan et al. 2000; unpubl. molecular data): 1 acquisition of hind gut fermentation and accompanying gut biota, 2 loss of *Blattabacterium*, 3 loss of flagellates, 4 acquisition of *Termitomyces*, 5 acquisition of soil-feeding. Termite taxa: *C* Cryptocercidae, *M* Mastotermitidae, *H* Hodotermitidae, *T* Termopsidae, *K* Kalotermitidae, *R* Rhinotermitidae, *m* Macrotermitinae, *t Foraminitermes*-group Major Clade termitids

complex nest structures (e.g. some *Coptotermes*). Again, the gut is simple, and somewhat cockroach-like, (Noirot 1995). The gut community is broadly as in (1) but without *Blattobacterium.*

3. The *Macrotermes* habitat. These are the group II fungus-gardening termites (Macrotermitinae) that have, in a sense, externalised the gut by the evolution of an obligate mutualistic relationship with the fungus *Termitomyces*. There are no flagellates in these guts and the hind guts have relatively high pH.
4. The *Foraminitermes-Nasutitermes* habitat. These guts are multi-chambered complex habitats (Noirot 2001), with probably considerable co-

axial variation in community structure (Friedrich et al. 2001; Schmitt-Wagner 2003). All have a relatively high pH in the hindgut (Bignell and Eggleton 1995). These guts have been poorly sampled taxonomically but appear to be dominated by spirochaetes and methanogens. They are without flagellates, and this is probably a key evolutionary innovation that has led to a major trophic diversification in the group. Most species within this group are soil-feeders, each feeding along a particular range of substrate humification, from humus (group III) to tightly bound soil organic matter (group IV). Soil-feeding appears to be the basal condition in this group. However, a significant minority of species have reverted to less humified substrates: grass, leaf litter, microepiphytes, dry dung and wood. This range of feeding preferences (IIf, III, IV, IIr) has led to very large differences in gut structure, and although the microbial communities of this range of gut structures has not been fully investigated, where they have been they do not appear to show an enormous range of variation that is tied in with feeding habit. For this reason I shall treat them all as a single heterogeneous habitat except for the very few examples when there is somewhat more extensive data available. I would be surprised, however, if future sampling does not reveal more extensive differences in the gut communities of Termitidae with very different feeding preferences (e.g. patterns discussed in Eggleton and Tayasu 2001).

Key events in the evolution of the extant habitats (Fig. 16.2) are therefore (1) acquisition of hindgut fermentation with associated symbionts, (2) loss of *Blattabacterium*, (3) loss of flagellates, (4) fungus gardening, (5) soil-feeding. In this chapter I will use these evolutionary events as a framework for what is known about the evolution of major symbiont components. I examine how the symbionts may have colonised the gut and the evolutionary relationships within the symbiont clades both within the gut and with non-gut inhabiting relatives. In the few cases where data are available I will also discuss evidence for co-evolution/co-cladogenesis between symbionts and their hosts.

16.5
Acquisition of Symbionts in Basal Dicytopterans

The ancestors of termites were most probably cockroach-like Dictyoptera feeding on dead plant material (Grimaldi 2001). Extant cockroaches, like other detrivores, commonly feed on faeces (‘coprophagy’) (Nalepa and Bandi 2000). This feeding substrate has the advantage that it is pre-conditioned by being softened and partly macerated, and perhaps most importantly, it is readily colonised by microbes (Anderson and Bignell 1980).

These microbes form a complex consortium, assist in the breakdown of the refractory parts of the dead plant material, and also provide a direct food source. For many detrivores this is as far as the process goes and most of the microbes found in the gut of, for example, woodlice are also found free-living. Once inside the gut, however, microbes are in a protected environment that can potentially evolve to suit the particular ecophysiological needs of both the microbes and the detritivores. This 'internalisation' of the gut and the evolution of mutualism would subsequently require conspecific coprophagy, to ensure that the symbionts could be passed from one individual to another (intraspecific coprophagy). In living cockroaches this occurs in gregarious synanthropic species (e.g. *Blattela germanica*, Kopanic and Schal 1997). It also occurs, even more strongly developed, in *Cryptocercus*-like species that form family units, where symbionts are passed from generation to generation by proctodeal trophallaxis, with adult cockroaches feeding immatures with flagellates directly from their rectal pouches. This adaptation to mutualism seems to be reflected in the life cycle of termite gut flagellates. In non-social cockroaches the flagellates (in the relatively few cases where they are found, see below) are found encysted in adult faeces, from where they can pass easily from one individual to another regardless of the individual's developmental stage. In contrast, the social system of *Cryptocercus* precludes transfer of protists by coprophagy (Cleveland et al. 1960). All termites with flagellates have proctodeal trophallaxis and this seems to be a key element in the evolution of the flagellate/termite symbiosis (Nalepa and Bandi 2000; Nalepa et al. 2001). When the flagellates are lost, termites change to feeding predominantly mouth to mouth (stomatodeal trophallaxis) using glandular secretions and it is not always as clear how horizontal transmission of symbionts is achieved (e.g. see Aanen et al. 2002).

Once flagellate symbionts colonised dictyopteran guts and then were passed only by trophollaxis, they enter into an intimate association with their hosts, which although perhaps not entirely predicated by vertical transmission, is undoubtedly in some senses co-evolutionary (i.e. there is 'a continuity in the identity of the microbes passed between generations'; Nalepa et al. 2001).

The evolution of a mutualistic relationship with symbionts derived from faeces seems to be supported by the close phylogenetic relationship between extant gut symbionts and faeces-associated clades (e.g. see the *Methanobrevibacter* section, below).

16.6 Evolution of Key Enzyme Systems: Endoglucanases and Nitrogenases

Endoglucanases. The text book view of termites has predominantly been of eusocial insects feeding on dead wood with the metabolic assistance of gut flagellate mutualists. The central argument of this view of termites is an evolutionary one: termites have never developed their own cellulase enzyme system. However, as researchers have looked further into the question of cellulose digestion in termites this view has been shown to be comprehensively wrong. The first evidence against termites' need for flagellate cellulases is mentioned above; no Termitidae have flagellates, but they all feed on cellulose-rich materials. This implies that termites do not need celluloytic flagellates, but clearly the cellulose degrading duties may have been taken over by prokaryotic symbionts. However, it turns out that all examined termites have their own endogenous cellulases and seem capable of breaking down cellulose in the absence of gut symbionts (Slaytor 2000). In fact there is growing evidence that production of cellulase may be a deeply rooting synapomorphy for bilateral animals (Lo et al. 2003a,b). In addition, termite cellulase are from a different gene family (glycosyl hydrolase family [GHF] 9) than parabasalid (GHF 45) cellulases, implying that horizontal gene transfer from parabasalids to termites is unlikely (Lo et al. 2003a,b).

Having said that, though, the gut symbionts clearly have some role. Termites die if they are defaunated (Inoue et al. 2000). However, the nature of this role has been made even less certain by recent work on flagellate cellulases (Li et al. 2003) showing that at least some of the flagellates in the gut of *Mastotermes* produce no detectable functional cellulases, but instead rely on the termite endogenous cellulases. It is possible that one of the main functions of flagellates in the termite gut is as a food source (Nalepa et al. 2001).

This is a good example of a pattern that may well be repeated in other symbiont groups, where there seems to be a lot of evolutionary change occurring, but this apparent major change overlays a physiologically rather stable system. In this case the loss of flagellates in the Termitidae has had enormous ecological consequences (e.g. trophic diversification and adaptive radiation within rain forest; Eggleton and Tayasu 2001; Davies et al. 2003) but the basic celluloytic processes (i.e. breakdown of cellulose plant cell walls using termite-produced cellulases) probably remain basically the same in *Cryptocercus* as in, say, *Nasutitermes,* a wood-feeding termitid (Slaytor 2000).

Nitrogenases. Termites need endoglucanases because many of they feed on microbially unconditioned dead wood, a high energy but very low nitrogen resource. Termites, especially single-piece nesters in dead wood, are potentially nitrogen-limited. For this reason, many termites have nitrogen-fixing bacteria in their guts (Tayasu et al. 1996, 1997). However, nitrogen-fixing bacteria have proved to be difficult to culture and so an alternative approach has been used: to sequence nitrogenase genes using cloning and PCR (Ohkuma et al. 1996). The gene that has been used is *nifH*, which encodes dinitrogenase reductase, and is evolutionarily conserved across a wide range of prokaryotes (Zehr et al. 2003). The most complete survey of termite nitrogenases is Ohkuma et al. (1999) who examined six lower termites (from the Kalotermidae, Termopsidae and Rhinotermitidae) and three termitids. They discovered all four major groups of the nifH gene within their study termites: the proteobacteria-cyanobacteria (proteo-cyano) group, the anaerobe group, the alternative *nif* methanogen (*anf*-methano) group, and the pseudo *nif* group. However, the proteo-cyano group sequences were rarer in clone libraries than the other three groups. In addition, the termite sequences were not a random subset of all known *nifH* groups but formed their own set of sequences clusters distinct from clusters found in other environments. Within each cluster there is almost no sequence overlap between different termite species. There also seems to be a general different between lower termites and termitids, with termitids having many more pseudo *nif* sequences than the other termites. The pseudo *nif* gene appears not to code for a nitrogen-fixing enzyme, and termitids do not appear to fix significant amounts of nitrogen (Tayasu et al. 1997). Other overall phylogenetic patterns are also evident in the data. However, the differences appear broadly statistical, rather than indicating clear co-cladogenesis. Given the feeding-group differences between the studied termites, it is likely that a significant amount of the observed, apparently phylogenetic, differences may be due to differences in feeding preference. However, the termitids feed on a range of substrates, which overlap with some of the lower termites' feeding substrates, yet their *nifH* profiles are more similar to each other than they are to the lower termites. This suggests a possible phylogenetic effect. The effects of history and feeding ecology are clearly difficult to unravel here, but this remains one of the few studies that examines an entire functional group of symbionts across the whole range of the termite phylogenetic tree.

16.7 Blattabacterium

Blattabacterium is an intracellular bacterial symbiont that lives in specially adapted cells (mycetocytes or bacteriocytes) within the fat body of

all cockroaches and *Mastotermes*. The symbiont seems to be transferred entirely vertically in parental eggs (Bandi and Sacchi 2000) and genotypes are uniform within a host species (Bandi et al. 1994). The genus appears to be monophyletic within the *Cytophaga-Flavobacterium-Bacteroides* (CFB) lineage of bacteria (Bandi et al. 1995).

Blattobacterium represents one of the more clear-cut examples of co-evolution within the termite + cockroach clade, probably because the relationship between host and symbiont appear to be obligately mutualistic (Cochran 1985) and because horizontal transfer from host to host is unlikely given the symbiont's life history. At a broad phylogenetic scale, Lo et al. (2003a,b) reconstructed the phylogeny of *Blattobacterium* (using 16S rDNA sequences) and of representatives of the cockroach-termite clade (using 18S rDNA, COII, 12 s rDNA and 16S rDNA sequences). They found highly significant levels of congruence between their trees, and concluded that almost strict co-cladogenesis had occurred. They were also able to put molecular clock estimates on the evolution of the 16S rDNA *Blattabacterium* sequences, suggesting that the [termite plus *Cryptocercus*] lineage diverged from its sister group, the Blattidae, ca.144 MYA.

The loss of *Blattabacterium* in all termites except *Mastotermes* has not yet been satisfactorily explained. *Blattabacterium* appears to have a role in the cockroach nitrogen economy, by recycling excretory uric acid into usable nitrogenous compounds. Gut symbiont bacteria may have taken over this role in more phylogenetically apical termite groups (Lo et al. 2003a, b), although the exact physiological details are still uncertain (Breznak 2000).

16.8 Parabasalids

These are flagellated protists thought, at least until recently, by many researchers to branch very early among the eukaryotes (within the so-called 'Archaezoa'; Cavalier-Smith 1993). They have no mitochondria (Cavalier-Smith 1987) and have RNA subunits intermediate in size between prokaryotes and eukaroytes (Wuyts et al. 2002). Their exact phylogenetic position is uncertain, although recent work suggests that the groups may be later branching within the Eukaryota than originally hypothesised (Silberman et al. 2002; Simpson et al. 2002). Oxymonad and parabasalid termite symbionts, although superficially similar, are now known not to be closely related (Moriya et al. 2001). Parabaslids may actually form a relatively early branching clade, while oxymonads appear to be closely related to *Trimastix*, a later branching protist group (Dacks et al. 2001).

A majority of parabasalids are found in the guts of termites and feed on recently dead plant material, essentially wood or dry grass. However,

other members of the clade are found in vertebrate and other invertebrate guts, while others are free-living in freshwater or marine habitats. Termites can harbour anything from a few to ca. 20 parabasalid species in a single gut, and different species of termites can have very similar parabasalid assemblages (Yamin 1979). This, together with a considerable intraspecific variation in symbiont assemblage composition (Kitade and Matsumoto 1993), suggests that strict co-evolution between host and symbiont cannot have occurred (Kane and Mueller 2002).

Parabasalids have been extensively investigated due both to their apparent phylogenetic position and their role in termite nutrition. Recently, the phylogenetics of the group has been studied quite extensively using 16S rDNA sequences (Keeling 2002; Gerbod et al. 2002). These studies have now been superseded by additional taxon sampling, and there are some tree rooting problems. In addition, none of the recent papers have been explicitly interested in the group in its more general evolutionary (i.e. habitat) context. For these reasons I present here a phylogenetic reconstruction using most of the available Genbank sequences (Fig 16.3).

The most parsimonious reconstruction of habitats (Fig. 16.3) clearly indicates that the basal habitat for Parabasalids is termite guts and that there have been several colonisation events from termites to (1) other metazoan guts and (2) free-living habitats. In most cases, the shift to (1) represents a change from strict specialists in termite guts to generalists in a range of vertebrate and invertebrate guts, often also including (possibly secondarily) termite guts. Non-*Cryptocercus* wood-feeding cockroaches appear only to have these generalist Parabasalids (e.g. *Monoceromonas* and *Tetratrichomastix* in *Parasphaeria boleiriana*; Brugerolle et al. 2003).

This evolutionary scenario initially appears paradoxical: parabasalids are a very ancient group and termites a relatively recent one (perhaps originating in the late Jurassic; Thorne et al. 2000). This implies, given the basal association between the two groups, that there must have been several hundreds of millions of years during which parabasalids were in some other, presumably termite-like, gut habitat. Such habitats are known to have existed in the form of basal cockroach-like Dictyoptera. These groups, unlike modern non-predatory Dictyoptera, had long piercing or drilling ovipositors and are assumed to have feed on woody plant material (Grimaldi 2001; Lo et al. 2003a, b). If these basal Dictyoptera were habitats for parabasalid flagellates then the present day termite gut habitats represent relict flagellate habitats of considerable evolutionary interest.

Within parabasalid clades found only in termites, there does not appear to be much evidence for strict co-cladogenesis. Most termite families are found as the host for most flagellate clades. However, there are some emerging patterns in the distribution of parabasalid taxonomic groups across termite groups (Table 16.2). First, the two earliest branching parabasalid

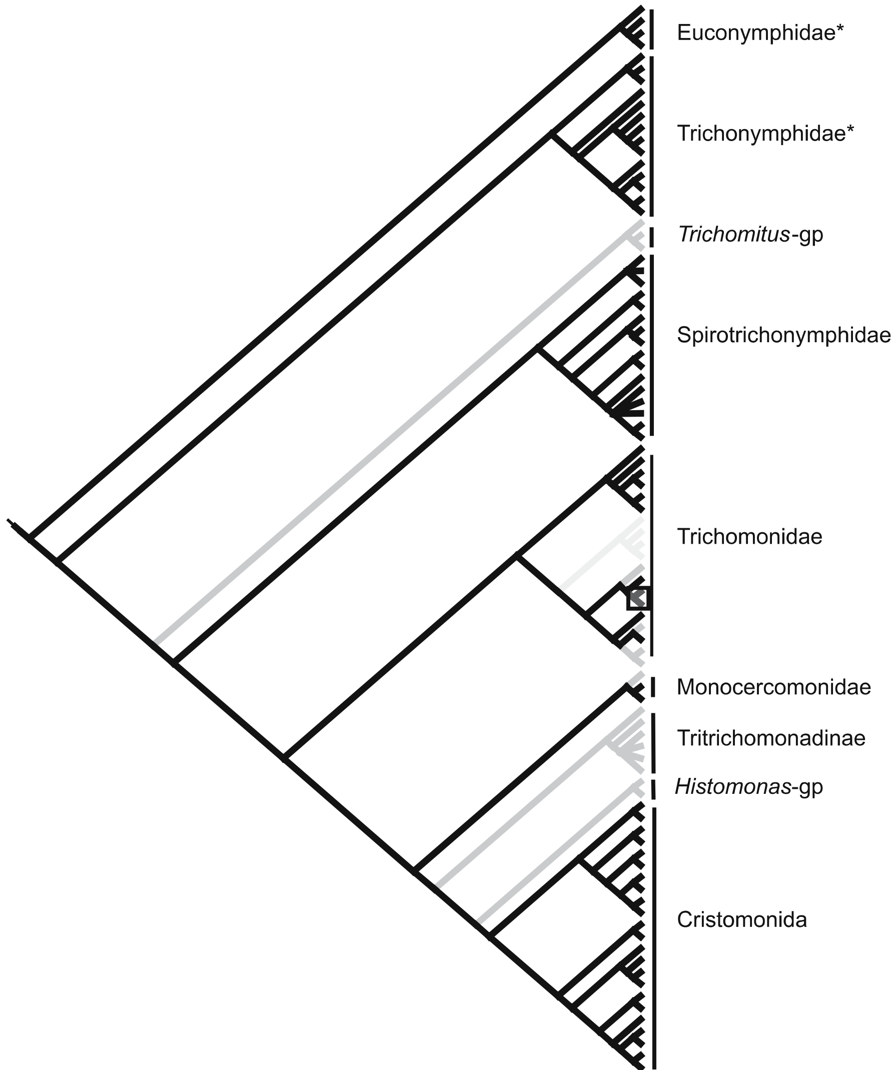


Fig. 16.3. Topology of phylogenetic tree for parabasalids derived using Bayesian methods on Genbank 16S rDNA data. The tree was generated using Mr Bayes with the following parameters: lset nst=6, rates=gamma, shape=0.6290; mcmc ngen=700000, printfreq=100, samplefreq=100 nchains=4. Outgroups (not shown on tree) used to root the tree were: *Acidolobus aceticus*, Uncultured CrenL25852 (Crenarcheota), *Aphrodite sulfiphila* (Euryarcheota) and *Giardia ardeae* (Diplomonadida). Habitat type is optimised onto the tree using simple parsimony: *black* branches=termite guts; *mid grey* branches=non-termite guts, predominantly vertebrates; *light grey* branches=free-living marine and freshwater habitats. The *square* indicates organisms with very large cell size. *Starred* groups are found in *Cryptocercus*.

Table 16.2. Matrix of parabasalid groups in different groups of termites (predominantly based on Yamin 1979)

	Cr	Ma	Ho	Te	St	Ka	Pa	Rh	IN	Fr	VE
With sequence data:											
Eucomonyphidae[a]	X			X		X		X			
Trichonymphidae[a]	X		X	X		X		X			
Trichomitus[b]											X
Spirotrichonymphida[a]			X	X		X	X	X			
Trichomonidae[b]		X	X	X		X	X	X	X	X	X
Monocercomonidae[b]			X	X		X	X	X	X		
Tritrichomonadinae[b]											X
Histomonas[b]											X
Cristamonadida[c]		X	X	X		X	X	X			
No sequence data:											
Rhizonymphidae[d]			X								
Kofodiidae[d]							X				
Staurojoeninidae[a]	X					X					
Teranymphidae[a]								X			
Spirotrichosomidae[a]	X				X						
Hoplonymphidae[a]	X						X				
Lophomonadidae[d]	X										
Cochlosomatidae[b]											X

Cr Cryptocercus, *Ma Mastotermes*, *Ho* Hodotermitidae, *Te* Termopsidae (part), *St Stolotermes* (Termopsidae), *Ka* Kalotermitidae (part), *Pa* Paraneotermes (Kalotermitidae), *Rh* Rhinotermitidae, *IN* invertebrate guts (including cockroaches), Fr free-living in aquatic habitats, VE vertebrate guts. Classification broadly follows Brugerolle and Lee (2000), except for the recognition of the Cristomonadida (sensu Brugerolle and Patterson 2001)

[a] Parabasalid order: Trichonymphida

[b] Parabasalid order: Trichomonida

[c] Parabasalid order: Cristomonadida = part of Trichonymphida (Joeniidae), part of Trichomonida (Devescovinidae, Calonymphidae), and Lophomonadida

[d] Parabasalid order: probably belongs in the Cristomonida

groups at the base of the tree are found in *Cryptocercus* (as well as other groups), suggesting a possible early ancestral relationship. Second, there is a strong similarity between the parabasalids found in *Cryptocercus* and *Stolotermes*. This potentially ties in well with morphological data that suggest that *Stolotermes* is an early branching termite. Third, *Paraneotermes* (Kalotermitidae) has an anomalous parabasalid composition, sharing some taxa with *Cryptocercus* and having some unique taxa. *Paraneotermes*, a desert termite, is unusual among Kalotermitidae in being an intermediate nester. Unfortunately we have very few parabasalid 16S sequences from *Cryptocercus*, *Stolotermes* or *Paraneotermes*.

Overall, the pattern of relationships does suggest a large degree of horizontal transfer of gut flagellates from one termite group to another, within the context of an essentially termite-specialised clade. It will be interesting to see how these postulated evolutionary transfers fit in with the old controversy concerning transfaunation – the possibility that flagellates can be passed from one host to another in a log within which they both nest. This hypothesis has had strong supporters (Thorne 1990, 1991) and opponents (Nalepa 1991).

Within such a long-established symbiosis the possibility exists that parabasalids may show parallel co-cladogenesis within sub-clades (i.e. that all or most existing parabasalid clades already existed in the ancestor of the termites/*Cryptocercus* clades). However, the data is too scanty to test this at present and would require a detailed phylogenetic analysis of at least one of the putatively monophyletic clades in Table 16.2 as well as their termite hosts.

16.9 Termitomyces

The Macrotermitinae (fungus-growing termites) form the most basal subfamily within the Termitidae, the family within which all symbiont flagellates have been lost. This loss of flagellates is correlated with a major diversification in feeding habits and some major changes in symbiont relationships. All Macrotermitinae have formed a mutualistic relationship with a basidiomycete fungus genus, *Termitomyces*. The fungus is grown within the nest chambers in a comb – an intricate honeycomb-like structure consisting of faecal matter upon which the fungal hyphae grow, and which forms an 'external rumen'. Worker termites defecate onto the comb to provide food for the fungus, and in turn they feed on the growing fungal mycelium and sporocarps. This is a very successful mutualism; in dry African savannahs fungus-growing termites are the most ecologically important decomposer organisms (Bignell and Eggleton 2000).

The most plausible explanation for the evolution of this mutualism is based on fungal invasion of carton termite nests (Donovan et al. 2001). A number of Rhinotermitidae (e.g. *Coptotermes*) build carton nests made of woody faecal material within soil mounds. Many of these nests are known to harbour significant populations of micro-organisms. It is very likely that worker termites feed on the carton material as microbial-conditioning tends to decrease the C:N ratio, thus helping the nitrogen balance of the colony. Fungal invasion of carton nests in *Coptotermes*-like ancestors of the Macrotermitinae may have led to the carton material becoming the main food source for the worker termites. In this case, the carton nest would

eventually no longer have a structural function, and therefore soil would have to be used as the only available building material for nests. The effect of all this would be to 'externalise' the gut (Nalepa et al. 2001) and this externalisation may also explain the loss of flagellates in the Termitidae gut (Eggleton and Tayasu 2001).

One major co-evolutionary study of the Macrotermitinae and *Termitomyces* has been published (Aanen et al. 2002). The authors examined species of Macrotermitinae and extracted fungi directly from the termite guts. They used Bayesian analysis of sequence data (COI) to construct both a termite and a fungal phylogenetic tree. These trees showed that the symbiosis originated in Africa and has evolved only once. The analyses found broad patterns of co-cladogenesis at higher taxonomic levels, but evidence for frequent host shifting. This suggests a complex mixture of vertical and horizontal acquisition of the symbionts. This is partly explained by the way the fungus is established in young colonies, with most species picking up initial spores from adjacent soils. Only *Microtermes* spp. and *Macrotermes bellicosus* show strict vertical inheritance from generation to generation.

16.10 Spirochetes

Spirochetes are very common in termite gut habitats (Breznak 2002) – very few, if any, other habitats have as high a morphological diversity and density of these bacteria (Ohkuma et al. 1999). They have been found in all studied termite species, although a full survey across all sub-clades of termites has never been attempted. Within termite guts they can be free-living within the gut, attached to the gut wall, or associated with oxymonad or parabasalid flagellates (either attached to the cell wall or entirely inside the protist cell). In some cases the flagellates act as 'false cilia' providing a propulsion mechanism for the protests ('motility symbiosis'). The role of spirochaetes is still not entirely clear, although some have been shown to be CO_2-reducing acetogens (Leadbetter et al. 1999) and others fix nitrogen (Lillburn et al. 2001); both processes would benefit the termite physiologically.

All known termite spirochete symbionts belong to the *Treponema* cluster (Berchtold and König 1996; Lillburn et al. 1999), and form two clearly defined lineages named cluster I and cluster II (Ohkuma et al. 1999). Cluster I groups close to *Spirochaeta stenostrepta* and *S. caldaria*, cluster II groups close to *Treponema bryantii*. Cluster I has been found in a wide range of termites (all that have been studied so far), cluster II has only been found in *Reticulitermes flavipes*, *R. speratus* and *Hodotermopsis sjoestedti*. In situ hybridisation studies have shown that cluster I species are found

both free-swimming within the termite gut and as ectosymbionts of gut flagellates, while cluster II is generally only found on the flagellates (Noda et al. 2003).

Cluster I shows some putative clades that are species-specific and some that may be family specific (e.g. groupings found only in Termitidae so far), although these groupings are not very well supported in the published trees (e.g. Fig. 16.1 of Ohkuma et al. 1999). Elsewhere in the cluster I tree, however, the tree topology suggests a very complex set of acquisitions and horizontal transfers that are not indicative of strict co-evolution of the spirochetes and their host.

Spirochetes within flagellates do not seem to be monophyletic: there appears to have been multiple independent acquisitions of spirochete attachment to gut protists (Noda et al. 2003). However, there does appear to be some host specificity: the spirochetes found in Pyrsonymphidae (*Dienympha* and *Pyrsonympha*) cluster separately from those in Oxymonadidae (*Oxymonas*).

Analyses of the full Genbank dataset (not shown) suggest at least two separate termitid clades. However, there is little evidence of co-evolution in the present, admittedly poorly taxon sampled, dataset, although there is some suggestion that species may have a unique set of symbionts (Lillburn et al. 2001).

16.11 Clostridiales

This bacterial group has a dauntingly high diversity in termite guts. The highest recorded phylogenetic diversity is from soil-feeding termites. In *Cubitermes orthognathus* there are clones from at least nine sub-groups of the Clostridiales (groups I, III, IV, XI, XIVa, XIVb, the *Spirobacter*-group, the *Sporomusa*-group, and one undefined, predominantly termite group) (Schmitt-Wagner et al. 2003). Clones derived (using a similar methodology) from *Reticulitermes speratus* (Hongoh et al. 2003) have a similar phylogenetic structure, although not all groups found in *Cubitermes* were recovered. This produces a massive dataset from just two species which, together with closely related environmental samples, is too large easily to analyse comprehensively. The analyses that are straightforward (using neighbour joining methods and BLAST searches on relatively short sequences) suggest a number of putatively termite-specific clades (not shown). This group, perhaps more than any other, indicates how complex the evolutionary ecology of the termite gut is likely to be.

16.12 Archaea

I will deal with these together as they form a more coherent evolutionary unit than the various bacterial groups. A large majority of the archaeal groups discovered in the guts of termites are methanogens classified predominantly within the Methanobacteriacae, Methanosarcinaceae and Methanomicrobiales. They are associated with the gut wall (especially in *Reticulitermes*; Leadbetter and Breznak 1996), with flagellates, and within the lumen of the gut. The main termite groups cluster close to *Methanospirillum* (Methanomicrobiales) and *Methanobrevibacter* (Methanobacteriaceae).

The systematics of *Methanobrevibacter* have been discussed in a recent paper (Dighe et al. 2004), where they analysed 16S rDNA sequences from a range of species, including several termite sequences. They propose a number of groupings, with a monophyletic 'termite gut symbionts' group as the sister group of a *curvatus* group (itself a symbiont of *Reticulitermes*) as well as individual gut symbionts within the *curvatus, arboriphilicus* and *filiformicus* groups. This broadly implied a termite-specific group made up of those four *Methanobrevibacter* groups; although their dataset had only one termitid. However, since the analyses in the Dighe et al. paper a number of *Methanobrevibacter* sequences from termitids have become available. Inclusion of those sequences, using Bayesian analysis, produces a superficially somewhat different tree (Fig. 16.4) (although comparison of the two trees is difficult given the variable lengths and different relative positions of the gene sequences used for the two trees). The reanalysis supports three clades with distinct properties: (1) a clade consisting of Dighe et al's *ruminiatum*, *smithii*, and *woesei* groups found predominantly in vertebrate guts/rumen and faeces; (2) a clade consisting of the *curvatus*, *filiformis* and 'termite gut symbionts' groups found in the guts of lower termites and associated with their flagellate protist symbionts; (3) a clade consisting of the *arboriphilicus* group, all existing termitid sequences and sequences from the endosymbiont ciliate *Nycotherus ovalis* found in the guts of cockroaches (Gijzen et al. 1991). This topology does not, in fact, contradict the Dighe et al. one as it only requires the addition of the termitid and ciliate sequences to the *arboriphilicus* group on the old tree to obtain a topology consistent with the new tree. In addition, the topology of the 'termite gut symbionts' group is essentially identical in the two trees: neither support the specific gut epithelium versus flagellate symbiont clades that Tokura et al. (2000) proposed, again, however, with a somewhat different dataset.

The relationship between cockroach ciliate methanogens and termitids, as suggested by the topology of the *arboriphilicus* part of the tree in Fig. 16.4,

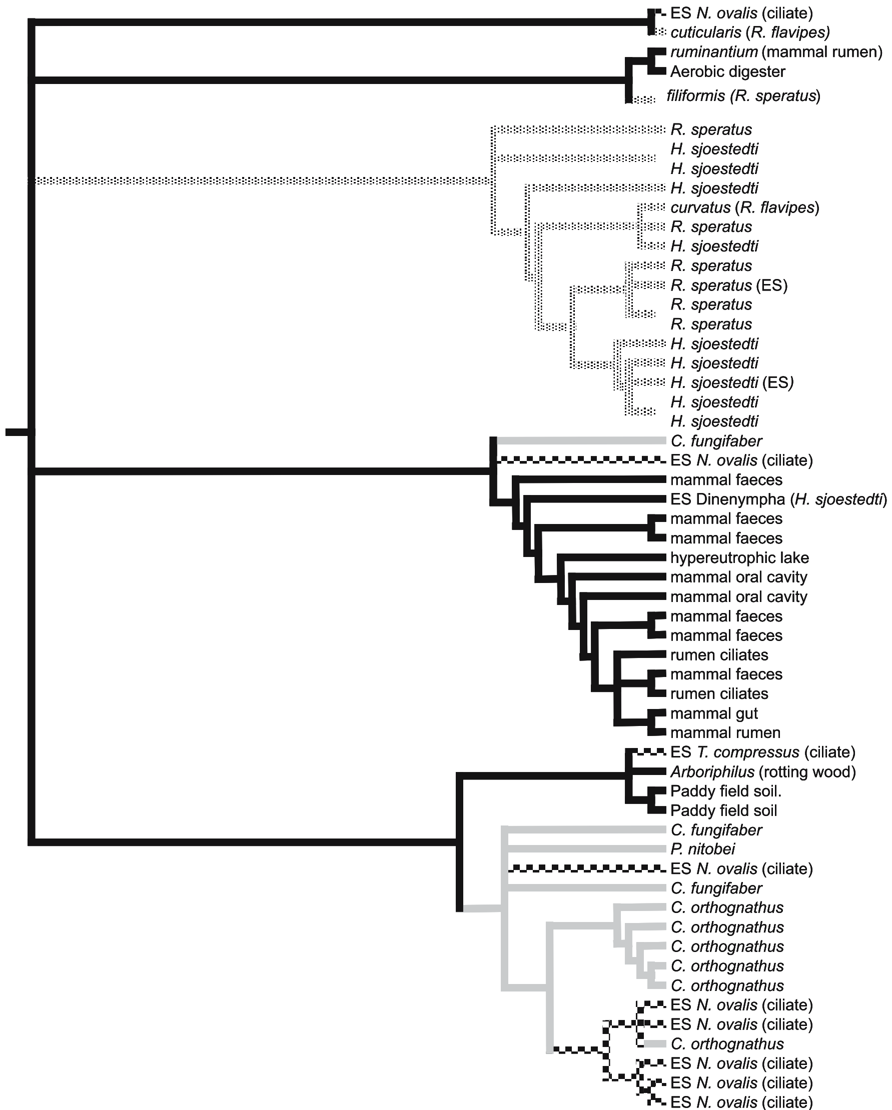


Fig. 16.4. Topology of phylogenetic tree for *Methanobrevibacter* derived using Bayesian methods on 16S rDNA Genbank data. The tree was generated using Mr Bayes with the following parameters: lset nst=6, rates=gamma, Shape=0.6690; mcmc ngen=300000 printfreq=100 samplefreq=100 nchains=4. Outgroup (not shown on tree) used to root the tree: *Methanococcus aeolicus*. Habitat type is optimised onto the tree using simple parsimony: *black* branches=vertebrate guts and faeces, *checkered* branches=endosymbionts of protists within ciliates in insect guts, *grey speckled* branches=in non-termitid termite guts (in lumen, on gut wall, or associated with flagellates), *dark grey* branches=in termitid guts. Phylotype names are replaced by names of habitat; *ES* endosymbiont

is obscure, but otherwise the hypothesis suggested here is of two major *Methanobrevibacter* colonising events in termite history, with one wave brought in by flagellates, the other introduced with soil-feeding, possibly with ingested soil. Within each group, however, there is not, as yet, good enough taxon sampling to examine possible co-evolutionary patterns.

A potentially termitid-specific clade is also found within the Methanomicrobiales, within a cluster close to *Methanospirillum hungatei* (Friedrich et al. 2001; Donovan et al. 2004 refer to it as 'termite archaeal group I'). These sequences have only so far been found in species of *Cubitermes*, *Pericapritermes nitobei*, and *Nasutitermes* sp. These termites have a range of feeding preferences: groups IV, III, and IIr, respectively. Therefore there seems to be no obvious ecological link between the presence of the gut symbiont clade and feeding preference. In addition, the data are too scanty to detect any relationship between the phylogenies of termite and symbiont. However, so far the symbiont clade has only been found in the *Foraminitermes* major clade.

Ancestral termitids appear likely to have had an intimate association with soil, either as soil feeders or by using soil to build their nests (Donovan et al. 2000). It therefore seems likely that termitid-specific methanogens originally colonised termite guts from the soil. However, it is not clear whether the soil still provides inocula for soil-feeding termite (horizontal transmission) or whether they pass methanogens (and other microbes) vertically from generation to generation. The distinction between these two methods is an important one, as vertical transmission is much more likely to lead to a long term evolutionary relationship between symbionts and their hosts than horizontal transmission (c.f. Macrotermitinae above).

A recent attempt has made to examine this question indirectly (Donovan et al. 2004). Samples were taken of soil-feeder (*Cubitermes fungifaber*) guts and the soil surrounding the sampled termites' mounds. Methanogens within guts and mounds were characterised across different soil types in Cameroon using a combined TRFLP and cloning technique. The results showed very large differences in the archaeal communities of the two microsites, with the termite gut profile varying very little with geographical position and soil type while the soil samples showed a much wider range of variation. This indicated that termite gut methanogen assemblages were not just subsets of the surrounding soil biota and therefore the data did not support the hypothesis that termite gut methanogens are derived from their food. However, the degree to which methanogens are transmitted vertically is still uncertain.

16.13 Conclusions

The overall history of termite gut mutualist clades is complex and needs to be examined against the evolutionary history of the gut habitats. This makes the problem a community or evolutionary ecology question as much as a co-evolutionary problem. There are clearly some apparently termite-specific groups, although there is always the possibility that the clusters may be broken up by subsequent, more comprehensive, taxon sampling.

Taxon sampling is an important problem, for both symbiont and host. What we know about the physiology and biochemistry of symbionts is strongly limited by what can be cultured in the laboratory. The only easily culturable bacterial phyla are the big four 'weed' groups (Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes), which predominate in non-molecular studies of termite gut bacteria (e.g. see Breznak 2000). In addition, most symbiont 16S rDNA gene sequences come from a small number of pest termite species (ca. 60% of the sequences in Table 16.1), implying that what we mostly know about, so far, are 'weeds growing within weeds'. We have, overall, 16S rDNA symbiont sequences from only 1% of termite species and 9% of the genera. In total this works out at roughly half a sequence per described termite species. This is nowhere near enough to represent a proper inventory of termite gut habitats.

Although the data are scant and noisy, it seem likely that major physico-chemical changes in the gut and in gut function, which are associated with important cladogenetic events, have supplied the raw material for colonisation events by symbionts into termite guts. The most important of these events was undoubtedly the assumption of hindgut fermentation in a primitive dictyopteran feeding on refractory dead plant material. Protist flagellates entered this system and brought with them a range of bacterial and archaeal symbiotic associates, including, perhaps, clades of spirochaetes and methanogens. A group like *Methanobrevibacter* shows this pattern: the taxa associated with lower termite guts are a mixture of free-living and flagellate-associated forms. These latter forms have not been recorded from termitids, all of whom have lost their intestinal flagellates. This pattern also applies to spirochetes: Cluster II spirochetes are associated with gut flagellates; they have not been recorded from any termitid.

The loss of flagellates in the Termitidae is correlated with an immediate diversification in feeding habits (Eggleton and Tayasu 2001). This has led to a number of new symbionts colonising the termite system. Macrotermitinae have a close relationship with *Termitomyces*. This relationship is relatively well characterised and does, at least in part, shows evidence for co-cladogenesis at higher taxonomic levels and also some degree of vertical transmission of symbionts. The assumption of soil-feeding in the

Foraminitermes major clade also completely changed the physico-chemical conditions within the gut and presented soil microbes with an excellent opportunity to colonise the new gut habitat. We do not yet know the degree to which these soil microbes have subsequently involved intimate co-evolved relationships with their hosts. However, there are a number of candidate clades that at the very least seem to be limited to termitid gut habitats.

Once inside the gut, it is tempting to generalise that co-evolution does not seem to be widespread between symbionts and termites. There are only two examples where co-evolution appears to be evident: *Blattabacterium*, where complete congruence appears to exist between the two phylogenies; and *Termitomyces* where there is a more complex set of horizontal and vertical evolutionary processes. In Parabasalids some horizontal transfer of symbionts seems irrefutable. However, this appears to be a very ancient symbiont:host system where phylogenetic and ecological relationships are likely to be overlaid with multiple evolutionary events that will make them hard to unravel. For other groups, such as spirochaetes and clostridia, taxon sampling is too incomplete for authoritative conclusions to be drawn.

There are some clues, however, that begin at least to suggest strong evolutionary interactions between termite and symbionts. In the case of methanogen groups where termites might reinoculate themselves from soil reservoirs, there is the counter evidence that wood-feeding taxa, which never have significant amounts of soil in their guts (Donovan et al. 2001) have methanogens from the same clade in their guts. Addressing the problem from the opposite direction, we can profitably look at the symbionts of soil-feeding insects that are distantly related to termites. Surveys of the gut assemblages of a humivorous Sacrabaeidae beetle larva, *Pachnoda ephippiata*, using 16S rDNA sequences, reveal a wide range of prokaryotic symbionts, but no sequences that cluster close to any of the putative termitid-specific groups (Egert et al. 2003). This suggests, even being very cautious, that these groups are not readily available as inocula in all soils.

The emerging picture is of a complex mix of apparently unchanging biochemistry and physiology alongside profound differences in functional ecology. Against this background some changes appear important, but may in a sense be quite trivial physiologically (loss of flagellates). Other changes have had enormous ecological impact (the evolution of fungus-growing termites, the evolution of soil feeding) but their evolutionary influence on gut communities is harder to define. My feeling is that these massive lifeway changes will have led to very different gut habitat communities, but that we have not yet looked at a wide enough range of either hosts or symbionts for complex evolutionary patterns to be clear. This task may be easier if we move towards a more holistic 'evolutionary community ecology' approach and away from the more narrowly defined search for 'co-evolution'.

Most termites live in tropical rain forests (Davies et al. 2003), which probably have the highest species densities of any ecosystem in the world. The guts of termites are equally qualified to be thought of as biodiversity 'hot spot', and more than that they are also evolutionary hot spots, carrying around an extraordinary consortium of organisms, some recently arrived, some relics of apparently ancient relationships. Contemporary phylogenetic studies will allow us to reconstruct the evolutionary histories of the termite hosts. A larger scale, and even more exciting prospect, is that we might eventually understand the construction, variation and evolutionary history of the gut community as a whole.

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Part III
Modern Methods for Studying Intestinal Methods

17 The Microbial Soil Flora: Novel Approaches for Accessing the Phylogenetic and Physiological Diversity of Prokaryotes

Alexander H. Treusch, Christa Schleper

17.1 Introduction

Soils are among the most diverse and interesting ecosystems on this planet. They harbor complex communities consisting of animals, plants and microorganisms with many interactions between them. "Soil organisms", including plants, are largely involved in essential global biogeochemical cycles like the C- and N-cycles. Key pathways like, for example N- and CO_2-fixation, denitrification, nitrification and many ways of degradation of organic material are mainly driven by soil organisms and especially microorganisms. Soils are also of interest because of their importance for agriculture, plant growth and bioremediation strategies. In contrast to their importance, the understanding of the diversity of soil microorganisms and the interactions between them are barely understood, mainly because of the overwhelming complexity of these ecosystems. The number of microorganisms in soil habitats, typically 10^9 cells/g, by far exceeds that in freshwater or marine habitats. Most of the microorganisms occur in the organically rich surface layers and in association with plant roots while they are less abundant in the underlying mineral soils. Soils contain different textures, depending on the varying proportions of clay, silt and sand particles, which influence the surface area available for microbial growth and the chemistry of the soil. Furthermore, plants and animals and varying physicochemical parameters, like temperature, pH, moisture and oxidizing/reducing conditions greatly influence the indigenous active microbial communities of different soil habitats. The residing microbial diversity is also influenced by the discontinuities in concentrations of, e.g. oxygen, which creates microhabitats of aerobic and anoxic zones that occur in relatively close proximity to each other. Therefore, obligate aerobes and anaerobes as well as facultative microorganisms are typically found living together in soils, each in its own microhabitat. While the abundance

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distribution of plant and animal species in samples from a wide variety of communities have been intensively studied during the past 100 years, just a very small fraction of < 1% of the microbial species living in soils is known (Dunbar et al. 2002). Instead of discussing the few well-described microorganisms that have typically been isolated from soils, this review focuses on recent results and technical developments that allow one to assess the huge microbial diversity in soil including novel cultivation strategies and molecular biological techniques.

17.2 The Modern Classical Approach

Since its beginning, microbiology depends on isolation and cultivation to describe species and their physiological properties, as it was first done by Robert Koch. Selective media and defined growth conditions are used, although these are very different from the conditions found in the ecosystem, in this case in soils. By now, around 5000 prokaryotic species have been cultivated and validly described with this approach (DSMZ 2004). Among them are the "classical" microbial species from soil, e.g. bacilli, streptomycetes and clostridia. But conventional cultivation techniques are stretched to their frontiers because they are selective and biased mostly by favoring fast growing organisms (Ferguson et al. 1984; Eilers et al. 2000). In order to circumvent this bias, new cultivation techniques have been developed that are based on low nutrition media or diluted media. Janssen et al. (2002) used 1:100 diluted nutrient broth (DNB) to culture and isolate members of novel lineages of the phyla Acidobacteria, Actinobacteria, Proteobacteria and Verrumicrobia from a bulk soil of an Australian pasture. They observed, using a serial dilution liquid culture technique described by Chin et al. (1999), that only 1.4% of the mean microscopically determined total cell count could be cultured, while solidification of DNB with agar or gellan gum resulted in cultivation of 5.2 and 7.5%, respectively. Sonication of the soil sample prior to dispersal raised the viable count to 14.1% when gellan gum DNB plates were used. In a follow-up study, refined methods were used to investigate the culturability of microorganisms in a 10 cm core of soil from a mixed rye grass and clover pasture (Sait et al. 2002). Here, culturability of the microorganisms decreased with increasing depth. A maximum of 19% of the microscopically countable cells produced colonies (0–2 cm section) with a minimum of 2.4% of the cells (8–10 cm section). From 71 of 210 selected colonies it was possible to obtain pure cultures by repeatedly fresh plating. The organisms were assigned to the bacterial phyla Acidobacteria, Actinobacteria, Bacteroidetes and Proteobacteria (alpha, beta and gamma subdivision). Beside isolates of well characterized soil bacteria, it was pos-

sible to obtain pure cultures of organisms belonging to groups with few, atypical or no cultivated representatives, e.g. the Acidobacteria.

Expanding the range of growth substrates (including the use of, e.g. soil extract), the same group reported the isolation of 350 soil microorganisms that could be assigned to 60 families in 9 bacterial phyla (Joseph et al. 2003). One-third of these 60 families has no validly named or described species, making the 93 isolates falling into these 20 families the first cultivated representatives. The isolation of these new species from soil led the authors to conclude that many organisms are culturable by random approaches.

Other new approaches attempt to simulate the natural environment in more detail than can be done in petri dishes or liquid cultures, under the presumption that if all chemical components of the natural environment are present, nearly every microbial species could be grown in culture. In particular, interactions between organisms that exchange metabolites and signaling molecules have not been considered in the past. Kaeberlein et al. (2002) were able to obtain pure cultures of new marine species relying on diffusion growth chambers, in which they embedded dilutions of extracted cells from a marine sandy sediment with agar. Sealing the chambers was done with 0.03 μm pore-size membranes, which were incubated on a sample from the same marine sediment in an aquarium filled with seawater. Driven by the same train of thought, Zengler et al. (2002) developed a method based on the encapsulation of single cells into gel microdroplets (GMDs). Culturing those encapsulated cells together in growth columns with continuous flow of fresh media simulated the natural environment, mainly by allowing the exchange of metabolites and signaling molecules. While focusing on a marine sample of the Sargasso Sea, they also showed cultivation of species from a soil sample in Ghana. Flow sorting of GMDs in which microcolonies had been grown resulted in the isolation of novel representatives from the alpha, beta and gamma subclasses of the Proteobacteria as well as Gram-positive bacteria.

Using those new cultivation approaches, thousands of additional species may be isolated. Hopefully it will be possible to get representative isolates of every lineage (predicted by molecular approaches) in pure culture. However, at present, it seems impossible to capture the whole microbial diversity from soils by cultivation, just because of the large number of species that are out there.

17.3 How Many Prokaryotic Species Live in Soil?

To answer this question it is necessary to define the term "species" first. In the prokaryotic world this is a tremendous task, as there are many differ-

ent opinions and systems. Before the use of molecular markers, classical species descriptions were done with phenotypic analyses based on the morphology of individual cells or colonies and on physiological and biochemical features, like optimal growth temperature, pH, media conditions, growth in the presence/absence of oxygen, sensibility to inhibitors and metabolic/catabolic activities. The major problems with these analyses are that they are difficult to standardize and often insufficient. Therefore, modern species definitions combine molecular parameters with phenotypic traits. Largely accepted molecular parameters to define a species are DNA similarities as defined by DNA re-association kinetics (Stackebrandt and Goebel 1994), G+C content (Lee et al. 1956) and rRNA sequence similarities (Woese and Fox 1977; Woese 1987). The Committee on Reconciliation of Approaches to Bacterial Systematics defined that a "species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5 °C or less T_m" (Wayne 1987). Empirically, it has been shown that organisms belonging to one species do not differ by more than 5 mol% in their G+C content, as do those differing by no more than 10 mol% belonging to the same genus (Rossello-Mora and Amann 2001). 16S rRNA sequence similarities can be defined to be higher than 95% on the genus level (Ludwig et al. 1998). A boundary for species circumscription could be 97% sequence similarity, as it has been observed that the genomic similarities in this case are above 70% (Amann et al. 1992; Stackebrandt and Goebel 1994). As mentioned above, the prokaryotic species definition is a difficult task and the presented definitions are pragmatic suggestions that are widely used and accepted (for review see Rosselló-Mora and Amann 2001).

For answering the question on how many species are in soil, new molecular technologies were used that do not depend on potentially biased cultivation procedures. Torsvik et al. (1990a) used DNA re-association kinetics to estimate species diversity in a top soil from a beech forest near Bergen, Norway. In the sample they counted 1.5×10^{10} bacteria per gram of dry soil by microscope observation, but only 4.3×10^{7} colonies were received by cultivation. This means that, as usual, less than 1% of the bacteria present had been cultivated. By extracting total DNA from the bacterial fraction and using it for DNA re-association kinetics, the heterogeneity was estimated to be 2.7×10^{10} bp (Torsvik et al. 1990a). Assuming an average genome size of 6.8×10^{6} bp for soil bacteria (Torsvik et al. 1990b), there were 4000 different bacterial genomes present in this sample (Torsvik et al. 1990a). Using the *Escherichia coli* genome with 4.1×10^{6} bp, the resulting genome equivalents present would go up from 4000 to 6000, which could represent 12,000–18,000 different species (Torsvik et al. 1996) depending on what average genome size is used. To estimate a correction factor comprising the species definition that only 70% of the DNA has to hybridize, Torsvik et al. (1990a) isolated 206 strains from the same soil and used their DNA for

re-association. This test gave an estimate of 20.6 different species when the DNAs used were in equal frequency. Looking at these results one can see a tenfold underestimate with the re-association method, leading to 40,000 species estimation in this specific sample (Dykhuizen 1998). Dykhuizen goes even further. Using estimations from animal and plant ecologists he defines a factor for taking into account different species frequencies and calculates the number of different bacterial species in a small quantity of soil to over half a million, by estimating 20,000 common ones.

With DNA–DNA re-association kinetics, a pasture soil was estimated to contain 3500–8800, and an arable soil to contain 140–350 genome equivalents (Ovreas and Torsvik 1998). This indicates varying complex microbial communities in soils of different composition and sampling sites. The questions – if there are soils with even higher diversity, if the same genomes are present in different soils and how the low abundant species are represented in these analyses – still need to be answered.

17.4 Molecular Approaches to Describe Microbial Diversity

DNA re-association kinetics are only answering the question of how many different genome equivalents are out there. What type of organisms they belong to and what their phylogenetic affiliation is remains unclear. Woese and coworkers (Woese and Fox 1977; Woese 1987) were the first to recognize that 16S and 18S rRNA sequences can be used to define species and their phylogenetic relationship, because every organism owns at least one copy of the small subunit rRNA gene. Today amplification of 16S rRNA genes from DNA directly extracted from environmental samples is widely used by microbial ecologists to describe microbial diversity independent of cultivation of the respective organisms. Sequence determination of the cloned products and reconstruction of phylogenetic trees resulted in a large and comprehensive dataset (Ludwig 1999). Only half of today's defined 52 bacterial phyla have cultivated representatives, the others are only represented by 16S rDNA sequences (Rappe and Giovannoni 2003).

Although, an impressive 112,000 different bacterial rDNA genes are currently found in the public databases (The Ribosomal Data Base Project II, June 2004, http://rdp.cme.msu.edu), most of which stem from environmental surveys; these still represent only a minor fraction of the actually existing diversity. Based on empirical data from 16S rDNA surveys from soils in Arizona, Dunbar et al. (2002) have constructed theoretical null models of bacterial species abundance and calculated that documentation of half the species community would require surveys of roughly 15,000–45,000 individuals per sample. However, in practice, usually only

a few dozen to several hundred clones per soil sample have been analyzed. Nevertheless, 16S rDNA surveys, even of small sample size, give insights into the occurrence of many novel and abundant lineages, most of which remain completely uncharacterized because no cultivated relatives exist. For example, the novel phylum of Acidobacteria (Barns et al. 1999) has been frequently detected in soil samples, where it often competes with Proteobacteria for representing the most abundant phylum in 16S rDNA clone libraries. It has been suggested from different studies that the ratio between the Proteobacteria and Acidobacteria might be indicative of the trophic level of the soil (see, e.g. Smit et al. 2001 and references therein).

Another unexpected finding was the detection of a novel lineage of Archaea, belonging to the kingdom Crenarchaeota in mesophilic habitats (DeLong 1992), because all cultivated Crenarchaeota known to date are extremely thermophilic organisms isolated from hot springs and hydrothermal vents. 16S rDNA sequences have not only been isolated from marine plankton but also frequently from soil ecosystems and freshwater habitats worldwide (e.g. Bintrim et al. 1997; Schleper et al. 1997; Ochsenreiter et al. 2003).

Potential drawbacks of the 16S RNA method are the different number of ribosomal operons within organisms, especially if abundances are being determined (Fogel et al. 1999), and the variation of rDNA sequences within one genome, although in cultivated species this is a rare finding (Mylvaganam and Dennis 1992; Nubel et al. 1996). Another challenge, especially when working with soil samples, is the need for purification of the nucleic acids from contaminating humic substances that can inhibit the PCR reaction (Tebbe and Vahjen 1993). The most basic problem, however, is that not all unknown sequences can be amplified. For 16S rRNA analyses it was believed, that targeting highly conserved regions with universal primers would potentially result in an unbiased amplification of all genes present in one sample. The enrichment of *Nanoarchaeum equitans*, a nanosized hyperthermophilic archaeon from a submarine hot vent, proved this theory wrong, as its unusual 16S rRNA gene could not be amplified with primers that were believed to be “universal” (Huber et al. 2002).

The diversity of amplified 16S rRNA genes of one environmental sample can be determined by denaturant gradient-based gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE). Both methods rely on the difference in mobility of DNA fragments of the same length but with different sequence compositions in denaturing polyacrylamide gels. In the case of DGGE the denaturants are urea and formamide (Fischer and Lerman 1983; Muyzer et al. 1993) and temperature is used in TGGE (Heuer et al. 1997). These methods are normally used when identities of the 16S RNA genes have been determined and diversity changes in the environment need to be monitored.

A similar method but with lower resolution is the amplified ribosomal DNA restriction analysis (ARDRA). PCR products of rDNA are hydrolyzed by one or more well chosen restriction endonucleases. The resulting fragments are separated on agarose gels, giving distinct bands for certain groups of organisms. Interpretation of the outcome is sometimes difficult because of large numbers of fragments, especially if a complex sample is analyzed. To modify this method, terminal restriction fragment length polymorphism (T-RFLP) was developed (Avaniss-Aghajani et al. 1994). Only the terminal fragments of restricted rDNA PCR amplificates are labeled and thus appear during separation. Shortcomings of both methods are the lower resolution power, because phylogenetically close relatives often have nearly identical fragment lengths.

Another technique used for community studies is the ribosomal internal spacer analysis (RISA) (Borneman and Triplett 1997). The internally transcribed spacer (ITS) between the 16S and 23S rRNA genes is amplified and its varying length in different species is used for species differentiation. However, the length of the spacer is not directly correlated with phylogenetic distance, e.g. species with similar length of spacers do not need to be close relatives (Garcia-Martinez et al. 1999). Using the ITS sequences for phylogenetic reconstructions is hampered by the limited number of database entries. This method is therefore mainly used for strain differentiation in clinical isolates of pathogenic bacteria (e.g. Perez Luz et al. 1998), although it has potential for environmental studies.

In conclusion, 16S rDNA sequencing remains the favored method for the determination of new species and their phylogenetic relation. Because of the high number of species present in many soils, fingerprinting techniques like DGGE and T-RFLP are used to monitor community changes under varying natural and anthropogenic influences. RISA is commonly used for strain separation and when higher resolutions are necessary.

17.5 The Current Picture of Prokaryotic Diversity in Soil

Around 52 identifiable major bacterial divisions (or phyla) are currently being reported (Table 17.1, first column), of which only 26 have cultivated representatives (add column 2 and 3 in Table 17.1, Rappe and Giovannoni 2003). Even within those 26 phyla, often only a few representatives have been cultivated, as, e.g. from Acidobacteria and Verrucomicrobia. Around 24 of all 52 phyla have been detected by molecular methods in soils (Table 17.1, column 5). Nine of those phyla have cultivated representatives from soil ecosystems (column 4). Not astonishingly, phyla with "classical" soil microorganisms like *Actinobacteria*, *Firmicutes* and *Proteobacteria* are

Table 17.1. Division-level bacterial diversity detected by cultivation approaches and molecular surveys

Bacterial phyla predicted by 16S rDNA comparisons[a]	Described by Woese in 1987[a]	Cultivated representatives after 1987[a]	In soil (cultivated representatives)[b]	In soil (molecular surveys)[c]
ABY1				
Acidobacteria		X	X	X
Actinobacteria	X		X	X
Aquificae		X		
Bacteroidetes	X		X	X
BD1–5 group				
BRC1				X
Caldithrix		X		
Chlamydiae	X			
Chlorobi	X			X
Chloroflexi (GNS)	X			X
Chrysiogenetes		X		
Coprothermobacter		X		
Cyanobacteria	X			X
Deferribacteres		X		
Deinococcus-Thermus	X		X	X
Desulfurobacterium		X		
Dictyoglomus		X		
Fibrobacteres		X		
Firmicutes	X		X	X
Fusobacteria		X		
Gemmatimonadetes		X	X	X
Guaymas1				
Marine Group A				
NC10				
Nitrospira				X
NKB19				
OP1				
OP3				X
OP5				
OP8				
OP9				X
OP10				X
OP11				X
OS-K				X
Planctomycetes	X		X	X
Proteobacteria	X		X	X
SBR1093				
SC3				X
SC4				X
Spirochaetes	X			

Table 17.2. (continued)

Bacterial phyla predicted by 16S rDNA comparisons[a]	Described by Woese in 1987[a]	Cultivated representatives after 1987[a]	In soil (cultivated representatives)[b]	In soil (molecular surveys)[c]
Synergistes		X		
Termite Group 1				X
Thermodesulfobacteria		X		
Thermotogae	X			
TM6				X
TM7				X
vadinBE97				
Verrucomicrobia		X	X	X
WS2				X
WS3				
WS5				
WS6				
Total: 53	12	14	9	25

[a] Based on Rappe and Giovannoni (2003)
[b] Compiled using the literature cited in this article
[c] Compiled using the literature cited in this article and using the *ARB* program package (Ludwig et al. 2004) with 24,000 aligned sequences

among them. A few strains from phyla that are expected to play important roles in soil ecosystems because of their abundance in molecular surveys, e.g. *Acidobacteria* and *Bacteroidetes*, have been isolated just recently from soils by advanced cultivation techniques (e.g. Sait et al. 2002).

The only cultivated archaeal species from soils are strictly anaerobic methanogens that have been isolated from anaerobic habitats, mostly flooded rice paddies. These organisms are of major interest as they are largely involved in production of the "greenhouse" gas methane. For a review on this special type of soil ecosystem see Liesack et al. (2000). The mesophilic crenarchaeota, which are distributed worldwide and abundant in soil ecosystems and certainly play an interesting role, remain uncultured to date. They can only be detected by molecular methods like 16S rDNA studies (e.g. Ochsenreiter et al. 2003) or metagenomic surveys (see below; Quaiser et al. 2002; Treusch et al. 2004).

17.6 Studying Physiological Diversity

While 16S rDNA surveys are suitable to give an inventory of the species living in soil they can seldom give clues about the ecological role of the or-

ganisms in the ecosystem. Therefore more and more culture-independent molecular studies concentrate on physiological properties by characterizing genes of key enzymes from metabolic processes. Of special interest in soil ecosystems is the nitrogen cycle, as many of its processes, like, e.g. nitrification, nitrogen-fixation and denitrification, are carried out here. In a study by Rösch et al. (2002), for example, the diversity of the genes for nitrogenase reductase (*nifH*), nitrous oxide reductase (*nosZ*), cytochrome cd_1- (*nirS*) and copper-containing nitrite reductase (*nirK*) in an acidic oak-hornbeam forest soil were analyzed. The richness of 16S rRNA genes as determined by an accompanying diversity study was contrasted by the relative low diversity of the functional genes studied. This showed that only a part of the species present were involved in denitrification and N_2-fixation processes. However, it could also be possible that the diversity of these genes is much higher in the environment but cannot be accessed with primers derived from the limited sequence space of cultivated species.

Monitoring the expression of functional genes can give clues about key metabolic processes during changing environmental conditions. Necessary for this purpose are technologies to extract mRNA from soils, which is a difficult but feasible task, as well as the quantitative real time PCR methods (e.g. Burgmann et al. 2003; Okano et al. 2004).

For ecological studies of functional gene expression high-throughput methods are required, so that large numbers of genes can be accessed in parallel and with successive replicates. DNA-microarrays seem to be the right tool to fulfill this task and the application to environmental questions is gaining ground (Guschin et al. 1997). Beside identification and genotyping of bacteria (Salama et al. 2000; Cho and Tiedje 2001), population genetics (Chakravarti 1999) and the detection and quantification of functional genes in the environment (Wu et al. 2001; Taroncher-Oldenburg et al. 2003) are the most promising applications.

To identify organisms which are metabolically active or consume specific compounds, a method commonly referred to as stable isotope probing is used. The addition of substrates labeled with stable or radioactive isotopes to a microbial community is followed by identification of the species with molecular methods after labeled DNA (e.g. Radajewski et al. 2000) or RNA (e.g. Manefield et al. 2002) is separated. Recently, the stable isotope approach was combined with the microarray technology to form the "isotope array" (Adamczyk et al. 2003).

All these techniques are hampered by one massive drawback: the primers and probes, used to detect environmental sequences, are primarily derived from microorganisms of the culturable world and might not reflect nor detect the existing diversity of functional genes in the environment.

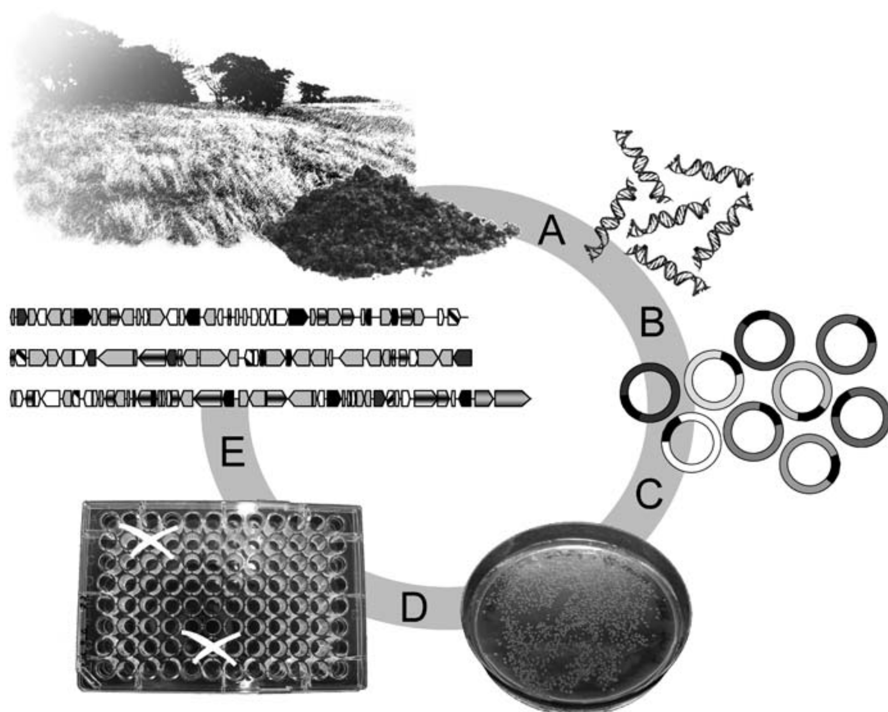

Fig. 17.1. Cloning of metagenomic soil libraries. DNA from a soil sample is isolated and purified from contaminating humic substances (*A*). Size-selected DNA is ligated with BAC or fosmid vectors and transformed into *E. coli* cells (*B* and *C*). After arrayed conservation of the obtained clones, sequence- or activity based screenings can be performed (*D*). Sequence analysis of identified clones gives insights into genomes of uncultivated microorganisms (*E*)

17.7 Environmental Genomic Studies

A novel and generic approach extending the possibilities of characterizing uncultivated microorganisms involves environmental genomic or "metagenomic" studies (Fig. 17.1). High molecular weight DNA is directly isolated from the ecosystem and cloned into fosmid or BAC vectors that replicate in *Escherichia coli*. The resulting complex, recombinant large-insert libraries are screened using phylogenetic molecular markers, like, e.g. the 16S rRNA gene of the organisms of interest. DNA-sequences of these clones provide insights into protein encoding genes that can give hints to specific physiological traits. This technique was first used by Stein et al. (1996) to characterize a 40-kb genomic fragment of a mesophilic planctonic marine crenarchaeote, and by Schleper et al. (1998) to gain genomic information

about the uncultivated marine archaeal symbiont *Cenarchaeum symbiosum*. The technique showed its full potential for answering ecological questions when Beja et al. (2000) discovered a new type of rhodopsin, named proteorhodopsin, encoded on a 130 kb genomic fragment from an uncultivated marine γ-proteobacterium, leading to the detection of an abundant novel bacterial physiology based on photosynthesis in the ocean (Beja et al. 2001; Man et al. 2003). Using the metagenomic approach for soil ecosystems is much more challenging because of the contaminating humic substances that are closely attached to the DNA and inhibit enzymes used in molecular biological methods. Many methods have recently been established to purify soil DNA from humic substances (e.g. Zhou et al. 1996; Krsek and Wellington 1999; Miller et al. 1999), but for metagenomic approaches involving the clowning of large inserts, this DNA additionally has to be of high-molecular weight. Rondon et al. (2000) published a method that resulted in high-molecular weight DNA from an agricultural soil. In two metagenomic libraries with ca. 1 Gbp of stored DNA, clones containing 16S rDNA of *Proteobacteria*, Cytophagales, low G+C Gram positives and Acidobacteria were identified. Furthermore, clones expressing DNase, amylase, lipase, hemolytic and antibacterial activities could be identified, indicating the high biotechnological potential of this approach. In order to isolate novel bioactive compounds, Courtois et al. (2003) constructed a 5000-clone library in an *Escherichia coli-Streptomyces lividans* shuttle cosmid vector and isolated at least 13 clones producing bioactive compounds.

To study the uncultured crenarchaeota from a sandy ecosystem, our laboratory has used a metagenomic approach and isolated several large fragments of 35 to 45 kb from fosmid libraries (Quaiser et al. 2002, Treusch et al. 2004, 2005). For this study, a new technology based on a two phase gel electrophoresis with polyvinylpyrrolidone (PVP) has been established to isolate and purify the DNA from humic substances. From the same ecosystem, 22 genomic fragments belonging to species of the phylum Acidobacteria were identified (Quaiser et al. 2003). Four out of six sequenced fragments were placed within group V, and two within group III of the Acidobacteria as determined by 16S rDNA comparisons. Sequences of fragments of the different groups varied in their G+C content of the protein-encoding regions by more than 10%, which was in good correlation with their phylogenetic placement. Although most encoded proteins could be assigned to "housekeeping" functions, some indicated special traits, e.g. a putative β-1′-2′-glucan synthetase, an intracellular polyhydroxybutyrate depolymerase and an operon with low but significant similarity to a biosynthetic cluster of *Streptomyces lincolnensis*. Furthermore, a cluster of genes was highly homologous and syntenic to a gene cluster found in species of the Rhizobiales, pointing to a recent horizontal gene transfer between these bacterial lineages.

For the recovery of more crenarchaeal genomic fragments not associated with 16S rRNA genes our group recently used a random fosmid end sequencing approach (Treusch et al. 2004). Three large-insert metagenomic libraries from two different ecosystems were analyzed by sequencing both ends of 2688 randomly chosen clones, resulting in 4 Mbp of soil metagenome sequence information. At least seven crenarchaeal clones were identified by their typical low G+C content and by matches of their end sequences to archaeal database entries. ORFs encoded on the sequenced fragments gave hints to the potential role of the crenarchaeota in the environment, because genes coding for submits of a putative ammonia monooxygenase were identified, indicating that these archaea are involved in nitrification processes (Treusch et al. 2005, Schleper et al. 2005) Additionally this study gave the first large scale insights into soil metagenomes.

Large-scale shotgun sequencing of small-insert metagenomic libraries has become practicable with dropping sequencing costs. Tyson et al. (2004) reported the near-complete reconstruction of the genomes of two uncultured species and the partial recovery of three others from an acid mine drainage biofilm. They were able to reveal potential physiological pathways that are helpful for the understanding of the biofilm community. However, this approach was only successful because the community was dominated by a few genomically distinct species.

Using this approach for a far more complex ecosystem, Venter et al. (2004) generated 1.045 billion base pairs of non-redundant sequence, still representing only a small part of the metagenome of the Sargasso Sea surface water microbial community. Using shotgun sequencing approaches on more complex ecosystems, such as soils, a much greater sequencing effort would be necessary.

Concluding, metagenomic studies of soil ecosystems have been proven to be possible and shed light on the genomic content and on potential physiological pathways. Thus, they deliver hints to potential roles of organisms present in soils that have been missed by cultivation approaches. Maybe these hints will help in getting some of these refractory organisms in culture. Looking at the potential of high-throughput sequencing of metagenomic libraries, as has been shown for simpler habitats, it might just be a matter of time until this is adopted for soil ecosystems.

17.8 Conclusions

Where are we heading? What are the questions we want to answer? The tremendous diversity of microbial species will leave scientists enough work for years to come. The main goal will be a better understanding

of the ecosystems “soils” and possible impacts of anthropogenic influences on them. For this purpose it might become necessary to use more integrated and large scale approaches on selected soil ecosystems, rather than analysing many different habitats with single methods. For example, taking a geobotanically well characterized soil and determining its microbial diversity by re-association kinetics will answer first how many species may be present. This knowledge would define how comprehensive the 16S rDNA diversity study should be in order to get a good overview of the most abundant species present in this soil. For the many not yet cultivated species, sequence determination of a part of the metagenome, that may be selected specifically for the organisms of interest, will give hints for possible physiological properties. Using the generated information will help in looking for active and dormant species in this habitat by mRNA analyses (“functional metagenomics”). Parallel cultivation approaches using the generated information will hopefully result in isolation of representatives of all major lineages present in this soil, which will open the possibility for “classical” microbial and biochemical analyses. Together, these approaches will lay the ground for studying species interactions and networks of dependences between organisms in a particular soil ecosystem. Integrated and high throughput approaches will be necessary if the species estimations of over half a million just in one soil are correct.

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18 Micromanipulation Techniques for the Isolation of Single Microorganisms

Jürgen Fröhlich, Helmut König

18.1 Introduction

A prerequisite for the biochemical and physiological investigation of microorganisms is the isolation and management of pure cultures. The only absolute criterion of purity for a bacterial culture is that it has been derived from the progeny of a single cell. Failure to apply this criterion may lead to much effort in proving the purity of a culture. All strains upon which research is to be based should therefore be rigorously purified before starting to investigate the properties of individual organisms (Johnstone 1969). Ecologically oriented microbiologists are faced especially with the problem of how to obtain a pure culture of certain microbial strains from their densely populated natural habitats. The methods used range from simple devices up to very complex machines.

The principal procedures for obtaining pure cultures of bacterial strains have not been greatly improved since Robert Koch (Koch 1881). The isolation according to conventional methods, like the separation of microorganisms by agar plates (Koch 1881) and agar shake tubes (cf. Pfennig and Trüper 1981), cannot avoid the fact that individual colonies are formed by cell aggregates. This occurs particularly with filamentous bacteria, which could easily agglomerate. More sophisticated electronic enumeration and sampling systems such as Coulter counter or flow cytometry cannot prevent the formation of cell aggregates. In addition to the isolation of single cells with optical tweezers (Huber 1999), there are alternative approaches, e.g. the "Bactotip" and "Membrane" methods for the micromanipulation of individual microorganisms. With the aid of these methods single cells can be picked out of a mixed population under direct visual control. The isolated aerobic or anaerobic species can be grown in pure culture or can be subjected to single cell PCR (Fröhlich and König 1999a, 2000; cf. Prescott et al. 2002; Fröhlich et al. 2002).

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18.2 Micromanipulation Techniques

18.2.1 Historical Perspective

Since the beginning of the twentieth century, several attempts have been made to improve the management of single cells by using micromanipulator techniques. Thereby, a suspension of an adjusted concentration of microorganisms was aspirated into a simple capillary so that a single cell was transferred statistically in a defined volume (Harbeck and Rothenberg 1995).

Moreover, a survey of the chief methods devised for single organism cultures was presented by Johnstone (1969, 1973). These include the block-cut method for the selection of an isolated organism on a lightly inoculated nutrient gel, formation of droplets with micropipettes, searching for those containing single organisms, and isolation by carrying the selected organisms across the sterile gel surface with a microneedle. Because of technical problems and disadvantages, these methods were not adopted for routine isolation.

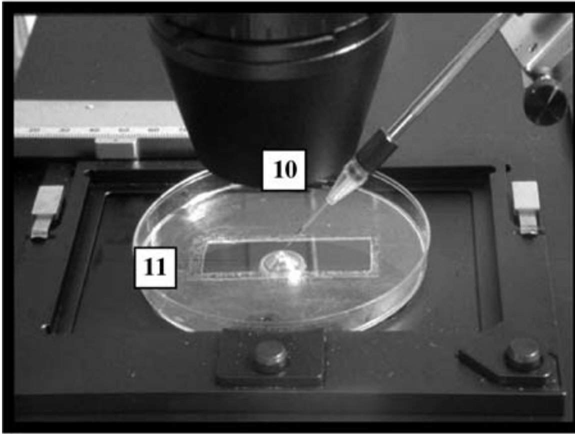
Other attempts to improve the management of single microbial cells by using micromanipulator techniques have been described in the literature. Either microneedles or microcapillaries were used for the separation of single bacterial cells (Skerman 1968; Bakoss 1970; Johnstone 1973; Thomsen et al. 2004). The techniques suggested more than 30 years ago were based on the state of art at that time. They involved several technical disadvantages, which hampered routine usage of the isolation techniques for a broad spectrum of prokaryotes in a microbiological laboratory. The magnification was limited and the transfer of single cells was hardly possible (Skerman 1968). It was designed for use with low power objectives (e.g. 10 ×) with a working distance of 7 mm or more. The instrument consists of a lens collar and magnetic tool-carrier. The lens collar was clamped onto the objective and it contained two steel slides which permitted the magnet tool-carrier to slide along freely. Knobs or microloops were the most useful tools for the isolation of cells from colonies on solid agar plates. By several operations cells were floated across the surface of solid media by lateral movement of the Petri dish and they were well separated from the original population. Attempts to lift single organisms in a loop for transfer were rarely successful. So far, this method has been applied for the isolation of large filamentous bacteria (Bradford et al. 1996), and cyanobacteria (Bowyer and Skerman 1968). Bakoss (1970) cloned single cells of leptospires with a micropipette connected to a syringe via a thin polyethylene tube, which was fastened to the holding clip of a micromanipulator. He used a syringe as a simple

pneumatic system. The disadvantage of this micromanipulator technique was that it was laborious. A mechanical micromanipulator with a microneedle was also used (Sherman 1973) to separate the four spores from a cluster in a yeast ascus. This technique was also applied for this purpose in our institute and it is also suitable for the separation of larger bacterial cells (>3 μm) by moving them onto an agar surface. Coccoid bacteria from the "corn cob" of human dental plaques were successfully isolated by Mouton et al. (1977) with microneedles designed to be a double angulated microhook as described by Johnstone (1973). Single selected spores of *Bacillus cereus* adhering to the glass point of capillary tubes were selectively removed from Petri dishes (Hamilton 1978). Micromanipulation was also successfully applied for the isolation of *Pedomicrobium* cultures from water samples (Sly and Arunpairojana 1987). Luttermann et al. (1998) described a micromanipulation method for transferring microobjects such as bacteria from agar plates with microcapillary tubes. An angulated capillary tube (angle of 90°) is positioned between the condenser and the objective. The agar plate with the selected bacteria is moved below the opening of the capillary tube with the microscope stage. The aspirated bacterium is placed on the surface of a solid medium or in liquid media in microtiter plates.

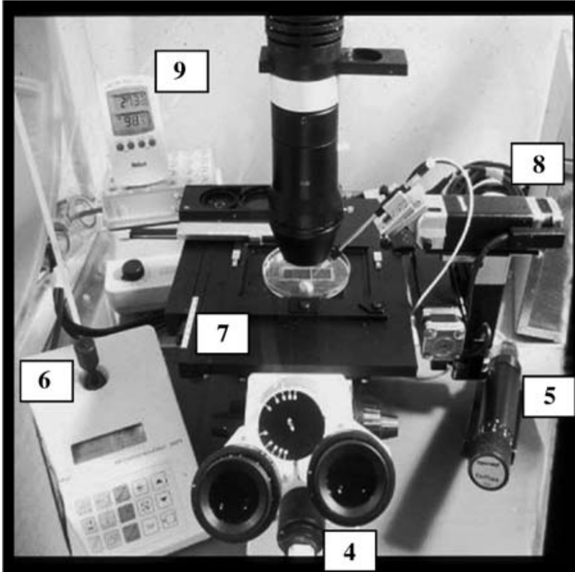
18.2.2 Modern Equipment

Since the sixties the technical equipment of micromanipulators has been greatly improved (Fig. 18.1). A long-distance objective (Zeiss, Oberkochen, Germany) with a magnification of 100-fold is now available. This allows manipulation at a magnification of 1000-fold and more with an inverse microscope. The capillary tools can be positioned quickly and precisely. The available pneumatic or hydraulic systems are very accurate pressure devices.

For the isolation of microbial cells a commercial micromanipulator (Eppendorf, model 5171) equipped with a pressure device (Eppendorf model 5246 plus or Cell Tram Oil) and mounted onto an inverse phase contrast microscope (Axiovert 25; objective CP "Achromat" 100x/1.25 Oil Ph2; Zeiss) is used (Bactotip method) (Fröhlich and König 1998, 1999a; Fröhlich et al.1998a, 1998b). The magnification is adjusted from 400× to 1000×. The micromanipulator is used according to the manufacturer's instructions (micromanipulator 5171: Operating Manual; Cell Tram Oil: Operating Manual, Transjector 5246: Operating Manual; Eppendorf, Hamburg, Germany). The diameter of the opening of the capillary tip can be adjusted to the size of the bacterial cell of interest. For the isolation of bacteria a sterile capillary tube ("Bactotip"; Fig. 18.2a, b) is used, which preferably possesses a bevelled

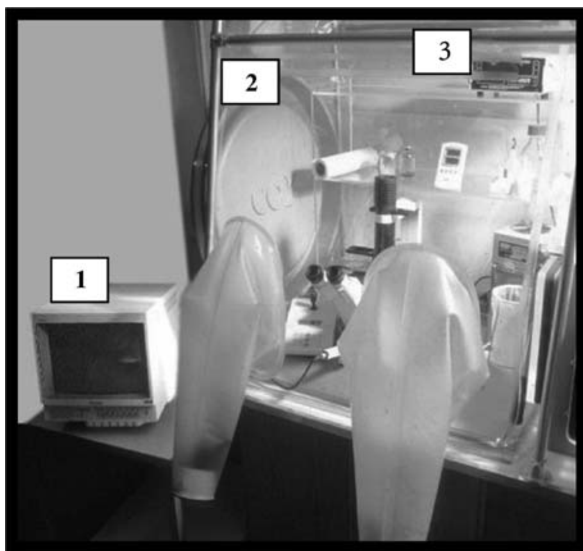


a



b

tip (angle 45°) usually with an opening of about 5–10 µm at the anterior end. The sterile Bactotips are produced by Eppendorf (Hamburg) or can be manufactured with a capillary-puller (Saur, Reutlingen, Germany) and a microgrinder (Saur, Reutlingen) using capillary tubes type GB 100 TF-8P (Science Products GmbH, Hofheim, Germany). The posterior end of the Bactotip is sealed with a droplet of sterile oil. If desired, the inner surface of the tip can be siliconized with dichlorodimethylsilane (Fluka Chemie AG, Buchs, Switzerland). This is advisable if the bacteria tend to adhere to glass surfaces. Our experiments show that desiccation and oxygen stress



c

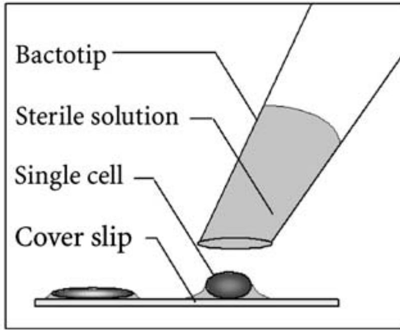
Fig. 18.1. Working station for the manipulation of single cells with a COY-chamber (**c**) for the aerobic and anaerobic isolation. The isolation of single cells is performed using an inverse microscope and a micromanipulator device (**b**). The spread cells are aspirated by the application of a Bactotip (**a**). Technical specifications: COY chamber (1), monitor (2), O_2/H_2 electrode (3), camera (4), Cell Tram Oil (5), joystick (6), inverse microscope (7), micromanipulator (8), thermometer/hygrometer (9), Bactotip (10), coverslip with spread bacteria (11)

(Krämer 1997) for the isolation of anaerobic and aerobic microorganisms can be avoided by using a glove box with a N_2/H_2 (95:5; v/v) atmosphere (COY chamber, Toepfer Lab Systems, Göppingen, Germany). The relative humidity in the chamber is adjusted from 95 to 100%. The microscope bulb is replaced by an optical fibre device (Schott, Mainz, Germany) which reduces the IR-radiation. The microscope is equipped with a CCD camera (Type AVTBC12CE (Zeiss) and a monitor (Type PM 95 B, Zeiss).

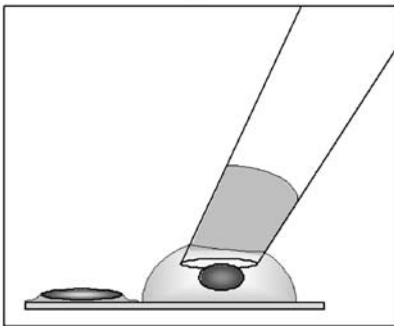
18.3 Isolation Techniques

18.3.1 Bactotip Method

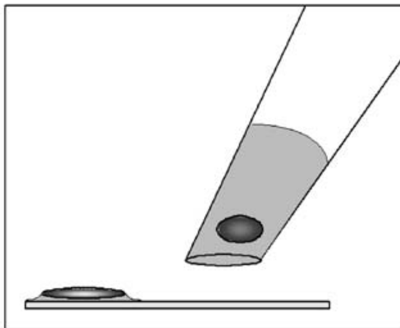
This technique is used when single cells are cultured in liquid media or genes are going to be amplified by single cell PCR (Fig. 18.2A; Fröhlich and



1. **Spread microorganisms on cover slip**

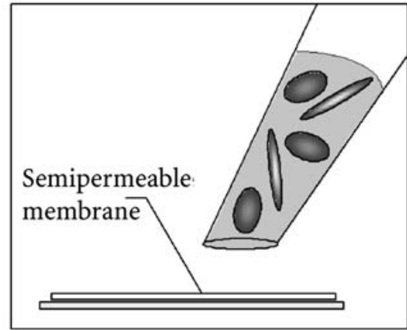


2. **Resuspend single cell**

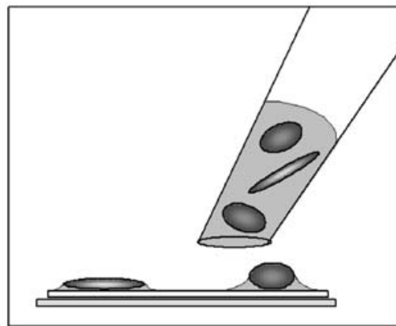


3. **Aspirate suspension**

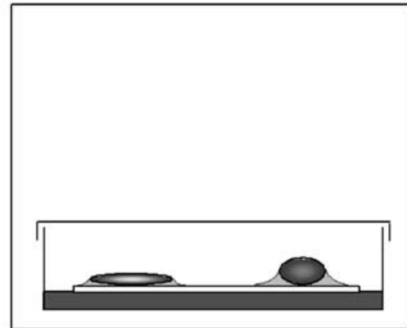
a



1. **Aspirate mixed culture**



2. **Spot single cell onto membrane**



3. **Transfer membrane to solid medium**

b

Fig. 18.2. Schematic drawings of **a** the Bactotip; **b** the membrane methods for the isolation of single cells

König 1999a). Cultures or complex mixtures of prokaryotic or eukaryotic strains are diluted in 1–10 ml phosphate buffered saline (1×PBS; 10 mmol/l sodium phosphate, 130 mmol/l sodium chloride, *pH* 7.0). An aliquot of the suspension (10 µl) is spread as a thin film on a sterile microscope coverslip (24×60 mm). A small volume of buffer or medium (ca. 0.1–0.2 µl) is aspirated into the capillary tube. When the opening of the Bactotip is brought close to the surface of a distinct microorganism, a droplet flows out of the tip and moistens the selected cell. The cell is suspended in the droplet after detaching from the glass surface and aspirated into the Bactotip. About ten single microbial cells are successively removed from the microscope slide within 30 min by aspirating them together with the droplet into the Bactotip. The withdrawn single cells can be transferred in Eppendorf reaction tubes or Hungate tubes (anaerobes) containing 0.3 ml of the corresponding liquid medium. The tubes are incubated at, e.g. 37 °C for 10–72 h (Fröhlich and König 1999a, 2000; cf. Prescott et al. 2002).

18.3.2 Membrane Method

For this method, see Fig. 18.2B and Fröhlich et al. 2002. In contrast to the Bactotip method, an appropriate dilution (ca. 0.1 µl) of a mixed culture is sucked into the capillary tube. The tip is brought close to the surface of a semi-permeable membrane (dialysis hose; Roth) and single cells are spotted under visual control on the membrane within a distance of 5 to 10 mm from each other. Subsequently, the membrane is removed with sterile tweezers and transferred onto a solid medium. Nutrients diffuse through the membrane and enable individual cells to grow up to colonies. In contrast to the Bactotip method, more cells can be isolated in a short time. The use of a dialysis membrane has the advantage of a very smooth surface compared to the application of agar layers, so that very small microorganisms can be separated without limitation of the visual control.

18.3.3 Efficiency of the Cloning Procedure

The efficiency-rate of the cultivation from freshly grown laboratory cultures was between 30% (*Escherichia coli*) and 70% (*Staphylococcus aureus*) (Fröhlich and König 1999a) and the isolation of lactic acid bacteria could be performed at a similar rate. The *Oenococcus oeni* strains B70 and B139^T could be isolated with an efficiency between 63 and 67%. Similar results could be obtained with the species *Lactobacillus brevis* (66%) *and Pediococcus damnosus* (70%). The fastidious anaerobe *Bifidobacterium bifidum*

could be micromanipulated anaerobically in the anaerobic chamber with a yield of 30% (Fröhlich et al. 2002). Thereby, the selection of the isolation method did not have any influence. The single cells grew up to a visible density or a visible colony in 10–72 h. A single cell could also be transferred onto solid media in Petri dishes as proved with *Bacillus cereus*. The colonies become visible after incubation over night at 37 °C. Furthermore, single cells (e.g. *Bacillus cereus*) were directly grown in the Bactotip.

Spreading of the bacterial suspension onto a microscopic slide after an appropriate dilution of the original culture was a prerequisite for the rapid isolation of the single cells, while the isolation of a single bacterial cell directly out of a droplet containing a suspension of a mixed microbial population was not successful. The cells should be transferred to the culture medium within 30 min after spreading. The application of the Bactotip method allows the transfer of single prokaryotic cells to different culture vessels such as Eppendorf reaction tubes, Hungate tubes, onto the surface of solid media in Petri dishes or to subject the isolates to single cell PCR. Cells were also directly grown in the Bactotip. The advantage of the Bactotip method compared to conventional isolation methods can be seen in the ability to pick out a single prokaryotic cell under direct visual control and to grow pure cultures of distinct aerobic and anaerobic cells directly out of a mixed natural or laboratory population in a relatively short time.

18.3.4 Described Applications

The highest number of positive culture tubes with leptospires obtained by Bakoss (1970) was 66.6%. Luttermann et al. (1998) obtained positive culture tubes in a range of 50–60%. We obtained the best results with laboratory cultures of *Staphylococcus aureus* and *Pediococcus damnosus* (70%), which may be due to their relatively high osmotolerance (*Staphylococcus aureus*: a_w=0.90–0.86) (Krämer 1997).

Since the cloning procedure described above was successfully applied to laboratory strains we isolated single bacterial cells from a complex natural population such as the termite gut (Bactotip method) (Fröhlich and König 1999a). The paunch contents (ca. 10 µl) of the termite *Mastotermes darwiniensis* were diluted and treated as described above. *Enterococcus* (*Enterococcus* sp. str. JF1; AJ132470) and *Sphingomonas (Sphingomonas* sp. str. JF2; AJ1232471) were directly isolated from the termite gut in one step.

If the opening of the tip is not larger than 0.5 µm, only very small cells may be picked up. With a capillary tube (inner diameter 0.5 µm; Femtotip II, Eppendorf, Germany), a *Mycoplasma alvi* related strain (AJ132469) was isolated from the cytoplasm of the Archaezoon *Koruga bonita* (Fröhlich and

König 1999a). The trichomonad, *Pentatrichomonoides scroa* (length 30 μm) (Brugerolle et al. 1994; Berchtold and König 1995), one of the smaller gut flagellates of *Mastotermes darwiniensis*, harbours about 50 endosymbiotic methanogens (Fröhlich and König 1999a,b). Cloned single cells of the endosymbiotic methanogen were used for the amplification of their SSU rDNA by a single cell seminested PCR. The methanogen (*Methanobrevibacter* sp.; AJ132468) isolated from *Pentatrichomonoides scroa* was related to both clone CD 3 identified in the gut contents of *Cryptotermes domesticus* (Ohkuma and Kudo 1998) *and Methanobrevibacter curvatus* (Leadbetter and Breznak 1996) isolated from the termite *Reticulitermes flavipes*.

For the correlation of 16S ribosomal DNA signature sequences with temperature-dependent growth rates of mesophilic and psychrotolerant strains of the *Bacillus cereus* group the strains could be easily micromanipulated by the Bactotip method (Prüss et al. 1999).

The Bactotip-method could also be applied for support of the investigation of the phylogenetic affiliation, characterization and in situ carbon metabolism of the bacteria that constitute phototrophic consortia (Fröstl and Overmann 2000; Glaeser and Overmann 2003).

Finally, several lactic acid bacteria and wine-related microorganisms could be micromanipulated with high efficiency rate (Fröhlich 2002).

18.4 Laser Micromanipulation Systems

18.4.1 Optical Tweezers

Ashkin et al. (1987) described the use of infrared laser beams (1,064 nm) for trapping and manipulation of biological specimens such as the single cells of *Escherichia coli* and *Saccharomyces cerevisiae*. This method was improved and successfully applied for the isolation of hyperthermophilic bacteria and archaea (Huber 1999; Huber et al. 1995).

A neodymium-laser is focused by a microscope objective. The movement of the microscope stage is computer-controlled. A rectangular glass capillary with a predetermined breaking point is used as separation chamber (inside dimensions, 0.1 × 1 mm; length, 10 cm), which is filled with fresh medium (90%) and the mixed microbial population (10%). A single selected cell is fixed with the laser beam and is separated from the mixed culture by moving the microscope stage. The capillary is broken at the predetermined breaking point and the single cell is transferred to the culture medium. The culture efficiency after an incubation time of up to 5 days was 20–100%. The isolation of dead cells could be prevented by application of fluorescent

dyes staining viable cells for example with bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Beck and Huber 1997). Photodamage can be reduced to background level under anaerobic conditions (Neumann et al. 1999). This method is a promising tool for the isolation of microorganisms, which cannot be obtained in pure culture by conventional methods.

18.4.2 Laser Microdissection

Schütze et al. (1998) described a laser pressure catapulting method (LPC), which uses a laser (Robot-Micro-Beam) for the microdissection and transfer of single cells. This method has been successfully applied for the isolation of single cells from human tissues. The specimens are spread on a sheath of a 1.35 μm thin polyethylene membrane. With the high photonic energy of a focused nitrogen laser a selected single cell is precisely circumscribed and the selected cell together with a small surrounding strip of the polyethylene membrane is cut out. The round polyethylene slip with the selected cell still adheres to the polyethylene membrane. The laser is then focused below the microdissected target cell and the microdissected sample is catapulted into the oil-dampened cap of a common Eppendorf reaction tube positioned above the sample with a laser shot of increased energy. The cells are subjected to single cell nested PCR. In principal, this method can be applied to cells of any size, but an application for the isolation of viable prokaryotes has not been published so far.

18.5 Conclusions

The above-mentioned techniques (Bactotip method, optical tweezers) allow the separation of a single bacterial cell in the presence or absence of oxygen under visual control. Many of the technical problems regarding the separation of a single bacterial cell have been solved. The remaining problems to be solved are now less of technical nature than choosing a suitable medium composition for growing bacterial cells of, e.g. an unknown systematic affiliation. Our experiments and also former observations (Skerman 1968) showed in addition that even individual cells of the same species (e.g. *Enterobacter*) differed in the number of grown cultures, depending on the manipulation technique used. Cells aspirated and transferred to fresh solid or liquid media with a capillary tube resulted in a lower number of grown cultures than cells which were separated from a colony and immedi-

ately moved over the surface of a agar layer as described by Skerman (1968) or Sherman (1973). The reason for this behaviour is not clear so far.

It has been known for over 40 years that bacterial populations with cells in a 'sleeping status' could be grown once again after the addition of sterile supernatant of a freshly grown culture (Harrison 1960; Postgate and Hunter 1963; Postgate 1976). In those early studies, the specific death rates of high-density bacterial populations were lower than those of low-density populations. A similar growth stimulating effect could be found with single cells by the addition of fresh culture supernatants. Also important is the volume of the medium into which a single bacterium is transferred, because the number of growing cultures decreased with increasing volume.

In recent years it has become clear that bacterial cells can communicate with each other via small signal molecules such as long chain fatty acid esters or *N*-acetyl-homo serine lactones (Clough et al. 1997; Flavier et al. 1997; Holden et al. 1999). Therefore, some of the difficulties in cultivating single isolated bacteria seemed possibly to be associated with a quorum-sensing effect. Thus, the manipulation technique is suitable for studying the effect of different concentrations of signal molecules on the growth behaviour of single cells. These few examples demonstrate that new cultivation approaches have to be applied to grow cloned single bacterial cells with higher efficiency.

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19 Localization and Visualization of Microbial Community Structure and Activity in Soil Microhabitats

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19.1 Introduction

Synecological studies reveal the interaction and interdependence of the physicochemical habitat conditions with the composition and activity of the organismic communities colonizing this habitat. The micro-sites as living space of microorganisms are, according to their dimension, in the micrometer range and their conditions change in space and time. In complex, structured environmental habitats like soils, the habitat quality is highly diverse and dynamic and hence it provides multiple and different selective forces driving the microevolution of organisms by selecting advantageous spontaneous mutations, gene rearrangements or even offspring of horizontal gene transfer events (Schloter et al. 2000; van Elsas et al. 2003). Synecology of microbial communities can only be resolved assessing the detailed community composition in micro-sites and the activity of cell assemblages or individual cells in these micro-sites at the micrometer range. The complex physical structure of soils can be resolved by separating particle size fractions and studying the colonization and activity in these micro-sites by physiological and molecular techniques (Sessitsch et al. 2001). For detailed microvisualizations, optical microscope techniques are applied, supported by specific labelling techniques and image analysis to yield the high resolution required. The confocal laser scanning microscope (CLSM) is a very useful tool to document the spatial arrangement of microbes and their in situ activities, as indicated by fluorescence labelling and reporter techniques (Schmid et al. 2004). Using the CLSM, the autofluorescence problem can be resolved in most habitats with complex structures or with autofluorescent particles. In this chapter, approaches for localization and microvisualization using physiological, molecular biological and

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microscope tools are summarized. Recent advances in specific labelling techniques for the in situ identification of microbes and their in situ activity are presented. Combining techniques, like the fluorescence in situ hybridization and microautoradiography, secondary ion mass spectrometry (TOF-SIMS) or stable isotope probing, new insights can be gained in specific in situ activities of microbial communities within micro-sites.

19.2 Localization and Microvisualization Approaches

The localization of microbial populations and activities in soils or other complex organic matrices can be performed in several different ways. One approach is the embedding of the entire sample in a resin to preserve the complete microstructure including the organisms; after ultrathin slicing the completely preserved structure can be analysed microscopically in detail; specific staining procedures can be applied before or after the embedding step. Alternatively, the samples are fixed and stained after embedding in agarose or gelatin or are studied without embedding and fixation under fully hydrated conditions. In a second approach, soil is disaggregated by, e.g. a mild ultrasonic treatment and different particle size fractions are prepared using repeated sieving and centrifugation steps (Stemmer et al. 1998). In general, four fractions are obtained from soils: the fraction "sand 1" with a particle size greater than 250 µm, the fraction "sand 2" with a particle size of 63–250 µm, the fraction "silt" with a particle size between 2 and 63 µm and the fraction "clay" with particle size of 2–0.1 µm. The spatial and physical size distribution patterns of carbon sources as well as the spatial restrictions for different sizes of micro-organisms in the soil porous structure control the location of microbial community members and associated enzymes. It is now evident that soil microorganisms as well as soil enzymes are heterogeneously distributed within the soil matrix. The individual properties of enzymes cause a preferential binding either to clay–humus–enzyme complexes or to particulate organic matter. Bacterial cell numbers and biomass are concentrated in the two smallest mineral soil particle fractions silt and clay (van Gerstel et al. 1996). The activities of typical bacterial enzymes like urease, invertase, and alkaline phosphatase were also highest in the silt and clay fractions. Xylanase activity and fungal-derived phospholipid fatty acids showed a very close relationship to the particulate organic matter of the coarse sand fraction (Kandeler et al. 1999a–c; 2000; Stemmer et al. 1999; Poll et al. 2003). This correspondence between the fungal enzyme producer with the enzyme and the substrate assures an efficient use of fungal substrates by the consumer (Kandeler et al. 2000). This was corroborated with the measurement of the

phospholipid fatty acid biomarkers and DGGE of 16S rDNA genes. Using T-RFLP-analysis, the diversity of bacterial communities was determined and again the highest bacterial diversity was found in the clay and silt fractions (Sessitsch et al. 2001; Poll et al. 2003). Most recently it could be shown using real time quantitative PCR for the “red-like” *cbbL*-gene of the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) of autotrophic bacteria, that this bacterial gene was exclusively localized in the silt and clay fractions in copy numbers of $10^6$to 10^7 per gram soil (Selesi et al. 2005; Selesi et al., in prep.). Using similar quantitative PCR-measurements, the gene for the bacterial subtilisin peptidase (*sub*) was found in 106 copies per gram soil (Bach et al. 2002), the *pmoA*-gene of methanotrophic bacteria in 5×10^6 copies per gram soil (Kolb et al. 2003).

For the microvisualization of microbial communities in their microhabitats, the confocal laser scanning microscopy (CLSM) has been successfully applied in combination with specific fluorescence staining or tagging techniques (Schmid et al. 2004). The major obstacle here is the high autofluorescence in environmental samples, e.g. by mineralogic and organic particles. Using proper fluorescence stains and excitation light in the long wavelength range (red and above), the out-of-focus autofluorescence problem can be solved by applying optical sectioning of the specimen in the CLSM in many cases. Projections of a series of such xy-scans result in an extended focus image or z-projection. The information contained in those scans is the basis for a three-dimensional reconstruction of a scanned specimen. Optical cross sections through a sample, perpendicular to the optical axis, can also be performed and are usually called z-scans or xz-sections. A recently developed CLSM system by Zeiss (Jena, Germany), the LSM 510 META, provides a unique scanning mode, by which the entire fluorescence spectrum is projected onto a 32-channel detector. The spectral signature for each pixel can now be detected, which allows the identification of characteristic emission spectra for fluorescent dyes and digital separation into channels reflecting dye distribution. Novel laser techniques, like dual photon excitation, have also been applied, which are especially useful for the examination of thicker specimens with reduced photobleaching (Lawrence et al. 2001). To retrieve quantitative data from the CLSM-pictures, digital image analysis approaches have been developed. Using the computer-controlled motorized stage, observation fields on the objective slide are precisely and reproducibly located and randomly selected for microscope observation and image acquisition (Kuehn et al. 1998). By spiking the samples with different known amounts of bacterial cells, serving as internal standard, this approach can also be used to determine the absolute number of cells in a complex sample (Daims et al. 2001a). In addition, digital image analysis tools are also available for the determination of the fluorescence intensity of probe-labelled cells in the FISH analysis (see below). This approach is

especially valuable to assess the cellular rRNA levels of particular cell populations (Daims et al. 1999). CMEIAS, computer software for the image analysis of bacterial morphotypes and spatial arrangements in bacterial communities, is of specific relevance for the detailed analysis of microbial communities visualized by CLSM images (Liu et al. 2001).

19.3 In Situ Composition Analysis of Bacterial Communities

19.3.1 The Fluorescence in Situ Hybridization (FISH) Technique

The Standard Technique. For the phylogenetic identification and characterization of prokaryotes, phylogenetic marker molecules, which are essential for cellular function, have been used. Besides the genes of b-subunit of the ATPase and the elongation factor Tu, the ribosomal RNA (rRNA) genes provide an ideal cellular macromolecule, reflecting the evolutionary history as molecular chronometer. The 16S and 23S-rRNA genes contain sequence motifs ranging from absolute conserved to highly variable stretches and can so serve as target molecules for phylogenetic classification and identification using complementary phylogenetic oligonucleotide probes (or polynucleotide probes; see sections "Amplification of the signal intensity" and "Recent development towards improved FISH techniques") in hybridization experiments. Using the fluorescence in situ hybridization (FISH) technique, prokaryotic cells can be identified without cultivation using the above-mentioned probes targeting specific regions either on the 16S or 23S-rRNA. Figure 19.1 summarizes the protocol which basically consists of a fixation step mostly using chemicals such as 4% formaldehyde or 50% ethanol, a hybridization step under stringent conditions, and a washing step to remove unspecific bound probes. The FISH analysis can be either performed on glass slides or in solution and typically uses probes with a length of 15–25 nucleotides which are covalently tagged mostly at their 5′ end with a fluorescent dye. Since the first application of this technique by DeLong et al. (1989), this technique was developed to the method of choice for reliable and rapid identification of micro-organisms in environmental samples (Amann et al. 2001; Moter and Göbel 2000). Nowadays, a large online database (Loy et al. 2003) provides an overview of more than 1100 published oligonucleotide probes for prokaryotic rRNAs suitable for FISH and Phylochip analyses (DNA microarray with gene probes) and their characteristics. Combined with a high spatial resolution, which is essential in complex environmental samples with disturbing autofluorescence material, confocal laser scanning microscopy (CLSM) in combination with

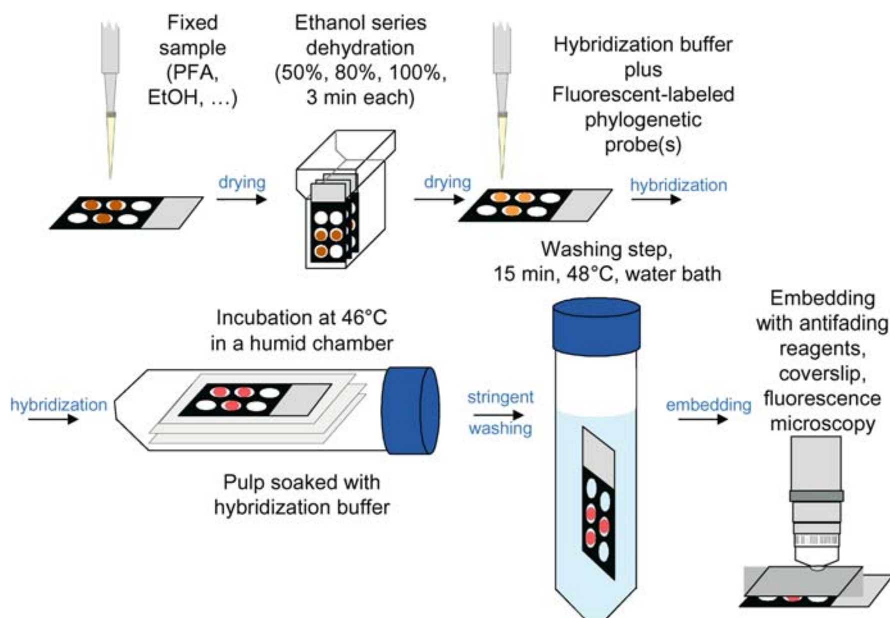


Fig. 19.1. Flow diagram of the major steps of FISH analysis

FISH analysis was successfully used for the in situ analysis of bacterial communities including uncultured/able organisms, giving insights in the spatial arrangements of microbial consortia (Amann et al. 1995; Daims et al. 2001b). The accumulated knowledge about the multiple applications of FISH analysis for the identification and characterization of prokaryotes in diverse habitats has been reviewed recently (Amann and Schleifer 2001; Bouvier and del Giorgio 2003; Wagner et al. 2003).

Obstacles and Limitations of the Standard Technique. Although it was successfully used in many studies, the standard FISH technique based on oligonucleotide probes has many limitations. One major problem is that not all bacterial and archaeal cells can be permeabilized using the same standard fixation protocols (Amann et al. 1995). Another serious limitation is the rather low sensitivity of the standard protocol using rRNA-targeted oligonucleotide probes covalently labelled with only a single fluorescence dye. This limits the application of standard FISH to the identification of prokaryotes with high cellular ribosome content, which usually are the minority in environmental habitats with low substrate availability. Furthermore, in complex habitats, the specific hybridization and washing conditions for unknown uncultured prokaryotes cannot be determined accurately. Since most of the data of fluorescence staining are obtained manually, the FISH analyses of complex samples is very tedious especially

to get quantitative data. The accuracy of this quantification approach is relatively low in densely colonized biofilms or other highly colonized habitats. Because of the high labour and time intensity, FISH-based studies seldom work with high sample numbers.

A general limitation comes also from the fact, that the rRNA is a target molecule with relatively slow mutation rate. Therefore, the differentiation between strains beyond the species level is not possible on the basis of 16S and 23S-rRNA hybridization analysis. FISH also does not provide information on the ecophysiological activity of a prokaryotic cell and the cellular RNA-content (measured as signal intensity of a cell after FISH analysis) does not always reflect the physiological activity.

19.3.2 Recent Developments Towards Improved FISH Techniques

Probe Design. The design of rRNA-targeted oligonucleotide probes with improved specificity and performance is a dynamic process and dependent on the continuously growing data base (Ludwig et al. 2004); the program is available under http://www.arb-home.de. In addition, several improved algorithms and computer programs for semi-automatic probe design have been developed recently (Ashelford et al. 2002; Pozhitkov and Tautz 2002). However, not all probes developed in silico for specific phylogenetic groups of bacteria work well in the FISH analysis. It has to be taken into account that the accessibility of the probe target sites on the 16S and 23S-rRNA molecules differ due to the three-dimensional structure of rRNA and the tight association with ribosomal proteins which causes high differences in the signal intensity in FISH analysis (Fuchs et al. 1998, 2000b). A first consensus accessibility map for prokaryotic 16S-rRNA has recently been developed by Behrens et al. (2003) comparing the in situ accessibility maps of *E. coli* with the Planctomycete *Pirellula*, the crenarchaeote *Metallosphaera sedula* and the eukaryote *Saccharomyces cerevisiae*. Since the probe accessibility of particular target sites may vary strongly in bacteria of quite different lineage, it is advisable to check, whether a selected probe may have been used successfully in related organisms using, e.g. the tool available at the ProbeBase website (Loy et al. 2003). It has been shown by Fuchs et al. (2000a) that certain target sites at the 16S-rRNA for oligonucleotide probes may be increased using unlabelled oligonucleotide probes that bind near to the probe-target sequence. The specificity of these so-called helper probes needs to be identical or broader than the specificity of the labelled probes and they must have a differentiation temperature (T_d) at least as high as the Td of the probe to avoid the dissociation of the helper probe at stringent hybridization conditions.

Signal-to-Noise Ratio. A series of innovative approaches to improve the signal to noise ratio for probe design are available, as reviewed by Wagner et al. (2003). Fluorescently labelled peptide nucleic acid (PNA) probes were reported to yield bright signals, targeting a site of the 16S rRNA with usually low accessibility for oligonucleotide probes (Perry-O'Keefe et al. 2001). Since the PNA pseudopeptides have an uncharged polyamine structure, low salt and high temperatures can be applied in the hybridization. The PNA probes may provide also the advantage for improved staining of Gram-positive bacteria and cyanobacteria. Another possibility of improving the specific signal intensity of the probe conferred fluorescence staining could use photo-induced electron transfer between the fluorescent dyes used for probe labelling and the nucleotides of the probe or target molecule (Marras et al. 2002). Although detailed experimental data on quenching effects in the FISH analysis are not yet available, this effect may cause problems in the design of probes and the application in chemically complex environments like soil micro-sites. Another reason for low signal-to-noise ratios of FISH-analysis in environmental samples is unspecific binding of labelled probes to sample material, leading to high background fluorescence. Self-ligating quenched probe pairs may have great potential in this respect. However, until now this approach has only been used for FISH analysis in pure cultures (Sando and Kool 2002).

Amplification of the Signal Intensity. A major obstacle of the FISH analysis in oligotrophic environmental sites with low nutrient availability is the low rRNA content of the probe-target cells giving only low or missing signals with fluorescently labelled oligonucleotide probes. Using mono-labelled oligonucleotide probes, the signal intensity of the detected cells is directly related to the number of target molecules in the cell. Therefore, cells with low rRNA-content cannot be detected which severely limits the successful application of FISH-analyses in soils (Chatzinotas et al. 1998). One solution could be the application of multi-labelled poly(ribo)nucleotide probes for FISH which are synthesized by incorporation of labelled nucleotides during in vitro transcription or PCR (DeLong et al. 1999; Karner et al. 2001; Trebesius et al. 1994; Zimmermann et al. 2001; Zwirglmaier et al. 2003a,b). It has been demonstrated clearly that polyribonucleotide probes enable to visualize a significantly higher percentage of prokaryotes in oligotrophic habitats as compared to FISH with mono-labelled oligonucleotide probes (Pernthaler et al. 2002c). However, polynucleotide probes have to be produced by cost- and labour-intensive PCR or by in vitro transcription in the laboratory. They are more sensitive to enzymatic degradation than oligonucleotide probes and due to their length (200–1000 base pairs) they cannot be designed as specific probes to discriminate closely related groups of prokaryotes.

Another very useful tool is the enzymatic signal amplification cascade using polyribonucleotide probes and *ca*talyzed *r*eporter *d*eposition (CARD–FISH; Fig. 19.2). In this approach horseradish peroxidase (HRP)-labelled oligonucleotide probes and tyramide signal amplification are used to enhance the signal intensities of hybridized cells significantly (Schönhuber et al. 1997). To allow the successful penetration of the large HRP-labelled oligonucleotides into the target cell a rigorous pre-treatment for cell wall permeabilization is necessary. Lysis of some cells could occur as consequence of this treatment which likely affects the composition of the microbial community understudy. This effect can be largely diminished by the use of an improved protocol for CARD–FISH in which the cells are embedded in low-gelling-point agarose before cell wall permeabilization (Pernthaler et al. 2002a). Studying North Sea plankton, 94% of the total cell counts were detectable by CARD–FISH, whereas only 48% of the cells were stained using the standard FISH protocol with the same probe carrying only one fluorescence label (Pernthaler et al. 2002a). The general applicability of CARD–FISH for multilayered biofilms or other complex

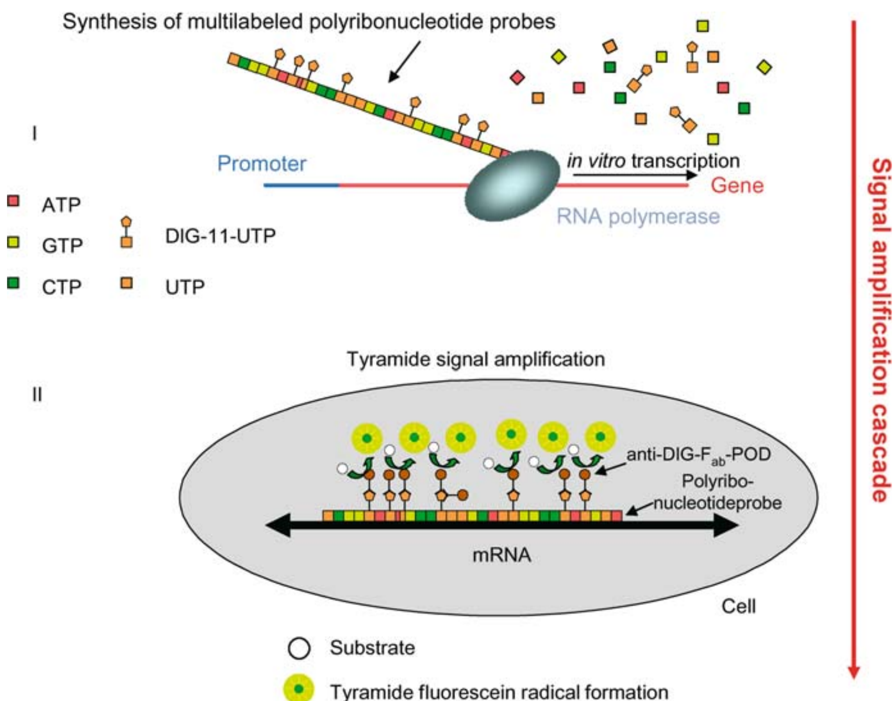


Fig. 19.2. "Signal amplification cascade" showing the synthesis of a DIG-labelled polyribonucleotide probe and its application in the CARD–FISH technique using tyramide signal amplification

structured microhabitats has to be proven still. Possibly a cryosectioning step of the microbial assemblage may be necessary. In addition, it has to be determined, whether thick and unusual cell wall structures such as in Gram-positive bacteria, Planctomycetes or Archaea are permeabilized as efficiently to give bright labelling.

Other Targets Than 16S-rRNA. Since the 16S-rRNA is rather conservative in structure the resolution of very closely related species or even strains of a given species is not possible. Using the larger 23S-rRNA, target sequences may be found with an improved resolution. However, since the data base of 23S-rRNA is much smaller compared to the 16S rRNA, in silico predictions are not as safe. A further possible alternative could be transcribed intergenic spacer regions of the rRNA operon. If the target bacteria are physiologically highly active and thus many spacer transcripts are available, the detection of bacteria via the ITS (internal transcribed spacer) is possible (Oerther et al. 2000; Schmid et al. 2001). If the transcription rate of the ITS is low, the in situ detection requires the use of CARD–FISH or other signal amplification methods.

Another RNA molecule being used as target for FISH analysis is the tmRNA or 10Sa RNA, which is encoded by the ssrA gene in *E. coli* and which is involved in the degradation of truncated proteins (Chauhan and Apirion 1989). This rather stable RNA molecule is present in a wide range of bacteria at about 1000 copies per cell. For some groups of bacteria, tmRNA could offer more suitable probe-target sites of differentiation than 16S-rRNA. Since the cellular concentration of tmRNA turned out to be too low for the detection with fluorescein-labelled oligonucleotide probes, tmRNA could be detected using CARD-FISH (Schönhuber et al. 2001). In future, other genes or even gene fragments on plasmids may also be identified in situ by FISH, applying polynucleotide probes by a procedure called RING–FISH (*r*ecognition of *in*dividual single *g*enes; Zwirglmaier et al. 2003b).

FISH-Analysis and Cytometry. Another obstacle for microscope analysis, even if supported by high throughput automated quantification and image analysis, is the limited sensitivity for detection of less abundant species. Cytometric analysis with many thousands of cells analysed could solve the problem, but the bacterial cells have to be desorbed from matrices and disaggregated from biofilm or microcolony assemblages. Approaches to reach this goal were reported by Hartmann et al. (2004) for desorption of bacteria from root surfaces. Similar approaches can also be applied to soils. However, modifications and improvements are obligatory for each type of matrix to achieve best results in desorption and disaggregation of bacteria.

The Cyclic rRNA Approach. Most bacteria from environmental samples are not cultivated yet, but they can be visualized and identified in situ using the "cyclic rRNA approach" using phylogenetic oligonucleotide probes (Amann et al. 1995; Ludwig et al. 1997). Since most of these sequences are not represented in the 16S ribosomal RNA database, it is necessary to clone the 16S-rRNA as only evidence of the existence of these novel type of bacteria. The 16S rRNA can be, e.g. transcribed in vivo using plasmids, e.g. with a T7 RNA polymerase promoter and *E. coli* host cells. The heterologeous rRNA transcripts can be investigated for their target-site accessibility and optimal hybridization conditions for probes targeting bacteria for which a pure culture is not available. Thus, Clone–FISH offers the fascinating possibility of rapidly screening environmental rRNA gene libraries for clones that carry the genes of interest (Schramm et al. 2002). When combined with flow cytometry (see below), this technique is able to enrich cells that carry the cloned rRNA genes with the respective probe target site, even if these clones are of very low abundance. This cloning approach could be extended to transcripts other than rRNA, opening the opportunity to raise the concentration of heterologous mRNA of functional genes (such as *nifH*, *amoA*, *dsrAB* or *cbbL*) cloned in *E. coli* to levels detectable by standard FISH procedures. This approach would allow rapid screening and sorting of functional gene libraries for genes of probe-defined target groups (see Sect. 4).

19.3.3 Immunological Techniques

Antibodies have long been used successfully as identification tools for specific micro-organisms. They can provide an extremely high resolution for in situ identification and discriminate microorganisms down to the strain level. As antibodies are a phenotypic marker, it is essential to use antigens as targets which are expressed stable under all environmental conditions. For example, it is know that lipopolysaccharides are expressed as a response to environmental conditions, and therefore may not serve as a good target for antigens (Schloter et al. 1995). Furthermore, a high specificity and sensitivity of the antibodies themselves for identification are needed. This specificity is mostly determined by the mode of creating the antiserum respective antibody: while polyclonal antisera have to be purified by, e.g. preincubation with cross-reacting cells to remove non-specifically binding antibodies to obtain a monospecific antiserum (Schloter et al. 1997), monoclonal antibodies usually are very highly specific because the hybridoma cell line, which is a fusion product of a cancer cell line and a B-lymphocyte produces only one type of antibody (for review see Schloter et al. 1995). Using

inactivated whole cells as antigens for immunization, antibodies are usually created against surface epitopes which have strain specificity (Schloter and Hartmann 1998). Highly specific antibodies provide a strain-specific detection which is not possible using the 16S-rRNA-probing technique, because this approach usually does not allow an identification below the species level. In contrast, it is very difficult to design antibodies that detect groups of microorganisms, like whole genera, because shared antigens are rare. Therefore, a combined labelling of cells with antibodies and 16S-rRNA probes does not only combine phenotypic and genotypic measurements, but also allows the detection of microbes at different taxonomic levels (Aßmus et al. 1997). Immunological techniques have been successfully applied also to label fungi in the environment. The immunological approach can also be used to investigate functional aspects of soil micro-organisms to study the expression if particular genes (see below).

19.4 In Situ Activity of Microbial Communities

19.4.1 In Situ Assessment of General and Specific Enzymatic Activities of Cells

The major goal in microbial ecology studies is the understanding of the function of microbes in their microhabitat. However, the phylogenetic analysis of microbes reveals very little about the contribution to the ongoing processes in a given micro-site. It is therefore a major challenge to develop and apply methods that determine activity and function of microbes at the single-cell level directly in natural samples. It is often assumed that high cellular ribosome content is an indicator of the general physiological activity of the respective cell, but recent data demonstrate that not only the current physiological state but also the physiological history determines the cellular ribosome content (Oda et al. 2000). Furthermore, at least some slow-growing chemolithoautotrophic bacteria such as aerobic and anaerobic ammonia oxidizers still posses high cellular ribosomes and eventually high rRNA concentrations and are therefore detectable by standard FISH protocols, even after extended periods of physiological inhibition or starvation (Wagner et al. 1995; Schmid et al. 2001). On the other side, bacterial cells might be highly active despite a low ribosome content and low FISH detectability like in the case of bacteria of the SAR86 lineage in marine samples (Pernthaler et al. 2002b). There is some evidence that the use of the ITS (intergenic transcribed spacer) region of the rRNA operon as target for fluorescently labelled oligonucleotide probes may more accurately link

general physiological activity with the FISH signal intensity (Oerther et al. 2000; Schmid et al. 2001).

Pernthaler et al. (2002b) developed an approach for the identification of prokaryotes undergoing DNA synthesis by visualizing rRNA content and incorporation of the uridine analogue bromodeoxyuridine into the DNA. Since the uptake of bromodeoxyuridine and its toxicity may be quite unpredictable for different bacterial groups in natural environments, it is to date questionable, whether this method is generally applicable in complex natural samples.

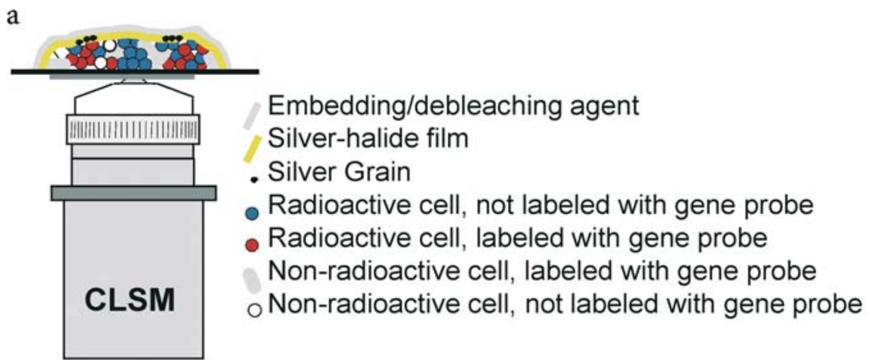
Enzymatic properties of single cells have been screened in the aquatic environment, biofilms and activated sludge by a new type of fluorogenic compound, ELF-97 (Molecular Probes, Eugene, OR), which is composed of sugar, amino acid, fatty acid or inorganic compounds such as sulfate or phosphate. This substrate is converted to a water-insoluble, crystalline, fluorescent product at the site of enzymatic hydrolysis, thus reporting the location of active enzyme when viewed by fluorescence microscopy (Larison et al. 1995). Van Ommen Kloeke and Geesey (1999) combined this technique with 16S rRNA oligonucleotide probe specific for the cytophaga–flavobacteria group to prove the importance of these microorganisms in liberation of inorganic ortho-phosphate in discrete bacteria-containing areas of the floc matrix in aerobic activated sludge. These new substrates have the future potential to be used for imaging the location of enzyme activities in micro-environments of soil Nedoma et al. 2003).

19.4.2 Isotope Tracer Techniques

The FISH–MAR Technique. The combination of FISH analysis with microautoradiography (FISH–MAR) provides information about both the phylogeny of the bacteria and an indication on their specific in situ activity (Lee et al. 1999; Oueverney and Fuhrmann 1999; Fig. 19.3). Using suitable isotopically labelled substrates (especially alpha- or weak beta-emitters,

▶ **Fig. 19.3.** FISH–MAR-technique.: **a** Schematic drawing of the simultaneous application of phylogenetic staining with FISH and micro-autoradiography producing dark silver grains at the locations of the accumulation of radioactively labelled substrate; **b** FISH analysis and CSLM image showing deposited silver grains at locations of uptake/adsorption of a radioactive substrate. Environmental sample (activated sludge) incubated with radioactive ^{14}C-acetate. *Left image* FISH image of corresponding *right image*, with beta-proteobacterial probe for *Rhodocyclus*-related bacteria PAO651 (cy3, *red*), gamma-proteobacterial probeGam42a (Fluos, *green*); and general bacteria probe EUB (cy5, *blue*). *Right image* Phase-contrast MAR image (uptake of radioactive acetate is seen as *darkened areas. Lower image* Overlapping image of corresponding MAR and FISH images

a



b

Gam42a cells show clear uptake of ^{14}C-acetate.

PAO651 cells no/weak uptake of ^{14}C-acetate.

10 µm

such as ^{14}C, ^{3}H), this technique is probably presently one of the best ways to determine and visualize indications for in situ physiological activity of bacteria directly in the environment. The alternative approach of combining FISH with CTC (5-cyano-2,3-tolyl-tetrazolium chloride) staining fails to detect many active cells (Nielsen et al. 2003a, b). Using the MAR–FISH technique, the substrate-uptake patterns in probe-defined bacteria could be investigated in situ in mixed natural communities on a single-cell level, even if the bacteria are not cultured yet (Gray et al. 2000; Ito et al. 2002; Lee et al. 2003, Rossello-Morá et al. 2003). For example, the abundance of in situ physiologically active iron-reducing bacteria could be quantified and assigned to phylogenetic groups by incubation with labelled acetate under appropriate conditions (Nielsen et al. 2002). MAR–FISH also allowed one to assess the contribution of certain bacterial groups to the cross productivity of an estuary by measuring the assimilation of ^{3}H-thymidine and ^{3}H-leucine (Cotrell and Kirchmann 2003). Furthermore, several novel insights into the complex phenomena in microbial ecology have also been revealed by this technique. For example, Lee et al. (2003) found not only differences in substrate uptake pattern between different probe-defined bacterial groups, but also within one type of a probe-defined bacterial group, suggesting that either differences in activity, or phylogeny of the probe-defined bacterial group (meaning that the gene probe is not specific enough), were prevalent. Rossello-Morá et al. (2003) found – using FISH–MAR technique – that bacteria behaved differently in the laboratory compared to in their natural environment, stressing the need to rely more on in situ activity measurements of bacteria in their natural environment than on laboratory culture studies. Thus, MAR–FISH enables to identify members of physiological groups of microorganisms in environmental samples and may help to close the gap to the general enzymatic measurements, which were recently also very much increased in sensitivity (Pritsch et al. 2004). Recently, a quantitative approach has been developed to determine the in situ substrate affinity (K_s) value of filamentous bacteria for the uptake of acetate in probe-defined cells in a complex system using the MAR-FISH approach (Nielsen et al. 2003a, b). Using the CARD–FISH protocol together with microautoradiography the detection of active bacteria was further improved and archaeal cells were found as the major physiological active fraction in the total bacterial population in the deep ocean taking up ^{14}C-labelled l-aspartic acid (Teira et al. 2004). Nevertheless, the FISH–MAR technique also shows some limitations, such as limited supply of suitable radiotracers and problems with interpretation of the uptake pattern, e.g. whether the uptake of the radiotracer is based on pure chemical adsorption or on real physiological uptake, and whether the substrate itself, or metabolic products of it, have been taken up. Thus, further improvements of this technique, or combination with other meth-

ods, such as stable-isotope-probing and full-cycle rRNA analysis, will have to be applied and further developed for a more thorough characterization of the in situ activity (Gray and Head 2001; Ginige et al 2004; Wagner 2004).

The TOF-SIMS Technique. Stable C and N isotopes have long been used to examine properties of various C and N cycling processes in soils (Boutton and Yamasaki 1996). However, relatively large sample sizes were needed for accurate gas phase isotope ratio mass spectrometric analysis. This limitation prevented to address C and N cycling issues on microbiologically meaningful scales. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) has been recently used to detect ^{13}C and ^{15}N assimilation by individual bacterial cells and to quantify N isotope ratios in bacterial assemblages and individual fungal hyphae (Cliff et al. 2002). This approach clearly has the potential to work with very small sample sizes and allowed one to locate N-assimilating microorganisms in soil and to quantify the ^{15}N content of cells that have assimilated ^{15}N-labeled mineral N. The ^{15}N content of fungal hyphae were significantly lower in regions where the hyphae were influenced by N-rich manure than in regions influenced by N-deficient straw. Thus, this approach is another promising tool to explore the factors controlling micro-site heterogeneities and microbial in situ activities in soils.

Stable Isotope Probing. In another approach using stable isotopes, the utilization of substrates labelled with the stable isotope ^{13}C is combined with the phylogenetic characterization of the microbes accumulating this substrate in the natural environment (Radajewski et al. 2000; Boschker and Middelburg 2002; Ginige et al. 2004). Stable-isotope probing (SIP) involves the incorporation of stable isotope-labelled substrates into cellular biomarkers, including DNA, RNA and phospholipid fatty acids. When ^{13}C-labeled DNA or RNA is used in the stable-isotope probing approach equilibrium density centrifugation is used to separate the DNA/RNA species enriched in ^{13}C due to the assimilation of ^{13}C-labelled substrate from the regular ^{12}C-labelled nucleic acids. The phylogenetic analysis is performed using 16S-rDNA/RNA PCR or reverse transcription-PCR and either denaturing gradient gel electrophoresis (Manefield et al. 2002) or by construction of clone banks (Lüders et al. 2004a,b). The present method is dependent on a rather efficient accumulation activity and labelling efficiency of the cells to be separated from the non-active cells. Utilization of the radioactive carbon isotope ^{14}C may increase both the sensitivity of the detection and the possibility to work with smaller sample volumes to get closer to microbial relevant scales.

19.4.3 Specific Fluorescence Labelling/Tagging Techniques

In Situ Gene Expression Studies Using Gfp/Rfp-Operon Fusions. To study in situ activities of individual cells and populations, so-called reporter constructs can be applied. For this reason, reporter genes coding for an autofluorescent compound (e.g. the green fluorescent protein, GFP) or for an enzyme giving raise for a colorimetric or fluorimetric reaction are fused to promotors of genes of interest. With the activation of promoters and such, the expression of particular genes can be studied in soil or rhizosphere microhabitats. Egener et al. (1999) demonstrated the activation of *nif* genes of diazotrophic bacteria of the genus *Azoarcus* in the rhizosphere. Steidle et al. (2001) showed the in situ perception of signalling compounds of the *N*-acetylhomoserinlactone (AHL) type produced by rhizosphere bacteria using a GFP-labelled *Pseudomonas putida* strain as AHL-reporter bacterium. The analysis of the fluorescently labelled microbes can be extended by double-labelling the bacterial population under study with another fluorogenic marker (e.g. the RFP-red fluorescent protein) to be able to assess the total population versus the activated subpopulation. Using the LSM510 META system (Zeiss, Oberkochen, Germany), the application of multiple fluorescence tagging became possible, because the instrument allows the separation of the multiple fluorescence signals in a spectrum-based manner.

Antibody Labelling of Enzyme Proteins. To study the expression of certain genes, the in situ assessment of the appearance of the enzyme protein is an important step towards the synecological understanding of microbes. This would also add the important proteomic aspect into ecological studies. The approach depends heavily on the availability of highly specific polyclonal or monoclonal antibodies. To find the right epitope, which is useful for antibody design, sometimes even artificial polypeptides are needed. Enzyme-specific antibodies are available, e.g. for the *nifHDK*-gene products using antibodies for the nitrogenase enzyme (Reinhold-Hurek and Hurek 2000), for the amoB-protein of ammonia oxidizers (Pink et al. 2001) and the dnirK-gene product (dissimilatory copper nitrite-reductase) (Bothe et al. 2000; Metz et al. 2002, 2003). Using either directly fluorescence labelled antibodies or the sandwich technique with secondary antibodies, bacteria cells synthesizing these enzymes were identified in situ using the CLSM or other highly resolving computer-assisted epifluorescence microscopy. Single bacterial cells expressing the enzyme of interest could be visualized specifically in situ on the surface of roots or inside root or plant tissues. By combining the antibody labelling of a functional enzyme with 16S-rRNA targeted probing, an in situ assessment of the structure and

function of microbial communities is possible. Furthermore, labelled cells can be sorted by flow cytometry and then further analyzed ex situ by any molecular technique.

19.5 Conclusions

In recent years, the localization and microvisualization of bacterial cells and their in situ activities in environmental samples have made tremendous progress. A future goal for methodological developments must be the application of these in situ techniques to the detection of fungal communities in the environment. On the one hand, the miniaturization and improvement of the sensitivity of enzymatic measurements and molecular biological techniques enabled new achievements in the synecological understanding of microbes on very small sample scales. On the other hand, the demonstration of in situ activities and the link to phylogenetically defined microbes using the FISH–MAR or TOF/SIMS techniques tshow promise in filling the gap between micrometer scale microbial ecology and the ecological understanding of processes on larger scales. These techniques provide rather robust and relatively rapid tools for the identification of the population structure and function of microbes in diverse environmental samples. However, for application to complex heterogenic samples, the spatial organization needs to be preserved in a refined way. The conservation of the three-dimensional structure in the micrometer range by the improvement of embedding techniques which still allow the fixation and hybridization procedure to occur at reliable precision would be very useful for further in depth studies in microbial ecology. In addition, the development of digital-image analysis tools to extract the three-dimensional spatial data from the investigated specimen would further support the in situ studies of organismic interactions.

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20 Microsensors for the Study of Microenvironments and Processes in the Intestine of Invertebrates

Andreas Schramm

20.1 Introduction

The intestines of invertebrates are frequently considered minute ecosystems or bioreactors (e.g., Brune 1998), teeming with microbial life and characterized by physicochemical conditions distinct from the environment in which the animals live. Only recently, and only for a small selection of invertebrates, the axially and radially highly structured nature of these intestines has become obvious (Brune and Friedrich 2000). These new insights were mainly due to microsensor measurements with high spatial resolution that revealed quite different physicochemical conditions in different gut compartments, and steep radial gradients within a given compartments. The description of the microenvironments of invertebrate intestines along with the in situ identification and localization of the microbial populations thriving in these different microhabitats is now regarded essential for further advancing our understanding of invertebrate gut microbiology. This chapter will (1) summarize measuring principles and types of microsensors available to date, and (2) discuss their application to measure inside the intestines of invertebrates.

20.2 Microsensors Available

With the introduction of the oxygen microsensor to microbial ecology almost 25 years ago (Revsbech et al. 1980), Niels Peter Revsbech opened up a whole new look at the habitat of microbes: in situ measurements on a microscale level. Since then, many other microsensors have been constructed and applied in laboratory and environmental settings (for reviews see, e.g., Revsbech and Jørgensen 1986; de Beer 1999; Buffle and Horvai 2000; Kühl and Revsbech 2001), and the term microsensor has

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been used in various ways. In this chapter, microsensors are defined as miniaturized sensors for measurements with a spatial resolution of less than 0.1 mm.

To date, there are three principally different microsensor types available: amperometric, potentiometric, and optical. In addition, microbiosensors can be constructed by combining one of the three with immobilized enzymes or whole bacterial cells.

Amperometric Sensors. These detect the current caused by electrochemical reactions (oxidation or reduction) of the analyte at the sensor tip. The reaction is driven by a difference in potential between sensing electrode and reference, and the reaction rate is proportional to the concentration of the analyte. In "Clark-type" sensors, the cathode/anode is protected by a semipermeable membrane against interference from the medium (Clark et al. 1953); the stability of the signal can be improved by an internal guard cathode (Revsbech 1989). A schematic drawing of a Clark-type amperometric sensor tip is shown in Fig. 20.1. The specificity of the sensor is determined by the applied potential difference, by the permeability of membranes in the sensor tip, or by specific redox mediators. Functional microsensors based on this principle are for O_2 (Revsbech 1989), N_2O (Andersen et al. 2001), H_2S (Kühl et al. 1998), H_2 (Witty 1991; Ebert and Brume 1997), HClO (de Beer et al. 1994), and a combined microsensor for O_2-N_2O (Revsbech et al. 1988). A special case of an amperometric sensor is the diffusivity sensor (Revsbech et al. 1998) which detects a tracer gas that diffuses out of a capillary to determine the diffusivity properties of the sample, which is required to calculate reaction rates from concentration profiles (Revsbech and Jørgensen 1986; de Beer and Stoodley 1999). The advantage of most amperometric microsensors is their reliability and relatively long lifetime (in the order of months); a serious disadvantage is the difficulty to construct them. Even the making of the simpler O_2 or H_2 sensors requires considerable experience, and the most complex sensors (e.g., for O_2-N_2O) could only be made by a few highly skilled experts.

Potentiometric Sensors. They detect an electrical potential at the tip generated by charge separation across a selective membrane. The potential is recorded against a reference electrode, usually a calomel or Ag/AgCl electrode. The selective membrane can be either of glass, solid state, or a liquid ion exchanger (LIX). An example for a full glass sensor is the pH glass microsensor (Thomas 1978; Revsbech et al. 1983), a miniaturization of the conventional pH glass electrode (Fig. 20.1). However, due to its relatively long sensing tip (ca. 100 μm), it has a low spatial resolution and is only of limited use for true microenvironmental studies. An AgS membrane sulfide sensor (Revsbech et al. 1983) has been used for studies of sulfur cycling in sediment, microbial mats and biofilms, but due to its limited accuracy and

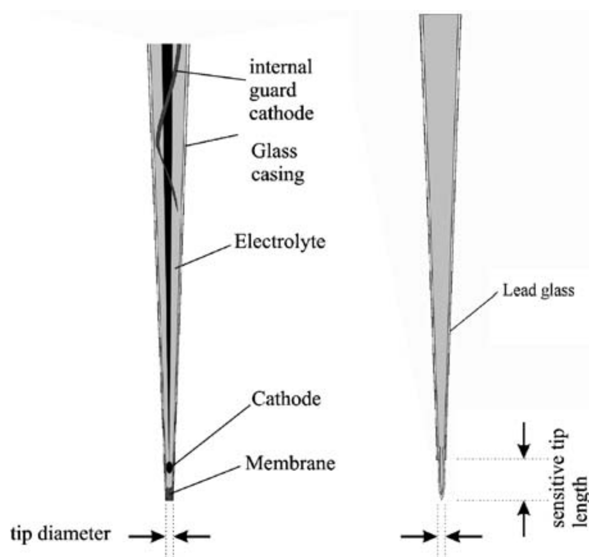

Fig. 20.1. Schematic drawing of microsensor tips of an amperometric, Clark-type O_2 sensor (*left*), and a potentiometric glass pH sensor (*right*). Typical tip diameters are 5–10 μm; however, the sensing tip of the pH sensor is much longer. Picture courtesy of Unisense A/S

lifetime it is being replaced by the more reliable amperometric H_2S sensor (except for applications in high-pH environments where almost all total sulfide occurs in the form of S^{2-}). The redox microsensor (Ebert and Brune 1997) is another example for a potentiometric sensor. LIX microsensors have been originally developed for intracellular measurements and can be made very small (< 1 μm). They are based on an ion-selective, liquid membrane in the sensor tip, which is ideally only permeable for one ion species. In practice, this holds only true for the LIX pH microsensors, which are also the most reliable and long-living LIX sensors available. Most other sensors exhibit more or less severe interference from other ions, which limits their application in marine or other high salinity habitats. In addition, the lifetime of most sensors is short (in the order of hours to days), and often significant drift is observed, requiring frequent re-calibration. However, LIX microsensors are easy to construct and can be made for very high spatial resolution. LIX sensors successfully applied in microbial ecology are for pH (de Beer et al. 1997b), NH_4^+ (de Beer and van den Heuvel 1988), NO_2^- (de Beer et al. 1997b), NO_3^- (de Beer and Sweerts 1989), Ca^{2+} (Amman 1986), and CO_2 (de Beer et al. 1997a; Zhao and Cai 1997).

Fiber-Optical Sensors. They are either used as so-called fiber-optic microprobes (Kühl et al. 1994) to directly measure light distribution and intensity (which is irrelevant in the context of gut microbiology and will not

be further discussed here), or as microoptodes that contain an indicator chemistry at the fiber tip that changes its absorption or its luminescence lifetime or intensity (Klimant et al. 1997). Optodes have a relatively large tip size (> 20 µm) but are considerably more stable and much easier to produce than microelectrodes. To date, O_2 (Klimant et al. 1995), pH (Kohls et al. 1997), and temperature (Holst et al. 1997) can be measured optically. In addition, a combined microcapillary injector/fiber-optic microprobe has been used to measure diffusivity and flow in biofilms (de Beer and Schramm 1999).

Microbiosensors. They combine electrochemical (or optical) sensors with a biological component, i.e., immobilized enzymes or bacterial cells. These convert analytes that cannot be directly measured (e.g., NO_3^-) into measurable products (e.g., N_2O). Alternatively, the consumption of, e.g., O_2 during conversion of the compound of interest (e.g., CH_4, fatty acids), can be followed. In general, the use of whole cells results in greater stability and longer lifetime of the sensor compared to immobilized enzymes. The drawback of the microbiosensors is their enormous complexity, which so far has restricted their production and application to only a few research groups. However, in the absence of new general measuring principles, microbiosensors bear the greatest potential to extend the spectrum of measurable compounds. To date, an enzyme-based microbiosensor has been described for glucose (Cronenberg et al. 1991), and whole cell-based sensors have been developed for CH_4 (Damgaard and Revsbech 1997), NO_2^-+NO_3^-+N_2O (Larsen et al. 1996, 1997), dissolved organic carbon (Neudörfer and Meyer-Reil 1997), NO_2^- (Nielsen et al. 2004), NH_4^+ (Bollmann and Revsbach 2005) and volatile fatty acids (Meyer et al. 2002); all these sensors consist of a bacterial reaction chamber placed in front of an amperometric sensor.

20.3 Microsensors in the Study of Invertebrate Guts

In principle, three levels of information can be obtained from microsensor measurements in invertebrate guts: (1) microscale information about the physicochemical conditions in the gut, (2) fluxes of substrates and products into and out of the gut, and (3) local activities along radial gradients in the gut.

Most microsensor studies of invertebrate intestines have been concerned with the first level, the microenvironment of the gut. Most commonly, its oxygen status has been recorded, e.g. in termites (Brune et al. 1995; Ebert and Brune 1997; Schmitt-Wagner and Brune 1999; Kappler and Brune 1999),

caterpillars and grasshoppers (Johnson and Barbehenn 2000), earthworms (Horn et al. 2003), beetle larvae (Lemke et al. 2003), and snails (Charrier and Brune 2003). In all cases, the gut center was anoxic, and in earthworms and snails oxygen could not be detected at all with the spatial resolution (50 μm) applied. Redox potential (Ebert and Brune 1997; Kappler and Brune 2002; Lemke et al. 2003), pH (Brune et al. 1995; Brune and Kühl 1996; Walker et al. 1996; Charrier and Brune 2003; Horn et al. 2003; Lemke et al. 2003), hydrogen partial pressure (Ebert and Brune 1997; Schmitt-Wagner and Brune 1999; Lemke et al. 2001, 2003; Charrier and Brune 2003), and N_2O (Horn et al. 2003) concentrations were also determined in selected invertebrates. Important results were the documentation of pH values more extreme then ever observed in pooled samples, and the localization of H_2 and N_2O consumption and/or production. As can be seen from the above list, the number of sensor types applied is limited, and several sensors of relevance for invertebrate gut microbiology (i.e., for CH_4, fatty acids, NO_3^-/NO_2^-, NH_4^+) await their first application in this context.

Determination of consumption or production rates (i.e., the second level of information) requires knowledge about the slope of the measured gradient through the diffusion boundary layer (e.g., Revsbech and Jørgensen 1986; de Beer and Stoodley 1999). Fluxes of oxygen and/or hydrogen could therefore be obtained from measurements in extracted termite guts that were embedded in agarose (Brune et al. 1995; Ebert and Brune 1997; Schmitt-Wagner and Brune 1999), thereby artificially creating a defined diffusion boundary layer. Alternatively, the extracted guts of cockroaches (Lemke et al. 2001), snails (Charrier and Brune 2003), and beetle larvae (Lemke et al. 2003) were irrigated with Ringer's solution during microsensor measurements.

Local conversion rates along radial gradients through the gut of invertebrates have not been reported yet; in most cases, the limited spatial resolution of the profiles would not have allowed the required calculations or computer-aided interpretations (for details how to obtain local conversion rates see, e.g., Revsbech and Jørgensen 1986; Berg et al. 1998; de Beer 1999); in addition, the diffusion characteristics of the gut are unknown. However, measurements at higher resolution appear possible in intestines (e.g., Johnson and Barbehenn 2000), and diffusivity can also be determined in situ with microsensors (Revsbech et al. 1998). In certain cases, therefore, the additional information of local conversion rates could aid in resolving structure-function relationships inside the gut.

20.4 Practical Considerations

20.4.1 Construction and Purchase of Sensors and Equipment

As stated above, the construction of microsensors, especially of the more complex amperometric and biosensors, requires considerable expertise and training; so unless a person is almost fully devoted to microsensor construction (and unless large quantities of microsensors are needed), it will usually be more rational to purchase sensors. To date, at least some of the amperometric (O_2, H_2S, H_2, diffusivity, and N_2O) and a few potentiometric (pH, redox) microsensors are commercially available, along with the instrumentation needed. Sources are Unisense A/S, Aarhus, Denmark (http://www.unisense.com) and Diamond-General, Ann Arbor, MI (http://www.diamondgeneral.com). Optodes and optoelectronic instruments are available from PreSens, Regensburg, Germany (http://www.presens.de). However, for the very complicated biosensors and for the simple but short-lived LIX microsensors, there is currently no alternative to "do-it-yourself". Valuable step-by-step instructions (in addition to the respective original publication of the sensor) can be found in Revsbech and Jørgensen (1986) and de Beer (1999). A special case is that of the pH microsensor: full glass sensors are commercially available, their use in invertebrate guts, however, may be problematic. In addition to the limited spatial resolution (due to the long sensing tip), the fusion of the pH-sensing tip with the lead glass (see Fig. 20.1) is somewhat fragile and tends to break in animals whose gut wall is difficult to penetrate or if there is a strong peristalsis (Andreas Brune, personal communication; Schramm, unpublished). This problem can easily be circumvented by use of the LIX pH microsensor, and the gain in this case is certainly worth the effort of constructing it.

20.4.2 Fixation of Animals, Gut Preparation and Measuring Conditions

Microsensor measurements with step sizes of 50 μm or below require samples that absolutely do not move; therefore, animals have to be fixed or at least sedated prior to measurements. In addition, measurements in whole animals (as shown in Fig. 20.2 for an earthworm) are very difficult if not impossible, e.g., due to a (for glass capillaries) impermeable chitin exoskeleton, or because gut compartments and the radial position of the sensor tip inside the gut cannot be localized without dissection (Brune et al. 1995). In such cases, measurements will have to be done on isolated or

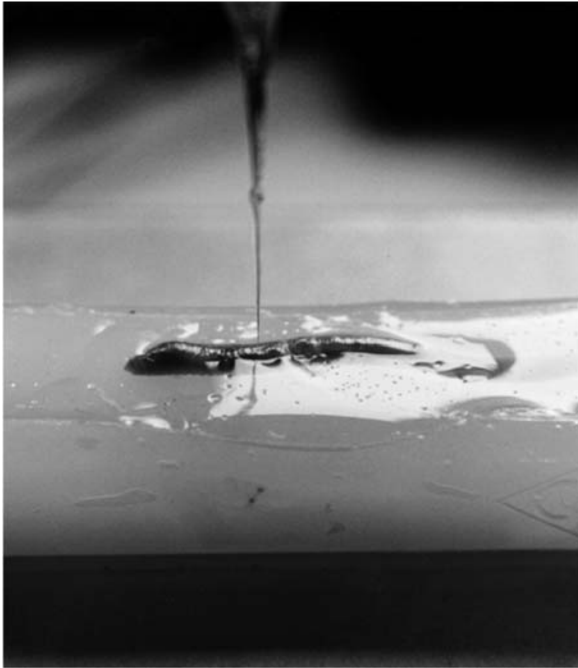

Fig. 20.2. Microsensor measurements in a whole, sedated earthworm that is partially embedded in agarose, leaving the dorsal side air-exposed. Picture courtesy of Marcus Horn

at least exposed guts (i.e., with the body wall open), which usually have to be embedded or submersed, e.g., in Ringer's solution, to avoid desiccation. It is then important to minimize (or at least recognize) artifacts that are introduced due to fixation, preparation, or embedding, and the appropriate procedures will have to be carefully chosen for each animal in question. Table 20.1 gives an overview of procedures that have been applied to different animals. Again, it has to be stressed that these procedures should not simply be transferred to other animals without careful evaluation of possible options and consideration of potential artifacts. For earthworms, e.g., we found in pre-experiments that sedation with CO_2 significantly changed the pH of the gut, while fixation by chilling ($-20\,^{\circ}C$) or $> 50\%$ ethanol changed the oxygen penetration depth into the earthworm body. Measurements on isolated earthworm guts resulted in the detection of oxygen in the originally anoxic gut (Schramm, unpubl.).

Likewise, Brune et al. (1995) and Lemke et al. (2001) discussed the effects of embedding isolated termite and cockroach guts, respectively, in agarose, which might limit tracheal diffusion of oxygen towards the gut but could also have the contrary effect since the animal itself is then unable to regulate

Table 20.1. Anesthetization and setup for microsensor measurements in the gut of invertebrates

Animals	Fixation/sedation	Measuring conditions	References
Termites	Decapitated without pre-treatment	Isolated gut, embedded in agarose-solidified Ringer's solution; defined headspace	Brune et al. (1995); Brune and Kühl (1996); Ebert and Brune (1997); Schmitt-Wagner and Brune (1999); Kappler and Brune (1999, 2002)
Caterpillars and grasshoppers	Ethyl acetate (5 min) or −20 °C (7–12 min)	Isolated gut, air-exposed	Johnson and Barbehenn (2000)
Cockroaches	N_2-CO_2(80:20, vol/vol), then decapitated	Isolated gut in air-saturated Ringer's solution or agarose-embedded; defined headspace	Lemke et al. (2001)
Earthworms	40% Ethanol	Whole worms, partially air-exposed	Horn et al. (2003)
Snails	Injection of 0.01% succinylcholine chloride	Isolated gut in air-saturated Ringer's solution	Charrier and Brune (2003)
Beetle larvae	N_2-CO_2(80:20, vol/vol), then decapitated	Isolated gut in air-saturated Ringer's solution	Lemke et al. (2003)

the tracheal oxygen supply. Charrier and Brune (2003) also speculated that oxygen uptake rates in helicid snails might be overestimated because the isolated intestines may have received a greater supply of oxygen. With so much uncertainty about the true in situ conditions, measurements under defined headspace (Ebert and Brune 1997; Lemke et al. 2001) might be an attractive option in order to elucidate minimum and maximum potential rates of certain processes, e.g., under air saturation as compared to anoxic conditions.

20.5 Conclusions

Microsensor measurements were essential in transforming invertebrate intestinal studies into modern microbiology and microbial ecology. Since

only a few selected animal species have been examined so far, and since several new sensors still await their application to invertebrate guts, we may expect many more exciting (and maybe again groundbreaking) discoveries by this technique within the next few years.

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